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Bionomic and Genetic Characterization of *Anopheles gambiae* from Ghana

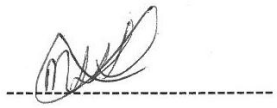
Maria Kaiser

This thesis is submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in fulfillment of the requirement for the degree of Doctor of Philosophy.

Johannesburg, May 2015

Declaration

I, Maria Louise Kaiser declare that this thesis is my own work. It is being submitted for the degree of Doctor of Philosophy in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.



26th day of May, 2015

Publications and presentations arising from this work

Peer reviewed publications:

1. Kaiser, M. L., Koekemoer, L. L., Coetzee, M., Hunt, R. H., Brooke, B. D. 2010. Staggered larval time-to-hatch in the major malaria vector *Anopheles gambiae* S form. *Malaria Journal* 9: 360.

2. Hunt, R.H., Fuseini, G., Knowles, S., Stiles-Ocran, J., Verster, R., Kaiser, M.L., Choi, K. S., Koekemoer, L.L. and Coetzee, M. 2011. Insecticide resistance in malaria vector mosquitoes at four localities in Ghana, West Africa. *Parasites and Vectors* 4: 107.

3. Kaiser, M.L., Duncan, F.D., Brooke, B, D. 2014. Embryonic Development and Rates of Metabolic Activity in Early and Late Hatching Eggs of the Major Malaria Vector *Anopheles gambiae*. *PLoS ONE* doi: 10.1371/journal.pone.0114381.

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2. Kaiser, M.L., Brooke, B.D., Koekemoer, L.L., Coetzee, M and Hunt, R.H. 2009. Larval time-to-hatch in the major malaria vector *Anopheles gambiae* is under genetic control. *Fourteenth Entomological Congress, Entomological Society of Southern Africa,* Stellenbosch, South Africa.

3. Kaiser, M.L., Brooke, B.D., Koekemoer, L.L., Coetzee, M and Hunt, R.H. 2009. Differences in levels of insecticide resistance between time-to-hatch phenotypes in the major malaria vector *Anopheles gambiae. University of the Witwatersrand Postgraduate Symposium,* Johannesburg, South Africa.

4. Kaiser, M.L., Koekemoer, L.L., Coetzee, M., Hunt, R.H. and Basil D Brooke. 2010. Time-to-hatch and insecticide resistance in *Anopheles gambiae* S form from Ghana. Wits Faculty of Health Sciences Research Day, University of the Witwatersrand, Johannesburg, and also presented at the National Institute of Communicable Diseases Academic Research Day. 2010. NICD, Johannesburg.

5. Brooke, B. D., Kaiser, M.L., Koekemoer, L.K., Coetzee, M and Hunt, R.H. 20-22 October 2010. Larval time-to-hatch and insecticide resistance in the major malaria vector *Anopheles gambiae* from Ghana. *Parasite to prevention: Advances in the understanding of malaria,* Edinburgh, UK.

6. Kaiser, M.L., Duncan F. Brooke B.D. 2011. Metabolic rate in *Anopheles gambiae* eggs from phenotypically characterised early and late time-to-hatch parents. *Fifteenth Entomological Congress, Entomological Society of Southern Africa,* University of the Free State, Bloemfontein, South Africa.

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2. Kaiser, M.L., Duncan F. Brooke B.D. October 2013. Embryonic development and rates of metabolic activity in early and late hatching eggs of the major malaria vector *Anopheles gambiae*. *Sixth Multilateral Initiative on Malaria conference*, International Convention Centre, Durban, South Africa.

Abstract

Malaria vector control relies principally on the use of insecticides. However, the increasing incidence of insecticide resistance threatens to undermine the effectiveness of this approach, necessitating resistance management strategies. Multiple insecticide resistance is becoming common in West Africa including Ghana. In particular, a population of the major malaria vector *Anopheles gambiae*, from the Ahafo region of Ghana, shows resistance to all classes of insecticide currently available for use in public health. Insecticide resistance is a primary adaptive characteristic of epidemiological importance although other adaptive traits such as staggered larval time-to-hatch may also be important. Typically, *An. gambiae* oviposits in small, temporary, sun-lit water bodies and eggs generally hatch 2-3 days post-oviposition. However, staggered distribution of hatching has previously been shown and was also observed in a newly colonized strain (GAH) from Ahafo. The broad aims of this project were therefore to assess and characterize multiple insecticide resistance in *An. gambiae* from Ahafo as well as to quantify and describe staggered time-to-hatch in a laboratory colony of this population in terms of its adaptive significance and pleiotropic effects on resistance.

WHO insecticide susceptibility bioassays conducted on wild-caught samples from Ghana and colonized material (GAH) indicated resistance to all insecticide classes. The presence of known metabolic resistance mechanisms as well as target-site insensitivity mutations were detected in GAH and are likely representative of the wild population from which the colony was derived. Staggered time-to-hatch was further investigated by monitoring hatching in GAH as well as in families reared from wild-caught females from Ghana and the Republic of the Congo. In addition, GAH was selected for early and late time-to-hatch and cross-mating experiments were conducted to determine the genetic heritability of this trait. The proportion of late hatching increased following selection, indicating that there is

a genetic component involved in time-to-hatch. Staggered distribution of hatching was observed both in GAH and wild families, some of which were exclusively early or late hatching. The effect of egg disturbance on time-to-hatch was also explored and was found to be necessary for optimal hatching. The association of insecticide resistance with selection for time-to-hatch was investigated and resistance profiles of the time-to-hatch selected strains differed significantly, suggesting a link between time-to-hatch and the expression of insecticide resistance, possibly as a consequence of pleiotropy. To further understand the mechanisms behind time-to-hatch, metabolic rates of early and late hatching strains were measured and embryonic development was qualitatively observed in the GAH time-to-hatch selected strains. It was found that embryos developed fully within four days regardless of subsequent hatch time and that some eggs are then able to delay hatching in a state of late embryonic diapause.

It is concluded that delayed hatch in *An. gambiae* is an adaptation to maximize reproductive output in an unstable aquatic environment. That this trait associates with resistance to insecticides suggests that enhanced phenotypic variation may be produced as a consequence of pleiotropy, providing a greater platform for resistance selection and resulting in increased ability of *An. gambiae* to adapt to an unstable environment.

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Ethics Approval

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TO WHOM IT MAY CONCERN:

Waiver: This certifies that the following research does not require clearance from the Human Research Ethics Committee (Medical).

Investigator: Maria Louise Kaiser (Student no 0420262N).

Project title: Bionomic and genetic characterization of the major malaria vector *Anopheles gambiae* from Ghana.

Reason: This study is on a mosquito vector of malaria. There are no human participants.

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Nomenclature/ Abbreviations

Ace-1^{R}- acetylcholine esterase-1 resistance mutation

ACT- Artemisinin combination therapy

AIDS- Acquired immune deficiency syndrome

DDT- Dichloro-diphenyl-trichlorethane

DEM- Diethyl maleate

DNA- Deoxyribonucleic acid

ELISA- Enzyme-linked immunosorbent assay

GABA- γ aminobutyric acid

GFATM- Global funds for AIDS, Tuberculosis and Malaria

GST- Glutathione S-transferase

IGS- Intergenic spacer

IPTp- Intermittent preventative treatment in pregnancy

ITN- Insecticide treated net

ITS- Internal transcribed spacer

IVM- Integrated Vector Management

Kdr- knockdown resistance mutation

LLITN- Long-lasting insecticide treated net

MOP- Malaria operational plans

PBO- Piperonyl butoxide

PCR- Polymerase chain reaction

PMI- President's Malaria Initiative

QTL- Quantitative trait locus

RBM- Roll Back Malaria

rDNA- ribosomal DNA

Rdl- Resistance to dieldrin mutation

RFLP- Restriction fragment length polymorphism

SINE- Short interspersed transposable element

SNP- Single nucleotide polymorphism

TPP- Triphenyl phosphate

VCRL- Vector Control Reference Laboratory

WHO- World Health Organization

WMR- World Malaria Report

Chapter 1: General Introduction

1.1 Malaria

There are five known human malaria parasites: *Plasmodium vivax*; *P. ovale*; *P. malariae*; *P. falciparum* and *P. knowlesi* of which *P. falciparum* is the most severe and can result in cerebral malaria without effective treatment. There were an estimated 584000 malaria deaths in 2013 (down from 660000 in 2012), 90% of which were in the WHO African region (World Malaria Report (WMR), 2014). Despite being a severe disease malaria is treatable and the chances of acquiring malaria can also be significantly reduced by employing preventative measures. Malaria prevention and control can be achieved through vector control, chemoprevention, microscopy, confirmation of diagnosis using rapid diagnostic tests and swift treatment of malaria cases using appropriate anti-malarials (WMR, 2014). The World Health Assembly (WHA) and the Roll Back Malaria (RBM) initiative aim, by 2015, to reduce malaria by 75% of that reported in 2000 and to have close to zero malaria deaths by this date as well (WMR, 2005; RBM, 2011). A further goal is to eliminate malaria in the WHO European region as well as in 10 new countries (RBM, 2011). According to the WHO (WMR, 2014), 55 of the 106 countries with ongoing malaria transmission in 2000 are projected to achieve the goal of 75% reduction in rates of malaria mortality, or maintain zero malaria deaths by 2015 (WMR, 2014).

1.2 Malaria Vector Species

There are approximately 70 species of the *Anopheles* genus that are capable of transmitting human malaria and 41 of these have been identified as dominant vector species capable of transmitting malaria at a level of public health concern (Hay *et al*., 2010). In Africa there

are four major malaria vectors. These are *An. funestus* of the *An. funestus* species group and *An. arabiensis, An. gambiae* and *An. coluzzii* of the *Anopheles gambiae* species complex. Other dominant, but more minor vectors are the salt water breeders *An. melas* and *An. merus* of the *An. gambiae* complex as well as *An. nili* and *An. moucheti* (Sinka *et al.*, 2010; Coetzee *et al.*, 2013a).

1.3 The *Anopheles gambiae* Species Complex

The *An. gambiae* species complex has recently been reviewed and currently consists of at least eight species (Coetzee *et al*., 2013a). These species are morphologically indistinguishable (Gillies and Coetzee, 1987). Extensive research on *An. gambiae sensu stricto* showed that it could be divided into 5 chromosomal forms (Mopti, Forest, Bissau, Bamako, and Savannah) (Coluzzi *et al*., 1985) as well as two molecular forms named M and S (della Torre *et al*., 2001). The division into chromosomal forms is based on polymorphic chromosomal inversions on the arms of chromosome 2 (Coluzzi *et al*., 1985) and the division into molecular forms is based on form specific ribosomal DNA (rDNA) single nucleotide polymorphisms (SNPs) in the intergenic spacer (IGS) and internal transcribed spacer (ITS) regions at the centromere of the X chromosome (della Torre *et al.*, 2001; Gentile *et al*., 2001). The divisions into chromosomal forms are thought to be linked to environmental adaptability or 'ecospeciation' (Coluzzi, 1985; Coluzzi *et al*., 2002, Ayala and Coluzzi 2005). The molecular forms, termed M and S until recently, were considered incipient species (della Torre *et al*., 2001). Although cross-mating between the forms does result in viable offspring when it occurs (Diabaté *et al.*, 2007), it generally does not normally happen in the field with hybrids found at low frequencies (della Torre *et al*., 2005), much less than expected if mating was at random. The study of molecular forms by

polymerase chain reaction (PCR) based methods indicated positive assortative mating between M and S forms (Favia *et al*., 1997, 2001; Fanello *et al*., 2002).

There are pre-mating reproductive barriers including spatial segregation of mating swarms (Diabaté *et al*., 2009) and the matching of wing beat frequency (Pennetier *et al*., 2010) resulting in assortative mating, but the hybrids obtained from laboratory crosses are fertile (Diabaté *et al*., 2007) which indicates the breakdown of pre-mating reproductive isolation in laboratory situations and a lack of post-mating reproductive isolation. It is possible that such hybrid forms carry a fitness cost which would act as a post-mating reproductive isolation mechanism (Wallace, 1889) in nature, or that chromosome rearrangements are protected from recombination assisting in maintaining and later increasing the separation between the forms (Ayala and Coluzzi, 2005; Slotman *et al*., 2006).

Incomplete reproductive isolation has been reported at the western margin of the *An. gambiae* range where the forms have recently come into contact (Caputo *et al*., 2008 and 2011, Weetman *et al*., 2012; Nwakanma *et al*., 2013). Lee *et al*. (2013), using divergence island SNPs, showed that the levels of introgression between the forms are dynamic over space and time in West and Central Africa- indicating that the levels of interbreeding between forms are higher than commonly thought (della Torre *et al*., 2005). In addition they provide evidence for selection against hybrid forms. The combination of these findings suggests speciation between these two forms despite gene flow (Lee *et al*., 2013). The 'Goundry' population from Burkina Faso has quite recently been described and consists of 36% M/ S hybrids (Riehle *et al*., 2011), in Hardy-Weinberg equilibrium, indicating high levels of interbreeding between the forms in this population. This population also differs in behaviour as the adults have not been found resting indoors as is

characteristic for this species, raising speculation as to whether this population may be another distinct genetic unit. The resting sites of adults of this population have not yet been discovered (Riehle *et al*., 2011).

Overall the research suggests that the M and S molecular forms fit the criteria for good species (Coetzee *et al*., 2013a). Based on molecular and bionomical evidence such as chromosomal inversions and pre-mating reproductive isolation mechanisms the forms have recently been named as separate species (Coetzee *et al* ., 2013a). *Anopheles gambiae* Giles is retained for *Anopheles gambiae* S form and the M form has been re-named *An. coluzzii* Coetzee and Wilkerson sp.n (Coetzee *et al*., 2013a). *Anopheles coluzzii* is believed to be the more recently diverged of the two species and occurs in more marginal habitats (Costantini *et al*., 2009). These two species differ in terms of distribution (but are often sympatric) (see Fig. 1 from Lanzaro and Lee, 2013), behaviour, preferred habitat (Lehmann and Diabaté, 2008; Sinka *et al*., 2010), insecticide resistance profiles (Dabiré *et al.*, 2008; Namountougou *et al*., 2012) and may differ in susceptibility to *P. falciparum* (Ndiath *et al*., 2011; Gnémé *et al*., 2013). Some evidence suggests that *An. coluzzii* can be further subdivided into breeding units (Slotman *et al*., 2007) and high levels of genetic differentiation within S form *An. gambiae* from Ghana has also been observed (Weetman *et al*., 2010). For the remainder of this thesis, *An. gambiae* will refer to the S molecular form and *An. coluzzii* to the M form.

1.4 *Anopheles gambiae* Complex Life History, Ecology and Behavior

Preferred habitat conditions for *An. gambiae* include more humid environments while *An. coluzzii* is more arid adapted (Lee *et al*., 2009, Lehmann and Diabaté, 2008; de Souza *et al*., 2010), although the species occur in sympatry in a large part of the occupied range

(Figure 1.1) (della Torre *et al*., 2005; de Souza *et al*., 2009; Lanzaro and Lee, 2013). Vector species composition and abundance may change across seasons or years in the same locality (Fontenille *et al*., 1997; Costantini *et al*., 2009).

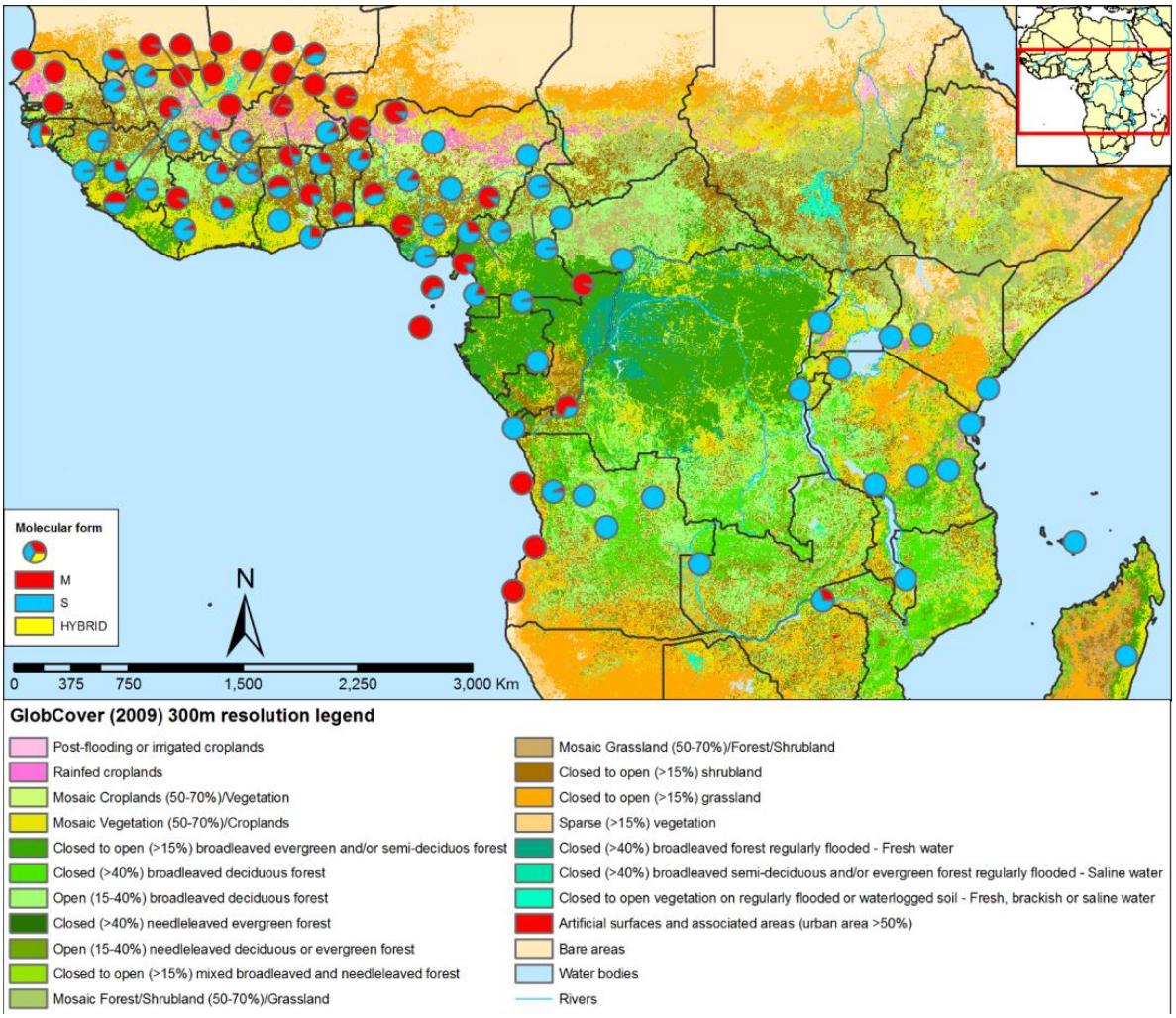


Figure 1.1: The distribution of *Anopheles gambiae* and *Anopheles coluzzii* (map from: Lanzaro and Lee, 2013).

Anopheles gambiae females must be mated to ensure fertilization of eggs and typically mate only once (Tripet *et al*., 2003). Mating occurs in male swarms that the females fly through (Charlwood and Jones, 1980). To mature her eggs a female must take a blood meal, either before or after mating (Charlwood *et al*., 2003). *Anopheles gambiae* females are typically endophagic and endophilic, entering dwellings to seek a host, acquire a blood

meal, and then rest indoors until ready to oviposit (Gillies and De Meillon, 1968). However, behavioural shifts to exophagy and feeding on non-human hosts may occur (Gillies and Coetzee, 1987; Lefèvre *et al*., 2009; Reddy *et al*., 2011). Female mosquitoes seek and select oviposition sites using several cues such as such as hydration of the oviposition substrate, the presence of competitors or predators and volatile compounds emitted by cyanobacterial mats and plant litter from reeds in the water (Huang *et al*., 2005; Munga *et al*., 2006; Rejmánková *et al*., 2005). *Anopheles gambiae* larval habitats are typically small, temporary, sun-lit water bodies such as hoof prints, tyre tracks and puddles while *An. coluzzii* is associated with larval habitats of larger water bodies such as rice paddies (Gillies and De Meillon, 1968; Tuno *et al*., 2005; Lehmann and Diabaté, 2008; Gimonneau *et al*., 2012). Once eggs have been oviposited most hatching takes place within two-to three days post-oviposition under optimal conditions (Service, 2000), although the time taken for eggs to hatch may be prolonged under certain conditions.

Larvae go through four instar stages over approximately 5 to 14 days depending on environmental and habitat conditions. Factors that affect the rate of larval development include food availability, temperature (Bayoh and Lindsay, 2004), competition, predation, larval density (Gimnig *et al.,* 2002), water quality as well as interactions between various factors (Lyimo, Takken and Koella, 1992). These factors are likely to affect the fitness of the emerging adults (Takken *et al*., 1998; Gimnig *et al.,* 2002; Ng'habi *et al.,* 2005; Oliver and Brooke, 2013). At the end of the larval stage a mobile pupa is formed. Adults emerge approximately 48 hours after pupation. Males normally emerge before females and they are usually smaller than females. *Anopheles* mosquitoes reach sexual maturity from approximately 12 hours (Mahmood and Reisen, 1982) to 72 hours post adult emergence (Chambers and Klowden, 2001), or once the males' genitals have rotated 180° (Oliva *et*

al., 2011). Large, newly-emerged females are more likely to mate before seeking a blood meal while smaller females are more likely to seek a blood meal before mating (Charlwood *et al*., 2003). Blood feeding followed by a single gonotrophic cycle generally requires approximately two days (Gillies, 1953). Malaria infected mosquitoes are more likely to bite or probe multiple times (Koella *et al*., 1998) and are more attracted to human odour than non-infected females (Smallegange *et al*., 2013), factors which improve their capacity to transmit malaria.

Traits such as time taken to adult maturity (Oliva *et al*., 2011), larval development time, adult longevity, and adult body size likely affect vector capacity (Lehmann *et al*., 2006). These traits may also have adaptive value particularly considering the transient habitats typically occupied by *An. gambiae*. Lehmann *et al.* (2006) found a genetic contribution to variation in larval development time, adult size and the longevity of starved adults.

1.5 Malaria Vector Control

Once anopheline mosquitoes were identified as the vectors of the parasite causing malaria during the separate works of Laveran, Ross and Grassi just before the end of the nineteenth century (Cox, 2010), efforts to control these vectors began. Previously some level of control had been achieved due to the draining of swamps and marshes as disease was associated with these areas and was thought to be due to bad air or ‘mal aria’ (Harrison, 1978). The earliest insecticidal compound used, after knowledge was gained on the indoor resting and feeding behaviour of mosquitoes, was natural pyrethrum (Park Ross, 1936). However, this did not last long in the environment and control was difficult with the need for weekly re-application of the insecticide (De Meillon, 1936).

For the past 50 to 60 years malaria vector control has relied heavily on indoor spraying of residual insecticides (IRS) and, more recently, the use of insecticide treated bed nets (ITNs) including long-lasting insecticide impregnated nets (LLINs). There is a very limited insecticide arsenal available for use in public health. No new insecticide classes have been approved for use in public health during the last 30 years (Zaim and Guillet, 2002, WHO 2013). Currently, only four classes of insecticide are permitted for vector control (WHO, 2013 (Table 1.1)).

1.6 Insecticide Classes Available for use in Malaria Vector Control

Table 1.1: Insecticides available for use in public health (WHO, 2013).

Insecticide Class	**Specific insecticides**
Organochlorines	DDT
Organophosphates	Fenitrothion Malathion Pirimiphos-methyl
Carbamates	Bendiocarb Propoxur
Pyrethroids	Deltamethrin, Permethrin, Lambda-cyhalothrin, Cyfluthrin Etofenprox

1.6.1 Organochlorines

The controversial insecticide DDT is an organochlorine. DDT was first discovered in 1939 by Paul Hermann Műller who was awarded the Nobel Prize in physiology or medicine in 1948 for "for his discovery of the high efficiency of DDT as a contact poison against several arthropods" (www.nobelprize.org). DDT was successfully used to control typhus

and malaria during the Second World War (Hays, 2000) and was the main insecticide used during the eighth World Health Assembly's (WHA) global malaria eradication campaign which spanned the period 1955-1969 (WHO, 1973). DDT can be manufactured relatively cheaply and offers a highly effective and affordable means of control in susceptible insects. DDT resistance was first detected in malaria vectors even before the start of the eradication campaign in 1951 (Livadas and Georgopulous, 1953). The production of DDT was banned in many countries during the 1970s after the publication of 'The Silent Spring' by Rachel Carson (Carson, 1962) which described environmental risks supposedly caused by DDT contamination such as the thinning of carnivorous bird egg shells. DDT enabled the elimination of malaria in certain countries during the global malaria eradication programme (see Mendis *et al*., 2009) and is still available for use in public health, particularly malaria vector control (WHO, position statement on DDT, 2011; WHO, 2013).

The cyclodiene insecticide dieldrin, a hydrogenated organochlorine was first produced by J. Hyman and Co. as an insecticide in 1948. Dieldrin was used in vector control from the 1950's (Hodgson, 2004). Dieldrin is no longer used for malaria vector control or any other application as it is highly toxic to vertebrates and invertebrates (http://chm.pops.int/TheConvention/ThePOPs). In addition, the development of resistance to the insecticide in malaria vectors was reported by Elliot and Ramakrishna (1956) soon after it was first introduced and became widespread (Busvine and Pal, 1969). High levels of dieldrin resistance are still recorded in several malaria vector populations despite the fact that it is no longer in use (Brooke *et al*., 2006; Koffi *et al*., 2013). However, this insecticide is used as a reference insecticide in vector surveillance and operational research (WHO, 2013), largely because resistance to dieldrin confers cross-resistance to the phenylpyrazole insecticide fipronil (Brooke *et al*., 2000; Kolaczinski and Curtis 2001).

Dieldrin targets the γ aminobutyric acid receptor (GABA) at neuromuscular junctions (Bermudez *et al*., 1991).

1.6.2 Organophosphate and Carbamate Insecticides

Organophosphates and carbamates were developed in the 1940's (Hodgson, 2004). Organophosphates are related to the nerve gases such as sarin. The use of organophosphates and carbamates in vector control began as an alternative to organochlorines (Busvine and Pal, 1969). Organophosphate resistance was first suspected in *Anopheles albimanus* in 1965 in Central America (Georghiou *et al*., 1972). Multiple resistance in *An. albimanus* to DDT, malathion and propoxur (representing the three insecticide classes in use at that time) was reported in central American countries as early as the late 1960s, and was largely attributed to the use of pesticides in agriculture (Pal, 1973).

Carbamate and organophosphate insecticides have similar modes of action and target acetylcholinesterase (AChE) in the synaptic clefts between neurons, preventing AChE from catalysing the hydrolysis of the neurotransmitter acetylcholine (ACh). This results in the accumulation of ACh at nerve synapses leading to continuous firing of action potentials, and eventually death (Fukuto, 1990). A major difference between these insecticide classes is the speed of hydrolysis which is rapid for carbamates and slow for organophosphates (Hodgson, 2004).

1.6.3 Pyrethroids

The pyrethroid insecticides are the most recent class of insecticides. The more stable synthetic pyrethroids, such as permethrin, were developed in the 1970s (Elliot and Janes, 1978). Synthetic pyrethroids are based on natural pyrethrum found in the flowers of Chrysanthemum plants (*Chrysanthemum cinerariaefolium* and *C. coccineum),* one of the earliest insecticidal compounds used in vector control. Pyrethroids are formulated to last longer than natural pyrethrum and are more effective. They are divided into two types based on the neurotoxic reactions they produce in mammals. Type I includes permethrin and type II includes cyano-pyrethroids such as deltamethrin and lambda-cyhalothrin (Soderlund and Bloomquist, 1989). Pyrethroids target the voltage gated sodium channel preventing the sodium ion channel from returning to a closed position which means action potentials are continuously fired which ultimately results in the insect's death (Hemingway and Ranson, 2000).

The only class of insecticides currently permitted for treating ITNs are pyrethroid insecticides. In addition, 77% of insecticides used for IRS are pyrethroids with DDT making up a further 20% (WMR, 2011). The reason pyrethroids have been used so extensively is due to their low mammalian toxicity, rapid and durable effect and low cost (WMR, 2011). Such widespread use of this one insecticide class is likely to result in resistant populations and there is now sufficient evidence to confirm that this has already occurred in multiple malaria vectors throughout the world (Hargreaves *et al.*, 2000; Czeher *et al*., 2008; Protopopoff *et al*., 2008; Padonou *et al*., 2012; Corbel and N'Guessan, 2013). The WHO World Malaria Report (2011) states that maintaining susceptibility to the four classes of insecticides available is critical. While resistance is very widespread it is not yet fixed in most target populations, and so maintaining some susceptibility is possible if

insecticides are used judiciously and monitoring is done in real time to identify emerging resistance. Resistance monitoring in areas where IRS or LLIN are being employed is now recommended annually or preferably every 6 months according to the Global Plan for Insecticide Resistance Management (WHO, 2012), which aims to track insecticide resistance globally (WMR, 2014).

There are other insecticides being tested for use in public health, however, these are not yet available. These include the pyrrole chlorfenapyr which inhibits mitochondrial electron transport (a novel mode of action not present in the current insecticidal arsenal) (Black *et al*., 1994), and the phenylpyrazole fipronil which targets the GABA receptor (Cole *et al*., 1993).

1.7 Indoor Residual Spraying of Insecticides

Indoor residual spraying (IRS) involves spraying the inside walls and eaves of houses with long-lasting insecticides to kill mosquitoes entering and resting indoors. Some of the insecticides also have repellant properties and may deter mosquitoes from entering sprayed structures. IRS is a highly effective vector control tool when correctly employed and has resulted in effective control (De Meillon 1936; Ghana PMI report, 2007; Pluess *et al*., 2010; Coetzee *et al*., 2013b) and elimination in some areas (Najera *et al*., 2011). Following organic pyrethrum extract, DDT was historically used for IRS. The now banned organochlorine insecticide dieldrin was also effective before widespread resistance and concerns over environmental impacts as a persistent organic pollutant stopped its use. Organophosphate and carbamate insecticides were soon introduced followed by longer lasting and more stable synthetic pyrethroids in the 1980s. No new insecticides have been approved for use in public health since the introduction of the synthetic pyrethroids at least

30 years ago (Zaim and Guillet, 2002). In Africa, all four insecticide classes are currently used for IRS. Coverage of IRS in the WHO African region rose from less than 5% to over 10% in 2005 and 2011 respectively. However, this has since decreased and in 2013 7% of the population at risk was covered by IRS (WMR, 2014). Globally only 3.5% of risk population at risk is covered by IRS (WMR, 2014).

1.8 Insecticide Treated Bed Nets

Long-lasting insecticide treated bed nets (LLINs) have been widely distributed for malaria vector control in recent years. The only class of insecticide currently approved for impregnating nets is the pyrethroids although alternatives are being sought (WHO, 2012). The nets function both by killing mosquitoes landing on them and attempting to feed off the human host sleeping underneath the nets, and by providing a physical barrier between the human host and the blood-seeking mosquito. Due to the repellant properties of pyrethroids the nets are also designed to repel mosquitoes. The ownership of at least one ITN per household in Africa has maintained an increasing trend, reportedly rising from 3% in 2000, to over 50% in 2013 (WMR, 2014). However, it is likely that not all owned nets are actually used (Kilian *et al*., 2013). While the nets, if used properly, can offer a level of protection against mosquito bites, there is concern about pyrethroid tolerance and increasing resistance within vector populations. This is because vectors are continuously exposed to low doses of pyrethroids in environments where nets are used (Trape *et al*., 2011). Such nets are likely responsible for some of the pyrethroid resistance we see today. In addition, the use of the nets cannot be controlled and there are reports of nets being used as bridal veils and fishing nets amongst others (Minakawa *et al*., 2008).

1.9 Insecticide Treated Plastic Sheeting

Insecticidal plastic sheeting has recently been developed and can be treated with all insecticide classes. This sheeting, also known as durable lining is used to line the walls of houses and can be seen as an alternative to IRS. It may also be used as an emergency vector control measure in refugee camps, simultaneously providing shelter in situations where people have been displaced (Burns *et al*., 2012).

1.10 Insecticide Resistance

Public health approved insecticides collectively target only two insect neurological sites: the sodium ion channel and the neurotransmitter hydrolyser acetylcholinesterase (Hemingway and Ranson, 2000). Reports of resistance to multiple insecticides in malaria vectors are not new (Pal, 1973) and are becoming common (Ranson *et al.*, 2009; Coetzee and Koekemoer, 2013). Insecticide resistance has been detected in a large number of *An. gambiae* populations, particularly in the West African region (Coetzee *at al*. 2006; Corbel *et al*., 2007; N'Guessan *et al*., 2007; Dabirè *et al*., 2008; Ranson *et al*, 2009; Hunt *et al*., 2011). The incidence of multiple resistance to different classes of insecticides is increasing and is currently a major concern for control programmes. The development of insecticide resistance may be attributed to selection pressure caused by IRS and LLINs as well as the use of agricultural pesticides (Pal, 1973; Lines 1988; Protopopoff *et al*., 2008). Mosaic coverage, rotational use of different insecticide classes and the use of insecticide mixtures or insecticides combined with synergists, are recommended for IRS to manage or circumvent the development of resistance in vector populations (WHO, 2012). There is an urgent need for novel insecticides or methods of vector control and to address this need the WHO Vector Control Advisory Group has been established shorten the process of proving and recommending innovative vector control tools and techniques (WHO, 2012).

1.11 Mechanisms of Insecticide Resistance

There are four mechanisms that confer resistance to insecticides. The two primary modes are metabolic detoxification and reduced target site sensitivity (Hemingway and Ranson, 2000). Three mechanisms that enhance the metabolism or detoxification of insecticides and five mutations that cause the insecticide target site to become insensitive to insecticides have been described in *An. gambiae*. Two other important mechanisms are vigour tolerance and behavioural resistance.

1.11.1 Metabolic Resistance

The metabolic system in all living organisms deals with a wide range of xenobiotics, food and waste products. Metabolic resistance to insecticides involves reducing the ability of an insecticide to interact with its target site by biochemical transformation. Three broad enzyme superfamilies have been associated with metabolic resistance namely: P450 monooxygenases, glutathione S-transferases (GSTs) and non-specific esterases (Hemingway and Ranson, 2000). The three enzyme superfamilies may function as resistance mechanisms either alone or in conjunction with each other.

1.11.1.1 Monooxygenase Based Resistance

P450 monooxygenases are involved in endogenous metabolism and metabolism of xenobiotics. In insects, P450 activities are essential for the synthesis and breakdown of juvenile hormones and steroid moulting hormones as well as for the metabolism of pheromones. They are known to be involved in resistance to nearly all insecticide classes (Hemingway and Ranson, 2000). The organophosphate and carbamate insecticides are commonly metabolized with the aid of P450s in insects that display metabolic resistance

(Hemingway and Ranson, 2000). These enzymes form part of the phase I group in detoxification and are usually the first line of defense (Hodgson, 2004b). P450 enzymes add an oxygen atom to a lipophilic compound (xenobiotic) which allows phase II enzymes to conjugate water soluble moieties, like glutathione, to the lipophilic compound thus rendering the toxin water soluble, allowing for excretion and preventing reabsorbtion. In insecticide resistant insects the P450s may be so active that the insecticide is metabolized before it reaches its target site (Berge *et al*., 1998). The specificity of such reactions is dependent on a P450 cytochrome. This protein has a peak absorption at 450nm when reduced and saturated with carbon monoxide (CO) (see Berge *et al*., 1998), hence the name. P450s are one of the most important protein super-families and are very diverse (Fukuto, 1990).

1.11.1.2 Esterase Based Resistance

Esterase enzyme systems, also part of the phase I group of metabolic enzymes, are important in conferring resistance to carbamate and organophosphate insecticides (Peiris and Hemingway, 1993; Hemingway and Karunaratne, 1998). Esterase enzymes have been divided into two main groups depending on whether they preferentially hydrolyse α (alpha) or β (beta) esterases. Esterases can confer resistance in insects through sequestration and accumulation of hydrolytic products. It has been found in resistant strains of mosquitoes that insecticide binding by enzymes (sequestration) is elevated in comparison to non-resistant strains suggesting that there has been positive selection pressure to maintain elevation of helpful alleles in insecticide resistant insects (Hemingway and Ranson, 2000). A non-elevated esterase mechanism has also been found to confer resistance to malathion (Hemingway and Ranson, 2000).

1.11.1.3 GST Based Resistance

Glutathione S-transferase (GST) enzymes (phase II metabolism) have been implicated in conferring resistance to several classes of insecticides through overexpression or increased enzyme activity. They are also involved in protection against oxidative stress, intracellular transport and the biosynthesis of hormones (see Enayati *et al*., 2005 review). Two groups of GSTs have been described in insects according to their location in the cell- microsomal and cytosolic- of which there are several classes (Hodgson, 2004b). Overexpression of GSTs has been associated with resistance to all classes of insecticides (Enayati *et al*., 2005). Dehydrochlorination of DDT, catalysed by GSTs, is an important detoxification mechanism in DDT resistant anophelines (Prapanthadara *et al*., 1995).

Highly elevated levels of GSTs have been found in pyrethroid resistant insect populations however, the role of GSTs in primary or secondary metabolism of pyrethroids has not been defined (Ranson *et al*., 2011). Vontas *et al.* (2001) hypothesized that GSTs may offer protection against pyrethroid induced oxidative stress and Kostaropoulos *et al.* (2001) suggest that GSTs may function as pyrethroid binding proteins thus sequestering the insecticide and reducing its toxic effects.

1.11.2 Reduced Target-site Sensitivity

In this type of resistance a mutation/s alter the insecticide target site rendering it insensitive or less sensitive to the effect of the insecticide. Insecticides that target the nervous system can all be affected by target site resistance (Hemingway and Ranson, 2000). The three known insecticide target sites that may contain mutations conferring resistance in *An. gambiae* are the sodium ion channel (*kdr* - knockdown resistance), the γ amino butyric acid

(GABA) receptor in the chloride ion channel (alanine 296 to glycine substitution - *rdl*), and acetylcholinesterase (*ace-1*^{R}) (Table 1.2).

Table 1.2: Mutations leading to reduced target site sensitivity confer resistance to specific insecticides in *Anopheles gambiae.*

Mutation name	Mutation(s) and location	Sequence	Insecticide affected
kdr	Two different substitutions in the voltage gated sodium channel gene 1.West African: leucine to phenylalanine substitution (L1014F) 2.East African: leucine to serine substitution (L1014S) 3. N1575Y	TTA- susceptible wild-type 1.TTT-west African resistant 2.TCA- east African resistant 3. found only on L1014F haplotypic background to date	DDT and pyrethroid insecticides
rdl	Alanine 296 to glycine substitution in the *Rdl* gene of the GABA receptor	GCA-susceptible GGA-resistant	Dieldrin
ace-1^{R}	Mutation in G119S of the acetylcholinesterase (AChE) gene	GGCT- susceptible AGCT-resistant	Carbamate and organophosphate insecticides

1.11.2.1 Knockdown Resistance – kdr

The knockdown resistance (*kdr*) target-site mutation was first described in *An. gambiae* from Burkina Faso by Martinez Torres *et al*. (1998). *Kdr* mutations occur in the voltage gated sodium ion channel. *Kdr* is now widespread and usually associates with resistance to pyrethroid insecticides and DDT (Donnelly *et al*., 2009). The best protection is recorded for the organochlorine DDT followed by the pyrethroids, permethrin (type I) and deltamethrin (type II) (Reimer *et al*., 2008; Brooke and Koekemoer, 2010). Two primary

mutations, L1014F (Martinez-Torres *et al*., 1998) and L1014S (Ranson *et al*., 2000a), that alter the voltage gated sodium channel preventing insecticide binding (Burton *et al*., 2011) have been described. They usually occur singly with the L1014F most common in West Africa and L1014S more common in East Africa. However, both mutations may occur together in individual mosquitoes (Pinto *et al*., 2006). Many studies implicate the presence of the knockdown resistance (*kdr*) mutation to the observed pyrethroid and/or DDT resistance (Czeher *et al*., 2008; Protopopoff *et al*., 2008; Padonou *et al*., 2012; Corbel and N'Guessan, 2013). However, the increased frequency of *kdr* following selection pressure does not always translate into an operational effect on malaria control programs (Ranson, 2011; WHO, 2012). Metabolic factors may also be concurrently involved (Brooke and Koekemoer, 2010; Mitchell *et al*., 2014).

A new mutation, N1575Y, associated with L1014F, has also recently been described (Jones *et al*., 2012a). This mutation occurs within the linker of domains III-IV at the site of a three amino acid sequence called the inactivation particle which closes the sodium channel pore of the voltage gated sodium channel (Jones *et al*., 2012a). N1575Y only occurs when L1014F is present. When the N1575Y mutation occurs with L1014F, survival to DDT and permethrin is significantly increased in comparison to those bearing the L1014F mutation alone. It is suggested that N1575Y compensates for the deleterious effects of the L1014F mutation or that it provides additional resistance to insecticides (Jones *et al*., 2012a).

1.11.2.2 Rdl

In *An. gambiae,* the resistance to dieldrin (*rdl*) mutation involves an alanine to glycine substitution in the GABA receptor of the chloride ion channels. The vector *An. arabiensis* carries an independent alternative alanine to serine substitution also associated with

resistance to dieldrin (Du *et al*., 2005). The WHO discriminating concentrations of 0.4% and 4% distinguish between heterozygous and homozygous dieldrin resistant mosquitoes respectively (WHO, 2013).

1.11.2.3 Ace-1^R

Mutations in the acetylcholinesterase gene of dipterans are widespread and confer resistance to organophosphate and carbamate insecticides. Weill *et al*. (2002) described a novel mutation (*ace-1^R*) in the acetylcholinesterase gene which confers resistance to carbamate and organophosphate insecticides in *Culex pipiens* and in *An. gambiae* (Weill *et al*., 2004).

1.11.3 Cuticular and Behavioural Resistance

Thickened cuticles are likely to enhance insecticide resistance or tolerance by slowing the rate of insecticide penetration across the cuticle. It has been shown that *Anopheles funestus* mosquitoes, which show tolerance to pyrethroid insecticides, have significantly thicker cuticles than susceptible *An. funestus* (Wood *et al*., 2010). In addition, mosquitoes may alter their behaviour (often by selecting an animal host or by feeding and resting outdoors) to reduce their exposure to insecticides (reviewed by Gatton *et al*., 2013).

1.12 Fitness Costs of Insecticide Resistance

It is generally believed that in the absence of insecticide exposure there is a net fitness cost in individuals that carry one or more resistance genotypes. However, it has been shown that in the case of multiple resistance mechanisms being present in one individual, these costs may offset each other. Berticat *et al*. (2008) investigated the effects of two target-site mutations, *ace-1^R* and *kdr*, conferring resistance to carbamate and pyrethroid insecticides

respectively, in *Culex quinquefasciatus.* Mosquitoes that carried either one or both of the mutations were monitored for fitness levels based on the probability of female mosquitoes reaching adulthood. They found that both of the resistance alleles were associated with a fitness cost in the absence of insecticide and that the *ace-1*^{R} mutation was associated with a higher fitness cost than *kdr*. However, the fitness cost, when both alleles were present in an individual, was significantly less than when *ace-1*^{R} only was present and slightly less than when *kdr* alone was present. This suggests that selection against resistance alleles will be lessened for those carrying both mutations than for those carrying only the *ace-1*^{R} mutation. However, it should be noted that resistance-associated mutations are highly conserved across insect taxa and are limited to a small number of loci. This is probably due to the fitness costs that they induce (see ffrench-Constant *et al*., 1998). Other studies have reported both costs and benefits associated with insecticide resistance mechanisms. Okoye *et al*. (2007) found that the number of eggs oviposited by pyrethroid resistant (metabolically conferred by P450s) *An. funestus*, as well as the proportion of these that survived to adulthood, was higher than that of an insecticide susceptible strain. However, the time taken for emergence to occur was longer in the resistant strain than in the susceptible strain. In general, metabolic resistance mechanisms are considered to confer less cost to fitness than mutations that reduce target-site sensitivity. However, the *rdl* mutation appears to persist in several *An. gambiae* populations despite its fitness cost (Rowland, 1991) and the long term ban of dieldrin. This may be attributable to linkage disequilibrium and the maintenance of the 2La chromosomal inversion (Brooke *et al*., 2002), that associates with adaptation to arid environments, and feeding behaviour (Coluzzi *et al*., 1979).

1.13 Rationale

The President's Malaria Initiative (PMI) has included IRS as an integral part of malaria vector control since April 2007. This inclusion was based on the IRS program implemented at the AngloGold Ashanti mining operation in Obuasi, Ghana, in 2004 (PMI-MOP Ghana, 2007). The NICD houses a colony of *An. gambiae* from Ahafo, Ghana (GAH colony), colonized by Prof. Richard Hunt in 2006. This colony was raised from wild-caught iso-female lines and has not undergone insecticide selection in the laboratory. The GAH colony contains insecticide resistance genes representative of the wild population in Ghana and is therefore ideal for the characterization of insecticide resistance to provide the information required to make informed decisions on the choices of insecticides to be used for IRS at the AngloGold Ashanti mine. The GAH colony also displays staggered larval time-to-hatch which has not been well studied in anophelines. Some eggs from a single batch hatch as normal, one to three days after oviposition, while the rest only hatch one to two weeks later. It was hypothesized that staggered larval time-to-hatch may have implications for vector control in terms of the expression of insecticide resistance and may also be associated with differences in physiological and reproductive fitness. Associations between insecticide resistance and staggered time-to-hatch may also have an impact on adaptation to different environments and could provide a broad base for adaptive selection within populations likely to be subjected to insecticidal control or environmental variability.

Furthering the study of potentially adaptive traits such as the time taken for eggs to hatch after oviposition (time-to-hatch) will add insight into the means by which the vectors maximize their chances of survival in unpredictable environments. Dissecting how these traits affect other life history or physiological traits of potential adaptive significance, will

improve the understanding of adaptive responses of vectors, and may assist in developing more effective or novel vector control strategies.

1.14 Aims and Objectives

The principal aim of this project was to characterize insecticide resistance in a wild population of *Anopheles gambiae* and to evaluate how insecticide resistance associates with other factors of adaptive significance using colonized material.

Specific Objectives:

1. Quantify and characterize insecticide resistance in a wild population of *Anopheles gambiae* from Ghana as well as in the GAH colony (Chapter 2)
2. Assess staggered time-to-hatch in wild-caught as well as colonized *An. gambiae* (Chapter 3)
3. Assess the effect of staggered time-to-hatch on the occurrence and expression of insecticide resistance phenotypes and mechanisms in *An. gambiae* from Ghana (Chapter 3)
4. Scan for microsatellite markers associated with staggered time-to-hatch and/insecticide resistance (Chapter 3)
5. Qualitatively describe the rate of embryonic development in *An. gambiae* eggs obtained from early and late time-to-hatch parents (Chapter 4)
6. Measure the metabolic outputs of *An. gambiae* eggs obtained from early and late time-to-hatch parents (Chapter 4)

Chapter 2: Insecticide Resistance in Ghanaian *Anopheles gambiae*

2.1 Introduction

Ghana is situated in West Africa and is bordered by Côte d'Ivoire, Burkina Faso, Togo and the Atlantic Ocean. The country was known as the Gold Coast before it achieved independence in 1957. It was also referred to as 'The White Man's Grave' as many Europeans who travelled there succumbed to malaria and other tropical diseases (www.worldatlas.com/webimage/countrys/africa/gh.htm, last accessed 12th December 2014). The country is divided into savanna zones in the north and south separated by a central forest zone. The population is just over 25 million people with 28% living below the international poverty line. The economy is predominantly mining and agricultural with cocoa being the biggest cash crop. However, rice, coffee, cassava, corn, shea nuts and bananas are also grown. Fishing and lumbering also generate income. Ghana is Africa's second largest gold producing nation. Recent oil production is expected to improve Ghana's economy. (CIA world factbook: https://cia.gov/library/publications/the-world-factbook/geos/gh.html, last accessed 6th January 2014).

2.1.2 Malaria and Malaria Control in Ghana

Malaria is holoendemic in Ghana and is the major cause of mortality, being responsible for 33% of mortality in children under five as well as 38% of outpatient illness (PMI-MOP Ghana, 2013). Environmental conditions in Ghana are ideal for vector breeding with average temperatures ranging from 26 °C to 29 °C and 1100-2100 mm of rainfall per annum (www.Ghanaweb.com/GhanaHomePage/geography/climate.php, last accessed on the 28th of April 2014). The major vectors of malaria in the West African region are

Anopheles gambiae, *An. coluzzii*, *An. funestus* and *An. arabiensis* (Sinka *et al*., 2010, Coetzee *et al*., 2013a).

Various methods have been used for malaria control in Ghana in the past including IRS, larval control, insecticide treated bed nets (ITNs – which started being distributed by the Roll Back Malaria programme in 1999), and personal protection such as pyrethroid-based mosquito coils. More recent malaria control programmes have been established by the President's Malaria Initiative (PMI) which was launched in 2005 and now includes 19 African countries as well as the greater Mekong sub-region in Thailand (http://www.pmi.gov/countries/index, last accessed 25th March 2014). The PMI, together with the Global Fund for AIDS, tuberculosis and malaria (GFATM), as well as other national and international groups, aim (by 2015) to halve malaria mortality in 70% of the at-risk population on the African continent. The groups most vulnerable to malaria are those living with HIV/AIDS (low prevalence of 1.5% in adults in Ghana (UNICEF, 2011)), pregnant women, and children under five. In order to achieve this goal the PMI will make use of the following four interventions: artemisinin-based combination therapy (ACT); intermittent preventative treatment in pregnancy (IPTp); insecticide treated nets; and indoor spraying with residual insecticides to achieve coverage of 85% of the population (PMI report for Ghana, 2013).

2.1.3 Newmont Goldmine, Ahafo

The fieldwork portion of this project was conducted at the Newmont goldmine in the Brong-Ahafo region of Ghana (Figure 2.1).



Figure 2.1: Map of Ghana. The approximate location of the Newmont goldmine in Ahafo is indicated by the red star. (Map from www.infoplease.com/atlas/country/Ghana.html, last accessed 6th January 2014. ©1997 Magellan Geographix).

The Newmont open-pit cyanide processing gold mine at Ahafo began operations on the 4th of July 2006 (www.Newmont.com). The mine is situated in the forest zone of the country and malaria poses a threat to employees, particularly to expatriates that have not previously been exposed to malaria.

The first survey of malaria vector species at Ahafo was conducted in 2006. It was established that the *An. gambiae* population was almost completely resistant to DDT and

dieldrin, highly resistant to deltamethrin, moderately resistant to bendiocarb and largely susceptible to malathion. On the other hand, *An. funestus* group were found to be fully susceptible to deltamethrin, malathion and bendiocarb (R. Hunt, Appendix 1). Based on these results it was recommended that an organophosphate insecticide be used for IRS and that the vector population should be carefully monitored for changes in levels of resistance to all classes of insecticides (R. Hunt). IRS was included in the malaria control program (PMI report for Ghana, 2007) at Ahafo for the first time in 2007 based on its successful use at the AngloGold/Ashanti mine in Obuasi, southern Ghana (Coetzee *et al*., 2006).

2.2 Aims and Objectives

The aim of this study was to determine baseline insecticide susceptibility levels of wild-caught *An. gambiae* and *An. funestus* from Ahafo, Ghana (2008) and in the GAH colony established from Ghanaian wild-caught mosquitoes from the 2006 collection, as well as to conduct vector incrimination and resistance characterization assays.

Specific objectives

1. Identify wild-caught mosquitoes to complex/species level using morphological keys in the field and to species level using diagnostic assays in the laboratory
2. Use the enzyme linked immunosorbent assay (ELISA) to test for the presence of *Plasmodium falciparum* circumsporozoite protein in blood-fed wild-caught females to estimate the percentage of the local mosquito population carrying *P. falciparum*
3. Screen wild-caught samples as well as GAH colony material for insecticide resistance using standard WHO bioassays
4. Use WHO wall cone bioassays to determine the efficacy of the insecticide used for IRS at the Newmont goldmine residences and offices in Ahafo, Ghana

5. Characterize insecticide resistance in the GAH colony established from Ghanaian wild-caught material using assays to detect the mechanisms conferring resistance to insecticides in this colony

2.4 Materials and Methods

2.4.1 Biological Material: 2006 Colonized Material

Anopheles gambiae caught in Ghana in 2006 and established as a colony (GAH) were used for insecticide resistance characterization. Mosquitoes were housed in the Botha De Meillon insectary at the Vector Control Research Laboratory (VCRL), Johannesburg, at standard insectary conditions and reared as per Hunt *et al*. (2005) on a mixture of ground dog biscuits (BEENO®) and yeast.

2.4.2 Biological Material: 2008 Field Collected Material

Mosquitoes were collected in villages surrounding the Ahafo goldmine complex (7°03.656N; 2°24.190W) in Ghana during June 2008 from inside dwellings after meeting with and gaining permission from house owners or residents. Collections were done by Richard Hunt, Maria Kaiser and Godwin Fuseini using an aspirator and torch. Collected mosquitoes were placed into polystyrene cups and provided with 10% sucrose solution prior to transportation to a temporary laboratory at the mining camp for insecticide susceptibility testing and identification after resting overnight.

2.4.3 Species Identification

Mosquitoes were initially identified to species level using morphological markers (Gillies and De Meillon, 1968; Gillies and Coetzee, 1987). This was done after testing wild-caught

females for susceptibility to insecticides. Once the mosquitoes were transported back to the laboratory DNA was extracted using the method of Collins *et al*. (1987) or the ZyGEM prepGEM^{TM} insect DNA extraction kit according to the manufacture's protocol. The molecular assay (Scott *et al*., 1993) was used to identify members within the *An. gambiae* species complex, followed by PCR to distinguish between molecular forms (Favia *et al*., 2001) now named as *An. gambiae* and *An. coluzzii* (Coetzee *et al*., 2013a). This PCR identification was also done on 20 individuals from the GAH colony to confirm that it was *An. gambiae s.s.* ("S form"). (For species identification and DNA extraction protocols see Appendix 2A.)

2.4.4 ELISA to Detect *Plasmodium falciparum*

An enzyme linked immunosorbent assay (ELISA) (Wirtz *et al*., 1987) was used to detect the presence of *P. falciparum* in 108 blood-fed wild-caught females (Ghaf mothers 1-55 and 53 females that had been exposed to insecticides in the field were tested). The heads and thoraces of the females were placed individually into 1.5 ml centrifuge tubes and ground in 50 µl of BB-NP-40. The remaining mosquito tissue was then rinsed off the pestles with 150 µl of BB to give a total volume of 200 µl. This was stored at -70°C until ready for use. Ninety six well clear plates (NUNC®) were used for ELISAs. The plates were coated with 50 µl of 5 ml phosphate buffered saline (PBS)/40 µl malarial antibody (MAb), covered with aluminium foil, and stored at 4 °C overnight. The coating was thoroughly aspirated the following morning and the plate filled with blocking buffer before incubation at room temperature for one hour. During the blocking buffer incubation step a positive control was prepared by adding 1 µl of *P. falciparum* positive solution to 50 µl of blocking buffer. This was stored on ice in a centrifuge tube until use. Negative controls were prepared as follows: four female mosquitoes from the GAH *An. gambiae* colony were

collected and ground in the same way as the experimental mosquitoes and kept at -70 °C until use.

Once the 1 hour incubation with blocking buffer was complete the plate was again thoroughly aspirated. The positive control was added to the first well of the ELISA plate and the four negative controls were split among the last seven wells. Experimental samples were then added to the other wells- all at 50 µl per well- before incubation for two hours at room temperature. Following incubation with sample the plate was washed twice with PBS-Tween. Tween is a surfactant and is used as a detergent to prevent non-specific binding of antibody. Thereafter 50 µl per well of 5.6 ml Blocking buffer with 10 µl malaria antibody (MAb) peroxidase was added to each well and the plate incubated for one hour at room temperature in the dark. During this incubation step the substrate was prepared and tested by adding 5 µl of MAb to 100 µl of substrate. This is to test whether the malaria antibody and the substrate are recognizing each other. If they do, the substrate will go green and the experiment can continue. After incubation in the dark the plate was washed three to four times with PBS-Tween and 100 µl of substrate was added changing the colour to green. Following addition of the substrate the plate was left to stand for an hour in the dark. Finally the plate was inserted into the plate reader and read at 420 nm. Any readings that were greater than twice the mean of the negative controls were scored as positives. Following the equation: X = ((the sum of the seven negative values)/7) x 2. ELISAs were repeated on all samples that scored positive and only samples that came up as positive on the repeat as well were considered positive.

2.4.5 WHO Insecticide Bioassays

Standard WHO (WHO, 1998; WHO 2013) bioassays were used to determine the insecticide susceptibility status of wild-caught mosquitoes. Approximately 25 female mosquitoes per test were exposed to diagnostic concentrations of insecticide for 1 hour (except fenitrothion - 2 hours) after which knockdown was recorded and mosquitoes were transferred to their respective holding tubes. Unexposed controls (treated in the same way as the test mosquitoes, but exposed to plain untreated paper instead of insecticide impregnated paper) were also included and no mortality was observed. During the holding period mosquitoes were provided with 10% sucrose solution. Mortality was scored at 24 hours post exposure.

Susceptibility tests were performed on the following insecticides at diagnostic concentrations in the field: DDT (4%); dieldrin (4%); deltamethrin (0.05%); malathion (5%); and bendiocarb (0.1%) (WHO, 1998; WHO, 2013). In addition to the field tests, samples of wild-caught mosquitoes were also exposed to 0.05% lambda-cyhalothrin, 0.75% permethrin (pyrethroids), 0.9% pirimiphos-methyl and 1% fenitrothion (organophosphates) at the laboratory in Johannesburg.

Samples drawn from the GAH colony were tested against the insecticides shown in Table 2.1 for insecticide susceptibility. These represent all four insecticide classes currently available for vector control.

Table 2.1: List of insecticides tested against samples of wild-caught *Anopheles gambiae* material from Ghana as well as from the GAH colony, showing diagnostic insecticide concentrations*, arranged according to class.

Insecticide class	Insecticides to be tested (concentration)
Organochlorines	Dieldrin (4%); DDT (4%)
Organophosphates	Malathion (5%); Fenitrothion (1%); Pirimiphos-methyl (0.9%)
Carbamates	Bendiocarb (0.1%); Propoxur (0.1%)
Pyrethroids	Permethrin (0.75%); Deltamethrin (0.05%)

*The diagnostic concentration is twice the concentration of insecticide which results in 100% mortality in susceptible mosquitoes and has been set as a standard for insecticide susceptibility testing by WHO.

2.4.6 Wall Cone Bioassays

The effectiveness of the pirimiphos-methyl insecticide formulation that had already been sprayed on the walls in the mining camp offices, the outside walls of the expatriate housing, and the inside walls of the Hotel Yanana, Sunyani was tested. These walls had been sprayed with the organophosphate insecticide between two months and one day before the cone bioassays took place. Tests were done using standard WHO cone bioassay equipment. Approximately 20 wild female mosquitoes were placed into each cone for a period of 30 minutes. After exposure, knockdown was recorded and all mosquitoes were transferred into holding cups and provided with 10% sucrose solution. Final mortality was recorded 24 hours post exposure.

2.4.7 Synergist Bioassays

Synergists are substances that, alone, are non-toxic, but when combined with an insecticide enhance its toxicity if the mechanism is metabolic-based. Synergists function by acting as substrates thereby blocking the enzymes involved in insecticide detoxification. The use of specific synergists allows one to determine which enzymes are involved in conferring resistance to the applied insecticide (Hemingway, 1981).

For each replicate fifty female mosquitoes were taken from the GAH colony. Half of these were exposed to the chosen synergist for one hour while the other half were placed into a holding tube lined with untreated paper. After the 1 hour synergist exposure both cohorts of mosquitoes were transferred to separate WHO tubes containing papers impregnated with insecticide for one hour after which knockdown was recorded. Mosquitoes were then transferred to separate holding tubes and provided with 10% sucrose solution. Final mortality was scored after 24 hours (as per standard WHO (2013) bioassays). At least five replicates were performed per synergist for each insecticide. A separate unexposed handling control (no synergist or insecticide exposure) was also included. Results were compared and analysed using two-sample t-tests or χ^2 tests (Statistix 7).

The synergists used were diethyl maleate (20% DEM), triphenyl phosphate (10% TPP) and piperonyl butoxide (4% PBO). These synergise GST, esterase and P450 (monooxygenase) enzyme activity respectively. Mosquitoes that survived synergist and insecticide bioassays as well as those that died following insecticide exposure only, were collected and stored on silica before they were screened for resistance associated mutations.

2.4.8 Molecular Screening for Resistance Associated Mutations

Individual mosquitoes were screened for *kdr*, *rdl* and *ace-1*^{R} mutations that are known to be associated with resistance to pyrethroids and DDT (Martinez-Torres *et al*., 1998; Ranson *et al*., 2000a), dieldrin (Du *et al*., 2005), and carbamates and organophosphates (Weill *et al*., 2004) respectively. Statistical comparisons between sample sets were performed using Statistix 7 (two-sample t-tests or χ^2 tests as suitable). The detection of the N1575Y mutation found in *An. gambiae* (Jones *et al*., 2012a) was not done because at the time of the experimentation this mutation was unknown.

2.4.9 DNA Sequencing and Cloning

Twenty-one female mosquitoes of the baseline GAH colony were screened for L1014F and the resultant products were sequenced. In addition the *rdl* fragment was cloned and sequenced for use as a positive control in the *rdl* hydrolysis probe assay and a sub-sample of the DNA screened for *ace-1*^{R} was sequenced to confirm the PCR results. Sequencing was done by Inqaba Biotech™. Cloning was done according to a standard protocol (see Appendix 2B).

2.4.10 Screening for Resistance Associated Mutations

2.4.10.1 Hydrolysis Probe (TaqMan®) Molecular Assays

Hydrolysis probe assays require a real time PCR machine to detect the fluorescence emitted by the minor groove binder (MGB) probes. Due to the instant visualization permitted by the use of real time technology there is no need to view amplified products by electrophoresis on a gel, effectively halving the time required to obtain results. Results are scored by reading the fluorescence values obtained. Two fluorescent dyes; VIC and FAM were used in the following assays. A substantial increase in VIC fluorescence indicates a homozygous susceptible individual, a substantial increase in FAM fluorescence- a homozygous resistant individual, and an intermediate increase of fluorescence in both probes indicates a heterozygote. The real time PCR machine used shows bi-directional scatter-plots of the fluorescence levels which assist in scoring individuals' genotypes (Figure 2.2).

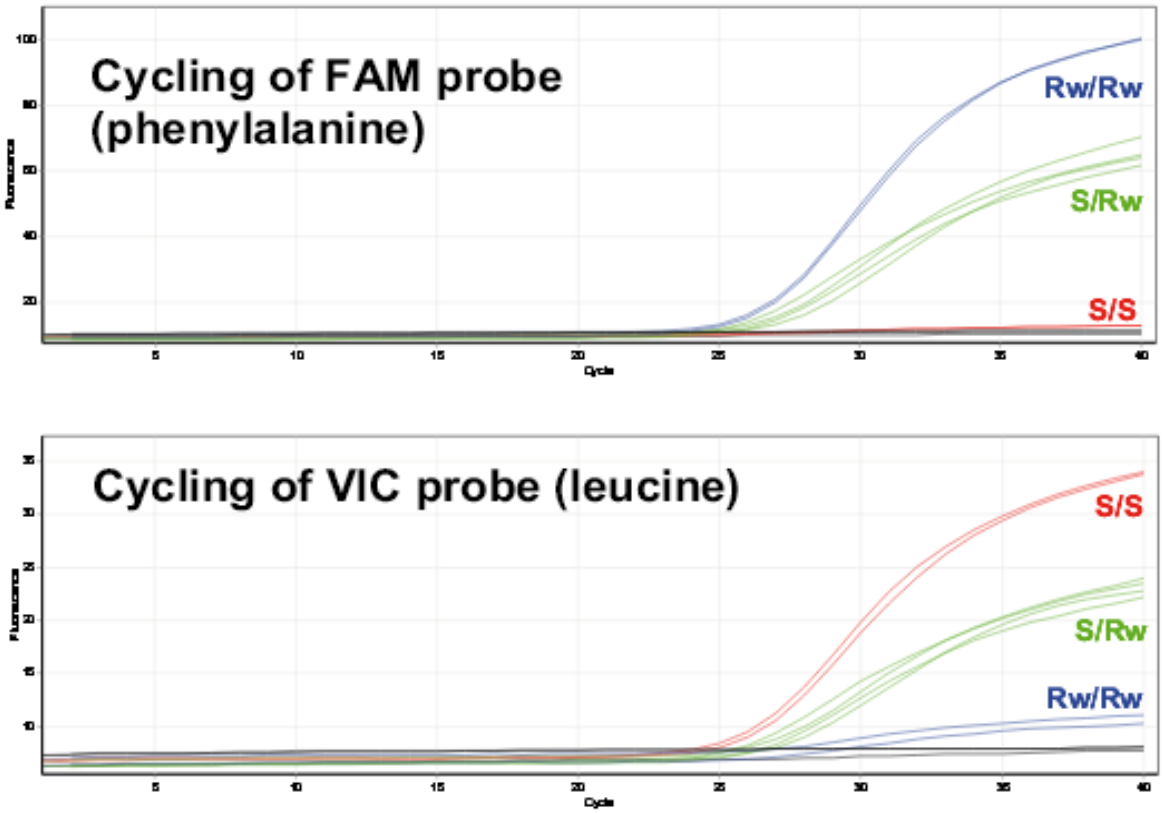


Figure 2.2: An example of the *kdr* fluorescence probe molecular assay (figure from Bass *et al*. (2008)). The fluorescence plots above show a simplified result to that seen when viewing a L1014F *kdr* fluorescence probe assay. The blue line indicates the FAM probe while the red line indicates the VIC probe. The green line indicates an intermediate response usually indicative that both VIC and FAM probes fluoresce at an intermediate level indicating a heterozygote for the L1014F (w-*kdr*) allele in such samples. Key: Rw- Resistant west *kdr* (L1014F *kdr* mutation present); S susceptible (wild type *kdr* genotype). Where samples are Rw/Rw it means that the individual tested is homozygous for the west *kdr* (L1014F) mutation, S/Rw indicates a heterozygote and S/S that the individual is homozygous susceptible (wild type).

Kdr

Two hydrolysis probe assays to detect the *kdr-w* (L1014F) and *kdr-e* (L1014S) mutations associated with resistance to pyrethroids and DDT have been developed by Bass *et al*. (2007, 2008). Both assays use the following primers:

KdrF: CATTTTTCTTGGCCACTGTAGTGAT; KdrR:

CGATCTTGGTCCATGTTAATTTGCA; and the VIC labelled WT probe: VIC-

CTTACGACTAAATTTC.

To detect L1014F the 6FAM labelled probe: 6FAM-ACGACAAAATTTC is used and to detect L1014S the 6FAM labelled probe: 6FAM-ACGACTGAATTTC is used.

The fact that the probes specific for L1014F and L1014S are labelled with the same fluorescent dye means that assays to detect these mutations must be performed separately. PCR cycling conditions for both assays are as follows: Ten minutes at 95 °C followed by 40 cycles of 95 °C for 10 seconds and 60 °C for 45 seconds. Assay conditions: for each sample tested the reaction contained the following: 0.75 to 1.5 µg DNA; 800 nM of each primer pair; 200 nM of each probe pair and 10 µl of Sensimix or IQ Supermix in a total volume of 20 µl.

Rdl

Rdl susceptible and resistant individuals were cloned for use as positive controls using the Du *et al*. (2005) PCR method. The hydrolysis probe assay developed by Bass *et al*. (2008) to detect A296G *rdl* mutation on *An. gambiae* was then used in molecular assays on dieldrin exposed samples that survived or died following exposure. The Bass *et al*. (2008) *rdl* assay employs the following primer pair:

GlyRdlF: TCATATCGTGGGTATCATTTTGGCTAAAT and;

GlyRdlR CGACATCAGTGTTGTCATTGTCAAG;

and probe pair: WT1: VIC-ACGTGTTGCATTAGG and;

Gly: 6FAM-ACGTGTTGGATTAGG.

Cycling conditions were: Ten minutes at 95 °C followed by 40 cycles of 95 °C for 10 s and 60 °C for 45 s. Each sample reaction contained the same proportions of reagents and genomic DNA as that described for the *kdr* hydrolysis probe assays.

2.4.10.2 A Restriction Fragment Length Polymorphism Assay to Detect the Ace-1^R Mutation Associated with Organophosphate and Carbamate Resistance

The restriction fragment length polymorphism (RFLP) PCR (Weill *et al*., 2004) was used to detect the *ace-1^R* resistance associated mutation in *An. gambiae*. DNA was extracted from samples that survived exposure to 0.1% propoxur or 0.1% bendiocarb as well as from samples that died following exposure. The *Alu*1 restriction enzyme (Fermentas International Inc., Canada, Catalogue #ER0011, supplied by Whitehead Scientific) digest was used to determine if the survivors had the *ace-1^R* mutation associated with carbamate resistance (Weill *et al*., 2004). *Alu*1 recognizes the sequence AG/CT. It restricts the sequence at these points generating fragments with blunt termini. If the *ace-1^R* mutation is present in the tested samples the restricted product sizes should be 120 bp and 74 bp respectively. In samples without the mutation the enzyme will not cut and the fragment will remain at 194 bp (Weill *et al*., 2004 (see figure 2.3)).

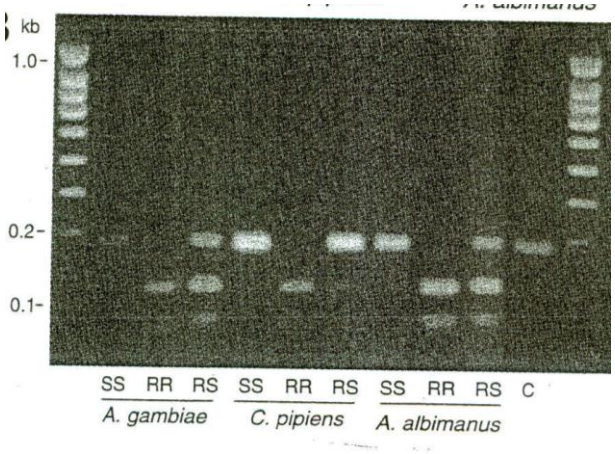


Figure 2.3: Weill *et al*. (2004) showing the restriction of DNA fragments in the presence of the *ace-1*^{R} gene target site mutation. SS= homozygous susceptible, RR= homozygous resistant, RS= heterozygous resistant.

2.5 Results

2.5.1 Species Identification

Mosquitoes were identified by hand lens in a temporary laboratory in Ghana using the key of Gillies and De Meillon (1968), and Gillies and Coetzee (1987). Most of the mosquitoes were morphologically identified as *An. gambiae* species complex (279/312- 89%) and a few as *An. funestus* group (39/312- 11%) mosquitoes. A sub-sample of 152 individuals that had been exposed to insecticide in the field and identified as *An. gambiae* species complex based on morphology were identified to species level (Scott *et al*., 1993). Of these, 10 samples showed no amplification and the rest were *An. gambiae* or *An. coluzzii*. One female specimen was *An. arabiensis***.** A sub-set of 86 samples that amplified as *An. gambiae* according to Scott *et al*. (1993) were further identified to individual *An. gambiae* or *An. coluzzii* species using the PCR of Favia *et al*. (2001). Of these 78 amplified successfully. *Anopheles coluzzii* made up 13% while the rest were *An. gambiae*. In addition, 19 GAH colony females screened for molecular form (species) were confirmed as *An. gambiae*.

2.5.2 *Plasmodium falciparum* ELISA

108 blood-fed wild-caught females were tested for *P. falciparum* circumsporozoite protein, of which 7.41% were positive. The majority of the *P. falciparum* circumsporozoite positive mosquitoes were from the sample of Ghaf mothers 1-55 (6 out of 8 positive mosquitoes).

2.5.3 Standard WHO Bioassays on Wild-caught Material

Based on WHO (2013) guidelines, wild-caught *An. gambiae* mosquitoes were almost completely resistant to DDT and dieldrin, highly resistant to the pyrethroid deltamethrin and moderately resistant to the carbamate bendiocarb and the organophosphate malathion (Table 2). Wild-caught mosquitoes were also exposed to lambda-cyhalothrin, permethrin and fenitrothion in the Johannesburg laboratory and were resistant to the two pyrethroids (19% and 15% mortalities respectively) and showed marginal resistance to the organophosphate fenitrothion (96% mortality).

Table 2.2: Percentage mortalities of Ghanaian wild-caught female *An. gambiae* mosquitoes exposed to four different classes of insecticide in the field during 2006* and in 2008. Key D = number of mosquitoes that died 24 hours after exposure, A= number of mosquitoes alive 24 hours after exposure.

Year	**2006***				**2008**			
Insecticide	**D**	**A**	**Total**	**% Mortality**	**D**	**A**	**Total**	**% Mortality**
DDT	0	35	35	*0*	4	61	65	*6.2*
Dieldrin	5	36	41	*12.20*	3	45	48	*6.25*
Deltamethrin	33	47	80	*41.25*	50	66	116	*43.1*
Malathion	54	0	54	*100*	107	10	117	*91.5*
Bendiocarb	52	17	69	*75.4*	87	22	109	*79.8*
Controls				*0**	0	42	42	*0*

*The 2006 assays were done by Professor Richard Hunt (see Appendix 1).

2.5.4 WHO Wall Cone Bioassays

Pirimiphos-methyl sprayed on the walls of different buildings at different times in and around the mining camp was ineffective at killing the mosquitoes regardless of how freshly sprayed the wall was (Table 2.3). The final cone bioassays performed at Kookooassie, the mine's senior staff village, were done only after the results of the other cone bioassays had been analysed and indicated a problem. The fact that, in the standard WHO bioassays, mosquitoes were susceptible to malathion but seemed to display high levels of resistance to pirimiphos-methyl sprayed on the walls, (both of which are organophosphates), aroused suspicion. Therefore, a batch of the mine's pirimiphos-methyl based insecticide was taken back to South Africa from Ghana. In Johannesburg, WHO cone bioassays on panels sprayed with mine's insecticide (1 g and 2 g active ingredient (ai) per m^2) were performed using 2-3 day old female mosquitoes from the susceptible colony, SUA (Table 2.4). Panels were sprayed by the South African Bureau of Standards. In addition standard WHO bioassays using WHO pirimiphos-methyl impregnated papers were done on wild-caught mosquitoes transported back to South Africa (Table 2.5).

Table 2.3: Percentage mortalities 24 hours after exposure to the mine's pirimiphos-methyl insecticide treated walls following standard WHO wall cone bioassays carried out in Ghana in June 2008. The date the walls were initially treated with insecticide as well as the date the cone bioassays were performed is indicated in parenthesis for each structure.

Hotel Yanana, Sunyani (sprayed on 28 April 2008, tested on 30 June 2008)					
Test	**30min kd**	**24hr Dead**	**24hr Alive**	**Total**	**% Mortality**
1	2	3	18	21	*14.2857*
2	0	1	17	18	*5.55556*
3	2	2	22	24	*8.33333*
control	0	0	19	19	*0*
HSLP building, Ahafo mine camp A (sprayed on 19 April 2008, tested on 27 June 2008)					
	30min kd	**24hr Dead**	**24hr Alive**	**Total**	**% Mortality**
1	0	0	28	28	*0*
2	1	2	22	24	*8.33333*
3	0	1	24	25	*4*
control	0	0	17	17	*0*
Kookooassie, Ahafo mine's Senior Staff Village (sprayed on 31 March 2008, tested 30 June 2008)					
	30min kd	**24hr Dead**	**24hr Alive**	**Total**	**% Mortality**
1	0	0	24	24	*0*
2	0	0	23	23	*0*
3	0	0	18	18	*0*
control	0	0	20	20	*0*
Kookooassie, Ahafo mine's Senior Staff Village (sprayed 1 July 2008, tested 2 July 2008)					
	30min kd	**24hr Dead**	**24hr Alive**	**Total**	**% Mortality**
1	0	0	19	19	*0*
2	0	0	21	21	*0*
3	0	1 (male)	20	21	*4.76191*
control	0	1	23	24	*4.16667*

The susceptible strain SUA showed very low levels of mortality to the insecticide used in Ghana. A functional insecticide should have resulted in 100% mortality (Table 2.4). Ghanaian mosquitoes previously exposed to pirimiphos-methyl during the cone bioassays on walls in Ghana, were exposed to the WHO diagnostic dose of pirimiphos-methyl and showed higher percentage mortalities than wild-caught mosquitoes that were not

previously exposed (Table 2.5). The susceptible strain SUA showed low levels of resistance to this insecticide according to the WHO (2013) criteria for determining insecticide resistance.

Table 2.4: Percent mortality 24 hours after exposure of the susceptible strain SUA to 1 g and 2 g ai/m^2 of pirimiphos-methyl from Ghana using cone bioassays.

Dose	24hr Dead	24hr Alive	Total	% Mortality
1g ai/m^2	4	45	49	*8.16*
2g ai/m^2	3	50	52	*5.77*
Unexposed control	1	21	22	*4.55*

Table 2.5: Percentage mortality achieved after exposing Ghanaian wild-caught mosquitoes to the WHO diagnostic concentration of 0.9% pirimiphos-methyl using standard WHO bioassays. The insecticide susceptible strain, SUA was used as a reference.

Samples tested	24 hr Dead	24 hr Alive	Total	% Mortality
Previously tested samples	16	34	50	*32*
Previously untested samples	4	44	48	*8.33*
Susceptible controls SUA	143	9	152	*94.08*

2.5.5 Insecticide Susceptibility Testing of the GAH *Anopheles gambiae* Colony using WHO Bioassays

The GAH colony displayed resistance to all four classes of insecticide. Overall, the colony was most susceptible to the organophosphates followed by carbamates, pyrethroids and organochlorines respectively (Figure 2.4).

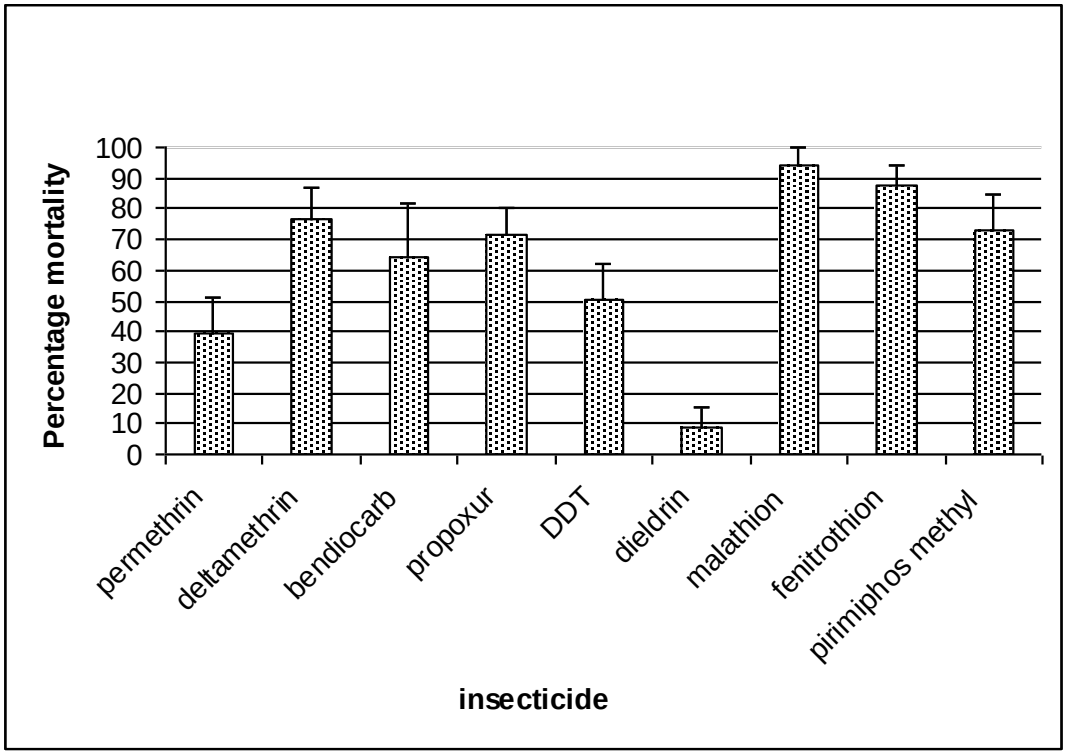


Figure 2.4: Mean percentage mortalities 24 hours post exposure with error bars of 3-5 day old *Anopheles gambiae* GAH colony females by insecticide. Mortality less than 98% indicates resistance should be suspected (WHO, 2013).

2.5.6 Synergist Bioassays on the GAH Colony

Synergist bioassays performed on baseline GAH indicated that monooxygenases are involved in pyrethroid detoxification (both deltamethrin and permethrin). In addition TPP significantly increased mortality in permethrin exposed samples. Resistance to deltamethrin was lost in the time (approximately four months) between the first synergist exposures performed using PBO and subsequent exposures using TPP and DEM. Thus no synergistic effect of TPP or DEM could really be observed as the mosquitoes were mostly susceptible to deltamethrin at this stage. None of the synergists had any significant effect on mortality when combined with any of the other insecticides tested (Table 2.6). Mortality in unexposed handling controls was 0-5% for all tests, within acceptable limits (WHO, 1998, 2013).

Table 2.6: Percentage mortality of *Anopheles gambiae* GAH either exposed to one of the following synergists: 4% piperonyl butoxide (PBO), 10% triphenyl phosphate (TPP) or 20% diethyl maleate (DEM) or unexposed to synergist prior to exposure to insecticides from all four classes. Percentage mortality for both unsynergised and synergised samples is given. P-values (calculated using two-sample t-tests) that indicate a significant synergistic effect are shown in underlined italics. Syn: samples exposed to synergist; unsyn: unsynergised samples.

Synergist		**4% PBO**		**10% TPP**		**20% DEM**	
Insecticide	**Syn/ unsyn**	**mean % mortality**	**p-value**	**mean % mortality**	**p- value**	**mean % mortality**	**p- value**
	Syn	27.46		39.6		38.37	
4% DDT	unsyn	27.48	~1	41.76	0.76	37.38	0.86
	Syn	11.7		13.76		12.15	
4% dieldrin	unsyn	10.83	0.81	9.967	0.13	12.65	0.9
0.75%	Syn	80.54		86.14		74.93	
Permethrin	unsyn	48.72	*<0.001*	56.11	*<0.01*	61.79	0.17
0.05%	Syn	95.32		94.12		100	
Deltamethrin	unsyn	72.52	*<0.01*	94.6	0.98	98	~1
	Syn	70.3		84.97		79.43	
0.1% Propoxur	unsyn	71.03	0.87	79.5	0.06	82.23	0.52
0.1%	Syn	77.74		78.84		68.99	
Bendiocarb	unsyn	71.56	0.29	71.36	0.16	72.43	0.66
0.9% Pirimiphos-methyl	Syn	33.39		86.65		74.65	
	unsyn	44.89	0.37	67.55	0.08	63.53	0.09

2.5.7 Molecular Screening for Known Mutations Associated with Insecticide Resistance in Anophelines

2.5.7.1 Kdr

There was a high proportion of L1014F in the unselected GAH baseline colony (Table 2.7).

Table 2.7: Knockdown resistance (L1014F) genotypes of mosquitoes from the *Anopheles gambiae* GAH colony determined by sequencing.

Genotype	Number
Homozygous susceptible	7 (33.33%)
Heterozygous	12 (57.14%)
Homozygous resistant	2 (9.52%)
Total sequenced	**21**

Kdr L1014F clearly associated with permethrin resistance ($\chi^2 = 18.47$, df = 1, $p < 0.01$) as well as DDT resistance ($\chi^2 = 43.43$, df = 1, $p < 0.01$) in GAH. In addition, exposure to the synergist PBO significantly increased the L1014F frequency amongst permethrin resistant GAH mosquitoes ($\chi^2 = 15.01$, df = 1, $p < 0.01$) (Table 2.8). The hydrolysis probe molecular assay to detect *kdr*-e (L1014S) was performed on 82 permethrin and DDT exposed GAH females and all were genotyped homozygous susceptible (SS).

Table 2.8: *Kdr* L1014F resistance phenotypes and genotypes of *Anopheles gambiae* GAH female mosquitoes exposed to permethrin, permethrin and PBO, or DDT.

Sample set	Phenotype	Genotype		
		RR	**RS**	**SS**
Permethrin + PBO survivors	Resistant	25/29	4/29	0
Permethrin only survivors	Resistant	8/12	3/12	1/12
Permethrin only dead	Susceptible	0	18/21	3/21
DDT only survivors	Resistant	13/21	8/21	0
DDT only dead	Susceptible	0	14/20	6/20

2.5.7.2 Rdl

The presence of the alanine 296-glycine (*rdl*) mutation in the GABA receptor strongly associated with survival following dieldrin exposure ($\chi^2 = 101.47$, df = 1, $p << 0.01$) (table 2.9).

Table 2.9: *Rdl* genotypes of baseline *Anopheles gambiae* GAH exposed to 4% dieldrin expressed as frequencies (proportions). The total number of each genotype observed against the total number of individuals successfully genotyped is indicated in brackets.

	Dieldrin susceptible phenotype	**Dieldrin resistant phenotype**
Genotype	**Frequency**	**Frequency**
SS	0.92 (23/25)	0
RS	0.08 (2/25)	0.55 (16/29)
RR	0	0.45 (13/29)

2.5.7.3 Ace-1^R

Twenty-eight GAH bendiocarb exposed survivors as well as 27 bendiocarb exposed females that died following exposure were screened for the *ace-1^R* mutation using the RFLP assay (Weill *et al*., 2004). Three of the 28 survivors were homozygous resistant and the remaining 25 survivors were heterozygous. All 27 mosquitoes that died following exposure to bendiocarb were homozygous susceptible (Table 2.10). These data show a strong association between *ace-1^R* genotype and response to bendiocarb exposure phenotype ($\chi^2 = 180$, df = 1, $p < 0.01$).

Table 2.10: *Ace-1^R* genotypes of baseline *Anopheles gambiae* GAH exposed to 0.1% bendiocarb expressed as frequencies (proportions). The total number of each genotype observed against the total number of individuals successfully genotyped is indicated in brackets.

	Bendiocarb susceptible phenotype	**Bendiocarb resistant phenotype**
Genotype	**Frequency**	**Frequency**
SS	1 (27/27)	0
RS	0	0.89 (25/28)
RR	0	0.11 (3/28)

2.6 Discussion and Conclusion

Species identification on mosquitoes collected around the mining camp confirmed that *An. gambiae s.s.* was the predominant vector. *An. coluzzii* comprised 13% of the samples. One

An. arabiensis female and a few *An. funestus* (by morphology) mosquitoes were also identified. *Anopheles gambiae* has been associated with smaller or more temporary environments due to larval adaptation in response to predators (Diabaté *et al*., 2005 and 2008) and this may be one of the reasons it dominated. However, species distribution and abundance may differ throughout the year and other vector species may be more abundant at different times or under different conditions (Fontenille *et al*., 1997; Onyabe and Conn, 2001; Minakawa *et al*., 2002).

The 7.41% sporozoite positivity detected by *P. falciparum* ELISA is far higher than the 3-5% positivity usually found in endemic areas. This may be because mosquitoes were collected from only a few dwellings. The presence of an infected person in one of the dwellings where many of the mosquitoes may have fed have could potentially caused a bias in infection results since some of the mosquitoes were bought back alive from the field and induced to lay eggs before being subjected to ELISA. This would have allowed sufficient time for the parasite to develop to the stage where it could be detected by the assay (8 to 15 days after an infective blood meal). Nevertheless, the high malaria infection rate in the mosquitoes is concerning and reflects the conditions at the mine at the time. Many expatriates at the mine had contracted malaria in 2008 when the survey was conducted and there was a medical evacuation from the mining camp of two patients who suffered severe malaria.

WHO insecticide susceptibility tests done in the field indicated resistance to the carbamate bendiocarb, the pyrethroid deltamethrin and the organochlorines DDT and dieldrin. The levels of resistance to the latter group were particularly high. WHO cone bioassays on insecticide sprayed walls in and around the mining camp showed extremely low levels of

mortality. The walls had been sprayed with an insecticide that contained pirimiphos-methyl, an organophosphate, as the active ingredient. This result was particularly strange since mosquitoes tested on malathion (organophosphate class) were susceptible (100% mortality). This led to a number of investigations including observing the technique of the spray-men, testing walls freshly sprayed with the insecticide, inspecting insecticide storage facilities and testing the susceptibility of wild-caught mosquitoes to diagnostic concentrations of pirimiphos-methyl as well as fenitrothion (also an organophosphate), using WHO standard bioassays in the laboratory in Johannesburg. The results of standard WHO bioassays using pirimiphos-methyl on known pre-exposed and potentially naive wild-caught females suggests that there is an additive dosage effect of pirimiphos-methyl. However, despite the dosage effect, the Ghanaian mosquitoes showed very high levels of resistance to pirimiphos-methyl (32% mortality in previously exposed mosquitoes, and 8.33% mortality for wild-caught mosquitoes not previously exposed). Exposure of the susceptible control SUA to the WHO pirimiphos-methyl impregnated papers confirmed that the insecticide was working (94% mortality). Some resistance to the organophosphate fenitrothion was detected in wild-caught mosquitoes (96% mortality). The differences in the levels of susceptibility observed in the wild-caught Ghanaian *An. gambiae* population to different insecticides within the organophosphate class suggest multiple resistance mechanisms for organophosphates. This has also recently been reported by Aïkpon *et al*. (2014). Numerous mechanisms can confer resistance to organophosphate insecticides in mosquitoes. Therefore a population may be largely resistant to one of the insecticides in a class but may not be equally resistant to all insecticides within that class, depending on the resistance mechanisms present. Resistance mechanisms for organophosphates include reduced target site sensitivity due to the *ace-1*^{R} mutation (Weill *et al*., 2004) and metabolically conferred resistance through malathion-specific esterases, as well as P450s

and GST enzymes (Hemingway 2000; Li *et al*., 2007). Due to the limited number of mechanisms conferring resistance, cross resistance between insecticide classes is common (Perera *et al*., 2008; Bisset *et al*., 1997).

The high rate of survival in older wild-caught mosquitoes exposed to pirimiphos-methyl is concerning. One would expect mortality in the mosquitoes that were tested in the laboratory to be higher than the mosquitoes that were tested in the field, because they were a few days older (Rajatileka *et al*., 2011). However, insecticide resistant blood-fed *An. funestus* females survive insecticide exposure better than non-blood fed females (Spillings *et al*., 2008), and the effect of multiple blood meals (which were provided to the wild-caught females in order to obtain eggs) on detoxification may have played a role in the markedly high survival observed in these Ghanaian mosquitoes which displayed resistance to all insecticide classes.

The insecticide storage facilities at the mine were examined. Insecticides were stored in a room away from the mining camp that did not have functional air-conditioning or any form of refrigeration. Temperatures in Ghana can easily exceed 35 °C which can result in a deterioration of insecticides. Storage in moist conditions may cause wettable powders to clump and lose insecticidal activity (Fred Fishel: www.extension.missouri.edu/explorepdf/agguides/g01921.pdf, last accessed 30th April 2014), which was also possible due to high humidity in Ghana. It was thus recommended that the insecticides be stored in a dry, refrigerated or air-conditioned facility below 25 °C. The insecticide used for spraying was all from the same batch so the possibility that that particular batch of insecticide had some problem in the manufacturing process cannot be ruled out. Temperature may have a marked effect on the longevity and effectiveness of

vector control insecticides, therefore it may be advisable to conduct bioassays more often on treated walls when temperatures exceed 25 °C for prolonged periods of time and possibly to reduce the intervals between each IRS treatment in such cases.

Resistance to multiple classes of insecticides in *An. gambiae* is a growing concern for malaria vector control (Corbel *et al*., 2007; N'Guessan *et al*., 2007; Dabiré *et al*., 2008). The *An. gambiae* laboratory colony GAH displayed resistance to all classes of insecticides currently available for use in public health. All three target site resistance mechanisms were found in the baseline colony. *Kdr* was found at a frequency of 0.62, *rdl* at 0.41 and *ace-1*^{R} at 0.28. Synergist bioassays showed the presence of two metabolic resistance mechanisms: P450s and esterases conferring resistance to deltamethrin and permethrin, and permethrin respectively. Synergist bioassays failed to implicate GSTs in conferring metabolic resistance in GAH. Although this colony is likely to show reduced genetic variation relative to the wild population from which it originated, multiple resistance in *An. gambiae* from Ghana has already been reported (Coetzee *et al*., 2006) and was also observed in the wild-caught material collected in Ghana and tested for insecticide susceptibility during this study and included in Hunt *et al*., (2011).

From the data presented in this chapter, mechanisms of resistance to pyrethroids in *An. gambiae* GAH include monooxygenase and esterase mediated detoxification as well as the L1014F *kdr* mutation. The correlation between *kdr* genotype and pyrethroid resistant phenotype was clear but not absolute, with small numbers of homozygous resistant (RR) genotypes occurring in the phenotypically susceptible samples and visa versa. These discrepancies coupled with the occurrence of RS heterozygotes in resistant and susceptible mosquitoes as well as the increased *kdr* frequency in synergised survivors, may suggest

that enzyme mediated detoxification plays an important role in the production of a measurable pyrethroid resistant phenotype (Brooke, 2008), or that other factors such as the presence of a SNP in the haplotypic background as described by Weetman *et al*. (2010), and the novel N1575Y mutation (Jones *et al*., 2012a) may play a role in enhancing the resistance phenotype. Resistance to DDT associated particularly closely with the assortment of the L1014F *kdr-w* mutation. Resistance to dieldrin associated closely with the *rdl* mutation while resistance to the carbamate bendiocarb associated closely with the *ace-1*^{R} mutation. The apparent scarcity of the RR (homozygous resistant) *ace-1*^{R} genotype in assayed adult mosquitoes may be attributable to an unusually high death rate of RR individuals at pupation as described by Djogbénou *et al*. (2010). Carbamate resistance and the low level of organophosphate resistance in the *An. gambiae* GAH colony is therefore likely mediated by the assortment of *ace-1*^{R} although metabolically mediated resistance likely plays a supporting role. High frequencies of *ace-1*^{R} have been recorded in *An. gambiae* populations in Burkina Faso indicating that caution should be employed when using carbamate and organophosphate insecticides for vector control in the West African region (Dabiré *et al*., 2009). A duplication of the *ace-1* gene has been reported which confers a more stable (or fit) resistant phenotype in *Culex pipiens* and higher levels of resistance in *Cx. pipiens* (Labbé *et al*., 2007) as well as in *An. gambiae* and *An. coluzzii* from Côte d'Ivoire (Djogbenou *et al*., 2009; Edi *et al*., 2014). The GAH colony was a relatively young colony (about 2 years old) and had not undergone insecticide selection in the laboratory when it was screened for insecticide susceptibility and the mechanisms conferring resistance. As such the colony is likely a good representation of the resistance mechanisms present in the wild-material from Ghana.

In comparing the colonized Ghanaian mosquitoes (GAH) to the wild-caught mosquitoes from which the colony was established in 2006 (Table 2.2 and Appendix 1), and wild mosquitoes caught two years later in 2008 (Table 2.2), some similarities can be seen. Deltamethrin and DDT resistance had decreased markedly in GAH during colonization, but appeared relatively stable in the wild-caught mosquitoes. Bendiocarb resistance was quite stable in the wild-caught mosquitoes and increased slightly in GAH. Malathion resistance was detectable at low levels in GAH and in the 2006 wild mosquitoes but was not seen in the 2008 wild mosquitoes. These results give some suggestions concerning the fitness costs of resistance to the different insecticides. Deltamethrin and DDT resistance costs were apparent due to the increased mortality seen in GAH which had not undergone insecticide selection in the laboratory, implying a fitness cost associated with *kdr* (Berticat *et al*., 2008; Brito *et al*., 2013; Alout *et al*., 2014). The fitness costs of bendiocarb and dieldrin resistance seem negligible or slight in this population as it was maintained in insectary conditions without insecticide selection pressure. Although reduced fitness has been reported with *ace-1*^{R} resistance genotypes (Berticat *et al*., 2008; Djogbenou *et al*., 2010), it is possible that bendiocarb resistance in this population has associated with compensatory mutations (Kulathinal *et al*., 2004) as a consequence of pleiotropy (Brooke and Koekemoer, 2010), or that under insectary conditions there are no costs associated with this mutation in this colony. It may also be that the combination of *kdr* and *ace-1*^{R} in GAH reduces the fitness cost of the individual mutations as described by Berticat *et al*. (2008). Resistance to dieldrin appeared stable in both wild collections as well as in GAH. As mentioned previously dieldrin resistance has been shown to persist at high levels in *An. gambiae* even without selection pressure (Brooke *et al*., 2006; Koffi *et al*., 2013). This may be due to the assortment of the resistant genotype with the 2LA "standard" inversion that has become fixed in some populations (Brooke *et al*., 2000; Brooke *et al*., 2002).

Resistance to the organophosphate malathion was detectable in 2006 but not in 2008. This suggests that the mechanism conferring resistance to pirimiphos-methyl does not confer cross resistance to malathion in this population, although these insecticides are from the same class (mosquitoes were resistant to pirimiphos-methyl used at the mine for IRS during the two year period between surveys, and GAH also showed resistance to pirimiphos-methyl). The susceptibility of mosquitoes to fenitrothion appeared equivalent to the levels seen for malathion so it likely that the resistance mechanism(s) for these insecticides overlap.

Although the number of malaria deaths has been decreasing in Ghana the number of admissions for malaria have shown an increasing trend since 2007 (WMR, 2014) even though malaria vector control efforts have increased. Resistance to all insecticide classes can be considered established in West Africa (Corbel *et al*., 2007; Okoye *et al*., 2008; Ranson *et al*., 2009; Namountougou *et al*., 2012) and poses a very serious threat to the future of vector control. There are a limited number of resistance mechanisms that operate on only four insecticide classes. This means that there is often cross-resistance between the different classes although this does differ for specific insecticides even within a single class. When choosing an insecticide/s for use in a particular setting it is therefore essential that the specific insecticides be used to test the susceptibility of mosquitoes from the targeted region. Various methods to slow or reduce the rate of insecticide resistance development or to 'break' resistance have been used or proposed, including rotations of insecticides, mosaic spraying, combinations of interventions and the use of insecticide mixtures (WHO, 2012). In addition the use of insecticide-synergist combinations to reduce metabolic resistance has been proposed (WHO, 2012). This is potentially dangerous because it is possible that the synergist may increase mortality in one insecticide but

decrease it in another depending on the mechanisms conferring resistance to the particular insecticides. This effect has been demonstrated for PBO against the phenylpyrazole insecticide fipronil (not currently approved for malaria vector control) in rice stem borers (Rider and LeBlanc, 2005), and in *Daphnia magna* where the action of the organophosphate insecticides parathion and malathion was antagonized (Huang *et al*., 2010). Perera *et al*. (2008) also reported an antagonistic effect of PBO on the function of organophosphates malathion and fenitrothion in anopheline malaria vectors in Sri Lanka. However, this does not seem to be a common occurrence as there are not many papers reporting this effect. Work with agricultural pesticides has also shown that the exposure of an insect to one insecticide followed by (or mixed with) another may not have the predicted effect and that insects may in fact become less susceptible, more susceptible or show no change in response to such combinations (or insecticide mixtures) than if they had been exposed to only one of the insecticides (pesticide interactions and compatibility- www.ovs.com/1_pesticide_interaction.htm, last accessed 9th May 2014). So monitoring the effects of potential mixtures or combinations of insecticidal control interventions should be done on field caught samples from the control area before considering this approach.

The development of multiple resistance in target vector populations is a serious threat to vector control. Thus, there is an urgent need for the development of new insecticides and other novel means of vector and parasite control to maintain current levels of malaria control and progress towards reduced global impact of the disease.

Chapter 3: Time-to-Hatch and Insecticide Resistance in *Anopheles gambiae*

3.1 Introduction

Larval hatching in *An. gambiae* generally occurs 2-3 days after oviposition depending on environmental conditions (Service, 2000). However, there are studies describing hatching occurring much later (Yaro *et al*., 2006; de Carvalho *et al*., 2002). Yaro *et al.* (2006) determined the distribution of hatching time in different water types for *An. gambiae* (S form) and *An. coluzzii* (M form) and showed that over 80% hatched within the first three days following oviposition regardless of water type. Between 5% and 16.8% hatched over the next four days while between 0.6% and 7.2% hatched after the first week. *Anopheles gambiae* produced significantly more hatchlings in the 3-7 day post-oviposition period than *An. coluzzii*, and *An. coluzzii* showed significantly higher levels of hatching one week post-oviposition than *An. gambiae*. It was also found that the type of water in which the eggs were kept had a highly significant effect on hatching with the distribution most distinct in puddle water. Yaro *et al*. (2006) concluded that larval time-to-hatch is determined by environmental conditions and that intraspecific variation in hatching time is an adaptation to survive variable conditions such as larval site flooding and desiccation. They also suggest that eggs are not passive and that the time taken for an egg to hatch is probably dependent on water factors such as bacterial composition and oxygen content.

Anopheles gambiae females are monandrous with 97% of wild females mating only once (Tripet *et al*., 2003). A female requires at least one blood meal before her eggs mature. It has been shown that larger females are more likely to produce eggs after one blood meal than smaller females (Lyimo and Takken, 1993). *Anopheles gambiae* typically oviposits in

small temporary larval habitats such as tyre tracks and hoof prints (Gillies and De Meillon 1968; Lehmann and Diabate, 2008; Gimonneau *et al*., 2012) that, by their nature, are prone to desiccation and re-flooding. The ability to stagger time-to-hatch in such conditions may therefore be beneficial.

3.1.1 Factors that Influence Hatching

Numerous factors have been shown to influence hatch timing. These include temperature and humidity, desiccation, and vibrations or disturbance. Bacterial and other factors in the water including the presence of predators or competitors have also been shown to influence hatching (see below).

3.1.1.1 Temperature and Humidity

Temperature is one of the most important factors influencing the hatching of anopheline eggs. It is commonly accepted that most eggs will hatch within 2-3 days at 27 °C (Service 2000). However, this is a generalisation. Beier *et al*. (1987) showed that eggs of *An. serengeti* would not hatch if kept above 34 °C, but percentage hatch at 27 °C and 17 °C was equivalent, although those eggs developing at 17 °C took longer to hatch. Studies on anopheline egg hatching and temperature indicate that the optimum temperature for hatching is between 22 °C and 27 °C, and that temperatures outside of this range reduce egg viability (Impoinvil *et al*., 2007, de Carvalho *et al*., 2002). However, it is likely that eggs adapt to the temperatures they experience in the field.

3.1.1.2 Desiccation

Anopheline eggs, unlike *Culex* and *Aedes* eggs, are generally considered unable to withstand desiccation (Service, 2000). However, eggs kept at least slightly moist have previously been shown to hatch up to 18 days after oviposition (Deane and Causey, 1943). Huang *et al*., (2006) showed that *An. gambiae* eggs kept in water or on damp soil can survive even at high temperatures while eggs kept on dry soil for more than a few hours were unlikely to hatch. However, it must be noted that this observation was also linked to temperatures that exceeded 41 °C. Beier *et al*. (1990) and Shililu *et al*. (2004) also studied the hatching of *An. gambiae* eggs in western Kenya. Beier *et al.* (1990) collected dry soil samples from the edges of temporary and permanent pools, animal hoofprint depressions and stream beds and immersed these in water. First instar larvae were observed from these soil samples two to five days after immersion. Shililu *et al.* (2004) observed that eggs on moist soil remained viable for up to 15 days. These studies demonstrate the ability of the egg stage of *An. gambiae* to resist desiccation while delaying hatching.

3.1.1.3 Egg Disturbance

Vibrations have been shown to induce hatching in *Aedes* mosquitoes. Vibrations simulate rainfall and trigger the eggs to hatch (Roberts, 2001). Vibrations may also be a cue for anopheline eggs to hatch. A form of disturbance likely to be experienced by eggs in an aquatic environment is periodic drying and subsequent inundation of oviposition/ larval sites when the water levels rise. Vitek and Livdahl (2009) studied the hatch response of *Ae. albopictus* to varied inundation frequencies and observed that delayed hatching was significantly lower in the treatment that experienced inundation less frequently.

3.1.1.4 Bacteria and other Factors of the Larval Site

The presence of bacteria in oviposition site water has been associated with *Aedes aegypti* egg hatching (Rozeboom, 1934). It was later established that a drop in the concentration of dissolved oxygen is a trigger for the hatching of *Aedes* eggs (Gjullin *et al*., 1941). This drop in oxygen concentration was found to be linked to microbial growth within the vicinity of the eggs. *Aedes* eggs are generally oviposited on damp substrates, such as leaf litter and mud (Service, 2000b) and complete embryogenesis within a few days (Vla, 1995). The eggs can withstand desiccation and do not hatch without a proper hatching stimulus. A recent study (Ponnusamy *et al*., 2011), on the effects of bacteria on hatching in *Ae. aegypti* found that bacteria could stimulate eggs to hatch even without a significant decline in dissolved oxygen. Bacterial density decreased from 0-4 hours in water with eggs. The authors suggested that this was likely due to feeding activity of the newly hatched larvae. Similar hatching stimuli may apply to anopheline eggs. Munga *et al*. (2013) found that habitats with fresh water were more attractive to ovipositing *An. gambiae* s.*l*. where females laid significantly more eggs in water aged 0 and 5 days than in water aged 10 or 15 days. Tuno *et al*. (2005) found that *An. gambiae* developed most successfully in open sun-lit habitats. In addition, Yaro *et al*. (2006) reported that the distribution of hatching differed significantly for different water types (distilled water, rock pool water, puddle water and rice paddy water) in eggs of *An. gambiae*, *An. coluzzii* and *An. arabiensis*.

3.1.1.5 Predator or Competitor Presence in Larval Habitats

One of the differences observed between the sibling species *An. gambiae* and *An. coluzzii* is the difference in their competitiveness in the presence of predators. *Anopheles gambiae* prefers predator-free larval environments normally comprising temporary habitats such as

those formed in flooded animal hoofprint depressions and tyre tracks. *Anopheles gambiae* has been shown to outcompete *An. coluzzii* in preferred smaller habitats such as puddles which are also less likely to house predators. However, in rice paddies the two species were comparable in terms of emergence success (Diabaté *et al*., 2005). When predators are present in these environments *An. coluzzii* outcompetes *An. gambiae* (Diabaté *et al*., 2008). Munga *et al*. (2013) found that habitats replenished with water every 10 days contained significantly more *An. gambiae s.l.* larvae than habitats replenished with water every 20 or 30 days. A contributing factor to this observation may be the significantly higher predator presence in habitats in which the water was replenished every 20 or 30 days than in the habitats where water was replenished more frequently (Munga *et al*., 2013). Recently Gotifrid *et al*. (2014) reported reduced hatchability of *An. gambiae* eggs in the presence of third instar larvae. The authors collected first instar (hatched) larvae every two hours. For larvae not recovered the eggs were considered preyed upon by the third instar larvae. It has been shown that *An. gambiae* complex mosquitoes will cannibalise larvae of conspecifics or closely related species (Koenraadt and Takken, 2003).

Gillet (1955a) also assessed several other factors and their relation to the timing of egg hatching in *Ae. africanus* and *Ae. aegypti*. The number of bloodmeals taken by the females; the number of times the females had been mated; the order in which the eggs from a female were deposited (at the beginning, middle or end of oviposition); the presence of a "hypothetical substance" on the eggs from the mother; and the orientation of the eggs once laid with respect to gravity were investigated. None of these factors had a significant influence on the time it took eggs to hatch. For the "hypothetical substance" from the female, this was tested by removing every second or third *Ae. africanus* egg as they were being oviposited and wiping these eggs on filter paper to potentially remove the maternal

substance. With eggs that underwent this treatment it was observed that they no longer adhered well to the filter paper if wet and would sink or float off (Gillet, 1955a). No differences in the rate or timing of hatching were observed in the eggs that were laid normally and the eggs that were wiped. In a second paper, Gillet (1955b) also showed that there was a genetic component involved in hatch timing.

3.1.2 Microsatellite DNA

Microsatellites are short sequences of tandem nucleotide repeats. They are located mostly in non-coding regions and are co-dominant. Mutation rates in many microsatellites are high (10^{-2} to 10^{-6} mutations per locus) due to the manner in which they are transcribed and microsatellites are often highly polymorphic (Schlötterer, 2000). These characteristics produce the diversity of alleles necessary for studies on ecological time scales (Schlötterer, 2000; Selkoe and Toonen 2006). Most of the variation in alleles is due to changes in the microsatellite length - the number of repeat units in an allele and the difference in length this causes between different individuals (Ellegren, 2004). Polymorphism of microsatellites in *An. gambiae* was shown by Lanzaro *et al*. (1995). An integrated genetic map including 131 microsatellites was developed by Zheng *et al*. (1996) for *An. gambiae*. Microsatellites have been used to determine *An. gambiae* and sibling species population structure (Lanzaro *et al*., 1995; Donnelly and Townson, 2000; Lehmann *et al*., 2003; Wang-Sattler *et al*., 2007; Maliti *et al*., 2014) and have also been used to map loci affecting insecticide resistance (Ranson *et al*., 2000b; Ranson *et al*., 2004) and refractoriness to parasite infection in malaria vectors (Niaré *et al*., 2002; Menge *et al*., 2006; Riehle *et al*., 2007). In addition, the oviposition behaviour of *An. gambiae* females has been inferred using microsatellite markers (Chen *et al*., 2006). Chen *et al*. (2006) indicated that 56% of females deposited eggs in multiple oviposition sites around a hut in Kenya based on the

maternity and relatedness of larvae in oviposition sites. Kilpatrick *et al*. (2007), using microsatellite markers, found that the genetic background of *Culex pipiens* may influence its preferred feeding behaviour.

3.2 Rationale

In 2006, in a newly colonized *An. gambiae* colony from Ghana (labelled GAH), variation in time-to-hatch was observed in eggs oviposited on the same day. First instar and third to fourth instar larvae were observed in the same rearing container approximately one week after oviposition (R. Hunt, personal communication). This situation was not seen in more established colonies, suggesting that delayed time-to-hatch in the GAH colony had been retained from the wild population from which this colony was derived, and that delayed hatch is an important adaptive trait under natural conditions.

3.3 Aims and Objectives

The aim of this research was to further assess the adaptive significance of larval time-to-hatch by investigating the possibility of a genetic component controlling time-to-hatch as well as by measuring the expression of insecticide resistance in association with time-to-hatch in the *An. gambiae* GAH colony.

Specific objectives were:

1. To select strains from the GAH *An. gambiae* colony for early and late time-to-hatch
2. To perform cross-mating experiments on the selected Early and Late time-to-hatch strains and monitor time-to-hatch in the hybrids as well as in the Early and Late time-to-hatch parental strains
3. To determine insecticide susceptibility of the GAH Early and Late time-to-hatch strains to the four classes of insecticides approved for use in public health
4. To further characterize insecticide resistance in the GAH Early and Late selected strains using assays to detect the mechanisms conferring resistance to insecticides
5. To determine the hatching distribution of batches of eggs oviposited by wild-caught single females from Ahafo, Damang and Tarkwa in Ghana as well as from Pointe Noire in the Democratic Republic of the Congo
6. To determine whether water disturbance has an effect on the hatchability of eggs obtained from the baseline GAH colony as well as from the selected Early and Late time-to-hatch strains
7. To scan for microsatellite markers associated with time-to-hatch or insecticide resistance in phenotypically characterized Ghanaian families reared from wild-caught females

3.4 Materials and Methods

3.4.1 Laboratory Colony Material

The GAH *An. gambiae* (S form) colony originating from Ghana and colonized in 2006 formed the basis of this study. SUA, an insecticide susceptible *An. coluzzii* colony from Liberia, was used as the susceptible reference strain in insecticide bioassays. All mosquitoes were reared in the Botha De Meillon insectary at the National Institute for Communicable Diseases, NHLS (Johannesburg, South Africa) at 27 °C, 75-85% relative humidity, in a twelve hour light: dark cycle with dusk and dawn transitions. Larvae were fed on a mixture of ground dog biscuits and brewer's yeast and adults received two to three blood-meals per week.

3.4.2 Wild-caught Material

Samples of *An. gambiae* were collected from Ahafo, Ghana (Ghaf), (7°03.656N; 2° 24.190W) in June 2008; Damang (5°30.992N; 1°52.022W) and Tarkwa (Ghag) (5° 22.383N; 2°01.017W), Ghana, in January 2009; Pointe Noire, Republic of the Congo (COGS), (4°40'31S; 11°58'14E) in March 2009. The samples from Ghana were used for various evaluations in conjunction with laboratory reared material as described below. The material from the Republic of the Congo served as a comparative *An. gambiae* sample from a different region.

3.4.3 Families Reared from Wild-caught Material

Most of the females caught in houses were already blood-fed and these were re-fed if required to produce individual egg batches. Numbered individual wild-caught, blood-fed females were placed into separate vials lined with moist filter paper and induced to lay eggs. Females were provided with cotton wool pads soaked in 10% sucrose. Females that

oviposited did so overnight and egg batches were rinsed into a bowl containing distilled water and labelled with the mother's identifier number, the batch number and the date of oviposition (the day before the eggs were found). Mothers that survived were returned to their vials and induced to lay eggs until death and those that died were stored on silica gel for later molecular analysis.

3.4.4 Species Identification of Wild-caught Mosquitoes and DNA Extractions

Wild-caught mosquito samples were transported to the VCRL and were initially sorted using the morphological keys of Gillies and Coetzee (1987). Those identified as members of the *An. gambiae* complex were identified to species using the *An. gambiae* species-specific PCR assay (Scott *et al*., 1993). All *An. gambiae sensu stricto* samples were further characterized as either M or S molecular form (now *An. coluzzii* or *An. gambiae* respectively) using the PCR assay of Favia *et al*. (2001). DNA extractions were performed using the method of Collins *et al*. (1987) or using an extraction kit (ZyGEMPrepGEM). (DNA extraction and species identification protocols are supplied in Appendix 2A.)

3.4.5 Early and Late Time-to-Hatch Selections

Mosquitoes were selected at the larval stage from the baseline GAH colony for early and late hatch phenotypes. For the initial selection larvae were allowed to develop for 7 to 10 days after which all fourth instar larvae were placed into a new bowl and marked GAH Early. Second and third instar larvae were not used and were returned to the GAH baseline colony. The first instar larvae and eggs that remained after 7 to 10 days were kept to establish the GAH Late strain. Larvae selected in this way were reared to adults. This process was repeated using several egg batches until enough adults had been produced to

start separate Early and Late time-to-hatch strains. These were blood-fed and maintained according to the standard insectary procedure. Eggs from each strain were harvested and the time-to-hatch selection procedure was continued. For the Early strain all larvae that hatched within four days of oviposition were removed from egg bowls and kept as GAH Early-hatch, the remaining eggs were transferred to the baseline colony. Conversely, the late strain was selected by transferring all larvae that hatched within four days of oviposition to the baseline colony and eggs that had not hatched by the fourth day after oviposition were kept as GAH Late-hatch. These strains were kept in separate, numbered generations. Selections continued through at least six generations before insecticide susceptibility and cross-mating experiments were conducted.

3.4.6 Cross-mating Experiments

In order to determine whether there is a genetic component associated with larval time-to-hatch, cross-mating experiments between the Early and Late time-to-hatch strains were set up. Early-hatch females were crossed with Late-hatch males and vice versa. The numbers of eggs produced from each cross were quantified. Eggs were monitored for hatching and the numbers of F1 hatchlings from each cross were recorded daily. F1 larvae from each cross were reared to adults and these were either back-crossed to the parental strains or were inter-crossed (Figure 3.1). Eggs and F2 hatchlings from each back-cross or inter-cross were monitored daily as described above. Egg production and larval hatching in the baseline GAH colony was concurrently monitored as a control.

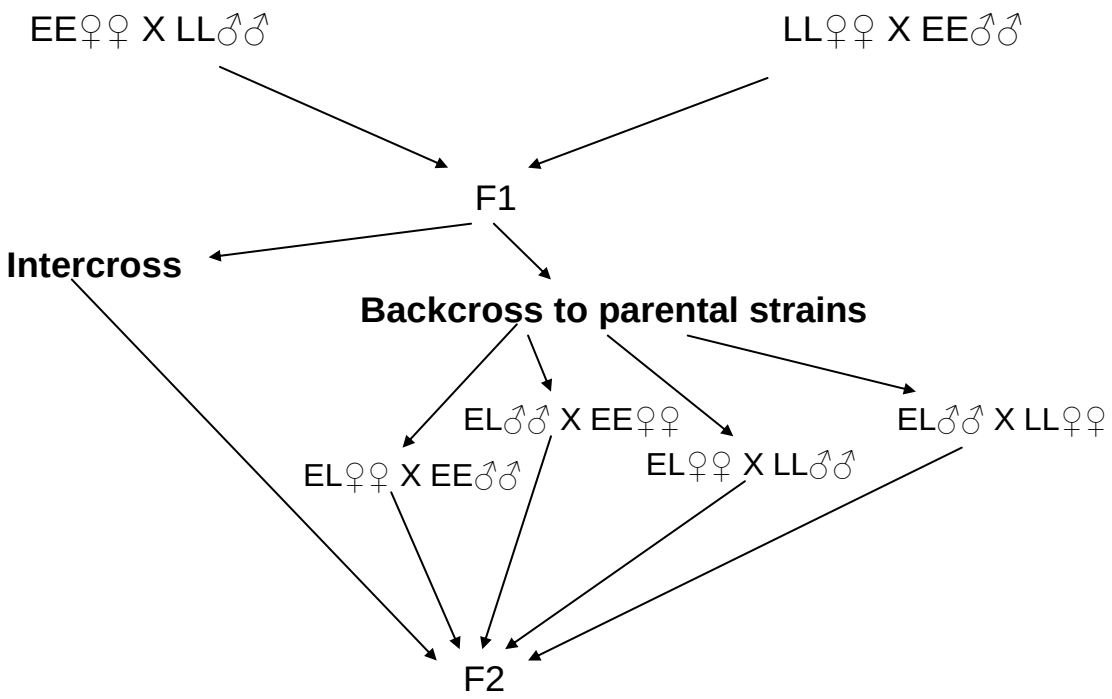


Figure 3.1: Schematic diagram of *Anopheles gambiae* GAH crosses between Early (EE) and Late (LL) hatch phenotypes, inter-crosses between hybrids (EL) and back-crosses to parental strains.

3.4.7 Determining Hatch Distributions of Eggs from Early and Late-Hatch Selected Strains, Cross-mating Experiments and the Baseline GAH *An. gambiae* Colony

Batches of eggs were collected from the baseline GAH colony as well as from the Early and Late time-to-hatch selected strains and rinsed into marked egg bowls. The date of oviposition was recorded and eggs were counted. Bowls were checked and any larvae present were counted and removed daily as for the cross-mating experiments. Any eggs that hatched within four days of oviposition were classified as early hatch and any that hatched after this were classified as late hatch. This was used as the cut off as earlier observations showed that the majority of eggs hatch within 4 days and the rest hatch in a staggered fashion for up to 22 days post-oviposition.

3.4.8 Determining Hatch Distributions of Families Reared from Wild-caught Females from Ghana and the Republic of the Congo

Blood-fed wild-caught females from Ghana and the Republic of the Congo were placed into individual vials lined with moist filter paper for oviposition. Cotton wool pads soaked in 10% sugar solution were provided. Mosquitoes were monitored daily for eggs and any produced were rinsed into individual egg bowls. Eggs were monitored daily for hatching and any hatchlings were counted and removed as for the cross-mating experiments. Those families reared from wild-caught females from Ahafo, Ghana, formed the pilot study from which the four-day cut off point for classifying the early and late hatch phenotypes was determined. This cut off point closely approximates with information from Yaro *et al.* (2006).

3.4.9 Determining the Effect of Density on Time-to-Hatch

The batches of eggs produced by wild-caught individual females that were used in hatch distribution analysis were also used to determine whether the number of eggs produced by a single female had an effect on the proportion of eggs that hatched, or on the proportion of eggs that hatched early. Data were grouped according to number of eggs laid and analysed using scatter plots and linear regression in Statistix 7 (Analytical Software, Tallahassee, FL, USA).

3.4.10 Determining the Effect of Water Disturbance on Egg Hatching in the Time-to-Hatch Selected Strains and Baseline Colony

An experiment to test the effect of disturbance on egg hatching from the two time-to-hatch selected strains and the baseline GAH colony was performed. Three batches (replicates) of eggs from each group were obtained and split for different treatments. One half of each egg

batch was rinsed into a larval bowl that contained a floating plastic ring and eggs were sprayed with water (disturbed) daily. The other half was also rinsed into a larval bowl but, the eggs were placed into the bowl so that they were contained within the floating plastic ring (Figure 3.2). This set up prevented the eggs from being stranded on the sides of the larval bowl and thus they required no disturbance to keep them in contact with the water. Both sets of larval bowls were monitored daily for hatchlings which were counted and removed. Each set of experiments lasted 25 days after which all eggs were disturbed to determine if any unhatched eggs would hatch, and later discarded. The proportion of eggs that hatched as well as the proportions of early and late hatching larvae per group was determined and analysed using two-sample t-tests or ANOVA (Statistix 7 Analytical Software, Tallahassee, FL, USA).

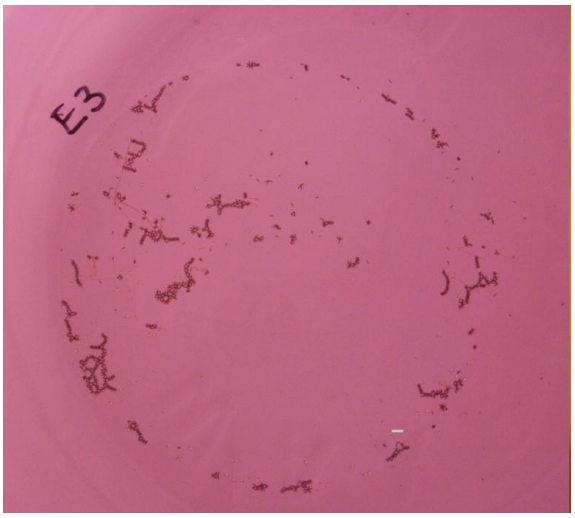


Figure 3.2: Experimental set-up used to determine the effects of disturbance on the hatching of eggs from the *Anopheles gambiae* Early and Late selected strains as well as from the baseline GAH colony. The photograph shows undisturbed eggs contained within a floating plastic ring.

3.4.11 Size Comparisons between Females from the Early and Late-Hatching Strains

Wing lengths of 30 females each from the Early and Late time-to-hatch selected strains were measured. Wing-length has been shown to be a good approximation of adult size when mosquitoes are reared in the same environment (Lyimo and Koella 1992; Koella and

Lyimo, 1996). Wing length was measured using one intact wing and measuring the length from the alular notch to the tip. Data were analysed using a two-sample t-test (Statistics 7 Analytical Software, Tallahassee, FL, USA).

3.4.12 Standard WHO Bioassays

Insecticide resistance bioassays were performed on all four classes of insecticides according to the standard WHO operating procedure (1998; 2013) described previously in Chapter 2.

3.4.13 Synergist Bioassays

Synergist bioassays on the time-to-hatch selected strains were performed using 4% PBO against: permethrin, bendiocarb and propoxur, and 10% TPP against: permethrin, dieldrin and DDT respectively, at diagnostic concentrations. The synergist DEM was not used as it is showed no significant synergistic effects in GAH for any of the insecticides tested. Synergist bioassays were conducted and analysed as described in Chapter 2.

3.4.14 Screening for Known Insecticide Resistance Associated Mutations

DNA was extracted from samples to be used in molecular assays according to the method of Collins *et al*. (1987) (Appendix 2B) or using a ZyGEMPrepGEM™ insect (ZyGEM Corp Ltd.) extraction kit following the supplied protocol. Individual mosquitoes were screened for *kdr* L1014F and *rdl* genotypes using hydrolysis probe molecular assays (Bass *et al*., 2007; Bass *et al*., 2008) as previously described. Data were analysed using Fisher's exact test, χ^2, or two-sample t-tests (Statistix7 Analytical Software, Tallahassee, FL, USA) as described in Chapter 2.

3.4.15 Microsatellite Detection

In a pilot study, 24 fluorescently labelled primer pairs were used to detect 24 microsatellites distributed across the genome (Table 3.1) in time-to-hatch and insecticide resistance characterized progeny from wild-caught females from Ghana. The families used were Ghaf 19, 20, 28 and 33 described previously. These were sorted according to time-to-hatch phenotype (early: up to and including the third day post-oviposition; intermediate: from the third day up to and including the 6th day post-oviposition; and late from the 7th day onwards) as well as on whether they survived or died following dieldrin or deltamethrin exposure. Family 19 was unusual in that it contained only later hatching progeny, while the other three families had both early and late hatching progeny.

3.4.15.1 SINE PCR

Before microsatellite amplification was conducted on the selected families the presence of DNA was determined by electrophoresing extracted DNA on an agarose gel. Short interspersed transposable element (SINE) typing (Santolomazza *et al*., 2008) to detect M and S molecular forms (*An. coluzzii* and *An. gambiae*) was conducted on 19 progeny from family 28 as well as the mother, and 6 samples each from families 19 and 20. Standard M and S form positive controls as well as a negative PCR reaction control were also included. All of these samples were confirmed as *An. gambiae* S form. The reaction conditions were as follows: kappa buffer 2.5 µl; dNTPs (10 mM) 0.5 µl; *taq* polymerase 0.17 µl; primer-1 1 µl; primer-2 1 µl in a total volume of 25 µl. PCR cycling conditions were- 95 ˚C for 5 minutes followed by 35 cycles of: 95 ˚C for 30 seconds; 54 ˚C for 30 seconds; 72 ˚C for 1 minute, and a final extension step of 72 ˚C for 10 minutes after which samples were stored at 10 ˚C until removed. PCR products, including a negative extraction control and known M and S positive controls, were electrophoresed on a 2.5% agarose gel. Fragment sizes were scored using a molecular weight marker (New England Biolabs).

3.4.15.2 Microsatellite PCR Conditions and PCR Duplexes

The microsatellites selected were amplified as duplexes in each PCR reaction so that two microsatellite regions could be amplified in a single reaction. The microsatellite loci selected and their primer sequences are given in Table 3.1. The PCR conditions for these reactions were: buffer 1.5 µl; dNTPs (10 µM) 0.3 ul; Primer 1F 0.2 µl; Primer 1R 0.15 µl; Primer 2F 0.2 µl; Primer 2R 0.15 µl; *taq* polymerase 0.2 µl; sample DNA 1.5 µl made up to a total volume of 15 µl with ddH_20. The forward primers (Sigma-Aldrich) were labelled with a fluorescent green or blue dye to enable viewing of the PCR products on the Beckman (Beckman Coulter) sequencer. The cycling conditions for the PCR were as follows: 95 ˚C for 5 minutes; 40 cycles of: 94 ˚C for 30 seconds; annealing temperature for 30s and 72 ˚C for 60 seconds followed by a final extension at 72 ˚C for 10 minutes. The annealing temperatures for the different primer pairs ranged from 55-63 °C. 0.5-2 ul of the PCR product for each sample was electrophoresed on the Beckman CEQ 8000 genetic analysis system with a size standard and allele sizes were scored using the fragment analysis kit (Beckman Coulter). Results were analysed using the Analysis Toolpak on Microsoft Excel. In addition results were analysed for deviations from Hardy-Weinberg equilibrium and the FST values determined using the free software Genepop (http://genepop.curtin.edu.au/genepop).

Table 3.1: Twenty-four microsatellite markers used to scan *Anopheles gambiae* samples from wild-caught material from Ghana. Markers are arranged according to their position on the chromosomes. The locus name and the primer sequences are given as well as the approximate length and the reference refers to the primer designer.

#	Position	Locus name	Primers (Sequence 5'-3') F/R	Reference
1	X	**AgXH36**	CGTATGTTTGCTAGGGGTGG/ GTCAAGAAATGGGCCACAGG	Zheng *et al* 1996
2		**AgXH7**	CACGATGGTTTTCGGTGTGG/ ATTTGAGCTCTCCCGGGTG	Zheng *et al* 1996
3		**AgXH678**	CCTCTCCCCAGAATCGGTAC/ AAGAGCAGAAACAACCGCAG	Zheng *et al* 1996
4	2R	**Ag2H79**	CGGGTAGCGCTAGAAGTATG/ AGAGAAATCTGCCGAAGGGG	Zheng *et al* 1996
5		**Ag2H799**	TTATGGGCAACTGCGGATGG/ CGTCGGTTTGATACATCTACG	Zheng *et al* 1996
6		**2RSI.5**	TTCTCGAAAGACTGCTCTGCTG/ ATTGGATCGAAAACGGTCTG	Liverpool Weetman
7		**Ag2H135**	AGGAGCATAACACATCGCCC/ CGCTCGTCAAAGAAACTGGC	Zheng *et al* 1996
8		**Ag2H786**	TGTGAAGCATTTCCTTGGCG/ TGCCCTTGAGTCGAGGTAGC	Zheng *et al* 1996
9	L	**Ag2H675**	CTGCCCCATTCAATTGCTCGGC/ CGTGACACTTTCAGGACACC	Zheng *et al* 1996
10		**Ag2H523**	CTCGTTAGGCGCTTGTGAAC/ CACTTCACGACTGTGAGCAC	Zheng *et al* 1996
11		**Ag2H787**	CGGGTCAAAGAAAACTCAGC/ GCATAAGAACGGCACATTGC	Zheng *et al* 1996
12		**Ag2H325**	CCGGTGTCCGTGTTG/ GCGCAAAGCAAATGACACG	Zheng *et al* 1996
13		**2L2LA**	GCATTGCGTTTGTGTGATTT/ GGTGGCGGTCTGTTTACACT	Liverpool Weetman
14	3R	**Ag3H128**	CGGGACGGCTAGATAAAGCG/ CCGGGCGACATAACCCACCC	Zheng *et al* 1996
15		**Ag3H812**	CTGGCCCATTTTGCATATGC/ TGCTCCACCCAAACCACATC	Zheng *et al* 1996
16		**Ag3H119**	GGTTGATGCTGAAGAGTGGG/ ATGCCAGCGGATACGATTCG	Zheng *et al* 1996
17		**3H33C1**	ATGAACACCACGCTCTCGG/ TTGCGCAACAAAAGCCCACG	Romans *et al* 1991
18		**Ag3H88**	TGCGGCGGTAAAGCATCAAC/ CCGGTAACACTGCGCCGAC	Zheng *et al* 1996
19		**Ag3H311**	CGGCAGAGGCGCTGCGGG/ CCCCGAAAAACGAAACGCAC	Zheng *et al* 1996
20	3L	**Ag3Hre242**	TTGGGTTCCCCACTAATGTC/ AATGTCAGTTCCGGTTGTCC	Zheng (Liverpool re-design)
21		**Ag3H765**	AGGCCAATGAGGTATCGAGC/ GCATGGCAACCGTTTTCTGC	Zheng *et al* 1996
22		**Ag3H577**	TTCAGCTTCAGGTTGGTCTC/ GGGTTTTTTGGCTGCGACTG	Zheng *et al* 1996
23		**Ag3H811**	AACCCACAGTACAGCTCGCG/ GTTGCTGCATACTAACCTCG	Zheng *et al* 1996
24		**Ag3L39A**	TGTTATGGGCTCGCATCTAA/ CGTTCCCCTACCTTTTTCAG	Dave/Deo Sciroko

3.5 Results

3.5.1 Species Identification of Wild-caught Samples

All wild-caught mosquitoes used in the time-to-hatch experiments were identified as *An. gambiae s.s.* (S form) except for Ahafo families (Ghaf 31, 33 and 49) which were *An. coluzzii* (M form).

3.5.2 Hatch Distributions of Wild-caught Mosquitoes

Most Ahafo families (Ghaf) showed both early and late hatching. Only one family (Ghaf 41) was entirely early hatching (3.6%) and ten families (Ghaf 1, 9, 15, 19, 22, 24, 27, 31, 35, and 42) were entirely late hatching (35.7%) (Figure 3.3). In this experiment 3 days post-oviposition was used as the cut-off between the early and late hatch phenotypes. The cut-off point was shifted to four days for all subsequent experiments as the majority of the larvae hatched within this period with the hatch proportion dropping off significantly after four days.

Most families reared from wild-caught females from the Republic of the Congo also showed both early and late hatching. Four families (22.22%) were entirely early hatching and none were entirely late hatching using four days post-oviposition as the cut-off point between time-to-hatch phenotypes (Figure 3.4).

All Ghana Damang and Tarkwa (Ghag) families showed both early and late hatching. The proportions of late hatching larvae per family (Figures 3.3 and 3.5) from Ghana (Ghaf and Ghag) were generally higher than those observed in the Congo families (COGS) (Figure 3.4).

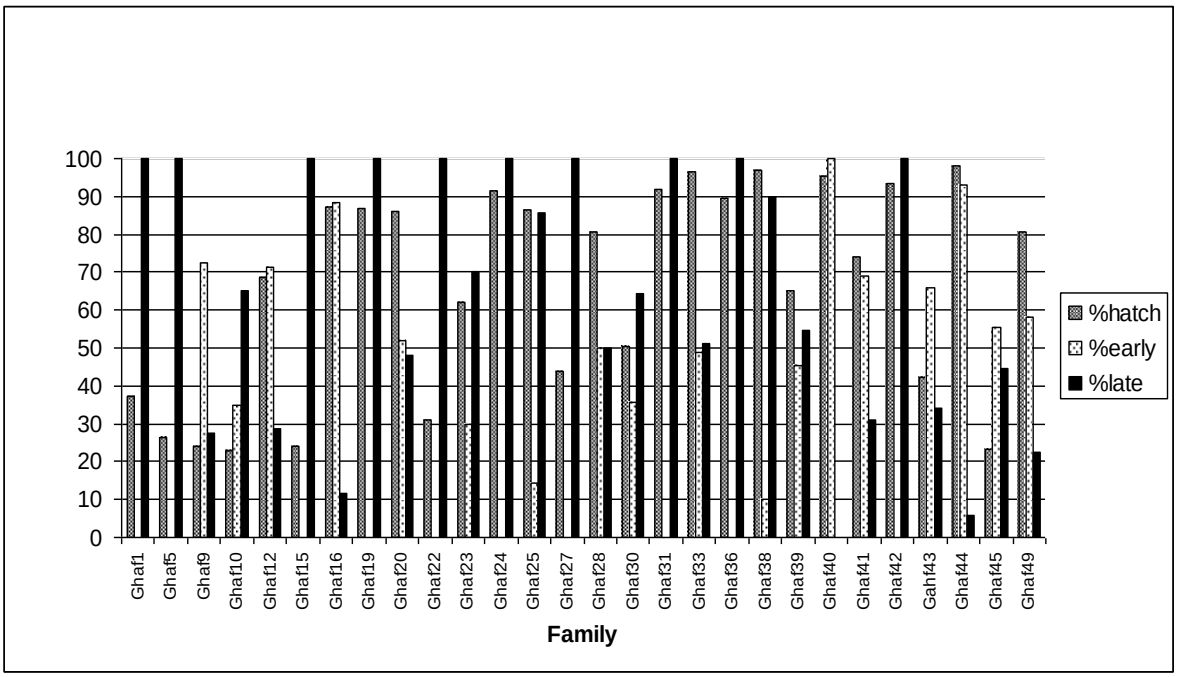


Figure 3.3: *Anopheles gambiae* families from Ahafo, Ghana. Overall larval hatch rate per family (percentage of eggs that hatched) and the proportions of larvae that hatched either early or late using three days post-oviposition as the cut-off between time-to-hatch phenotypes are shown.

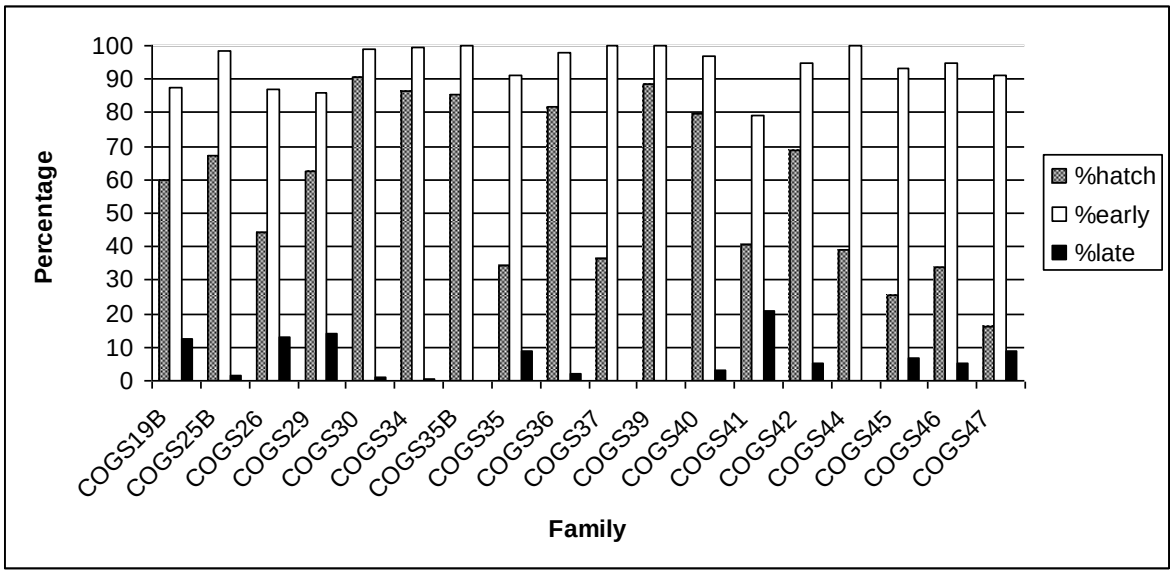


Figure 3.4: *Anopheles gambiae* families from Pointe Noire, Republic of Congo. The overall larval hatch rate per family (percentage of eggs that hatched) and the proportions of larvae that hatched either early or late using four days post-oviposition as the cut-off between time-to-hatch phenotypes are shown.

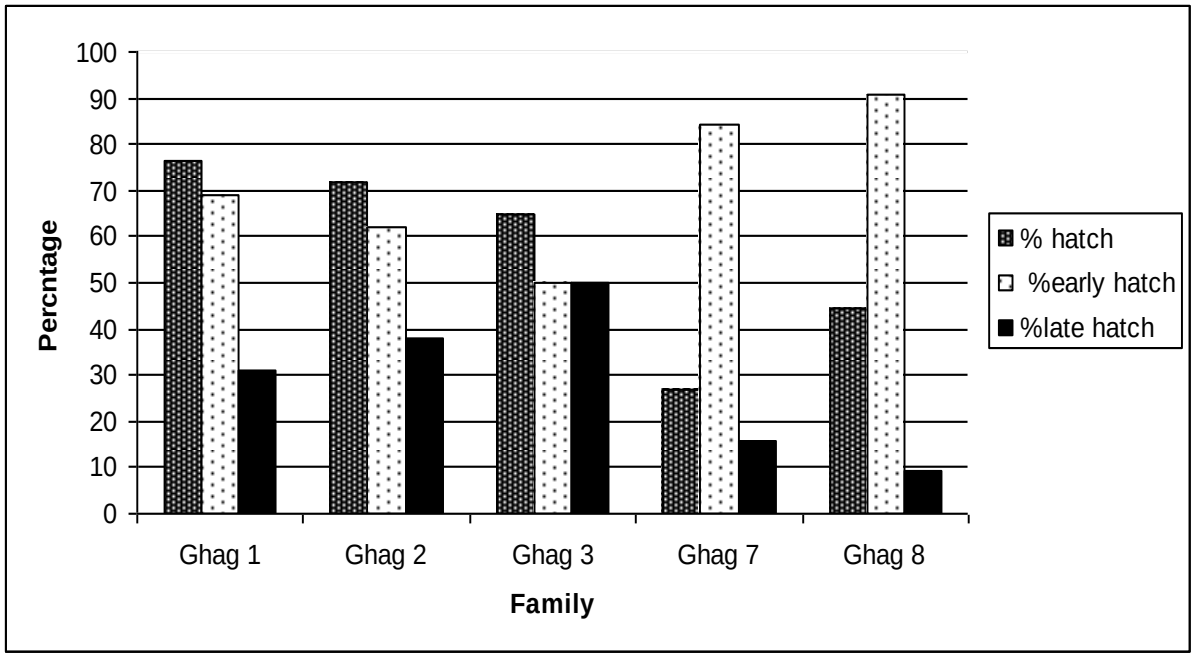


Figure 3.5: *Anopheles gambiae* families from Damang and Tarkwa, Ghana. Overall larval hatch rate per family (percentage of eggs that hatched) and the proportions of larvae that hatched either early or late using four days post-oviposition as the cut-off between time-to-hatch phenotypes are shown.

3.5.3 Hatch Proportions in Cross-mating Experiments and Selected Strains

The overall proportions of F1 and F2 progeny that hatched from the crosses, back-crosses and intercrosses, as well as progeny from the unselected GAH colony and time-to-hatch selected strains did not vary significantly (ANOVA, $p > 0.05$) (Figure 3.6). Between 400 and 3000 eggs per cross or selected strain were monitored in total. The latest hatch occurred 22 days post-oviposition, but in general hatching after 9 days was uncommon.

The majority of larvae hatched early across all batches (Figure 3.7). The highest proportions of late hatching larvae were recorded in the Late-hatch selected strain and in the F2 progeny of both back-crosses to the parental strains. These late hatching proportions were numerically higher than those recorded in the GAH base-line colony, the Early-hatch selected strain, the F1 hybrid progeny of Early-hatch crossed with Late-hatch and F2

progeny of the hybrid intercross. However, these differences were not significant for untransformed data (ANOVA: $p > 0.1$; $df = 5$; $F = 2.29$), and were only significant at lower confidence for arcsine-transformed data ($p = 0.08$). In summary, these data do not correlate with any hypothetical models based on Mendelian inheritance of a single genetic factor controlling larval time-to-hatch, but suggest that larval time-to-hatch phenotypes can be selected for (Figure 3.7).

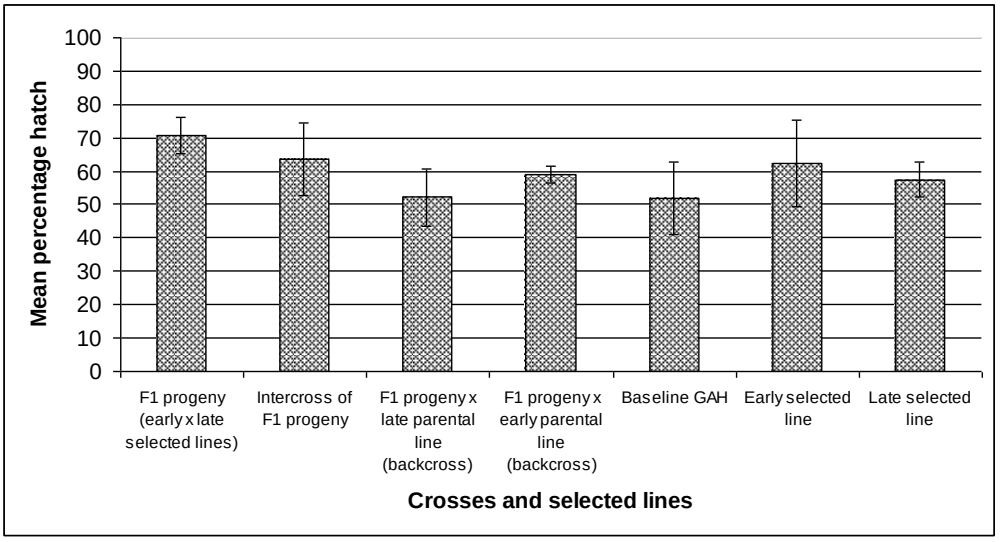


Figure 3.6: Overall hatch rate did not differ significantly for crosses and selected lines.

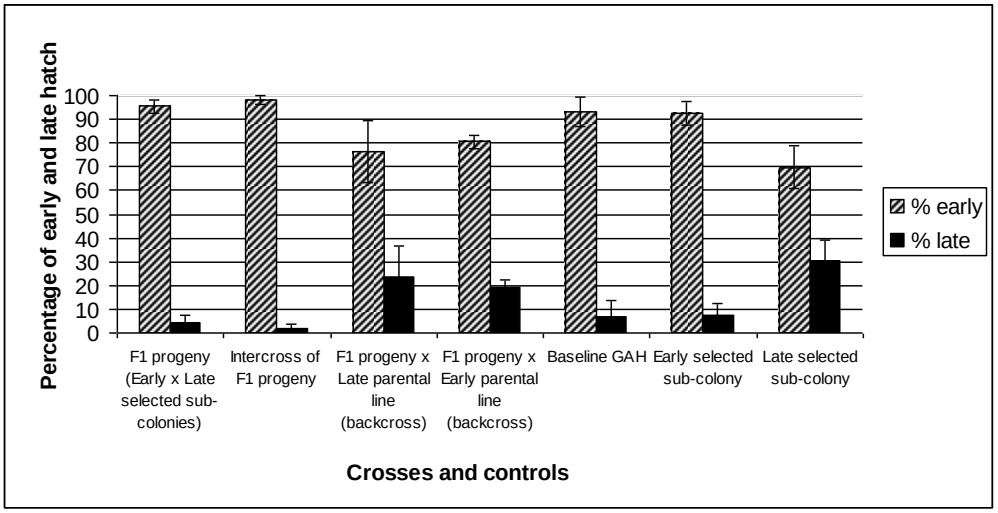


Figure 3.7: Proportions of early and late hatching *Anopheles gambiae* larvae per cross, GAH laboratory colony (baseline) and time-to-hatch selected (Early and Late) strains.

3.5.4 Density Dependent Effects on Hatching

Linear regressions were performed on each group of wild-caught families (Ghaf, Ghag and COGS) to test for an association between the number of eggs laid per female and the proportion of eggs that hatched, or the number of eggs laid per female and the proportion of eggs that hatched early. No significant trends were detected (Table 3.2) suggesting that the number of eggs laid by a female does not influence the proportion that hatch or the proportion of eggs that hatch early. For scatter-plots see Appendix 3.

Table 3.2: The number of eggs laid by single females does not affect the proportion of eggs that hatch or the proportion of egg that hatch early. P-values obtained by performing linear regressions on *Anopheles gambiae* families reared from wild-caught females. The number of families per collection is given in brackets.

Mosquitoes	**Ghaf** (27)		**Ghag** (5)		**COGS** (17)	
Statistics	**P**	**r^2; f; df**	**p**	**r^2; f; df**	**p**	**r^2; f; df**
Number of eggs/ proportion hatched	p = 0.42	r^2 = 0.026 f = 0.67 df = 26	p = 0.29	r^2 = 0.351 f = 1.62 df = 4	p = 0.57	r^2 = 0.023 f = 0.35 df =16
Number of eggs/ proportion early hatch	p = 0.52	r^2 = 0.017 f = 0.44 df = 26	p = 0.76	r^2 = 0.037 f = 0.12 df = 4	p = 0.63	r^2 = 0.015 f = 0.24 df = 16

3.5.5 The Effect of Water Disturbance on Hatching

The total numbers of eggs monitored ranged from 1600 to 3200 eggs divided between three replicates per experiment. Significantly fewer eggs from the undisturbed experiments hatched compared to the disturbed experiments (Table 3.3), indicating that egg disturbance is necessary for optimal hatching (two-sample t-tests: $p < 0.01$, $t = 13.25$, $df = 4$ for GAH baseline; $p = 0.01$, $t = 4.11$, $df = 4$ for the Early time-to-hatch strain; $p < 0.01$, $t = 10.24$, $df = 4$ for the Late time-to-hatch strain). In addition, there was a significant difference in the total percentage of eggs that hatched in the undisturbed experiments between the Early and Late time-to-hatch selected strains, and between the Early time-to-hatch selected strain and

the GAH baseline colony ($p < 0.01$, $t = 4.63$, $df = 4$ and $p = 0.01$, $t = 4.55$, $df = 4$, respectively) with significantly more hatching occurring in the Early time-to-hatch strain (Table 3.3). No significant differences in total hatch were observed between groups for the disturbed experiments.

Table 3.3: Mean egg hatch proportions as well as late and early hatch proportions in unselected and time-to-hatch selected strains of *Anopheles gambiae* GAH. Data are given as mean percentages and are sorted according to whether or not eggs were disturbed prior to hatching. SD = Standard deviation.

Colony/ strain	Hatch rate/ time-to-hatch proportion	Disturbed (%)	SD	Undisturbed (%)	SD
GAH base	Mean hatch Proportion late Proportion early	78 20.15 79.85	7.22%	20.4 29.87 70.13	2.15%
Early-hatch	Mean hatch Proportion late Proportion early	75 5 95	6.63%	47.23 25.4 74.6	9.99%
Late-hatch	Mean hatch Proportion late Proportion early	90.2 19.9 80.1	9.9%	11.4 37.8 62.2	8.99%

3.5.6 Size Comparisons between Females from the Early and Late Time-to-Hatch Selected Strains

There was no significant difference in wing length between females from the Early-hatch strain (3.46 mm; SE: 0.032 mm) and females from the Late-hatch strain (3.42 mm; SE: 0.038 mm) (two-sample t-test; $t = 0.86$; $df = 1$; $p = 0.39$).

3.5.7 Insecticide Susceptibly Bioassays in Early and Late GAH Time-to-Hatch Selected Strains

Time-to-hatch selected strains were exposed only to those insecticides that the baseline GAH colony showed resistance to according to WHO guidelines (WHO, 1998) (see

Chapter 2) which excluded malathion and fenitrothion. The levels of resistance in the larval time-to-hatch selected strains differed significantly from each other ($P < 0.05$ based on two-sample t-tests) through all insecticides tested with the exception of the pyrethroid deltamethrin against which neither strain showed resistance (Figure 3.8). Resistance to deltamethrin was lost in GAH in the time (approximately four months) between the first synergist exposures performed using PBO and subsequent exposures using TPP and DEM. The Early time-to-hatch strain showed significantly higher levels of resistance to the carbamates bendiocarb and propoxur as well as to the organophosphate pirimiphos-methyl than the Late time-to-hatch strain. This trend was reversed in response to exposure to DDT, dieldrin and permethrin, with the Late time-to-hatch showing significantly higher levels of resistance (Figure 3.8, Table 3.4). The response of the baseline GAH colony to the insecticides tested is also given in Figures 3.8 b and c for comparative purposes.

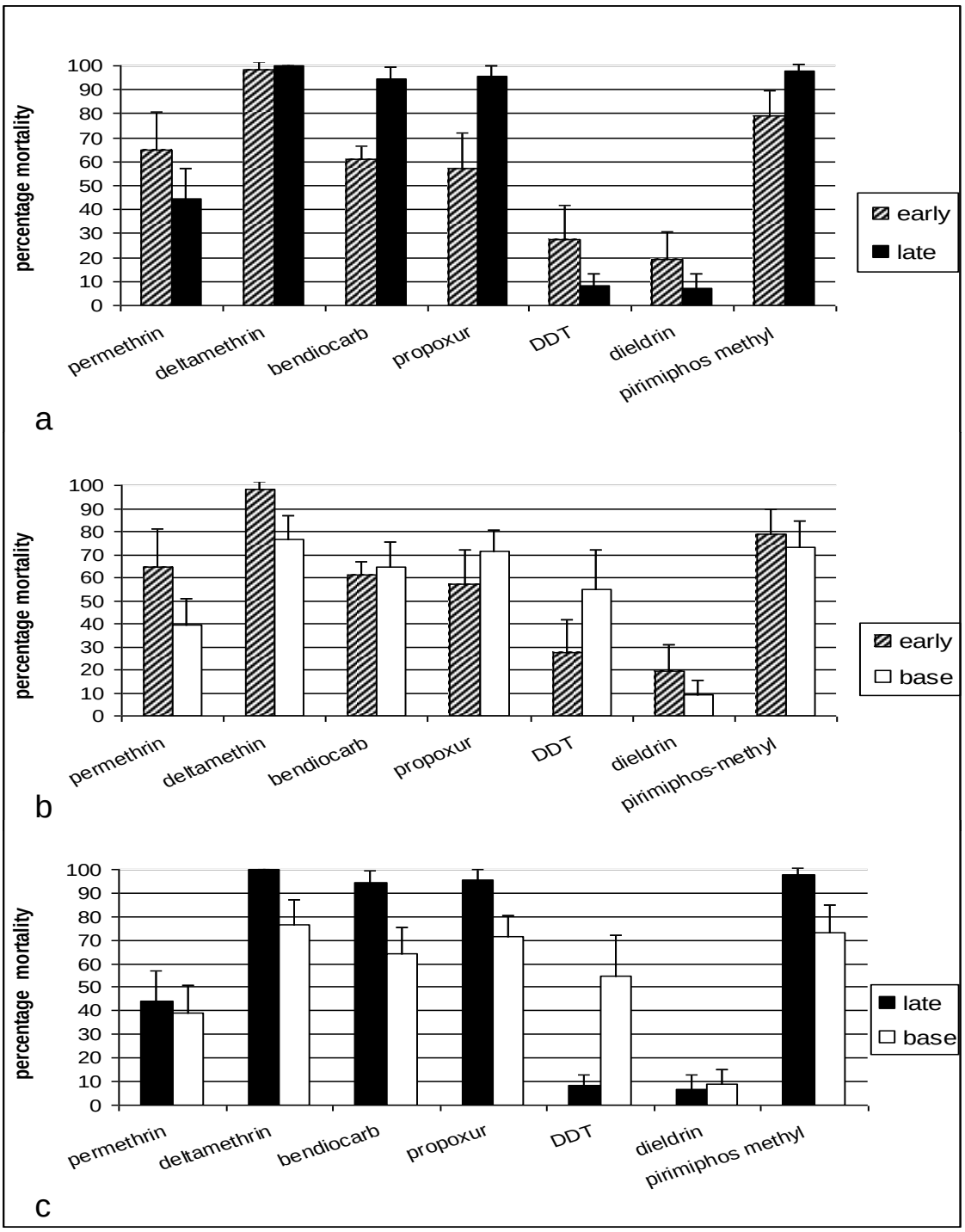


Figure 3.8: a) Resistance profiles of the *Anopheles gambiae* GAH Early and Late time-to-hatch selected strains. b and c) Resistance profiles of the GAH Early and Late strains compared to the baseline colony indicating a shift in insecticide resistance profiles with the selection for time-to-hatch. Standard deviations from the means are shown.

Table 3.4: P and t values obtained following two-sample t-tests comparing resistance to different insecticides between the *Anopheles gambiae* GAH Early and Late selected strains. Significant values are indicated in italics. Seven to ten replicates (df range 6-9) of approximately 25 female mosquitoes were tested per insecticide. Sample sizes per insecticide ~175-250. Where mosquitoes showed full susceptibility a sample size of 50-75 (2-3 replicates) was used.

Comparison	**Early vs. Late**		**Early vs. Baseline**		**Late vs. Baseline**	
Insecticide	**p**	**t**	**p**	**t**	**p**	**t**
Permethrin 0.75%	*< 0.01*	2.97	*< 0.01*	4.02	0.21	1.28
Deltamethrin 0.05%	susceptible	NA	*< 0.01*	6.48	*<< 0.01*	NA
Bendiocarb 0.1%	*<< 0.01*	-13.14	0.9129	0.10	*<< 0.01*	8.61
Propoxur 0.1%	*<< 0.01*	-7.59	*< 0.05*	-2.52	*<< 0.01*	6.08
DDT 4%	*< 0.01*	4.00	*< 0.01*	-3.81	*<< 0.01*	-8.12
Dieldrin 4%	*< 0.05*	3.14	*< 0.05*	2.87	0.51	-0.66
Pirimiphos-methyl 0.9%	*< 0.01*	-4.20	0.30	1.09	*<< 0.01*	5.24

3.5.8 Synergist Bioassays on Time-to-Hatch Selected GAH Strains

Four percent PBO significantly synergised permethrin resistance in GAH Late ($p = 0.02$; $\chi^2 = 29.39$, df =10) but did not induce 100% mortality. DEM was not used as a synergist on the selected strains as it had no effect on the baseline GAH colony. This effect was not detected in the Early time-to-hatch strain as resistance to pyrethroids had diminished over approximately four months between the initial susceptibility testing on baseline GAH and the selected strains and the synergist bioassays. Over 90% of the Early time-to-hatch strain did not survive permethrin exposure after the four month gap. TPP induced no significant reductions in insecticide resistance expression in the time-to-hatch strains when challenged against permethrin, dieldrin or DDT. PBO did not synergise permethrin, bendiocarb or propoxur resistance in the Early time-to-hatch strain. PBO was not tested against bendiocarb and propoxur in the Late time-to-hatch strain as this strain was largely susceptible to these two insecticides.

3.5.9 Screening for Resistance Associated Mutations

3.5.9.1 Kdr

The hydrolysis probe molecular assay (Bass *et al*., 2007) was used to genotype samples for the L1014F *kdr* mutation. These samples were drawn from the GAH colony and the larval time-to-hatch selected strains. Sample sizes ranged from 18 to 30. All mosquitoes assayed were characterized by insecticide exposure bioassay as either insecticide resistant or susceptible. Some were further characterized by their response to insecticide exposure following pre-exposure to PBO. There was significant variation in *kdr* allele frequency between samples (Figure 3.9), showing clear associations between permethrin resistance and *kdr* frequency. However, several homozygous resistant RR genotypes were recorded in the permethrin susceptible samples and a small proportion of homozygous susceptible SS genotypes were recorded in the permethrin resistant samples. Pre-exposure to PBO significantly increased the *kdr* frequency amongst permethrin resistant samples in the GAH baseline colony and GAH Early time-to-hatch strain (Fisher's exact test: $\chi^2 = 15.01$; $p < 0.01$ and $\chi^2 = 10.49$; $p < 0.05$, respectively) but not in the GAH Late time-to-hatch strain. The baseline GAH colony was also screened for L1014S mutation but this was not detected (see Chapter 2).

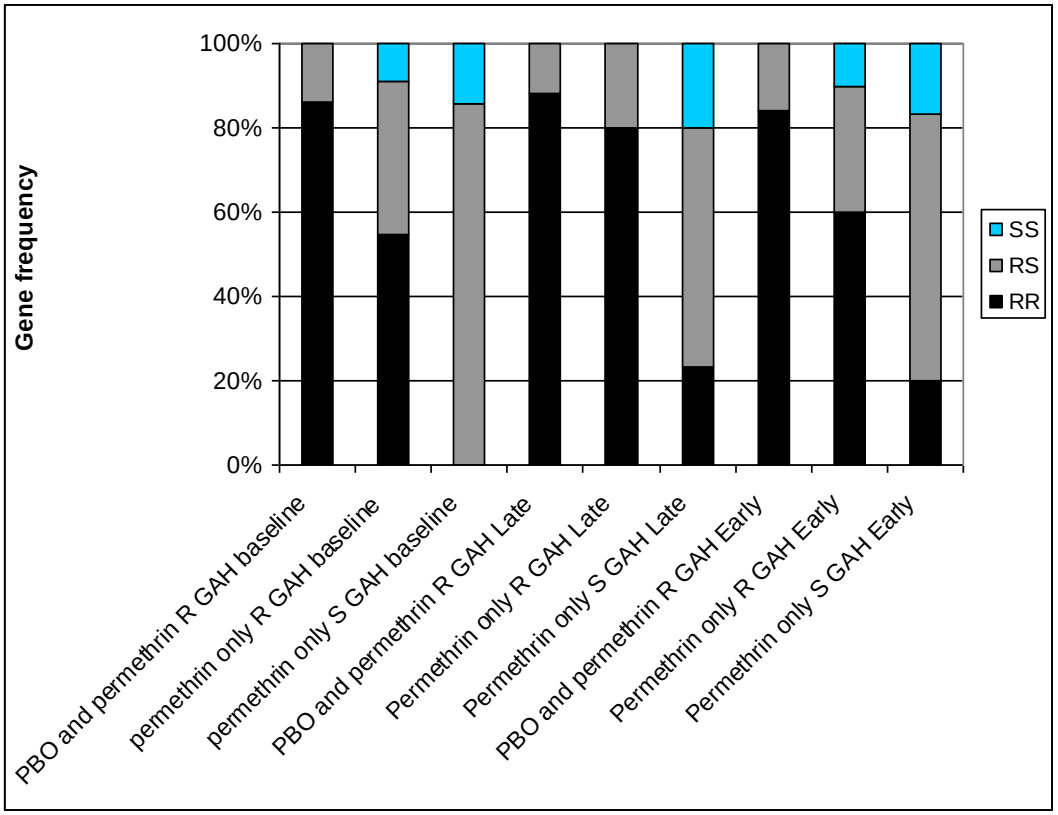


Figure 3.9: Proportions of L1014F *kdr* genotypes sorted by response to insecticide exposure phenotype (resistant or susceptible) from either the GAH baseline colony or the time-to-hatch selected strains exposed to permethrin only or permethrin and PBO. R = resistant, S = susceptible.

3.5.9.2 Rdl

A sample of *An. gambiae* GAH females were characterized as either dieldrin resistant or susceptible following exposure to 4% dieldrin. Sample sizes ranged from 22-29 mosquitoes. The alanine 296-glycine (*rdl*) GABA receptor mutation was detected by hydrolysis probe assay (Bass *et al*., 2008) and there was a clear association between *rdl* genotype and response to dieldrin phenotype in both the time-to-hatch strains as well as the baseline colony (Fisher's exact test: GAH Late: $p < 0.001$, Pearson's $\chi^2 = 108$; GAH Early: $p < 0.001$, Pearson's $\chi^2 = 67.89$; GAH Base: $p < 0.001$, Pearson's $\chi^2 = 98.13$) (Figure 3.10). In addition, GAH Late survivors (R) had significantly higher frequencies of the *rdl* mutation than GAH Early survivors (Fisher's exact test: $p = 0.03$, Pearson's $\chi^2 = 5.21$),

reflecting the differences in their susceptibility to dieldrin in the WHO bioassays (see Figure 3.8a).

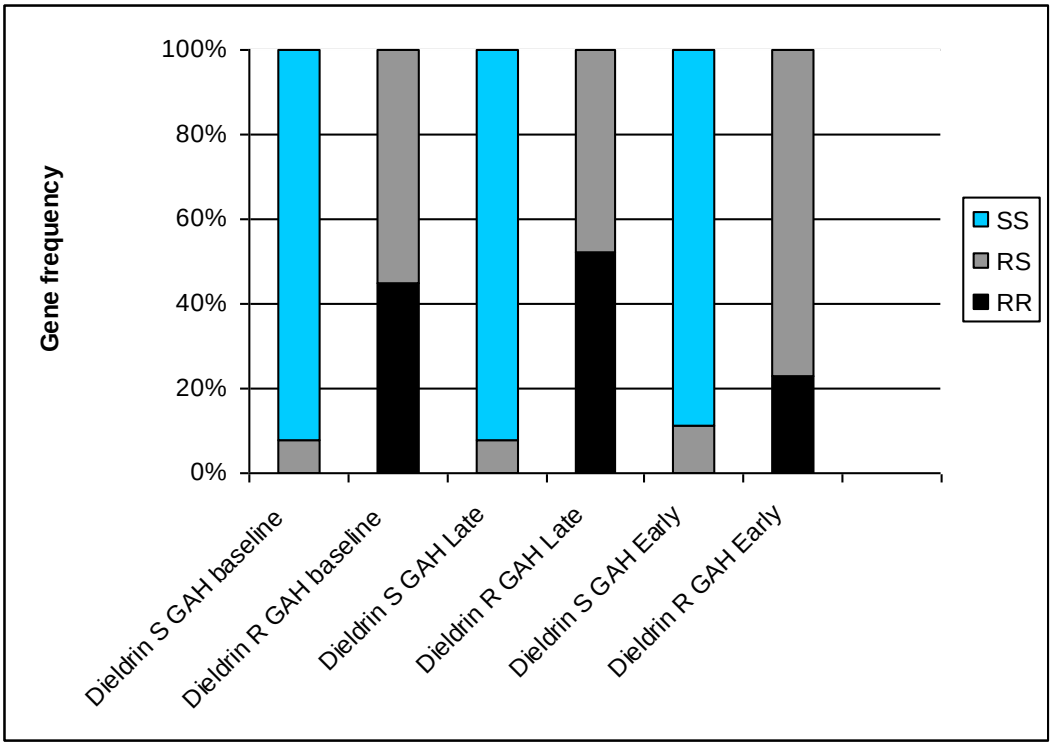


Figure 3.10: Proportions of alanine 296-glycine (*rdl*) genotypes assorted according to phenotype after response to 4% dieldrin exposure of female *Anopheles gambiae* from baseline GAH and time-to-hatch selected strains. S = susceptible, R = resistant.

Ace-1^{R} was not screened for in time-to-hatch strains although it was detected in the baseline colony (see Chapter 2).

3.5.10 Microsatellite Detection

All the microsatellites except for Ag3L39A were successfully amplified by PCR and subsequently run in the Beckman analyser. Some optimisation, relating mostly to annealing temperature, was required in order to acquire a single amplicon. Analyses were performed by David Weetman (Vector Group, Liverpool School of Tropical Medicine, Pembroke Place, Liverpool, UK). Discrepancies between expected and observed heterozygotes indicated possible scoring errors for more than half of the microsatellite loci.

FST values indicated that there was a high degree of genetic differentiation in these samples at certain loci (AG2H675 and AG3H811) however, scoring errors were also suspected (Figure 3.11).

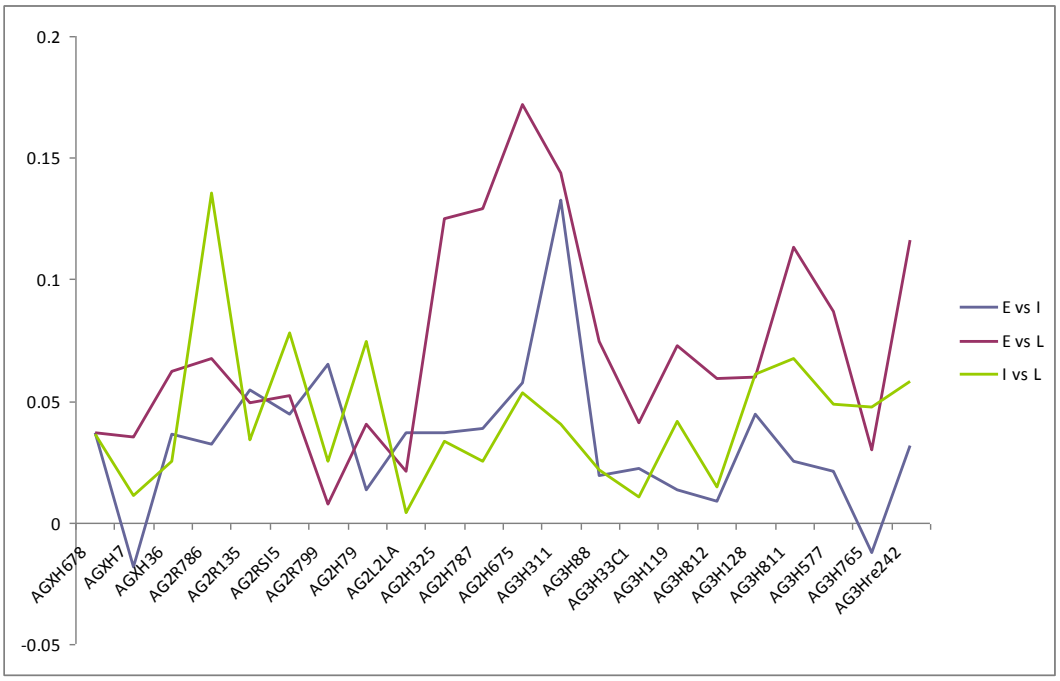


Figure 3.11: Plot of FST values for all amplified microsatellites indicating high variation at some loci. Key E=Early selected; I=Intermediate selected; L=Late selected.

3.6 Discussion and Conclusion

The majority of the *An. gambiae* GAH eggs hatched within four days of oviposition. However, the proportion of late hatching eggs increased following selection for this phenotype suggesting that, in addition to environmental factors (Yaro *et al*., 2006), there may be a genetic component controlling larval time-to-hatch. Cross-mating between the time-to-hatch phenotypes, supported by hybrid or inter-crosses and back-crossing of hybrids to the parental stains, gave ambiguous results suggesting that this genetic component may be multi-factorial. A genetic influence on variation in time-to-hatch is further supported by the observation that some of the wild-caught *An. gambiae* families from Ghana and the Republic of the Congo were exclusively early or late hatching.

Mosquitoes reared in the laboratory may display less variation in time-to-hatch due to unintentional selection pressure. The variation recorded in response to insecticide exposure assays also correlates with variation in *rdl* frequencies between the time-to-hatch selected strains, showing that selection for time-to-hatch inadvertently affected the frequencies of those factors influencing insecticide resistance.

Pyrethroid resistance decreased in GAH (Chapter 2) as well as the time-to-hatch selected strains (Chapter 3) during the months that elapsed between conducting standard WHO susceptibility bioassays and the synergist bioassays. This suggests a fitness cost associated with the mechanism conferring pyrethroid resistance in GAH. *Kdr* would be the likely choice as it is commonly associated with resistance to pyrethroid insecticides and is known to confer fitness costs in the absence of insecticide selection (Berticat *et al*., 2008; Brito *et al*., 2013; Alout *et al*., 2014). However, resistance to DDT increased during the same time period making this possibility unlikely, especially as *kdr* most closely associated with resistance to DDT (Chapter 2). This implies that other resistance mechanisms may be associated with maintaining resistance to pyrethroids even when *kdr* is present (Reimer *et al*., 2008; Brooke, 2008; Donnelly *et al*., 2009; Brooke and Koekemoer, 2010), that also likely confer a significant fitness cost in the absence of insecticide selection pressure. This warrants further investigation.

Although the adaptive significance of staggered larval time-to-hatch post-oviposition has not been quantified, it is highly likely that this characteristic has evolved in response to the variable nature of preferred *An. gambiae* larval sites (Yaro *et al*., 2006). These sites are typically small, highly variable in terms of water quality, subject to large fluctuations in temperature, and are susceptible to desiccation and flooding. That variation in hatching

time is ubiquitous across *An. gambiae* populations is supported by the quantification of this characteristic in geographically diverse samples from the Republic of the Congo and Ghana. Hatch time plasticity/heterogeneity within laboratory colonies may be lost due to unintentional selection pressure. This may be particularly true for eggs that hatch late. Larvae are transferred from smaller to bigger bowls as the larvae develop and unhatched eggs that remain on the side of the egg bowl are often discarded.

The data further show that the proportion of eggs that hatch as well as the proportions of eggs that hatch either early or late do not depend on the initial number of eggs laid per *An. gambiae* female. This suggests that delayed larval hatch is not an adaptation in response to over-crowding or to avoid competition for resources. Additionally, water disturbance induces a significantly higher rate of hatching in *An. gambiae*.

A high degree of variation was detected in the wild-caught samples genotyped for microsatellites despite the fact that the samples were drawn from only four families which were caught in the same region in Ghana, in villages surrounding the mine. Analysis of the microsatellite data revealed some problems. In particular, there were too many allele sizes present per family indicating either contamination with unrelated mosquitoes or mis-scoring (this was supported by the discrepancies observed for many of the loci between the levels of expected and observed heterozygosity). The scoring was repeated but did not significantly alter the results. A possible source of error is that Ghaf 33 family was *An. coluzzii* (M form) while the remaining three families were *An. gambiae s.s*. Due to time constraints the PCR assays and allele detection in the Beckman could not be repeated. This would ideally have been done to check any suspect alleles including those that were out of range and null alleles. The high number of different allele sizes observed could be

explained by multiple fathers although this is highly unlikely in anophelines as the majority of females have been shown to mate only once in a lifetime (Tripet *et al*., 2003). Allele size variation is more likely an effect of mis-scoring, amplification of some non-specific PCR products, or possibly contamination of mosquito families although care was taken during rearing to prevent such contamination. Microsatellites can be difficult to score correctly for several reasons such as variation in DNA sequence (mutation near the marker), low quality or quantity of DNA (the quality of the DNA in this study was confirmed by SINE PCR), biochemical artefacts (+A tail), and human error (Pompanon *et al*., 2005). A repeat of this study using crosses and back-crosses would help determine whether there are microsatellite markers useful for detecting time-to-hatch phenotypes and their assortment with insecticide resistance profiles. This pilot study indicated that the different hatching phenotypes may correlate to actual genetic differences. However the study was inconclusive.

The presence of insecticides in fluctuating concentrations significantly alters the chemical environment in which mosquitoes breed and rest. A significant adaptive response to the presence of multiple insecticide classes necessarily involves the development of multiple resistance mechanisms whose effectiveness may be enhanced by intra-population variation in the expression of insecticide resistance in association with the expression of time-to-hatch phenotypes. The variation in the expression of insecticide resistance in association with selection for larval time-to-hatch described here may induce this kind of enhanced adaptive plasticity as a consequence of pleiotropy. In this scheme cohorts of mosquitoes are able to complete the aquatic life stages in a variable larval environment giving rise to an adult population with enhanced variation in the expression of insecticide resistance.

Enhanced variation inadvertently produced in this way offers a wider platform for the selection of resistance to insecticides.

Successfully managing insecticide resistance in malaria vector control should take into consideration the biological and adaptive variation inherent within the target vector populations. Effective malaria control can then be achieved by adopting an evidence-based approach which incorporates the principles of the judicious use of insecticides in a broader Integrated Vector Management (IVM) system.

Chapter 4: Metabolic Rate and Embryonic Development in Early and Late Hatching Eggs of *Anopheles gambiae*

4.1 Introduction

In *Anopheles gambiae*, intra-species adaptive plasticity that enhances population survival in variable or unpredictable environments also inadvertently maintains malaria parasite transmission. Some of these adaptive traits that might affect vector capacity include larval development time, adult body size and adult longevity. These are postulated to be under the control of genetic and environmental factors in approximately equal measure in *An. gambiae* under laboratory conditions, although environmental effects are likely of greater importance under natural conditions (Lehmann *et al*., 2006).

As eggs are fertilized during oviposition by sperm stored in the female's spermatheca after mating, a single batch of eggs oviposited in one sitting is fertilized at approximately the same time and development of the embryo commences once the eggs are oviposited (Clements, 2000). Eggs normally hatch once embryogenesis is complete which is within two to three days post-oviposition in optimal conditions in *An. gambiae.* However, some eggs are able to hatch two weeks or more after oviposition (de Carvalho *et al*., 2002; Yaro *et al*., 2006 and see Chapter 3) increasing their risk of desiccation, particularly as anopheline eggs normally do not cope well with desiccation. The ability to delay or stagger larval time-to-hatch is therefore a possible adaptive trait that increases reproductive output despite the increased risk of desiccation in an unstable environment (Beier *et al*., 1990; Minakawa *et al*., 2001; Shililu *et al*., 2004; Yaro *et al*., 2006).

Delayed Development in Mosquitoes

Many mosquito species are able to delay development. This can be at the egg, larval or adult stages, although diapause normally only occurs at one of the stages within a species, with a few exceptions (Denlinger and Armbruster, 2014). Delayed development can be a direct response to adverse conditions that can be terminated immediately upon return of suitable conditions (quiescence), or it may occur before the actual onset of severe conditions and may persist even if conditions for continued development are suitable (diapause). Metabolic rate depression is a defining characteristic of diapause (Hahn and Denlinger, 2011). Diapause in temperate regions is known to be under control of day length; however, in tropical regions environmental cues may be more numerous with biotic factors such as food availability also involved (Denlinger, 1986).

In mosquitoes, embryo survival in the egg stage during diapause is as a pharate first larval instar (fully developed embryo waiting to hatch) (Hurlbut, 1938; Vinogradova, 2007). *Aedes albopictus* from the southern USA showed a strong egg diapause response when mothers were exposed to short days, while populations of the same species in Brazil either lost diapause or (the southern-most populations) had only a small but significant percentage of eggs remaining dormant (Lounibos *et al*., 2003). In general, diapause incidence increases with increasing distance from the equator.

In anophelines, diapausing (overwintering) larvae (Vinogradova, 2007; Kim *et al*., 2009) and adult females in temperate regions (Tauber *et al*., 1986), and the tropics (Omer and Cloudsley-Thompson, 1968; Denlinger, 1986; Lehmann *et al*., 2010), have been described. Diapause winter eggs that remained viable for seven months have been described in one anopheline species, *An. walkeri* (Hurlbut, 1938).

Because diapause is initiated in anticipation of adverse conditions, it is normally maternally regulated when expressed in the embryo (Mosseaue and Dingle 1991, Denlinger and Armbruster, 2014). Diapause responds to natural (Lounibos *et al*., 2003; 2011) and artificial (Scheiner and Istock, 1991) selection, making it an important adaptive trait. Resistance to desiccation in *Aedes albopictus* is higher in diapause than in non-diapause eggs (Sota and Mogi, 1992, Urbanski *et al*., 2010), and may have selected for retention of diapause in populations of *Ae. albopictus* in Florida, USA (Lounibos *et al*., 2011). The ability to delay egg development in the malaria vector *An. gambiae* could have important ecological consequences and may have implications for vector control if the conferred adaptive advantage directly or indirectly facilitates malaria transmission as a consequence of enhanced survival.

Anopheline eggs kept at least slightly moist have previously been shown to hatch up to 18 days after oviposition (Deane and Causey, 1943). Huang *et al.* (2006), in Kenya showed that *An. gambiae* eggs kept in water or on damp soil survive even at high temperatures (41 °C). In addition first instar larvae were observed two to five days after flooding dry soil samples (obtained near potential oviposition sites) in water (Beier *et al*., 1990), and viable eggs were obtained after up to 15 days on moist soil (Shililu *et al*., 2004). Minakawa *et al*. (2001), also in western Kenya, studied oviposition site preference in *An. gambiae* and found that the order of preference was flooded soil, moist soil, dry soil and a blank dish. Mosquitoes showed a considerable preference for the flooded soil. However, when dry season conditions were simulated by providing only moist soil, dry soil and an empty dish, the majority of eggs were laid on the moist soil substrate. The authors suggest that this is a strategy to maintain populations through the dry season in combination with the knowledge

that *An. gambiae* embryos can survive on moist soil for several days. These studies clearly demonstrate the ability of some eggs to survive in a desiccating environment for a period ranging from a few days to more than two weeks. However, Yaro *et al.* (2006) quantified the distribution of hatching times in *An. gambiae*, *An. coluzzii* and *An. arabiensis* in different water types, but without desiccation, and found that most (89%) of the eggs hatched early during the second and third days post-oviposition, ten percent hatched between days four and seven and one percent hatched after the first week. They also found that eggs that hatched early developed to adulthood faster and produced smaller adults than late hatchers. Kaiser *et al*. (2010) and Ebrahimi *et al*. (2014) have confirmed that desiccation is not a requirement for delayed hatching and a proportion of eggs may be programmed to delay hatching or to hatch in response to a different number or intensity of egg disturbance events or hatching triggers (inundation with water, rainfall, agitation). Delayed embryo development has been extensively studied in flood water mosquitoes (*Aedes* species) which can delay hatching for several months (Gillet, 1955a). There is a comparative scarcity of information available for anopheline mosquitoes.

Evidence of a genetic association with staggered larval time-to-hatch in *An. gambiae* is based on time-to-hatch phenotypic selections and cross-mating experiments (Kaiser *et al*., 2010). This evidence also includes an association between larval time-to-hatch and the assortment of insecticide resistance phenotypes in a laboratory colony of *An. gambiae*, showing that selection for either early or late larval time-to-hatch affects the frequencies of those factors associated with insecticide resistance as a consequence of pleiotropy. Based on these data it is proposed that staggered larval time-to-hatch occurs because a proportion of individuals are genetically predisposed to hatch late which coincidentally offers a broader phenotypic platform for the selection of insecticide resistance (Kaiser *et al*., 2010).

However, the processes that enable some *An. gambiae* eggs from the same batch to hatch substantially later than others are unknown. The aim of this study was to investigate the mechanism of staggered larval time-to-hatch by examining embryo development and metabolic output in *An. gambiae* eggs drawn from early and late hatching mothers.

4.2 Materials and Methods

4.2.1 Biological Material

The *Anopheles gambiae* colony (GAH), as described in Chapter 2 was used for all experiments. This colony has undergone selections for early and late time time-to-hatch and has been described previously (see Chapter 3 and Kaiser *et al*., 2010). Cross-mating experiments indicated that time-to-hatch did not follow a Mendelian mode of inheritance although selection for late time-to-hatch increased the proportion of late hatching larvae to 30%. Both selected strains, however, still contained early and late time-to-hatch eggs (Kaiser *et al*., 2010).

4.2.2 Time-to-Hatch Monitoring

Eggs from the Early and Late time-to-hatch selected strains, as well as eggs from the baseline GAH colony used for the selections, were monitored for time-to-hatch. This was done to determine whether the selections previously described (Kaiser *et al*., 2010) still had an effect on the time-to-hatch phenotype. Experiments were set up in the Botha de Meillon insectary and maintained at standard conditions. Five batches of eggs from both the baseline colony and the Early time-to-hatch strain were monitored. For the Late time-to-hatch strain, 7 batches of eggs were used as the egg batches obtained from this strain

were smaller because of fewer egg producing adults at this stage. Eggs were monitored daily for time-to-hatch by counting and removing hatchlings.

4.2.3 Egg Collection and Storage for Egg Metabolic Rate Experiments

At least three batches of eggs obtained from the Early and Late time-to-hatch strains were collected on different days after allowing a group of females to oviposit over a period of two hours. The eggs were then left to melanize for one hour after removal before rinsing the eggs onto filter paper. The water was allowed to drain prior to sealing the eggs on the filter paper, in an appropriately labelled plastic bag for storage until experiments started. Eggs were stored in the laboratory at ambient temperature during summer months. In experiments conducted during the colder months the eggs were kept in an incubator set at 25 °C.

4.2.4 Egg Metabolic Rate Measurements

Metabolic rates of sets of 150 eggs were measured every 24 hours up to 8 days old. At least four replicates per age group were used. The total metabolic output of batches of 150 eggs was measured by determining the amount of CO_2 emitted by the eggs in a closed system using a CO_2 analyser. CO_2 measurements were obtained using an infrared CO_2 analyser (LI-CO 6262, Li-Cor, Lincoln, NE, USA) in a similar way to that described by Woods and Singer (2001). Thirty ml glass syringes (Becton Dickson, Franklin Lakes, NJ, USA) with a small hole drilled through the wall of each syringe closest to the top (plunger end) served as respiratory chambers. A set of 150 eggs from either Early or Late time-to-hatch parents was placed onto a 1 cm^2 piece of moist filter paper using a very fine paintbrush (Herbert Evans). The eggs on filter paper were then placed into the respiratory chamber. A three-way stopcock was placed between the syringe and the needle and, to prevent leakage, connections were sealed with petroleum jelly. A total of three syringes

were used during each experiment. Initially, one syringe contained no eggs (control) to obtain a baseline reading and the remaining two syringes contained sets of 150 eggs. Thereafter each syringe served as its own control and one group of eggs from the Early or Late-hatch strains was placed into each syringe consecutively so that two or three replicates per strain and per egg batch were measured on the same day. This method was adopted as readings differed slightly between syringes. (See Figure 4.1 for experimental set-up).

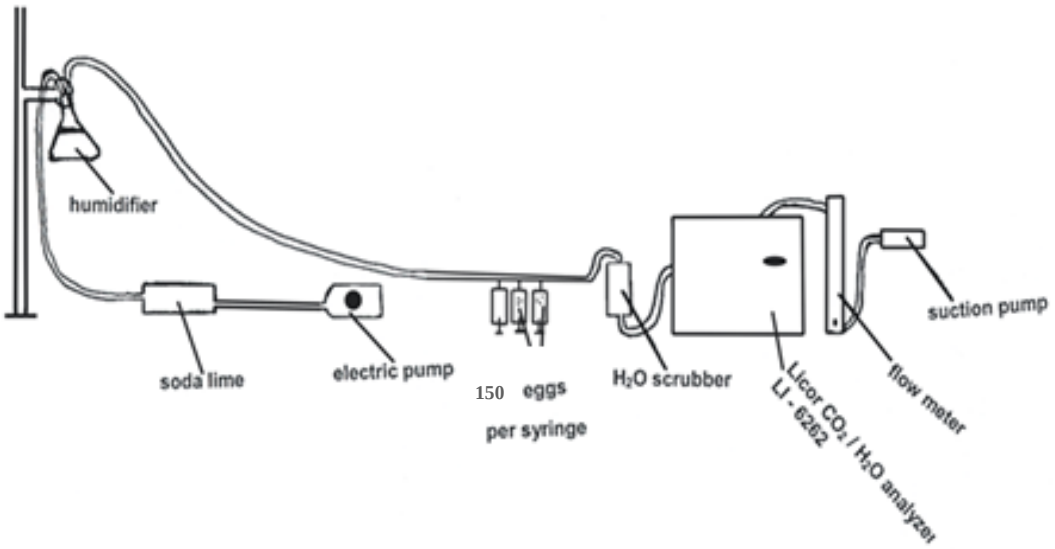


Figure 4.1: Experimental design for measuring metabolic rate of eggs (as successfully used for locust eggs). Due to the small size of the anopheline eggs 150 eggs per syringe were tested. Image used with permission from I. Kambule.

Humid CO_2 free air was obtained by pumping room air through a soda lime scrubber followed by a humidifier. After the eggs had been placed into the syringes they were flushed with the humid CO_2 free air for five minutes. Once flushing was complete the plunger was depressed to the 20 ml mark, blocking the hole so that the purged air was

flushed through the needle. Eggs were left in the sealed CO_2 free chamber for 1 hr. Air scrubbed of CO_2 and water using soda lime and magnesium perchlorate, respectively, was drawn through the CO_2 analyser at a rate of 100 ml/min. After the 1 hour, 5 ml air boluses from each syringe were inserted into the air stream, before the water scrubber. This was done for each syringe in sequence. This process was repeated twice (3 measurements per syringe) and the amount of CO_2 in the injected samples converted from parts per million (ppm) to ml by integration of the CO_2 curve and then multiplied by 1000 to give a value in µl. Data were recorded and analysed using DatacanV (Sable systems, Las Vegas, NV, USA). Comparisons of CO_2 output between syringes as well as between time-to-hatch egg batches were based on two-sample t-tests using Statistix 7 (Tallerhassee, USA).

4.2.5 Observing Embryo Development in Fixed and De-chorinated Eggs

Eggs were collected from mixed age groups of Early or Late time-to-hatch adults that had received at least two blood meals prior to egg collection. Eggs were stored in distilled water until they reached the developmental age at which viewing was required. Eggs were collected on a strip of filter paper and covered in a few drops of FAA fixative solution (2.5 ml formaldehyde; 25 ml of absolute ethanol; 2.5 ml of glacial acetic acid made up to 50 ml using distilled water) for at least 30 minutes. Once eggs had been fixed, the filter paper strip with eggs was placed into a 1.5 ml reaction tube and covered with one ml of decalcifying solution. The decalcifying solution was as described by Trpiš (1970), modified slightly and used to decalcify the chorion so that embryos could be viewed under the microscope. The decalcifying solution contained 2 ml sodium hypochlorite and 1 ml glacial acetic acid made up to 50 ml with distilled water. This successfully fixed the eggs preventing hatching and bleached the chorion so that embryos could be viewed under the microscope. The date the eggs were collected, the age of the eggs in hours or days post-

oviposition and the cage of adults the eggs were collected from were noted. This ensured that at least three batches of eggs from different cages were collected for viewing. The embryo of each egg was viewed with a Zeiss Stereo Discovery V12 microscope at 150 times magnification. Digital images of embryos were obtained and scale bars inserted. Embryos were compared by time post-oviposition by qualitatively describing the level of embryo development based on whether segmentation was visible as well as the overall degree of development.

4.3 Results

4.3.1 Time-to-Hatch Monitoring

Five to seven batches of eggs were obtained from each of the time-to-hatch strains as well as from the baseline colony. The sample sizes were 3692, 2381 and 5189 for the Early and Late-hatch strains, and the baseline GAH colony respectively. In all cases the majority of the eggs hatched on the second day post-oviposition. The baseline colony then showed less hatching on day 3 post-oviposition following which hatching occurred in a staggered fashion with most of the eggs hatching within 6 days (Figure 4.2A). The last recorded hatching in the baseline colony was on day 18 post-oviposition, as was also the case in the Late time-to-hatch strain (Figure 4.2B) although the numbers were very small (three and one, respectively). The mean times to last hatch were 10.2 (± 5.02), 5 (± 1.41) and 13.6 (± 2.43) days for the baseline, the Early time-to-hatch and the Late time-to-hatch strains respectively. These differences were statistically significant (ANOVA: $f = 10.39$, $df = 2$, $p \leq 0.01$). The primary difference was in a comparison between the Early and Late time-to-hatch strains (two-sample t-test: $t = 7$, $df = 1$, $p << 0.01$). There were no significant differences in the mean times to last hatch between the baseline GAH colony and the Late

time-to-hatch strain (two-sample t-test: t = 1.56, df = 1, p = 0.15) or between the baseline GAH colony and the Early time-to-hatch strain (two-sample t-test: t = 2.23, df = 1, p = 0.08). There were no differences in the total number of eggs that hatched between the baseline colony and the time-to-hatch strains (means ranged from 58.55-64.05%).

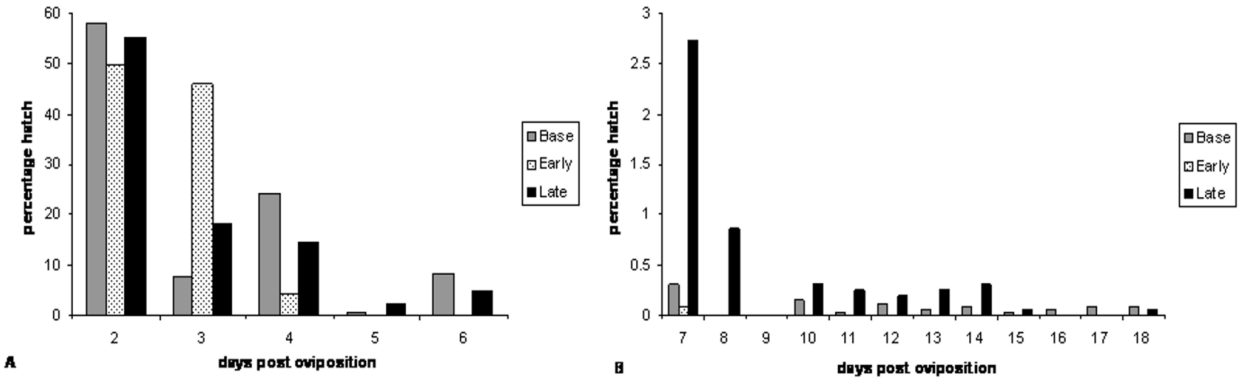


Figure 4.2: Proportional hatch rate of eggs from the *Anopheles gambiae* GAH baseline colony as well as the Early and Late time-to-hatch selected strains. A) from days two to 6 post-oviposition and B) from days 7 to 18 post-oviposition. Note the difference in scale on the y-axis.

4.3.2 Egg Metabolic Rate

The amount of CO_2 produced by eggs per age did not differ significantly between the Early and Late time-to-hatch strains (ANOVA: $p > 0.05$ for all comparisons, Table 4.1). In general, egg metabolic rate tended to decrease as the eggs aged up to day 5 post-oviposition after which a slight increase in metabolic rate was observed from days 6 to 8, particularly for the eggs from the Late time-to-hatch strain. The highest metabolic rate for both groups of eggs was recorded 24 hours post-oviposition (Figure 4.3). Based on linear regression, the metabolic output of eggs from the Early time-to-hatch strain decreased significantly with age ($f = 6.35$, $df = 1$, $p = 0.04$; $r^2 = 0.48$). The data from the Late time-to-hatch strain did not show a significant trend with age ($f = 1.20$, $df = 1$, $p = 0.31$; $r^2 = 0.15$). On day 3 the eggs obtained from Early time-to-hatch selected parents produced a

significantly higher amount of CO_2 than the eggs obtained from the Late time-to-hatch parents (two-sample t-test: t = 2.42, df = 1, p = 0.02) while the reverse was true at day 7 post-oviposition (t = -2.06, df = 1, p <0.05). Otherwise there were no significant differences in metabolic rate between the strains.

Table 4.1: Linear regressions and analysis of variance (ANOVA) indicators obtained from trend analysis of CO_2 outputs with age by syringe, as well as comparisons between the CO_2 outputs of batches of eggs obtained from the Early and Late time-to-hatch selected *Anopheles gambiae* strains. The degree of freedom for all tests was 1.

Regressions	**r^2**	**p**
s2e-age	0.52	*0.02*
s3e-age	0.26	0.15
s2l-age	0.42	0.06
s3l-age	0.03	0.68
sce-age	0.48	*0.04*
scl-age	0.15	0.31
ANOVA	**F**	**p**
s2e-s2l	0.14	0.71
s3e-s3l	0.28	0.61
s2e-s3e	0.07	0.79
s2l-s3l	1.72	0.21
sce-scl	0.07	0.79

*s2 = syringe 2; s3 = syringe 3; sc = syringes combined; e = eggs from the Early time-to-hatch strain; l = eggs from the Late time-to-hatch strain.

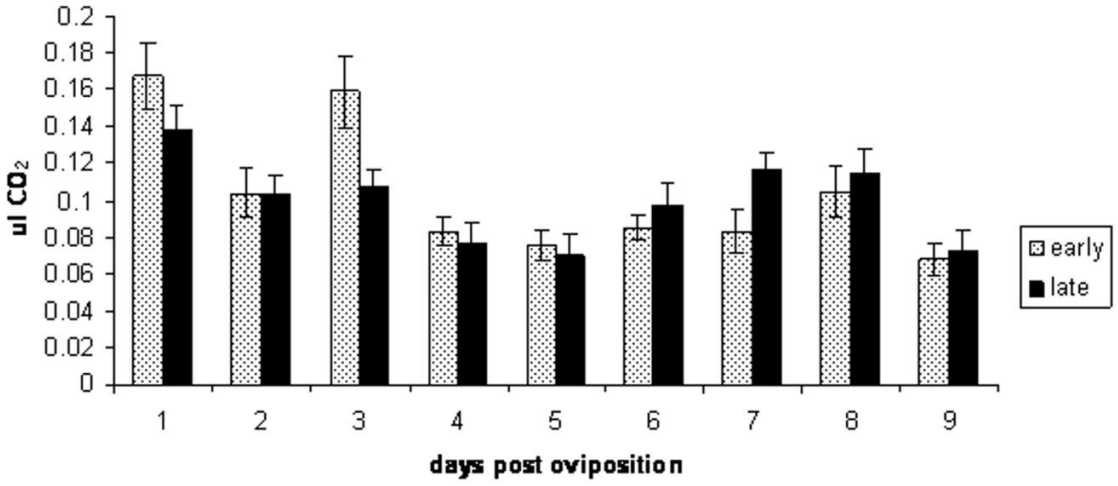


Figure 4.3: CO_2 output (µl) of eggs obtained from Early and Late time-to-hatch selected *Anopheles gambiae* strains by age. Standard error bars are shown.

4.2.4 Qualitative Embryo Development Observations

Approximately 30 images of eggs per age group (1-8 days post-oviposition) were obtained from both Early and Late time-to-hatch selected strains. Using the degree of definition in embryo segmentation as a visual cue for development, no obvious differences were observed in the rates of embryo development between the eggs from the Early and Late time-to-hatch strains (Table 4.2 and Figure 4.4). Most of the embryos from both groups of eggs were fully developed by two to three days post-oviposition. Both groups also had eggs that hatched on day two post-oviposition. The majority of eggs hatched between days two and four as previously described. Many embryos did not develop. The mean rate to full embryonic development of photographed embryos across the ages 24 hours to 8 days was 44.88% in the Early time-to-hatch strain and 46.75% in the Late time-to-hatch strain.

Table 4.2: Qualitative description of the degree of development of unhatched embryos by age post-oviposition for eggs obtained from Early and Late time-to-hatch selected *Anopheles gambiae* parents. The total percentages of developed and developing embryos are shown in bold. # = number of images taken.

Days post-oviposition	**Early time-to-hatch** #	Description	**Late time-to-hatch** #	Description
4 – 6 hrs	31	15 white granular with lateral floats often visible. The rest were white granular with some clearer sections possibly the start of tissue differentiation. Many had a large round tissue mass in the central region.	48	20 were white granular. Most were white-granular on the anterior and posterior ends, some with a clearer region in the centre.
1 Day	58	**83%** showed form and evidence of segmentation, often with a gap between the embryo and the shell. Head and body could be defined in most cases.	86	**63%** showed form and segmentation. The rest were granular white or patched white, some with the central tissue mass.
2 Days	34	**44%** showed clear form and segmentation (most did not fill the whole shell cavity). Hatching had begun.	31	**71%** showed clear form and segmentation or appeared to be developing.
3 Days	30	**36.7%** were fully developed taking up the whole shell cavity or close to fully developed	27	**18.5%** were fully developed taking up the whole shell cavity
4 Days	31	**58.1%** were fully or very close to fully developed	31	**48.4%** fully or close to fully developed
5 Days	27	**48.1%** fully developed or close to fully developed	39	**38.5%** fully or close to fully developed
6 Days	30	**20%** were fully developed or close to fully developed	28	**43%** were fully or nearly fully developed
7 Days	27	**26%** were fully developed or close to fully developed	31	**58.1%** were fully developed or close to fully developed
8 Days	28	**43%** were fully developed or nearly fully developed	33	**36%** were fully or close to fully developed

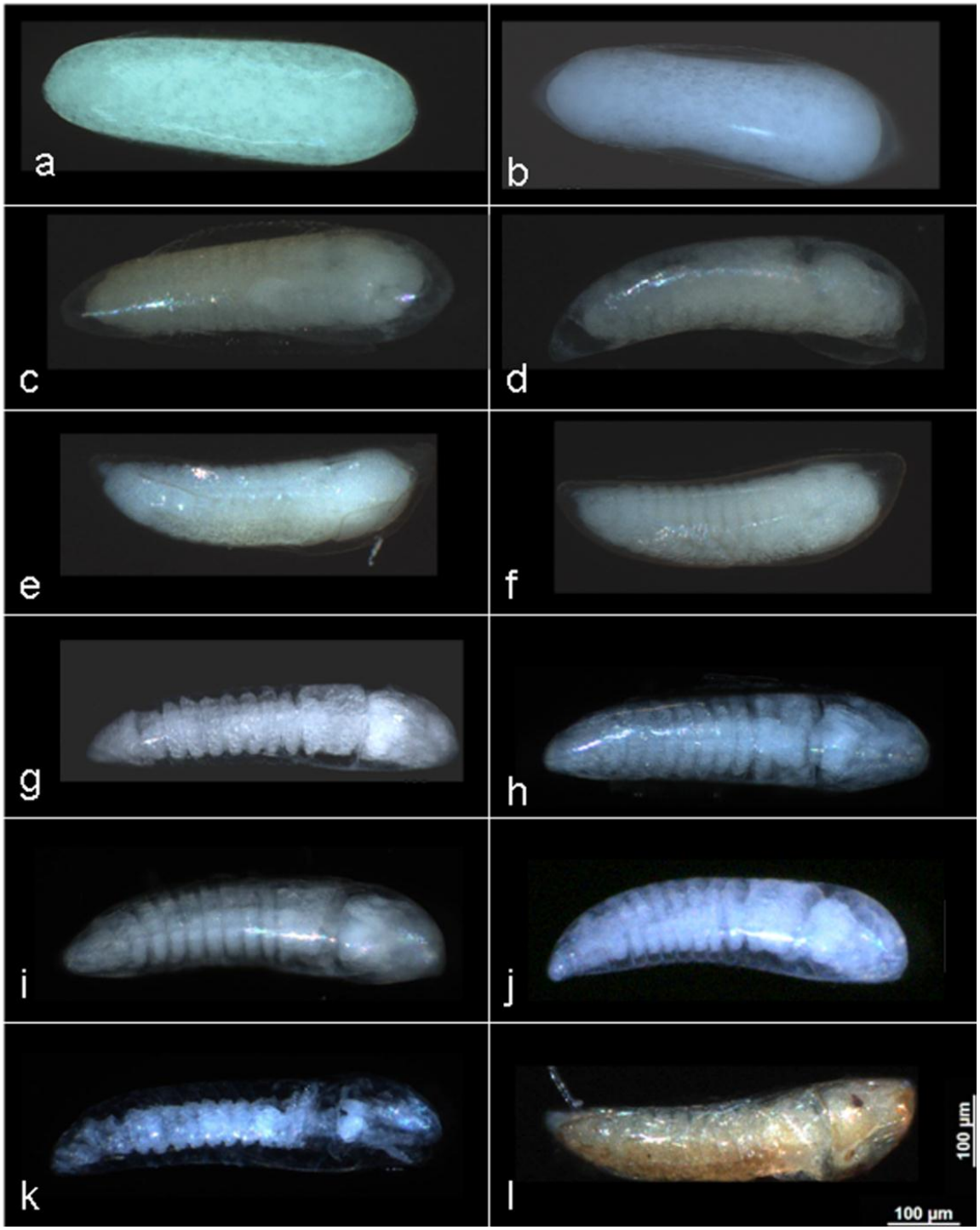


Figure 4.4: Embryos photographed at different ages and developmental stage post-oviposition: eggs obtained from the Early time-to-hatch strain are on the left and eggs from the Late time-to-hatch strain on the right - a) and b) 4 hours post-oviposition; c) and d) 20 hours post-oviposition; e) and f) 24 hours post-oviposition; g) and h) 48 hours post- oviposition; i) and j) 4 days post-oviposition; k) and l) 6 days post-oviposition.

4.4 Discussion and Conclusion

The Late time-to-hatch selected strain showed a later mean time to last hatch than the Early time-to-hatch strain. Under standard insectary conditions, eggs from both time-to-hatch strains tended to hatch early (four days post-oviposition and earlier) although the Late time-to-hatch strain showed a higher proportion of eggs that hatched 5 days post-oviposition and later. In both strains a very small proportion of eggs hatched up to 18 days post-oviposition.

The ability to delay hatch when conditions for hatching are not immediately suitable is an important adaptive trait in some mosquitoes - *Aedes* species in particular (Gillet, 1995a and 1955b; Perez and Noriega, 2013). Although dormancy in tropical insects is known, it is less often considered (Denlinger, 1986). The delayed or staggered time-to-hatch described here for the *An. gambiae* baseline and selected strains has also been observed in families reared from wild-caught *An. gambiae* females collected in Ghana and the Republic of the Congo (Kaiser *et al*., 2010), as well as from colonized and wild-caught mosquitoes from Kenya (Beier *et al*., 1990; Minakawa *et al*., 2001; Shililu *et al*., 2004), showing that this trait is not unique to laboratory strains or particular ecological zones, although considerable differences are expected to be seen between different populations. Staggered time-to-hatch likely carries adaptive significance in *An. gambiae* (Yaro *et al*., 2006; Kaiser *et al*., 2010).

The metabolic rates of eggs from the Early and Late time-to-hatch selected strains only showed significant variation at three and 7 days post-oviposition. Woods *et al*. (2005) showed that metabolic rates in eggs of the moth, *Manduca sexta*, were generally low at the beginning of development and then increased more or less continuously until hatching

occurred at the end of development (within 2-3 days). However, Kambule *et al*. (2011) showed that the metabolic rate of non diapause locust eggs continued to increase until the eggs hatched while the metabolic rate of diapause eggs was consistently lower. The highest metabolic rates in the *An. gambiae* eggs were recorded at 24 hours post-oviposition which corresponds to the stage in development when embryos undergo a 180° rotation around the longitudinal axis during the germ band retraction phase (Monnerat *et al*., 2002). Prior to this most of the development has already occurred with intersegmental furrows visible and the ventral aspect of the embryo facing the flattened dorsal side of the shell (Monnerat *et al*., 2002). Metabolic rates then tended to decrease with time post-oviposition in the selected strains suggesting that late hatching eggs likely enter a state of diapause prior to hatching.

Wilkinson *et al*. (1978) found that 29% and 1% of *An. dirus* eggs survived after 21 days and 92 days respectively on moist filter paper. An experiment by Darrow (1949) on *An. quadrimaculatus* eggs showed that eggs removed from water within 9 hours of oviposition could not resist desiccation. However, those removed from water at 10-13 hours post-oviposition did develop desiccation tolerance and 27% of these eggs hatched after exposure to 0% RH for 12 hours. Darrow (1949) also removed eggs from water at 23.5 hours post-oviposition and returned them to water after 24 and 36 hours respectively. The eggs subjected to this treatment hatched approximately 23.5 hours after being returned to water suggesting that embryonic development ceases during desiccation and then continues at the normal rate. In this study, the eggs used for metabolic rate experiments and monitored for time-to-hatch post-oviposition, hatched almost immediately after being returned to water. This occurred in all eggs except for 24 hour old eggs, indicating that, similar to (Darrow, 1949), eggs can only begin to hatch approximately two days after

oviposition when the embryo is fully developed. This suggests that the eggs at ages two days and older were ready to hatch when taken for use in experiments, but that hatch was delayed.

Qualitative embryo development observations were somewhat hampered by the hatching of the majority of eggs between two and four days post-oviposition, which made the acquisition of unhatched eggs for age groups older than 4 days difficult. As a result many batches of eggs were sampled to acquire adequate sample sizes of late hatching eggs. There were no consistent morphological differences observed between eggs obtained from the two strains in terms of the level of embryo development by age group or between the number of embryos that were fully developed per age group. This may be partly due to the fact that both strains contain mostly early time-to-hatch eggs, with the Late time-to-hatch strain showing a higher proportion of late hatchers. In general, all embryos that developed fully did so within four days post-oviposition, supporting the metabolic output data which suggests that late hatching eggs enter a state of diapause once the embryos are fully developed.

Time-to-hatch in anopheline mosquito eggs is generally believed to be fixed so that it occurs at the completion of embryo development. This is normally at approximately 50 hours old in optimal conditions (Darrow, 1949; Vargas *et al*., 2014). However, various factors, temperature in particular, are known to affect that rate of embryo development and hatching (Impoinvil *et al*., 2007). It makes sense that eggs are able to adapt to some extent to different hatching conditions in response to risks and opportunities. Parents are also able to influence hatch timing, so the assumption that hatch timing is relatively fixed is not always true (see review by Warkentin, 2011). The current study found that the embryos

develop within the first two to four days post-oviposition with most hatching immediately and a small proportion delaying hatching. The delayed hatch appears to be accompanied by a fairly constant and reduced metabolic rate in some eggs. As all eggs were exposed to the same environment this phenomenon cannot be considered quiescence but rather is assumed to be late embryonic diapause as seen in the Gypsy moth *Lymantria dispar* (Leonard, 1968) and some temperate mosquito species (Vinogradova, 2007).

Hatch timing may influence other traits such as insecticide resistance and fitness. Recently Perez and Noriega (2013) showed that extended quiescence (eggs induced to hatch after 10 weeks post-oviposition instead of after just less than one week for short quiescence) affected performance and reproductive fitness of adult *Aedes aegypti* females as well as the nutritional status of their progeny via maternal effect. Specifically, the females from the eggs that underwent extended quiescence survived 10% longer, laid more eggs and produced 14% more viable offspring when reared on a sub-optimal diet of 3% sucrose solution. However, the reproductive success of females reared from extended quiescent eggs was dramatically affected by stress in the larval environment in the form of metal contamination. The authors claim that intrapopulation variation in the sensitivity of individuals to environmental cues is what leads to asynchronous hatching and suggest that phenotypic plasticity results as a consequence of pharate (waiting to emerge or hatch) first instar larvae. That variation in sensitivity to environmental stimuli may lead to asynchronous hatching is supported by Ebrahimi *et al*. (2014) who found that all *An. gambiae* eggs in their experiments required agitation to hatch and that some eggs required more agitation events than others. This is compared to instalment hatching or bet hedging observed in *Aedes* mosquitoes where only a proportion of eggs will hatch in response to a given stimulus (Gillet, 1955a). Ebrahimi *et al*. (2014) point out that this requirement for

agitation to induce hatching may be an advantage in mass rearing situations where a large number of synchronously developing mosquitoes are needed. Most studies looking at delayed hatching of *An. gambiae* complex eggs have done so in conjunction with desiccation conditions. Whether the delayed hatching phenotypes observed in this study may be more desiccation tolerant than early hatching phenotypes remains to be investigated.

An association between insecticide susceptibility and staggered time-to-hatch in *An. gambiae* has previously been described (Kaiser *et al*., 2010 and Chapter 3). These studies suggest that variation in one trait (such as staggered time-to-hatch) may affect and even enhance variability in other traits that affect reproductive and physiological fitness as well as environmental adaptability. This may occur as a result of resource re-allocation or as a consequence of pleiotropy. It is likely that there are interactions between environmental and genetic factors that influence time-to-hatch in mosquitoes (Gillet 1955b; Lehmann *et al*., 2006; Kaiser *et al*., 2010). These factors in turn may influence subsequent life history traits and may even affect behaviour as a consequence of pleiotropy. It would be interesting to determine whether eggs associated with more permanent larval sites such as those of *An. funestus* are more or less likely to hatch as soon as embryos are fully developed without the requirement of a disturbance. Beier *et al*. (1990) indicate that *An. funestus* is less able to resist desiccation and this may also translate to the ability to delay hatching.

It is concluded that all viable embryos in *An. gambiae* develop to full maturity at the same rate and that a proportion are able to delay hatching. As it has previously been shown that it is possible to at least partially select for late time-to-hatch, this characteristic is likely to

involve genetic as well as environmental factors. This study supports several others (Beier *et al*., 1990; Shililu *et al*., 2004; Yaro *et al*., 2006) in which it was also concluded that delayed time-to-hatch in *An. gambiae* is likely an adaptation to maximize reproductive output despite the increased risk of desiccation in an unstable aquatic environment.

Chapter 5: Final Discussion and Conclusions

Resistance to all classes of insecticides tested was recorded in the wild-caught samples from Ghana and in the GAH colony established two years previously. This data set adds to the increasing incidence of resistance to multiple insecticides which is a major cause for concern and provides a strong rationale for thoughtfully implementing the principles of insecticide resistance management (WHO, 2012).

The resistance phenotypes recorded in the GAH laboratory colony are attributed to combinations of target site insensitivity (L1014F, *rdl* and *ace-1*^{R}) and metabolic resistance mechanisms (P450's and esterases) and are likely representative of the wild-population from which the colony was derived as no insecticide selection pressure was applied to the colony in the laboratory. No GST mediated detoxification was implicated based on synergist assays using DEM. However, GST upregulation has been reported in response to blood feeding in the GAH colony (Spillings, 2012). The upregulated GST in GAH was GSTD3 (Spillings, 2012) which has also been found upregulated in a sample of DDT resistant *An. arabiensis* from Burkina Faso (Jones *et al*., 2012b) as well as in DDT resistant *An. coluzzii* in Cotonou in Benin (Djébé *et al*., 2014) supporting the request for validation of this GST in conferring resistance to DDT in these vectors (Djébé *et al*., 2014). These data in combination with the discrepancy observed between the levels of DDT and pyrethroid resistance in the presence of *kdr* in GAH, emphasise the importance of metabolic detoxification mechanisms in the production of resistance phenotypes and further illustrate how most resistance phenotypes are mediated by several mechanisms working in concert (Reimer *et al*., 2008; Brooke, 2008; Brooke & Koekemoer, 2010; Aïkpon *et al*., 2014; Edi *et al*., 2014; Mitchell *et al*., 2014).

Any environmental adaptations that enhance the general reproductive and physiological fitness of malaria vectors at the individual and population levels have either direct or indirect epidemiological implications. Although insecticide resistance is the primary adaptive characteristic of interest in the context of vector control, other adaptive characteristics may also have implications in terms of how vector populations respond to changes in their environment. Staggered time-to-hatch was identified as an adaptation that likely enhances reproductive output in unstable larval environments and that may also affect insecticide resistance expression at the population level, depending on how staggered time-to-hatch is mediated. The data presented here show that early and late time-to-hatch can be selected for, implying a genetic component. However, selection was not complete and did not follow a Mendelian model of inheritance based on crosses and subsequent back-crosses between the Early and Late time-to-hatch selected strains. Nevertheless, the data suggest a genetic component controlling delayed hatch, and that environmental factors are also important. Moderate to high heritability estimates for larval development time, adult size and longevity of starved *An. coluzzii* adults were shown by Lehmann *et al*. (2006), lending further support to the likelihood that there is a genetic component contributing to variation in time-to-hatch. That the degree of heritability was not clear from the present study may be due to the way in which selections were performed. There was no buffer period between the selections. Allowing a gap of a day or two between selections, instead of a single time-point cut-off for early and late time-to-hatch could possibly have increased our ability to detect the magnitude of a genetic contribution to time-to-hatch.

Disturbance (in the form of rinsing eggs with water) significantly increased the proportion of hatching, particularly in the late-time-to-hatch selected strain. This may provide a clue

as to the environmental conditions needed to select for delayed time-to-hatch. In a simplified scheme, environments with standing water and a low risk of disruption are likely to favour immediate (early) hatching once the pharate first larval instar has reached maturity, with only a small proportion hatching late. Conversely, small larval sites prone to desiccation and flooding likely favour staggered time-to-hatch as a strategy to enhance the probability of survival under conditions of larval site instability. Under these conditions, females producing eggs with a greater proportion of late hatchers can be described as hedging their bets and ultimately enhancing their fertility.

That staggered time-to-hatch is not a once-off laboratory effect was confirmed by monitoring time-to-hatch in batches of eggs obtained from wild-caught females from Ghana and the Republic of the Congo. The hatching distribution closely approximated that shown by Yaro *et al.* (2006). Egg density had no significant effect on time-to-hatch in *An. gambiae* (Chapter 3), as has since been independently confirmed by Ebrahimi *et al*. (2014).

Selection for early or late time-to-hatch in GAH affected the resistance profiles of the selected strains possibly as a consequence of pleiotropy (Kaiser *et al*., 2010). The Early time-to-hatch strain showed significantly higher levels of resistance to the carbamates as well as to the organophosphate pirimiphos-methyl than the Late time-to-hatch strain. The reverse was observed in response to exposure to the organochlorines and permethrin, with the Late time-to-hatch showing significantly higher levels of resistance than the Early time-to-hatch strain to these insecticides. Genes associated with stress response have been shown to be differentially expressed in response to insecticide exposure in mosquitoes (Vontas *et al*., 2005) as well as in diapausing *Culex pipiens* (Robich *et al*., 2007), although this was measured in adult females. This could potentially explain some of the differences

observed in the insecticide resistance profiles between the two selected strains. It is expected that early and late time-to-hatch individuals may also differ significantly in other respects. For example, differences in size, starvation resistance and longevity between *Ae. aegypti* adult females raised from short quiescence (hatched within one week of oviposition) and extended quiescence eggs (kept in the insectary for 10 weeks before hatching) were reported by Perez and Noriega (2013). Females raised from extended quiescence eggs were smaller and showed increased tolerance to starvation and increased longevity. In addition, the reproductive strategy between females reared from extended quiescence eggs and those reared from short quiescence differed. Specifically, rates of follicular resorbtion on a sub-optimal diet of 3% sucrose were 39% and 22% for females reared from short and long quiescence eggs respectively. Under these conditions the long quiescence females produced 14% more viable offspring than short quiescence females. On the other hand when larvae from short and extended quiescence eggs were exposed to metal contamination the resulting adult females from the long quiescence eggs produced significantly fewer eggs and viable offspring than those from the short quiescence eggs (Perez and Noriega, 2013). A maternal effect of reduced starvation tolerance of newly hatched larvae from the females from extended quiescence eggs was also observed (Perez and Noriega, 2013). These pleiotropic effects may at least be partially due to genetic linkages and consequent linkage disequilibrium between the controlling factors of each phenotype, further supporting the likelihood that staggered time-to-hatch carries a genetic component.

The rate of embryonic development in eggs from the Early and Late time-to-hatch strains did not vary significantly. On average, viable embryos were fully developed within two to four days post-oviposition regardless of time-to-hatch. This is similar to the developmental

time observed in other mosquito species (Vla, 1995). In addition, when embryonic diapause occurs in mosquitoes it is at the stage of the pharate first larval instar (Vinogradova, 2007; Denlinger and Armbruster, 2014). The metabolic rate (CO_2 output) of eggs from the time-to-hatch selected strains also did not vary. The only significant differences recorded were at days 3 and 7 post oviposition. Three days after oviposition is commonly when most of the hatching occurs so the high CO_2 output in the Early time-to-hatch strain at this age was expected. The increased CO_2 output at day 7 post-oviposition in eggs from the Late time-to-hatch strain is less easily explained. However, when looking at the distribution of time-to-hatch between the selected strains, the Late time-to-hatch strain does show a spike in hatching at 6-8 days post-oviposition (Chapter 4)- corresponding to the increase in metabolic rate observed at day 7 post-oviposition. Alternatively, some insects do have regular periods where metabolic rate increases during the diapause state possibly as a consequence of undergoing damage repair, boosting stress responses and expelling metabolic end products (see review: Hahn and Denlinger, 2011). These results suggest that all embryos develop at more or less the same rate and that a small proportion then enter a state of diapause based on a genetic predisposition to hatch late. Thus, time-to-hatch is based either on genotype or on an external stimulus such as water disturbance or heat shock.

A limitation of the metabolic rate study was the inability to know prior to experiments the relative proportions of the eggs tested that were alive or dead. It is likely that as a batch of eggs ages a higher relative proportion of non-viable embryos accrues. However, the observations of embryo development across the ages 2 to 8 days old showed non-significant differences in the proportions of embryos that appeared to be fully developed or developing and those that appeared to be non-viable.

Survival in the egg stage for several weeks may have distinct and very important benefits in unstable environments. Malaria vector distribution is limited by rainfall and temperature (Craig *et al.*, 1999). In the Ahafo region of Ghana, from where the GAH colony originates, the rainfall is seasonal with a major rainfall season from March to June and a minor season from September to November. There is a short dry season mainly in August and a long one from December to March. In the eggs from wild-caught females from the Republic of the Congo, situated nearer the equator, there was less variation in hatch timing within batches of eggs, possibly reflecting comparatively less environmental variability for this area. Several studies have indicated that late hatching eggs may survive the length of the short dry season as eggs have also been shown to resist desiccation to a degree (Minakawa *et al*., 2001; Koenraadt *et al*., 2003; Shililu *et al*., 2004). Various cues may be used by insects to predict changing seasons even in tropical regions where the seasonal changes may not be very marked (Denlinger, 1986). Small differences in day length, temperature, cloud cover and humidity may be sufficient signals to induce higher proportions of a population to enter a diapause state or to produce more offspring capable of entering such a state. Biotic factors such as nutrient availability or salt composition of oviposition sites may also play a role (Denlinger, 1986), although embryonic diapause in mosquitoes is proposed to be mainly maternally regulated (Denlinger and Armbruster, 2014). In Ghana day length can vary by over 45 minutes (www.dateandtime.info/sunriseandsunset) during the year which may, in combination with other factors, also provide a cue to the mosquitoes at various life stages (Denlinger, 1986) to shift developmental pathways. The knowledge that *An. gambiae* eggs can delay hatching may have an impact on models designed to predict the dynamics of malaria vector populations and disease transmission.

From the combined results of the metabolic rate measurements as well as the embryo development observations it was concluded that embryos develop fully and are capable of hatching within four days post-oviposition. While the majority do hatch within this time some eggs are predisposed to delay hatching, entering a diapause state with associated reduction in metabolic rate with age. This delay is considered as diapause because all eggs were reared in the same insectary conditions normally considered optimal for mosquito development. This egg diapause has been shown to last from several days for up to three weeks in the laboratory even in conditions considered optimal for hatching (eggs on the water surface, optimal temperature and humidity). The assortment of insecticide resistance profiles in association with early or late time-to-hatch provides an example of heterokairy where individuals from a single batch can follow different developmental pathways (Spicer and Burggren, 2003) and give rise to different phenotypes.

Summary Conclusions

The ability to develop along different pathways and produce phenotypically diverged individuals (in terms insecticide resistance in this study) from a single batch of eggs likely helps explain why *An. gambiae* is such an efficient malaria vector and a constant challenge to control. Small changes in developmental pathways can lead to greater phenotypic variation as a consequence of pleiotropy. The enhanced variation produced in this way may provide a broader platform for selection by factors such as insecticide pressure and environmental conditions resulting in improved adaptability. Better ecological understanding of *An. gambiae* may provide insights into the development of novel control strategies and may ultimately help elucidate the processes that allow *An. gambiae* populations to adapt to their prevailing environmental conditions.

Appendices

Appendix 1: Mine Baseline Malaria Vector Survey 2006

BASE-LINE MALARIA VECTOR SURVEY – AHAFO MINE – GHANA
7 – 18 DECEMBER 2006

Richard H. Hunt

Introduction

A mosquito survey was conducted at the Ahafo mine in central Ghana over ten days. The survey was designed to assess insecticide susceptibility levels in the malaria vector mosquitoes in the vicinity of the Newmont mine.

The current malaria vector control programme is using the pyrethroid insecticide Bifenthrin 10% wettable powder for indoor residual spraying. I did not see the spraying in operation but from what I was told it appeared that they were probably under-dosing. The larviciding is being done with Vectobac in a granular formulation. There is a large "swing fog" machine that can be mounted on a vehicle for general fogging. There are also two "Mosquito Magnet" devices – these units do not work on African malaria mosquitoes and can be discarded.

Infrastructure

Newmont was responsible for transport, accommodation, laboratory space and field assistants. All these facilities were provided efficiently, resulting in an enjoyable and productive stay.

Environment

Ahafo is characterised by dense to degraded vegetation and extensive secondary forest especially along the banks of small streams. The "water dam", an artificial lake, dominates the aquatic environment close to the mine village and main mine infrastructure. The shallow, well-vegetated edges of the lake provide breeding places for mosquitoes. The local inhabitants of the area were very vocal about the increase in the mosquito population since the construction of the dam. Strangely, they also claimed that the water had resulted in a marked increase in the snake population. Most wheel ruts and roadside sun lit puddles contained large numbers of *Anopheles gambiae* group larvae (Fig 1). This type of breeding site makes a significant contribution to malaria transmission in the more 'urban' environment of the larger villages.

The badly polluted drains and cess pools in the urban environment do not contribute to malaria transmission, but do provide breeding sites for the vectors of elephantiasis (filariasis). Water containers in the domestic environment and used vehicle tyres provide breeding sites for the Yellow Fever mosquito *Aedes aegypti.* (Fig. 2)

The senior staff housing is located in a swampy area with stagnant pools and slow moving drainage channels. These need on-going larval control measures. The stagnant ponds also provide a potential risk of bilharzia transmission, particularly as they are a temptation to the children living in the staff village. Transmission of this disease is by direct contact with water containing the parasite so swimming, paddling and fishing are high risk activities.

Mosquito Collections and Susceptibility Testing

Collections were made at 10 sites. GPS readings were as follows:

Kwakwukyei	N 07°03.280 W 02°23.350
Ibrahim Aduku	N 07°03.329 W 02°23.410
Kwadionikrom	N 07°03.394 W 02°22.777
Opanin Kwaku Addaekrom	N 07°03.457 W 02°23.197
Ola Resettlement	N 06°59.552 W 02°24.134 — funestus.
Water Dam (Chief)	N 07°03.656 W 02°24.190
Obenkurom	N 06°56.140 W 02°24.588 — funestus.
Donkorkrom	N 06°55.530 W 02°25.894
Yamfo-Newmont House	N 07°12.890 W 02°14.352
Kenyasi I	N 06°58.468 W 02°22.583

Mosquitoes were collected by aspirator from the walls, roofs and furnishings of bed rooms in dwellings ranging from rural traditional huts to more sophisticated structures such as those in the resettlement area at Ola (Fig. 5) near Kenyasi II. Considerable variation was found in the numbers of mosquitoes per house. This is normal, reflecting variation in the numbers of people per room, proximity to breeding sites, personal hygiene and usage of personal protection methods such as insecticide treated bed nets and mosquito coils.

The mosquitoes were stored in insulated containers and transported to the "laboratory" that was set up in the health and safety building (Fig. 4). There they were sorted into groups, *Anopheles gambiae* group and *Anopheles funestus* group, and were tested separately for insecticide susceptibility. Samples were exposed to the following insecticides:

DDT (organochlorine)
Dieldrin (cyclodiene)
Deltamethrin (pyrethroid)
Malathion (organophosphate)
Bendiocarb (carbamate)

Exposures were carried out in standard WHO test kits using test papers supplied by WHO. The exposure time was 1 hour and final mortality was recorded 24 hours post-exposure. Two sets of controls were run, both giving 100% survival showing that mortality in the tests was due to the insecticides and not to some extraneous environmental factor.

Results

A total of 455 *An. gambiae* group and 34 *An. funestus* were tested for insecticide susceptibility. Results are as follows:

Species	**Insecticide**	**# Dead**	**# Alive**	**Total**	**% Mortality**
An. gambiae	DDT	4	61	65	6.2
	Dieldrin	3	45	48	6.25
	Deltamethrin	50	66	116	43.1
	Malathion	107	10	117	91.5
	Bendiocarb	87	22	109	79.8
An. funestus	Deltamethrin	3	0	3	100
	Malathion	28	0	28	100
	Bendiocarb	3	0	3	100

The *Anopheles funestus* sample was very small and the majority were tested on Malathion after high resistance to the other insecticides had been clearly demonstrated in the *An. gambiae* group.

These results are calculated from the raw field data assuming that all mosquitoes of the *An. gambiae* group are in fact *An. gambiae sensu stricto* – preliminary identifications on survivors indicate that this is the case, but we have yet to determine which molecular form is present. It is possible, therefore, that the results will have to be adjusted in the light of the laboratory identifications when they are to hand.

The DDT and dieldrin results appear to be a common feature in Ghana. This is not the first time that a field sample gives virtually 100% survival on both of these insecticides and similar results were obtained at the Newmont mine at Akyem. While these results obviously eliminate the use of either of these insecticide classes (organochlorines and cyclodienes) for malaria control in the area, they raise questions as to the nature of the resistance mechanism that should be the subject of a research programme. Cross-resistance between classes of insecticides occurs depending on the mechanism responsible for the resistance, e.g. DDT and pyrethroid resistance based on a genetic mutation known as *kdr*. Information on possible cross-resistance is essential when planning implementation strategies for malaria vector control.

Appendix 2A: DNA extraction and species identification PCR protocols

All plastic ware (pipette tips, centrifuge and reaction tubes) used for DNA extractions were autoclaved for 20 minutes at 121 °C and allowed to cool before use.

Collin's (1987) DNA extraction

Extraction solutions:

1. Grinding buffer:

The following was included: 1600 µl 1M NaCl; 1.095 g Sucrose; 2400 µl 0.5 M EDTA; 1000 µl 10% SDS; 2000 µl 1 M Tris-Cl (pH 8.6), made up to a total volume of 20 ml using distilled water.

2. 8 M KAc (Potassium Acetate):

7.854 g KAc in 10 ml distilled water.

Collin's (1987) extraction protocol:

The heating block was turned on and set to 70 °C allowing half an hour to reach temperature prior to use. Samples were transferred individually to a 1.5 ml centrifuge tube using forceps and were ground dry as finely as possible using a pestle in. Two hundred µl grinding buffer was then added while removing the pestle in order to rinse off any mosquito tissue adhering to the pestle. The centrifuge tubes containing the ground samples were then incubated for 30-45 minutes at 70 °C. Centrifuge tubes were then removed from the heating block, 28 µl KAc was added and mixed by flicking the centrifuge tube. Following this, tubes were chilled on ice for 30 minutes prior centrifugation for 15 minutes at 13000 rpm to precipitate cell debris. The supernatant from each sample was transferred into clean 1.5 ml centrifuge tubes and the pellets discarded. Four hundred µl ice cold ethanol (100%) was added to the supernatant and mixed, then incubated overnight at minus 20 °C. The following day the samples were centrifuged for 30 minutes at 13000 rpm before

the ethanol was pipetted off and discarded. 200 µl of 70% ice cold ethanol was added to the pellet to remove salt, and re-centrifuged 13000 rpm for 30 minutes. The 70% ethanol was then pipetted off and the pellet allowed to air dry on the bench before being resuspended in 200 µl of TE buffer. Extraction negatives as well as species positives from the insectary were included in each batch of extractions.

Extracted DNA was stored at 4 ˚C or -20 °C until required.

Scott *et al*. (1993) *An. gambiae* complex PCR

One leg per mosquito sample was added to a reaction tube containing: 1.25 µl PCR buffer; 1.25 µl dNTPs (deoxynucleotide solution); 0.5 µl $MgCl_2$; 0.5 µl QD primer (CAGACCAAGATGGTTAGTAT); 1 µl each of primers: UN (GTGTGCCCCTTCCTCGATGT); ME (TGACCAACCCACTCCCTTGA); GA (CTGGTTTGGTCGGCACGTTT); AR (AAGTGTCCTTCTCCATCCTA) and 0.1 µl r(recombinant)*Taq* polymerase (Takara) in a total volume of 12.5 µl. Primer working stock concentrations were at 10 µM. For each set of reactions performed a reaction without a mosquito leg was included as a negative control as well as positive controls for each species: *An. quadriannulatus*; *An. merus*; *An. gambiae* (or *An. coluzzii);* and *An. arabiensis*.

PCR cycling conditions were as follows:

Two minutes at 92 °C followed by 30 cycles of 94 °C for 30 seconds; 50 °C for 30 seconds; 72 °C for 30 seconds and followed by extension 72 °C for 5 minutes and held at 8 °C until removed.

Favia *et al*. (2001) M/S form PCR

One µl of sample DNA was added to a reaction tube containing: 2.5 µl 10x PCR buffer; 2.5 µl 4 mM dNTPs; 2 µl each of primers R3 (GAATTCTAGGGAGCTCCAG), R5 (CCAATCCGAGCTGATAGCGC), Mopint (GCCCCTTCCTCGATGCAT) and B/S int (ACCAAGATGGTTCGTTGC), and 0.2 µl of r*Taq* polymerase made up to 25 µl with PCR grade water. Primer working stock concentrations were at 10 µM. A negative extraction control (no DNA) was included for all sets of samples done as well as positive controls from M and S species characterized colonies maintained in the insectary.

PCR cycling conditions were as follows:

Ten minutes of 94 °C followed by 25 cycles of: 94 °C for 30 seconds; 62 °C for 30 seconds and 72 °C for 30 seconds, and a final extension of 72 °C for 7 minutes. Samples were held at 8 °C until removed.

Agarose gels, electrophoresis and visualization

PCR products were visualized by mixing 10 µl of PCR product from each sample mixed with 2 µl of loading buffer containing bromophenol blue was added to each well of a 2.5% or 1.5% agarose gel (made using 1 x Tris Acetate EDTA (TAE) and stained using ethidium bromide). At least one well on each gel contained 8 µl of a 100 kb DNA ladder (O'Range ruler- Fermentas) in order to size the PCR products and negative and positive control were also included for each batch of samples. Gels were electrophoresed at 100 volts until separation of the ladder was great enough to clearly distinguish PCR product sizes. Gels were viewed under an ultraviolet transilluminator light in a gel documentation system (Vacutec).

Appendix 2B: Cloning of the *rdl* fragment to obtain *rdl* positive controls

Screening for the *rdl* mutation is not common practice in the laboratory therefore there were no positive controls. The hydrolysis probe assay primers used to amplify the area containing the *rdl* mutation amplified a region of only 98 bp. Direct sequencing of this small PCR product was not an option. An attempt was made to develop other primers to amplify a larger region surrounding the mutation; however, the first attempt was unsuccessful, yielding only high amounts of primer dimers. Following this, it was decided to conduct a conventional PCR using primers from the hydrolysis probe assays and to then extract this fragment after electrophoresis on a 2.5% agarose gel using the QIaquick gel extraction kit (Qiagen, GMBH, Germany supplied by Southern Cross Biotechnology, Cape Town, S.A). PCR cloning was used to amplify the sequence amplified by the TaqMan® *rdl* primers. This yielded a repeat of the fragment amplified allowing one to view the sequence after sequencing of the cloned PCR product.

The following method was employed to clone the PCR product amplified by the hydrolysis probe *rdl* primers after initially viewing the PCR product on a 2.5% agarose gel to confirm that an amplicon of the expected size was amplified: The pGEM®-T Easy vector system (Promega, Madison, WI, USA, supplied by Whitehead Scientific, Cape Town) was used for the ligation step of the cloning procedure. An insert: vector ratio of 6:1 was used after quantifying DNA in two samples: GAH dieldrin exposed survivor number 1; and GAH dieldrin exposed survivor number 10. These samples were scored as RR and RS genotypes respectively in the hydrolysis probe assay and we wanted to confirm this result by sequencing the samples so that they could be used as standard controls in any subsequent *rdl* molecular assays. The ligation reactions consisted of: 50 ng pGEM®-T Easy vector; 20 ng of insert/ 8 ng control insert DNA (ligation positive control)/ nothing (background

control); 3 Weiss units of T4 DNA ligase; 5 µl of 2X Rapid ligation buffer made up to a total volume of 10 µl with distilled water. Once the reactions were set up they were stored at 4 °C for 36 hours before continuing with the transformation step.

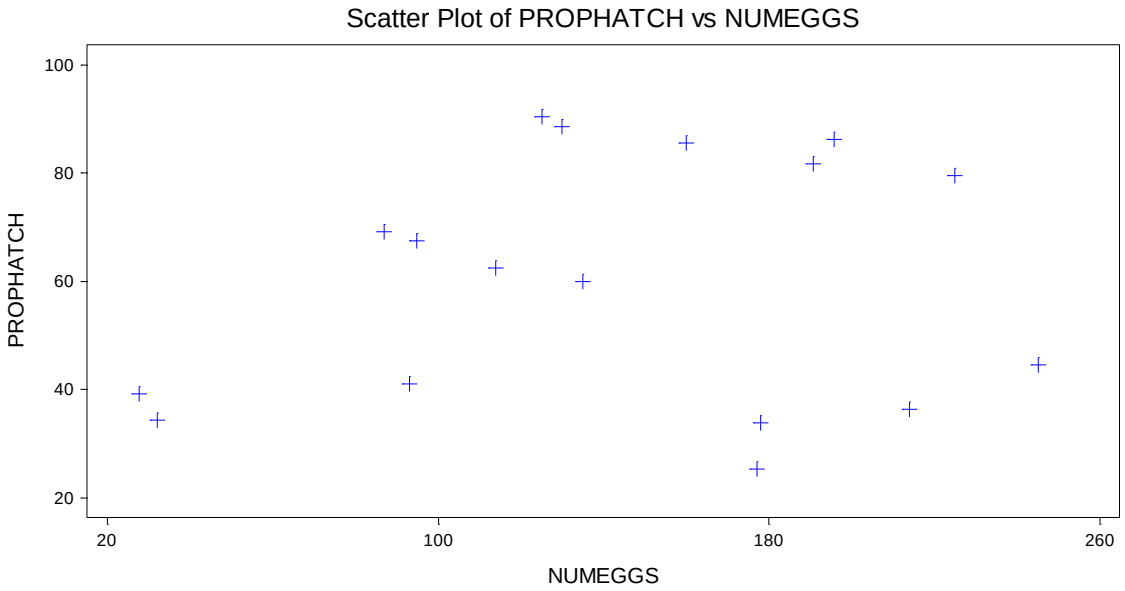
The transformation section of the cloning procedure was done using E. Cloni® chemically competent cells (Lucigen Corporation, Middleton, WI, USA, supplied by Inqaba Biotechnical Industries, Pretoria). These cells have been pre-optimized for transformation by heat shock. Before transformation took place agar pates were prepared with 500 ml autoclaved YT agar (YT powder included with E.Cloni® cells). Three hundred microlitres of isopropyl β -D-1 thiogalactopyranoside (IPTG), 800 µl bromo-chloro-indolyl-galctopyrnoside (X-gal) and 1 ml of ampicillin (AMP) was added and mixed into the YT agar before it was poured into Petri dishes under sterile conditions and allowed to cool to room temperature. Before transformation began one sterile, 1.5 µl centrifuge tube per reaction was placed on ice to chill and the ligation reactions were heat inactivated at 70 °C for 15 minutes. The E. Cloni® cells were removed from the -70 °C freezer and allowed to thaw completely on ice. Fifty µl of competent cells were added to each centrifuge tube and 10 µl of each heat inactivated ligation reaction was added to their respective centrifuge tubes and mixed gently with the pipette tip. The reactions were then incubated on ice for 30 minutes before heat shocking in a 42 °C water bath for 45 s. Following heat-shock, cells were incubated on ice for 2 min with 950 µl of recovery medium (supplied with the E.Cloni® chemically competent cells) per centrifuge tube. Tubes were shaken at 230 rpm for one hour in an incubator at 37 °C. Two hundred µl of transformed cells were plated onto YT agar plates previously prepared, under sterile conditions. Controls included a ligation control as well as a background control. Plates were sealed with parafilm and

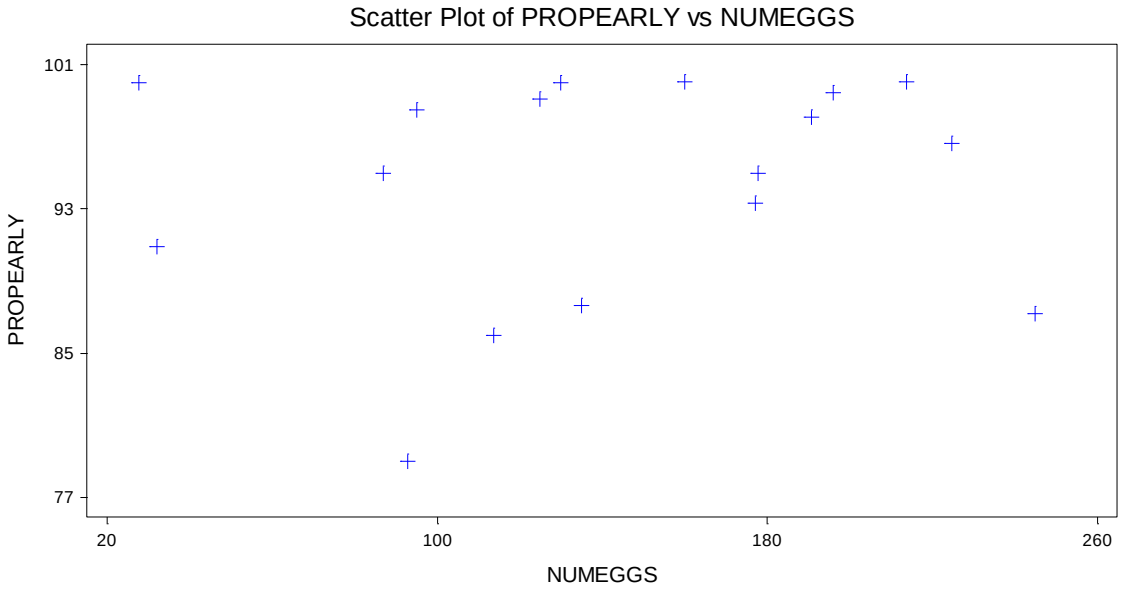
incubated overnight at 37 °C. Culture plates were thereafter transferred to a 4 °C fridge for two hours prior to colony selection, to allow colonies to develop colour.

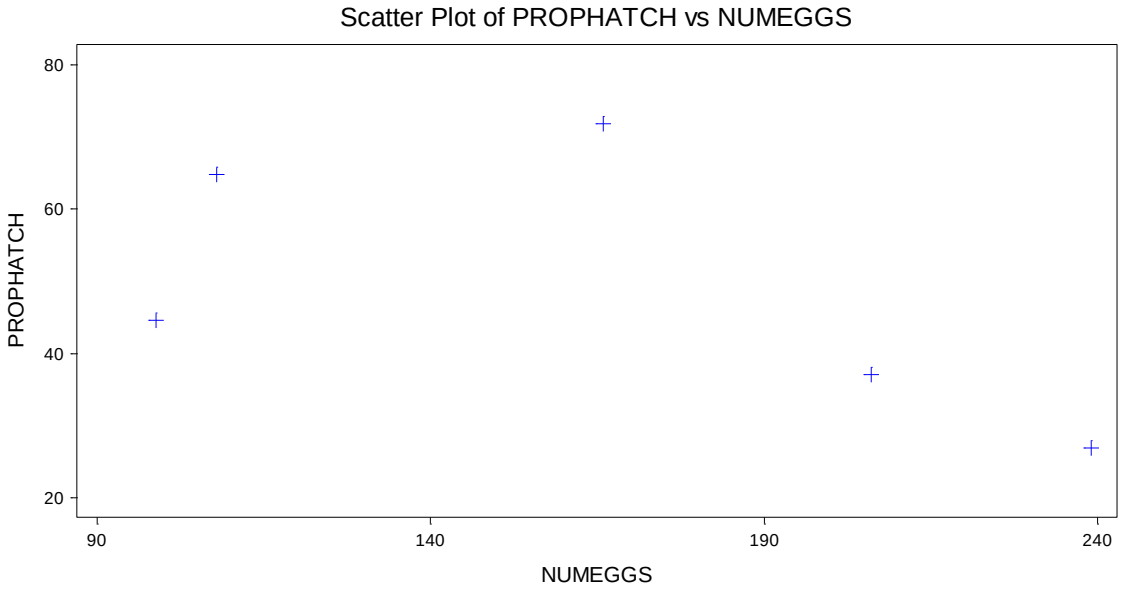
While culture plates were stored in the fridge for two hours, lysogeny broth (LB) (10 g/l tryptone; 5 g/l yeast extract; 10 g/l NaCl) with 50 µl/ml of AMP was prepared. Three µl of this mix was placed into each of twenty 15 ml Falcon tubes. A PCR reaction containing: 200 µM dNTPs; 1.5 mM $MgCl_2$; 1X PCR buffer; 6 pM of each primer (SP6: 5´-TATTTAGGTGACACTATAG-3´; and
T7: 5´-TAATACGACTCACTATAGGG-3'); and 1.25 units of *Taq*, and made up to a volume of 10 µl, was prepared. Single and circular, transformed colonies (white), were randomly selected from culture plates by scraping them off the culture plate with a pipette tip. The pipette tip was then swirled into a labelled 0.2 ml tube containing the PCR mixture and the tip ejected into a correspondingly labelled Falcon tube. A PCR with the following conditions: 94 °C for 2 min; 25 cycles of: 94 °C for 30 s; 55 °C for 30 s and 68 °C for 45 s; 72 °C for 1 min, was performed. The LB inoculates (in 15 ml Falcon tubes) were incubated at 37 °C and shaken at ~ 200 rpm, overnight. Thereafter cells in LB were concentrated by centrifugation at 13000 rpm for 1 min and the supernatant discarded, followed by a second centrifugation at 13000 rpm for 1 min. Three hundred and 20 µl of the supernatant from the second centrifugation step was kept and the rest discarded. Four hundred and eighty µl of 50% glycerol, and the 320 µl of supernatant previously kept aside, were used to re-suspend the concentrated cells. Glycerol stocks were stored at -70 °C.

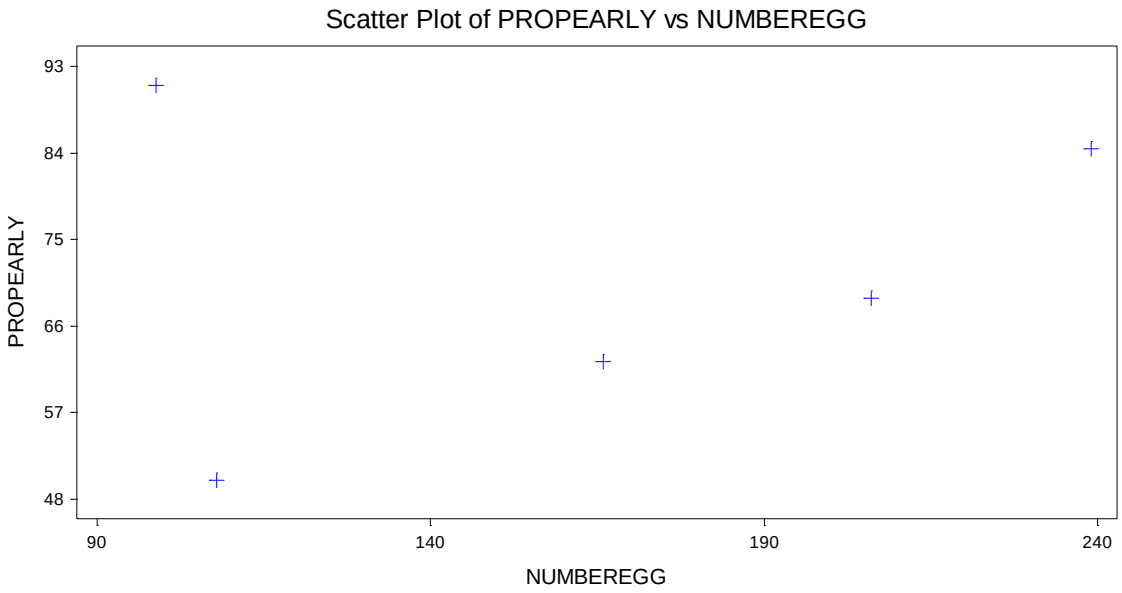
Appendix 3: Effect of density on time-to-hatch in wild-caught families: scatter-plots.

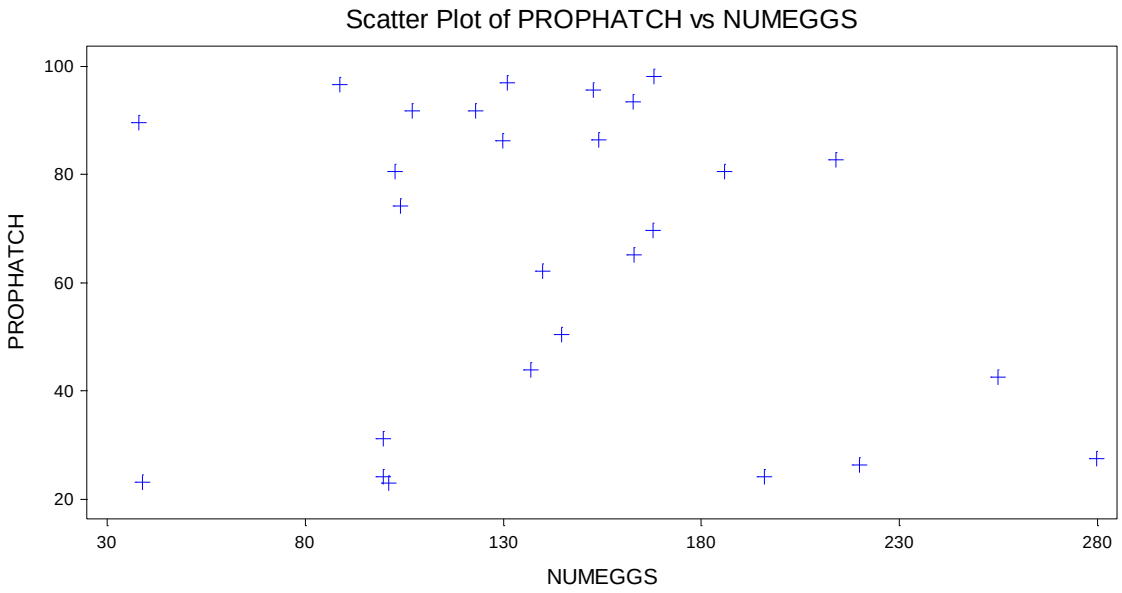
COGS

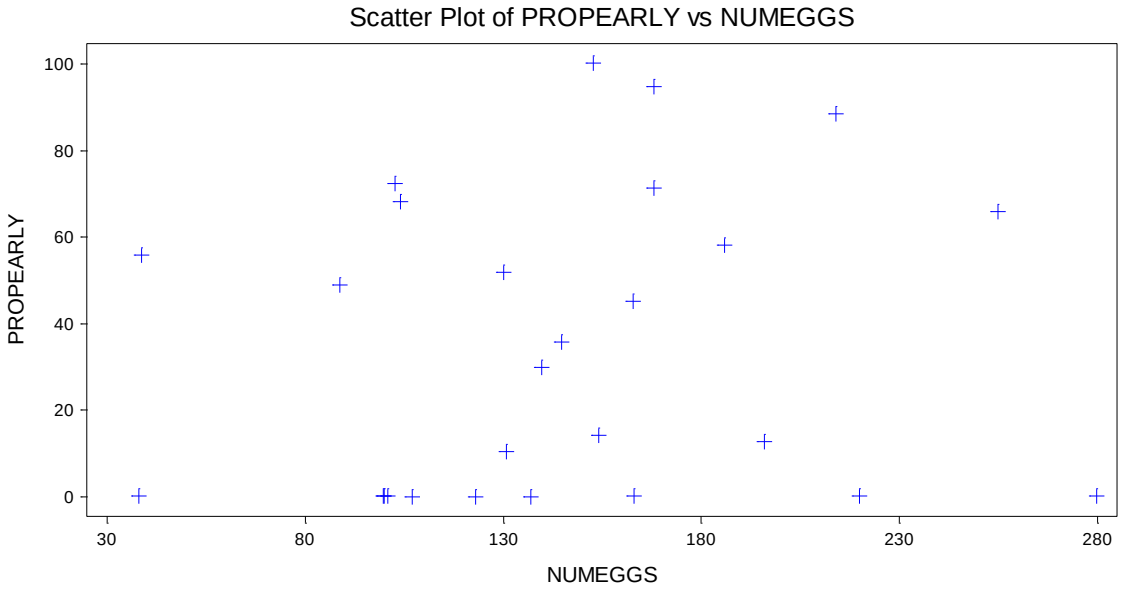





Scatter Plot of PROPHATCH vs NUMEGGS
PROPHATCH
80
60
40
20
90
140
190
240
NUMEGGS


Scatter Plot of PROPEARLY vs NUMBEREGG
PROPEARLY
93
84
75
66
57
48
90
140
190
240
NUMBEREGG


Scatter Plot of PROPHATCH vs NUMEGGS
PROPHATCH
NUMEGGS
100
80
60
40
20
30
80
130
180
230
280


Scatter Plot of PROPEARLY vs NUMEGGS
PROPEARLY
NUMEGGS
100
80
60
40
20
0
30
80
130
180
230
280

Appendix 4: Hunt *et al*., 2011. Insecticide resistance in malaria vector mosquitoes at four localities in Ghana, West Africa.

Hunt *et al. Parasites & Vectors* 2011, **4**:107
http://www.parasitesandvectors.com/content/4/1/107



RESEARCH Open Access

Insecticide resistance in malaria vector mosquitoes at four localities in Ghana, West Africa

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Abstract

Background: Malaria vector control programmes that rely on insecticide-based interventions such as indoor house spraying with residual insecticides or insecticide treated bed nets, need to base their decision-making process on sound baseline data. More and more commercial entities in Africa, such as mining companies, are realising the value to staff productivity of controlling malaria transmission in their areas of operation.
This paper presents baseline entomological data obtained during surveys conducted for four mining operations in Ghana, West Africa.

Results: The vast majority of the samples were identified as *Anopheles gambiae* S form with only a few M form specimens being identified from Tarkwa. *Plasmodium falciparum* infection rates ranged from 4.5 to 8.6% in *An. gambiae* and 1.81 to 8.06% in *An. funestus*. High survival rates on standard WHO bioassay tests were recorded for all insecticide classes except the organophosphates that showed reasonable mortality at all locations (i.e. > 90%). The West African *kdr* mutation was detected and showed high frequencies in all populations.

Conclusions: The data highlight the complexity of the situation prevailing in southern Ghana and the challenges facing the malaria vector control programmes in this region. Vector control programmes in Ghana need to carefully consider the resistance profiles of the local mosquito populations in order to base their resistance management strategies on sound scientific data.

Background

Malaria remains today the biggest killer of children in Africa [1] and it demands increased attention from control authorities in affected countries. Multi-national corporations operating in Africa recognize the burden malaria places on their staff and its impact on their commercial operations [2,3]. There is an increasing move by these multi-nationals to control malaria within the boundaries of their activities, and in many cases, extending this control to the surrounding communities [4,5].

The traditional methods of protecting work-forces using fogging, prophylaxis, repellents and handing out insecticide treated bed nets to workers (ITNs), have clearly not resulted in the desired outcome and companies are now implementing malaria control through the use of indoor residual house spraying [2,3]. Key to the success of these measures is knowledge of the local vector populations, the species identity, role in transmission and susceptibility to the four classes of insecticides approved by the World Health Organization for use in vector control. Baseline surveys to collect this information need to be carried out prior to implementation of malaria vector control interventions and on-going susceptibility surveys done to guard against the development of increasing insecticide resistance. This is becoming more important as insecticide resistance increases and spreads across Africa.

This paper reports on four such baseline surveys carried out at mining operations in Ghana, West Africa.

Materials and methods

Study sites

Ghana has a relatively high rainfall, situated between 5° and 12° North of the Equator with typical tropical temperatures. The vegetation at the sites sampled was dense degraded forest, mixed with agricultural land. Formal

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cultivation of cocoa and oil palm is interspersed with secondary forest and subsistence farming with cassava, maize, pineapples, etc. The following gold mining sites (Figure 1) were surveyed for a maximum of two weeks each between 2006-2010:

Obuasi: (6°11'36"N, 1°39'29"W), a town of approximately 150,000 people with local inhabitants living in and around the mining operations. Gold has been mined at Obuasi for over 100 years, much of it opencast mining. Originally owned by Ashanti Gold Mines, the mine is now operated by AngloGold/Ashanti who have instituted an extensive malaria control programme in the area for the past four years. The mosquitoes used in this survey were collected outside but adjacent to the Obuasi malaria vector control area in July 2010.

Tarkwa/Damang: (5°18'N, 1°59'32"W) Two new mines in this area operated by AngloGold/Ashanti and Randgold Resources were sampled in May 2007 and March 2009.

Ahafo: (7°1'33"N, 2°20'27"W), a Newmont Mines operation, was sampled in December 2006 and June 2008.

Akyem: (6°20'24"N, 1°0'28"W) also operated by Newmont Mines, was sampled in June 2006.

Mosquito collections

Local villages on the outskirts of the mining operations were visited and permission sought from the chief or local householders to search their houses for indoor resting mosquitoes. Houses were searched at random and those found to yield good numbers of mosquitoes were revisited repeatedly to ensure sufficient samples for the susceptibility tests. The collections were carried out in the mornings, usually up until midday except when mosquitoes were very scarce and collections had to be continued until mid-afternoon. All mosquitoes were collected by hand using an aspirator. Live mosquitoes were identified morphologically as belonging to either the *Anopheles gambiae* complex or the *An. funestus* subgroup and used in insecticide susceptibility tests. They were subsequently stored on dry silica gel for laboratory processing.

WHO insecticide susceptibility tests

The WHO protocol [6] was used for testing susceptibility to the four classes of insecticides approved for vector control (pyrethroids, carbamates, organophosphates and organochlorines) except that age and physiological status of the mosquitoes was unknown. Treated test papers with the WHO diagnostic dosages were supplied by the

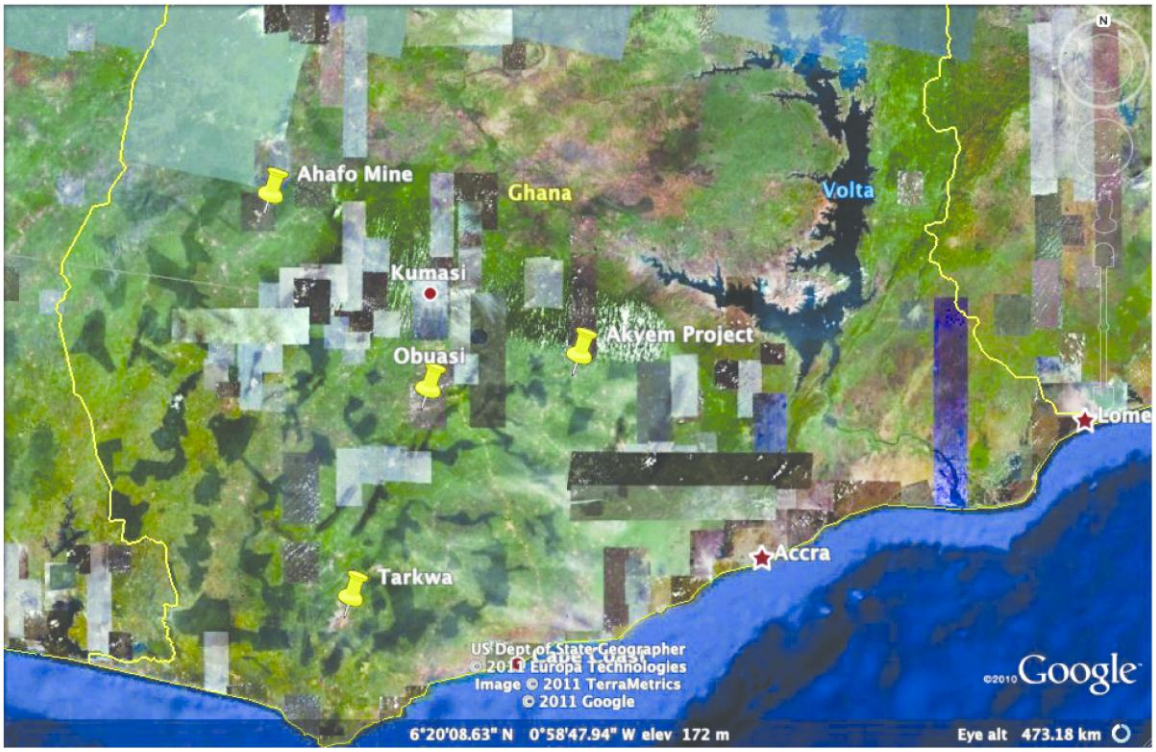


Figure 1 Google Earth map showing the localities of the four mining sites surveyed in this study.

WHO Collaborating Centre in Penang, Malaysia (Table 2). Cohorts of ~25 mosquitoes were exposed for 1 hour to the discriminating dose (except for fenitrothion which requires a 2-hour exposure [7]) and held for 24 hours before final mortality was recorded. Control mosquitoes were handled in the same way but not exposed to insecticides and were added to the following day's collections to maximise the numbers tested, particularly when population density was low. Treated papers used in the field were tested in the laboratory using known susceptible mosquitoes to check for efficacy. The dead and alive mosquitoes were stored separately for species identification. WHO criteria for susceptibility are: 98-100% mortality - susceptible; 80 - 97% mortality - resistance suspected and more investigations required; 0 - 79% mortality - resistance confirmed.

Laboratory analysis

Mosquitoes were processed for species identification using the standard protocols for the *An. gambiae* complex [8,9] and the *An. funestus* group [10]. DNA was sourced from legs and wings of individual mosquitoes, either directly by grinding in buffer or using the Collins *et al*. extraction method [11]. *Plasmodium falciparum* parasite detection was carried out on dissected heads and thoraces of wild females using the enzyme-linked immunosorbent assay (ELISA) of Wirtz *et al*. [12]. The target site *kdr* resistance mutations were assayed in mosquitoes exposed to pyrethroids (from the same DNA samples used for species identification) using the Taqman assay [13] for both East and West African mutations.

Results

The collection of indoor resting mosquitoes is very haphazard. Some houses in a village proved to be very productive with lots of mosquitoes present while other houses were not. Where treated bed nets in good condition were present, no mosquitoes were found in the bedrooms but on some occasions many mosquitoes were found resting inside the treated nets as well as in the adjacent living rooms, behind chairs or under tables, obviously avoiding the bedrooms due to the repellent effect of the treated bed nets [14].

Of the 1704 specimens of the *An. gambiae* complex (76.4% of 2229) that were successfully identified, all but one was *An. gambiae s.s*. (a single *An. arabiensis* was identified from Ahafo). A subsample of these (n = 862) was further characterised for the molecular forms and most were identified as the S molecular form (93.4%) (Table 1). The molecular M form was found in low numbers at Ahafo (6.8% of 220) and Tarkwa (8.3% of 459) while M/S hybrids (n = 4) were found only at Tarkwa. *Anopheles funestus s.s.* was the only member of the group identified from all localities (Table 1) except for a single specimen of *An. leesoni* being found at Tarkwa.

The results of the susceptibility tests for 2229 *An. gambiae s.l.* are given in Table 2. In all cases the control mortality was less than 5%, therefore not requiring adjustment with Abbott's formula [6]. Only the organophosphates showed good efficacy across all localities. High frequencies of resistance were recorded to pyrethroids, DDT and carbamates.

Table 1 Species identification and infection rates with *Plasmodium falciparum* circumsporozoite protein

	funestus	*arabiensis*	*gambiae s.s.*	S form	M form	M/S hybrids
Obuasi						
No. Identified	181	0	633	160	0	0
% infected	1.81* (4/221)	-	-	4.9* (13/267)	-	-
Tarkwa						
No. Identified	78	0	475	417	38	4
% infected	3.8 (3/78)	-	4.7 (17/365)	4.5 (15/333)	6.25 (2/32)	0
Ahafo						
No. Identified	34	1	461	205	15	0
% infected	2.9 (1/34)	-	8.3 (19/229)	8.6 (16/187)	15.4 (2/13)	-
Akyem						
No. Identified	100	0	134	23	0	0
% infected	8.06 (5/62)	-	5.03 (8/159)	-	-	-

*These infectivity rates for Obuasi are taken from Coetzee *et al*. [2] for comparison as no ELISAs were carried out in 2010.

Table 2 Results of exposure of wild *Anopheles gambiae* S form adults to insecticide treated papers using the WHO susceptibility test

Locality	Insecticide class	Treated papers	Length of exposure	N	% Mortality 24 h post-exposure
Obuasi	Pyrethroids	0.05% Deltamethrin	1 hour	64	89.1
		0.05% Lambda-cyhalothrin		60	76.7
		0.15% Cyfluthrin		62	66.1
		0.75% Permethrin		65	38.5
	Carbamates	0.1% Bendiocarb	1 hour	61	93.4
		0.1% Propoxur		59	89.8
	Organophosphates	1.0% Fenitrothion	2 hours	60	96.7
	Organochlorines	4.0% DDT	1 hour	60	31.7
Tarkwa	Pyrethroids	0.05% Deltamethrin	1 hour	152	56.6
	Carbamates	0.1% Bendiocarb	1 hour	113	74.3
	Organophosphates	5.0% Malathion	1 hour	210	98.6
	Organochlorines	4.0% DDT	1 hour	39	5.4
		4.0% Dieldrin		14	0
Akyem	Pyrethroids	0.05% Deltamethrin	1 hour	173	75.1
	Carbamates	0.1% Bendiocarb	1 hour	56	37.5
	Organophosphates	5.0% Malathion	1 hour	51	98.0
	Organochlorines	4.0% DDT	1 hour	53	5.7
		4.0% Dieldrin		56	0
Ahafo	Pyrethroids	0.05% Deltamethrin	1 hour	186	45.1
		0.05% Lambda-cyhalothrin		27	19.0
		0.75% Permethrin		41	15.0
	Carbamates	0.1% Bendiocarb	1 hour	178	77.6
	Organophosphates	5.0% Malathion	1 hour	171	95.4
		1.0% Fenitrothion	2 hours	29	93.1
	Organochlorines	4.0% DDT	1 hour	100	3.1
		4.0% Dieldrin		89	9.2

Each day's exposures were run with at least 20 control mosquitoes. All controls showed < 5% mortality negating the need for the use of Abbott's formula [6].

All the *An. funestus* tested were 100% susceptible to deltamethrin (n = 166) and malathion (n = 251) at all four localities. Samples from three localities were tested on bendiocarb and resistance was detected only at Obuasi (71.4% mortality, n = 56 [2]) but sample sizes were small at the other two sites (Tarkwa 53, Ahafo 3). DDT resistance was recorded at Obuasi (60.9% mortality, n = 23 [2]) but insufficient samples for testing this insecticide were collected at the other sites.

The *kdr* results are given in Table 3. As expected for *An. gambiae* S form in this area of West Africa, *kdr* frequencies of the West African mutation, Leucine to Phenylalanine (L1014F), were high. The East African mutation, Leucine to Serine (L1014S), was not found in any of the specimens. Only five specimens of *An. gambiae* M form were available (from Tarkwa) for *kdr* analysis and one was heterozygous for L1014F. The single M/S hybrid that survived exposure to deltamethrin was homozygous RR.

The *Plasmodium falciparum* infection rates are given in Table 1 with Obuasi rates taken from the published literature [2]. Since only a small number of the Akyem *An. gambiae s.s.* were further identified to S form, the infection rate is given for "*gambiae s.s.*". The highest infection rates were recorded at Ahafo with over 8% for the S form and for the pooled *"gambiae s.s."*. The rate

Table 3 Knockdown resistance (*Kdr*) mutations (L1014F) in wild *Anopheles gambiae*, exposed to pyrethroid insecticide treated papers, from four localities in Ghana.

Locality	Molecular form	Resistant Phenotype	RR	RS	SS	% *kdr* frequency
Obuasi	S	Resistant	11	1	0	95.8
		Susceptible	12	31	8	53.9
Ahafo	S	Resistant	20	0	0	100
Akyem	S	Resistant	17	18	3	68.4
		Susceptible	14	7	6	64.8
Tarkwa	S	Resistant	56	1	0	99.1
		Susceptible	10	0	0	100

Genotypes RR = homozygous resistant, RS = heterozygous resistant/susceptible and SS = homozygous susceptible.

of 15% for the M form is to be treated with caution given the small sample size (n = 13). Infection rates for *An. funestus* were much lower than *An. gambiae* at three of the four sites but higher for *An. funestus* at Akyem.

Discussion

The most common species encountered was *An. gambiae* S form, with very few M form and only one *An. arabiensis* identified. Since the surveys were carried out on an *ad hoc* basis and usually timed for the middle of the rainy season, it is possible that the species composition varies depending on the time of the year. *Anopheles funestus* appeared to play a secondary role as a vector except at Akyem. It has a scattered distribution and was found only at sites where suitable swampy breeding habitats were abundant.

All insecticide exposures were carried out on wild female mosquitoes of unknown age and physiological status. Since knowing the age of the samples is useful for early detection of developing resistance, when resistance is already established and as high as in the present study, the age effect [15] becomes less important. Furthermore, while age effect has been demonstrated for DDT resistance in *An. gambiae* [15] and permethrin resistance in *An. funestus* [16,17], it is not known whether the same applies to the organophosphates and carbamates for these species.

Mosquitoes of all physiological stages were collected, from unfed, to fully fed and gravid. The status of each individual was not recorded which means that susceptibility results give an overall view of resistance at each locality and cannot be broken down into physiological groups for comparison. Blood feeding is known to enhance the ability of female mosquitoes to survive exposure to some insecticides in some vector species (e.g. *An. funestus* [18]) but this may not be true for others (e.g. *An. gambiae* [19] except that this study did not expose freshly blood fed females but rather females that had been given a blood meal 6 days prior to exposure). The direct testing of wild females, as opposed to F-1 adults, is therefore pertinent for operational control even if it leads to an over-estimation of true (genetic) resistance in a population. Choice of insecticide for control interventions must be based on the number of mosquitoes that can survive exposure to any given insecticide regardless of age or physiological status.

Where very low mortality rates were recorded in the first round of testing, no further tests were conducted on that insecticide resulting in small sample sizes for some of the assays. In many cases mosquitoes were hard to come by and samples were used judiciously to provide maximum data on possible candidate insecticides for use in vector control by the various mines. As stated above, the surveys were conducted for only a short period at each locality, at times of the year that may not have been conducive to sampling other vector species such as *An. gambiae* M form, *An. arabiensis* and *An. funestus*. It is possible that these species can be found in greater numbers at other times of the year and that they may have different insecticide resistance profiles.

In general, the data confirm that resistance to one member of a class of insecticides is good evidence that resistance to other chemicals in the same class will occur. This is certainly the case with the pyrethroids tested here and is cause for concern. This insecticide group contains the only insecticides approved for use on bed nets. This valuable vector control strategy, where the person to be protected also serves as the bait to lure the mosquito into absorbing a lethal dose of insecticide, thus achieving the 'mass killing' effect [20], can no longer be relied upon. The diminishing effectiveness of this control tool comes at a time when international support for malaria control is significantly improving bed net coverage in Africa. During the study conducted at Ahafo in 2008 it was commonplace to find *An. gambiae* mosquitoes fully blood fed and resting inside long-lasting nets during the day. These observations were not quantified, but the study by N'Guessan *et al.* [21] in neighbouring Benin provides data for this phenomenon and highlights a very real problem facing control programmes in this region of West Africa.

It is probable that the increasing use of ITNs in the area is impacting on the levels of resistance to pyrethroids and DDT. In 2000, Kristan *et al.* [22] collected *An. gambiae s.s.* (no molecular identifications done) from the Tarkwa area and reported 100% 24-hr mortality on deltamethrin, 99.2% on permethrin and 94% after an 80 min exposure on DDT. This is in sharp contrast to the results presented here where 24-hr mortality was 56.6% on deltamethrin and only 5.4% on DDT from samples collected seven years later. It is obvious that this population has undergone considerable selection pressure and that resistance has increased exponentially over a very short period. On the other hand, the susceptibility results published for Obuasi in 2006 [2] show much lower mortality rates than the 2010 results given here for pyrethroids and carbamates. This may be due to the 2010 collections coming from rural villages outside of the mining area whereas the 2006 results were based on mosquitoes collected inside Obuasi town (before IRS was implemented) where inhabitants commonly use treated bed nets, mosquito coils and aerosols thereby increasing the selection pressure for resistance. The very low levels of mortality on 0.75% permethrin at Obuasi (38.5%) and Ahafo (15%) are of grave concern for the continued use of permethrin-treated bed nets.

Yawson *et al*. [23] report high frequencies of the Leu-Phe *kdr* mutation in Ghana populations with 100% recorded from Kumasi which is just north of Obuasi and in close proximity to Ahafo and Akyem (Figure 1). In the present study, of 63 specimens from Obuasi processed for *kdr*, 61.9% carried the Leu-Phe mutation, with 91.7% of the deltamethrin survivors being homozygous *kdr*. In Ahafo there was 100% *kdr* in 20 surviving individuals and in Akyem the overall frequency of *kdr* was 66.9%. Other recent studies in the vicinity of Accra also showed high *kdr* frequencies [24-26]. The almost 100% *kdr* mutations recorded from Tarkwa, including those samples that were susceptible to deltamethrin, indicates that a metabolic mechanism must play a role in conferring resistance to pyrethroids and that *kdr* on its own is not sufficient to confer resistance to an individual mosquito.

Working in northern Ghana near Navrongo, Anto *et al*. [27] reported worrying trends in *An. gambiae* and *An. funestus* survival on four pyrethroids and DDT. Despite their assertion that the vectors were all susceptible to these insecticides, in some instances survival was as high as 20%. In the limited number of specimens that were processed in the laboratory, no *kdr* mutations were found and all *An. gambiae* specimens were identified as the M molecular form. This suggests that a metabolic mechanism is responsible for the resistance observed in this region of Ghana as well.

Conclusions

The data presented here clearly indicate that insecticide resistance is widespread and often at very high frequencies, usually sufficiently high to preclude the use of several of the few insecticides approved by WHO for malaria control. Resistance management strategies are therefore critical for vector control programmes in this region.

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Authors' contributions

RHH and MC were responsible for the design of the projects, data analysis and drafting the manuscript. GF, SK, JS-O and RV were responsible for the field logistics, data collection and drafting of the manuscript. MK, KSC and LLK were responsible for the laboratory data collection and analysis and drafting of the manuscript. All authors read and approved the final version of the manuscript.

Competing interests

The authors declare that they have no competing interests.

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Staggered larval time-to-hatch and insecticide resistance in the major malaria vector *Anopheles gambiae* S form

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Abstract

Background: *Anopheles gambiae* is a major vector of malaria in the West African region. Resistance to multiple insecticides has been recorded in *An. gambiae* S form in the Ahafo region of Ghana. A laboratory population (GAH) established using wild material from this locality has enabled a mechanistic characterization of each resistance phenotype as well as an analysis of another adaptive characteristic - staggered larval time-to-hatch.

Methods: Individual egg batches obtained from wild caught females collected from Ghana and the Republic of the Congo were monitored for staggered larval time-to-hatch. In addition, early and late larval time-to-hatch sub-colonies were selected from GAH. These selected sub-colonies were cross-mated and their hybrid progeny were subsequently intercrossed and back-crossed to the parental strains. The insecticide susceptibilities of the GAH base colony and the time-to-hatch selected sub-colonies were quantified for four insecticide classes using insecticide bioassays. Resistance phenotypes were mechanistically characterized using insecticide-synergist bioassays and diagnostic molecular assays for known reduced target-site sensitivity mutations.

Results: *Anopheles gambiae* GAH showed varying levels of resistance to all insecticide classes. Metabolic detoxification and reduced target-site sensitivity mechanisms were implicated. Most wild-caught families showed staggered larval time-to-hatch. However, some families were either exclusively early hatching or late hatching. Most GAH larvae hatched early but many egg batches contained a proportion of late hatching larvae. Crosses between the time-to-hatch selected sub-colonies yielded ambiguous results that did not fit any hypothetical models based on single-locus Mendelian inheritance. There was significant variation in the expression of insecticide resistance between the time-to-hatch phenotypes.

Conclusions: An adaptive response to the presence of multiple insecticide classes necessarily involves the development of multiple resistance mechanisms whose effectiveness may be enhanced by intra-population variation in the expression of resistance phenotypes. The variation in the expression of insecticide resistance in association with selection for larval time-to-hatch may induce this kind of enhanced adaptive plasticity as a consequence of pleiotropy, whereby mosquitoes are able to complete their aquatic life stages in a variable breeding environment using staggered larval time-to-hatch, giving rise to an adult population with enhanced variation in the expression of insecticide resistance.

Background

Malaria is holoendemic in Ghana and is responsible for an estimated 22% of mortality in children under five, as well as 9% of maternal deaths [1], a situation that is mirrored in much of West Africa.

The major vectors of malaria in the West African region are *Anopheles gambiae*, *Anopheles funestus* and *Anopheles arabiensis* [2]. *Anopheles gambiae* is the nominal member of the *An. gambiae* complex and is subdivided into 5 informally named chromosomal forms [3] and two molecular forms, M and S [4]. Recent evidence suggests that the M molecular form can be further subdivided into two distinct breeding units [5]. Insecticide resistance has been detected in a large number of

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An. gambiae populations, particularly in the West African region [6-9]. The incidence of multiple resistances to insecticides is increasing and poses a threat to malaria vector control. This is because there are currently only four classes of insecticides available for use in malaria control and these collectively target only two insect neurological sites. In a mosquito survey carried out for the AngloGold Ashanti gold mine in Obuasi, Ghana, resistance to multiple insecticides was detected in *An. gambiae* and *An. funestus* [10].

Larval hatching in *An. gambiae* generally occurs 2-3 days after oviposition depending on environmental conditions [11]. However, there are descriptions of hatching occurring much later in a non-uniform way [12,13]. Yaro *et al* [12] determined the distribution of hatching time in different water types for *An. gambiae* M and S forms and showed that over 80% hatched within the first three days following oviposition regardless of water type. Between 5% and 16.8% of the total number of eggs hatched over the next four days while between 0.6% and 7.2% hatched after the first week. The S form produced significantly more hatchlings in the 3-7 day post oviposition period than the M form, and the M form showed significantly higher levels of hatching one week post-oviposition than the S form. It was also found that the type of water in which the eggs were kept had a highly significant effect on hatching with the distribution most distinct in puddle water. Yaro *et al* [12] concluded that larval time-to-hatch is determined by environmental conditions and that intraspecific variation in hatching time is an adaptation to survive variable conditions such as breeding site flooding and desiccation. They also suggest that eggs are not passive and that the time taken for an egg to hatch is probably dependent on water factors such as bacterial composition and oxygen content.

Here the adaptive significance of larval time-to-hatch is further assessed by investigating the possibility of a genetic component controlling time-to-hatch as well as the expression of insecticide resistance in association with time-to-hatch in *An. gambiae*.

Methods

Laboratory colony material

The GAH *An. gambiae* S form colony originating from Ghana and colonized in 2006 formed the basis of this study. SUA, an insecticide susceptible *An. gambiae* colony from Liberia, was used as the susceptible reference strain in insecticide bioassays. All mosquitoes were reared in the Botha De Meillon insectary at the National Institute for Communicable Diseases, NHLS, Johannesburg. Conditions were maintained at approximately 25°C with 75-85% relative humidity in a twelve-hour light: dark cycle with 30 min dusk and dawn transitions. Larvae were fed on ground dog biscuits and yeast and adults received three blood meals per week.

Wild caught material

Samples of *An. gambiae* were collected from Ahafo, Ghana (7°03.656N; 2° 24.190W), in June 2008; Damang (5°30.992N; 1°52.022W) and Tarkwa (5° 22.383N; 2° 01.017W), Ghana, in January 2009; Pointe Noire, Republic of the Congo (4°40'31S; 11°58'14E), in March 2009. The samples from Ghana were used for various evaluations in conjunction with laboratory-reared material as described below. The material from the Republic of the Congo served as a comparative *An. gambiae* sample from a different region.

Field collections

Mosquitoes were collected in Ghana and the Republic of the Congo from inside human dwellings using a torch and aspirator. Mosquitoes were placed in polystyrene cups and were provided with a 10% sugar solution prior to transportation.

Species identification

Wild-caught mosquito samples were transported to the NICD and were initially sorted using morphological keys [14,15]. Those identified as members of the *An. gambiae* complex were identified to species using the *An. gambiae* species-specific PCR assay [16]. All *An. gambiae sensu stricto* samples were further characterized as either M or S molecular form by PCR [17].

Larval early and late hatch selections

GAH colony mosquitoes were selected at the larval stage according to early and late hatch phenotypes. During initial selections larvae from the base colony were allowed to develop for approximately 10 days, at which point all fourth instar larvae were removed and placed into a new bowl labelled GAH Early Hatch. The second and third instar larvae were returned to the baseline colony. All first instar larvae at 10 days as well as remaining unhatched eggs were pooled as GAH Late Hatch. Larvae selected in this way were reared through to adults. This process was repeated using several egg batches until separate Early and Late Hatch adult sub-colonies had been produced. These were maintained and blood-fed according to the standard procedure. Eggs from each sub-colony were harvested and the time-to-hatch selection procedure was repeated. All larvae that hatched within four days of oviposition from the Early Hatch sub-colony were kept as GAH Early Hatch while the remaining eggs were transferred to the baseline colony. The Late Hatch sub-colony was selected by transferring all larvae that hatched within four days of oviposition to the baseline colony so that all larvae

that hatched subsequently remained as GAH Late Hatch. These selections continued for at least six generations before cross-mating experiments or insecticide susceptibility bioassays were conducted.

Cross-mating experiments

In order to determine whether there is a genetic component associated with larval time-to-hatch, cross-mating experiments between the Early and Late Hatch sub-colonies were set up. Early Hatch females were crossed with Late Hatch males and vice versa. The numbers of eggs produced from each cross were quantified. Eggs were monitored for hatching and the numbers of F1 hatchlings from each cross were recorded daily. F1 larvae from each cross were reared to adults and these were either back-crossed to the parental strains or were intercrossed. Eggs and F2 hatchlings from each back-cross or intercross were monitored daily as described above. Egg production and larval hatching in the baseline GAH colony was concurrently monitored as a control.

Determining hatch proportions of eggs from early and late hatch selected sub-colonies, cross-mating experiments and the baseline *An. gambiae* GAH colony

Eggs from each sub-colony, the baseline colony and the cross-mating experiments were collected and placed in marked egg bowls. The date of oviposition was recorded for each egg batch and the numbers of eggs quantified. Bowls were checked every morning and any larvae present were counted and removed. Hatchlings that emerged within four days of oviposition were classified as early hatch and those that emerged four days or more post-oviposition were classified as late hatch. Four days post oviposition was used as the cut-off point between early and late hatch as initial observations on the progeny of wild-caught mosquitoes showed that the majority of eggs hatch within four days while the rest tend to hatch in a staggered fashion for up to 22 days post oviposition.

Determining larval time-to-hatch distributions in families reared from wild-caught *An. gambiae* females from Ghana and the Republic of the Congo

Blood-fed, wild-caught *An. gambiae* females from Ghana and the Republic of the Congo were individually placed in vials lined with moist filter paper for oviposition. Cotton wool pads soaked in a 10% sugar solution were provided. Vials were monitored daily for eggs. All eggs produced were placed in egg bowls by family and were monitored daily for hatching. Hatchlings were counted and removed as described for the cross-mating and time-to-hatch selection experiments. Those families reared from wild-caught females from Ahafo, Ghana, formed the pilot study from which the four-day cut off point for classifying the early and late hatch phenotypes was determined. This cut off point closely approximates with information from Yaro *et al* [12].

Effect of egg density on hatch success and time-to-hatch in *An. gambiae*

The batches of eggs produced by each wild-caught female that was used in the hatch distribution analysis were also used to determine whether the number of eggs produced by a single female had an effect on the proportion of eggs that hatched, or on the proportion of eggs that hatched early. Data were grouped according to number of eggs laid and analysed using scatter-plots and linear regression in Statistix 7 (Analytical Software, Tallahassee, FL, USA).

The effect of water disturbance on egg hatching in *An. gambiae* GAH

An experiment to test the effect of disturbance on egg hatching from the time-to-hatch selected sub-colonies and the baseline GAH colony was performed. Three batches (replicates) of eggs from each group were obtained and divided into two cohorts per batch for different treatments. One cohort was rinsed into a larval bowl that contained a floating plastic ring, but the eggs were not specifically contained within this ring and so could be stranded on the sides of the bowl due to water evaporation. These eggs were sprayed with water (disturbed) daily. The other cohort was also rinsed into a larval bowl so that all the eggs were contained within the floating plastic ring. This ring prevented the eggs from being stranded on the sides of the larval bowl and thus they did not require any disturbance to keep them in contact with the water. The disturbed and undisturbed egg batches were monitored daily for hatchlings, which were counted and removed. Each set of experiments lasted 25 days after which all eggs were disturbed to determine if any unhatched eggs would hatch, and later discarded. The total number of eggs that hatched as well as the proportions of early and late hatching larvae per group was determined and analysed using two-sample t-tests or ANOVA (Statistix 7).

Insecticide susceptibility assays

In order to test for associations between insecticide resistance phenotypes and larval time-to-hatch, adult insecticide susceptibility assays were performed on samples drawn from the *An. gambiae* GAH colony as well as from the larval time-to-hatch sub-colonies. Susceptibility to four classes of insecticide was assessed (see Table 1) according to the standard WHO procedure [18]. Approximately 125 adult female mosquitoes between the ages of two and five days, divided into five

Table 1 Insecticides used for adult insecticide susceptibility tests against the Anopheles gambiae laboratory colony GAH as well the larval time-to-hatch selected sub-colonies.

Insecticide class	Insecticides used (concentration)
organochlorines	dieldrin (4%); DDT (4%)
organophosphates	malathion (5%); fenitrothion (1%); pirimiphos methyl (0.9%)
carbamates	bendiocarb (0.1%); propoxur (0.1%)
pyrethroids	permethrin (0.75%); deltamethrin (0.05%)

Insecticides are listed by class with diagnostic concentrations in parentheses.

replicates of 25 mosquitoes each, were exposed to diagnostic concentrations of each insecticide (Table 1) for one hour (except for fenitrothion which has an exposure period of two hours) using WHO test kits and insecticide treated filter papers. Knockdown was recorded at the end of the exposure period after which all mosquitoes were transferred to holding tubes for 24 hours during which they were provided with a 10% sugar solution. Mean percentage mortalities 24 h post exposure were recorded. Controls included exposures to untreated filter paper and treated paper efficacy was confirmed by exposing samples drawn from the insecticide susceptible reference *An. gambiae* strain, SUA, to the insecticide treated papers. Data were analysed by performing two sample t-tests using Statistix 7 (Analytical Software, Tallahassee, FL, USA).

Synergist bioassays

A set of enzyme synergists was used to test for associations between enzyme activity and the expression of insecticide resistance in the *An. gambiae* GAH laboratory colony and the time-to-hatch selected sub-colonies. Enzyme synergists can be employed in this manner because they are recognized as substrates by those enzyme systems implicated in insecticide detoxification [19].

The synergists used were 20% diethyl maleate (DEM), 10% triphenylphosphate (TPP) and 4% piperonyl butoxide (PBO). These generally synergize glutathione S-transferase (GST), esterase and monooxygenase enzyme activity respectively. However, the specificity of each synergist is likely to be affected by the metabolic pathways associated with resistance and cross-reaction can occur. Approximately 25 adult female mosquitoes, 2 to 5 days old, were exposed to synergist for one hour immediately followed by exposure to a diagnostic concentration of insecticide for 1 h (2 h for fenitrothion). A similar sample was concurrently exposed to insecticide only for 1 h. Controls included concurrent exposure to synergist only as well as exposure to untreated paper. Mortalities were recorded 24 h post exposure. At least five replicates per synergist for each insecticide were performed. Mosquitoes that survived synergist bioassays, as well as those that died following insecticide exposure only, were collected and stored on silica before undergoing molecular screening for target site mutations associated with insecticide resistance. Final mortalities from the synergized samples were compared to their corresponding unsynergized samples using two sample t-tests (Statistix 7).

Screening for reduced target site sensitivity mutations

DNA was extracted from samples to be used in molecular assays according to the method of Collins *et al* [20] or using a ZyGEM PrepGEM™ insect (ZyGEM Corp Ltd.) extraction kit following the supplied protocol. Individual mosquitoes were screened for *kdr*, *ace-1*^{R} and *Rdl* genotypes. Data were analysed using Fisher's Exact test, χ^2, or two sample t-tests (Statistix7).

Hydrolysis probe molecular assays

The *kdr* and *Rdl* TaqMan® hydrolysis probe molecular assays [21,22] were used to detect *kdr* (L1014F and L1014S) and *Rdl* mutations associated with resistance to pyrethroids and DDT (*kdr*) and resistance to dieldrin (*Rdl*). All primers used in the real time experiments were supplied by Inqaba Biotechnical Industries, Hatfield, Pretoria, and probes were supplied by Applied Biosystems Inc, Forster City, CA, USA. *Kdr* and *Rdl* homozygous (RR and SS) and heterozygous (RS) positive controls as well as no template controls were included in all assays.

Restriction fragment length polymorphism assay to detect the *ace-1*^{R} mutation

The *ace-1*^{R} mutation in acetylcholinesterase 1 has previously been associated with organophosphate and carbamate resistance in *An. gambiae* [23]. DNA was extracted from samples that survived exposure to 0.1% bendiocarb as well as from samples that died following exposure. The *Alu*1 restriction enzyme (Roche Diagnostics, Basel, Switzerland) digest was used to genotype samples for *ace-1*^{R} [23].

Results

Species identification of wild-caught and colony samples

All wild-caught mosquitoes used in the time-to-hatch experiments were identified as *An. gambiae s.s.* Of the Ghana Ahafo families, two (Gahf 33 and 49) were M form whilst the remaining 26 were S form. All Republic of the Congo (COGS, N = 18) and Ghana Damang and Tarkwa (Ghag, N = 5) families were S form. The GAH laboratory colony was confirmed as S form.

Larval time-to-hatch distributions of wild-caught families

Most Ghana Ahafo families (Gahf) showed bimodal hatching. Only one family (Gahf 41) was entirely early hatching (3.6%) and ten families (Gahf 1, 9, 15, 19, 22,

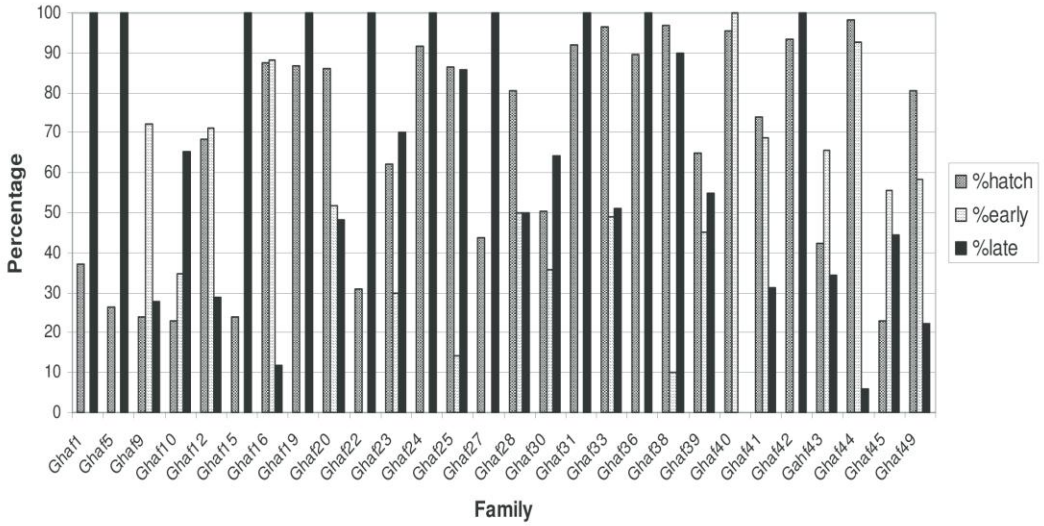


Figure 1 ***Anopheles gambiae*** **families from Ahafo, Ghana**. Overall larval hatch rate per family (percentage of eggs that hatched) and the proportions of larvae that hatched either early or late using three days post oviposition as the cut-off between time-to-hatch phenotypes are shown.

24, 27, 31, 35, 42) were entirely late hatching (35.7%) (Figure 1). In this experiment, three days post oviposition was used as the cut-off between the early and late hatch phenotypes. The cut-off point was shifted to four days for all subsequent experiments as the majority of the larvae hatched within this period following which hatch proportions dropped off significantly.

Most families reared from wild-caught females from the Republic of the Congo (COGS) showed bimodal hatching. Four (22.22%) were entirely early hatching and none were entirely late hatching using four days post oviposition as the cut-off point between time-to-hatch phenotypes (Figure 2).

All Ghana Damang and Tarkwa (Ghag) families showed bimodal hatching. The proportions of late hatching larvae per family (Figure 3) were generally higher than those observed in the COGS families.

Larval time-to-hatch proportions in laboratory colonies and progeny of cross-mating experiments

The overall proportions of F1 and F2 progeny that hatched from the crosses, back-crosses and intercross as well as progeny from the unselected GAH colony and time-to-hatch selected sub-colonies did not vary significantly (ANOVA, $P > 0.05$). Between 400 and 3000 eggs per cross or selected sub-colony were monitored in total. The latest hatch occurred 22 days post oviposition, but in general hatching after 9 days was uncommon. The vast majority of larvae hatched early across all batches (Figure 4). The highest proportions of late hatching larvae were recorded in the Late Hatch selected sub-colony and in the F2 progeny of both back-crosses to the parental sub-colonies. These late hatching proportions were numerically higher than those recorded in the GAH laboratory colony, the Early Hatch selected sub-colony, the F1 hybrid progeny of Early Hatch crossed with Late Hatch and F2 progeny of the hybrid intercross. However, these differences were not significant for untransformed data (ANOVA: $P > 0.1$), and were only significant at lower confidence for arcsine-transformed data ($P = 0.08$). In summary, these data do not correlate with any hypothetical models based on Mendelian inheritance of a single genetic factor controlling larval time-to-hatch, but suggest that larval time-to-hatch phenotypes can be selected for (Figure 4).

Effect of egg density on hatching

Linear regressions were performed on each group of wild-caught families (Gahf, Ghag and COGS) to test for an association between the number of eggs laid per female and the proportion of eggs that hatched, or the number of eggs laid per female and the proportion of eggs that hatched early. No significant trends were detected ($P > 0.05$) suggesting that the number of eggs laid by a female does not influence the proportion that hatch or the proportions of eggs that hatch early.

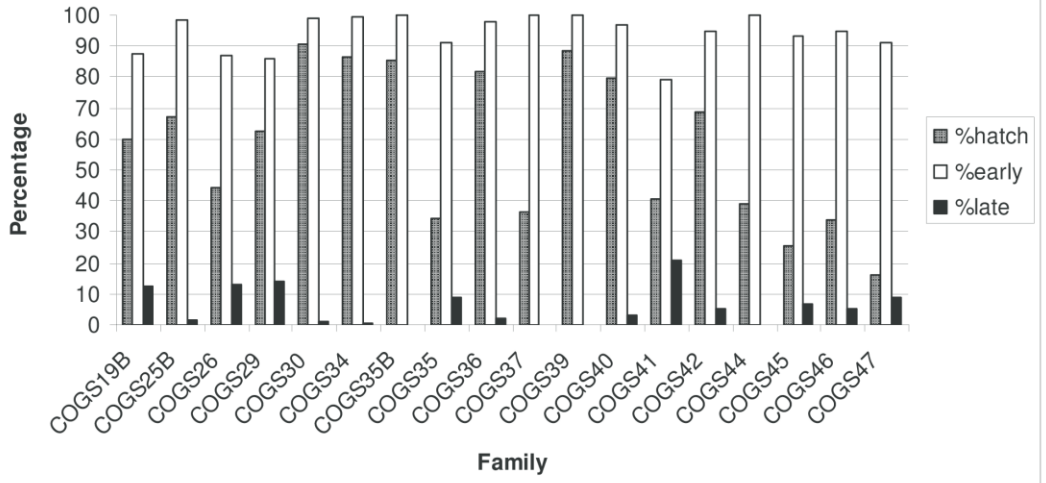


Figure 2 *Anopheles gambiae* families from Pointe Noire, Republic of Congo. The overall larval hatch rate per family (percentage of eggs that hatched) and the proportions of larvae that hatched either early or late using four days post oviposition as the cut-off between time-to-hatch phenotypes are shown.

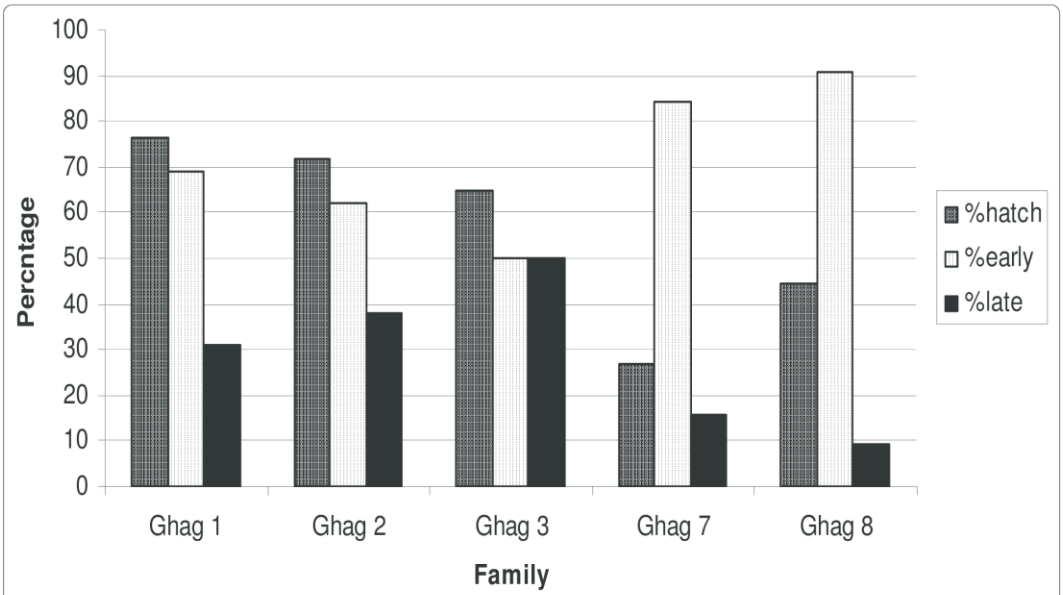


Figure 3 *Anopheles gambiae* families from Damang and Tarkwa, Ghana. Overall larval hatch rate per family (percentage of eggs that hatched) and the proportions of larvae that hatched either early or late using four days post oviposition as the cut-off between time-to-hatch phenotypes are shown.

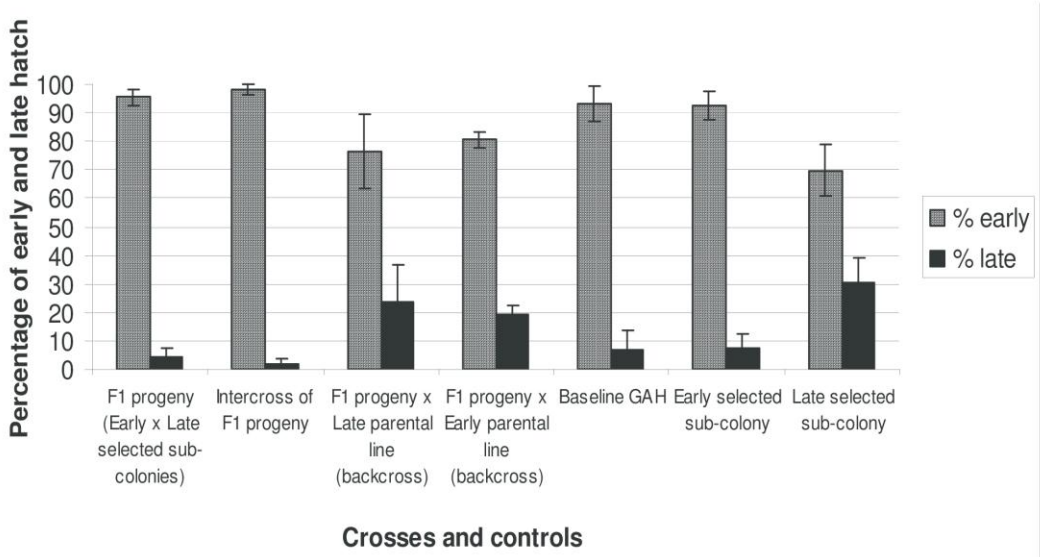


Figure 4 Proportions of early and late hatching *Anopheles gambiae* larvae per cross, GAH laboratory colony (baseline) and time-to-hatch selected (Early and Late) sub-colonies.

Effect of water disturbance on egg hatching

The total numbers of eggs monitored ranged from 1,600 to 3,200 per experiment, divided between three replicates per experiment. Significantly fewer eggs from the undisturbed experiment hatched compared to the disturbed experiment, indicating that egg disturbance is necessary for optimal hatching (two sample t-tests: $P < 0.01$ for GAH baseline; $P = 0.01$ for the Early Hatch sub-colony; $P < 0.01$ for the Late Hatch sub-colony) (Table 2). In addition, there was a significant difference in the total percentage of eggs that hatched in the undisturbed experiment between the Early and Late Hatch selected sub-colonies, and between the Early Hatch selected sub-colony and the baseline colony (two sample t-tests: $P < 0.01$ and $P = 0.01$, respectively) with significantly higher proportions of hatching occurring in the Early Hatch sub-colony. No significant differences in total hatch were observed between groups for the disturbed experiment. There were significantly higher proportions of late hatching larvae in the Late Hatch selected sub-colony compared to the Early Hatch selected sub-colony and vice versa regardless of whether the eggs were disturbed or not (two-by-two contingency tables: $P < 0.05$ in all cases) (Table 2), showing significant assortment of hatching phenotype with time-to-hatch sub-colony selection.

Table 2 Mean egg hatch proportions as well as late and early hatch proportions in unselected and time-to-hatch selected sub-colonies of Anopheles gambiae GAH.

Colony/sub-colony	Hatch rate/time-to-hatch proportion	Disturbed (%)	Undisturbed (%)
GAH base	Mean hatch	78	20.4
	Proportion late	20.15	29.87
	Proportion early	79.85	70.13
Early Hatch	Mean hatch	75	47.23
	Proportion late	5	25.4
	Proportion early	95	74.6
Late Hatch	Mean hatch	90.2	11.4
	Proportion late	19.9	37.8
	Proportion early	80.1	62.2

Data are given as mean percentages and are sorted according to whether or not eggs were disturbed prior to hatching.

Insecticide susceptibility assays

Based on WHO criteria [18], *An. gambiae* GAH showed various levels of resistance to permethrin and deltamethrin (pyrethroids), bendiocarb and propoxur (carbamates), DDT and dieldrin (organochlorines) and pirimiphos methyl (organophosphate). Resistance to the organophosphates fenitrothion and malathion is also suggested (Figure 5).

The larval time-to-hatch selected sub-colonies differed significantly from each other through all insecticide resistance phenotypes with the exception of the pyrethroid deltamethrin against which neither sub-colony showed resistance (Figure 6). The Early Hatch selected sub-colony showed significantly higher levels of resistance to the carbamates bendiocarb and propoxur as well as to the organophosphate pirimiphos methyl than the Late Hatch selected sub-colony (two sample t-tests: $P < 0.01$ in all cases). This trend was reversed in response to exposure to DDT, dieldrin and permethrin, with the Late Hatch selected sub-colony showing significantly higher levels of resistance (two sample t-tests: $P < 0.01$ in all cases).

Synergist bioassays

Pyrethroid resistance was significantly synergized by PBO and TPP in *An. gambiae* GAH. These data implicate esterases and P450 monooxygenases in pyrethroid metabolism in Ghanaian *An. gambiae*. No other insecticide resistance phenotypes reduced in expression following exposure to synergists (Table 3).

Pyrethroid resistance was significantly synergized by PBO in the Late Hatch selected sub-colony (two sample t-test: $P = 0.02$). This effect was not detected in the Early Hatch selected sub-colony as resistance to pyrethroids had diminished by this generation with over 90% of the Early Hatch selected mosquitoes not surviving permethrin exposure. TPP and PBO induced no significant reductions in insecticide resistance expression in the time-to-hatch selected sub-colonies when challenged against the other insecticides listed in table 3. DEM was not assayed against the time-to-hatch selected sub-colonies as it induced no effect on insecticide resistance in the GAH baseline colony.

Screening for reduced target site sensitivity mutations *kdr*

Samples genotyped for the L1014F *kdr* mutation were drawn from the GAH laboratory colony and the larval time-to-hatch selected sub-colonies. Sample sizes ranged from 18 to 30. All mosquitoes assayed were characterized by insecticide exposure bioassay as either insecticide resistant or susceptible. Some were further characterized by their response to insecticide exposure following pre-exposure to PBO. There was significant variation in *kdr* allele frequency between resistant and susceptible samples (Figure 7) whereby clear associations between *kdr* and permethrin resistance (GAH baseline $\chi^2 = 18.47$, $P < 0.01$; Late Hatch $\chi^2 = 35.07$, $P < 0.01$; Early Hatch $\chi^2 = 11.41$, $P < 0.01$) as well as *kdr* and DDT resistance ($\chi^2 = 43.43$, $P < 0.01$) were detected.

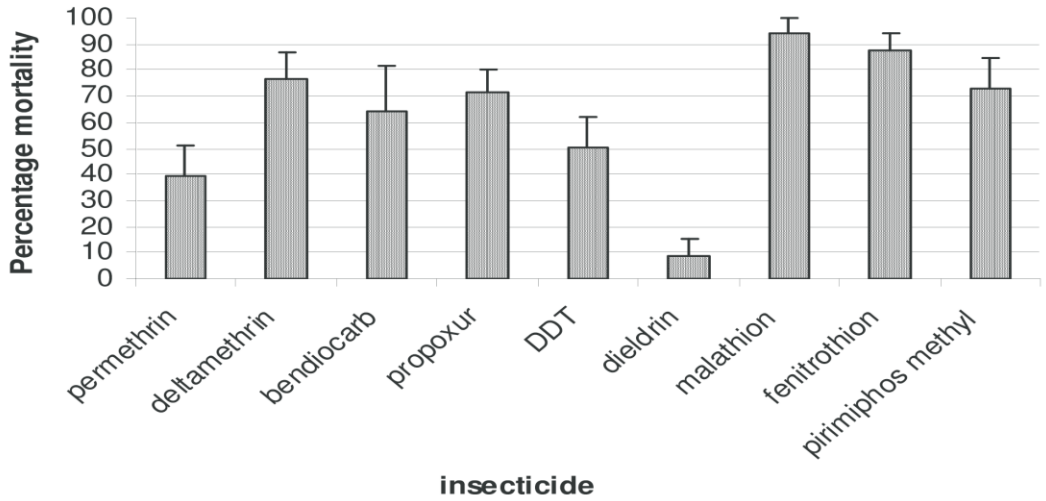


Figure 5 Mean percentage mortalities of 2-5 day old *Anopheles gambiae* laboratory colony (GAH) females 24 h post exposure to listed insecticides. Assays were based on the standard WHO bioassay method for testing adult susceptibility to insecticides (WHO, 1998).

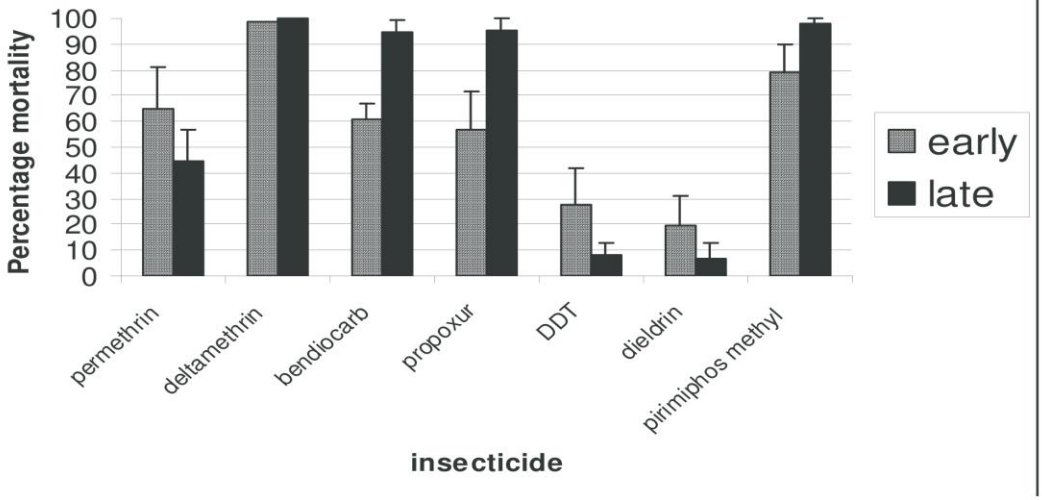


Figure 6 Mean percentage mortalities of 2-5 day old *Anopheles gambiae* time-to-hatch selected sub-colony (GAH) females 24 h post exposure to listed insecticides. Assays were based on the standard WHO bioassay method for testing adult susceptibility to insecticides (WHO, 1998).

Table 3 Mean percentage mortalities of PBO, TPP or DEM synergized and unsynergized samples of 2-5 day old Anopheles gambiae laboratory colony (GAH) females 24 h post exposure to listed insecticides (syn = synergized; unsyn = unsynergized. Bolded p-values are significant at 95% confidence intervals).

Synergist		4% PBO		10% TPP		20% DEM	
Insecticide	Syn/unsyn	mean % mortality	t-test p	mean % mortality	t-test p	mean % mortality	t-test p
4% DDT	syn	27.46	0.99	39.6	0.76	38.37	0.86
	unsyn	27.48		41.8		37.38	
4% dieldrin	syn	11.70	0.81	13.8	0.13	12.15	0.89
	unsyn	10.83		9.9		12.65	
permethrin	syn	80.54	**< 0.01**	86.14	**< 0.01**	74.93	0.16
	unsyn	48.72		56.11		61.78	
deltamethrin	syn	95.32	**< 0.01**	94.12	0.98	100	~1
	unsyn	72.52		94.60		98	
propoxur	syn	70.30	0.87	84.98	0.06	79.43	0.52
	unsyn	71.03		79.5		82.23	
bendiocarb	syn	77.74		78.8	0.16	68.99	0.66
	unsyn	71.56	0.29	71.36		72.43	
pirimiphos methyl	syn	33.39	0.37	86.65	0.08	74.65	0.09
	unsyn	44.89		67.56		63.53	
bendiocarb	syn	77.74		78.8	0.16	68.99	0.66
	unsyn	71.56	0.29	71.36		72.43	
pirimiphos methyl	syn	33.39	0.37	86.65	0.08	74.65	0.09
	unsyn	44.89		67.56		63.53	

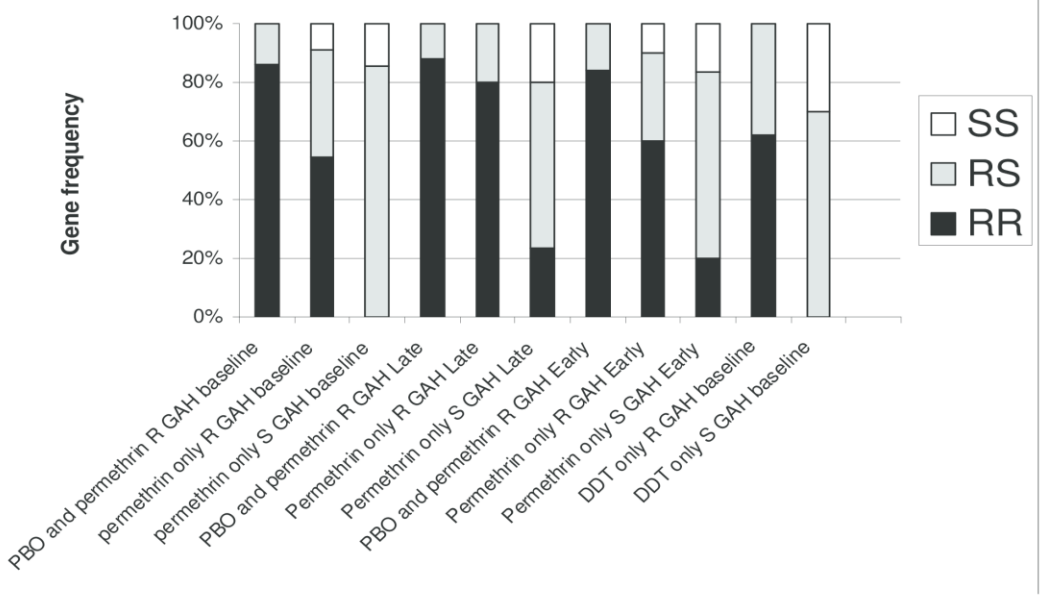


Figure 7 Proportions of L1014F *kdr* genotypes sorted by response to insecticide exposure phenotype (resistant or susceptible). All samples were comprised of female *Anopheles gambiae* laboratory reared mosquitoes from either the GAH baseline colony or the time-to-hatch selected sub-colonies exposed to permethrin only, permethrin and PBO, or DDT only. R = resistant, S = susceptible.

However, several homozygous resistant RR genotypes were recorded in the permethrin susceptible samples and a small proportion of homozygous susceptible SS genotypes were recorded in the permethrin resistant samples. Pre-exposure to PBO significantly increased the *kdr* frequency amongst permethrin resistant samples in the GAH baseline colony (χ^2 = 15.01, P < 0.01) and the GAH Early Hatch sub-colony (χ^2 = 10.49, P < 0.01) but not in the GAH Late Hatch sub-colony (χ^2 = 0.24, P > 0.05).

The hydrolysis probe molecular assay [21] was also used to genotype the L1014S *kdr* mutation in 82 permethrin and DDT exposed GAH females. All were genotyped as homozygous susceptible SS.

Rdl

A sample of *An. gambiae* GAH females were characterized as either dieldrin resistant or susceptible following exposure to 4% dieldrin. The alanine296-glycine (*Rdl*) GABA receptor mutation was detected by hydrolysis probe assay [22] and there was a clear association between *Rdl* genotype and response to dieldrin phenotype in both the time-to-hatch selected sub-colonies (Early Hatch χ^2 = 67.89, P < 0.01; Late Hatch χ^2 = 108, P < 0.01) as well as the base colony (χ^2 = 52.06, P < 0.01) (Table 4). In addition, GAH Late survivors showed significantly higher frequencies of the *Rdl* mutation than GAH Early survivors (χ^2 = 5.21, P < 0.05). Exposure to PBO prior to dieldrin exposure did not increase mortality in GAH (two sample t test: P = 0.81) (Table 3), as was previously observed in another *An. gambiae* colony [24], suggesting that P450 monooxygenases do not play a significant role in resistance to dieldrin in GAH.

ace-1^R

Twenty-eight bendiocarb exposed *An. gambiae* GAH female survivors and 27 bendiocarb exposed females that died following exposure were screened for the *ace-1^R* mutation using the RFLP assay [18]. Three of the 28 survivors were scored as homozygous resistant and the remaining 25 survivors were scored as heterozygous. All 27 mosquitoes that died following exposure to bendiocarb were scored as homozygous susceptible. These data show a strong association between *ace-1^R* genotype and response to bendiocarb exposure phenotype (χ^2 = 180; P < 0.01). The apparent scarcity of the RR (homozygous resistant) genotype may be attributable to an unusually high death rate of RR individuals at pupation as previously described [25].

Discussion

Resistance to multiple classes of insecticides in *An. gambiae* populations is a growing concern for malaria vector

Table 4 Numbers and proportions of alanine296-glycine (Rdl) Anopheles gambiae GAH female genotypes sorted according to response to dieldrin exposure phenotype (resistant or susceptible).

	GAH base Susceptible	GAH base Resistant	GAH Late Susceptible	GAH Late Resistant	GAH Early Susceptible	GAH Early Resistant
Genotype	Frequency	Frequency	Frequency	Frequency	Frequency	Frequency
SS	23/25 = 92%	0	23/25 = 92%	0	24/27 = 89%	0
RS	2/25 = 8%	16/29 = 55.17%	2/25 = 8%	12/25 = 48%	3/27 = 11%	17/22 = 77%
RR	0	13/29 = 44.83%	0	13/25 = 52%	0	5/22 = 23%

S = dieldrin susceptible *Rdl*$^{+}$, R = dieldrin resistant *Rdl*.

control [6]. The *An. gambiae* laboratory colony GAH shows resistance to all classes of insecticide currently available for use in public health. Although this colony is likely to show reduced genetic variation relative to the wild population from which it is derived, multiple insecticide resistance in *An. gambiae* has previously been reported from Ghana [10].

From the data presented here, mechanisms of resistance to pyrethroids in *An. gambiae* GAH include monooxygenase and esterase mediated detoxification coupled with the L1014F *kdr* mutation. The correlation between *kdr* genotype and pyrethroid resistance phenotype was clear but not absolute, with small numbers of homozygous resistant (RR) genotypes occurring in the phenotypically susceptible samples and vice versa. These discrepancies, coupled with the occurrence of RS heterozygotes in resistant and susceptible mosquitoes as well as an increase in *kdr* frequency in PBO synergized samples, suggest that enzyme mediated detoxification also plays an important role in the production of a measurable pyrethroid resistance phenotype [26,27]. Resistance to DDT associated particularly closely with the assortment of L1014F *kdr*. Resistance to dieldrin associated closely with the *Rdl* mutation while resistance to the carbamate bendiocarb associated closely with the *ace-1*^{R} mutation. Carbamate resistance and the low level of organophosphate resistance recorded in the *An. gambiae* GAH colony is therefore likely mediated by the assortment of *ace-1*^{R} although enzyme mediated detoxification may play a supporting role. High frequencies of *ace-1*^{R} have been recorded in *An. gambiae* S form populations in Burkina Faso indicating that caution should be employed when using carbamate and organophosphate insecticides for vector control programs in the West African region [28].

The majority of *An. gambiae* GAH eggs hatched within four days after oviposition. However, the proportion of late hatching eggs increased following selection for this phenotype, suggesting that, in addition to environmental factors [12], there may be a genetic component controlling larval time-to-hatch. Cross-mating between the time-to-hatch phenotypes, supported by hybrid inter-crosses and back-crossing of hybrids to the parental strains, gave ambiguous results suggesting that this genetic component may be multi-factorial. A genetic influence on variation in larval time-to-hatch is further supported by the observation that some of the wild-caught *An. gambiae* families from Ghana and the Republic of the Congo were exclusively early or late hatching. Importantly, there was also significant variation in the expression of insecticide resistance between the time-to-hatch phenotypes. The variation recorded in the response to insecticide exposure assays also correlates with variation in *kdr* and *Rdl* frequencies between the time-to-hatch selected sub-colonies, showing that selection for time-to-hatch inadvertently affected the frequencies of those factors controlling insecticide resistance.

Although the adaptive significance of staggered larval time-to-hatch post oviposition has not been quantified, it is highly likely that this characteristic has evolved in response to the variable nature of preferred *An. gambiae* breeding sites [12]. These sites are typically small, are highly variable in terms of water quality, are subject to large fluctuations in temperature and are susceptible to desiccation and flooding. That variation in larval time-to-hatch is ubiquitous across *An. gambiae* populations is supported by the quantification of this characteristic in geographically diverse samples from the Republic of the Congo and Ghana.

These data further show that the number of eggs that hatch in total as well as the proportions of eggs that hatch either early or late do not depend on the initial number of eggs laid per *An. gambiae* female. This suggests that delayed larval hatch is not an adaptation to avoid over-crowding or competition for resources. Additionally, water disturbance induces a significantly higher rate of egg hatching in *An. gambiae*.

Conclusions

The presence of insecticides in fluctuating concentrations significantly alters the chemical environment in which mosquitoes breed and rest. A sufficient adaptive response to the presence of multiple insecticide classes necessarily involves the development of multiple resistance mechanisms whose effectiveness may be enhanced

by intra-population variation in the expression of resistant phenotypes. The variation in the expression of insecticide resistance in association with selection for larval time-to-hatch described here may induce this kind of enhanced adaptive plasticity as a consequence of pleiotropy. In this scheme cohorts of mosquitoes are able to complete their aquatic life stages in a variable breeding environment using staggered larval time-to-hatch, giving rise to an adult population with enhanced variation in the expression of insecticide resistance. Enhanced variation inadvertently produced in this way offers a wider platform for the continued development of resistance to insecticides.

Successfully managing multiple insecticide resistance in malaria vector control should involve an appraisal of the biological and adaptive variation inherent within target vector populations. Effective malaria control can then be achieved by adopting an evidence-based approach, which incorporates the principles of judicious insecticide use in a broader Integrated Vector Management (IVM) system.

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Authors' contributions
MLK conducted all the experiments, analysed the results and drafted the manuscript; LLK assisted in the design and analysis of the molecular experiments and drafting of the manuscript; MC and RHH assisted with the design of the project and the drafting of the manuscript; BDB designed the project, assisted with data interpretation and statistical analysis and completed the drafting of the manuscript. All field collections were conducted under guidance of RHH. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

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Appendix 6: Kaiser *et al*., 2014. Embryonic Development and Rates of Metabolic Activity in Early and Late Hatching Eggs of the Major Malaria Vector *Anopheles gambiae*

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RESEARCH ARTICLE

Embryonic Development and Rates of Metabolic Activity in Early and Late Hatching Eggs of the Major Malaria Vector *Anopheles gambiae*

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Abstract

Anopheles gambiae eggs generally hatch at the completion of embryo development; two-three days post oviposition. However, staggered or delayed hatching has been observed whereby a single batch of eggs shows marked variation in time-to-hatch, with some eggs hatching 18 days post oviposition or later. The mechanism enabling delayed hatch has not been clearly elucidated but is likely mediated by environmental and genetic factors that either induce diapause or slow embryo development. This study aimed to compare metabolic activity and embryonic development between eggs collected from sub-colonies of the baseline *Anopheles gambiae* GAH colony previously selected for early or late time-to-hatch. Egg batches from early and late hatch sub-colonies as well as from the baseline colony were monitored for hatching. For both time-to-hatch selected sub-colonies and the baseline colony the majority of eggs hatched on day two post oviposition. Nevertheless, eggs produced by the late hatch sub-colony showed a significantly longer mean time to hatch than those produced by the early hatch sub-colony. The overall proportions that hatched were similar for all egg batches. CO_2 output between eggs from early and late hatch sub-colonies showed significant differences only at 3 and 7 days post oviposition where eggs from the early hatch and the late hatch sub-colony were more metabolically active, respectively. No qualitative differences were observed in embryo development between the sub-colonies. It is concluded that all viable embryos develop to maturity at the same rate and that a small proportion then enter a state of diapause enabling them to hatch later. As it has previously been shown that it is possible to at least partially select for late hatch, this characteristic is likely to involve genetic as well as environmental

factors. Delayed hatching in *An. gambiae* is likely an adaptation to maximise reproductive output despite the increased risk of desiccation in an unstable aquatic environment.

Introduction

Anopheles gambiae sensu stricto is the nominal member of the *Anopheles gambiae* species complex. There are currently eight recognized members in this complex [1] of which three: *An. coluzzii* Coetzee and Wilkerson sp.n (previously *An. gambiae* M form), *An. gambiae* Giles (previously *An. gambiae* S form) and *An. arabiensis* Patton, are major African malaria vectors [2, 3].

In *An. gambiae*, intra-species adaptive plasticity that enhances population survival in variable or unpredictable environments also inadvertently maintains malaria parasite transmission. Some of those adaptive traits that likely affect vector capacity include larval development time, adult body size and adult longevity. These are postulated to be under the control of genetic and environmental factors in approximately equal measure in *An. gambiae* under laboratory conditions, although environmental effects are likely of greater importance under natural conditions [4].

An often ignored trait that may affect total development time to adulthood as well as the quality of individuals produced in terms of overall fitness is time-to-hatch. *Anopheles gambiae* are monoandrous and in nature 97% of females mate only once [5]. Oviposition occurs once a female has consumed enough blood to mature her eggs and has selected a suitable oviposition site. The selection of oviposition sites by anopheline females is influenced by factors such as water type and land cover type, and the larval site selected also affects egg hatchability [6, 7]. Larval habitats typically selected by *Anopheles gambiae* tend to be small, temporary water bodies such as water-filled tyre tracks and hoof prints [8]. They can thus be described as unstable because they are prone to periodic desiccation and flooding.

As eggs are fertilized during oviposition by sperm stored in the female's spermatheca after mating, a single batch of eggs oviposited in one sitting is fertilized at approximately the same time and development of the embryo commences once the eggs are oviposited [9]. Eggs normally hatch once embryogenesis is complete which is within two to three days post oviposition in optimal conditions in *An. gambiae*. However, some eggs are able to hatch two weeks or more after oviposition [10, 11] increasing their risk of desiccation even though anopheline eggs normally do not cope well with desiccation. Hatching in mosquito eggs has been shown to be affected by several other factors including temperature [12], vibrations [13], a drop in O_2 concentration [14] (which may be caused by several factors) and the presence of bacteria [15]- although the majority of this work has been done on *Aedes* species.

Anopheline eggs kept at least slightly moist have previously been shown to hatch up to 18 days after oviposition [16]. Huang *et al.* [17] showed that *An. gambiae* eggs kept in water or on damp soil can survive even at high temperatures while eggs kept on dry soil for more than a few hours are unlikely to hatch, although it must be noted that this observation was also linked to temperatures that exceeded 41℃.

Beier *et al* [18] and Shililu *et al* [19] independently studied hatching of *An. gambiae* s.l eggs on soil in Kenya. Beier *et al* [18] collected dry soil samples from animal hoofprint depressions, the edges of temporary and permanent pools, and stream beds. Two to five days after flooding these samples in water, first instar larvae were observed. No larvae were seen in soil samples taken from stream banks or ploughed fields which are unlikely sites for oviposition. Shililu *et al* [19] found that eggs remained viable for up to 15 days on moist soil and that hatch was influenced by the rate of soil drying, the type of soil and egg age. Minakawa *et al* [20], also in western Kenya, studied oviposition site preference in *An. gambiae* and found that the order of preference was flooded soil, moist soil, dry soil and a blank dish. Mosquitoes by far preferred the flooded soil. However, when dry season conditions were simulated by providing only moist soil, dry soil and an empty dish, the majority of eggs were laid on the moist soil substrate. The authors suggest that this is a strategy to maintain populations through the dry season in combination with the knowledge that *An. gambiae* embryos can survive on moist soil for several days. These studies clearly demonstrate the ability of some eggs to survive in a desiccating environment for a few days to more than two weeks. However, Yaro *et al.* [11] quantified the distribution of hatching times in *An. gambiae*, *An. coluzzii* and *An. arabiensis* in different water types, but without desiccation, and found that most (89%) of the eggs hatched early during the second and third days post oviposition, ten percent hatched between days four and seven and one percent hatched after the first week. They also found that eggs that hatched early developed to adulthood faster and produced smaller adults than late hatchers. Kaiser *et al* [21] and Ebrahimi *et al* [22] have confirmed that desiccation is not a requirement for delayed hatching and a proportion of eggs may be programmed to delay hatching or to hatch in response to a different number or intensity of egg disturbance events or hatching triggers (inundation with water, rainfall, agitation). The ability to delay or stagger larval time-to-hatch is therefore likely an adaptive trait that increases reproductive output despite the increased risk of desiccation in an unstable environment [11, 18, 19]. This trait has been extensively studied in flood water mosquitoes (*Aedes* species) which can delay hatching for several months [23, 24]. There is a comparative scarcity of information available for anopheline mosquitoes.

Evidence of a genetic association with staggered larval time-to-hatch in *An. gambiae* is based on time-to-hatch phenotypic selections and cross-mating experiments [21]. This evidence also includes an association between larval time-to-hatch and the assortment of insecticide resistance phenotypes in a laboratory colony of *An. gambiae*, showing that selection for either early or late larval time-to-hatch affects the frequencies of those factors associated with insecticide

resistance as a consequence of pleiotropy. Based on these data it is proposed that staggered larval time-to-hatch occurs because a proportion of individuals are genetically predisposed to hatch late which coincidentally offers a broader platform for the selection of insecticide resistance [21].

However, the processes that enable some *An. gambiae* eggs from the same batch to hatch substantially later than others are unknown. The aim of this study was to investigate the mechanism of staggered larval time-to-hatch by examining embryo development and metabolic output in *An. gambiae* eggs drawn from early and late hatching mothers.

Materials and Methods

Biological material

The *Anopheles gambiae* colony (GAH) established using wild-caught females from Ahafo, Ghana, in 2006 was used for all experiments. This colony has undergone selections for early and late time time-to-hatch and has previously been described [21]. Cross-mating experiments [21] indicated that time-to-hatch did not follow a Mendelian mode of inheritance although selection for late time-to-hatch increased the proportion of late hatching larvae to 30%. Both sub-colonies, however, still contained early and late time-to-hatch eggs. The mosquitoes were reared in the Botha de Meillon insectary at the Vector Control Research Laboratory of the National Institute for Communicable Diseases, NHLS, Johannesburg, South Africa. Insectary conditions were maintained at approximately 25℃ and 75–85% relative humidity with a 12 hour light: dark cycle with 30 minute dawn and dusk transitions. Larvae were provided with ground dog biscuits (BEENO) and yeast. Adult mosquitoes were maintained as per Hunt *et al.* [25] on a ten percent sucrose solution and females received two to three blood meals per week.

Time-to-hatch monitoring

Eggs from the early and late time-to-hatch selected sub-colonies as well as from the baseline colony from which the sub-colonies were drawn were monitored for time-to-hatch. This was done to determine whether the selections previously described [21] still had an effect on the time-to-hatch phenotype. Experiments were set up in the Botha de Meillon insectary and maintained at standard conditions. Five batches of eggs were monitored each for the baseline colony and the early time-to-hatch selected sub-colony. For the late time-to-hatch selected sub-colony, 7 batches of eggs were used as the egg batches obtained from this colony were smaller because there were fewer egg producing adults. Eggs were monitored daily for time-to-hatch by counting and removing hatchlings.

Egg collection and storage for egg metabolic rate experiments

At least three batches of eggs obtained from the early and late time-to-hatch selected sub-colonies were collected on different days after allowing a group of females to oviposit over a period of two hours. The eggs were then left to melanize for one hour after removal before rinsing the eggs onto filter paper. The water was allowed to drain prior to sealing the eggs on the filter paper, in an appropriately labelled plastic bag for storage until experiments started. Eggs were stored in the laboratory at ambient temperature during summer months. In experiments conducted during the colder months the eggs were kept in an incubator set at 25℃ (humidity was not controlled, but was raised above ambient by placing a beaker of water for evaporation in the incubator).

Egg metabolic rate measurements

The metabolic rates of sets of 150 eggs were measured every 24 hours up to 8 days old. Eggs were drawn from the larval time-to-hatch selected *An. gambiae* sub-colonies. The total metabolic output of batches of 150 eggs was measured by determining the amount of CO_2 emitted by the eggs in a closed system using a CO_2 analyzer. CO_2 measurements were obtained using an infrared CO_2 analyzer (LI-CO 6262, Li-Cor, Lincoln, NE, USA) in a similar way to that described by Woods & Singer [26]. Thirty ml glass syringes (Becton Dickson, Franklin Lakes, NJ, USA) with a small hole drilled through the wall of each syringe closest to the top (plunger end) served as respiratory chambers. A set of 150 eggs from either early or late time-to-hatch parents was placed onto a 1 cm^2 piece of moist filter paper using a very fine paint brush (Herberts and Evans). The eggs on filter paper were then placed into the respiratory chamber. A three-way stopcock was placed between the syringe and the needle, and to prevent leakage, connections were sealed with petroleum jelly. A total of three syringes were used during each experiment. Initially one syringe contained no eggs (control) to obtain a baseline reading and the remaining two syringes contained sets of 150 eggs. Thereafter each syringe served as its own control and one group of early eggs and one group of late eggs was placed into each syringe consecutively so that two or three replicates per sub-colony and per egg batch were measured on the same day. This method was adopted as readings differed slightly between syringes.

Humid CO_2 free air was obtained by pumping room air through a soda lime scrubber followed by a humidifier. After the eggs had been placed into the syringes they were flushed with the humid CO_2 free air for five minutes. Once flushing was complete the plunger was depressed to the 20 ml mark, blocking the hole so that the purged air was flushed through the needle. Eggs were left in the sealed CO_2 free chamber for 1 hr. Air scrubbed of CO_2 and water using soda lime and magnesium perchlorate, respectively, was drawn through the CO_2 analyzer at a rate of 100 ml/min. After the 1 hour 5 ml air boluses from each syringe were inserted into the air stream, before the water scrubber. This was done for each syringe in sequence. This process was repeated twice (3 measurements per syringe) and the amount of CO_2 in the injected samples converted from parts per million

(ppm) to ml by integration of the CO_2 curve and then multiplied by 1000 to give a value in µl. Data were recorded and analysed using DatacanV (Sable systems, Las Vegas, NV, USA). Comparisons of CO_2 output between syringes as well as between time-to-hatch egg batches were based on two-sample t tests using Statistix 7 (Tallerhasse, USA).

Observing embryo development in fixed and de-chorinated mosquito eggs

Eggs were collected from mixed age groups of early or late time-to-hatch adults that had received at least two blood meals prior to egg collection. Eggs were stored in distilled water until they reached the developmental age at which viewing was required. Eggs were collected on a strip of filter paper and covered in a few drops of fixative solution: 3.6 M formaldehyde; 0.87 M glacial acetic acid; and 8.5 M absolute ethanol (FAA), for at least 30 minutes. Once eggs had been fixed the filter paper strip with eggs was placed into a 1.5 ml reaction tube and covered with one ml of decalcifying solution. The decalcifying solution was as described by Trpis [27] modified slightly and used to decalcify the chorion so that embryos could be viewed under the microscope. The decalcifying solution contained 0.59 M sodium hypochlorite and 0.35 M glacial acetic acid in distilled water. This successfully fixed the eggs so as to prevent hatching and bleached the chorion so that embryos could be viewed under the microscope. The date the eggs were collected, the age of the eggs in hours or days post oviposition and the cage of adults the eggs were collected from were noted. This ensured that at least three batches of eggs from different cages were collected for viewing. The embryo of each egg was viewed with a Zeiss Stereo Discovery V12 microscope at 150 times magnification. Digital images of embryos were obtained and scale bars inserted. Embryos were compared qualitatively by time post oviposition by qualitatively describing the level of embryo development based on whether segmentation was visible as well as the overall degree of development.

Results

Time-to-hatch monitoring

Five to seven batches of eggs were obtained from each of the time-to-hatch selected sub-colonies as well as from the baseline colony. The sample sizes were 3692, 2381 and 5189 for the early time-to-hatch selected sub-colony, the late time-to-hatch selected sub-colony and the baseline colony respectively. In all cases the majority of the eggs hatched on the second day post-oviposition. The baseline colony then showed less hatching on day 3 post oviposition following which hatching occurred in a staggered fashion with most of the eggs hatching within 6 days post oviposition (Fig. 1A). The last recorded hatching in the baseline colony was on day 18 post-oviposition, as was also the case in the late time-to-hatch selected sub-colony (Fig. 1B) although the numbers were very small (three and

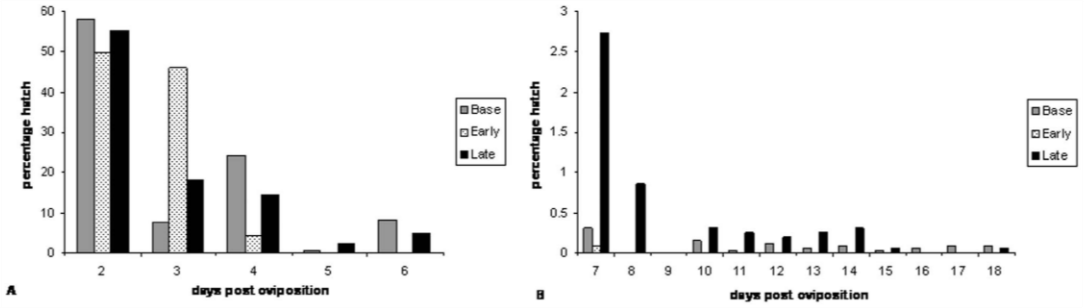


Fig. 1. Proportional hatch rate of eggs from the *Anopheles gambiae* baseline colony as well as the early and late time-to-hatch sub-colonies, A) from days two to 6 post oviposition and B) from days 7 to 18 post oviposition.

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one, respectively). The mean times to last hatch were 10.2 ($\pm$5.02), 5 ($\pm$1.41) and 13.6 ($\pm$2.43) for the baseline colony, the early time-to-hatch sub-colony and the late time-to-hatch sub-colony respectively. These differences were statistically significant (ANOVA: $F=10.39$, $DF=2$, $p\leq0.01$). The primary difference was in a comparison between the early and late time-to-hatch sub-colonies (two sample t-test: $t=7$, $DF=1$, $p<<0.01$). There were no significant differences in the mean times to last hatch between the baseline colony and the late time-to-hatch sub-colony (two sample t-test: $t=1.56$, $DF=1$, $p=0.15$) or between the baseline colony and the early time-to-hatch sub-colony (two-sample t-test: $t=2.23$, $DF=1$, $p=0.08$). There were no differences in the total number of eggs that hatched between the baseline and the time-to-hatch selected sub-colonies (means ranged from 58.55–64.05%).

Egg metabolic rate

The amount of CO_2 produced by age did not differ significantly between the early and late time-to-hatch sub-colonies (ANOVA: $p>0.05$ for all comparisons, Table 1). In general, egg metabolic rate tended to decrease as the eggs aged up to day 5 post oviposition after which a slight increase in metabolic rate was observed from days 6 to 8, particularly for the eggs from the late-time-to-hatch sub-colony. The highest metabolic rate for both groups of eggs was recorded 24 hours post oviposition (Fig. 2). Based on linear regression, the metabolic output of eggs from the early time-to-hatch sub-colony decreased significantly with age ($F=6.35$, $DF=1$, $p=0.04$; $r^2=0.48$). The data from the late time-to-hatch colony did not show a significant trend with age ($F=1.20$, $DF=1$, $p=0.31$; $r^2=0.15$). On day 3 the eggs obtained from early time-to-hatch selected parents produced a significantly higher amount of CO_2 than the eggs obtained from the late time-to-hatch selected parents (two sample t-test: $t=2.42$, $DF=1$, $p=0.02$) while the reverse was true at day 7 post oviposition ($t=-2.06$, $DF=1$, $p<0.05$).

Table 1. Linear regressions and analysis of variance (ANOVA) indicators obtained from trend analysis of CO_2 outputs with age by syringe as well as comparisons between the CO_2 outputs of batches of eggs obtained from the early and late time-to-hatch selected *Anopheles gambiae* sub-colonies.

Regressions	R^2	P
s2e-age	0.52	*0.02*
s3e-age	0.26	0.15
s2l-age	0.42	0.06
s3l-age	0.03	0.68
sce-age	0.48	*0.04*
scl-age	0.15	0.31
ANOVA	**F**	**P**
s2e-s2l	0.14	0.71
s3e-s3l	0.28	0.61
s2e-s3e	0.07	0.79
s2l-s3l	1.72	0.21
sce-scl	0.07	0.79

The degree of freedom for all tests was 1.
*s2 = syringe 2; s3 =syringe 3; sc = syringes combined; e= eggs from the early time-to-hatch sub-colony; l= eggs from the late time-to-hatch sub-colony.

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Qualitative embryo development observations

Approximately 30 images of eggs per age group (1–8 days post oviposition) were obtained from both early and late time-to-hatch selected sub-colonies. Using the degree of definition in embryo segmentation as a visual cue for development, no obvious differences were observed in the rates of embryo development between the eggs from the early and late time-to-hatch sub-colonies (Table 2 and Fig. 3). Most of the embryos from both groups of eggs were fully developed by two to three days post oviposition. Both groups also had eggs that hatched on day two post oviposition. The majority of eggs hatched between days two and four as previously described. Many embryos did not develop. The mean rate to full

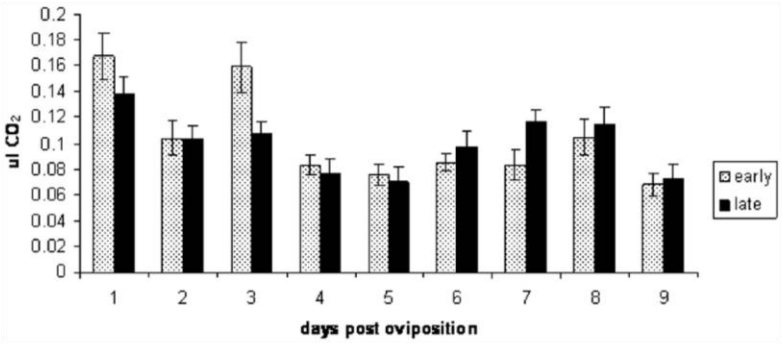


Fig. 2. CO_2 output (μl) of eggs obtained from early and late time-to-hatch selected *Anopheles gambiae* parents by age. Standard error bars are shown.

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Table 2. Qualitative description of the degree of development of unhatched embryos by age post oviposition for eggs obtained from early and late time-to-hatch selected *Anopheles gambiae* parents.

Days post oviposition	Early time-to-hatch		Late time-to-hatch	
	No. Images taken	Description	No. Images taken	Description
4–6 hrs	31	15 white granular with lateral floats often visible. The rest were white-granular with some clearer sections, possibly the start of tissue differentiation Many had a large round tissue mass in the central region.	48	20 were white granular. Most were white-granular on the anterior and posterior ends with a clearer region in the centre of material (only one embryo).
1 Day	58	**83%** showed form and evidence of segmentation, often with a gap between the embryo and the shell. Head and body could be defined in most cases.	86	**63%** showed form and segmentation. The rest were granular white or patched white, some with the central tissue mass
2 Days	34	**44%** showed clear form and segmentation (most did not fill the whole shell cavity). Hatching had begun.	31	**71%** showed clear form and segmentation or appeared to be developing.
3 Days	30	**36.7%** were fully developed taking up the whole shell cavity or close to fully developed	27	**18.5%** were fully developed taking up the whole shell cavity
4 Days	31	**58.1%** were fully or very close to fully developed	31	**48.4%** fully or close to fully developed
5 Days	27	**48.1%** fully developed or close to fully developed	39	**38.5%** fully or close to fully developed
6 Days	30	**20%** were fully developed or close to fully developed	28	**43%** were fully or nearly fully developed
7 Days	27	**26%** were fully developed or close to fully developed	31	**58.1%** were fully developed or close to fully developed
8 Days	28	**43%** were fully developed or nearly fully developed	33	**36%** were fully or close to fully developed

The total percentages of developed and developing embryos are shown in bold.

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embryonic development across the ages 24 hours to 8 days was 44.88% in the early time-to-hatch sub-colony and 46.75% in the late time-to-hatch sub-colony.

Discussion and Conclusions

The late time-to-hatch selected sub-colony showed a later mean time to last hatch than the early time-to-hatch colony. Under standard insectary conditions, eggs from both time-to-hatch sub-colonies tended to hatch early (four days post-oviposition and earlier) although the late time-to-hatch colony showed a higher proportion of eggs that hatched 5 days post-oviposition and later. In both sub-colonies a very small proportion of eggs hatched up to 18 days post-oviposition.

The ability to delay hatch when conditions for hatching are not immediately suitable is an important adaptive trait in some mosquito species- *Aedes* species in particular [19, 20]. Although dormancy in tropical insects is known, it is perhaps not often considered [28]. The delayed or staggered time-to-hatch described here for the *An. gambiae* baseline and sub-colonies has also been observed in families reared from wild-caught *An. gambiae* females collected in Ghana and the Republic of the Congo [21] as well as from colonised and wild caught mosquitoes from Kenya [18, 19, 20], showing that this trait is not unique to laboratory strains or

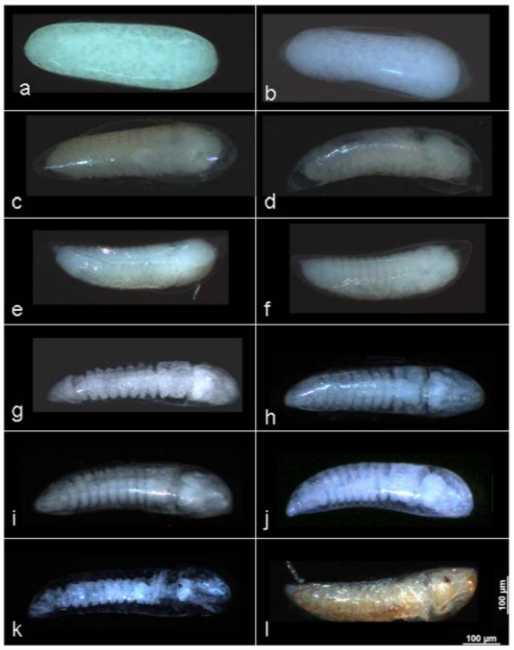


Fig. 3. Embryos photographed at different ages and developmental stage post oviposition. a) early 4 hours post oviposition b) late 4 hours post-oviposition c) early 20 hours post oviposition d) late 20 hours post oviposition e) early 24 hours post oviposition f) late 24 hours post oviposition g) early 48 hours post oviposition h) late 48 hours post oviposition i) early 4 days post oviposition j) late 4 days post oviposition k) early 6 days post oviposition l) late 6 days post oviposition.

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particular ecological zones although considerable differences are expected to be seen between different populations. Staggered time-to-hatch likely carries significant adaptive significance in *An. gambiae* [21].

The metabolic rates of eggs from the early and late time-to-hatch selected sub-colonies only showed significant variation at three and 7 days post-oviposition. Woods *et al.* [29] showed that metabolic rates in eggs of the moth, *Munduca sexta*, were generally low in the beginning of development and then increased more or less continuously until hatching occurred at the end of development (within 2–3 days). However, Kambule *et al.* [30] showed that the metabolic rate of nondiapause locust eggs continued to increase until the eggs hatched while the metabolic rate of diapause eggs was consistently lower. The highest metabolic rates in the *An. gambiae* eggs were recorded at 24 hours post oviposition which corresponds to the stage in development when embryos undergo a 180° rotation around the longitudinal axis during the germ band retraction phase. Prior to this most of the development has already occurred with intersegmental furrows visible and the ventral aspect of the embryo facing the flattened dorsal side of the shell [31]. Metabolic rates then tended to decrease with time post oviposition in the

sub-colonies suggesting that late hatching eggs likely enter a state of diapause prior to hatching.

Wilkenson *et al.* [32] found that 29% and 1% of *An. dirus* eggs survived after 21 days and 92 days respectively on moist filter paper. An experiment by Darrow [33] on *An. quadrimaculatus* eggs showed that eggs removed from water within 9 hours of oviposition could not resist desiccation. However, those removed from water at 10–13 hours post oviposition did develop desiccation tolerance and 27% of these eggs hatched after exposure to 0% RH for 12 hours. Darrow [33] also removed eggs from water at 23.5 hours post oviposition and returned them to water after 24 and 36 hours respectively. The eggs subjected to this treatment hatched approximately 23.5 hours after being returned to water suggesting that embryonic development ceases during desiccation and then continues at the normal rate. In this study the eggs used for egg metabolic rate experiments and monitored for time-to-hatch post oviposition hatched almost immediately after being returned to water. This occurred in all eggs except for 24 hour old eggs, indicating that, similar to [33], eggs can only begin to hatch approximately two days after oviposition when the embryo is fully developed. This suggests that the eggs at ages two days and older were ready to hatch when taken for use in experiments, but that hatch was delayed.

Qualitative embryo development observations were somewhat hampered by the hatching of the majority of eggs between two and four days post oviposition which made the acquisition of unhatched eggs for age groups older than 4 days difficult. As a result many batches of eggs were sampled to acquire adequate sample sizes of late hatching eggs. There were no consistent morphological differences observed between the two groups of eggs in terms of the level of embryo development by age group or between the number of embryos that were fully developed in eggs from the two time-to-hatch selected sub-colonies by age. This may be partly due to the fact that both sub-colonies contain mostly early time-to-hatch eggs, with the late time-to-hatch colony showing a higher proportion of late hatchers. In general, all embryos that developed fully did so within four days post oviposition, supporting the metabolic output data which suggests that late hatching eggs enter a state of diapause once the embryos are fully developed.

Time-to-hatch in Anopheline mosquito eggs is generally believed to be fixed so that it occurs at the completion of embryo development. This is normally at approximately 50 hours old in optimal conditions [33,34]. However, various factors, temperature in particular, are known to affect that rate of embryo development and hatching [12]. It makes sense that eggs are able to adapt somewhat to different hatching conditions in response to risks and opportunities. Parents are also able to influence hatch timing, so the assumption that hatch timing is relatively fixed is not true (see review by Warkentin, [35]). The current study found that the embryos develop within the first two to four days post oviposition with most hatching immediately and a small proportion delaying hatching. The delayed hatch appears to be accompanied by a fairly constant and reduced metabolic rate in some eggs. As all eggs were exposed to the same

environment this phenomenon can not be considered quiescence but rather is assumed to be late embryonic diapause as seen in the Gypsy moth *Lymantria dispar* [36] and some temperate mosquito species [37].

Hatch timing may influence other traits such as insecticide resistance and fitness. Recently Perez and Noriega [38] showed that extended quiescence (eggs induced to hatch after 10 weeks post oviposition instead of after just less than one week for short quiescence) affected performance and reproductive fitness of adult *Aedes aegypti* females and the nutritional status of their progeny via maternal effect. Specifically, the females from the eggs that underwent extended quiescence survived 10% longer, laid more eggs and produced 14% more viable offspring when reared on a sub-optimal diet of 3% sucrose solution. However, the reproductive success of females reared from extended quiescent eggs was dramatically affected by stress in the larval environment in the form of metal contamination. The authors claim that intrapopulation variation in the sensitivity of individuals to environmental cues is what leads to asynchronous hatching and suggest that phenotypic plasticity results as a consequence of pharate (waiting to emerge or hatch) first instar larvae. That variation in sensitivity to environmental stimuli may lead to asynchronous hatching is supported by Ebrahimi *et al* [22] who found that all *An. gambiae* eggs in their experiments required agitation to hatch and that some eggs required more agitation events than others. This is compared to instalment hatching or bet hedging observed in *Aedes* mosquitoes where only a proportion of eggs will hatch in response to a given stimulus [23]. Ebrahimi *et al* [22] point out that this requirement for agitation to induce hatching may be an advantage in mass rearing situations where a large number of synchronously developing mosquitoes are needed. Most studies looking at delayed hatching of *An. gambiae* complex eggs have done so in conjunction with desiccation conditions. Whether the delayed hatching phenotypes observed in this study may be more desiccation tolerant than early hatching phenotypes remains to be investigated.

An association between insecticide susceptibility and staggered time-to-hatch in *An. gambiae* has previously been described [21]. These studies suggest that variation in one trait (such as staggered time-to-hatch) may affect and even enhance variability in other traits that affect reproductive and physiological fitness as well as environmental adaptability. This may occur as a result of resource re-allocation or a as consequence of pleiotropy. It is likely that there are interactions between environmental and genetic factors that influence time-to-hatch in mosquitoes [4, 21, 23]. These factors in turn may influence subsequent life history traits and may even affect behaviour as a consequence of pleiotropy. It would be interesting to determine whether eggs associated with more permanent larval sites such as those of *An. funestus* are more or less likely to hatch as soon as embryos are fully developed without the requirement of a disturbance. Beier *et al* [18] indicate that *An. funestus* is less able to resist desiccation and this may also translate to the ability to delay hatching.

It is concluded that all viable embryos in *An. gambiae* develop to full maturity at the same rate and that a proportion are able to delay hatching. As it has

previously been shown that it is possible to at least partially select for late time-to-hatch, this characteristic is likely to involve genetic as well as environmental factors. This study supports several others [11, 18, 19] in which it was also concluded that delayed time-to-hatch in *Anopheles gambiae* is likely an adaptation to maximise reproductive output despite the increased risk of desiccation in an unstable aquatic environment.

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Author Contributions

Conceived and designed the experiments: FDD BDB MLK. Performed the experiments: MLK. Analyzed the data: MLK FDD BDB. Contributed reagents/materials/analysis tools: FDD BDB. Wrote the paper: MLK FDD BDB.

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