Insecticide resistance monitoring - a review of current methodology

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Henry Frempong Owusu
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Prof. Dr. Marcel Tanner, Dr. Pie Müller, und Dr. John Gimnig

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Prof. Dr. J. Schibler

Dekan der Philosophisch-

Naturwissenschaftlichen Fakultät

Summary

Introduction: Insecticide resistance continues to pose a serious threat to the control of vector-borne diseases. In the last decades, it has spread across Africa and many countries with high transmission of mosquito-borne diseases. A major handicap in the efforts to control resistance is the limited availability of routine and reliable data, a situation which arises from the fact that many countries with ongoing transmission of vector-borne diseases do not perform routine data collection, or in areas where data are available, there are high levels of inconsistencies in the reported data. Although WHO has put in place guidelines to be followed in performing bioassays to detect resistance, the guidelines are not standard operating procedure and leave room for discretion. Taking into account the importance of effective vector control and the limitation on the number of insecticide classes available, preserving the susceptibility of malaria vectors to the present classes of insecticides is essential in maintaining effective malaria control. The evolution of resistance to insecticides could endanger current and future achievements in controlling malaria. Therefore, the need for proper monitoring interventions equipped with well laid out guidelines cannot be overlooked.

Aims and objectives: The main aim was to review existing methodologies employed in insecticide resistance monitoring and identify factors that lead to inconsistencies in data generated in vector control strategies. This overarching aim is divided under three main objectives:

i. to assess the effect of bioassays on the test outcome;
ii. to assess the influence of the rearing conditions of mosquitoes on bioassay outcomes; and
iii. to assess the effect of inter laboratory variability on the outcome of the test.

Methods: Using laboratory-bred mosquito colonies, we performed susceptibility experiments with the principal diagnostic bioassays against insecticides mostly used in public health for the control of the major vectors involved in disease transmission to assess the robustness of the bioassays. We also bred mosquito larvae under different conditions to evaluate the effect of changes in environmental factors on the susceptibility of the adults to insecticides. The data generated was extended to a mathematical model to estimate the effects of larval population

density on adult survival. The major sources of inter-laboratory differences in data generated in insecticide resistance monitoring activities were also tested by performing the WHO susceptibility assay at multiple centres.

Results: The results indicate that the WHO susceptibility and CDC bottle bioassays which are generally used interchangeably for both field and laboratory evaluations of insecticide resistance are highly inconsistent in generating the same results on the same mosquito population. The WHO cone assay also produces different results when the assay is performed at different angles. We also found the breeding conditions during the larval stage significantly affect the susceptibility status of the adult mosquito to insecticides. The mathematical models also showed that larval density significantly affects adult survival.

Conclusion: Results from this thesis reinforces the call for proper insecticide resistance monitoring tools and practices. While the problem of insecticide resistance is on the rise, the lack of effective and reliable methods to detect and monitor resistance remains a major concern.

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1. Introduction

This chapter gives a succinct overview of the life cycle, general characteristics and biology of mosquitoes. It highlights their public health importance and gives detailed information on the current practices employed in mosquito control. The problem of insecticide resistance is introduced and an outline of the organisations and structures in place to monitor and curb the threat is also provided here. The research questions and motivations for this dissertation are given in the last section and are put in context to the provided background information.

1.1. Our nemesis, the Mosquito

The word mosquito originates from the Spanish word "mosca" and the diminutive "ito", meaning "small fly". They are small, with an average adult size of 3-6 mm, but there is nothing small about their importance in public health and in the ecosystem. They are a major nuisance with their buzzing sound and biting and blood sucking behaviour. But most importantly, they transmit some of the deadliest diseases of humans and animals [1]. Due to their ability to successfully adapt to a wide variety of habitats, mosquitoes have a cosmopolitan distribution; occurring throughout the tropic and temperate regions and into the Arctic Circle. They are absent only from a few islands and Antarctica. They have also been found at areas with elevations of 3,500 m, as well as mines with depths of 1,250 m below sea level [2]. The larvae inhabit a wide range of water bodies. With the exception of marine habitats with high-salt concentration [3], they can colonize temporary, permanent, clean, large or small, stagnant, fresh, brackish, clear, turbid and polluted water bodies as well as small accumulations of water such as leaf axils, buckets, old tyres and hoof prints. The adults have also been found to survive aircraft conditions [4–6], making it easy for them to be transported and introduced to new places by this means. There are about 3,530 species of mosquitoes, which are classified into the order Diptera, family Culicidae and the subfamilies Toxorhynchitinae (toxorhynchites), Anophelinae (anophelines) and Culicinae (culicines). As members of the "true flies", they are characterized by fully-developed functional fore-wings and vestigial hind wings reduced to form a pair of small knob-like balancing organs called halteres (Figure 1.1). Mosquitoes are set aside from other true flies by their forward-projecting proboscis extending from the head and scales on the thorax, abdomen, legs, wing veins and posterior margin of the wings [2].

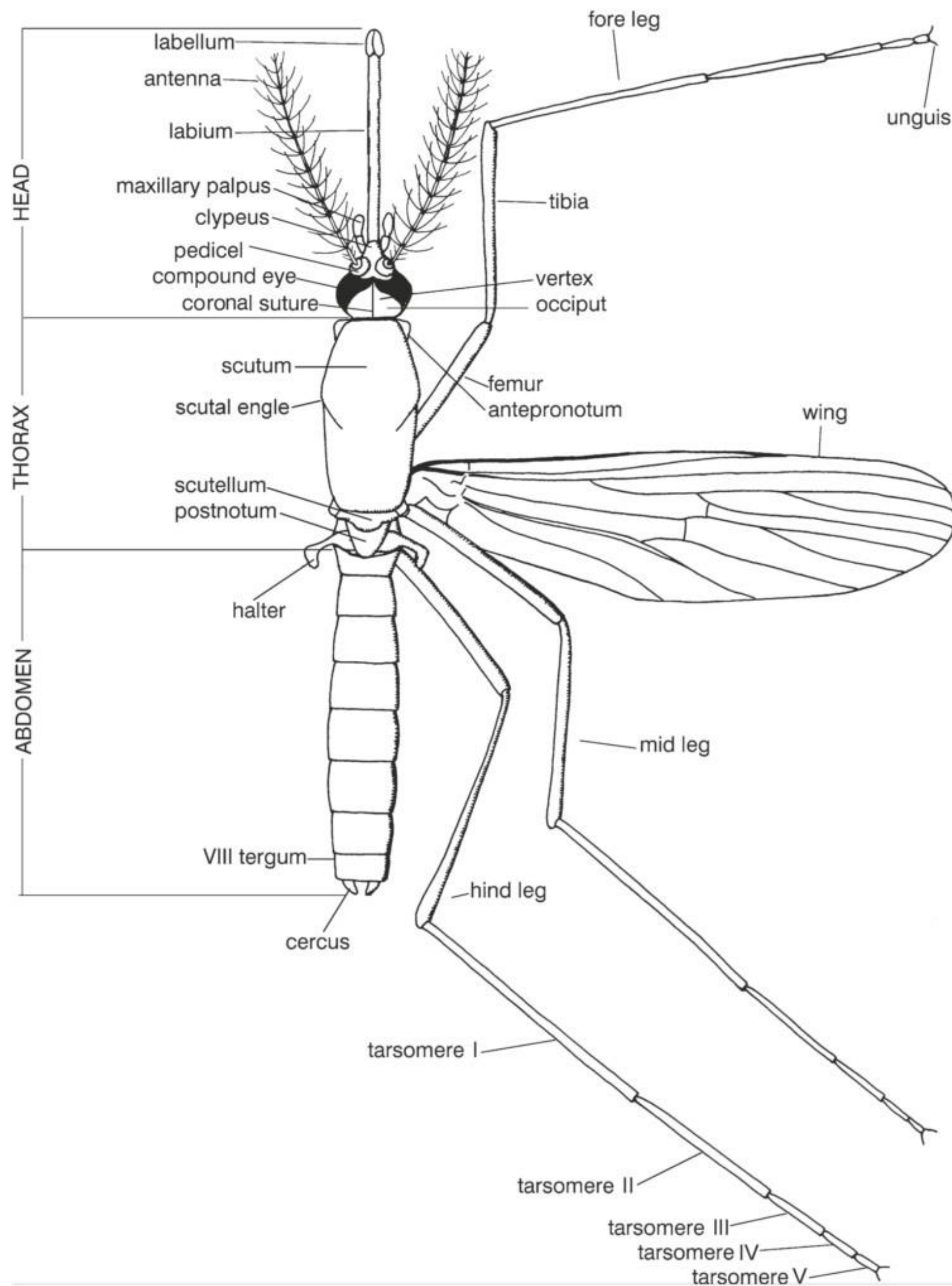

Figure 1.1: A labelled diagram showing the parts of an adult mosquito [7]

1.2. Life cycle

Mosquitoes have a holometabolous development, also known as complete metamorphoses. Their life cycle is made up of four distinct stages; egg, larva, pupa, and adult. The first three

stages require water for proper development and the adults are terrestrial. The larval stage consists of four instars (Figure 1.2).

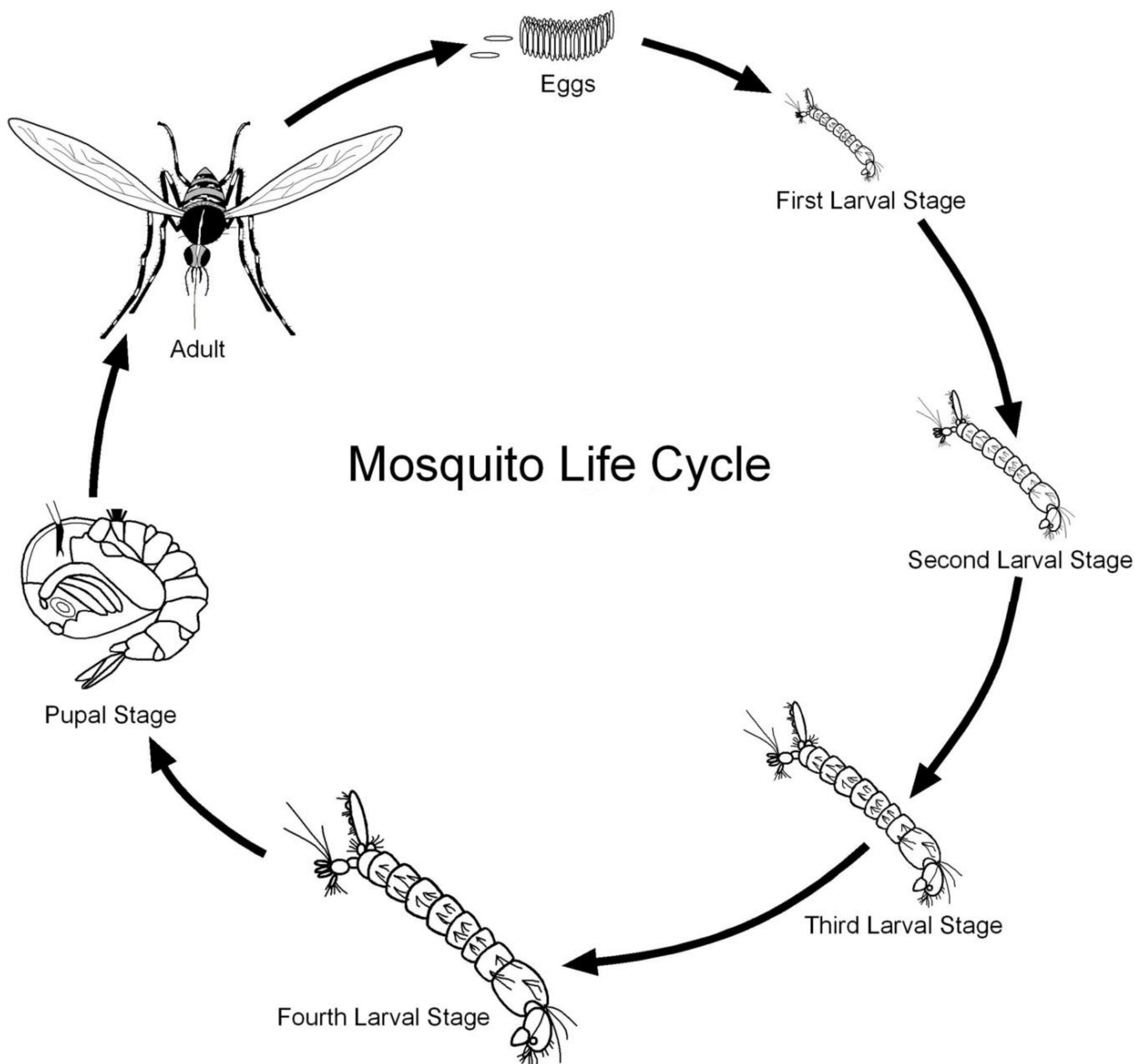

Figure 1.2 : The stages in the life cycle of a mosquito

1.2.1. **Mating and blood feeding**

Mating usually occurs shortly after the emergence of adults. Males and females are at different stages of sexual maturity when they emerge. Due to the need for the inversion of

the male hypopygium before they are ready to mate, they are not sexually mature at emergence. This usually takes about a day, therefore males usually emerge a day or two before the females to synchronize sexual maturity in the population [7]. Many species of mosquitoes typically mate in swarms [8–10] although some other species can mate without swarming [11]. In *Opifex fuscus* for example, the males seize pupae before the adult breaks from the pupal skin and mating occurs during emergence [12]. Swarms are aggregations of males sometimes in large numbers over projecting objects such as piles of wood or rubbish, wells and grass at low light intensities, usually at sunset [13]. These objects are termed markers and it is still unknown how and what factors influence the choice of the substance for swarming, although the markers have been found to characteristically either provide a contrast with the surroundings or break the uniformity of a landscape [8]. In the swarms, males face into the wind and fly in oscillatory patterns over the marker. Females fly into the swarms singly. Their lower wing-beat frequencies are detected by the males through their plumose antennae and they leave the swarm *in copula*. During mating, the male deposits spermatozoa in the bursa copulatrix of the female which is then stored in the spermathecae. The male secretes the hormone matronae which prevents the female from further mating. The female stores enough sperm in the spermathecae to fertilize subsequent egg batches without the need for further copulation.

Most mosquito species are anautogenous, meaning after insemination the female mosquito needs a blood-meal to obtain the necessary nutrients for oogenesis to be completed. For this reason, only female mosquitoes bite, and the males are therefore unable to transmit diseases. Host-seeking behaviour may differ between and within species based on the season and the availability of certain hosts. Recognition and location of a host largely involves responses to odour in addition to thermal and visual stimuli [14]. Mosquitoes can also respond to CO_2 and other cues such as lactic acid, octanol and acetone.

After a blood-meal, the abdomen appears bright red and dilated (Figure 1.3). Digestion of the blood is temperature-dependant and can last from 2 – 3 days in the tropics to 7 – 14 days in colder temperatures [2]. As the blood is digested, the eggs in the ovaries also enlarge and at the mid-point, the abdomen appears whitish posteriorly due to the eggs and dark reddish anteriorly due to the blood-meal (Figure 1.3). The mosquito is then said to be half-gravid. When digestion of the blood meal is completed, the female enters the gravid stage where the

abdomen is dilated and whitish due to the fully developed eggs. The search for a suitable oviposition site then begins. After laying eggs, the female takes another blood meal and subsequently lays another batch of eggs. This cycle of blood-feeding, egg maturation and oviposition is referred to as the gonotrophic cycle and it is repeated several times in the female's lifetime.

Figure 1.3 : A female Anopheles mosquito immediately after taking a blood meal (left) and a half gravid female mosquito (right)[7]

1.2.2. Eggs and oviposition

Freshly laid mosquito eggs are soft and whitish, but they sclerotize and darken within 1–2 h. Generally, the eggs are brown or blackish and measure about 1 mm or less in length. They are elongate and bounded by a thick shell. Females can lay up to about 500 eggs in one oviposition [7] and several different egg laying behaviours have been identified in the of various mosquito species. Eggs can be laid singly, such as in *Anopheles*, or in batches. *Culex* lay their eggs in rafts made up of several hundred eggs attached together in a boat-shaped structure. In addition, both *Anopheles* and *Culex* deposit their eggs directly into water. Members of the *Aedes*, *Haemogogus* and *Psorophora* genera on the other hand do not lay directly on water surfaces. The eggs are rather deposited just above water lines and other damp surfaces such as mud, because unlike the eggs of *Anopheles* and *Culex*, the eggs of the members of these genera can survive desiccation. The eggs enter into diapause (dormancy) and hatch under certain environmental stimuli such as temperature and/or availability of water. Mosquito eggs hatch within 2-3 days under tropical conditions, but may take up to about 14 days or even longer in lower temperatures.

1.2.3. **Larvae**

The larvae of mosquitoes are legless (apodous) and the body is divided distinctly into the head, the thorax and the abdomen. The head is well developed and possesses a pair of lateral antennae located near the anterior end, a pair of compound eyes and mouth-parts. The thorax is roundish and has long and prominent hairs. It is conspicuously bulbous and bigger than both the head and the abdomen, a feature that easily distinguishes mosquito larvae from other aquatic insects. There are ten abdominal segments which also possess branched and unbranched hairs, though only nine are visible. The last segment differs in shape and has hair structures which form the caudal setae and ventral brush.

The larvae of all mosquito species require water to survive, but they also need to breathe atmospheric oxygen to live. For this reason, most mosquito species need to come to the surface of the water to acquire oxygen. Air is taken in through spiracles located on the abdomen. In the Culicinae and Toxorhynchitinae subfamilies, these spiracles are at the end of a sclerotized tube called siphon. They hang at an angle from the water surface with the tip of their siphon inserted into the surface film to breathe. The siphons of *Coquillettidia* and *Mansonia* are modified for piercing and they obtain oxygen by inserting their siphon into the roots or stems of aquatic plants and thus do not need to surface. The larvae of most species are generally filter feeders. The beating of their head brushes generates water currents which carry food particles towards the mouth. They usually feed on organic matter, bacteria, yeasts, protozoans and other micro-organisms but a few species have carnivorous and cannibalistic larvae [7].

The larva goes through four instars, with the shedding of skin (molting) and increase in size at the end of each. The length of the larval stage normally takes 5-14 days, but this can be even shorter or longer depending on environmental conditions. The fourth instar molts into the comma-shaped pupa or tumbler.

1.2.4. **Pupae**

Like the larval stage, all mosquito pupae are aquatic. This is the transitional stage between the aquatic immature stages and the terrestrial adult stage of the life cycle. At this stage, there is no feeding and larval organs are digested away whereas special cells that had remained dormant during the larval stage start to multiply and form the organs of the adult insect. The body of the pupa has two distinct parts. The head and thorax are characteristically fused to

form the cephalothorax which has a pair of respiratory trumpets on the dorsal surface. The cephalothorax is attached to an elongate abdomen which terminates in a pair of oval flattened structures termed paddles. Mosquito pupae also breathe atmospheric oxygen. They spend most of their time at the water surface taking in oxygen through the respiratory trumpets. Similar to the larvae, the pupae of *Coquillettidia* and *Mansonia* insert their trumpets into roots and stems of aquatic plants to obtain oxygen. The pupal stage normally lasts between 2-3 days in tropical conditions but may go up to about 12 days or more in colder temperatures [2]. At the end of the pupal stage, the skin splits on the dorsal surface of the cephalothorax and the adult crawls out.

1.2.5. **The Adult**

After emergence, the adult seeks a protective environment in its surroundings to allow its wings to complete development and dry. The body of the adult has three distinctive parts; the head, the thorax and the abdomen (Figure 1.1). On the head is a pair of large kidney-shaped compound eyes, in between the eyes is a pair of filamentous, segmented antennae. The antennae of males are generally characterised by the presence of many long hairs which give the antennae a feathery appearance, as compared to the whorls of short hair found in the females (Figure 1.4). Hence, mosquitoes can be easily sexed using the antennae. Located just below the antennae is a pair of palps which are used to sense the characteristics of potential foods. A forward projecting long proboscis which contains the mouth parts arises between the palps. The thorax is connected to the head and it is the point of attachment of the wings, the halteres and three pairs of legs. The thorax is also covered dorsally and ventrally with numerous scales and the arrangements and colours of the scales give many species their distinctive colours. The abdomen hangs from the thorax and serves as the stomach and lungs. Small openings called spiracles line both sides of the abdomen for gaseous exchange. There are 10 abdominal segments, though only the first seven and eight are visible. Segments IX and X are reduced and modified into a pair of cerci in the female and claspers in the male, forming part of the external genitalia. The thorax and abdomen in Anopheles mosquitoes are characteristically held at 45˚ when at rest as compared to the parallel orientation in other species, making it easy for them to be distinguished (Figure 1.5). The adult is the reproductive stage of the mosquito.

Figure 1.4: Male (left) and female (right) Culex mosquitoes [15]

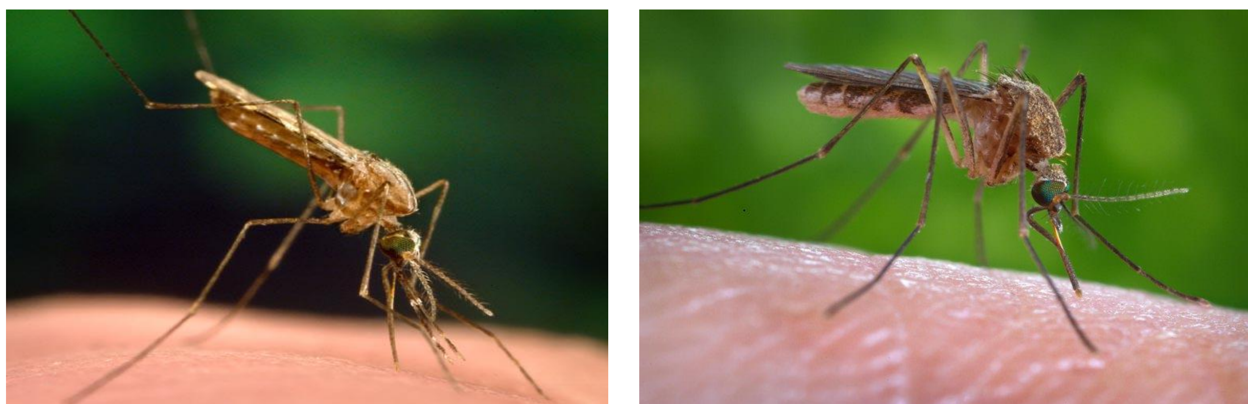

Figure 1.5:Anopheles mosquitoes (left) rest with the abdomen held at about 45˚ to the resting surface compared to the parallel orientation of Culex (right) and other species [16, 17].

1.3. Why are mosquitoes important?

Mosquitoes pose one of the biggest public health threats because of their importance as vectors of a wide range of viral and parasitic diseases which affect both humans and animals. They are considered the most dangerous animals confronting mankind in regards of the mortality and morbidity caused by the diseases they transmit. More than half of the world's population lives under the risk of becoming infected by mosquito-borne diseases such as malaria, dengue, yellow fever, Japanese encephalitis, lymphatic filariasis, chikungunya and West Nile fever [7]. The World Health Organization (WHO) estimates about 214 million cases of malaria and 438,000 deaths out of which 306,000 were children in 2015 [18]. There are also about 2.5 billion people living in more than 100 dengue endemic countries and areas with up to 50 million infections annually leading to about 500,000 dengue haemorrhagic fever cases and 22,000 deaths most of which are children [19]. Although approximately three quarters of the world's mosquito species occur in the humid tropics and subtropics, they also cause a substantial nuisance and occasionally transmit pathogens to humans in temperate areas as well [20]. The most important pests and vectors are mostly found in the genera *Anopheles, Aedes, Culex, Mansonia, Psorophora, Haemagogus* and *Sabethes* [2].

The knowledge of the involvement of mosquitoes in the transmission of deadly diseases is relatively new. These blood-sucking insects have been around for more than 100 million years [21], with the oldest known sample with a similar anatomy to modern species found in a 79-million-year-old Canadian Cretaceous amber [22]. But it was not until the 19th and 20th centuries that they were implicated in the transmission of deadly diseases through the extensive works of several scientists. Prior to the discovery, a host of theories and opinions about how these diseases were transmitted were held which included the ideas that malaria was transmitted by miasma (bad air or night air) or the drinking of contaminated water and yellow fever by damaged coffee beans lying on the wharves or the turning over of soil in construction work. The idea of a connection between mosquitoes and diseases had existed since early Roman times, but Joseph Nott is credited to be the first to clearly make that connection [23] when he suggested in 1848 that mosquitoes were involved in transmitting the of yellow fever. Nevertheless, the first demonstration of the man–mosquito component in the transmission of a human pathogen was made by Patrick Manson, who through his series of

experiments in 1877, established that *Wuchereria bancrofti* is transmitted by the *Culex* mosquito [24].

In August 1881, Carlos Finlay presented his hypothesis that mosquitoes were the agents of the transmission of yellow fever [25] to the Royal Academy of Medical, Physical and Natural Sciences in Havana after extensively studying the anatomy and behaviour of the *Culex* mosquito [26]. His paper was dismissed. But based on his theory, The American Yellow Fever Commission which was led by Walter Reed proved that yellow fever was indeed transmitted by mosquitoes [27]. During the closing years of the 19th century, the separate works of Ronald Ross and Battista Grassi also led to the discovery that mosquitoes, and for that matter, *Culex* and *Anopheles,* were the vectors of avian and human malaria respectively [23–28].

Mosquitoes transmit diseases as a result of their blood sucking behaviour. They acquire the pathogens as they take up a blood meal from an infected vertebrate. During blood feeding, it is necessary that the blood remains in a liquid state. To achieve this, saliva containing anticoagulants is injected into the puncture as the mosquito feeds. A pathogen-carrying mosquito then transfers the infective stages to the next host through the saliva.

1.4. Mosquito control

Systematic efforts for mosquito control started in the early years of the 20th Century, following the discovery of the connection of mosquitoes to several major diseases. Towards the end of the Spanish-American War at the close of the 19th century, William Gorgas, a member Medical Department of the US Army was sent to Havana as Chief Sanitary Officer. Still believing that yellow fever was caused by filth, his initial approach was to improve on sanitation in Havana. He quickly redirected his approach to target the mosquito when the findings of the work of Reed were publicized in 1901. The strict mosquito control program which included punishment for having mosquito larvae on a domestic property, the quarantine and treatment of infected patients [27], and draining and filling of fields where water collected led to drastic reductions in mortality and consequently elimination of yellow fever and malaria [29]. After his success in Havana, he was assigned to take charge of sanitary in Panama during the construction of the Panama Canal which had seen thousands of workers’ lives lost to tropical diseases including malaria and yellow fever. Using a similar approach as in Havana, malaria and yellow fever were successfully brought under control [29]. The successful application of

reducing vector population and limiting mosquito-human contact set the stage for mosquito control.

1.5. Mosquito control today

Vector control in the present day involves a variety of methods. While a significant amount of attention has been given to other interventions such as proper housing [30–35],the greater proportion rely on larval source management and the use of chemical adulticides to control larvae and adults respectively.

1.5.1. Larval source management

Before the Second World War, environmental control was the predominant method for the control of the proliferation of mosquitoes in the attempt to reduce vector borne diseases. Larval source management (LSM) is the targeted management of aquatic habitats that are potential mosquito breeding sites, with the objective to reduce the number of the immature stages of mosquitoes [36]. Long-lasting insecticide-treated mosquito nets (LLINs) and indoor residual spraying (IRS) target the adult vector population. LSM is a complimentary component with the purpose of providing further suppression of transmission by targeting the aquatic stages of disease vectors, leading to reductions in the populations of both outdoor and indoor biting vectors. LSM comes in four different types, namely habitat modification, habitat manipulation, larviciding and biological control.

1.5.1.1. Habitat modification

In habitat modification, the environment is permanently altered. WHO defines it as “a form of environmental management consisting of any physical transformation that is permanent or long-lasting of land, water and vegetation, aimed at preventing, eliminating or reducing the habitats of vectors without causing unduly adverse effects on the quality of the human environment” [37]. Alteration of the environment as a means of mosquito control has been a key component efforts to control vector-borne diseases over the years. It was the main method applied before the introduction of pesticides. Proper irrigation systems, drainage, land filling and grading, land levelling and transformation and impoundment margins are all examples of habitat modification exercises [36].

1.5.1.2. Habitat manipulation

Habitat manipulation is "any form of environmental management consisting in any planned recurrent activity aimed at producing temporary conditions unfavourable to breeding of vectors in their habitats" [37]. It differs from habitat modification in the sense that it needs to be repeated to maintain efficacy and it is also usually aimed at a particular vector species. It involves activities such as stream flushing, shading, removal of aquatic vegetation, straightening and steepening of shorelines, regulating water level in reservoirs, changing water salinity, draining of swamp areas and exposure to sunlight [36].

1.5.1.3. Larviciding

Larviciding refers to the regular application of biological or chemical insecticides to larval habitats to control mosquitoes [38]. There are currently about 48 countries around the globe that use larval control as a malaria intervention [18]. Several different types of larvicides are available for use. There are the oils and surface agents which are surface films such as mineral oils and alcohol-based surface products which kill larvae by suffocation or specific toxicity. Their activity is generally short-lived, lasting for only a few hours or days. There are currently no formal WHO recommendations regarding the use of oils as surface agents for the control of larvae [36]. Synthetic organic compounds that interfere with the nervous system of larvae are also used as larvicides. The most common in use are fenthion, malathion, pirimiphos methyl and temephos. WHO does not recommend the use of pyrethroids such deltamethrin and permethrin for the control of mosquito larvae due to the possibility of selection for pyrethroid resistance in the adult population and their harmful effects on non-target organisms such fish, crustaceans and other aquatic animals. Bacterial larvicides are toxins obtained from bacteria which are highly efficacious and specific against mosquito larvae. The most widely used bacterial larvicides come from *Bacillus thuringiensis* var. *israelensis* (Bti) and *B. sphaericus* (Bs), otherwise known as *Lysinibacillus sphaericus*, which are naturally occurring, spore-forming bacteria found in aquatic environments and soil worldwide. During sporulation, they produce highly specific endotoxins which when ingested, are only lethal to the larvae of mosquitoes, black flies and closely related flies. *Bs* produces more target specific toxins with longer residual activity than *Bti* [36]. Another type of larvicides, called insect growth regulators, interrupt the normal growth and development of the vector. They fall under two main groups. Juvenile hormone analogues such as methoprene and pyriproxyfen prevent the

progress of larvae and pupae into adults, whereas chitin synthesis inhibitors, such as difulbenzuron and triflumeron, kill larvae upon moulting. Larvicides come in different formulations such as water dispersible granules, emulsifiable concentrate, wettable powder, suspension concentrate, pellets, briquettes and granules.

1.5.1.4. Biological

Biological control is one of the earliest methods used in mosquito control. It involves the introduction of natural enemies such as predators, parasites or other disease-causing organisms into larval habitats [36]. Its application dates back to the 19th century when attempts were made to introduce predators such as dragon flies [39] to kill mosquitoes. The discovery of DDT saw a shift to heavy use of chemicals but after increase in environmental issues and public awareness led to more controlled use of chemical control, attention was once again turned to biological control in the 1960s and 1970s [7]. A detailed knowledge and understanding of the biology of the control agent and how it interacts with the ecosystem is a very important requirement for the successful use biological control. While there are a large variety of available biological control agents for mosquitoes, including those for the adult stage, most biological control efforts target the larval stage. Fish remain the oldest and better-known organisms which have been successfully used in mosquito control [40]. *Gambusia affinis*, also known as the mosquito fish, is the best known fish used for this purpose. It has been used for more than 100 years and remains one of the best options available [41] for biological control. *G. holbrooki* (eastern mosquito fish) and *Poecilia reticulata* are also widely used.

1.5.2. Chemical control of adult mosquitoes

Chemical insecticides have played a significant role in the control of mosquitoes for many years and still form the backbone of most vector control programmes [42]. The extinction of many vector-borne diseases can be attributed to the use of chemical insecticides [40]. Presently, available insecticides for public health application belong to four major classes; organophosphates, organochlorines, carbamates and pyrethroids. The use of insecticides for the control of mosquitoes is mainly in the form of indoor residual spraying, impregnated mosquito nets or household fabrics such as curtains [43] or as larvicides [36].

1.5.2.1. Indoor residual spraying (IRS)

Together with insecticide treated nets, IRS is one of the primary vector control interventions for preventing and reducing the transmission of malaria [44, 45]. The WHO defines IRS as "the application of long-acting chemical insecticides on the walls and roofs of all houses and domestic animal shelters in a given area, in order to kill the adult vector mosquitoes that land and rest on these surfaces" [45]. The primary objective of IRS is to reduce the density and life span of vector mosquitoes in order to interrupt transmission. IRS has a long history in malaria control. It was very instrumental in the efforts to control malaria between the 40s and 70s together with DDT. Malaria control programmes such as the Malaria Eradication Programme which contributed to significant reductions in the global burden and eradication of malaria from certain regions such as Europe were based on IRS [45]. It continues to be very important in vector control today, with some 116 million people worldwide protected by IRS in 2014. Despite its popularity, the use of IRS has seen a decline in recent years. The global estimate of the proportion of the population at risk protected by IRS dropped from 5.7% in 2010 to 3.4% in 2014. Lack of government commitment and financial support to sustain IRS efforts over the long term, fears of insecticide resistance and the debate on the effects of DDT on the environment and on human health have been cited among other reasons for the decline in ITS use [18].

1.5.2.2. Insecticide-treated nets (ITNs)

The use of mosquito nets as a protection against biting insects has been in existence since historical times and their purpose was to prevent disturbances by blood-sucking insects in order to achieve uninterrupted sleep rather than preventing diseases [46]. Currently bed nets have become a very integral component of vector control, especially in the prevention of malaria. Insecticide treated nets serve the double function of providing a physical barrier and reducing vector density by killing the adult when it makes contact with the net. The WHO estimates that 55% sub-Saharan Africa sleep under an ITN as at 2015, a marked increase from the less than 2% in 2000 [18]. Presently, only pyrethroids are used in ITNs due to their high insecticidal activity and low mammalian toxicity. Earlier instalments of ITNs were treated by dipping them in the desired insecticide and re-treatments were needed at least once a year for optimal performance. The turn of the 21st century saw a new generation of ITNs, the LLINs, which have the insecticide incorporated into the fibres of the net at factory level and do not

require re-treatment. The insecticides in these nets can withstand multiple washes and are gradually released to the surface of the netting fibres over time [47]. In an effort to maintain efficacy in pyrethroid-resistant populations and prevent the development of resistance in susceptible strains, new generations combination nets that utilise alternative or multiple classes of insecticides or synergists [48–51] are being implemented or researched with some success [46]. LLINs are expected to retain efficacy for as long as the average lifespan of the net, which is about 4 to 5 years. Due to the success of ITNs in effectively reducing malaria morbidity and mortality [52], efforts are being made to improve the coverage of bed net access in malaria endemic countries, with the aim of achieving universal coverage. In 2014, over 189 million nets were delivered to countries in sub-Saharan Africa, the highest number delivered in a single year [18]. According to WHO estimates, about 200 million nets would be required each year to achieve universal access to ITNs under ideal conditions. However, due to net loss and current distribution patterns, an estimated 300 million new nets would be required per year to ensure coverage of all persons at risk of malaria.

1.5.3. **The problem of resistance**

After the discovery of the insecticidal properties of DDT by the Swiss scientist Paul Müller in 1939 [53], it was extensively used during and after World War II for nearly three decades. During this period, the control of mosquitoes was highly successful and it was believed that malaria would be eradicated. But this was not to be, as among other reasons, the development of high levels of resistance to DDT and ecological issues led to the discontinuation and ban of its use in the 70s. Resistance to insecticides has remained one of the biggest problems in the fight against vector-borne diseases up to today. Defined as “the situation in which the vectors are no longer killed by the standard dose of insecticide (they are no longer susceptible to the insecticide) or manage to avoid coming into contact with the insecticide” [54], the development of resistance is a result of the exposure of mosquito populations to selection pressure from insecticides. The phenomenon was first described in 1914 by the entomologist A. L. Melander when he observed that sulphur-lime, an otherwise potent insecticide in use at the time, was no longer effective against the San Jose scale (*Quadraspidiotus perniciosus*) [55]. Insecticide resistance in mosquitoes is currently wide spread in the major disease vectors. According to WHO, there is resistance to at least one insecticide in one malaria vector in 60 countries and to insecticides from two or more

insecticide classes in 48 countries out of the 78 countries with malaria transmission from which data on resistance monitoring available since 2010 [18]. Resistance has also been widely reported in *Aedes* [56–60] and *Culex* [61–64] which are also responsible for the transmission of a host of major diseases.

Insecticides enter the body of the insect by penetrating the cuticle to its site of action, which may be a nerve tissue, an essential enzyme or a receptor protein. The insecticide becomes lethal when enough molecules have successfully bound to the target site to reach the threshold concentration [7]. There are several mechanisms developed by the insect to disrupt this pathway. The insects may undergo behavioural changes such as adopting outdoor biting instead of indoor and avoid contact with the insecticide. Cuticular permeability can also be reduced by alterations that lead to thickening of the cuticle, therefore reducing the rate of penetration of the insecticide. Resistance also results from biochemical mechanisms which involve new or elevated levels of metabolic enzymes which lead to more efficient detoxification of the insecticide (metabolic resistance) or alterations in the insecticide binding site which results in the inability of the insecticide to bind (target site resistance). Three enzyme families, the esterases, the P450 monooxygenases and the glutathione S-transferases (GSTs) have been identified to be principally responsible for the development of metabolic resistance. Target site resistance occurs as a result of alterations in acetylcholinesterase (AChE) which renders it insensitive. It could also be due to the substitution of alanine residue by a serine or more rarely to a glycine in the GABA receptor resulting in insensitivity to the insecticide or mutations in the voltage-gated sodium channel which is often referred to as “knock-down resistance” (kdr) due to the rapid „knock-down‟ effect of pyrethroids [65].

1.5.3.1. Insecticide resistance monitoring

WHO is at the centre stage in terms of monitoring insecticide resistance. There are several divisions of WHO such as the WHO Global Malaria Programme and the WHO Pesticide Evaluation Scheme (WHOPES) which provide standard methodologies and guidelines for carrying out vector control activities [36, 54]. WHOPES is heavily involved in the maintenance of the effectiveness of insecticides and their formulations against the major disease vectors. Set up in 1960 with the approval of the World Health Assembly, the primary role of WHOPES is “to promote and coordinate the testing and evaluation of pesticides of interest to public health, including chemosterilants, pathogens, and hormone-like compounds, as well as

repellents and attractants" [42]. It provides the current standard guidelines and instructions for conducting field and laboratory tests for the monitoring and evaluation of products to ensure the efficacy, operational acceptability and safety of public health insecticides.

1.5.4. The contribution of mathematical modelling to the control of vector borne diseases

The use of mathematical models to understand the disease transmission dynamics within and between hosts and parasites has become an integral part of the study and control of in infectious diseases. Ronald Ross made the earliest attempt at using mathematical models to explain malaria transmission [76]. He used equations to support his claim that malaria can be eradicated by reducing the number of mosquitoes. He developed a simple model which is now known as the classical "Ross model" [77] explaining the relationship between the number of mosquitoes and incidence of malaria in humans. Macdonald further extended Ross' model, introducing other factors that influence the transmission [78]. The Ross-McDonald model has seen a lot of revisions and several new indices have been introduced into the modelling of malaria transmission [79–81]. Presently, mathematical models are very instrumental in the control of vector-borne diseases and have been used to predict and/or explain all aspects of disease scenarios in all the major disease vectors [82–88].

1.5.5. Identified research questions

1.5.5.1. The problem?

As previously described in the sections above, insecticide resistance is widespread and continually on the increase [66]. The magnitude of the problem is compounded by the limited data at the disposal of scientists working to contain the threat due to the fact that at present, most insecticide resistance monitoring activities are performed ad hoc. The lack of dependable data arises from two causes. Many countries where transmission of vector-borne diseases occurs have yet to carry out adequate regular susceptibility testing [54]. On the other hand, there are significant inconsistencies in testing and reporting [67]. In effect, data is either missing or unreliable. This has made it difficult to estimate the true extent to which resistance to insecticides is spread and also to evaluate the impact of resistance on efforts to reduce or eliminate the burden of vector-borne diseases. The limited availability of reliable routine

monitoring data also makes the process of informed decision-making on managing insecticide resistance complicated and difficult.

1.5.5.2. The approach

The problem described above is complex and requires a collective and extensive effort to rectify. This thesis focused on the second aspect of the problem to identify factors that significantly contribute to the inconsistencies observed in data generated from insecticide resistance monitoring activities. To find answers to the question, the overall aim was divided into three objectives which looked at various players involved in the insecticide resistance monitoring process; the methodology, the mosquito itself and the facilities performing the tests.

In our first objective, we identified the primary bioassays used in the generation of data for insecticide resistance monitoring activities. In the laboratory and field evaluations of insecticide resistance in mosquito populations, the Centers for Disease Control and Prevention (CDC) bottle and WHO susceptibility bioassays are the principal diagnostic tests used in the detection and reporting of insecticide resistance. While data generated from these two assays are reported on the same scale and used interchangeably, no efforts have been made to test the level and/or consistency of agreement between these two tests. The WHO cone assay is used for a similar purpose, though its function is more specialized for the testing of insecticide treated bed nets and treated surfaces. It serves the double purpose of being used to test insecticidal activity as well as excito repellent properties of compounds. Especially due to its use for testing treated surfaces, the test is performed at various angles. That coupled with its use to test excito repellent properties makes it imperative to identify whether the angle at which the test is performed has any influence on the outcome. This information is very essential because to generate reliable data, it is vital to know whether the outcome was a result of the test material or rather an error from the test setup.

The second objective focused on the physiological aspects of the mosquito that could contribute to the outcome of the bioassays. Though the observed resistance phenotype of a mosquito population could result from a collection of several factors, mosquitoes are generally evaluated based on their genetic makeup and the physiological contributions are largely ignored. The interactions of the mosquito with the environment has been found to contribute significantly to the life history traits [68–72] and changes in conditions during the

larval stage has been found to produce marked effects on the adult [71, 73]. But little has been done [74, 75] on how the environment contributes to insecticide resistance in the adult mosquito. We therefore investigated the effects of temperature, nutrition and crowding during the larval stage on the susceptibility of the adult to insecticides. As an additional step, the effects of changes in conditions at the larval stage on adult survival and for that matter malaria transmission were assessed using mathematical models (introduced in section 1.5.4).

And thirdly, we looked factors that could contribute to inter-laboratory differences in data. Apart from the factors discussed above, a host of other factors such as transportation and storage of working materials, infrastructure, personnel and testing conditions such as temperature could contribute to differences in testing and reporting. We performed an inter-laboratory study to determine the essential factors that contribute to inconsistencies of the results between laboratories using the same strain of mosquitoes obtained from the same source and insecticide-treated papers acquired from the same source.

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2. Comparability between insecticide resistance bioassays for mosquito vectors: time to review current methodology?

Henry F Owusu[1, 2*], Danica Jančáryová[1, 2], David Malone [3]and Pie Müller [1, 2]

[1]Epidemiology and Public Health Department, Swiss Tropical and Public Health Institute, Socinstrasse 57, PO Box,CH-4002 Basel, Switzerland.

[2]University of Basel, Petersplatz 1, CH-2003 Basel, Switzerland.

[3]Innovative Vector Control Consortium, Liverpool School of Tropical Medicine, Pembroke Place, Liverpool L3 5QA, UK.

*Corresponding author

Email addresses:

HFO: henry.owusu@unibas.ch

DJ: danica.jancaryova@unibas.ch

DM: david.malone@ivcc.com

PM: pie.mueller@unibas.ch

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2.1. Summary

Background Insecticides play an integral role in the control of mosquito-borne diseases. With resistance to insecticides on the rise, surveillance of the target population for optimal choice of insecticides is a necessity. The Centers for Disease Control and Prevention (CDC) bottle assay and the World Health Organization (WHO) susceptibility test are the most frequently used methods in insecticide resistance monitoring. However, the two bioassays differ in terms of insecticide delivery and how insecticide susceptibility is measured. To evaluate how equivalent data from the two assays are, we compared the two methods side-by-side.

Methods We did a literature search from 1998 to December 2014 to identify publications that performed both assays on the same mosquito population and compared the results. We then tested the WHO and CDC bioassays on laboratory strains of *Aedes aegypti*, *Anopheles stephensi*, *An. gambiae* and *An. arabiensis* with different insecticide resistance levels against permethrin, λ-cyhalothrin, DDT, bendiocarb and malathion. In addition, we also measured the relationship between time-to-knockdown and 24 hours mortality.

Results Both published data and results from the present laboratory experiments showed heterogeneity in the comparability of the two bioassays. Following their standard procedures, the two assays showed poor agreement in detecting resistance at the WHO cut-off mark of 90% (Cohen's κ = 0.06). There was better agreement when 24 hour mortality was recorded in the CDC bottle assay and compared with that of the WHO susceptibility test (Cohen's κ = 0.5148). Time-to-knockdown was shown to be an unreliable predictor of 24 hours mortality.

Conclusion Even though the two assays can detect insecticide resistance, they may not be used interchangeably. While the diagnostic dose in the WHO susceptibility test does not allow for detecting shifts at low or extreme resistance levels, time-to-knockdown measured in the CDC bottle assay is a poor predictor of 24 hours mortality. Therefore, dose-response assays could provide the most flexibility. New standardized bioassays are needed that produce consistent dose-response measurements with a minimal number of mosquitoes.

Key words: Mosquitoes, insecticide, resistance, susceptibility, CDC bottle assay, WHO susceptibility assay, bioassay.

2.2. Background

Since the resistance of insects to insecticides was first described by Melander in 1914 [1], it has emerged as a major topic for research and discussion in public health because its presence in disease vectors is one of the major obstacles to the reduction of the burden of vector-borne diseases in endemic countries. Over the last decades, resistance of mosquito vectors to insecticides has increased significantly [2, 3] and continues to pose a great threat to the success of chemical control interventions and the control of mosquito-borne disease as a whole. It is known to be present in nearly two thirds of the countries with on-going malaria transmission, in all major vector species and to all available classes of insecticides [4]. Resistance has been detected in at least one insecticide in use for the control of malaria in not less than 64 countries where malaria is endemic [5].

Efforts to curb the threat of insecticide resistance are being scaled up, with one of the most recent steps being the launch of the Global Plan for Insecticide Resistance Management in Malaria Vectors (GPIRM) by the World Health Organization (WHO) [4]. The GPIRM strongly advocates incorporation of insecticide resistance management measures into every vector control programme, even in the absence of resistance. Of utmost importance to these measures is the reliability of the data generated from monitoring and evaluation activities and this is further emphasised by the five pillars outlined in the collective strategy against insecticide resistance in the GPIRM. The acquisition of data largely depends on susceptibility bioassays. The data from these assays are relied on to provide information on the impact of resistance on current interventions and vice versa, leading to an informed choice on strategies to adopt in prevention and management. Although the link between bioassay data and epidemiological failure of a control programme is not well established [6], insecticide susceptibility bioassay data are an indicator if targeted mosquitoes respond well to the insecticides in use [7]. Unfortunately, it is common to find inconsistent testing and reporting of resistance in published data [6], which could be due to several factors including the choice of assay.

The standard bioassay for the detection of resistance in mosquito vector populations is the WHO susceptibility test [8]. This is a direct response-to-exposure analysis that uses discriminating concentrations to distinguish between resistant and susceptible mosquito

populations [8]. In 1998, an alternative to the WHO assay, a time-mortality analysis known as the Centers for Disease Control and Prevention (CDC) bottle assay, was developed by Brogdon and McAllister [9]. Currently, both assays are being widely used both in the field and in the laboratory for routine monitoring of resistance. Though both assays measure insecticide susceptibility, they differ in several aspects and as a result, each has some advantages over the other [10]. The CDC bottle assay might be chosen over the WHO susceptibility assay because it can be carried out without the need of ordering specialised equipment, which may be difficult to procure. The assay also provides the convenience of evaluating different concentrations of an insecticide as well as the possibility of using synergists, which allows for fast and inexpensive assessment of metabolic resistance mechanisms.

While the CDC bottle assay may be customised to individual needs, the WHO susceptibility assay is less prone to problems with quality assurance and control. Test kits and insecticide-impregnated papers may all be obtained from a centralised source, thus reducing the introduction of variability. This also makes it easier to trace the source of problems, should they arise. It is also easier to separate dead and live mosquitoes, allowing further analysis on these two groups.

The growing rate of insecticide resistance emphasises the need for data of good quality, and with the widespread use of the two bioassays for resistance detection, we set out to assess the extent to which they are interchangeable and how much time-to-knockdown is a proxy for 24 hours mortality. First, we reviewed published data to evaluate the existing evidence of comparability between the two assays. We subsequently compared the two assays side-by-side, following their published guidelines on characterised laboratory mosquito strains. Finally, we carried out laboratory experiments to investigate the relationship between time-to-knockdown and eventual 24 hours mortality, which are the basic end points of the CDC bottle assay and the WHO susceptibility assay, respectively.

2.3. Methods

2.3.1. Literature review

We conducted a literature search in compliance with the PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) guidelines [11] and systematically searched the National Library of Medicine's PubMed, ISI Web of Science, EMBASE and the Cochrane library to identify publications that performed susceptibility assays on adult mosquitoes. We searched studies that were published between 1998, when both bioassays have been in use, and December 2014. We used the search terms "Culicidae" in combination with "insecticide resistance" or "insecticide susceptibility". Our interest was in publications that conducted both the CDC bottle and WHO susceptibility assays on the same mosquito populations following their standard protocols. We therefore excluded publications which only collected susceptibility data on larvae, those that performed neither the CDC nor the WHO assay and those that performed only one of the two assays.

2.3.2. Mosquito strains

A total of seven laboratory maintained strains were used in the experiments (Table 2.1). Mosquitoes were reared at temperature and relative humidity ranges of 26 - 28°C and 60 - 74%, respectively, in a 12:12 hours day:night regime. All the strains listed above were used in the evaluation of the relationship between time-to-knockdown and 24 hours mortality except JHB and NDJA which were excluded from the comparison between the CDC and WHO assays due to non-existence of standard concentrations for testing *Culex spp* and insufficient mosquito numbers in NDJA. The KISUMU1 and JHB strains were established in our insectary in 2011. The STI and ROCK strains were also established in 1971 and 1978, respectively. All other mosquitoes were acquired as eggs and the emerging adults were then used for the assays.

Table 2.1: Mosquito strains used in the insecticide susceptibility assays

Species	Strain	Source	Resistance status	Resistant to	Known resistance mechanism
An. gambiae s.s.	KISUMU1	MR4 (MRA-762)	S	NA	NA
An. gambiae s.s.	VK7	LSTM	R	Pyrethroid and DDT	kdr
An. arabiensis	NDJA	LSTM	R	Pyrethroid and DDT	Metabolic
Cx. quinquefasciatus	JHB	MR4	R	Pyrethroid and DDT	Not known
An. stephensi	STI	LSHTM	R	Pyrethroid and DDT	Suspected metabolic
An. gambiae	ZAN/U	MR4	R	DDT	Metabolic
Ae. aegypti	ROCK	JHU	R	Reduced susceptibility to DDT	Not known

"R" indicates resistant and "S" indicates susceptible
MR4 - Malaria Research and Reference Reagent Resource Center
LSTM - Liverpool School of Tropical Medicine
LSHTM - London School of Hygiene and Tropical Medicine
JHU - Johns Hopkins University

2.3.3. Insecticides

Five different insecticides, representing the four classes available for public health applications against adult mosquitoes were used: the carbamate bendiocarb, the organochlorine DDT, the organophosphate malathion and the pyrethroids permethrin (25:75 cis:trans) and λ-cyhalothrin. All insecticide solutions used in the CDC bottle assay were self-prepared. Bendiocarb, DDT and malathion were purchased as analytical grades from Sigma-Aldrich® (Switzerland), while permethrin and λ-cyhalothrin were kindly provided by Syngenta Crop Protection AG (Switzerland). All insecticide treated filter papers for the WHO susceptibility tests were sourced from the WHO Pesticide Evaluation Scheme (WHOPES) through Universiti Sains Malaysia based in Penang, Malaysia.

2.3.4. CDC bottle assay

The preparations, diagnostic doses, exposure time (Table 2.2) and assay procedure were all as recommended by the CDC guidelines [12]. Prior to performing the assays, 250ml SIMAX bottles (Kavalierglass Co. Ltd., Czech Republic) were coated the previous evening with the desired insecticide dissolved in acetone. Three to five-day-old non blood-fed female mosquitoes were introduced into the treated bottles and observed for knockdown up to a maximum of 120 minutes. To allow for the evaluation of the relationship between time-to-knockdown and 24 hours mortality, mosquitoes were individually exposed till they were

knocked down. Once knockdown occurred, the mosquito was immediately transferred into a small 30ml plastic beaker. The beaker was then covered with a small piece of cotton mosquito net and labelled with a unique identification number. The mosquito's id and time-to-knockdown were recorded. Each mosquito was provided with 10% sugar solution and held for 24 hours after which mortality was recorded. Temperature and relative humidity recorded during the laboratory testing ranged from 25.9 – 29.6°C and 59 - 77%, respectively.

2.3.5. WHO susceptibility test

The WHO susceptibility tests were performed according to the latest published guidelines [8]. Three to five-day-old non blood-fed female mosquitoes were exposed in batches of 24 to 27 individuals to insecticide-treated filter papers at the WHO discriminating concentrations and exposure times [13] (Table 2.2). After exposure, mosquitoes were transferred back into the holding tube, provided with 10% sugar solution and kept for 24 hours after which mortality was recorded. As per WHO definition, a mosquito was scored in both assays as alive if it was able to fly, irrespective of the number of legs still intact and dead, or knocked down, if immobile or incapable of flying or standing in a coordinated manner [8].

Table 2.2: Mosquito strains and insecticides used in the WHO susceptibility test and the CDC bottle assays

Strain	Insecticide	Diagnostic concentration		Diagnostic exposure time (min)		Estimated insecticide concentration on surface (μg/cm²)	
		WHO (%)	CDC (μg/bottle)	WHO	CDC	WHO	CDC
KISUMU1	permethrin	0.75	21.5	60	30	27.53	0.089
	λ-cyhalothrin	0.05	12.5	60	30	1.84	0.052
	bendiocarb	0.1	12.5	60	30	3.67	0.052
	malathion	5	50	60	30	183.50	0.21
	DDT	4	100	60	45	146.8	0.41
VK7	permethrin	0.75	21.5	60	30	27.53	0.089
	λ-cyhalothrin	0.05	12.5	60	30	1.84	0.052
STI	permethrin	0.75	21.5	60	30	27.53	0.089
	λ-cyhalothrin	0.05	12.5	60	30	1.84	0.052
	bendiocarb	0.1	12.5	60	30	3.67	0.052
	malathion	5	50	60	30	183.50	0.21
	DDT	4	100	60	45	146.80	0.41
ZAN/U	permethrin	0.75	21.5	60	30	27.53	0.089
	DDT	4	100	60	45	146.80	0.41
ROCK	permethrin	0.25	15	60	30	9.18	0.062
	λ-cyhalothrin	0.03	10	60	30	1.10	0.041
	bendiocarb	-	12.5	-	30	-	0.052
	malathion	0.8	50	60	30	29.36	0.21
	DDT	4	75	30	45	146.80	0.31
JHB	permethrin	-	21.5	-	30	-	0.089
	λ-cyhalothrin	-	12.5	-	30	-	0.052
	bendiocarb	-	12.5	-	30	-	0.052
	malathion	-	50	-	30	-	0.21
	DDT	-	100	-	45	-	0.41

Insecticide in μg per cm² was calculated in the CDC assay by dividing the amount in μg per bottle by the estimated surface area of the inner wall of the glass bottle. In the WHO assay, it was calculated based on information provided in the guidelines **[25]**.

2.3.6. Data analysis

All data analysis was done in the freely available statistical software package R, version 3.0.2[14]. The level of significance was set at α = 0.05.

For the interpretation of the bioassay results and, in accordance with the current WHO guidelines [8], we applied a 90% mortality or knockdown threshold as the cut-off value to score resistance. The guidelines for the CDC bottle assay also refer to the WHO definition of resistance [12]. In addition to the 90% cut-off value, we also compared the two bioassays at 98% level because, according to WHO, this would be the rate below which resistance is suspected [8].

In the laboratory study, we compared the two tests for agreement in two different ways. First we compared the outcome measures as defined by their protocols (i.e. 24 hour mortality in the WHO and knockdown at the diagnostic time in the CDC bottle assay) and called this the "standard comparison". Secondly, the two assays were compared using 24 hours mortalities measured in both assays. We called this comparison the "24 hours comparison". Comparisons were done between the two assays for each mosquito strain and insecticide combination. A single comparison for a single strain and insecticide is here referred to as a "pair".

Cohen's Kappa (κ) [15] was calculated to quantify the magnitude of agreement between the WHO susceptibility test and the CDC bottle assay, both in the data extracted from the literature search and bioassays conducted in the present study. κ accounts for agreement taking place only by chance beyond simple per cent agreement calculations. Its values are interpreted as poor ($\kappa \leq 0$), slight ($0 < \kappa \leq 0.2$), fair ($0.2 < \kappa \leq 0.4$), moderate ($0.4 < \kappa \leq 0.6$), substantial ($0.6 < \kappa \leq 0.8$) and almost perfect agreement ($0.8 < \kappa \leq 1.0$) [16].

In addition to the two end points of percentage mortality and knockdown, we also investigated whether the two assays produced similar patterns in the cumulative number of mosquitoes knocked down as a function of time. For example, if strain A was knocked down quicker than strain B against permethrin in the WHO susceptibility assay, would the same be observed in the CDC bottle assay, and vice versa? If this were the case, it would suggest that the two bioassays yield similar outcomes qualitatively despite their differences, including insecticide concentrations. For their comparison, the knockdown curves for all mosquito strains were analysed using the Kaplan-Meier survival function [17, 18]. First it was determined, separately for the two bioassays, whether there was any difference between the

strains tested for a particular insecticide. If so, the order in which the insecticide knocked down the mosquitoes was compared between the two assays. Lines which appeared to overlie or were very close to one another were further analysed for differences.

Finally, the relationship between 24 hours mortality and time-to-knockdown was investigated by generalised linear mixed-effects models (GLMM) with a logit link function. We used data from tests against permethrin, λ-cyhalothrin and DDT because of very high levels of susceptibility in all the strains to malathion and bendiocarb. Owing to differences in the action times of insecticides and the different susceptibility levels of the strains, we analysed data from the different strains separately for each insecticide. Twenty-four hours mortality was predicted by time-to-knockdown, with the day of testing included as a random effect term to account for correlations within the same day. The GLMMs were modelled using the R package lme4 [19, 20].

2.4. Results

2.4.1. Literature review

The database search pulled out a total of 6,536 records which were systematically screened to remove publications that were not relevant to our study. After the removal of duplicates, 3,773 eligible records were reviewed. Nine publications in which the two assays were performed on the same mosquito population were identified. Results from the identified studies showed mixed outcomes. The agreement ranged from poor to perfect. Three publications had perfect agreement in the strains tested, two showed poor agreement and the rest were moderate to substantial (Table 2.3).

Table 2.3: Comparison between WHO susceptibility and CDC bottle assay data from the literature search

Study	Country	Species tested	Insecticides tested	CDC diagn. dose used	Pairs (*N*)	*K*	Quoted protocol
Perea et al.[21]	Peru	*An. albimanus*	deltamethrin	Determined by authors	2	1.00	1998
Hargreaves et al.[24]	South Africa	*An. funestus*	permethrin	Determined by authors	21	-0.02	1975
Matowoet al.[22]	Tanzania	*An. arabiensis*	permethrin	Determined by authors	2	1.00	1981
Aïzoun et al.[10]	Benin	*An. gambiae*	deltamethrin, bendiocarb	CDC recommended	12	1.00	1998
Fonseca-González et al.[32]	Columbia	*Ae. aegypti*	deltamethrin, cyfluthrin, permethrin, etofenprox, malathion, fenitrothion, DDT, λ-cyhalothrin	Determined by authors	96	0.82	1981, 1998
Ocampo et al.[33]	Columbia	*Ae. aegypti*	deltamethrin, λ-cyhalothrin, malathion, fenitrothion, Bendiocarb, DDT	Determined by authors	46	0.55	1981
Fonseca-González et al.[34]	Columbia	*An. darlingi*	deltamethrin, λ-cyhalothrin, fenitrothion, malathion, DDT	Determined by authors	24	0.70	1981, 1998
Fonseca-González et al.[35]	Columbia	*An. nuneztovari*	deltamethrin, λ-cyhalothrin, malathion, fenitrothion, DDT	Determined by authors	24	0.52	1981, 1998
Ochomo et al.[23]	Kenya	*An. gambiae* s.s.	permethrin, deltamethrin, bendiocarb	CDC recommended	3	0.00	1998

2.4.2. WHO susceptibility test vs. CDC bottle assay from present study

2.4.2.1. Comparison of mortality rates

In the present laboratory study, we had a total of 18 pairs of results available for comparison between the CDC bottle assay and the WHO susceptibility assay. Here, the two assays showed variations in the degree of agreement at the various levels of comparison (Table 2.4). In the standard comparison, agreement in detecting resistance was only slight at both the 90% (κ= 0.06) and 98% (κ = 0.01) cut off marks. In the 24 hours comparison, the agreement improved to moderate at 90% (κ = 0.51) and also at 98% (κ = 0.58).

Table 2.4: Comparison between WHO susceptibility and CDC bottle assay data in the present study

Strain	Insecticide	N		% 24 hours mortality		% KD at CDC diagn. time	Status 90%		Status 98%	
		WHO	CDC	WHO (95% CI)	CDC (95% CI)		W	C	W	C
ROCK	Permethrin	110	100	96.4 (90.6 - 98.8)	96.0 (89.8, 98.7)	100	S	S	R	S
	λ-cyhalothrin	108	100	99.1 (94.3 - 100)	75.0 (65.6 - 82.5)	100	S	S	S	S
	Malathion	101	100	100 (96.4 - 100)	100 (96.4 - 100)	100	S	S	S	S
	DDT	118	100	67.0 (58.0 - 74.8)	75.0 (65.6 - 82.5)	100	R	S	R	S
KISUMU1	Permethrin	102	100	99.0 (94.1 - 100)	90.0 (82.3 - 94.6)	99	S	S	S	S
	λ-cyhalothrin	108	100	98.2 (93.1 - 99.9)	88.0 (80.0 - 93.1)	100	S	S	S	S
	Malathion	105	100	100 (96.6 - 100)	100 (96.4 - 100)	91	S	S	S	R
	Bendiocarb	107	100	100 (96.6 - 100)	100 (96.4 - 100)	100	S	S	S	S
	DDT	110	100	100 (96.7 - 100)	98.0 (92.5 - 99.9)	99	S	S	S	S
STI	Permethrin	104	102	71.2 (61.8 - 79.0)	52.9 (43.3 - 62.3)	100	R	S	R	S
	λ-cyhalothrin	95	100	32.6 (24.1 - 42.6)	16.0 (10.0 - 24.6)	100	R	S	R	S
	Malathion	110	100	100 (96.7 - 100)	100 (96.4 - 100)	82	S	R	S	R
	Bendiocarb	108	100	100 (96.6 - 100)	100 (96.4 - 100)	100	S	S	S	S
	DDT	104	100	47.1 (37.8 - 56.6)	60.0 (50.2 - 69.1)	99	R	S	R	S
	Permethrin	100	100	78.0 (68.8 - 85.0)	91.0 (83.5 - 95.3)	96	R	S	R	R
VK7	λ-cyhalothrin	49	40	93.9 (82.7 - 98.4)	95.0 (82.4 - 99.4)	100	S	S	R	S
ZANU	Permethrin	103	100	100 (96.5 - 100)	87.0 (78.8 - 92.3)	100	S	S	S	S
	DDT	108	100	96. 3 (90.5 - 98.8)	67.0 (57.3 - 75.4)	100	S	S	R	S

Wilson's method [31] was used to calculate the confidence intervals in the 24 hours mortalities.

2.4.2.2. Comparison of knockdown curves

The order and rate of knockdown in the various strains and insecticides was not always the same in both tests (Figure 2.1). The survival analysis showed that the order and patterns of the lines of cummulative knockdown of the strains for the various insecticides were all statistically significant in both assays (p-values; for WHO:< 0.01 for all insecticides, for CDC: < 0.01 for all insecticides except bendiocarb, $p = 0.05$). Lines which were very close or overlying also showed statistically significant differences in the rates of knockdown except for STI, KISUMU1 and ZANU tested against permethrin (WHO; $\chi^2 = 4.2$ $p = 0.121$, CDC;$\chi^2 = 10.8$ $p <0.01$) and KISUMU1, VK7 and STI against λ-cyhalothrin (WHO; $\chi^2 = 126$, $p <0.001$, CDC;$\chi^2 = 0.9$, $p = 0.65$).

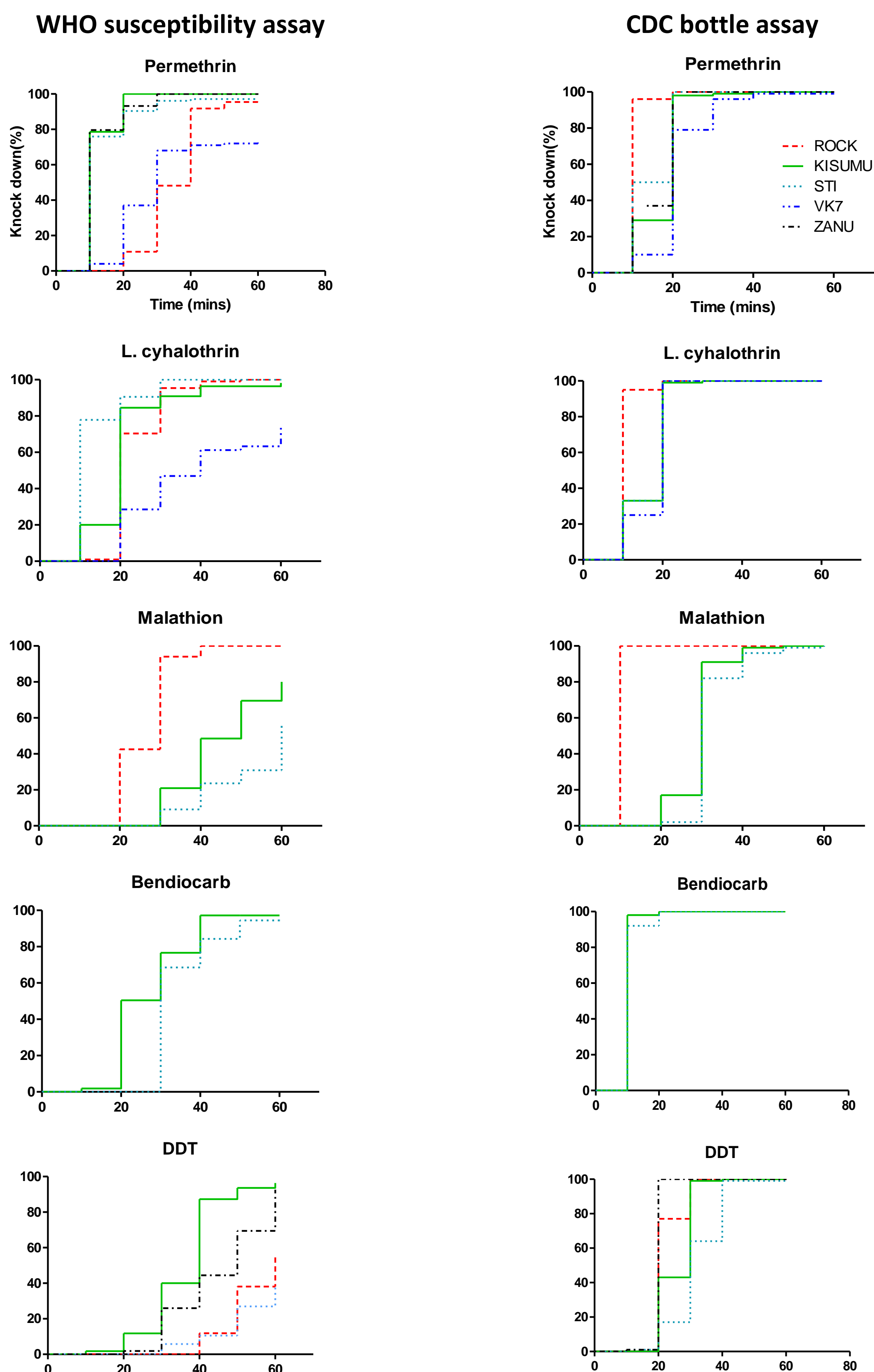

Figure 2.1: Comparison of cumulative knockdown rates in the WHO susceptibility test (left panels) and the CDC bottle assay (right panels). Knockdown was scored at 10 minutes intervals

2.4.2.3. 24 hours mortality as a function of time-to-knockdown

A total of 2,405 mosquitoes from seven strains were tested against five insecticides (Table 2.1). Time-to-knockdown was a very poor predictor of 24 hours mortality. In all the strains tested, it only showed a significant association with 24 hours mortality when ROCK was tested against DDT (OR = 0.87, p = 0.02, 95% CI = 0.77 – 0.98) and VK7 against permethrin (OR = 0.78, $p < 0.001$, 95% CI = 0.68 - 0.90). Table 2.5 shows a summary of the odds ratios and significance levels in each strain and insecticide combination.

A plot of the time it takes to knockdown 50% of the test population (KDT_{50}) from the various strains and insecticides against mortality showed no clear pattern, corroborating the results from the GLMM models (Figure 2.2). The KDT_{50} values ranged from 4 minutes in the treatment of ROCK and VK7 with bendiocarb to 28 minutes in the treatment of VK7 with DDT.

Table 2.5: Summary of the relationship between time-to-knockdown and 24 hours mortality in the various strains given for each insecticide

Strain	Insecticide	N	OR	p	95% CI
JHB	Permethrin	100	0.99	0.72	0.94 – 1.03
	λ-cyhalothrin	100	0.99	0.59	0.94 - 1.03
	DDT	101	1.0	0.59	0.97 - 1.05
STI	Permethrin	102	1.09	0.28	0.93 - 1.27
	λ-cyhalothrin	100	0.84	0.12	0.66 - 1.05
	DDT	100	0.97	0.29	0.91 - 1.03
ROCK	Permethrin	100	0.67	0.31	0.35 - 1.40
	λ-cyhalothrin	100	1.18	0.26	0.88 - 1.58
	DDT	100	0.87	0.02	0.77 – 0.98
ZANU	Permethrin	100	1.07	0.56	1.35 - 0.85
	DDT	100	0.96	0.64	0.79 - 1.16
VK7	Permethrin	100	0.78	<0.001	0.68 - 0.90
	λ-cyhalothrin	40	0.25	0.14	0.04 - 1.57
	DDT	18	0.94	0.25	0.84 – 1.04
KISUMU1	Permethrin	100	1.13	0.37	0.87 - 1.47
	λ-cyhalothrin	100	0.96	0.73	0.75 – 1.22
	DDT	100	0.84	0.38	0.56 - 1.24

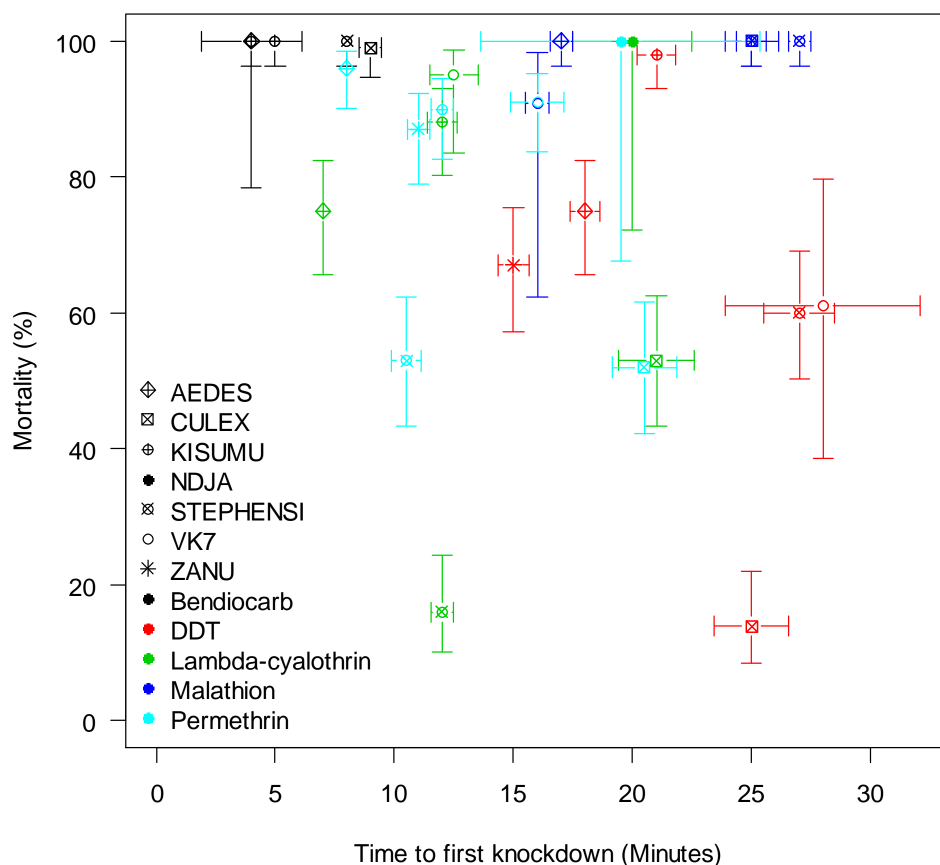

Figure 2.2: Summary plot showing the relationship between 24 hours mortality and the time it takes to knockdown 50% of the population (KDT_{50}) for all strains and insecticides tested in the present laboratory study. Symbols show point estimates and 95% confidence intervals for mortality are computed after Wilson [31] and those for KDT using the boxplot function.

2.5. Discussion

Knowing the insecticide susceptibility or resistance status is key in choosing the appropriate intervention to control any mosquito population. Many factors influence the outcome of insecticide resistance monitoring exercises and the importance of the choice of the assay cannot be overlooked. The results from our study show that although the CDC bottle assay and WHO susceptibility test are mutually used to detect insecticide resistance, the two must not be used interchangeably because the agreement between the two is inconsistent. This could be explained by the fact that the two assays differ in their basic setup. The WHO susceptibility assay, carried out in 125mm length x 44mm diameter tubes, measures 24 hours mortality by exposing mosquitoes to a known standard concentration for a fixed period of

time, usually 1 hour. The insecticides are delivered through impregnated filter papers and the standard concentration is twice the lowest concentration that produced 100% mortality systematically from a baseline analysis of a susceptible strain [8]. Intended to be a simple, easy-to-use assay for field work due to its economical and convenient setup, the CDC assay measures the length of time it takes for an insecticide to "kill" a sample of adult mosquitoes exposed to a known concentration. It is presented in 250 ml glass bottles and the insecticide delivery is by coating the inner walls of the glass bottle [12]. Although in both assays the insecticides are dissolved in acetone, the insecticide carriers differ due to the addition of oil to the mixture in the WHO assay. The insecticide is therefore carried by the oil since acetone is volatile.

From our literature search, the WHO susceptibility assay was the more widely used assay. A notable observation from the search was that the version of the WHO assay protocol quoted as the one followed for the methodology differed between publications, with some recent publications still quoting the 1981 version (Table 2.3). Even though there have not been a lot of major changes in the procedure in the various versions, there have been updates in some details and recommended concentrations. Despite a lot of studies employing the use of one or the other of the two assays, only nine have used them both on the same population. Aïzoun *et al*. [10] did a direct comparison of the two tests. While there was high consistency observed in their study, our findings suggest the opposite. Their results might have been influenced by the high susceptibility to bendiocarb and high *kdr* frequency, which resulted in high resistance to deltamethrin in the field populations they used, as opposed to the heterogeneity in our resistance phenotypes. It is also not very clear whether the number of pairs also had an influence on the agreement. Perea *et al*. [21] and Matowo *et al*. [22] both observed almost perfect agreement with two pairs while Ochomo *et al*. [23] found a poor agreement with three pairs. On the other hand, Hargreaves *et al*. [24] also recorded a poor agreement with 21 pairs.

A shortfall of our study was our inability to test all of our strains against all of the present insecticides due to lower numbers available in certain strains. This led to some results being excluded from the comparison as the numbers were considered too low. Moreover, it was not feasible to run all assays simultaneously to exclude daily variations. However, we were careful to maintain equal rearing and testing conditions and randomised test sequences. In addition,

we included a random term in the GLMMs to account for correlations within a test day in the analysis of 24 hours mortality as a function of time-to-knockdown.

Several factors could have been the cause of the discrepancies observed in the published and present data. It is not clear what impact the concentration used in the CDC assay, whether self-established or CDC recommended diagnostic doses, has on the agreement of the two assays (Table 2.3). For example, Aïzoun *et al*. [10] and Ochomo *et al*. [23] both used the CDC recommended concentrations but had different agreement levels. It could be argued that the low number of pairs (i.e. 3) in Ochomo *et al*. introduced a bias. Nevertheless, our results, having 18 pairs, still yielded disagreements between the two assays. One explanation might be the difference between the applied concentrations in the two assays. According to WHO [25] the filter papers are prepared such that a treatment at 1% contains 36.7 µg insecticide per cm^2. Standard filter papers treated with permethrin, for example, would then contain about 300 times more insecticide per cm^2 than in the CDC bottle assay (Table 2.2). But despite the gap in concentrations, the amount of insecticide available to the mosquito in the WHO assay is not clear. These figures are calculated based on the surface area and not the volume of the filter paper, which unlike the glass surface, absorbs the insecticide carrier used in the preparation. This is evident in the fact that during preparation, insecticides are applied on one side of the filter paper, the “right” side, but the “wrong” side also contains enough insecticide to kill mosquitoes. Although concentrations are higher in the WHO assay, this might not be reflected in the insecticide availability due to the method of delivery. This could also explain why the order and rates of knockdown were inconsistent between the two assays in our study. The curves in the CDC assay were steeper, indicating a faster knockdown rate than in the WHO assay. The statistical significance of the Kaplan-Meier survival function also shows that these assays are not only inconsistent in the output of mortality, but also knockdown. With the WHO diagnostic concentrations in use for over a decade [26], it might be time to review them. This could provide a good opportunity to bring the two assays in synchrony. A possible solution to the current inconsistent agreements could be for WHO and CDC to come together to re-calibrate both assays using the same population of mosquitoes. In doing so, the concentrations and exposure times that would provide the same level of mortality can be derived for each assay.

Evidently missing in the CDCs setup is a 24 hour holding period. Though this makes the assay rather short and rapid, we believe that some level of resistance, especially metabolic resistance could be missed in the CDC assay due to the lack of a recovery time. This was observed in the results obtained from our laboratory tests, especially in the STI strain (Table 2.4). At both the 90% and 98% knockdown threshold κ was very low. This may be explained by the fact that the CDC assay scored most of the strains as susceptible due to the high knockdown rates. This increased the agreement in scoring the colony as susceptible but not as resistant. Holding the mosquitoes from the CDC assay for 24 hours significantly increased the agreement from poor to moderate due to the recovery in some strains. No holding period in the CDC assay also means that "dead" mosquitoes are recorded only during the period of exposure. According to the WHO guidelines, knockdown is recorded during the exposure period and mortality is recorded 24 hours post exposure. Therefore, by applying this definition, the CDC assay only measures knockdown, and the WHO assay measures mortality. This then raises the question of how much 24 hours mortality may be explained by time-to-knockdown. From our results, the relationship between the two is rather unreliable, feeding into the long standing question of which is the best way to measure resistance. As previously observed, in the presence of very high resistance, time-response assays have not been very useful [27, 28].

With time-to-knockdown being rather a poor predictor of mortality, a better way of measuring 24 hours mortality could be the use of dose-response curves. These curves are dynamic, provide more flexibility and are more informative. With current assays the downside is, however, the requirement of higher mosquito numbers. For example, according to the WHO guidelines, at least 600 mosquitoes would be required, a number often difficult to collect in the field. A single diagnostic concentration (LC_{99}) may remain unchanged in the presence of low numbers of homozygous resistant individuals. The LC_{50}, however, might shift with growing numbers of heterozygous resistant individuals if the mutation has a co-dominant effect [29]. A comprehensive dose-mortality therefore, would enable the detection of any early indications of emerging resistance. This would also facilitate the comparison of susceptibility over time within or between populations [30]. While being sensitive to low levels of decreased sensitivity, likewise, it would still capture changes at the high end of resistance [27].

Practically, a remaining challenge will be the choice of concentrations at which 24 hours mortality is measured in order to make sound dose-response curve estimates.

2.6. Conclusion

This study shows that though the two assays can both successfully detect insecticide resistance, they may not be used interchangeably due to the high level of inconsistencies in the agreement between them. Since a lot of factors go into the results obtained from bioassays, the choice of assay is very important. At a time when the WHO is putting a lot of emphasis on monitoring and evaluation, the choice of the assay should be based on the data and information required rather than which is readily available. Given their various advantages over the other, they will be very beneficial to control programs if their purposes are re-defined and adopted for specific functions in insecticide resistance monitoring and evaluation. With time-to-knockdown being a poor predictor of mortality, turning to dose-response assays could provide the most flexibility as it eliminates the dependence on diagnostic thresholds and therefore can also capture subtle changes that happen below, or above, that concentration. We recommend that new standardised bioassays are developed that produce consistent dose-mortality estimates with a minimal number of mosquitoes required.

2.7. Competing interests

The authors declare that they have no competing interests.

2.8. Authors' contributions

HFO, DM and PM conceived the idea of the project. HFO, DJ, DM and PM participated in the development of the experimental design. HFO and DJ performed laboratory experiments and literature search. HFO and PM analysed and interpreted the data. HFO drafted the manuscript and all authors contributed to the draft. All authors read and approved the final manuscript.

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3. How important is the angle of tilt in the WHO cone bioassay?

Henry F. Owusu* and Pie Müller

Epidemiology and Public Health Department, Swiss Tropical and Public Health Institute, Socinstrasse 57, PO Box, CH-4002 Basel, Switzerland

University of Basel, Petersplatz 1, CH-2003 Basel, Switzerland

*Corresponding author

Email addresses:

HFO: henry.owusu@unibas.ch

PM: pie.mueller@swisstph.ch

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3.1. Abstract

Background The World Health Organization (WHO) cone bioassay plays an integral role in the evaluation of the efficacy of long-lasting insecticidal nets as well as insecticides used in indoor residual spraying. The test is used on a variety of treated substrates, such as pieces of bed nets, mud, cement and wood. The cone setup assumes a wide variety of angles under different settings in which it is applied. However, the guidelines provided for the performance of the assay do not specify the angle at which the test must be performed.

Methods Laboratory colonies of Anopheles gambiae Kisumu-1 and Anopheles stephensi STI were tested in the WHO cone bioassay at four different angles (0°, 45°, 60° and 90°) following the WHO guidelines against net pieces of Olyset Plus and Netprotect. The tests were repeated after 20 washes of the nets. Individual mosquitoes were also exposed at 0° and 60° and the amount of time each spent in contact with the net was recorded.

Results Mosquitoes spent more time on the net at 60° as compared to 0° (coefficient = 45.8, 95 % CI 34.6–55.6, $p < 0.001$) and were more likely to die when the test was done at 45° (OR 3.3, 95 % CI 1.7–6.3, $p = 0.001$), 60° (OR 3.1, 95 % CI 1.7–5.9, $p < 0.001$) and 90° (OR 6.0, 95 % CI 1.9–18.5, $p = 0.002$) as compared to 0°.

Conclusion The angle at which the test is performed significantly affects the amount of time mosquitoes spend resting on the nets, and subsequently mortality. Angle must thus be considered as an important component in the performance of the assay and duly incorporated into the guidelines.

Key words

Mosquitoes, Long-lasting insecticidal net, Insecticide resistance.

3.2. Introduction

Insecticide-treated bed nets (ITNs) have been shown to effectively reduce malaria morbidity and mortality [1] and consequently, the last years have seen many intervention programmes being put in place to distribute and promote the use of long-lasting insecticidal nets (LNs) in malaria endemic countries [2–5]. As a key strategy of the Roll Back Malaria initiative [6], LNs have been and continue to be implemented as part of national malaria control programmes around the world. With the increasing demand for treated bed nets and the ever present threat of the development of resistance to insecticides comes the need to ensure the best products are available for use. As a result, new bed net products are evaluated for efficacy, durability and operational acceptability prior to approval.

A very important feature of the efficacy testing of mosquito nets is the World Health Organization (WHO) cone bioassay [7]. It serves as a pivotal tool on which a lot of decisions made in efficacy studies are based. WHO instructs that nets would first have to meet the criteria of WHO cone bioassay before they are passed to go through phase II testing [7]. It is the recommended assay for testing the efficacy and irritant or excito-repellent properties of insecticide-treated substrates. It also plays a pivotal role in IRS as it is used to test formulations of insecticides on various substrates such as mud, cement, plywood and other materials commonly used for building [8]. In 2013 the WHO Pesticide Evaluation Scheme (WHOPES) published the latest version of the Guidelines for laboratory and field-testing of long-lasting insecticidal nets [7] which replaced the earlier version published in 2005 [9]. The document outlines the procedure for testing LNs and provides the current set of instructions for performing the WHO cone bioassay. Even though its purpose is to provide specific, standardised procedures for testing LNs to harmonise testing procedures in order to generate data for registration and labelling of such products [7], it leaves some important details in the instructions to the discretion of the personnel conducting the experiment, which could potentially influence the outcome of the test. One such detail is the angle at which the set-up should be held during exposure. To test samples of treated bed nets, the assay is set up by attaching WHO polyvinyl chloride (PVC) cones to the net sample and the two are secured together by two plastic panels with circular holes cut in them to accommodate the cone and expose the netting. These panels are held together by two metallic binder clips. The guidelines do not provide any information on the positioning of the assembly. The only mention of the

angle is in the caption of the figure illustrating the cone assay, where it states that the holding board on which the assembly rests is slanted at an angle of 45˚. The angle is completely ignored in the case of using the assay on sprayed surfaces where the PVC cone is taped to the surface of interest, in which case the angle could range from a flat table (0˚), through an upright wall (90˚) to an upside down ceiling (180˚). The assay heavily depends on the mosquitoes making contact with the net or surface being tested. Due to the behaviour of mosquitoes, the angle of testing could lead to less contact with the surface, subsequently leading to significant fluctuations in the outcome of the assay.

On this background, we investigated the possible effects the angle of testing has on the outcome of the WHO cone bioassay on mosquito nets by performing the test following the recommended guidelines [7] at four different angles. The difference in behaviour as a function of angle was also evaluated.

3.3. Methods

3.3.1. **Mosquitoes**

Mosquitoes of two laboratory-bred Anopheles colonies were used in the experiment; the pyrethroid susceptible *Anopheles gambiae* Kisumu-1 and the pyrethroid resistant *Anopheles stephensi* STI. The Kisumu-1 (MRA-762) strain was obtained from the Malaria Research and Reference Reagent Resource Center in 2011. This is a standard strain used in cone bioassays to evaluate if a LN meets WHOPES specifications [8]. In addition, as occasionally new products are also evaluated against insecticide resistant mosquitoes, the pyrethroid resistant STI colony was also included. The colony was originally obtained from the London School of Hygiene and Tropical Medicine in 1971. The larvae were fed with finely ground fish food Tetramin (Tetra GmbH, Germany) and the resulting adults were maintained on 10 % sugar solution at temperature and relative humidity ranges of 26–28 °C and 60–74 %, respectively, in a 12:12 h day:night regime.

3.3.2. **Insecticide treated nets**

To account for the repellent/irritant effect, Olyset Plus® and Netprotect®, impregnated with permethrin and deltamethrin, respectively were used. Olyset Plus is produced by Sumitomo Chemicals Co. Ltd. (Japan) and is made of knitted polyethylene thread incorporated with 2 % (w/w) permethrin combined with 1 % piperonyl butoxide. Netprotect, manufactured by

BestNet A/S (Denmark), is also a polyethylene LN and it is impregnated with 0.18 % (w/w) deltamethrin.

3.3.3. WHO cone bioassay

The bioassay was performed according to the WHOPES guidelines [7]. The set-up was prepared by cutting approximately 25 cm x 25 cm pieces of netting from each net type. Four WHO plastic cones were attached to each piece of net and held together by two plastic boards which were clamped together with two binder clips. The assembly was held at one of the test angles; 0˚ (flat on the table), 45˚, 60˚ and 90˚ (Figure 3.1). Using an aspirator, five to eight non blood-fed females aged two to five days were introduced into each cone and the holes were plugged by pieces of cotton. The mosquitoes were exposed for 3 minutes and subsequently transferred into labelled 150 ml plastic holding cups and provided with 10% sugar solution. Knockdown and mortality were recorded 60 minutes and 24 hours after exposure, respectively. Mosquitoes were scored as alive if they were able to fly, irrespective of the number of legs still intact and dead, or knocked down, if immobile or incapable of flying or standing in a coordinated manner [10]**.** An untreated net was used as a control and was tested each day the bioassay was performed. The bioassays were performed at temperature and relative humidity ranges of 25.9 – 29 °C and 58 - 73 % respectively.

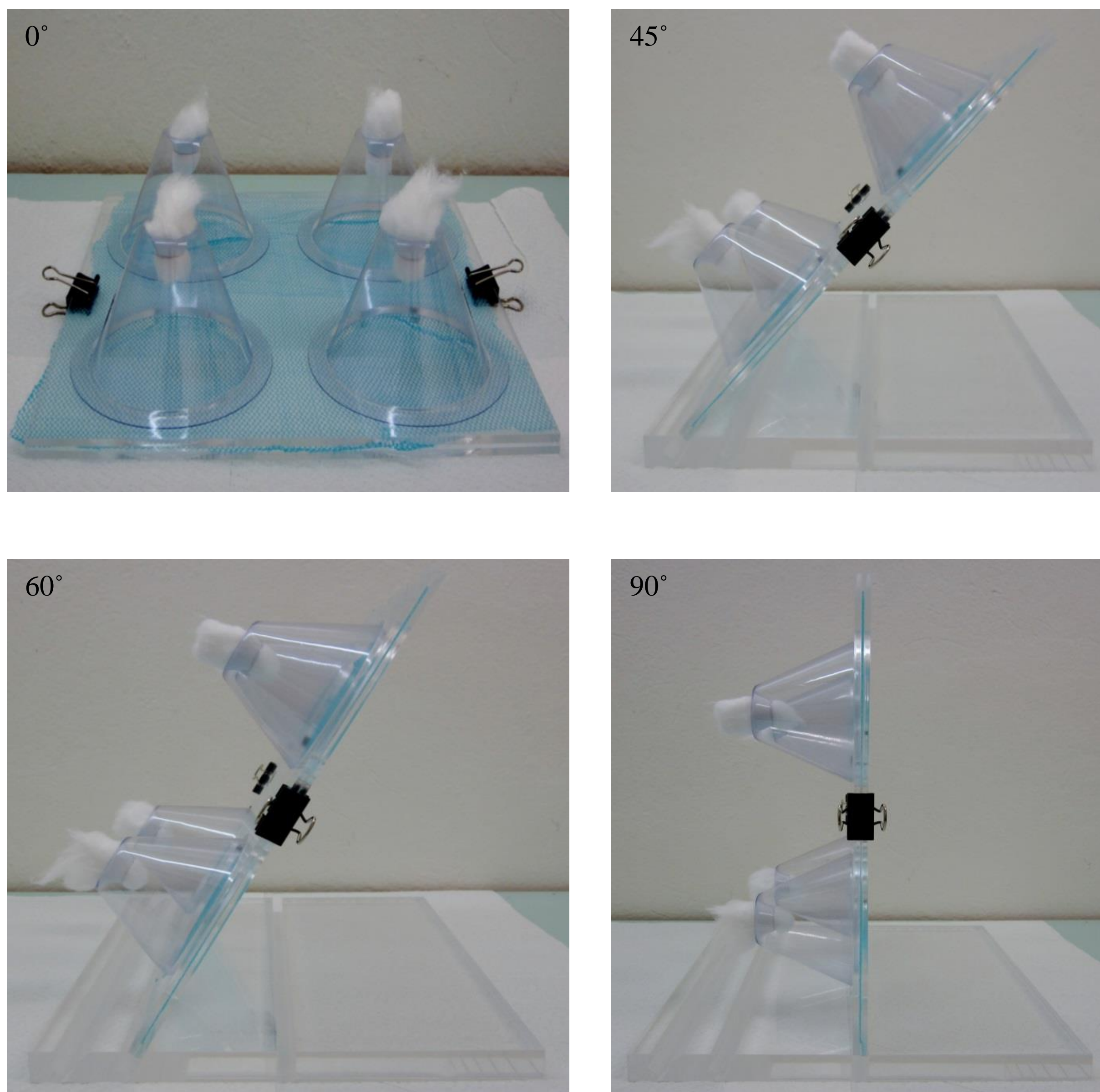

Figure 3.1: Cone bioassay of LNs. The holding board was slanted at different angles of 0°, 45°, 60° and 90°

3.3.4. Washing procedure

After the first round of testing, the net sample pieces were washed a total of 20 times each, following the WHO recommended procedure for washing nets for laboratory testing [7]. Individual net pieces were introduced into 0.5 l of deionised water in a 1 l beaker. Each net was washed in a separate beaker designated to that net to avoid cross-contamination. Just before washing, 1 g of the WHO-recommended soap "savon de Marseille" was added and fully dissolved. The beaker was immediately put in an incubator set at 30 °C and shaken at 155

movements per minute for 10 minutes. The nets were then removed, rinsed twice in clean deionised water for 10 min at the conditions given above. After washing, they were dried at room temperature and stored at 27 - 29 °C in the dark between washes. Washing was done at a minimum of seven day intervals to allow regeneration of insecticides. Testing was repeated as described above with the washed nets.

3.3.5. Time spent in contact with the net

Based on the results from the angle experiments, mosquitoes were exposed to nets elevated at 60° or flat at 0° and their behaviour inside the cone was observed to evaluate the correlation between time spent in contact with the nets and mortality. Mosquitoes were introduced individually into the cone setup and observed for their behaviour during the 3 min of exposure. Within this period, recordings were made on the amount of time the mosquito spent flying around, resting on the net, on the cotton plug and on the side of the PVC cone. As in the experiment described above, mosquitoes from each strain were exposed to washed and unwashed pieces of Olyset Plus and Netprotect. A total of 15 individuals were tested for each combination of net product, net state (i.e. washed or unwashed), mosquito strain, and angle. To minimize the effect of the act of blowing the mosquitoes into the cone on the behaviour, each mosquito was gently blown onto the net.

3.3.6. Data analysis

The data were analysed by generalized linear mixedeffects models (GLMM) in the freely available statistical software package R [11], version 3.1.2 and the R package lme4 [12, 13]. The day of testing was included as a random intercept in the models to account for correlations within the same day. The level of significance was set at $\alpha = 0.05$.

Logistic regressions were used to assess the effect of the angle on knockdown and 24 h mortality. Angle 0° was used as the reference for comparison. In addition to the angle, strain and the net product were also included as fixed factors in the models and the data was analysed separately for washed and unwashed nets. The amount of time the mosquitoes spent resting on the net was analysed using a linear regression. Time spent was predicted by angle, mosquito strain, net product and state of the net (i.e. washed or unwashed). The R package lmerTest [14] was used to generate the p values for the estimates in the GLMMs.

3.4. Results

3.4.1. Effect of angle on knockdown and mortality

A minimum of 150 mosquitoes were exposed for each strain, net type, net state and angle combination. Table 3.1 summarizes the mortality recorded in the various groups. In the unwashed nets, knockdown rates were very high (Table 3.1; Figure 3.2) and there was no difference detected between the two strains (odds ratio = 0.3, 95 % CI 0.01–1.03, p = 0.06). As shown in Table 3.2, in the washed nets, knockdown rates were significantly lower in Olyset Plus than in Netprotect (OR 0.3, 95 % CI 0.25–0.36, p < 0.001). Holding the test unit at 45° (OR 0.7, 95 % CI 0.5–0.9, p = 0.004) and 90° (OR 0.6, 95 % CI 0.4–0.7, p < 0.001) produced significantly lower knockdown rates as compared to 0°, but there was no statistically significant difference at 60° (OR 0.9, 95 % CI 0.7–1.1, p = 0.26). Figure 3.2 shows a plot of the mean knockdown rates recorded for each combination.

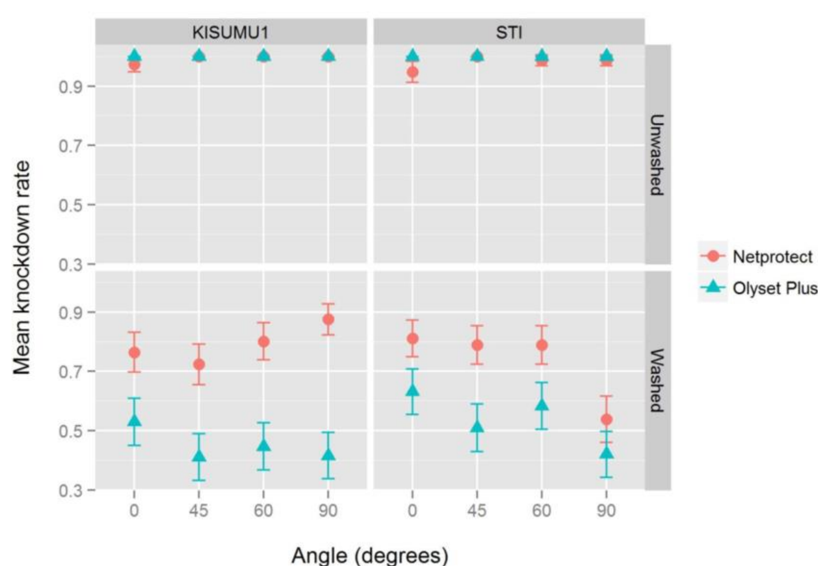

Figure 3.2: Knockdown rates at the various angles recorded in the KISUMU-1 and STI strains against washed and unwashed nets. The *points* and the *whiskers* represent mean knockdown rates and 95 % confident intervals, respectively

Mortality was generally lowest at 0° (Figure 3.3; Table 3.1). The highest mortality was usually at 45° or 60°, and a closer look showed that statistically, there was no significant difference in the mortality between the two angles in washed (OR 0.9, 95 % confidence interval = 0.5–1.5, p = 0.6) and unwashed nets (OR 1.0, 95 % CI 0.6–1.5, p = 0.9). On the unwashed nets, mosquitoes were more likely to die on Olyset Plus (OR 1.9, 95 % CI 1.5–2.5, p

< 0.001) than on Netprotect (Table 3.2), while this observation was reversed in the washed nets (OR 0.5, 95 % CI 0.3–0.8, $p = 0.008$). Mortality was significantly increased when the tests were performed at 45° (OR 3.3, 95 % CI 1.7–6.3, $p = 0.001$), 60° (OR 3.1, 95 % CI 1.7–5.9, $p < 0.001$) and 90° (OR 6.0, 95 % CI 1.9–18.5, $p = 0.002$) as compared to 0°. There were no observed interactions between any of strain, angle and net product. After 20 washes there was no statistically significant difference between the mortality obtained at 0° and 90° (OR 1.4, 95 % CI 0.8–2.3, $p = 0.212$). However, mortality was significantly higher at 45° (OR 2.7, 95 % CI 1.7–4.3, $p < 0.001$) and 60° (OR 1.9, 95 % CI 1.1–3.1, $p = 0.01$). There was a significant interaction between the net product and the angle of assay (interaction term OR at 45° = 0.3, $p < 0.001$; 60° = 0.2, $p < 0.001$; 90° = 0.2, $p < 0.001$; Likelihood-ratio test: $\chi 2 = 14.4$, $p = 0.002$), indicating that the effect of angle on mortality is different for the different net products.

The ambient temperature and relative humidity measured during testing did not have any significant effect on the test results in neither the washed (temperature: OR 1.0, 95 % CI 0.4–2.8, $p = 1.0$; relative humidity: OR 0.8, 95 % CI 0.6–1.0, $p = 0.10$) nor the unwashed (temperature: OR 0.9, 95 % CI 0.5–1.9, $p = 0.86$; relative humidity: OR 1.0, 95 % CI 0.8–1.2, $p = 0.91$) nets.

Table 3.1: Summary of the number of mosquitoes tested, knockdown and mortality recorded in the test groups

Strain	Net brand	State of net	Angle (degrees)	No. tested	% Knockdown (95% CI)	% Mortality (95% CI)
KISUMU-1	Olyset	Unwashed	0	153	100 (97.6 – 100)	90.2 (84.5 -94.0)
			45	154	100 (97.6 – 100)	96.8 (92.6 -98.6)
			60	154	100 (97.6 – 100)	98.1 (94.4 – 99.3)
			90	161	100 (97.7 – 100)	100 (97.7 – 100)
	Netprotect		0	151	97.4 (93.4 - 99.0)	86.8 (80.4 - 91.3)
			45	157	100 (97.6 – 100)	96.8 (92.8 - 98.6)
			60	155	100 (97.6 – 100)	98.1 (94.5 - 99.3)
			90	156	100 (97.6 – 100)	100 (97.6 – 100)
	Olyset	Washed	0	152	53.0 (45.0 – 60.8)	13.3 (8.7 - 19.6)
			45	151	41.1 (33.6 – 49.0)	14.6 (9.8 - 21.1)
			60	150	44.7 (36.9 - 52.7)	8.0 (4.6 - 13.5)
			90	154	41.6 (34.1 - 49.5)	5.2 (2.7 - 9.9)
	Netprotect		0	153	76.5 (69.2 – 82.5)	26.8 (20.4 - 34.3)
			45	163	72.4 (65.1 - 78.7)	54.6 (46.9 - 62.1)
			60	155	80.2 (73.2 – 85.6)	44.8 (37.3 - 52.7)
			90	153	87.6 (81.4 - 91.9)	41.8 (34.3 - 49.8)
STI	Olyset	Unwashed	0	155	100 (97.6 – 100)	70.3 (62.7 – 77.0)
			45	166	100 (97.7 – 100)	86.1 (80.1 - 90.6)
			60	155	100 (97.6 – 100)	87.1 (80.9 - 91.5)
			90	161	100 (97.7 – 100)	77.0 (69.9 - 82.8)
	Netprotect		0	154	94.8 (90.1 - 97.3)	42.2 (34.7 - 50.1)
			45	165	100 (97.7 – 100)	77.0 (70.0 - 82.7)
			60	153	98.7 (95.4 – 99.6)	73.9 (66.4 - 80.2)
			90	154	98.7 (95.4 – 99.6)	61.7 (53.8 – 6.90)
	Olyset	Washed	0	152	63.2 (55.3 - 70.4)	4.0 (1.8 - 8.3)
			45	151	51.0 (43.1 – 58.8)	2.0 (0.7 - 5.7)
			60	151	58.3 (50.3 - 65.8)	2.7 (1.0 - 6.6)
			90	157	42.0 (34.6 - 49.9)	2.6 (1.0 - 6.4)
	Netprotect		0	153	81.2 (74.3 - 86.6)	8.4 (5.0 - 13.9)
			45	152	78.9 (71.8 - 84.7)	12.5 (8.15 - 18.7)
			60	152	78.9 (71.8 - 84.7)	13.8 (9.2 - 20.2)
			90	158	53.8 (46.0 - 61.4)	6.3 (3.5 - 11.3)

Table 3.2: A summary of the outputs from the logistic regression models explaining the predictors of mortality and knockdown

Net state	Outcome	Explanatory variable	Odds Ratio	95% CI	p-Value
Unwashed	Mortality	Product (Olyset Plus)	1.9	1.5 - 2.5	< 0.001
		Strain (STI)	0.1	0.04 – 0.2	< 0.001
		Angle (45°)	3.3	1.7 - 6.3	0.001
		Angle (60°)	3.1	1.7 - 5.9	< 0.001
		Angle (90°)	6.0	1.9 - 18.5	0.002
Washed		product (Olyset Plus)	0.5	0.3 - 0.8	0.01
		Strain (STI)	0.06	0.03 - 0.12	< 0.001
		Angle (45°)	2.7	1.7 - 4.3	< 0.001
		Angle (60°)	1.9	1.1 - 3.1	0.01
		Angle (90°)	1.4	0.8 - 2.3	0.212
Washed	Knockdown	Product (Olyset Plus)	0.3	0.25 - 0.36	< 0.001
		Strain (STI)	1.1	0.89 – 1.3	0.5
		Angle (45°)	0.7	0.5 - 0.9	0.004
		Angle (60°)	0.9	0.7 - 1.1	0.26
		Angle (90°)	0.6	0.4 - 0.7	< 0.001

Netprotect, KISUMU-1 and the angle 0˚ were the reference levels (intercepts) in the coefficients. No model was ran on knockdown in the unwashed nets due to the high levels of knockdown observed

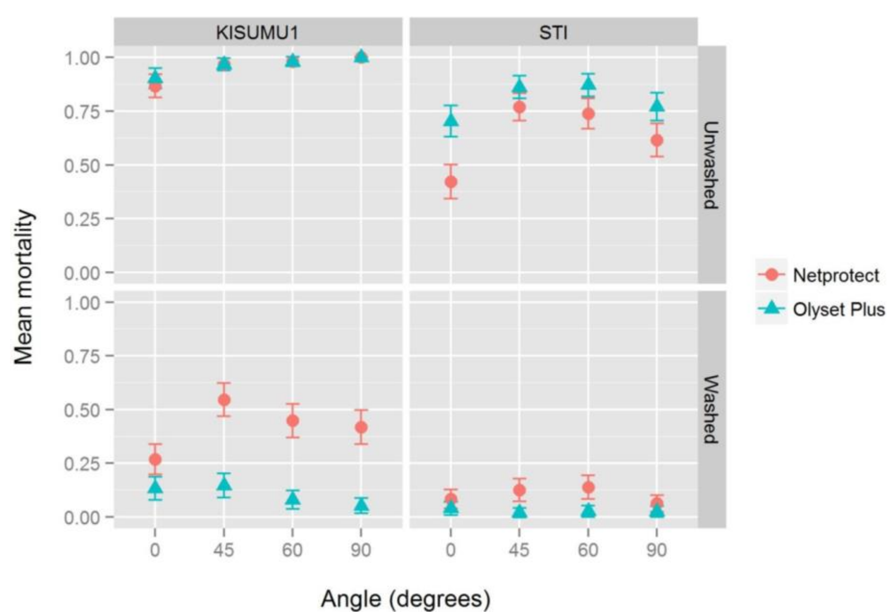

Figure 3.3: Mortality at the various angles recorded in the KISUMU-1 and STI strains against washed and unwashed nets. The *points* and the *whiskers* represent mean mortalities and 95 % confident intervals, respectively

3.4.2. Time spent in contact with the net

This experiment was carried out in order to examine whether the lower mortality rates observed at 0° could be explained by the amount of time the mosquitoes spent resting on the net samples. The two angles that produced the highest and the lowest mortality values were chosen. The highest mortalities were recorded at 45° and 60°, but because there was no statistically significant difference between the two angles, 60° was chosen, while the lowest mortality was recorded at 0°. A total of 240 mosquitoes were tested; 15 individuals from each strain against washed and unwashed pieces of the two net products at 0° and 60°. Cumulatively, mosquitoes spent more time in contact with the net at 60° (16,682 s) than at 0° (11,259 s) and more time in flight at 0° (8321 s) than at 60° (3883 s). They also spent more time on the cotton at 0° (1212 s) than at 60° (148 s). Figures 3.4 and 3.5 show graphical presentations of how both strains spent the time inside the cones. Indeed, the multiple linear regression model (Table 3.3) showed that mosquitoes spent more time in contact with the net at 60° than at 0° (coefficient = 44.8, 95 % CI 34.3–55.3, $p < 0.001$). The effect of the mosquito strain was also significant, with the resistant STI strain spending more time on the net than the susceptible Kisumu-1 (coeff = 24.9, 95 % CI 2.8–47.1, $p = 0.05$). The type of net (coeff = −11.3, 95 % CI −31.7 to 9.0, $p = 0.3$) and whether washed or not (coeff = 12.2, 95 % CI −2.4 to 26.7, $p = 0.1$) did not have a significant influence. A 3-min side-by-side video showing mosquitoes exposed at 0° and 60° is provided as an Additional file 1.

Table 3.3 The output of the linear regression model predicting the time spent by the mosquitoes resting on the net

Explanatory variable	coefficient	p-Value	95% CI
Product	-8.4	0.1	-19.0 - 2.2
Strain	34.9	< 0.001	24.3 - 45.4
Angle	0.8	< 0.001	0.6 - 0 .9
Net state	-10.1	0.06	-20.7 - 0.5

Netprotect, KISUMU1 and the angle 0˚ were the reference levels in the coefficients

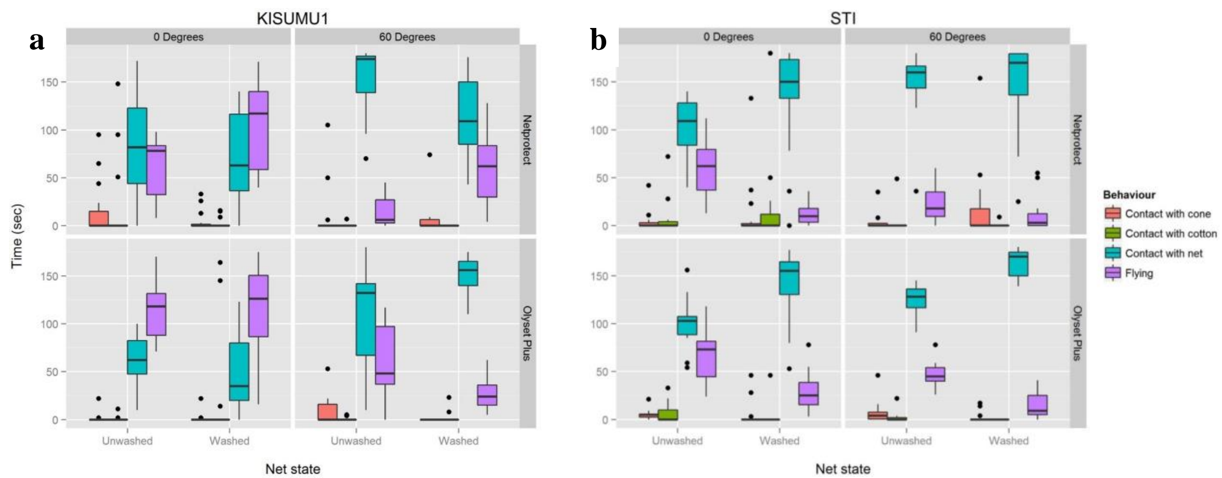

Figure 3.4: Boxplot of the time distribution in the observational experiment in KISUMU-1 (a) and STI (b). The *boxes* represent the interquartile distances (IQD), while the center lines through each box show the medians. The *dots* indicate outliers and the *whiskers* extend to the extreme values of the data, calculated as ±1.5 × IQD from the median

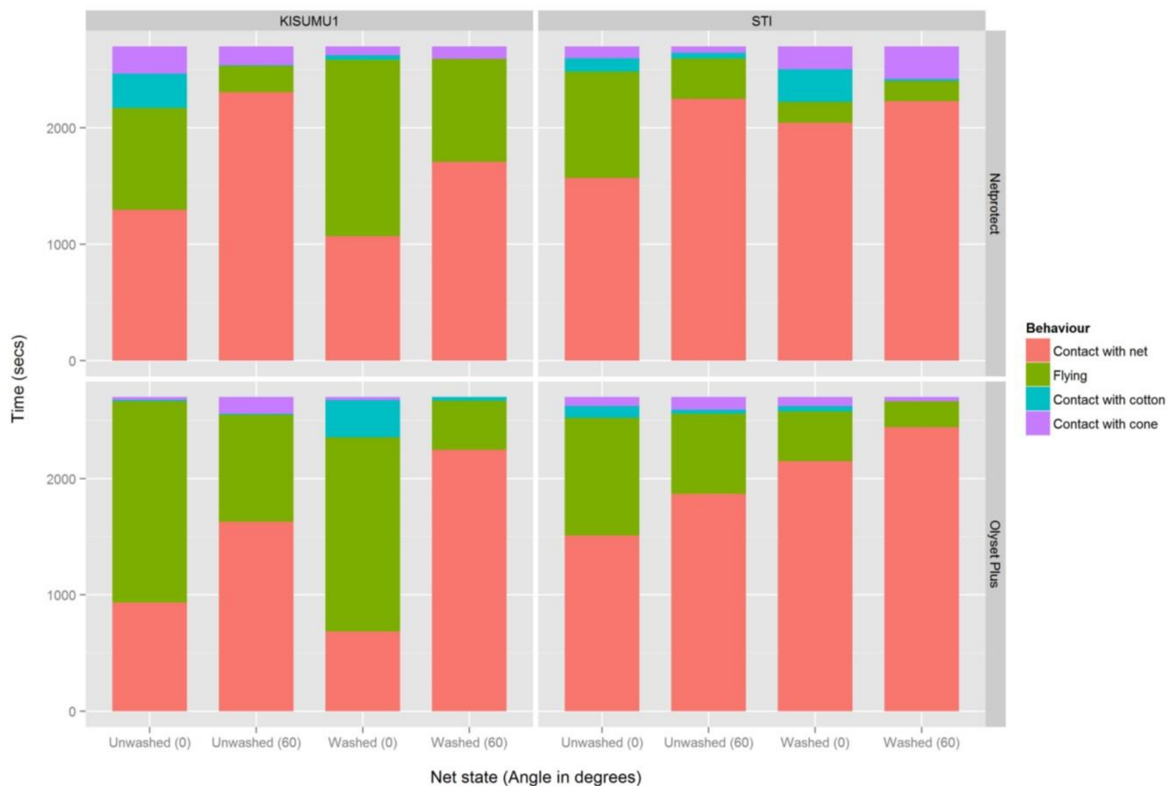

Figure 3.5: Proportion of the cumulative time the mosquitoes spent flying, resting on the net, cone and cotton within the 3 min of exposure

Figure 3.6: Some mosquitoes spent a substantial amount of time resting on the cotton and not in contact with the net.

3.5. Discussion

The results from the current study provide evidence that the angle at which the WHO cone bioassay is performed considerably affects the time mosquitoes spend in contact with the net, and subsequently 24 h mortality. The cone assay is heavily depended on as the main test for the determination of the bioefficacy in terms of insecticidal activity and irritant or excito-repellent properties throughout the three WHOPES phases of the efficacy assessment of LNs. It serves as the first test in phase I trials and is used to generate information on the efficacy and wash-resistance of the nets and to assess the interactions between the insecticide and the netting fibre such as regeneration time [7]. An assay of such importance requires specific instructions on details pertaining to the procedure rather than leaving it to the performer's convenience. The importance of the angle at which the assay is performed has been largely ignored, although this should be considered an essential feature of the assay. According to WHO [15], the cone assay is recommended as the assay of choice for insecticidal activity because it directly measures the amount of insecticide available to contact and kill mosquitoes and a net is expected to produce a mortality of ≥80 or ≥95 % knockdown to fulfil the WHO

efficacy requirements. This implies that the time the mosquitoes spend in contact with the net is very important for this purpose. Yet, as data from this study show, for the assay to effectively measure this parameter, the guidelines need to be updated to incorporate the angle of testing. Due to the assay's function of measuring irritancy, it makes sense that the WHO cone has enough room to accommodate the behaviour of irritated mosquitoes. On the other hand, this could also provide enough space for flying which could affect residual efficacy measurements. In the observational experiment, no mosquitoes were observed to have spent all the 3 min in flight but six individuals were recorded, all at 0°, that did not spend any time resting on the net and 11 that rested for less than 30 s. Given that these mosquitoes were introduced individually, factoring in the disturbance due to the presence of other mosquitoes as is the case in the standard procedure and the presence of excito-repellent effects could possibly result in more mosquitoes making no contact at all with the test material when testing on a flat surface. Currently, a net which fails to meet the requirements of the cone assay in phase I undergoes the tunnel test to measure the mortality and success of blood-feeding of host-seeking mosquitoes. This is a step to ensure that the efficacy of nets are not underestimated due to high excito-repellent effects of certain insecticides [7]. The tunnel test is very instrumental for this purpose due to its ability to capture the activity of slow acting compounds [16]. A major downside of the tunnel test however, is the use of live animals as baits which may vary in how attractive they are to mosquitoes. The baits are kept in very small confinements in such a way that they cannot move or feed within the 12–15 h exposure period.

Although there are guidelines in place to ensure that animals used for testing are not mistreated, they could be subjected to unpleasant and cruel violations if the test is not performed properly. While the two assays play very important roles in the evaluation process, optimizing and adjusting the methodology of the cone assay to improve reliability would save energy and resources and ensure the tunnel test is only turned to when actually needed. If cone tests are used for intrinsic insecticidal activity when screening, for example, for new compounds or formulations, separate assays could also be developed such that the setup of the assay reduces the space inside the cone to minimize flying and force contact with the test material.

In addition to mosquitoes flying around, individuals that spend time on the cone and the cotton plug were also recorded in both experiments (Figure 3.6), therefore avoiding contact with the net for significant periods of time. This is one area which could also be improved, design-wise, to minimize the non-treated surfaces inside the cone. In an attempt to reduce the chances of mosquitoes resting on the cone instead of the treated nets, some studies [17] put net flaps inside the cones. While this could work well and ensure increased contact with the net, it reduces the comparability of results between studies and laboratories. The wire-ball test, an alternative to the cone assay, overcomes the problem of mosquitoes resting on untreated surfaces. It consists of a cubical 15 cm x 15 cm x 15 cm or two intersecting circles of about 15 cm diameter wire frame around which the piece of netting is wrapped [8], thereby reducing the amount untreated surfaces inside the exposure space. However, before this test can be used, it has to be calibrated with the WHO cone assay [8].

Aside from the evaluation of LNs, the cone assay is also used for testing the insecticide bioavailability and residual activity of insecticide-treated substrates such as housing materials after indoor residual spraying (IRS) [8] and durable wall linings [18] in small-scale (Phase II) and large-scale (Phase III) WHOPES field trials. For this purpose, the cones are attached to the walls and ceilings of the experimental huts or houses. This automatically results in different angles that could range from 0° (flat) to 180° (upside down). Although in this study only nets were tested and the effects at 180° were not evaluated, it is expected that the variation in the angles will result in a similar outcome in other substrates. The mosquitoes are exposed for a longer period of 30 min, but an increased number of 10 mosquitoes could result in individuals being affected by both the angle and the presence of other flying mosquitoes, thereby making less contact with the treated surface. This situation could be even worse in the presence of an irritant. The observation that the susceptible Kisumu-1 strain spent less time in contact with the net as compared to the resistant STI strain (Figure 3.5) suggests that any irritancy property of the insecticide had a stronger effect on the susceptible mosquitoes.

While the data from the current study show that mosquitoes tend to spend more time on the net at 60°, there is a valid argument for performing the assay at 0°. A recent study showed that most of the activities and net contact of host-seeking mosquitoes occur on the top side of the net [19]. Therefore, testing at 0° could provide a realistic assessment of the kill ability of the nets.

Apart from the angle, another detail which could influence the amount of time the mosquito spends in contact with the net is how they are blown into the cone. It is not yet defined whether the mosquitoes have to be blown directly onto the net or rather blown to fly freely in the cone. This could affect the resting or flying behaviour of the mosquitoes in the cone and could influence the amount of time they spend in touch with the net. Making initial contact with the net upon introduction into the cone could also result in mosquitoes picking up insecticide, which could make a difference in the observed mortality, especially in the case of testing high concentrations of insecticide. This hypothesis would be in agreement with Sternberg et al. [20] who also suggested that accidental contact of mosquitoes to treated materials beyond exposure periods could alter the outcome of the assay.

3.6. Conclusion

From the results of this study, the inclination at which the test is performed is rather an important component of the assay and changing the angle leads to inconsistencies in the outcome. The WHOPES guidelines should be explicit in defining a working angle in all instances where the cone assay is used for the evaluation of treated substrates. From the data shown here, performing the test at an inclined angle results in more contact with the treated surface than on a flat surface and the 45° implicitly suggested in the WHOPES guidelines is set at a comfortable working slope. Clearly stating this angle in the WHOPES guidelines as the standard working angle is highly recommended.

3.7. Additional file

https://static-content.springer.com/esm/art%3A10.1186%2Fs12936-016-1303-9/MediaObjects/12936_2016_1303_MOESM1_ESM.avi

Additional file 1. Side-by-side video showing how two mosquitoes exposed at 60° (left) and 0° (right) behaved in the 3 min of exposure.

3.8. Authors' contributions

HFO and PM conceived the idea of the project and developed the experimental design. HFO performed laboratory experiments. HFO and PM analysed and interpreted the data. HFO

drafted the manuscript and PM contributed to the draft. Both authors read and approved the final manuscript.

3.9. Acknowledgements

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3.10. Competing interests

The authors declare that they have no competing interests.

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4. Insecticide susceptibility of Anopheles mosquitoes changes in response to variations in the larval environment

Henry F Owusu[1, 2, 3], Nakul Chitnis[1, 2] and Pie Müller[1, 2]*

[1]Epidemiology and Public Health Department, Swiss Tropical and Public Health Institute, Socinstrasse 57, PO Box, CH-4002 Basel, Switzerland

[2]University of Basel, Petersplatz 1, CH-2003 Basel, Switzerland

[3]Department of Immunology and Infectious Diseases, Harvard T.H. Chan School of Public Health, 665 Huntington Ave, Boston, MA 02115, U.S.A

*Corresponding author

Email addresses:

HFO: henry.owusu@unibas.ch

PM: pie.mueller@swisstph.ch

NK: nakul.chitnis@swisstph.ch

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4.1. Abstract

Insecticide resistance threatens the success achieved through vector control in reducing the burden of malaria. An understanding of insecticide resistance mechanisms would help to develop novel tools and strategies to restore the efficacy of insecticides. Although we have substantially improved our understanding of the genetic basis of insecticide resistance over the last decade, we still know little of how environmental variations influence the mosquito phenotype. Here, we measured how variations in larval rearing conditions change the insecticide susceptibility phenotype of adult *Anopheles* mosquitoes. *Anopheles gambiae* and *A. stephensi* larvae were bred under different combinations of temperature, population density and nutrition, and the emerging adults were exposed to permethrin. Mosquitoes bred under different conditions showed considerable changes in mortality rates and body weight, with nutrition being the major factor. Weight is a strong predictor of insecticide susceptibility and bigger mosquitoes are more likely to survive insecticide treatment. The changes can be substantial, such that the same mosquito colony may be considered fully susceptible or highly resistant when judged by World Health Organization discriminatory concentrations. The results shown here emphasise the importance of the environmental background in developing insecticide resistance phenotypes, and caution for the interpretation of data generated by insecticide susceptibility assays.

Key words

Mosquitoes, environment, insecticide resistance, nutrition, temperature, larval density

4.2. Introduction

Insecticide resistance in mosquitoes, especially in malaria endemic regions, has increased dramatically in the last decade[1,2]. With only four classes of insecticides available for adult mosquito control, and only pyrethroids accepted for long lasting insecticidalnets (LLINs), the World Health Organization (WHO) has scaled up efforts to control the rapid spread of resistance. These efforts were strengthened with the launch of the Global Plan for Insecticide Resistance Management in 2012[3].

Attempts to reduce the likelihood of the development of resistance to insecticide as well as to improve the success rates of interventions against disease vectors have led to the promotion of an integrated approach to the control of vector-borne diseases[4]. These combined interventions have been shown to be effective in significantly reducing malaria morbidity and mortality[5]. Until the 1950s, larval source management (LSM) was the principal malaria control method[6]. The discovery of chemical insecticides saw LSM relegated to the background, but the recent increased preference for combined interventions has led to a resurgence of its application, and today, there is renewed interest in LSM to reduce mosquito populations[6]. There is strong evidence showing that eliminating breeding sites and targeting immature stages of mosquitoes significantly impact the incidence and prevalence rates of mosquito-borne diseases[7–10]. As a result, the WHO accordingly recommended larviciding as an important supplement to core interventions in 2012[11]. The growing interest in LSM also brings into the spotlight the interaction of the larval stage with the environment, since the implementation of LSM largely involves either manipulation or modification of the environment in which the larvae live[6]. All LSM activities aim at reducing larval population numbers, which directly or indirectly alter the level of competition for space and resources available to the surviving individuals.

The mosquito is dependent on the environment for the completion of its life cycle[8], and the conditions encountered during the early developmental stages may have strong downstream effects on the life history traits. The larval stage is essential in the life cycle of the mosquito because it is the only immature stage where feeding takes place, making it important for the accumulation of nutritional reserves for the development of the adult in the pupal stage[12,13]. The responses of the larval stage to varying conditions could therefore play

an important role in population size regulation, and understanding these responses will contribute significantly to effective control measures. Mosquito life history traits such as larval survival, adult fecundity, longevity, susceptibility to viruses and size as well as biting behaviour have been shown to be affected by temperature, nutrition and population density during the larval stage in both field and laboratory populations[14–19]. At the centre of how the larva-environment-adult complex plays out is the role of body size in determining observed phenotypes. Changes in the larval stage can result in faster maturing offspring with larger body sizes[20,21] which have been argued to live longer, therefore increasing their potential to transmit diseases[22]. Adult body size, and for that matter weight, has been shown to influence several biological traits such as rates of gaseous exchange and metabolic rates[23]. The emerging interest in the importance of the larval stage has led to several studies investigating the impact of the environmental breeding conditions during the larval stage on different life history traits of the imago[24–27]. Little has been done, however, to understand and quantify its relevance to insecticide resistance. The complex nature of insecticide resistance requires a thorough knowledge and understanding of the environmental, physiological and genetic interactions that interplay between the larval and adult stages to produce observed phenotypes.

Currently, there are still a number of elementary questions that remain unanswered regarding which factors play a role in producing an observed phenotype in the susceptibility of mosquitoes to insecticides and the magnitude of their effects. The extent to which insecticide susceptibility test results are dependent on body size is not well understood[28]. While previous studies have shown that larval nutrition and adult size correlate with susceptibility of the adults to insecticides[29,30], nutrition might not be the only factor involved and other elements may equally affect susceptibility or contribute to the effect of nutrition. The effect of breeding conditions on susceptibility may also vary between susceptible and resistance strains, due to differences in energy and resource usage and needs.

This study investigated the influence of temperature, nutrition and crowding during the larval stage and adult body weight on the susceptibility of *Anopheles gambiae* KISUMU1 and *A. stephensi* STI adults to insecticides. The implications of the observed effects for insecticide resistance monitoring are discussed.

4.3. Results

Mosquito larvae were reared under two levels (high and low) each of temperature, nutrition and population density in a factorial experimental design. The emerging adults were tested against a previously established LC_{50} for permethrin in the WHO susceptibility assay. The estimated permethrin LC_{50} values for the STI and KISUMU1 colonies were 0.125% (95% confidence interval, CI: 0.023–0.255%) and 0.068% (95% CI: 0.018–0.118%), respectively. The observed 24 hour mortality against these LC_{50} values in the WHO assay varied between the treatment groups. In the STI strain, the highest mortality was recorded in treatment group “c” (low temperature, low nutrition and high larval density) at 79.7% and the lowest was 43.4% in group “b” (low temperature, high nutrition and low density). With 67.3%, group “ab” (high temperature, high nutrition and low density) had the lowest mortality in the susceptible KISUMU1 strain and group “ac” (high temperature, low nutrition and high density) had the highest mortality at 97.7%. A summary of the odds ratios (OR), p-values and 95% confidence intervals from the regression models in the two species is given in Table 4.1. From the models, nutrition had the biggest influence on mortality. Lower amounts of larval nutrition significantly increased adult mortality in both strains, with ORs of 4.4 (95% CI = 2.7–7.1, $p < 0.001$) in the KISUMU1 strain and 3.0 (95% CI = 2.2–4.1, $p < 0.001$) in STI. Larvae growing at lower levels of population density and temperature were found to be protective for the adults, in both the KISUMU1 (temperature: OR = 0.4, 95% CI = 0.2–0.7, $p = 0.003$; density: OR = 0.3, 95% CI = 0.1–0.5, $p < 0.001$) and the STI (temperature: OR = 0.7, 95% CI = 0.5–0.9, $p = 0.02$; density: OR = 0.5, 95% CI = 0.4–0.7, $p < 0.001$) models. While there was no interaction between any of the factors in the STI model, there was a significant interaction between temperature and population density in the KISUMU1 model (OR = 3.1, 95% CI = 1.3–7.3, $p = 0.01$). This indicates that the effect of temperature on mortality was different at low population density from the effect at high density in KISUMU1.

Table 4.1: The output of the regression model for the effect of the factors on mortality in the two colonies

Colony	Factor	Odds ratio	95% CI	p value
STI	Nutrition (low)	3.0	2.2 – 4.1	< 0.001
	Temperature (low)	0.7	0.5 – 0.9	0.02
	Density (low)	0.5	0.4 - 0.7	< 0.001
KISUMU1	Nutrition (low)	4.4	2.7 – 7.1	< 0.001
	Temperature (low)	0.4	0.2 – 0.8	0.005
	Density (low)	0.3	0.1 - 0.5	< 0.001
	Interaction: Temp x Density	2.8	1.3 - 6.2	0.01

After the WHO susceptibility assay, dead mosquitoes were separated from live ones and their dry weights were measured. The recorded weights varied considerably across the treatments in both colonies (Figure 4.1) and ranged from 0.088 to 0.965 mg with a mean of 0.361 mg (95% CI = 0.356–0.368 mg). In both the STI (OR = 0.0002, CI = 0.00004–0.0009, $p < 0.001$) and KISUMU1 (OR = 0.00003, 95% CI = 0.00004–0.0002, $p < 0.001$) strains, heavier mosquitoes were significantly more likely to survive the treatment with permethrin (Figure 4.2).

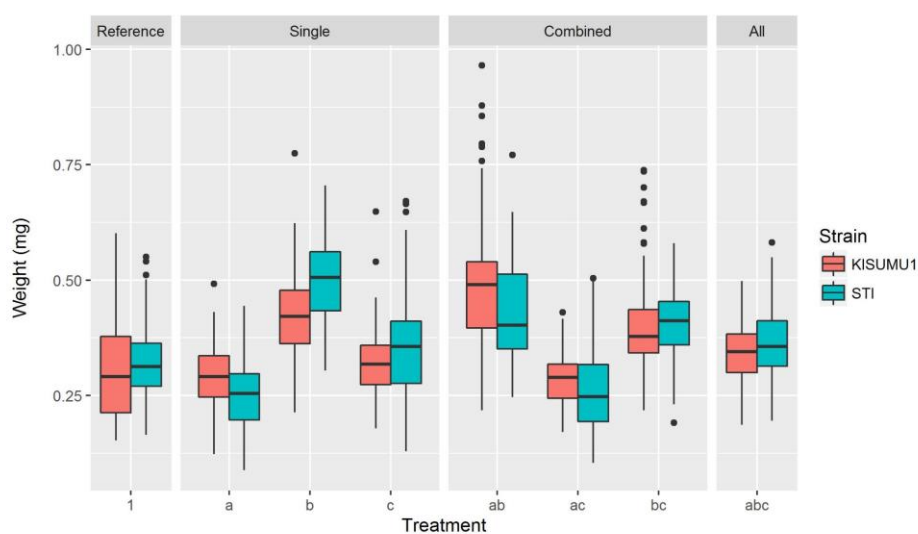

Figure 4.1: The temperature and humidity controlled cabinet used for weighing dried mosquitoes (a) and the weighing scale (b)

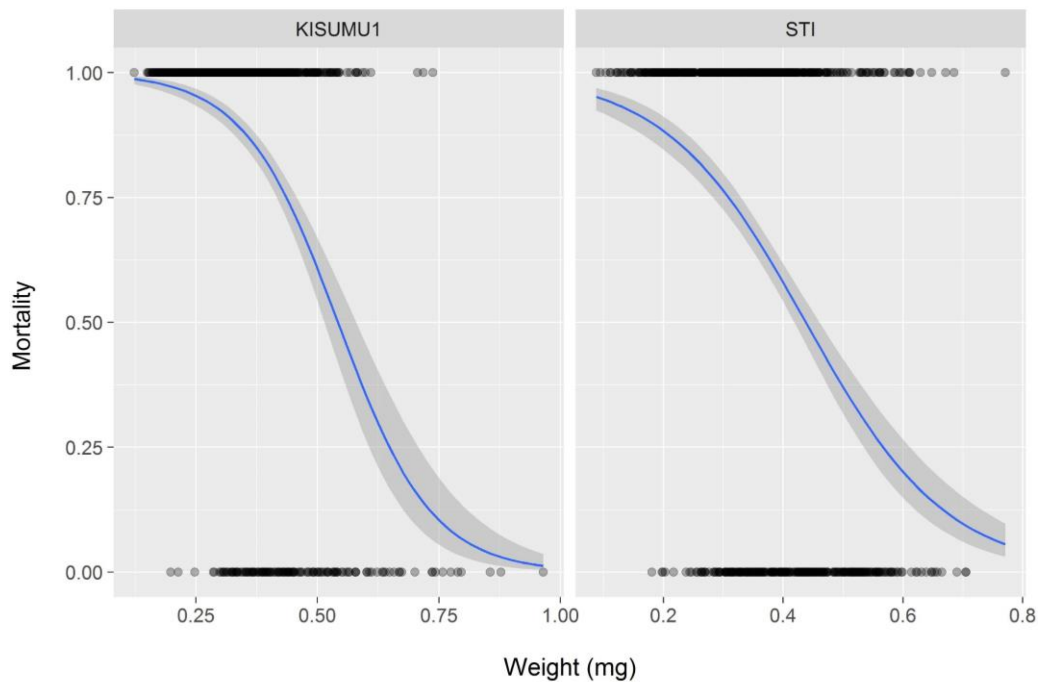

Figure 4.2: Mortality as a function of body weight. Dots represent individual mosquitoes (1 = dead, 0 = alive) and the lines show the predicted odds and the 95% confidence intervals (shaded areas) of dying as a function of weight.

Following the data obtained from the breeding experiment, nutrition turned out to be the most prominent factor affecting weight. We therefore adjusted the amount of food fed to the larvae to produce two new groups of mosquitoes (well fed and starved) with extreme body sizes to evaluate the practical implications of body size on susceptibility to insecticides. The "well fed" group was provided with 4x the amount given to the standard group per larva (Table 4.2) whereas the starved group was given ¼ of the standard amount. Emerging adults were tested in a dose-response assay and logistic regressions were used to estimate the LC_{50} values of the two groups.

Table 4.2: Feeding protocol used under standard rearing conditions at 27 °C and a density of 300 larvae per tray. In the case of extended development time, the larvae were fed 0.4 mg on alternate days

Day	Larval stage	Amount of food per larva (mg)
1	Hatching L1	0.1
2	L1/L2	No feeding
3	L2	0.1
4	L2/L3	No feeding
5	L3	0.4
6	L3/L4	No feeding
7	L4	0.4
8	L4/pupae	No feeding

In both strains, the well fed mosquitoes appeared larger than their starved counterparts as illustrated by the two specimens in Figure 4.3. The predicted LC_{50} values (Table 4.3) showed that in both strains, the estimates for the two groups were higher in the well fed and lower in the starved groups than the values obtained in the reference groups (0.068% for KISUMU1 and 0.125% for STI). In KISUMU1, the predicted values for the well fed and starved groups were 0.079% and 0.062% respectively. There was a bigger shift in LC_{50} in the resistant STI strain in favour of the larger mosquitoes. The well fed group had an LC_{50} of 0.67% as compared to 0.103% for the starved group, a 6.5 fold increase.

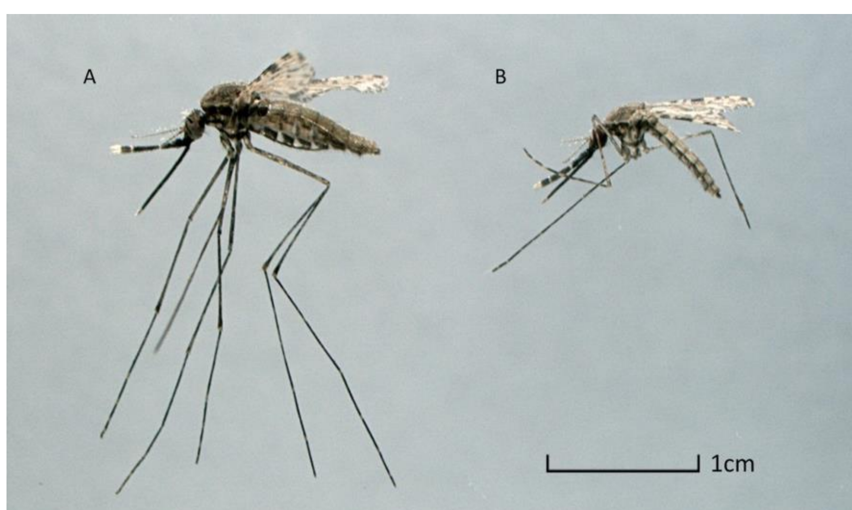

Figure 4.3: Examples of a well fed (A) and a starved (B) individual of the *A. stephensi* STI strain from the dose-response assay.

Table 4.3: Predicted values of LC_{50} and mortality at 0.75% permethrin concentration from the two treatment groups compared against the observed mortality in the reference group. The figures for the standard group were observed values obtained from WHO insecticide susceptibility bioassays. [1]The nutritional amount is given as the ratio of food provided as compared to the standard condition in Table 4.4

Strain	Group	Nutritional amount[1]	LC_{50} [%] (95% CI)	Mortality at 0.75% permethrin (95% CI)
KISUMU1	Starved	0.25	0.062 (0.03, 0.121)	0.996 (0.994 – 1.0)
	Standard	1.00	0.068 (0.018, 0.118)	0.99 (0.941 – 1.0)
	Well fed	4.00	0.079 (0.011, 0.148)	0.998 (0.997 – 1.0)
STI	Starved	0.25	0.103 (0.005, 0.201)	0.98 (0.97 – 0.99)
	Standard	1.00	0.125 (0.023, 0.255)	0.71 (0.62 – 0.79)
	Well fed	4.00	0.670 (0.486, 0.854)	0.65 (0.59 – 0.71)

From the dose-response curve, the predicted mortality at 0.75% permethrin, the WHOPES discriminatory concentration [28], showed a change in mortality between the groups in the STI strain but not in the KISUMU1 strain (Table 4.3, Figure 4.4). The change is so dramatic that when starved, the population would be considered susceptible (i.e. 98% mortality), while in the presence of abundant nutrition the colony becomes resistant (i.e. 65% mortality) to permethrin.

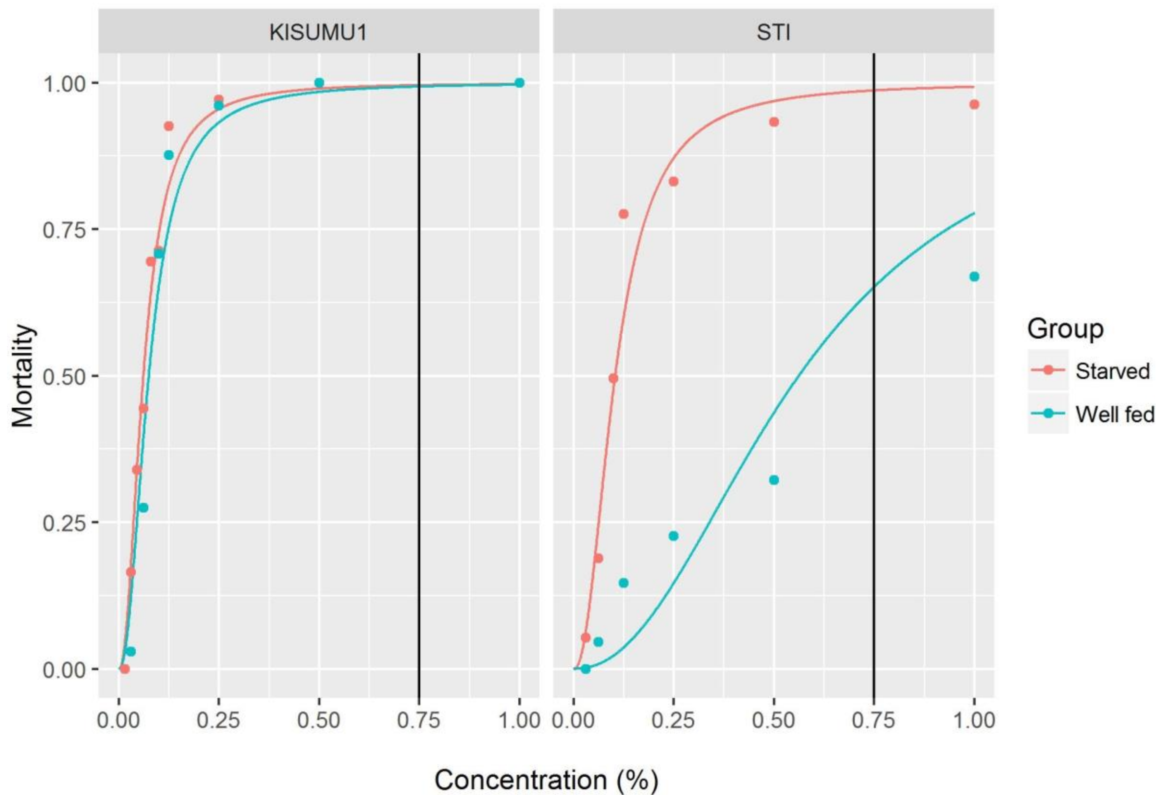

Figure 4.4: Dose-response curves for showing the mortality as a function of permethrin concentration for the starved and well fed groups. The dots show the summary mortalities measured at different insecticide concentrations on the filter papers in the WHO insecticide susceptibility assays. The lines are the predicted curves based on the statistical models.

4.4. Discussion

The data presented here show that larval breeding conditions in *Anopheles* mosquitoes considerably influence the susceptibility of the adults to permethrin, and that adult dry weight is a very strong predictor of 24 hours mortality.

The effects of changes in larval breeding conditions on the fitness of the adult mosquito has been the subject of multiple studies. Both field and laboratory based studies, have shown that changes in environmental conditions during the larval stage significantly affect several life history traits such as larval survival[31] and adult longevity[18]. The current study demonstrates that temperature, crowding and availability of nutrition during the larval stages directly or indirectly modulate 24 hours mortality in the adult when exposed to permethrin. There is strong evidence that decreasing larval density and higher nutrition lead to improved survival and faster development time in the immature stages as well as increased biting

frequencies, longer survival, wing length and blood meal duration leading to increased vectorial capacity in the adult[13,15,32–34]. In relation to insecticide resistance, the current data are consistent with the findings of two previous studies in which *Anopheles* mosquitoes were exposed to DDT after varying larval nutrition[29,30]. It was noted that nutrition might be the most important factor and that the effect of density could be compensated by the availability of food. From the data presented here, larvae that were bred under high density with high nutrition had lower mortality than those at low density and low nutrition.

In the current study, as an intermediate outcome, dry weight had a very strong association with mortality in both the insecticide resistant and the susceptible strains. Like in previous studies, at high population density, emerging adults were smaller[25,35] and more susceptible to insecticides. The effect of crowding could be the result of physical interactions rather than chemical, as suggested by Roberts & Kokkinn[36] who observed that moving larvae constantly bumping into others and causing waves of disturbances in the feeding larvae, are the main cause of the crowding effect. Consistent with previous findings[37,38], the heaviest groups in both species were observed when nutrition was high and larval density was low. Just as in some preceding studies[16,39,40], mosquitoes bred at the lower temperature weighed heavier than those reared at the higher temperature, although some studies found no association[41]. In his review, Atkinson found that about 90% of aquatic organisms showed a reduced body size at a higher temperature, and noted that this seemed paradoxical as higher temperatures would be expected to increase metabolism[42]. Possible explanations could be that organisms in higher temperatures have a higher metabolic rate and are also more active, thereby expending more energy, which results in the smaller body sizes or 32 ˚C is rather in the zone where larval development is on the decline and closer to the upper lethal temperature[43]. Although this study was conducted in the laboratory, the levels of the conditions experimented are not far off from field conditions. Larval breeding sites vary in size and it will not be unusual to have large numbers of larvae crowded in a small habitat or a few with abundant food. The temperature values chosen have also been recorded in field mosquito habitats[44,45].

As the focus on the effects of the environment on mosquito dynamics intensifies, the role of body weight in these dynamics is also becoming more apparent and it may hold an important key in future mosquito control strategies. It may be the most important

physiological proxy in the observed interactions between the environment and the life history traits, since the majority of the beneficial traits have been observed in bigger mosquitoes. In addition to larger offspring being more tolerant to insecticides as demonstrated here, they have also been shown to be more efficient vectors by being better at finding hosts [34] and having as much as a three-fold relative increase in transmission potential than smaller mosquitoes[32]. Although larger males might not be the most successful in finding partners during mating[46], their female counterparts survive better and have a higher probability of being inseminated and producing more egg batches than smaller ones[22].

The role of weight in defining mortality after treatment with insecticide might be linked to the amount of nutritional reserves available. Both wing length and weight are manifestations of body size[38]. But for the purpose of this study, dry weight was measured. This is because mosquitoes with similar wing size could still differ in weight even when bred under the same conditions due to their differences in the accumulation of proteins and lipids[47]. Studies have shown that the energy reserves in mosquitoes are size-dependent[13] and larvae bred on high nutrition have higher reserves than those on a lower diet [48]. In other studies, resistant mosquitoes were found to contain less energy reserves than susceptible ones[49,50], suggesting that they use up the energy reserves to activate processes that overcome the toxins.

As much as dry weight is strongly associated with mortality, it is an interesting observation that there is no such association between weight and time-to-knockdown (Supplementary Information online). This emphasises that the time-to-knockdown and mortality are not directly linked [51] but rather more complex and the two endpoints do not share the same predictors.

A shortfall of this study is that changes at the genetic level were not measured. Lehmann[38] indicated that there is substantial genetic contribution to phenotypic variations observed in adaptive traits. It would therefore be valuable to know how the phenotypic changes observed are genetically controlled. It would also be important to find out what kind of resistance mechanism is acquired, if any. As mentioned, it has been shown before that changes during the larval stage influences the insecticide susceptibility of the adult mosquito[29], yet, this study went a step further by looking at both single and combined factors and as well as providing an

insight into the practical implications of the effects by showing that in a resistant strain, LC_{50} can increase by about 6 fold when food is abundant.

The effect of incomplete larviciding on the population dynamics of mosquitoes is a very important subject for discussion; the fate of larvae that survive control activities will influence the overall impact of larviciding on malaria transmission, since the surviving larvae will develop under conditions of low density and high nutrition. From this and other laboratory studies[29,30] larval conditions affect insecticide resistance and the practical impact on vector control cannot be ignored. While resulting larger mosquitoes have several advantages, this study shows that the resistance threshold is significantly shifted in their favour as well. The predicted mortalities at the WHO discriminating concentration for permethrin (i.e. 0.75%) suggest that a change in weight has little effect in the apparent absence of insecticide resistance mechanisms but does have a considerable impact in the presence of resistance mechanisms. A further step in understanding the dynamics would be to evaluate field populations. Energetic reserves have been found to vary in different directions between field and laboratory populations[52,53] but a similar impact of weight on resistance as we found in this study may be expected.

The results of this study have considerable consequences for product development involving screening of insecticide resistant lab colonies. Different breeding conditions may result in divergent adult phenotypes. These factors may contribute to inter- and even intra-laboratory inconsistencies in bioassay results upon which vector control product efficacy assessments are based. Laboratories must be charged with maintaining high consistency in the density and amount of nutrition provided to larvae and regularly control weight or size of adults to ensure consistent test mosquitoes. It is, therefore, highly recommended that such practices are included in test guidelines and quality control procedures.

The findings from this study strengthen the importance of environmental contributions to the expression of resistance to insecticides in mosquitoes. The need to give environmental factors more attention in addition to the genetic background of mosquitoes, particularly in using resistant strains is strongly advocated.

4.5. Methods

4.5.1. Mosquito colonies and standard rearing conditions

Laboratory colonies of a pyrethroid resistant *A. stephensi* (STI) and a pyrethroid susceptible *A. gambiae* s.s. (KISUMU1, MRA-762) strains[54] were used. The STI colony was obtained from the London School of Hygiene and Tropical Medicine in 1971 and the KISUMU1 strain was obtained from the Malaria Research and Reference Reagent Resource Center (MR4) in 2011. Adult mosquitoes were maintained in plastic 30 cm x 30 cm x 30 cm BugDorm cages (MegaView Science, Taiwan) on 10% sucrose solution at a temperature of 26 - 28 °C and a relative humidity of 60 - 74% in a 12:12 hours day:night regime. The females were fed with pig blood and 72 hours later, each cage was provided with an 80 mm diameter crystallizing dish, filled to a depth of about two centimetres with water to collect the eggs. To prevent the eggs from sticking to the glass and drying out, the rim of the dishes were lined with filter paper. The egg dishes were left in the cages for 24 hours after which they were removed and the harvested eggs were left in the dishes to hatch. Three hundred newly hatched larvae were then transferred using a Pasteur pipette into 30 cm x 19 cm x 8 cm larval trays filled to a depth of 1 cm with tap water treated with AquaSafe (Tetra, Germany). The larvae were fed on ground Tetramin fish food (Tetra, Germany) according to the protocol in Table 4.2.

4.5.2. Larval rearing experiments

In a preliminary experiment, dose-response curves for the KISUMU1 and STI strains were estimated to define the lethal concentration at which 50% of the mosquito population would be killed (LC_{50}) using the WHO susceptibility assay kit[28]. Three to five-day-old females obtained from larvae reared under the standard rearing conditions (see above) where exposed in WHO tubes to filter papers (Whatman No. 1) impregnated with permethrin (25:75 cis:trans ratio) at concentrations of 1%, 0.5%, 0.25%, 0.125%, 0.0625%, 0.0313% and a negative control containing no insecticide for 1 hour. The filter papers were prepared from mixtures of insecticides dissolved in acetone and Dow Corning 556 Silicon fluid. At each concentration, a minimum of 100 female mosquitoes were exposed in batches of 24 to 33 individuals. After the exposure, mosquitoes were transferred from the test to the holding tube, provided with 10% sucrose solution and held for 24 hours after which mortality was recorded.

In the next experiment, mosquito larvae were reared under different rearing conditions and the emerging adults were exposed to the established LC_{50}. The LC_{50} guaranteed that the effect of a rearing condition on the insecticide susceptibility could be measured in both directions, that is, either an increase or a decrease in permethrin susceptibility. Adult mosquitoes were reared as described above, and newly hatched larvae were split into different larval trays and each tray subjected to a specific rearing condition (i.e. treatment) to investigating the effects of temperature (A), population density (B) and the amount of food per larva (C) as well as their combinations. Each of the three factors was set at two levels, low and high, yielding 8 different combinations of rearing conditions (Table 4.4). Temperature was set either at 24 °C or 32 °C. Population density had a low level of 150 larvae per tray (half the standard conditions) and a high level of 600 larvae per tray (twice the standard conditions). Nutrition was either half or double the standard amount of food indicated in Table 4.2. The amount of food was adjusted for the larval density in order to separate the effects of nutrition from that of population density. The trays were then placed in a thermostatic cabinet (AQUALYTIC, Germany; Model AL654) to regulate air temperature, while the water level was checked daily and topped up when necessary. Temperature was continuously monitored with a Log 32 TH Data Logger (Dostmann Electronic, Germany). Emerging adult females were split by treatment and transferred to separate 30 cm × 30 cm × 30 cm plastic cages. Three to five-day-old adult females were exposed in batches of 20 to 28 to filter papers treated with the previously established LC_{50} in the WHO susceptibility test. After an exposure period of 1 hour, the mosquitoes were transferred into a holding tube, provided with 10% sucrose solution and held for 24 hours after which mortality was scored. Each treatment combination was replicated in at least 4 trays and a minimum of 100 adult mosquitoes were tested for insecticide susceptibility.

Table 4.4: Larval rearing conditions used in the factorial experiment. [1]The food ratio indicates the ratio of the amount of food fed to the larvae as compared to the standard rearing conditions (Table 4.2). In the reference treatment all factors were set at the lower levels.

Treatment	Rearing condition	Temperature (°C)	Food/density ratio[1]	Larvae per tray
1	Reference	24	0.5	150
a	High temperature	32	0.5	150
b	High food	24	2.0	150
c	High density	24	0.5	600
ab	High temperature, High food	32	2.0	150
ac	High temperature High density	32	0.5	600
bc	High food, High density	24	2.0	600
abc	High temperature, High food High density	32	2.0	600

Based on the outcome of the larval rearing experiment, a second rearing experiment was carried out in which larvae from each colony were reared under the conditions that yielded the least and the most susceptible adults in order to assess the practical implications rearing conditions would have on the dose-response and the interpretation of WHO insecticide susceptibility assay data at the discriminatory concentration. Again a minimum of 100 females in batches of 22 to 32 individuals were exposed for 1 hour at the permethrin concentrations used to establish the initial dose-response curve. After the exposure, mosquitoes were transferred from the test to the holding tube, provided with 10% sucrose solution and held for 24 hours after which mortality was recorded.

4.5.3. Measuring relationship between mortality and body weight

In order to measure the relationship between mortality and weight, dead and alive mosquitoes from the susceptibility assays were separated and their dry body weight measured. In preparation for the weighing, mosquitoes were transferred to punctured 1.5 ml Eppendorf tubes. While dead mosquitoes were transferred directly, live mosquitoes were first killed in a −20 °C freezer. The tubes were then left over silica gel in a sealed Tupperware box for at least 14 days to ensure complete and uniform drying. To avoid irregularities in dry weight introduced by re-absorption of atmospheric moisture, the weighing was carried out in a temperature and humidity-controlled cabinet. The mosquitoes were moved into the cabinet containing the balance at least 24 hours prior to weighing to condition them to the pre-set temperature and relative humidity. The temperature and relative humidity ranged between

21–25 °C and 30–40%, respectively. Once conditioned the mosquitoes were weighed to the µg using a UMX2 micro balance (Mettler Toledo, Switzerland).

To further understand the relationship between weight and mortality, we tested mosquitoes to investigate whether dry weight predicts time-to-knockdown in a modified CDC bottle assay[51]. The findings are provided as Supplementary Information which is available online.

4.5.4. Data analysis

All data analysis was done in the freely available statistical software package R, version 3.2.0[55]. The LC_{50} values for the susceptibility assays were calculated on the basis of a predicted dose-response curve which was fitted assuming a binomial distribution and a logit link function. The effects of temperature, nutrition and density on mortality were analysed by generalised linear mixed-effect models (GLMM)[56]. In the GLMM, mortality was predicted by the independent variables temperature, population density and nutrition which were treated as fixed terms. Each treatment was replicated at least four times and the tray number was introduced into the model as a random term to account for variability among trays. The GLMMs were modelled using the lme4 package in R[56,57]. The relationship between mortality and weight was predicted by a logistic regression. The practical implications of different larval breeding conditions on the susceptibility of adults to insecticides were modelled by dose-response curves in logistic regressions which assumed a quasibinomial distribution. The models were run separately for the two species. The significance level was set at $\alpha = 0.05$. All graphs were generated with the package ggplot2[58].

4.6. Acknowledgments

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4.7. Author Contributions

H.F.O., N.C. and P.M. conceived the idea of the project and were all involved in the development of the experimental design. H.F.O. performed laboratory experiments and together with P.M. analysed and interpreted the data. H.F.O. drafted the manuscript and all authors contributed to the writing of the manuscript. All authors read, reviewed and approved the final manuscript.

4.8. Competing financial interests

The authors declare that they have no competing interests.

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5. A mathematical model to estimate the effects of larval density on adult mortality

Henry F. Owusu and Nakul Chitnis

Epidemiology and Public Health Department, Swiss Tropical and Public Health Institute, Socinstrasse 57, PO Box, CH-4002 Basel, Switzerland

University of Basel, Petersplatz 1, CH-2003 Basel, Switzerland

5.1. Introduction

Malaria continues to pose a significant public health threat in spite of intensified efforts, causing about 438,000 annual deaths globally [1]. The current global approaches to protecting individuals and populations from malaria focus on vector control to reduce transmission, chemoprevention which is directed at blood-stage infection in humans and prompt diagnosis and treatment of cases. Vector control has remained an integral part of measures to control mosquito-borne diseases since the close of the 19th and beginning of the 20th centuries [2, 3]. It is largely implemented through the use of insecticide-treated mosquito nets (ITNs) and indoor residual spraying (IRS). Despite the success of these strategies in reducing malaria morbidity, supplementary measures are still needed to maintain the momentum. One downside of ITNs and IRS is that they mainly target indoor biting vectors. Recently, larval source management is gaining more attention as a supplementary strategy [4], in that reducing larval habitats leads to a decrease in the populations of both indoor and outdoor biting vectors, consequently resulting in further suppression of transmission. The increase in the popularity of larval control has led to an increase in the focus on the importance of the larval stage to the population dynamics of the mosquito. As shown by several studies, the larval stage has proved to be a very important stage of the mosquito where changes in conditions affect the life history traits of the mosquito [5–9].

It is of utmost importance to fully understand the effect of incomplete larval control, which results in changes in the amounts and balance between population and nutrition. With the majority of the cases and deaths from malaria occur in the temperate regions of Africa [1], climate, and for that matter, temperature is also a very driver of malaria [10]. The interactions of the vector with these factors are very complex. To gain a better understanding, laboratory investigations are not enough to fill in all the gaps. The use of mathematical models provides an essential means to simulate and evaluate data which are otherwise impossible or difficult to collect. Ross was the first to develop mathematical models to explain malaria transmission [11] and since then, models have become central in malaria control. With its growing application in public health, it is emerging as an important tool for planning and developing malaria control strategies. Some models have been used to explain the effects of temperature alone malaria transmission [12], whereas others have looked at climate change and other

interventions [13] as well as the dynamics of malaria and mosquito populations and species distribution and seasonal variations [14, 15].

In this study, we assessed the effects of breeding conditions of the larval stage on the mortality of adults in *Anopheles* mosquitoes using a basic description of the larval and adult populations and incorporating density dependent (nutrition and crowding) and density independent (temperature) factors.

5.2. Methods and results

The models consisted of a series of differential equations based on data on life history traits collected from laboratory bred anopheles gambiae KISMU1 strain and the simulations were run in the programming software R [16].

5.2.1. State variables and parameters

The model looked at the dynamics of the larval (L) and adult (N) populations. A two-step approach was used to estimate the effect of larval conditions on adult survival. In the first step, ordinary differential equations (ODE) were used to estimate the change in adult population with the assumption that adult mortality is constant. Delay differential equations (DDE) were then used in the second step to assess the effect of past larval density on adult mortality. A lag time of 10 days was introduced and cumulative larval density over the lag period was given as D. Tables 5.1 and 5.2 show definitions and estimates of the parameters and variables. The adult rate of laying eggs was calculated based on the average number of eggs laid by 25 female mosquitoes kept individually in one oviposition cycle. The length of the lag period τ_1, the duration of the larval period δ and the proportion of larvae that survive to pupation β were assumed based on data from my master thesis. The proportion of eggs hatching into larvae [17] was estimated from literature. The adult and larval population numbers were assumed. All other parameters were calculated mathematically.

Table 5.1: Definitions of the state variables

State variable	Definition
N	Number of adults
L	number of larvae
D	cumulative larval population density

Table 5.2: Definitions of the parameters and variables

Param/var	Definition	Estimate
ε	reciprocal of larval development time	0.09
	Proportion of eggs hatching into larvae	0.9
μ	Adult mortality rate	0.04
t	Time	NA
τ_1	Time since hatching of larvae	11.5
τ_2	time since adult emergence	0
f	function relating larval density to adult mortality	
φ	adult rate of producing female larvae from eggs	30
δ	duration of larval period	11.5
β	proportion of larvae that survive to pupation	0.8
μ_L	density independent larval death rate	0.02
μ_{2L}	density dependent larval death rate	0.001
$\tau_1$and τ_2	limits of time where larval density affects adult fitness	NA
$g(s)$	weighting function for adult mortality	1
α	constant factor weighting adult mortality	
ω	the factor translating larval density into adult death rate	0.000004

5.2.2. **Equations**

The impact of larval density in the past on the current adult mortality rate was given by:

$$\mu(t) = \alpha \int_{t-\tau_1}^{t-\tau_2} g(s) f\big(L(s)\big) ds$$

In the simplest case, $g(s) = 1$ and $f(x)= x$. Therefore:

$$\mu(t) = \alpha \int_{t-\tau_1}^{t-\tau_2} \big(L(s)\big) ds$$

The adult and larval populations were given by the equations:

$$\frac{dN}{dt} = \varepsilon L - \mu(t) N \text{ .... (1)}$$

and:

$$\frac{dL}{dt} = \varphi N - \mu_L L - \mu_{2L} L^2 - \varepsilon L \text{ .... (2)}$$

5.2.3. **Step 1- ODE system when adult mortality is constant with larval density**

Assuming the average span of *Anopheles* mosquitoes in the lab is about 25 days in the lab [18] and the larval duration is 11.5 days (from Master thesis), the adult mortality rate, μ will be given by $\frac{1}{25} = 0.04$ and $\varepsilon = \frac{1}{11.5} = 0.09$. At the equilibrium state, the changes in the larval and adult populations are 0. That is;

$$\varepsilon L - \overline{\mu} N = 0 \text{ .... (3)}$$

And

$$\varphi N - \mu_L L - \mu_{2L} L^2 - \varepsilon L = 0 \text{ .... (4)}$$

Finding N in equation (3), substituting it into equation (4) and solving for L leads to three sets of equations which relate ε, μ_L and μ_{2L};

$$\frac{1}{\varepsilon + \mu_L + \mu_{2L} L^*} = \delta \text{ .... (5),}$$

$$\frac{\varepsilon}{\varepsilon + \mu_L + \mu_{2L} L^*} = \beta \text{ .... (6) and}$$

$$\varphi \frac{\varepsilon}{\overline{\mu}} - \mu_L - \varepsilon = \mu_{2L} L^* \text{ .... (7)}$$

where $\overline{\mu}$ and L^* are the mortality rate and larval population number at the equilibrium points. If the proportion of larvae that will develop into pupae in the absence of density dependence is estimated at 0.8, the density independent larval density can be estimated as $\frac{0.09}{0.8} - 0.09 = 0.02$. The density dependent larval mortality rate is therefore given by $\frac{1.14}{L}$. assuming an equilibrium point of 800 for larvae, the death rate is 0.001.

The expected female adult offspring produced by a single female adult through her lifetime in the absence of density dependence, R_0 was calculated by the equation;

$$R_0 = \varphi \times \frac{1}{\overline{\mu}} \times \frac{\varepsilon}{\varepsilon + \mu_L} \text{ .... (8)}$$

This equation would be interpreted as R_0 = (adult rate of producing female larvae from eggs) x (expected lifespan) x (probability of egg surviving to adulthood). A female would therefore be expected to produce about 615 females in her lifetime. The scenario of this dynamics with initial values of 10 larvae and 1000 adults is shown in Figure 5.1.

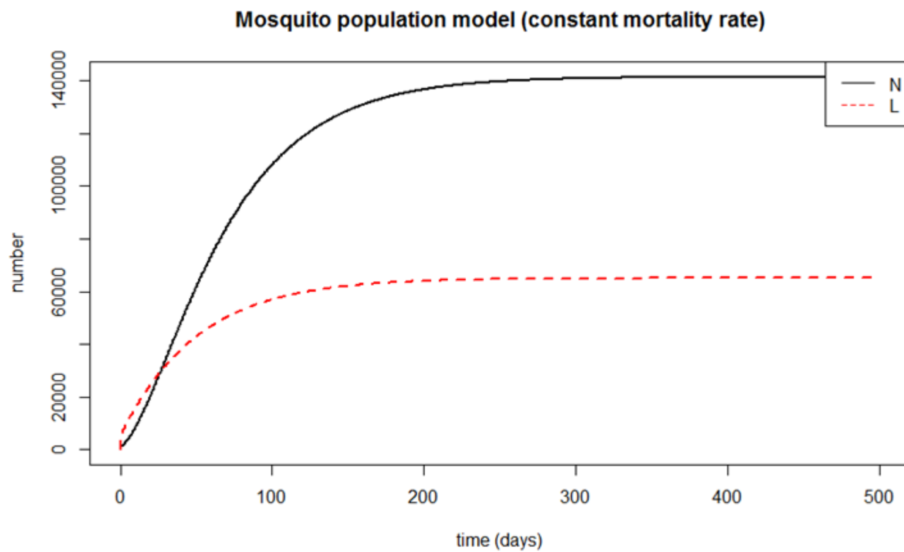

Figure 5.1: the numerical simulation of the ODE model assuming a constant adult mortality rate

5.2.4. **Step 2- Integro-differential equations**

The change in adult population based on the previous larval density is given by;

$$\frac{dN}{dt} = \varepsilon L - (\omega \int_{t-\tau_1}^{t-\tau_2} g(s) f\big(L(s)\big) ds) N \text{ .... (9)}$$

Assuming $g(s) = 1$ and $f(x) = x$, the change in adult population based on the previous larval density was given by

$$\frac{dN}{dt} = \varepsilon L - \omega \left(\int_{t_0}^{t} L(s) ds \right) N \text{ .... (10)}$$

To solve the equation, we let the cumulative larval density over time be given by D;

$$\mathrm{D}(t) = \int_{t_0}^{t} L(s) ds \text{ .... (11)}$$

Therefore, by accounting for the effect of larval density in the past, the three population states are explained by the equations;

$$\frac{\mathbf{dN}}{\mathbf{dt}} = \boldsymbol{\varepsilon}\mathbf{L} - \boldsymbol{\omega}\mathbf{DN} \text{ .... (14),}$$

$$\frac{\mathbf{dL(t)}}{\mathbf{dt}} = \boldsymbol{\varphi}\mathbf{N(t)} - \boldsymbol{\mu}\mathbf{L(t)} - \boldsymbol{\mu}_{\mathbf{2L}}\mathbf{L^2(t)} - \boldsymbol{\varepsilon}\mathbf{L(t)} \text{ .... (15)}$$

$$\frac{\mathbf{dD(t)}}{\mathbf{dt}} = \mathbf{L}(\mathbf{t} - \boldsymbol{\tau}_2) - \mathbf{L}(\mathbf{t} - \boldsymbol{\tau}_1) \text{ .... (16)}$$

Using the same parameter values as in the ODE model above, τ_1 and τ_2 were defined as the time since larvae emerged and the present time and had values of 11.5 and 0 respectively. At equilibrium, the conversion of larval density to adult mortality ω is given by $\frac{\overline{\mu}}{D^*}$, where D^* is the cumulative larval density at equilibrium. If $D^* = 800 \times 11.5$, then $\omega = 0.000004$. The graph for equations (14) – (16) are shown in Figure 5.2.

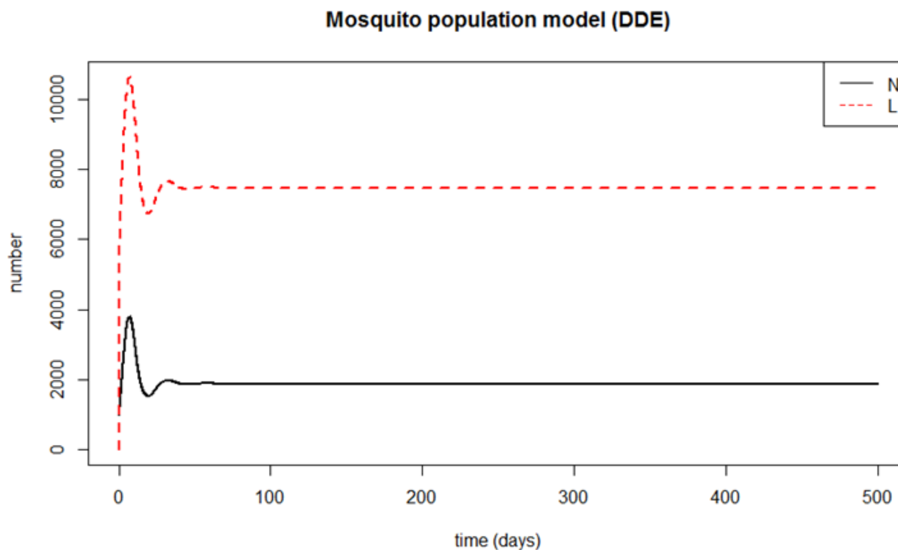

Figure 5.2: the numerical simulation of the DDE model showing the effect of previous larval density on adult mortality

5.3. Discussion and conclusion

Whiles a lot of success has been achieved through the current malaria control methods of IRS, ITNs and larval control, a lot of gaps remain to be filled on details in mosquito population dynamics and malaria transmission. Vector population dynamics involve a lot of complex interactions that are driven by both biotic and abiotic factors. The need to identify the role of each is very important in understanding the vector and how it can be controlled. The interaction of the mosquito with the environment is also complex. There are a lot of concerns about the impact global warming will have on the transmission of malaria [10, 19, 20, 25]. In our model, we looked at how environmental factors influence the adult survival and thus malaria transmission. Mosquito density does not only depend on temperature. The importance of nutrition, density and competition and breeding sites in the life cycle of the mosquito have been studied [21, 22]. It is known that very few organisms die out of senescence, with predators, disease and other hazards more responsible for mortality than old age [23]. The success of transmission of malaria from person to person is highly dependent on the on the survival of the adult. According to Miller et al, daily survival of the adult is the most important parameter and must be known with precision in order to make quantitative predictions [24]. It is however difficult to accurately estimate or directly measure the mortality rate and for that matter the life span of mosquitoes in the wild. In our model, we use parameter estimates from laboratory-bred mosquitoes to make estimates on how the larval

density affects adult survival. A clear difference between the two models shown in Figure 5.1 and Figure 5.2 is that in the absence of density dependent mortality, the adult population grows and reaches equilibrium around 140,000. When adult mortality is affected by larval mortality, the growth of the adult population quickly levels off around 2000. The effect of larval density on the adult is evident because at the equilibrium point, $N = \frac{\varepsilon L}{\omega D}$. The expected life span of the adult mosquito, $\frac{1}{\omega D}$ therefore decreases with an increase in accumulated larval density.

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6. A multi-centre study on the WHO susceptibility assay

Henry F Owusu and Pie Müller

Epidemiology and Public Health Department, Swiss Tropical and Public Health Institute, Socinstrasse 57, PO Box, CH-4002 Basel, Switzerland

University of Basel, Petersplatz 1, CH-2003 Basel, Switzerland

Email addresses:

HFO: henry.owusu@unibas.ch

PM: pie.mueller@unibas.ch

6.1. Introduction

The control of vector-borne diseases relies heavily on the use of chemical insecticides to suppress the mosquito vector. Over the last decades, the rise in the resistance of mosquito vectors to insecticides poses an enormous threat to the success of the control of mosquito-borne diseases [1–3]. Resistance to pyrethroids, the only class of insecticide used in the treatment of bednets, has been reported in many countries in Africa [4].

Generally, continual increase in the frequency of resistant individuals in a vector population leads to a reduction in the efficacy of the choice of insecticide, which might result in the need to replace the intervention. It is therefore essential to thoroughly monitor the development of resistance to insecticides routinely for effective management and successful prediction of the impact of resistance on vector control strategies. This requires the generation of sound and reliable data on the presence of resistance and characterization of the mechanisms involved.

The World Health Organization (WHO) susceptibility test [5] remains the standard bioassay for detecting resistance phenotypes in mosquito populations. It is a direct response-to-exposure analysis that uses discriminating concentrations and a set time period to distinguish between resistant and susceptible mosquito populations. The mosquitoes are held for a period of 24 hours post exposure, after which mortality is read. The bioassay may be used to monitor field population, to characterise laboratory strains or to test new active ingredients.

A recurrent concern with the use of the assay has been the observed variability in testing and reporting of published data [6]. Several factors could account for the intra- and inter-laboratory differences observed in available data. Reports on the performance of the assay when intralaboratory conditions are altered have been published [7–9], but data stemming from the interlaboratory variability in external quality-control schemes are not available.

This study was designed to determine the variability observed when measuring susceptibility of mosquitoes to insecticides in different laboratories, using test papers, standard operating procedure (SOPs) and mosquito populations acquired from the same source. The aim was to determine the effect of factors such as shipping of equipment, storage

and laboratory personnel on the efficacy on insecticide treated papers and output of routine insecticide resistance testing.

6.2. Methods

6.2.1. Study sites

We identified seven institutions in Europe and Africa that focus on mosquito control research and have the necessary facilities to breed mosquitoes and perform insecticide resistance assays (Table 6.1). The group leaders were contacted and upon agreement to participate, the necessary protocols, documents and insecticide treated papers were sent to all the participants. Two of the study sites were located in Europe, two in East Africa and three in West Africa.

Table 6.1: Institutions that accepted to take part in the multi-centre study.

Institution	Country
Centre de Recherche Entomologique de Cotonou (CREC)	Bénin
Centre Suisse de Recherches Scientifiques en Côte d'Ivoire (CSRS)	Côte d'Ivoire
Ifakara Health Institute (IHI)	Tanzania
Institute de Recherche en Sciences de la Santé (IRSS)	Burkina Faso
Kilimanjaro Christian Medical Centre (KCMC)	Tanzania
Liverpool Insect Testing Establishment (LITE)	UK
Swiss Tropical and Public Health Institute (SwissTPH)	Switzerland

6.2.2. Mosquitoes

Sites were instructed to register and order eggs of the susceptible laboratory strain *Anopheles gambiae* KISUMU1 (MRA-762) from the Malaria Research and Reference Reagent Resource Center (MR4). Once received, the eggs were reared to adults and maintained on 10% sugar solution until testing. Participating laboratories followed their routine laboratory practices in larval breeding such as number of larvae per tray and the type and amount of larval food provided.

6.2.3. Insecticides

Mosquitoes were tested against several concentrations of permethrin and bendiocarb. Filter papers impregnated with standard concentrations of 0.75% permethrin and 0.01% bendiocarb (standard papers), together with their respective controls (control papers) were sourced from the WHO Pesticide Evaluation Scheme (WHOPES) through Universiti Sains Malaysia based in Penang, Malaysia. For each insecticide, the Liverpool Insect Testing Establishment (LITE) prepared five papers with serially diluted concentrations and one control (test papers). The test papers were blindly coded and sent to each site with the dates of preparation and expiry. The coded papers were unblinded after all laboratories had completed testing for data analysis (Table 6.2).

Table 6.2: The insecticides, their concentrations and the working codes used in the study.

Insecticide	Concentration	Code
Bendiocarb	0.05	B
Bendiocarb	0.025	X
Bendiocarb	0.0125	M
Bendiocarb	0.00625	D
Bendiocarb	0.00313	Y
OP Control	0.0	U
Permethrin	0.4	C
Permethrin	0.2	A
Permethrin	0.1	Z
Permethrin	0.05	F
Permethrin	0.025	H
PY Control	0.0	N

6.2.4. Susceptibility assay

All laboratories the standard WHO susceptibility assay using provided test papers. The assay was performed according to the WHO guidelines [5]. Three to five-day-old non blood-fed female mosquitoes were exposed to the insecticide-treated filter papers at the WHO recommended exposure time of 60 minutes. Knockdown was recorded every 10 minutes and after exposure, the mosquitoes were transferred back into the holding tube. They were

provided with 10% sugar solution and kept for 24 hours after which mortality was recorded. From the results of the bioassay with the test papers, the LC_{50} and LC_{99} values were estimated for each insecticide to evaluate the practical implications of the different laboratories on the bioassay outcome. After testing was completed, the insecticide papers were sent to the Liverpool School of Tropical Medicine (LSTM), United Kingdom, for High Performance Liquid Chromatography (HPLC) analysis to measure the amount of insecticide in the filter papers.

6.2.5. Data analysis

All data analysis was done in the freely available statistical software package R, version 3.2.0 [10]. The LC_{50} and LC_{99} values for the susceptibility assays were calculated on the basis of a predicted dose-response curve which was fitted assuming a binomial distribution and a logit link function. All graphs were generated with the package ggplot2 [11]. A one-way analysis of variance (ANOVA) was used to evaluate the difference between mean mortalities when mosquitoes were tested against the WHO standard and control papers.

6.3. Results

All the selected laboratories received the insecticide treated papers and test protocols. However, due to various administrative difficulties, only CSRS, LITE and SwissTPH successfully completed testing for all the test papers provided. For confidentiality, the data presented here is blinded and the three laboratories have been assigned the codes A, B and C.

A summary of the mortalities obtained against the test papers is shown in Figure 6.1. When the mosquitoes were tested with the standard WHO permethrin papers, sites A and B obtained 100% mortality is all the replicates. Site C recorded 100% mortality in two replicates and 92% and 96.2% mortality in the other two. However, there was no statistically significant difference in the mean mortality across the three laboratories at neither the standard concentration ($p = 0.1453$) nor PY control papers ($p = 0.7$). All three laboratories recorded 100% mortality in all replicates when testing against the WHO standard bendiocarb papers.

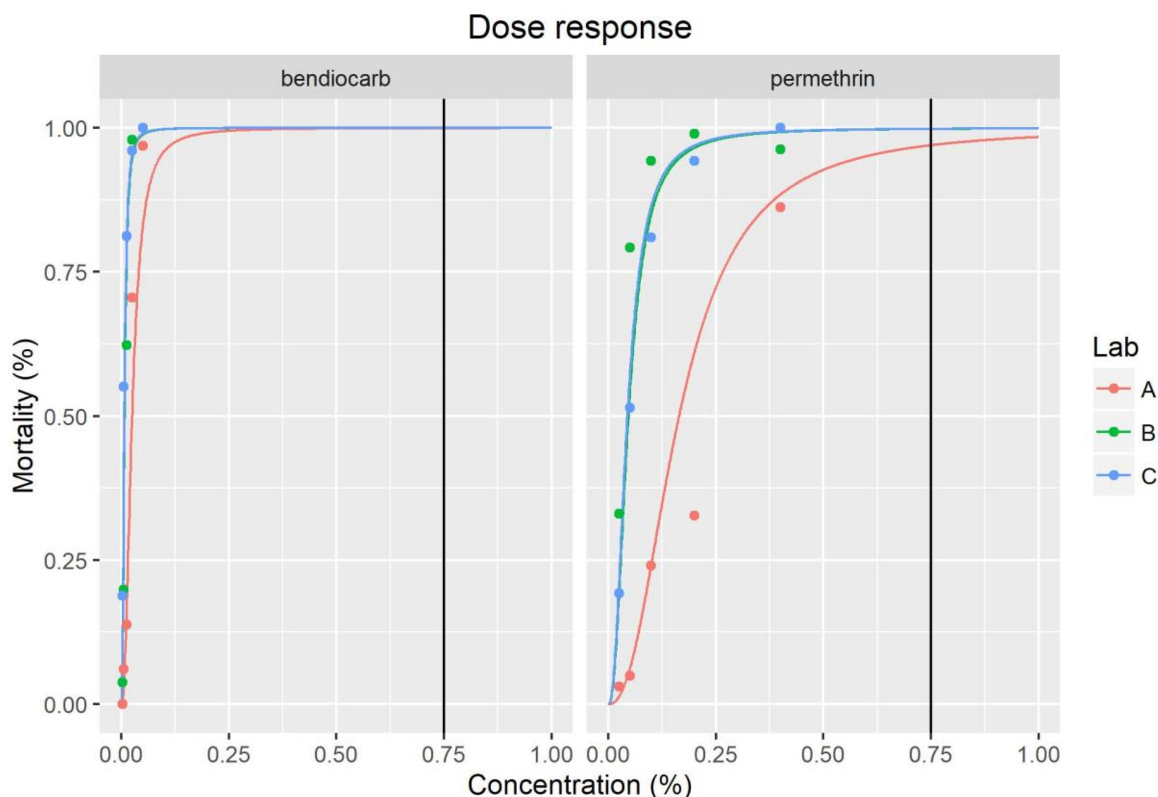

Figure 6.1: Dose-response curves for showing the mortality as a function of insecticide concentration for the three laboratories. The dots show the summary mortalities measured at different insecticide concentrations on the filter papers in the WHO insecticide susceptibility assays. The lines are the predicted curves based on the statistical models

The estimated LC_{50} and LC_{99} values showed significant variation across the three laboratories in both insecticides. A summary of the estimates is provided in Table 6.3.

Table 6.3: The estimated LC_{50} and LC_{99} values from the three laboratories.

Institution	Insecticide	LC_{50}	LC_{50} [%] (95% CI)	LC_{99}	LC_{99} [%] (95% CI)
A	permethrin	0.211	0.186 - 0.240	2.189	1.369 - 3.498
	bendiocarb	0.019	0.018 - 0.021	0.079	0.060 - 0.103
B	permethrin	0.031	0.027 - 0.036	0.281	0.186 - 0.424
	bendiocarb	0.010	0.009 - 0.011	0.042	0.032 - 0.054
C	permethrin	0.049	0.044 - 0.055	0.421	0.293 - 0.606
	bendiocarb	0.006	0.005 - 0.007	0.046	0.032 - 0.066

Only the test papers from site C were successfully analysed by HPLC to estimate the amount of insecticide contained in the treated papers. Table 6.4 shows the HPLC results and the WHO

estimates of the amount of active insecticide per m^2 test papers should contain at the given percentages.

Table 6.4: HPLC analysis showing the insecticide content of the filter papers from site C. [1]WHO estimates that a 1% treatment filter paper would contain 367 mg/m^2 insecticide.

Insecticide	Paper	Concentration (%)	Insecticide content (mg/m2)	WHO estimated content[1] (mg/m2)
Permethrin	PC	0.4	144.8	146
	PA	0.2	79.8	73.4
	PZ	0.1	39.3	36.7
	PF	0.05	21.5	18.4
	PH	0.025	10.3	9.2
	PN	0	0.0	0
	WHO standard	0.75	295.3	275.3
	WHO PY control	0	0.0	0
Bendiocarb	BB	0.05	18.8	18.4
	BX	0.025	8.6	9.2
	BM	0.0125	4.7	4.6
	BD	0.00625	2.5	2.3
	BY	0.00313	1.3	1.1
	BU	0	0.0	0
	WHO standard	0.1	44.4	36.7
	WHO OP control	0	0.0	0

6.4. Discussion

The data generated from this study was, unfortunately, not sufficient to fully answer the questions we had at the beginning of the study. Due to administratve difficulties, four laboratories could not complete the breeding and bioassay testing. Some sites were unable to acquire the necessary permits to import the mosquito eggs. From the limited data generated from the three sites, there was significant variation in the LC_{50}s when their 95% confidence intervals are compared. Results from sites B and C were closely related. However, site A had consistently higher LC_{50} and LC_{99} values in both insecticides. This could be attributed to the fact that sites A and C completed the last replicate of the bioassay testing 12 months and four

months respectively, after site B had completed testing. Therefore, the longer duration of time between sites A and B resulted in a loss of efficacy of insecticide papers at site A.

Despite the differences in LC_{50} estimates between sites B and C, the LC_{99} values show that the mosquitoes are still susceptible at the WHO standard concentrations of 0.1% and 0.75% for bendiocarb and permethrin respectively. On the other hand, the LC_{99} values of site A are 3 fold and 4 fold higher than the standard concentrations of bendiocarb and permethrin. This suggests that the data from site A will could characterise a rather susceptible population as resistant.

Although data was only available from one set of insecticide papers, the HPLC analysis of the filter papers showed that the amount of insecticide per m^2 in the LITE-prepared test papers were consistently closer to the WHO estimates for the given percentages than the standard papers obtained from WHO. This may be because the same individual prepared these papers and thus, the sources of inconsistencies are minimized. According to WHO estimates [12], a 1% insecticide treated filter paper would contain 367 mg/m^2 insecticide. Therefore, at 0.75%, the standard WHO standard permethrin paper would contain 275.3mg/m^2 and the bendiocard paper would contain 36.7mg/m^2 at 0.1%. However, our analysis showed that the standard permethrin and bendiocard papers contained 20mg and 7.7mg more insecticide per m^2 than the WHO estimates. While these differences might not necessarily affect the overall detection of resistance in our study, it indicates that there are inconsistencies in the insecticide contents of the standard papers and call for more stringent regulation and quality control assessment.

6.5. Conclusion

Although the data generated here are limited, there is still evidence that interlaboratory differences affect routine insecticide susceptibility assays. The data showed more variation in the LC_{50} estimates between the laboratories than in the LC_{99} values. This indicates that interlaboratory factors could affect routine susceptibility bioassays in the presence of small shifts in resistance to insecticides. The data also provides an argument for the proper quality control monitoring of WHO standard insecticide papers.

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7. Discussion

This PhD thesis was a quality assessment of the current methods used in insecticide resistance monitoring and mosquito control. The main aim was to review these methodologies and identify factors that lead to inconsistencies in data generated in insecticide resistance monitoring. It also looked to provide some data which though are very basic and crucial, are unavailable for vector control. The overarching aim can be divided under three main objectives:

iv. to assess the effect of bioassays on the test outcome;
v. to assess the influence of the rearing conditions of mosquitoes on bioassay outcomes; and
vi. to assess the effect of inter laboratory variability on the outcome of the test.

The work involved laboratory colonies of mosquitoes from the three important genera *Anopheles, Aedes and Culex* and insecticides from the four WHO recommended classes available for public health adulticides. The work in the thesis covered basic methods in measuring resistance as well as the physiological state of the mosquito and the extent of inter-laboratory differences.

7.1. Insecticide resistance testing bioassays

Using laboratory-bred colonies, three very important diagnostic tools were evaluated to test their robustness. The CDC bottle [1], WHO susceptibility [2] and WHO cone [3] bioassays are the three most important diagnostic assays used in field and laboratory measurement of resistance in mosquito populations.

The CDC bottle and WHO susceptibility bioassay are used routinely and interchangeably. We convincingly showed that the two tests are highly inconsistent in detecting resistance at both 90 % and 98 % (Chapter 2) [4]. We arrived at our conclusion through a comprehensive comparison which looked at previously published data [5–12] and results from our laboratory experiments which directly compared the two tests using Cohen's Kappa (κ) [13]. The tests covered five different mosquito strains and five insecticides with at least one from each class. By examining the amount of insecticide available to the mosquito during testing, we pointed out the significant effect the basic differences in the solvents and insecticide delivery have on

the outcome of the tests. Though it was expected, one of the interesting findings was that introducing a 24 hour holding period in the CDC bottle assay improves the agreement between the two tests from slight agreement (90 % κ = 0.06, 98 % κ = 0.01) to moderate (90 % κ = 0.51, 98 % κ = 0.58) at both cut-off marks. This was an important finding because it highlighted the need for the introduction of the recovery period in the CDC bottle assay which would improve the assay's ability to detect metabolic resistance.

One missing piece of the puzzle when it comes to the use of the two assays is whether they measure the same parameters. By definition, the CDC assay measures time-to-knockdown and the WHO susceptibility assay measures 24 hours mortality. The question of how well the time it takes for a mosquito to be knocked down by the insecticide predicts whether it actually dies is very basic. Unfortunately, this information was not available. We tested mosquitoes of different resistance status against insecticides with different times of action to show that time-to-knockdown does not reliably predict 24 hours mortality.

Though we managed to provide data that answered several basic questions, our data would have been more conclusive if we had a broader range of resistance phenotypes across the mosquito populations. Insecticides have different modes of action as well as how long it takes for them to take effect. Mosquitoes also exhibit different adaptations to insecticide. Therefore, having enough colonies which exhibited the different methods of insecticide resistance would have improved our study a lot. We particularly did not have enough mosquitoes exhibiting strong knockdown resistance. This of course would have very much shown how well the two assays fair in the face of knock-down resistance. It would also have shown how time-to-knockdown predicts mortality when the times are much longer.

After showing some inconsistencies in the performance of the CDC bottle and WHO susceptibility bioassays, we also evaluated the WHO cone bioassay. As the main assay for measuring insecticide resistance in the evaluation of mosquito nets, its importance in the process of getting a net product on the market is underrated. Using two different insecticides, susceptible and resistant mosquito strains, we assessed the influence the angle of tilt of the test on the outcome of the assay. While the assay has been extensively used for different purposes at different angles, we showed for the first time that the angle at which the assay is performed has an influence on the data. We showed that tests that are performed on a flat surface produce a reduced mortality rate compared to those performed at an angle. For the

first time, we also demonstrated that mosquitoes spend less time in contact with the test material when the test is performed on a flat surface. This should be a very useful piece of information for the performance of the assay. This is because is used in testing both insecticidal activity and excito repellency. Therefore, showing that the mosquitoes avoid the test material as a result of the basic setup of the test is very instructive.

This study could have been improved by having more angles, in order to establish the relation between change in angle and change in mortality. That way, we would have an idea of how much change in mortality is expected. We could also have tested serial washes of the nets so that we would be able to see how mortality changes with each wash.

The evaluations of these bioassays showed that while the basic bioassays have been in use over long periods of time, their basic set-ups and the quality of data they generate are below par. These assays form the core of insecticide resistance monitoring and the most important decisions in the control of resistance to insecticides are based on data generated from the assays. In line with other studies [15], we show that these assays are inconsistent and not robust. With the current state of the wide spread of resistance to insecticides and the limited availability of data from parts of malaria endemic areas [16], the need for high quality tools and data to counter the growing threat of resistance cannot be over emphasised. One of the major challenges facing malaria control programmes is to keeping track of the spread of insecticide resistance. There is a high level of lack of resources and expertise to regularly monitor resistance. There is also the problem of the lack of comprehensive and reliable data bases [17]. The establishment of IR Mapper [18] and VectorBase [19] provide some hope for an improved management of insecticide resistance data. But these databases are almost useless if the basic tools generating the data are unreliable and the quality of data cannot be guaranteed. The main problem with the performance of these bioassays is that the provided methodologies and the guidelines lack enough streamline to ensure consistency and reproducibility of data. Diagnostic assays are still valuable and informative in detecting the prevalence of resistance in a population. The information they provide is, however, limited and in their current state they are vulnerable to heterogeneity. New bioassays are clearly needed to address the numerous problems associated with the current ones. Toé et al [14] also called for an immediate consensus on an appropriate method for monitoring resistance,

complete with concise guidelines in the face of the alarmingly high resistance levels being detected in some vector populations.

7.2. Breeding conditions

The objective of this study was to investigate how the breeding conditions of the larval stage affect susceptibility of the adult to insecticide. This was another step to investigate which other factors alter results of susceptibility assays aside the bioassays themselves. We looked at the effect of nutrition, larval density and temperature in a susceptible strain and a resistant strain. A lot of work has been done to look at the effect of larval conditions on the life history traits of mosquitoes [20–26], but very few have done so with respect to insecticide resistance [27, 28]. A significant difference between our study and the previous ones is that we quantified the decrease in susceptibility of bigger mosquitoes to insecticides in a dose-response assay. In the monitoring of insecticide resistance, considerations are always given to the genetic makeup of the mosquito populations. Not so much is attributed to the physiology contributions. Is a mosquito resistant only because of its genetic makeup? Or are bigger mosquitoes more resistant to insecticides than their skinny counterparts? These questions are too often ignored. We show that the physiological aspects might be as important as the genetic constitution. Adults that emerge from larvae that grew up in the absence of intense competition and abundance of food were bigger and more tolerant to insecticides. We could show that in a resistant strain, the LC_{50} can increase by as much as 6 fold when food is abundant.

The impact of temperature on malaria is very important, especially when global warming is concerned. Scientists disagree on the impact of global warming on the spread and transmission of malaria [29]. Some insist that increasing temperatures will result in the re-introduction of malaria to presently colder malaria-free regions such as Europe and America. Others on the other hand disagree and are of the opinion that the absence of mosquitoes in these regions is not a result of the lower temperatures since malaria still persisted in Europe during the cooler “little ice age” periods in the 16th and 17th centuries [30]. They argue that the major decline began in the warmer second half of the 19th century and the elimination of malaria in Europe was rather down to improved drainage and reduction of breeding sites as well as deliberate control of mosquitoes [29]. While the debate is ongoing about whether or

not malaria will spread with increasing temperature, we should also be concerned about how increasing temperature will contribute to insecticide resistance. From our study, mosquitoes bred at higher temperatures were smaller and the smaller mosquitoes were more susceptible to insecticides. While this is very informative, performing the experiment at more temperature values would help us further understand how temperature affects resistance.

In addition to the laboratory experiments, we presented a model for the mosquito life cycle that explores the effects of density-dependent and density-independent factors at the larval stage on adult survival. In its simples form, the model showed that the continual increase in larval density will ultimately lead to a reduction in the expected life expectancy of the adult. This in itself is instructive. The model could still be further improved by the introduction of more parameter and variables such as size of the larval habitat, weight of the adult mosquito and duration of the egg stage. The larval stage could also be further explored to identify how the dynamics of each of the instar stages is affected by the previous one. Nevertheless, the model presents a good foundation upon which a more elaborate and expansive one can be built.

7.3. Inter laboratory differences

Even with well-structured bioassay setups and well defined protocols and standard operating procedure, there are other inter-laboratory factors that could significantly contribute to inconsistencies in insecticide resistance monitoring data. Several of the factors discussed above are as a result of differences in laboratory practices. The choice of the bioassay to use is decided in part by the geographical location of the laboratory in question. The CDC bottle assay is used more frequently in some regions while the WHO susceptibility assay is the test of choice in other regions. Currently, all equipment for the performance of the WHO susceptibility assay are sourced from the Universiti Sains Malaysia based in Penang, Malaysia. This means that the test papers are shipped to various geographical locations across the globe. The efficacy of the impregnated papers could be affected due to varying conditions during transportation and storage conditions, infrastructure and testing conditions at the testing facility. Different laboratories also adapt different protocols and feeding regimes for breeding mosquitoes.

In our study, seven institutions agreed to test susceptible mosquito colonies ordered from the same source with insecticide papers prepared at the same source with the WHO susceptibility assay. Out of the seven, only three successfully completed testing all insecticide papers provided. The limited data obtained suggests that the efficacy of the treated papers decline over time. There was also evidence that standard WHO papers might contain inconsistent amounts of insecticide per m^2, which could ultimately alter bioassay outcomes. The data also showed more variation in the LC_{50} estimates between the laboratories than in the LC_{99} values. This indicates that interlaboratory factors could affect routine susceptibility bioassays in the presence of small shifts in resistance to insecticides.

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8. Conclusion

Whereas this thesis was not rocket science with Nobel Prize- winning findings, it gives very useful contributions to the global control and management of insecticide resistance. The problem of resistance is very complex and there is no one-size-fits-all solution which can be adopted. But like all big problems, one of the first steps in finding a solution is to go to the grass roots and identify basic short comings in the current setup. We focused on the reliability of insecticide resistance data, which forms the foundation upon which any attempts at controlling the problem of resistance is based. We have successfully identified and pointed out a host of factors that contribute to the heterogeneity and unreliability of data and in the process, we have also provided very basic but highly useful data which will be very vital in restructuring the approach to resistance monitoring strategies.

The CDC and WHO bioassays, the principal tests used to detect resistance, are highly inconsistent in producing the same results on the same population of mosquitoes and time-to-knockdown is not a proxy for mortality. The current reliance on a single diagnostic dose for the detection of insecticide resistance is inadequate and limited in terms of how much information it provides. The simultaneous/interchangeable use of the two diagnostic assays which have not been calibrated against each other is rather creating more confusion in data reporting and reliability. A single assay, one such as a dose-response assay which will use fewer mosquitos but provide extensive details on the resistance profile of a population is highly needed.

The effect of the larval stage on the population dynamics and adult survival in mosquito vectors must be accorded more importance. As shown by the experiments and mathematical models, adult survival is very much dependent on changes in environmental factors during the larval stage. Identifying the right factors to manipulate to give the right effect will ultimately be valuable in the control of both outdoor and indoor biting vectors. Additionally, there are currently no streamlined and comprehensive guidelines on how to setup and breed laboratory colonies. The importance of laboratory strains cannot be underestimated because they serve as a proxy for field strains and are extremely useful in generating data that would otherwise be difficult to collect on field populations. A uniform breeding protocol adopted by all laboratories will ensure a certain level of physiological baseline which will improve the comparability of data across laboratories.

All these findings call on WHO to extensively review the current protocols and guidelines available for diagnostic assays and provide new ones for the maintenance and breeding of laboratory-kept mosquito populations. Results here have shown that the current setup of the bioassays in use make them vulnerable to heterogeneity. As demonstrated in the cone assay, a "small and negligible" detail such as the angle at which the test is performed can lead to a significantly altered output and subsequently misleading interpretation of the data.

There is a lot of money being invested in interventions such as LLINs and IRS, the effect of which is that mosquitoes are continually being exposed to insecticides. Inevitably, the development of resistance will therefore remain a major threat. Whereas indisputable evidence that resistance to insecticides is compromising control strategies is lacking at the moment, it can also not be denied that there are indicators. With the current state complexities in measuring insecticide resistance, we are still far off from knowing the actual extent and impact of insecticide resistance. The lack of evidence however, must not be misinterpreted as evidence of no impact.

As researchers, we have a responsibility to develop, evaluate, and implement new products and provide an evidence-based strategy on how best to deploy them such that the development and spread of resistance to insecticides are minimized in order to ensure our goal of significantly reducing the burden of vector-borne diseases is reached. Yet, if we are to make substantial progress in controlling insecticide resistance, we must invest a lot more in the tools available for data generation and maximize their outputs. Any sound insecticide resistance management strategy must be built on robust, reliable, and routinely collected data. As it stands now, critical gaps in knowledge on the contributing factors and consequences of insecticide resistance need to be filled. I may not get a Nobel Prize, but hopefully I have planted a seed that will set in motion a series of actions and events that will lead to significantly improved tools and practices and consequently, data quality in insecticide resistance management in the next years.

9. Appendix 1 - STANDARD OPERATING PROCEDURE

Issued: First Edition

TITLE: Mosquito rearing – *Anopheles gambiae* s.s. KISUMU1

9.1. Introduction:

Anopheles gambiae, like other mosquitoes, goes through four developmental stages; three aquatic developmental stages and one terrestrial adult stage. The aquatic stage comprises the egg, the larva and the pupa. The egg hatches into the larva which goes through four larval stages before pupating. The pupa is a non-feeding stage where organs of the adult develop. The development from eggs to adults takes approximately 11-14 days, depending on larval density and amount of additional diet.
This SOP outlines the rearing procedure.

AUTHORS: Henry F. Owusu and Danica Jančáryová

Signature:………………………………….. Date:……………………….

AUTHORISED BY: Dr Pie Müller

Signature:………………………………….. Date:……………………….

DISTRIBUTION:

9.2. INSTRUCTIONS

9.3. Safety

- Escape of mosquitoes from the cages should be avoided. Free flying mosquitoes must not be tolerated.
- Utmost care must be taken to avoid contamination of or by other colonies.

9.4. Principle

The rearing cycle starts with the deposition of eggs into bowls half-filled with water placed in the cages, followed by the hatching of the eggs into larvae. The water with the freshly hatched larvae is transferred into a new tray (stock trays) filled with 0.6 litres of water to guarantee a water depth of 1 cm. Depending on the larval stage, a different amount of food is fed to the larvae. Approximately 8 days after hatching from the egg, pupation takes place. The pupal stage lasts 2 days and then the imagos (adult mosquitoes) emerge. Imagos are then fed with pig blood, completing the life cycle.

9.5. Material and reagents

a) Plastic mosquito cages (44.5 x 51 x 38cm)
b) egg dish (80 mm diameter)
c) Trays (size 32 x 26.5 cm)
d) Metal spoon for food
e) Nets to cover trays
f) Ground fish food (Tetramin)
g) Tally Counter
h) Dechlorinated water (water treated with Aquasafe®
i) 10% sugar solution
j) Blender
k) Filter paper (Filter discs, grade 1289)
l) 70 mm diameter plastic cups (for pupae)
m) Cotton wool
n) Parafilm
o) Aspirator system
p) Battery-operated hand pooter
q) A pair of scissors
r) Masking tape and permanent marker

s) 25 ml Erlenmeyer flask

9.6. Method

All mosquito colonies are colour-coded to avoid contamination. All equipment used for *An. gambiae* KISUMU are labelled with green-coloured tapes.

9.6.1. Egg collection

- Fill egg dish halfway with clean dechlorinated water
- Cut 2 filter papers into two each and use the halves to line the inside of the bowl to avoid eggs getting stuck to the side such that each piece is partly submerged
- place egg dish into the mosquito cage

9.6.2. Setting up the eggs

- Remove the egg dish from the mosquito cage the day after they were placed into the cage (see schedule).
- Fill a clean larva tray with 600ml of AquaSafe-treated water from the green tank
- Bleach the eggs (refer to bleaching protocol)
- Take a piece of parafilm (10 x 10cm) and cut out a large square (8 x 8cm) in the middle such that only about 2cm of the outer edges are left.
- Put the parafilm in the water and pour the bleached eggs into the centre of the parafilm (this makes the eggs stick to the parafilm and prevents them from sticking to the sides of the tray and drying out)
- Cover the tray with a mosquito net
- Label the tray with date and generation
- On day 2, split larvae into 300 per tray

9.6.3. Feeding larvae

Larvae are fed with Tetramin fish food that has been ground into a powder using the coffee blender. After blending, it is sieved to remove larger particles. The amount fed to the larvae each day is based on the developmental stage of the larvae and given in Table 9.1.

Table 9.1: Summary of feeding schedule for the larvae

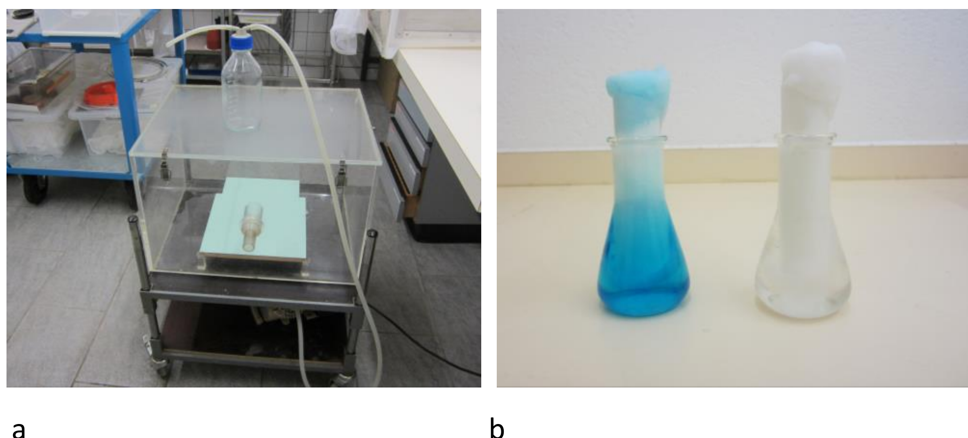
Day	Stage	Amount of diet
1	L1	60 mg (1/2 spoon)
2	L1/L2	120 mg (1 spoon)
3	L2 (split up larvae; 300 per tray)	No additional diet needed
4	L2/3	120 mg (1 spoon)
5	L3	90 mg (2/3 spoon)
6	L3/4	No additional diet needed
7	L4	No diet needed/ or 40 mg
8	L4/pupae	120 mg (1 spoon)

Figure 9.1: 90 mg of Tetramin

9.6.4. Setting up adult cage

- When pupae appear, pick them out using the aspirator system (Figure 9.2a) and transfer them into a plastic cup **NB: On Mondays, adults need to be collected with a hand pooter from the larval trays into adult cages**
- Place plastic cup containing pupae into the cage for adults to emerge directly into the cage
- Fill an Erlenmeyer flask to 2/3. One with 10% sugar solution.
- Roll a piece of cotton wool in filter paper such that the cotton sticks out on both ends of the rolled filter paper. When giving sugar solution, add a bit of sugar solution on the tip of the cotton wool to make it wet
- Insert the rolled filter paper into the Erlenmeyer flask (Figure 9.2b)

- Place the sugar solution in the mosquito cage
- Fill another Erlenmeyer flask to about 2/3 with de-chlorinated water and repeat the steps as for the sugar solution. For easy identification, water is coloured blue with food colour (Figure 9.2b).



a b

Figure 9.2: (a) the aspirator system for collecting pupae. The tube to the left of the bottle is used to suck the pupae which are collected in the bottle and that to the right is connected to the suction. (b) Erlenmeyer flasks filled with water (coloured blue) and 10% sugar solution

9.6.5. Blood feeding

For the females to lay eggs, feed with pig blood twice a week (schedule 1; Table 9.2) or three times a week (schedule 2; Table 9.3), depending on the demand for adult mosquitoes. For the blood-feeding procedure, refer to SOP.

9.6.6. Breeding schedule

9.6.6.1. Schedule 1

Monday: set up larval tray, transfer adults from larva tray into adult cage and place new egg bowls into adult cages.

Tuesday: Remove the egg dish from the cage. Bleach the eggs and set up larval tray. Blood-feed and replace sugar solution. Split larvae set up on previous Friday

Wednesday: Feed larvae

Thursday: Feed larvae. Place new egg bowl in cage and split larvae set up on Tuesday

Friday: Remove the egg dish from the cage. Bleach the eggs and set up larva tray. Blood-feed and replace sugar solution

NB: Feeding of larvae should be based on the feeding protocol

Table 9.2: Weekly Mosquito rearing schedule 1-overview

Tasks	Mon	Tue	Wed	Thu	Fri
Remove egg bowls from cages and set-up new trays (setting up)		X			X
Feed larvae	X	X	X	X	X
Remove adults from trays	X				
Blood meal		X			X
Replace sugar solution		X			X
Add new egg bowls to cages	X			X	
Count larvae and divide trays		X		X	
Clean trays	X		X		X

9.6.6.2. Schedule 2

Monday: Blood feed adults (after 14:00), set up larva tray, transfer adults from larva tray into adult cage and place new egg bowls into adult cages.

Tuesday: Remove the egg dish from the cage. Bleach the eggs and set up larva tray. Blood-feed and replace sugar solution. Split larvae set up on previous Friday

Wednesday: Blood feed adults (after 14:00), feed larvae

Thursday: Feed larvae. Place new egg bowl in cage and split larvae set up on Tuesday

Friday: Remove the egg dish from the cage. Bleach the eggs and set up larva tray. Blood-feed adults (after 14:00) and replace sugar solution

NB: Feeding of larvae should be based on the feeding protocol

Table 9.3: Weekly Mosquito rearing schedule 2-overview

Task	Mon	Tues	Wed	Thurs	Fri
Remove egg bowls from cages and set-up new trays (setting up)		X			X
Feed larvae	X	X	X	X	X
Remove adults from trays	X				
Blood meal	X		X		X
Replace sugar solution		X			X
Add new egg bowls to cages	X			X	
Count larvae and divide trays		X		X	
Clean trays	X		X		X

9.6.7. **Cleaning of larva trays**

Larva trays should be washed with hot water. If needed use a tiny amount of soap and make sure to rinse thoroughly.

9.6.8. **Disposal of mosquitoes**

Disposal of mosquitoes, both adults and immature, should be done by freezing.

- **Larvae/pupae**: Sieve out larvae and put them in a plastic cup. Leave the cup in a deep freezer for at least 24hrs to ensure freezing.
- **Adults**: use a Vacuum cleaner to suck up misquotes. Remove the suction bag from the cleaner and put in a deep freezer. Leave in the freezer for at least 24 hrs to ensure adequate freezing.
- Dispose dead mosquitoes in the garbage bin

9.6.9. **Precautions**

- Remove dead mosquitoes in the egg bowl before bleaching
- Avoid overfeeding of larvae. This can lead to the formation of a biofilm on the water surface or contaminations resulting in death of the larvae
- Always check water levels in the larval trays and top up whenever necessary
- When feeding larvae, stir water or shake the tray to avoid the formation of a biofilm on the water surface. This results in death of the larvae.

9.6.10. Preparation of Reagents

9.6.10.1. Sugar solution

- Place a clean, empty weighing paper on the scale and zero it
- Using a clean spatula, weigh 10 g of sugar
- Add to 100 ml deionised water (white tap) and stir until completely dissolved.
- Store in a clean, labelled and dated bottle in the fridge.

9.6.10.2. Dechlorinated water

- Fill water tank with tap water.
- Add Aquasafe® (12 ml/100 litres)
- Prepare at least a day before use

10. Curriculum Vitae

Personal information

Full name	Henry Frempong Owusu
Address	36 South Huntington Ave, 02130 Boston, USA
Telephone	+18572434704
E-mail	henry.owusu@unibas.ch
Date and place of birth	29 December 1981, Akwatia, Ghana
Nationality	Ghanaian
Civil status	Single
ResearcherID:	B-8434-2017

Education

Ph.D. in Epidemiology – Epidemiology and Public Health Department, Swiss Tropical and Public Health Institute, University of Basel.

Basel, Switzerland, May 2012 – March 2016.

Dissertation: *Insecticide resistance monitoring - a review of current methodology*

Supervision: Dr. Pie Müller and Dr. Nakul Chitnis

Thesis defense: 03/03/2016

Examination committee: Prof. Dr. Marcel Tanner, Dr. Pie Müller, and Dr. John Gimnig

MSc. in Infection Biology and Epidemiology - Swiss Tropical and Public Health Institute, University of Basel. Basel, Switzerland, September 2010 – 17/02/2012.

Thesis: *Environmental determinants of life history traits of mosquitoes and their susceptibility to permethrin.*

Supervisor: Dr. Pie Müller

Description: Course work concentrated in methods in epidemiology, immunology and drug development, international health, Statistics, mathematical modeling of infectious diseases, study design and data analysis.

BSc. in Medical Laboratory Technology/Biomedical Sciences - Kwame Nkrumah University of Science and Technology. Kumasi, Ghana, 2001 –2005.

Description: Coursework in Haematology, Chemical Pathology, Anatomy and Physiology, Cellular Pathology, Microbiology and Medical Entomology.

Professional Experience

Postdoctoral fellow, Harvard T.H. Chan School of Public Health. Boston, USA. 01/06/2016 – Present.

Advisor: Prof. Flaminia Catteruccia.

Projects: Evaluating the genetic basis of the refractoriness of *Aedes aegypti* mosquitoes to human malaria parasites; Evaluating the effects of larval nutrition on adult fitness and malaria transmission in *Anopheles gambiae*

Epidemiologist, Swiss Tropical and Public Health Institute. Basel, Switzerland. September 2010 – March 2016

Work focused on quality assessment of insecticide resistance monitoring tools in mosquito control through systematic literature reviews, laboratory experiments and review of current guidelines which address specific issues of current work procedures to evaluation and develop succinct Standard Operating Procedures (SOPs). The overall aim is to fill gaps in working procedures and improve upon the field applicability of laboratory procedure to provide quality insecticide resistance management in mosquito vectors.

Biomedical Scientist/Medical Laboratory Technologist, Agogo Presbyterian Hospital. Agogo, Ghana. 2007 – 2010.

Supervision of Technical officers and Technical Assistants in the Laboratory.

Performing Biochemical (Liver & Kidney/Renal Function Tests, Lipid and Cardiac profiles), haematological, microbiological and parasitological assays on human samples for diagnosis.

Performing External Quality Assessment (UKNEQAS and Thistle) and Internal Quality Control in Biochemistry, and Heamatology

Performing CD4/CD3/CD8 Count on patient samples and trained HIV councellor.

Biomedical Scientist/Medical Laboratory Technologist, Sefwi Wiawso Government Hospital. Sefwi Wiawso, Ghana. 2006 – 2007.

Head of the Laboratory department

Performing haematological, microbiological and parasitological assays on human samples for diagnosis.

Screening and processing of blood for safe transfusion.

Biomedical Scientist/Medical Laboratory Technologist, Effia Nkwanta Regional Hospital. Takoradi, Ghana. 2005 – 2006.

Assistant to the Head of department

Rotation through the departments of Clinical Chemistry, Bacteriology, Haematology, Parasitology and Transfusion science

Special training

Swiss School of Public Health (SSPH+)

23rd – 24th February 2015: Introduction to the statistical software R; an introductory course on the use of the statistical programming software R

28th – 30th April 2014: Writing a Journal Article… and Getting it Published

25th – 26th April 2013: Systematic review and meta-analysis; a practical approach to the methods and practices of perform quality systematic reviews and meta-analysis

Liverpool School of Tropical Medicine

Vector Population Biology and Control; understanding population biology as applied to vector ecology with an emphasis on the practical methods used and the relevance for epidemiology and control

Swiss Tropical and Public Health Institute

23rd December 2010: Essentials in Drug Development and Clinical Trials

Agogo Presbyterian Hospital

5th June 2009: Post HIV exposure prophylaxis(PEP)

28th March 2009: International Conference on Harmonisation, Good Clinical Practice – Refresher course

19th - 25th May 2008: Agogo Presbyterian Hospital HIV/AIDS Counsellors Training Workshop

14th - 5 April 2008: International Conference on Harmonisation, Good Clinical Practice; Training for Malaria Vaccine Initiative Clinical studies

Conferences

Temperature, nutrition and population density during the larval stage influence the susceptibility status of adult Anopheles mosquitoes. Poster presentation, AvecNet Management Committee and Scientific Meeting. Manchester, UK. May 2016

Measuring insecticide resistance in mosquitos. Oral presentation, Monday Students' Seminar, Swiss Tropical and Public Health Institute. Basel, Switzerland. 22 February 2016

Comparability between insecticide resistance bioassays for mosquito vectors: time to review current methodology? Oral presentation, 2015 annual conference of the American Society of Tropical Medicine and Hygiene. Philadelphia, USA. 27 October, 2015

Breeding conditions influence susceptibility to insecticides in mosquitoes. Poster presentation, 2015 annual meeting of the American Society of Tropical Medicine and Hygiene. Philadelphia, USA. 26 October, 2015

Breeding conditions influence susceptibility to insecticides in mosquitoes. Oral presentation, 9th European Congress on Tropical Medicine and International Health (ECTMIH). Basel, Switzerland 08 September 2015

Comparability between insecticide resistance bioassays for mosquito vectors: time to review current methodology? Roll Back Malaria Vector Control Working Group meeting. Geneva, Switzerland. 27 January 2015.

Breeding conditions influence susceptibility to insecticides in mosquitoes. Joint meeting of the German Society for Parasitology, the German Society for Tropical Medicine and International Health and the Swiss Society of Tropical Medicine and Parasitology. Zurich, Switzerland. 18 July 2014.

Host seeking in insects; how mosquitoes find us, Swiss Tropical and Public Health Institute. Basel, Switzerland. 14 April 2014

Measurements of insecticide susceptibility depend on the choice of standard bioassays and larval rearing conditions, 6th Multilateral Initiative on Malaria (MIM) Pan-African Malaria Conference. Durban, South Africa. 2013.

A comparison of the WHO cylinder and CDC bottle bioassays, AvecNet Management Committee and Scientific Meeting. Rome, Italy. 18 September 2013

Measurements of insecticide susceptibility depend on the choice of standard bioassays and larval rearing conditions, Oral presentation. Swiss Society of Tropical Medicine and Parasitology, Meeting for Doctoral Students in Parasitology and Tropical Medicine. Basel, Switzerland. 30 October 2013.

Insecticide resistance monitoring; what data is needed? Oral presentation. Swiss Society of Tropical Medicine and Parasitology, Meeting for Doctoral Students in Parasitology and Tropical Medicine. Bern, Switzerland. 12 December 2012.

Fellowship awards

Early Postdoc.Mobility grant – Swiss National Science Foundation

Publications

Henry F. Owusu, Danica Jančáryová, David Malone and Pie Müller. Comparability between insecticide resistance bioassays for mosquito vectors: time to review current methodology? Parasites & Vectors. **2015** Jul 7;8 (1):357. http://parasitesandvectors.biomedcentral.com/articles/10.1186/s13071-015-0971-6

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https://www.nature.com/articles/s41598-017-03918-z

References

Prof. Flaminia Catteruccia, Department of Immunology and Infectious Diseases, Harvard T.H. Chan School of Public Health, Boston, USA.

E-mail: fcatter@hsph.harvard.edu

Telephone: +16174321773

Professor Marcel Tanner – Director emeritus, Swiss Tropical and Public Health Institute

Email: marcel.tanner@unibas.ch

Telephone: +41 61 284 82 87

Dr. Pie Müller, Head of Vector Control, Swiss Tropical and Public Health Institute

E-mail: pie.mueller@unibas.ch

Telephone: +41 61 284 82 41

Prof. Thomas Smith, Head of Health Systems Research and Dynamical Modelling Unit, Swiss Tropical and Public Health Institute

E-mail: thomas-a.smith@unibas.ch

Telephone: +41 61 284 82 73