

**The Effect of Dieldrin Exposure on *Anopheles gambiae*
(Diptera: Culicidae): Fitness, Fecundity and Fertility.**

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of
Master of Science in Medicine.**

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Candidate's Declaration

I, Ryan Anthony Ingram Norton declare that this dissertation is my own work. It is being submitted for the degree of Master of Science, at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.

The 12th day of January, 2012

Publications and presentations arising from dissertation

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Abstract

The effect of dieldrin exposure on fecundity, fertility and longevity was investigated in two populations of the African malaria vector *Anopheles gambiae* s.s. A wild representative strain and a laboratory strain were used. Experimental procedures were designed and optimised to obtain the maximum amount of data for individual females. In some instances, novel experiments had to be designed to answer specific research questions as no similar studies were available in the literature. A compilation of reference photographs depicting the different stages of oocyte growth in *An. gambiae* females was produced to assist in the identification of growth phases. Fecundity was shown to be reduced in dieldrin exposed females with close correlation to egg retention. Oocyte developmental rate was reduced in both populations of mosquitoes after exposure to dieldrin. Longevity was observed to decrease in the wild strain and remained unchanged in the laboratory strain when compared to the unexposed controls. Fertility was generally decreased, however dieldrin exposure appeared to have no negative affect on mating or spermatozoan viability before or after mating in male testes or female spermathecae respectively. In summary this study showed that exposure to dieldrin had subtle effects on the biology of these mosquitoes, but would probably not have a detrimental effect on a population as a whole.

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ABBREVIATIONS

ANOVA	Analysis of variance
Bz	Benzodiazepines
CNS	Central nervous system
COGS	Resistant wild <i>Anopheles gambiae</i> field collected population
DDT	Dichloro - diphenyl - trichloroethane
DNA	Deoxyribonucleic acid
GABA	Gamma aminobutyric acid
GAH	Resistant laboratory <i>Anopheles gambiae</i> strain
GST	Glutathione - S - transferase
IRS	Indoor residual spraying
OEH	Ovarian ecdysteroidogenic hormone
PBM	Post blood meal
PBS	Phosphate-buffered saline
Rdl/Rdl	Homozygous dieldrin resistance mutation
Rdl/s	Heterozygous dieldrin resistance mutation
RR	Homozygous resistant
RS	Heterozygous resistant
SS	Homozygous susceptible
SUA	Susceptible laboratory <i>Anopheles gambiae</i> strain
WHO	World health organisation

Chapter 1

Literature Review

1.1 Introduction

Mosquitoes are well known for their nuisance and are notorious for spreading diseases to humans and animals throughout the world. Malaria is one of the mosquito-borne diseases, having caused countless deaths in the past century and has been reported to kill over 800 000 people annually in the tropics (WHO, 2010). Current malaria distribution maps indicate that almost all countries falling within 15 degrees North and South of the equator are high risk areas for disease transmission (excluding Australia) (Figure 1.1). The major malaria parasite, *Plasmodium falciparum*, is responsible for the majority of malaria cases in Africa. In 2008, 109 countries were endemic for malaria, including 45 countries within the African region (WHO, 2008). Approximately 80% of clinical malaria cases in Africa occurred in only thirteen countries with over half of them in Nigeria, Democratic Republic of the Congo, Ethiopia, United Republic of Tanzania and Kenya (WHO, 2008). Malaria is closely associated with poverty stricken countries found in the tropics and has been shown to be a major cause of economic burden (Gallup and Sachs, 2001). Malaria tends to thrive where human populations struggle, and it is this factor that is causing an increase in the disease burden. The main reasons attributed to this are population movements into infected areas, agricultural practices involving water bodies such as irrigation schemes and dams, deforestation and the deterioration of public health systems in poor countries (Sachs and Malaney, 2002). The changing climate has also been attributed as one of the factors augmenting the growth and spread of malaria due to pronounced El Niño cycles and global warming (Sachs and Malaney, 2002).

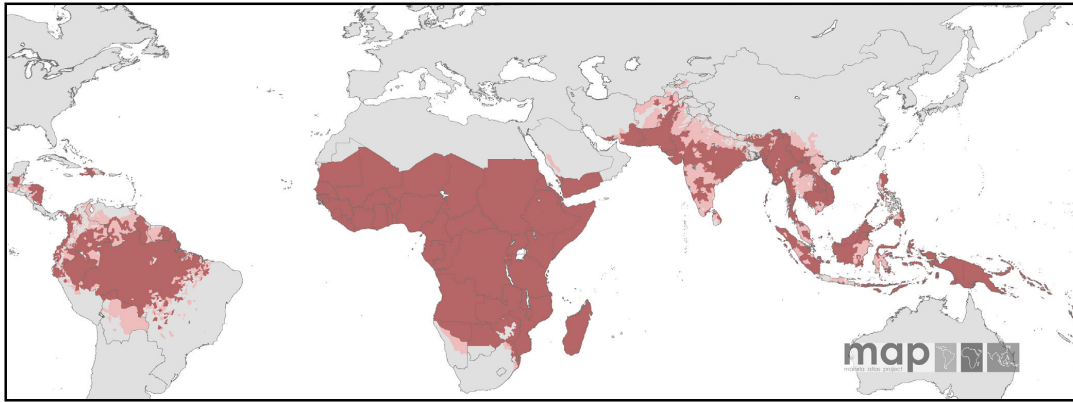


Figure 1.1 *Plasmodium falciparum* malaria risk defined by annual parasite incidence (PfAPI). Modified from Guerra *et al.* (2008). The dark red areas indicate stable risk (PfAPI > 0.1 per thousand per annum), the pink areas represent the unstable risk (PfAPI < 0.1 per thousand per annum) and the grey areas show no risk.

Three of the main African malaria vectors are found within the genus *Anopheles*. *Anopheles funestus* is a member of the *An. funestus* group, while the remaining two vectors (*An. gambiae* and *An. arabiensis*) belong to the *Anopheles gambiae* complex, (Gillies and De Meillon, 1968; Gillies and Coetzee, 1987). This complex currently consists of seven different species, six having been named and the seventh currently being described (Gillies and Coetzee, 1987; Hunt *et al.* 1998). The species are *An. gambiae* Giles, *An. arabiensis* Patton, *An. quadriannulatus* species A Theobald, *An. quadriannulatus* species B (Hunt *et al.* 1998), *An. bwambae* White, *An. melas* Theobald and *An. merus* Dönitz. The two latter species are saltwater breeders whereas the others breed in freshwater. *Anopheles bwambae* is found only in the mineral springs of Uganda (Gillies and De Meillon, 1968; Gillies and Coetzee, 1987). Two molecular forms, M and S, within *An. gambiae* have been identified (Favia *et al.* 1997). Recent investigation has shown that the M molecular form can be further divided into two distinct breeding groups (Slotman *et al.* 2007). These species are all morphologically indistinguishable (Gillies and Coetzee, 1987) and can only be identified to species level using specific molecular

techniques. *Anopheles funestus* and *An. arabiensis* do not form part of this research and will therefore not be discussed in more detail.

1.2 Biology of *Anopheles gambiae*

Anopheles gambiae is one of the most persistent malaria vectors in Africa, transmitting the parasite *Plasmodium falciparum* quickly and efficiently throughout human populations in malaria endemic areas. This species has a distribution that falls within a band situated diagonally across the continent from North-West Africa down to South-East Africa and includes Madagascar. *Anopheles gambiae* is absent from all areas North-East of Uganda and the Kenya highlands as well as areas South-West of Mozambique including most of South Africa and Namibia (Sinka *et al.* 2010).

1.2.1 Early developmental stages (Aquatic stages)

Mosquitoes are holometabolous insects as they undergo a complete metamorphosis occurring at the pupal stage of development. There are four different developmental stages in a mosquito life cycle: eggs, larvae, pupae and adults. The adult females oviposit in aquatic habitats where the first three stages of the life-cycle are completed (Gillies and Coetzee, 1987).

A few days post-oviposition, the eggs hatch. The rate of hatching is different between species and there may be early and late hatching observed in some egg batches due to the conditions they are exposed to (Gillies and De Meillon, 1968; Kaiser *et al.* 2010). The larvae develop in four instar stages of growth with successive instars being significantly bigger due to moulting that occurs between each instar (Gillies and Coetzee, 1987). Larvae feed continuously until pupation which starts at about 10–14 days after hatching,

in optimal conditions (Bayoh and Lindsay, 2003). The growth rate of the larvae can be affected by a number of factors such as temperature, nutrition and population density (Gimnig *et al.* 2002; Bayoh and Lindsay, 2003). Larval development occurs over a range of temperatures bordered by a lower developmental threshold (16 °C) and an upper lethal temperature (34 °C) (Bayoh and Lindsay, 2003). This range varies between species. Furthermore, a positive correlation between developmental time and temperature can be found within this range (Bayoh and Lindsay, 2003).

Aquatic habitats chosen by each female will vary from one location to another and thus food sources may vary correspondingly, according to the environment in which they are situated (e.g. Larvae found in a natural pond will have access to detritus in the form of dead leaves from trees, if present, and microorganisms in the form of algae). The larvae in turn use adaptations to utilise the available food source and use it as efficiently as possible. Food items found in these aquatic habitats may consist of micro-organisms, detritus and bio-films, giving rise to different modes of feeding being employed by the larvae (Clements, 1999). The larvae feed on available food sources using four main actions. Collection and filtering of suspended food items found near the surface of the water is the most characteristic of *Anopheles* species. Other mosquito species utilise much more active feeding regimes whereby food particles are either gathered, scraped off a surface, shredded up or predated upon (Clements, 1999). Most mosquito larvae will also compete intraspecifically in the form of cannibalism when larvae from different instar stages are present together in one water body (Koenraadt and Takken, 2003; Koenraadt *et al.* 2004).

As the larvae grow in size, they shed their exoskeletons three times between instars until they reach the fourth and final instar. From here the larvae become pupae. The pupal stage is the developmental period at which metamorphosis occurs and is a non feeding phase. The pupae, however, are aware of the surroundings and can actively escape predation. The pupal developmental rate is temperature dependent and usually lasts about 1-3 days (Bayoh and Lindsay, 2003). The adult mosquitoes generally emerge in the evening dusk period and fly to the nearest form of cover to rest and allow their cuticle to harden (Clements, 1999).

1.2.2 Adult mosquitoes

The adult stage is characterised by flight and a decrease in the reliance on the aquatic habitat for survival. The sequence of emergence in *Anopheles* mosquitoes in the early stages of this phase has been observed to be dominated by males (Personal observation). However, this may be an artefact associated with rearing in insectary conditions. *Anopheles gambiae* males will only respond to females 12-36 hours after emerging due to the male genitalia having to rotate before they become sexually active (Clements, 1999). In the first 30-60 hours of adulthood, *An. gambiae* females are non-receptive to males (Clements, 1999). Avoidance of mating is accomplished by the bending of the terminalia by the females or the physical removal of males by kicking (Clements, 1999). Mating usually takes place once in the lifetime of *An. gambiae* females as spermatozoa are stored in protective spermathecae in quantities sufficient to fertilise all eggs produced. For mating to occur, males and females of the same species need to locate each other. Mating will take place in several ways including swarming by males, assemblage at biologically significant locations or the approach of an individual female by a male. Usually the two former behaviours are favoured and found to be more common (Clements, 1999). Males

of *An. gambiae* have been found to swarm at a certain period during dusk whilst females actively fly through the swarm. This swarming behaviour enhances mating success and is an efficient mate-finding mechanism (Charlwood *et al.* 2000). Close range mate recognition in the form of wing-beat frequency matching has been shown to occur in *An. gambiae* within incipient species, namely the M and S molecular forms (Pennetier *et al.* 2010).

Male and female mosquitoes require different nutritional components for the various metabolic demands that need to be fulfilled. Plant sugars provide an important energy source for both sexes and are essential for survival specifically for the males of the species. These sugars in the form of sucrose are primarily found in floral nectar but are also found in extra-floral nectaries, damaged fruits, vegetative tissues and honeydew (Gary and Foster, 2004).

Female mosquitoes feed primarily on blood, ingesting up to twice their body weight after one feed, and are capable of separating the plant sugars and blood in the digestive tract. The sucrose/nectar is stored in the crop, whilst the blood is transported into the midgut. This allows the females to replenish their energy reserves as well as obtain a blood meal at any given time (Clements, 1999). The acts of host-finding, blood feeding, developing oocytes and ovipositing eggs have been described by Beklemishev (1959) as a single gonotrophic cycle. Female mosquitoes may undergo a number of gonotrophic cycles in their lives, thereby producing hundreds of eggs.

In order to produce fully developed eggs, females need to ingest at least one blood meal after mating (Gillies and De Meillon, 1968, Fiil 1976; Clements, 1992). Chambers and

Klowden (2001) have shown that the mating status of *Anopheles* females does not appear to influence blood feeding behaviour. Therefore whether the females have mated or not, they may still blood feed.

Anopheles gambiae females are anautogenous and thus require a blood meal for the maturation of their oocytes. Autogenous mosquito species (eg. *Aedes atropalpus* and *Culex pipiens molestus*) are able to produce the first batch of eggs without an initial blood meal. Thereafter, these species also need a blood meal for subsequent egg production. There is evidence that, within a species, the quality of larval nutrition determines the stage of development of the ovarian follicles at emergence (Clements, 1992). Autogenous mosquito species may revert to an anautogenous state if insufficient larval nutrition has resulted in limited primary egg development (Bock and Milby, 1981; Steinwascher, 1982; Packer and Corbet, 1989). To complete the first gonotrophic cycle, two blood meals may be required however, subsequent cycles normally only require one blood meal for oocyte maturation and this is known as gonotrophic concordance (Bertram, 1962).

If a small amount of blood is ingested by the female due to interrupted feeding, a pre-gravid condition may occur. In this scenario more than one blood meal is needed for the maturation of the primary oocytes (Lyimo and Takken, 1993). It has also been shown that laboratory reared mosquitoes that have been provided with adequate larval nutrition, can develop the first batch of eggs after feeding to repletion only once (replete feeding is the term given to mosquitoes that imbibe as much blood as their bodies can withstand) (Gillies, 1954; Fiil, 1976).

Most *Anopheles* species will feed primarily on mammals. However some have been observed to feed on birds, reptiles and fish (Tempelis, 1975). The need for vertebrate blood for the production of eggs is the sole factor leading to parasite transfer thereby enhancing the malaria vectorial capacity of the mosquito species (Takken and Constantini, 2006). Anautogenous species use odour signals originating from the host, when searching for blood (Takken and Knols, 1999). *Anopheles gambiae* is anthropophilic and mainly feeds indoors. Most biting occurs between midnight and dawn when the host defences are at the lowest (i.e. people are sleeping) (Gillies and DeMeillon, 1968).

After blood feeding, the blood protein haemoglobin is quantitatively converted to haematin, which is defecated at the end of the digestion period (Briegel, 1980). The production of haematin, an excretory product, is characterised by defecation of brown/black deposits on the resting surfaces. Haematin excretion can be used as an indicator of the amount of haemoglobin in the blood meal and can be correlated with egg production for individual mosquitoes (Hogg and Hurd, 1995a).

The females rest in sheltered environments while the oocytes mature. Most *An. gambiae* females rest indoors where the host is located (Gillies and De Meillon, 1968). This behaviour allows the females refuge from outdoor predators when they are at their most vulnerable with the developing oocytes.

Once the oocytes are fully developed the next stage in the gonotrophic cycle is the selection of a suitable habitat for the female to oviposit her egg batch. The location of these habitats occurs through behavioural responses and olfactory and visual cues

(Clements, 1999). After assessing the water quality using olfactory and gustatory cues (Clements, 1999), the female will either lay her eggs or move on to find a more suitable oviposition site.

1.2.3 Mosquito reproductive morphology and oogenesis

The reproductive system of *An. gambiae* females includes two fusiform ovaries that are situated dorsally in the abdomen at approximately the fifth abdominal segment. The development of the ovaries and oocytes contained within has been described by Bertram (1962) and subsequently by Clements (1992) based on the work performed by Christopher (1911). The development has been divided into different stages and phases of maturity. The ovaries of a newly emerged female are usually about 1mm long and appear translucent. The ovarian follicles - which are epithelial sacs containing the oocyte and nurse cells - at this stage (Figure 1.2) are at the previtellogenic resting phase of development (1) (Clements 1992). No further growth takes place until the mosquito has obtained a blood meal. Development of the first follicle in the ovarioles proceeds as the blood meal is digested (Bertram, 1962; Clements, 1999).

The initiation phase (2) begins with blood feeding and lasts a few hours, terminating with the release of ovarian ecdysteroidogenic hormones (OEH) (Clements, 1992). The release of OEH stimulates vitellogenesis. Vitellogenesis is the term given to all of the processes of yolk formation. The major protein found in yolk bodies is called vitellin with a secretory precursor vitellogenin. Vitellogenin is released by the fat bodies and diffuses into the oocytes via receptor-mediated endocytosis stimulating the trophic phase (3) of development (Clements, 1992). The trophic phase is characterised by the uptake and accumulation of vitellogenin in the ooplasm of the developing oocytes. The oocytes grow

to their final size and assume their final form in the post-trophic phase (4). During the process of oviposition the mature oocytes pass through the common oviduct, being fertilised as they pass the spermathecal duct. The entry of the spermatozoid via the micropyle stimulates the completion of meiosis and the female gamete is transformed into an egg containing an embryonic larva (Clements, 1992). After oviposition the chorion (egg shell) darkens rapidly.

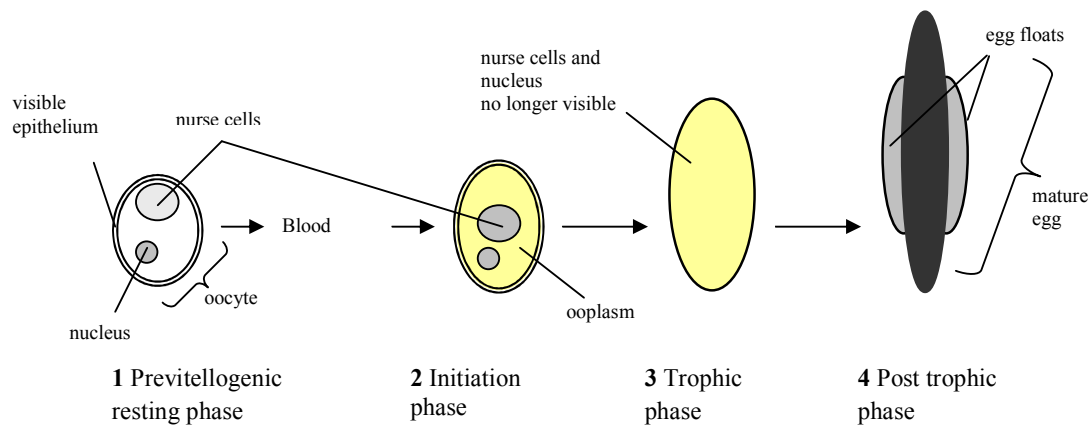


Figure 1.2. Flow diagram of the developmental phases associated with oocyte growth.

1.2.4 Mosquito fecundity and fertility

Female mosquito reproduction is influenced in two ways: 1) by the number of ovarioles present in the ovaries (Hurd *et al.*, 1995) and 2) by the teneral body size of the female (measured by wing length) (Briegel, 1990a). Briegel, (1990a) identified a minimum wing length of 3 mm, below which the first blood meal did not induce oogenesis in *An. gambiae*. Fecundity is usually measured as the sum of oviposited and mature eggs still inside the female at the end of the first gonotrophic cycle (Hogg and Hurd, 1995b).

Fertility of mosquitoes is usually indicated by oviposition, as the females do not usually oviposit without having mated first (Clements, 1992). As the eggs pass through the

common oviduct they are individually fertilised by stored male spermatozoa from the spermatheca which travel through the spermathecal duct to fertilise the eggs. Hatch rate is used to measure the difference in fertility between individuals or between two or more consecutive egg batches.

1.3 Insecticides and other interventions in vector control

The struggle to control malaria vectors began as far back as the early 1900's (Tren and Bate, 1999). The first form of malaria vector control was the removal of stagnant water puddles and swamps which harboured mosquito larvae. In the 1930's, South Africa, along with numerous other countries, began to use the larvicide Paris Green (copper (II)-acetoarsenite) which led to a dramatic decrease in the incidence of malaria between 1932 and 1938 (Tren and Bate, 1999).

Early control of malaria in the form of indoor spraying was conducted in the 1930's using pyrethrum (De Meillon, 1936). This trend was followed by the use of organochlorines such as Dichloro-diphenyl-trichloroethane (DDT) and dieldrin in the 1950's for indoor residual spraying (IRS) (Patel and Rao, 1958). The organochlorines were found to linger in the environment without losing their activity and therefore remained effective in sprayed houses for long periods of time. High levels of resistance to the organochlorine, dieldrin have previously affected the control of malaria vectors and are still observed in current wild populations of certain vector species (Etang *et al.* 2003).

Insecticides that have been used in vector control programmes belong to four different chemical classes - organochlorines, organophosphates, carbamates and pyrethroids. The use of pyrethroid insecticides has increased in recent years whereas the use of

organochlorines and some of the organophosphates has decreased due to the associated negative effects in non-target organisms and resistance in target organisms. DDT is still used in certain situations where no other alternatives can be found, provided the World Health Organisation (WHO) guidelines and recommendations are adhered to. DDT is conditionally approved under the Stockholm convention on persistent organic pollutants (WHO, 2006).

Currently, vector control includes indoor residual spraying combined with the use of insecticide treated nets. Other forms of vector control that originated for use in agriculture, include biocontrol where naturally occurring insect pathogens or insect hormones have been used to reduce vector populations. Specific strains of bacteria have been developed to infect the midgut of the larval stages of the vectors. *Bacillus thuringiensis israelensis* and *B. sphaericus* are two such bacteria that have been described as potential pest-specific biocides (Majori *et al.* 1987; Karch *et al.* 1991; Skovmand and Sangogo 1999; Fillinger *et al.* 2003). Insect fungal pathogens have also been investigated as potential bio-control agents. *Metarhizium anisopliae* as well as *Beauveria bassiana* - two well-known entomopathogenic fungi - have been shown to have great potential as vector control agents (Zimmerman 1993; Scholte *et al.* 2006; Farenhorst *et al.* 2008; Kikankie *et al.* 2010). Finally, insect growth regulators have been used more recently as a form of vector control. Analogues of the juvenile hormone (juvenoids) such as methoprene and pyriproxyfen (Yapabandara *et al.* 2001), as well as chitin synthesis inhibitors, such as diflubenzuron, triflumuron and novaluron have been used to prevent the complete metamorphosis of mosquitoes, killing the pupae at the emergence stage of development (Shahabuddin *et al.* 1993; Shen and Jacobs-Lorens, 1997).

1.4 Resistance to insecticides

According to the Insecticide Resistance Action Committee (IRAC) (www.irac-online.org), “Resistance is the selection of a heritable characteristic in an insect population that results in the repeated failure of an insecticide product to provide the intended level of control when used as recommended”. Mosquitoes have a relatively short life cycle and each individual female can produce hundreds of progeny. Their short generation time means that changes which can bring about resistance can be established in a population in a very short time period, provided they are under some sort of selection pressure (Feyeresein, 1995). Selection refers to the differential survival and reproduction of genotypes. The high generation turnover and large population size, combined with genetic recombination, enables the mosquitoes to adapt and increase the likelihood of an inherited resistance mechanism in the progeny (Pinto *et al.* 2007).

The degree of resistance in malaria vector populations is dependent on the length and frequency of insecticide exposure, the type of insecticide used, the vector characteristics and the volume of insecticide used (Hemingway and Ranson, 2000). There are a number of different resistance mechanisms used by insect vectors, including: insecticide metabolism (esterase based resistance, glutathione-S-transferase-based resistance, monooxygenase-based resistance) and target-site resistance (acetylcholinesterase, GABA receptors and sodium channels).

1.5 Dieldrin

Dieldrin is a cyclodiene insecticide belonging to the chlorinated hydrocarbon (organochlorine) class of insecticides. Dieldrin was originally produced by J. Hyman and

Co. in 1948 as an insecticide. It was developed as an alternative to DDT and was first employed in the spring of 1954 by the Plant Pest Control branch of the United States Department of Agriculture to control a local infestation of Japanese beetles, *Popilla japonica* in Illinois (Scott *et al.* 1959).

Dieldrin was introduced for malaria control in a number of African localities in the 1950's and was one of the predominant insecticides in use from 1955 to 1969 (Patel and Rao, 1958; Trigg and Kondrachine, 1998). It has been reported that 9, 301 million pounds of dieldrin were used for public health purposes in 55 countries around the world, during the period between 1954 and 1958 (Hays and Laws, 1991).

This chemical was also introduced as a pesticide against ticks in Africa in 1961 and was used by farmers on livestock as well as a seed dressing and the treatment of soil in the agricultural environment (Patel and Rao, 1958; Wandiga 2001). In addition to this, dieldrin was also used for timber preservation, termite-proofing of plastic and rubber coverings of electrical and telecommunications cables, of plywood and building boards and as a termite barrier in building construction (Scott *et al.* 1959).

The extensive use of dieldrin was successful in many areas. By the mid 1960's however, the negative impacts caused by dieldrin were starting to be observed. High mortality amongst non-target organisms such as small mammals and birds was associated with exposure to this insecticide (Scott *et al.* 1959). Many adverse effects on humans were also observed as it is absorbed by the skin as well as through the gastrointestinal tract and is stored in fatty tissue where it is slowly excreted into the rest of the body (WHO, 1975, De Kom and Dewan, 2007). The harmful effects and resistance to dieldrin eventually became

so widespread (Ramakrishna and Elliot, 1959; Georghiou and Metcalf, 1963) that the insecticide was banned in the 1970s in most countries (De Kom and Dewan, 2007). The use of dieldrin was only banned in Africa during 1992, but despite the ban, the chemical was still found to be available in local Kenyan markets and environment by Wandiga (2001) at the time of publication. As this dissertation focuses principally on the effect of dieldrin on mosquito fitness, other insecticides will not be discussed in detail.

Cyclodiene resistance is historically widespread and has accounted for almost 60% of reported cases of insecticide resistance (Georghiou, 1969; Thompson and Steich, 1993). Dieldrin resistance in *An. gambiae* has been reported in Mali (Toure, 1984), Burkino Faso (Majori *et al.* 1987), Ghana, Benin and Kenya (Brown, 1986), Senegal, Cote d'Ivoire, Guinea, Mauritania and Madagascar (Coetzee *et al.* 1999) and Cameroon (Etang *et al.* 2003). Resistance to dieldrin in *An. gambiae* was first observed in 1955, 18 months after a malaria control pilot project was performed in western Sokoto, northern Nigeria (Ramakrishna and Elliot, 1959). Resistance has been maintained for over four decades in several West African populations of *An. gambiae* and has been associated with target-site resistance (Du *et al.* 2005; Brooke *et al.* 2006). It has been suggested that resistance to dieldrin in strains of *An. gambiae* is persistently maintained at a high level in the absence of selection pressure because the mutation is contained within a chromosomal inversion (Brooke *et al.* 2000). These authors have shown that there is cross resistance to fipronil (a phenyl pyrazole insecticide) which has the same target site as dieldrin (Brooke *et al.* 2000). However due to fipronil being a relatively new insecticide, the cross resistance would have only occurred recently, suggesting that resistance of previously observed dieldrin resistance in the field would have been caused by illegal use of the insecticide.

Genetically, dieldrin resistance in *An. gambiae* has been observed to be semi dominant (Davidson, 1956) or dominant (Davidson and Hamon, 1962) depending on whether the heterozygotes survive exposure to 4% dieldrin for one hour. It has been shown by Davidson (1956) that these genotypes can be identified, using different concentrations of dieldrin. Homozygous susceptible mosquitoes (SS) can be killed by 0.4 % dieldrin concentration and heterozygous mosquitoes (RS) with a 4 % concentration of dieldrin leaving only the homozygous resistant individuals (RR) alive. It was observed however that the heterozygotes could also be resistant to the 4 % dieldrin concentration (Davidson and Hamon, 1962) which therefore means that when exposed to 4% dieldrin, the resulting survivors should all be either heterozygous or homozygous resistant. Generally heterozygous resistant individuals tend to be at an advantage as they possess the resistance to insecticides from the resistant parent, combined with the higher fecundity of the susceptible parent (Campanhloa *et al.*, 1991).

1.6 Dieldrin resistance mechanisms in *Anopheles gambiae*

1.6.1 Chloride ion channels and GABA receptors

A chloride ion channel (Figure 1.3a) is a macromolecular protein passageway that spans the lipid bilayer of the cell membrane (Ackerman and Clapham, 1997). A γ -amino-butyric-acid (GABA) receptor (Figure 1.3b) is a heteromultimeric ligand-gated chloride ion-channel located in the insect's central nervous system (CNS) and in neuromuscular junctions (Bermudez *et al.* 1991). There are three known types of GABA receptors, namely: GABA_A, GABA_B and GABA_C (Enna and Möhler 2007). The GABA_A receptor is associated with resistance to chemical pesticides in invertebrates (Feyereisen, 1995).

Most GABA receptors are made up of five individual subunits of at least three distinct types (Figure 1.3b and Figure 1.4). The most common subunit types are α , β , and γ , however others such as δ , ϵ , θ , and π have been observed in different receptor types (Martin *et al.* 2002). In normal cell functioning, the inhibitory neurotransmitter, GABA (agonist), binds to a receptor protein subunit on the chloride channel, causing it to open. When the channel opens, negatively-charged chloride ions flow into the nerve or muscle cell, down an electrical potential gradient (Ackerman and Clapham, 1997). This flow of ions lowers the electrical potential of the cell decreasing the frequency of neural firing in response to other stimuli (Chalmers, 1993). Benzodiazepines (Bz) act synergistically with GABA (Figure 1.3b) to increase the effect of agonist-mediated activation of the receptor (Martin *et al.* 2002).

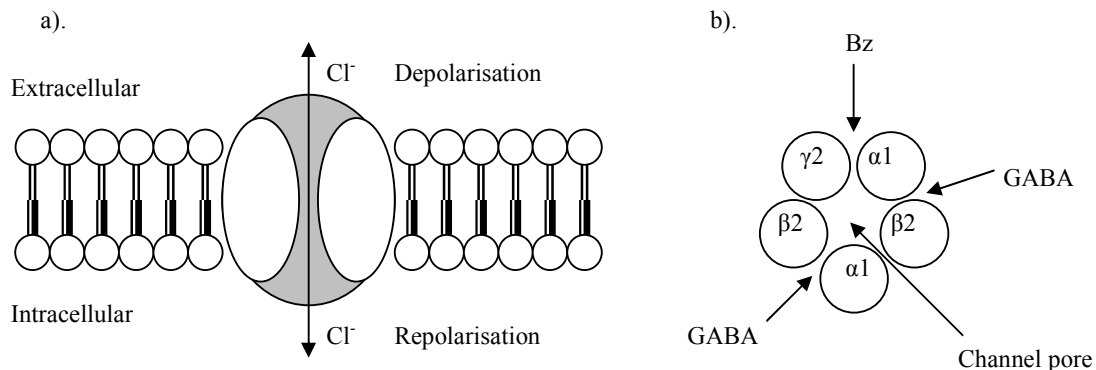


Figure 1.3. Chloride ion channel and GABA receptor. **a).** Sectional view of a chloride ion channel showing the pathway of chloride ions in and out of the cell (adapted from Ackerman and Clapham, 1997). **b).** Plan view of a GABA_A receptor indicating the pentameric orientation of the subunits (adapted from Martin *et al.* 2002).

The channel opening time and frequency are dependent on the concentration of agonist and antagonists present (Martin *et al.* 2002). The GABA receptor can be blocked by the antagonist, picrotoxin, non-competitively (Martin *et al.* 2002). In susceptible insects, numerous insecticides including the cyclodiene dieldrin, target chloride channels of the

nervous system, blocking them, preventing any flow of chloride ions as well as antagonizing the inhibitory neurotransmitter GABA (Hemingway *et al.* 1998; Hemingway and Ranson, 2000). The expected neuronal target of dieldrin is the picrotoxin binding site on GABA_A (Feyereisen, 1995). Death is therefore caused by prolonged, uncontrolled firing of nerve cells (hyper-excitation), convulsions and paralysis (Tanaka *et al.* 1984; Chalmers 1993; Bloomquist 1999; Payne *et al.* 2001). Insects or specifically mosquitoes can become resistant to dieldrin mainly through two mechanisms, target-site insensitivity or metabolic resistance.

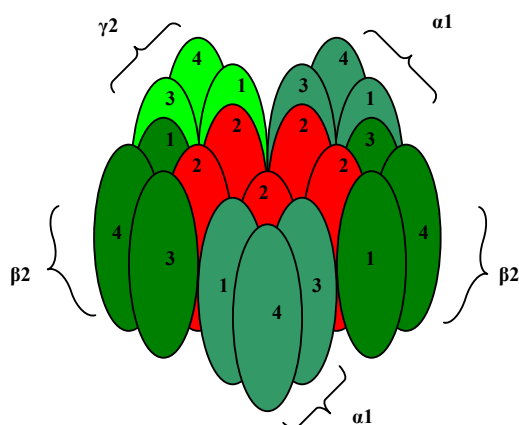


Figure 1.4. Structure of a GABA receptor, showing the five distinct subunits composed of four proteins each (No. 1-4). The proteins represented in red, line the channel pore and it is within these structural proteins, where the conformational change occurs, due to a mutation (adapted from Martin *et al.* 2002).

1.6.2 Target - Site Insensitivity

Target-site insensitivity or target-site resistance was first characterised in cockroaches (Kadous *et al.* 1983) and later in mosquitoes and houseflies (Tanaka and Matsumura, 1986). The cyclodiene resistance gene is known as *Rdl* (resistant to dieldrin) and codes for a subunit of a GABA gated chloride ion channel which contains a novel receptor subtype (Thompson and Steich, 1993). This resistance locus consists of 9 exons. The seventh exon contains an alanine residue, first described as alanine³⁰² in *Drosophila*

melanogaster (Ffrench-Constant *et al.* 1994). In *D. melanogaster* and other subsequent insect species (Thompson and Steich, 1993; Ffrench-Constant *et al.* 2000; Hemingway and Ranson, 2000), a single base substitution, replacing the alanine with a serine, in the second membrane spanning region of the channel subunit, lining the ion pore, has been associated with cyclodiene resistance (Feyereisen, 1995) (Figure 1.5).

Resistance to dieldrin in anophelines is inherited in a Mendelian fashion with evidence of two resistance alleles assorting at the same locus, one dominant and the other co-dominant (Davidson, 1956; Hemingway *et al.* 1998; Hemingway and Ranson, 2000). In resistant individuals, the alanine residue (alanine²⁹⁶ in *An. gambiae*), found in the chloride ion channel pore of the protein is replaced by a glycine residue (Du *et al.* 2005). Thus resistance is conferred by the replacement of a single amino acid in the chloride ion channel pore of the protein. This mutation leads to insensitivity towards blockage caused by dieldrin allowing for the maintenance of normal chloride channel function when exposed (Kadous *et al.* 1983; Feyereisen, 1995; Ffrench-Constant *et al.* 2000).

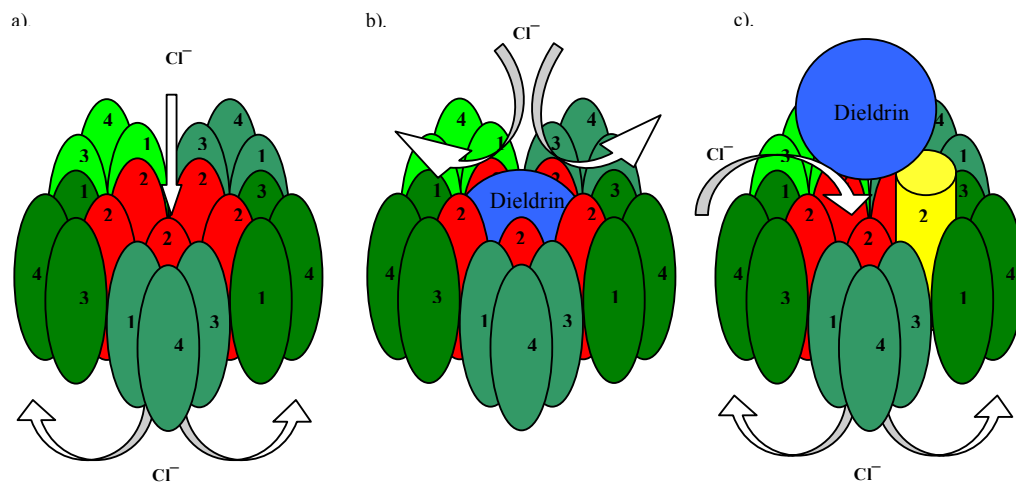


Figure 1.5. Structure and functioning of a GABA receptor. **a).** A GABA receptor indicating normal chloride channel functioning. **b).** A dieldrin molecule blocks the chloride ion channel pore, preventing the flow of chloride ions into or out of the cell in a susceptible individual. **c).** An insensitive GABA receptor with a mutation caused by target-site resistance allowing normal channel functioning (adapted from Martin *et al.* 2002).

1.6.3 Metabolic Resistance

Metabolic-based resistance mechanisms are important in conferring insecticide resistance by changing the rate at which the insecticide is detoxified (Hemingway *et al.* 1998). Thus far, a total of three enzyme super families that are involved in the metabolism of the four major insecticide classes have been identified. These include cytochrome P450's (Bergé *et al.* 1998), the glutathione-S-transferases (GST's) (Ottea and Plapp, 1984) and the carboxylesterases (Devonshire, 1977). These enzymes act by rapidly metabolising the insecticide to non-toxic products, or by rapid binding and slow sequestration of the insecticide (Hemingway *et al.* 1998). The up-regulation of these enzymes due to gene amplification or regulatory changes in gene expression results in resistance being conferred. The up-regulation of esterases usually occurs via gene amplification. Gene amplification is a process whereby additional copies of chromosomal sequences are made (including the functional genes) (Feyereisen, 1995). These copies of the amplified gene must be transcribed and translated into functional protein products for the expression of the resistance mechanism to be successful (Feyereisen, 1995). GST's in mosquitoes most often confer resistance to the organochlorine insecticides (Prapanthadara *et al.* 1996; Yu, 1996). Changes in gene expression appear to be involved in GST's and cytochrome P450's (Feyereisen, 1995). The malaria vector *An. gambiae* carries a gene (GSTe2) that is associated with metabolic insecticide resistance (David *et al.* 2005). The gene is over expressed in individuals resistant to cyclodienes causing an overabundance in gene product which codes for an enzyme that dehydrochlorinates the insecticide (Ranson *et al.* 2001; Ortelli *et al.* 2003; Hemingway *et al.* 1998).

1.7 Rationale

Blood feeding and the subsequent reproductive success of female anopheline mosquitoes are highly complex and could be affected by many different factors. Hunt (1984) observed that resistant *An. gambiae* females that survived exposure to dieldrin were subsequently incapable of continuing normal feeding and reproductive cycles. A preliminary study showed that exposed resistant *An. gambiae* females did have a tendency to feed to repletion numerous times, but fecundity was generally decreased (Norton *et al.* 2008). Subsequent work on *An. gambiae* showed that when dead resistant females were dissected, numerous oocytes in different stages of development were present. This indicated that females had all developed oocytes but most died before they could oviposit the eggs. Possible changes in the reproductive biology of the female mosquitoes after exposure may have important implications for vector control, as a reduced egg yield in resistant females means lower vector population numbers and therefore decreases the likelihood of malarial transmission.

1.8 Aim

A fitness assessment comparing fecundity, fertility and longevity in exposed dieldrin resistant malaria vectors has never been reported. This study aims to assess the effect of dieldrin exposure on fitness, by measuring fecundity, fertility and longevity of resistant *An. gambiae* homozygous (*Rdl/ Rdl*) or heterozygous (*Rdl/ S*) females. Fecundity from here on will refer to the number of eggs produced by an individual female. In this study, fecundity has been divided into ‘total fecundity’ and ‘ovipositional fecundity’. These terms refer to the total amount of eggs present in the ovaries in addition to those oviposited (total fecundity) and the number of eggs oviposited (ovipositional fecundity).

Fertility will refer to the number of eggs that hatch. These parameters will be measured to identify the reason/s behind the reduced fecundity that has been observed previously in this mosquito species.

1.9 Objectives

- 1.9.1. Observe the effect of dieldrin exposure on fecundity, fertility and longevity of a resistant wild strain of *An. gambiae* (COGS).
- 1.9.2. Test the effect of dieldrin exposure on the spermatozoan fertility in a dieldrin resistant laboratory strain of *An. gambiae* (GAH).
- 1.9.3 Identify and observe the effect of dieldrin exposure on the different stages of oocyte and ovary development in dieldrin resistant strain (GAH).
- 1.9.4. Observe the effect of dieldrin exposure on fecundity, fertility and longevity in a dieldrin resistant laboratory strain (GAH).

Chapter 2

Dieldrin exposure of F₁-progeny of wild-caught *Anopheles gambiae* from West Africa and optimisation of experimental techniques.

2.1 Introduction

A pilot study was carried out on a population of *Anopheles gambiae* mosquitoes from a group of F₁-progeny originating from 25 wild-caught females from the Republic of the Congo. This wild material was also used in a larger study (see Koekemoer *et al.* 2011) and therefore only 25 wild-caught individuals were available for this preliminary investigation. The F₁-progeny of the females (COGS- Wild resistant *An. gambiae* population) were used, as these offspring would be genetically similar to wild-caught individuals and indicative of resistance present in the wild populations. It is important to remember that even though there were hundreds of progeny, they only represented 50 wild individuals (including the males they mated with) and therefore the genetic diversity was relatively limited. The supporting argument however, is that these individuals would represent a more realistic picture of the natural environment compared to a laboratory strain and therefore the results obtained are important when investigating what is happening in the field with regards to resistance and other biological functioning in these vectors.

2.1.1 Aims

The investigation reported in this chapter aimed to test the effect of dieldrin exposure on the fecundity of wild *An. gambiae* females by observing changes in certain biological functions such as, haematin excretion frequency, oviposition, blood feeding, and longevity between unexposed and exposed female mosquitoes. The methods used were based on previous methods that have been described by other authors (WHO, 1998; Briegel

1980/1990a/1990b; Lyimo and Takken, 1993; Meadows 1968 and Freaan 2007), which are covered in the sections below, as well as novel approaches. The new methods were optimised in this chapter and used for experimentation in subsequent chapters.

The following objectives were investigated:

1. Bioassay data from the F₁ - progeny were collected to determine susceptibility to dieldrin .
2. Blood feeding frequency and excretion were monitored and compared between unexposed and exposed cohorts.
3. The effect of dieldrin exposure on fecundity, fertility and longevity were measured.

2.2 Materials and Methods

2.2.1 Background of the Colonies

Two *Anopheles gambiae* strains were used in the experiments. These strains were maintained in the Botha de Meillon insectary of the Vector Control Reference Unit (VCRU), at the National Institute for Communicable Diseases (NICD) situated in Sandringham, Johannesburg, South Africa. The COGS (Resistant wild *Anopheles gambiae* population) was established at the beginning of the study from a wild-caught sample of females collected in March 2009 from Pointe Noire, Republic of the Congo (4°40'31S; 11°58'14E). This colony was maintained for use as a representative field population. However, the resistance of COGS to dieldrin was reduced due to the lack of insecticide selection pressure. Dieldrin resistance in this colony has been attributed to target-site resistance (Rdl and L1014F/ L1014S kdr) (Koekemoer *et al.* 2011). The colony was subsequently discarded from further experimentation, as only females that survived exposure to dieldrin were required. The data obtained from the F₁ generation in this chapter however, were valuable in designing and further optimising subsequent experimental procedures. SUA (Susceptible laboratory *Anopheles gambiae*) was established from wild specimens obtained in Liberia in 1996 (with no insecticide exposure history). This colony was maintained in the insectary with no insecticide selection pressures and used as a dieldrin-susceptible reference control strain for the bioassays, to confirm the efficacy of the insecticide-treated papers.

2.2.2 COGS

2.2.2.1 F₁-Progeny

The eggs of 25 *An. gambiae* females were pooled together and allowed to hatch. The larvae were fed on a constant diet of ground dog biscuits (BEENO®) and brewer's yeast.

To prevent larval density from affecting the developmental rate, the larvae were split up into smaller groups and placed into larger bowls (from 2 litres to 10 litres) as they grew in size. The pupae were left to emerge and the adults were sexed within the first 12 hours after eclosion to prevent mating from occurring before exposure. Males and females were placed into separate cages and provided with a 10% sucrose solution as a source of nourishment and to prevent dehydration.

2.2.2.2 *F₈-Progeny*

The *F₈*- progeny of this strain were only used to test if dieldrin resistance was being maintained in the laboratory environment. All methods used were the same for the *F₁*-progeny.

2.2.3 *Dieldrin Exposure Bioassays*

Resistance to insecticides in *An. gambiae* is a much more common occurrence in present times than it has been in the past. When working with insect vector populations it is necessary to confirm the resistance profile (types and concentrations of insecticides affecting the test subject) for the organism being used, so that future control methods are carried out using informed decisions about the type of insecticides to be used and which are appropriate. In the case of malaria vectors, insecticide susceptibility bioassays are performed to obtain susceptibility or resistance data that determines how resistant or susceptible a mosquito population is.

Cohorts of four-day-old males and females were exposed to 4% dieldrin separately in same sex exposures according to the World Health Organisation protocol for testing insecticide resistance in adult anophelines (WHO, 1998). Groups of 25 mosquitoes of the same sex

were aspirated into WHO holding tubes (Figure 2.1) and allowed one hour to reach equilibrium with the holding tube environment. The mosquitoes were then exposed to insecticide treated papers for one hour. The knock-downs were recorded every five minutes for the first 20 minutes and every 10 minutes for the rest of the hour (a knock-down indicates a mosquito that is observed to be susceptible to the insecticide at the time of exposure and unable to maintain normal biological function). The mosquitoes that fell to the bottom of the bioassay tubes, unable to get back up, were scored as “knock downs”. The mosquitoes were held in the holding tubes to recover with access to a 10% sucrose solution and the 24 hour mortality was recorded the following day.

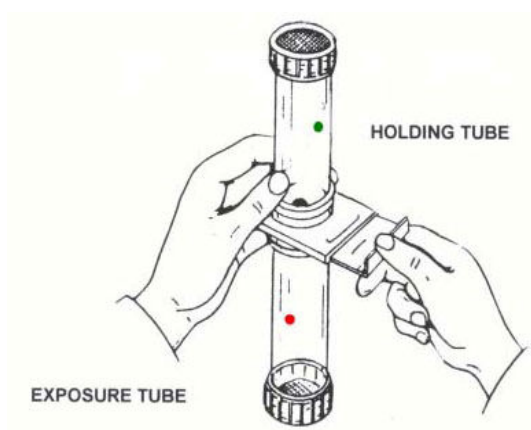


Figure 2.1. Apparatus used for performing bioassays (WHO, 2002). The holding tube (top) is connected to the exposure tube (bottom) by a slide. The slide is moved out to allow the mosquitoes to be blown through to the exposure tube and then closed. After a one hour exposure, the mosquitoes are blown out of the exposure tube and back into the holding tube where they remain for 24 hours.

2.2.4 Combining of Survivors and Mating

Male and female survivors of the exposure (section 2.2.3) were combined into five litre cages and provided with a 10% sucrose solution as previously described. An unexposed control cohort of the F_1 -progeny was maintained in parallel with the exposed cohort. The mosquitoes were given one week for mating to occur, allowing a large percentage of the females to be inseminated.

2.2.5 *Blood feeding*

The females in the cages (one week post exposure) were allowed to blood feed to repletion on anaesthetised guinea pigs, as is routinely done for all the mosquito colonies under the supervision of the NHLS animal unit and their ethics committee,. Repletion refers to the voluntary cessation of feeding by a female. Only females that had taken their first blood meal were used in the subsequent experiments, as the blood meal initiates the development of the primary oocytes. Replete feeding was confirmed by capture of the individual in the glass tube of an aspirator and illuminated from behind with a flashlight. The transmitted light shows up the blood meal contained within the midgut of the abdomen, which is observed as being transparently red and distended.

2.2.6 *Haematin excretion frequency*

Directly after the first blood meal each female was placed into a separate oviposition vial. The vials contained a moist piece of filter paper shaped to fit the vial. This ensured that the female could rest comfortably on the side as well as oviposit as naturally as possible. The filter paper was kept moist at all times with distilled water. After digestion of the blood meal, the female mosquito deposits droplets of excreta in the form of haematin and urea (Briegel, 1980). This was observed as a brown discolouration on the filter paper. The filter papers of each vial were monitored daily and the total frequency of excretion was recorded for the entire longevity of each female. Each filter paper was changed after excretion so that subsequent excretion events could be verified and counted.

2.2.7 *Oviposition and fecundity*

The females in the oviposition vials were fed three times a week for the duration of the experiment. The success of feeding was monitored using the technique described

previously, with females containing any blood, being recorded as “fed” and females showing no trace of blood, as “unfed”. The number of blood meals taken per female was recorded for both the unexposed and exposed cohorts.

Any eggs that were oviposited on the filter paper were transferred into a small round larval bowl containing 250 ml distilled water and counted. All egg batches were kept separate from each other, including those from females that oviposited more than once (indicating more than one gonotrophic cycle) to record the success of hatching. The oviposition frequency for each female was also recorded.

Ovipositional fecundity in the unexposed and exposed cohorts was measured by counting the number of eggs oviposited per female and total fecundity as the total amount of eggs matured per female. The latter measurement was obtained by dissecting out any retained eggs from the ovaries of the females after they had died and adding this number to the amount oviposited per female.

2.2.8 *Body size*

Body size has been shown to be directly proportional to fecundity in *Anopheles gambiae* (Briegel, 1990a) and wing length has been shown to be directly correlated with body size (Lyimo and Takken, 1993, Briegel, 1990b). This study has therefore used wing length as an indicator of body size. The wing length of each female was measured by taking the best wing specimen and placing it onto a slide using clear mounting fluid. Once dried, the specimen was further covered in a thin layer of varnish to ensure a durable finish. The wing lengths were measured at 200 x magnification, using an eyepiece micrometer mounted on a dissecting microscope (Wild, Heerbrugg, M5-71661).

2.2.9 Longevity and post-mortem dissections

The longevity of each female was recorded as the number of days from emergence to death. Post-mortem dissections of the females were performed to observe the ovarian development and to count any eggs still present in the ovaries. The ovaries were dissected out in phosphate buffered saline (PBS) following methods described by Meadows (1968) and transferred to a drop of distilled water for photography. Using a fine dissecting needle, the ovaries were torn open to release the individual oocytes which were organised into a single layer on top of the microscope slide for easy counting. No photographic equipment was available for the dissecting microscope at the time of research and therefore the photographs were taken using a Sony Ericsson K810i and W995 through the ocular lens of a dissecting microscope following the procedure described by Frean (2007). The number of retained eggs was counted using the acquired jpeg image and analySIS® LS Research Soft imaging system by Olympus soft imaging solutions, Münster, Germany.

2.2.10 Data Analysis

All statistical data analyses were performed using Statsoft Statistica 8.0 analytical software (Tulsa, Oklahoma, USA). The bioassay results of the exposures to 4% dieldrin were represented in a bar graph and two-sample t-tests for independent samples were performed between the cohorts of first and eighth generations.

Haematin excretion frequency was plotted as a bar graph and analysed using a two - sample t - test. The correlation of haematin excretion frequency with blood meal numbers was plotted as a regression line graph. The number of individuals within a specific blood meal group and correlated excretion frequency was represented using a frequency plot.

Overall fecundity was represented in a bar graph and a one-way analysis of variance (ANOVA) was performed. An LSD post-hoc test was performed to test for significance between the ovipositional and total fecundity of the unexposed and exposed cohorts.

The wing lengths were placed into groups of significantly different sizes to prevent the effect of size from influencing with observed fecundity however, there were no significant differences between the recorded fecundity and therefore it was decided to rather combine the fecundity data. The sample sizes for each wing length group were also too small for an analysis of variance to be carried out and were therefore represented as the number of individuals for each wing length group.

The longevity was represented in a bar graph and significance testing was performed using a two-sample t-test for dependent (correlated) samples.

2.3 Results

2.3.1 Bioassays

The results of dieldrin exposure to the F₁-progeny indicated a low percentage mortality in both the male and female cohorts (Figure 2.2). The females however, showed slightly more variation than the males. This susceptibility test indicated that there was far less than 80% mortality for the F₁-generation, which according to the WHO indicates resistance when testing with discriminating doses (WHO, 1998).

The dieldrin resistance was observed to rapidly decrease after the subsequent colonisation of these mosquitoes. The males and females of the F₁-progeny displayed a mean mortality of 25.0 ± 2.4 % and 22.2 ± 8.4 % percent after 24 hours respectively. Susceptibility testing

was performed on the 8th generation of colonised COGS progeny and was observed to increase dramatically (Figure 2.2). The female cohort showed 82.6 ± 4.6 % mean percentage mortality and the male cohort, 70.6 ± 4.6 % mean percentage mortality. These results indicate a significant difference in resistance after colonisation in both the males ($t_{(4; 75)} = -12.478$; $P < 0.01$) and females ($t_{(3; 75)} = -10.933$; $P < 0.001$). The females of the F_8 generation were recorded to have a significantly higher mortality than the males of the same generation ($t_{(4; 75)} = -12.478$; $P < 0.01$).

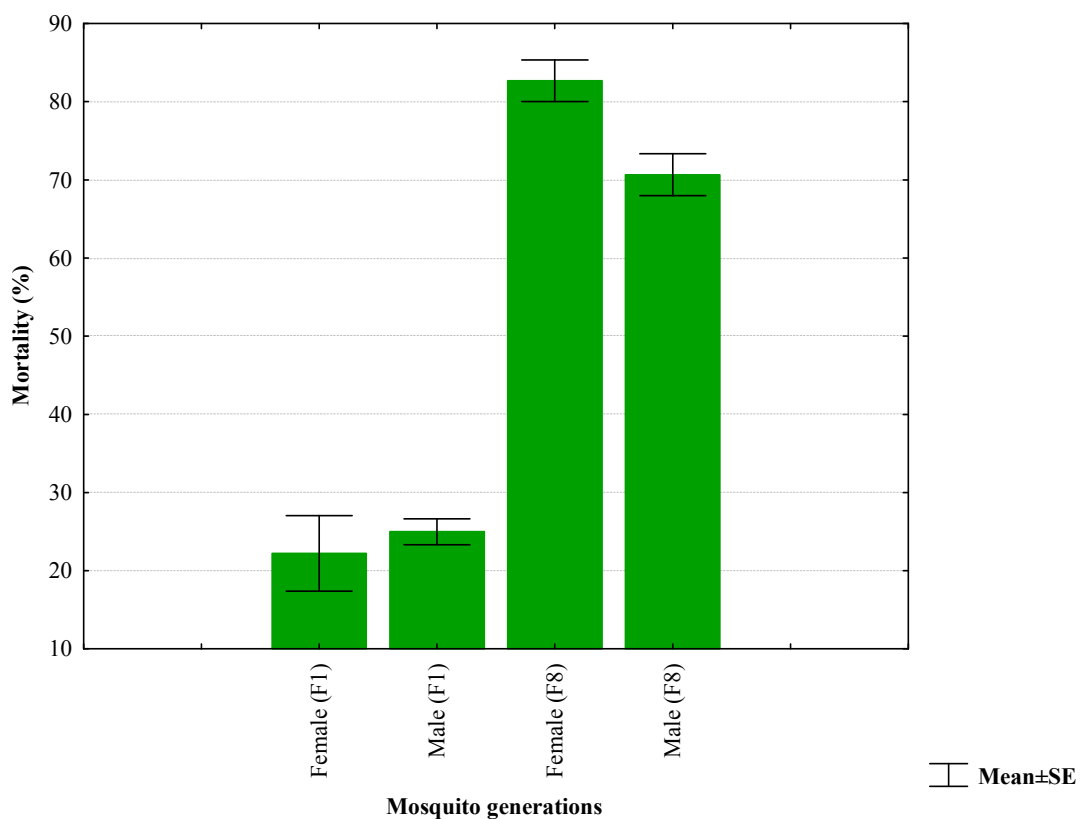


Figure 2.2. The mean percentage mortality of F_1 *An. gambiae* (COGS), exposed to 4% dieldrin for one hour. The males and females were exposed separately. The above graph shows the susceptibility to dieldrin in the F_1 -generation and the F_8 -generation. The latter was obtained from colonised material (COGS) that had not been placed under any insecticide selection pressure in the laboratory ($n=75$, per sex, per generation).

2.3.2 *Haematin excretion frequency*

The overall haematin excretion frequency (Figure 2.3) was tested for the F₁- generation. The unexposed group averaged 4.08 times per lifetime and the exposed group averaged 3.66 times per lifetime indicating no significant difference between the two groups tested ($t_{(59; 60)} = 0.78$; $P > 0.05$).

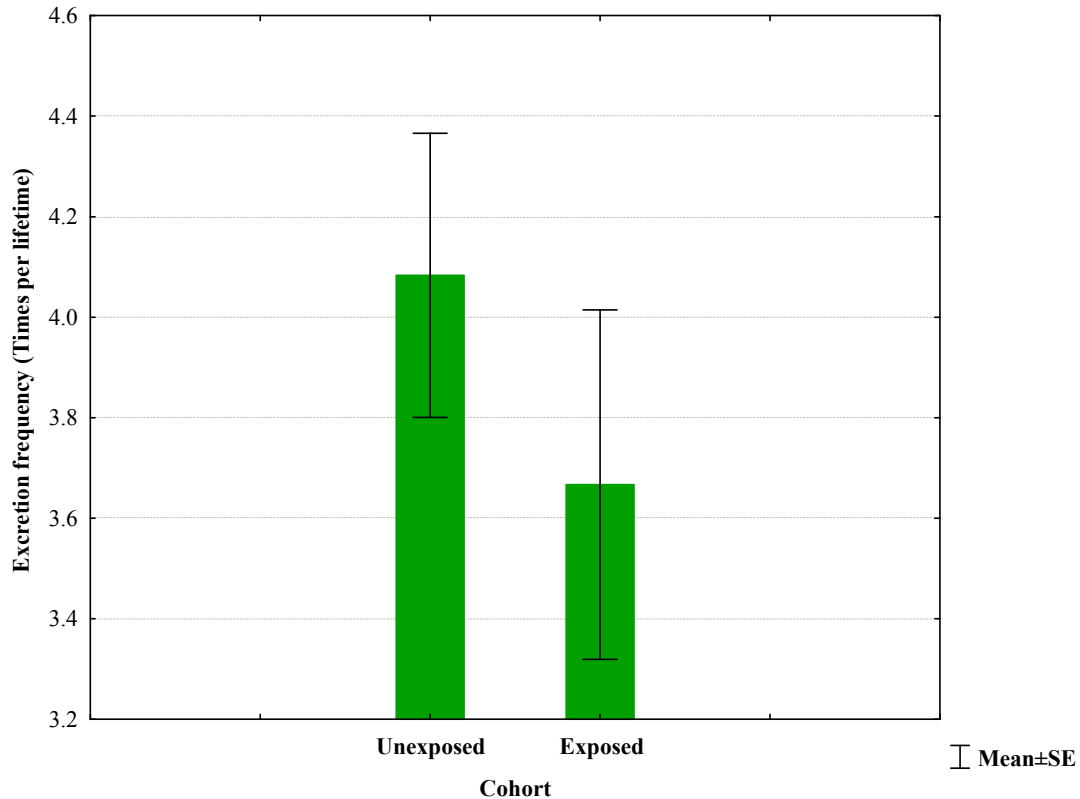


Figure 2.3. Excretion frequency observed in the unexposed and exposed F₁ cohorts of COGS.

There was a positive correlation of excretion frequency and blood meal number for both the unexposed and exposed groups (Figure 2.4), indicating that excretion positively correlates to the number of blood meals taken. The largest proportion of females (as indicated by the blue oval) were found to imbibe four to five blood meals in the unexposed cohort and three to four blood meals with similar corresponding excretion frequencies for the exposed cohort. The excretion frequency corresponded with the blood feeding activity.

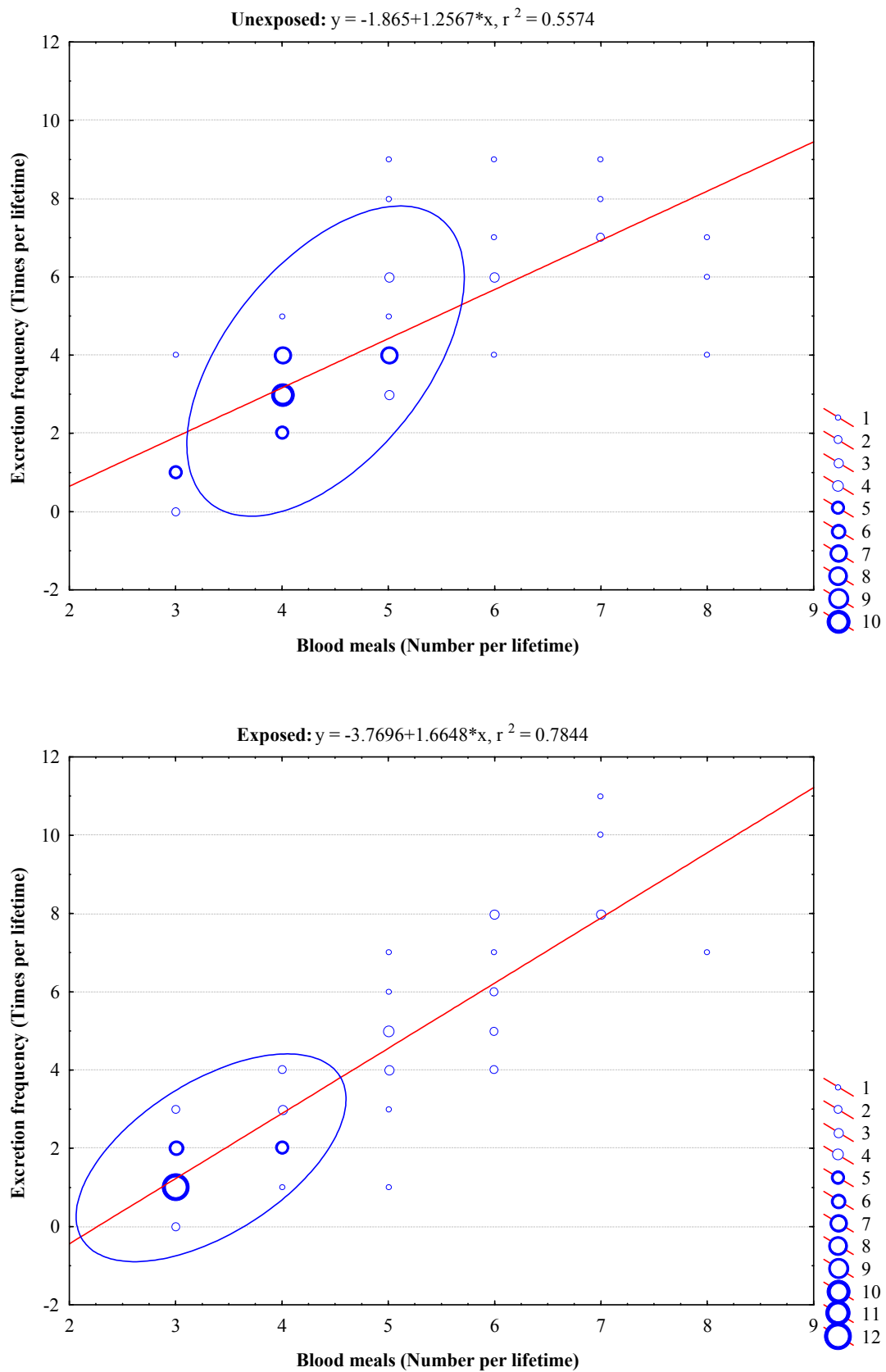


Figure 2.4. Blood meal frequency compared with excretion frequency in the unexposed (top) and exposed (bottom) cohorts. The numbers of females that occur in each group are represented by the frequency key on the right.

2.3.3 Fecundity and fertility

The number of eggs oviposited, retained and the total number of eggs matured (combined number of retained and oviposited eggs) are represented in Figure 2.5 as fecundity. The unexposed and exposed cohort both produced very similar numbers of eggs in total. In the unexposed cohort, 11.6 % of females oviposited while 62 % of the females matured eggs but never oviposited. The exposed cohort showed similar results with 6.7 % of females ovipositing and 62 % of females having matured eggs.

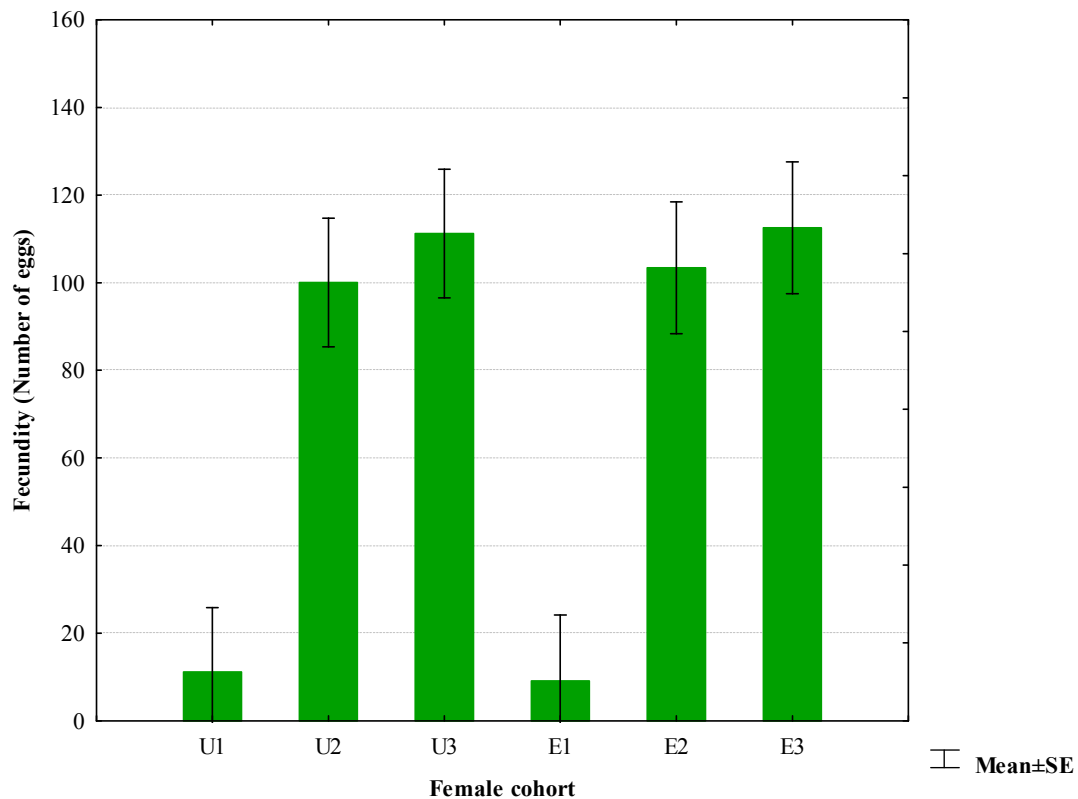


Figure 2.5. Number of eggs oviposited (1), retained (2) and total number produced (3) by unexposed (U) and exposed (E) females. (n = 60; unexposed), (n = 60; exposed).

The unexposed group oviposited significantly less eggs than were matured ($(F_{3, 89}) = 1.912$; $P < 0.05$). The exposed group also oviposited fewer eggs than were matured but the

difference was not significant ($(F_{3, 89}) = 1.912; P > 0.05$). Overall, the average number of retained eggs and the total number of eggs matured by females from both cohorts was very similar and only the ovipositional fecundity differed. These results are representative fecundities from females of all wing length sizes. The measurement of fertility of the eggs oviposited by both groups of females could not be calculated as none of the eggs hatched in either of the cohorts and mating status was not confirmed. During the post-mortem dissections the percentage of females that had matured secondary oocytes was recorded as 16.66% for the unexposed group and 13.13% for the exposed group.

2.3.4 Wing length measurements

Figure 2.6 shows a wing from a female *Anopheles gambiae* indicating how the measurement of wing length was performed. Figure 2.7 gives the wing sizes of all the females from the F_1 – progeny, divided up into significantly different groups. The ranges of wing lengths of the unexposed and exposed cohorts were found to contain 9 groups that were significantly different in size when compared ($(F_{16, 68}) = 91.533, P < 0.01$).

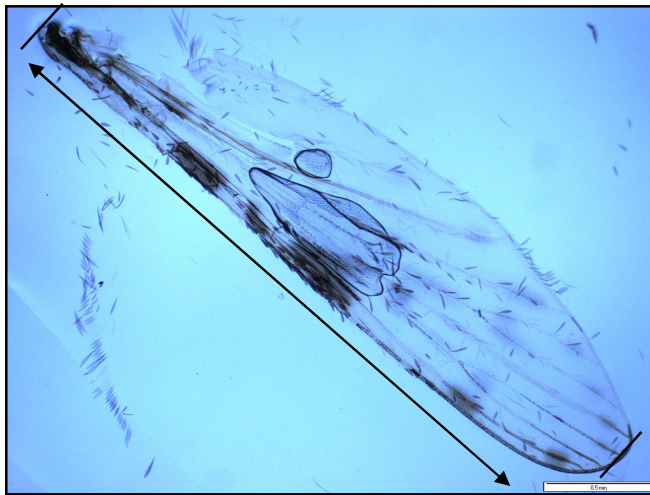


Figure 2.6. A wing specimen from *An. gambiae* glued to a slide for measurement. The distance between the two black lines at the very ends of the wing was measured.

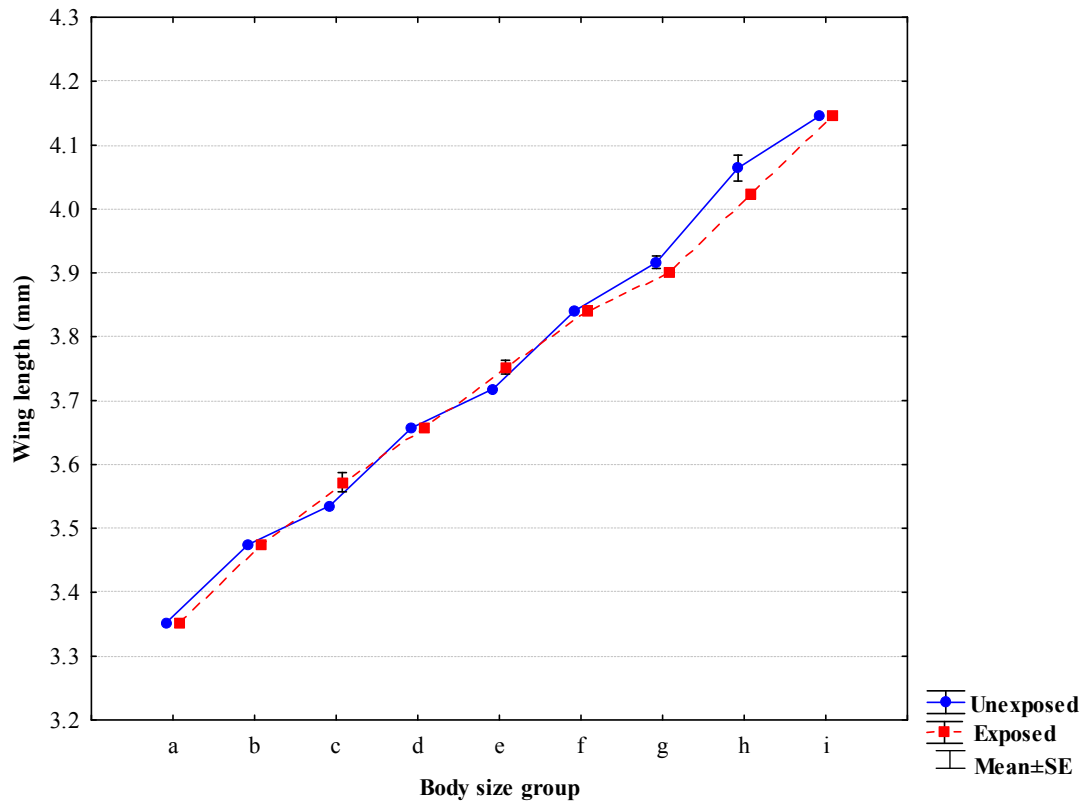


Figure 2.7. Wing lengths of female mosquitoes that were significantly different from each other. The y-axis indicates wing length which is an indirect measure of body size and the x-axis represents the different body size groups (based on wing length). a = 3.3-3.4 mm; b = 3.4-3.5 mm; c = 3.5-3.6 mm; d = 3.6-3.7 mm; e = 3.7-3.8 mm; f = 3.8-3.9 mm; g = 3.9-4.0 mm; h = 4.0-4.1 mm and i = 4.1- 4.2 mm.

2.3.4.1 Fecundity according to wing length

The number of individuals recorded for each wing length of the unexposed cohort of COGS are presented in Figure 2.8 along with the ovipositional and total fecundity. The distribution of females is very close to normal as shown by the slightly skewed bell curve. The ovipositional fecundity was negatively correlated with wing length ($R^2 = 0.036$; $P > 0.05$). This is a very weak correlation and is probably due to the low sample sizes recorded. The total fecundity shows a stronger positive correlation with wing length ($R^2 = 0.380$; $P > 0.05$) but no significant differences were observed. The figure also indicates that a large proportion of the eggs that were matured were not oviposited. A higher proportion of of the

eggs were matured by the larger females however, the smaller females were recorded to oviposit more eggs.

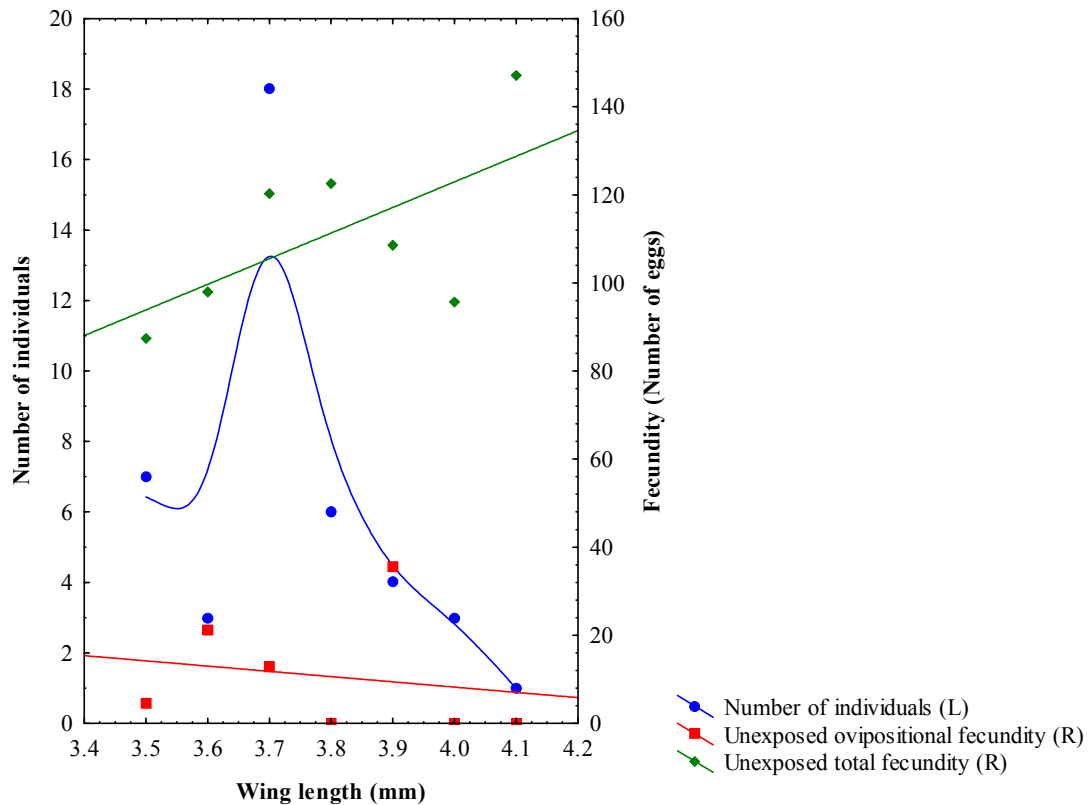


Figure 2.8. The number of individual females recorded for each wing length and the corresponding ovipositional and total fecundity data for the unexposed cohort of *An. gambiae* COGS F₁- females.

The exposed cohort of COGS (Figure 2.9) was observed to have a right skewed distribution of individuals within the wing length groups indicating that there was a higher number of larger individuals present within this cohort. The ovipositional fecundity was observed to be weakly correlated with body size ($R^2 = 0.017$; $P > 0.05$) but indicated that hardly any eggs were oviposited by individuals in any of the groups at all. The total fecundity recorded for the females showed a much stronger positive correlation with wing length ($R^2 = 0.2$; $P > 0.05$).

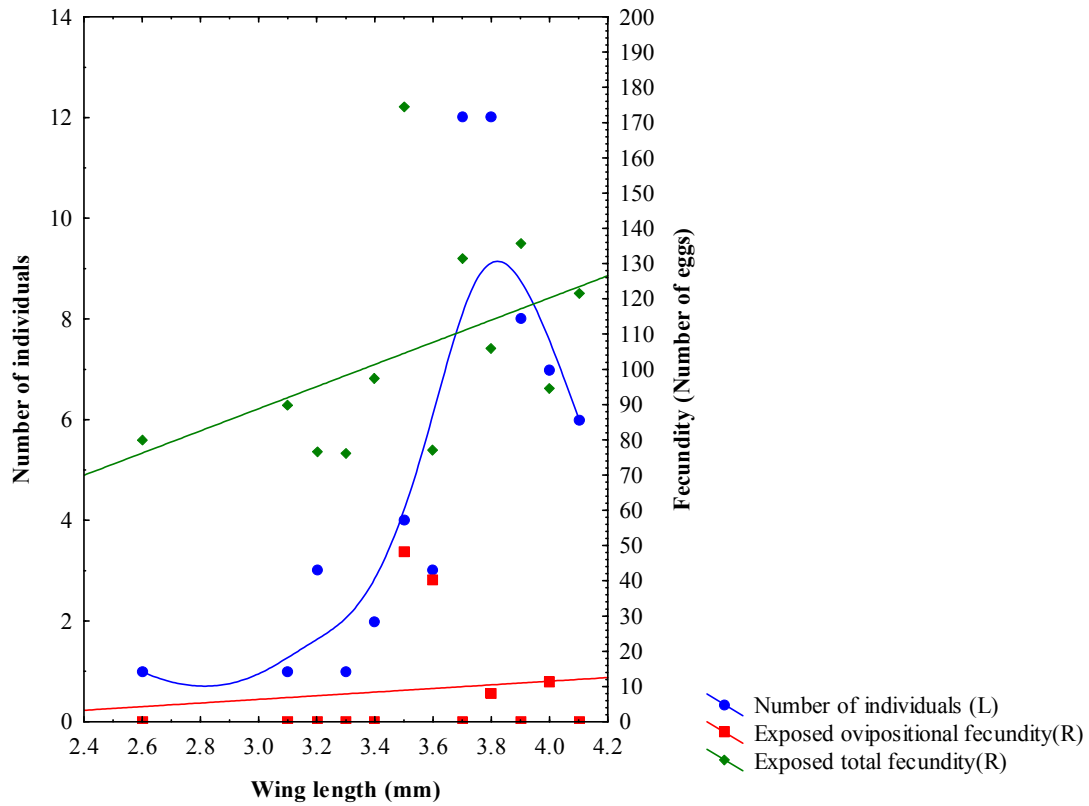


Figure 2.9. The number of individual females recorded for each wing length and the corresponding ovipositional and total fecundity data for the exposed cohort of *An. gambiae* COGS F₁- females.

2.3.5 Longevity

The mean longevity of the unexposed females (Figure 2.10) was longer than the exposed females but not significantly ($t_{(59; 60)} = 1.912$; $P > 0.05$). It can be observed from the graph that the mean difference is roughly three days.

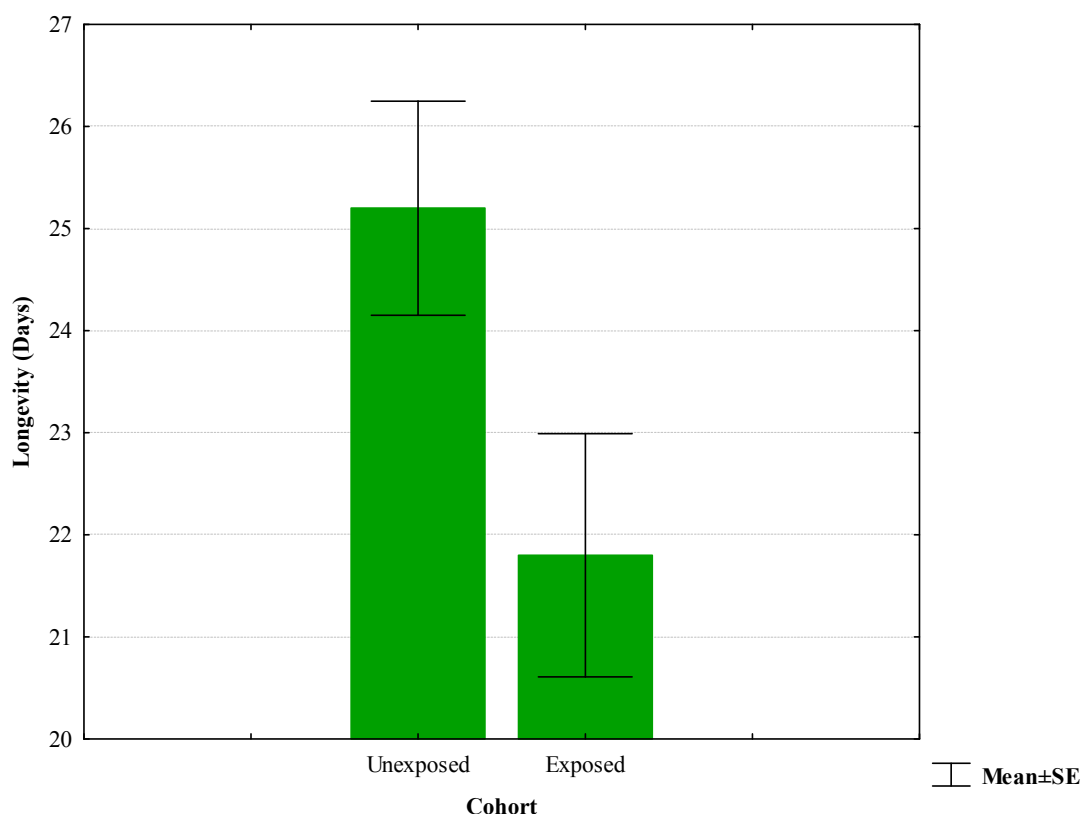


Figure 2.10. The mean longevity of individual females from unexposed and exposed cohorts (n = 60 for each cohort).

2.4 Discussion

The bioassay data were clearly indicative that the COGS mosquito strain had rapidly lost its resistance to dieldrin by the 8th generation due to a lack of insecticide selection pressure combined with the bottleneck effect of colonisation. The low mortality levels recorded initially, indicated the strain to be resistant. After eight generations of colonisation dieldrin mortalities were significantly higher than those recorded in the F₁-generation. There was a significant difference in mortality observed between the males and females of the different generations. The males showed slightly less mortality in the 8th generation. Normally the opposite is observed (WHO, 1998), with females showing higher resistance than the males, hence the standard protocol for exposing adult female anophelines (females are also more

important as they are the vectors, not males) (WHO, 1998). This difference may therefore be attributed to the fact that these mosquitoes were from a wild population and may display a larger percentage of heterogeneity within the group, validating the disparity. Another possibility is that the resistance genes have drifted out of the population at a faster rate in the females than the males.

The dieldrin exposure results indicate that the mosquitoes of the COGS strain were resistant to dieldrin under possible selection pressure in the wild, however when in the laboratory, under no insecticide selective pressures, the resistance was almost completely lost. It is possible that the resistance mechanism was placing a fitness cost on this strain when in the insectary and it may have therefore been selected against causing the reduction in resistance that was observed. The dieldrin resistance in COGS has subsequently been attributed to reduced target site sensitivity (*Rdl*) (Koekemoer *et al.* 2011), although a metabolic mechanism cannot be excluded in the current study. There is a possibility, that even though dieldrin was banned for commercial use in Africa in 1992, it is still being used illegally. Stock piles of dieldrin that have not been destroyed make easy targets for people who could sell it in local markets (Wandiga, 2001). The fact that the resistance was lost in such a short space of time ($\pm$ 16 weeks) indicates that dieldrin (or the related chemical, Fipronil) may still be in use in The Republic of the Congo.

The significant difference observed in the low number of eggs oviposited by the unexposed females was observed as egg retention when the dissections were performed. These females were maturing their oocytes but were dying before ovipositing their eggs. This egg retention was not brought on by premature death as the average longevity for the unexposed group was 25 days, more than enough time for a female to oviposit all her eggs

(Figure 2.10). The differences in egg numbers due to egg retention observed in the exposed cohort were not significant when compared to the unexposed cohort, however, still indicated that something was preventing the females from ovipositing. There is the possibility that some females could have drowned on the water before ovipositing all of their eggs. Daily observations that were made, do not support this theory completely, as a number of the females were found dead clutching to the side of the filter paper away from the water. The mating status of these females was not confirmed, so the absence of oocyte fertilisation cannot be ruled out as a possibility for reduced fecundity or egg retention.

The most important aspect of this experiment was the effect of dieldrin exposure on the fecundity of the females. It has been shown by Lyimo and Takken (1993) that body size is directly proportional to fecundity in *An. gambiae*. Due to natural variation, not all females are the same size and therefore the effect of these different body sizes would have an impact on the observed fecundity and therefore the effect of exposure alone, would be compromised. The large amount of variation observed in both total and ovipositional fecundity is indicative of possible instability and variation within the mosquito population or due to the small sample size for some wing length classes. Likely explanations for this could be the natural heterogeneity found within populations of individuals. This wild population may have a high frequency of variant alleles causing the observed variance (Lewontin and Hubby, 1966).

Variation in a natural population allows for the “survival of the fittest”, which means that those mosquitoes that have naturally occurring resistance due to random genetic mutations, will stand an increased chance of survival after insecticide exposure. Compared to an older more homogenous laboratory strain of mosquitoes, a wild strain may not be adapted to

such controlled conditions and therefore the altered traits present in these individuals may be amplified and observed in an easily altered fitness trait like fecundity.

The ovipositional fecundity for the unexposed cohort was not expected as the negative correlation with body size does not make sense. A possible reason for this may be attributed to the mosquitoes adapting to the laboratory environment. All other fecundity values from the unexposed and exposed cohorts followed the normal biological trend of increased fecundity with body size (Briegel, 1990b). The lower longevity observed in the exposed cohort is indicative that the exposure may have affected the females. Decreased longevity after exposure to any form of pesticide was an expected result as these chemicals are designed to harm the target organism and ultimately kill it. The longevity observed for the exposed cohort when compared to the unexposed cohort indicated the extent of resistance in these mosquitoes and their subsequent resilience to unfavourable conditions. Bielza et al., 2008 explain that insecticide-resistant strains of insects often show reduced fitness compared with susceptible ones in the absence of selection pressure and this is a perfect example.

2.5 Recommendations for further experiments

The above mentioned results indicate that there are a multitude of variables that need to be considered for future experimentation.

- It is essential that all variables having any significant impact on fecundity were controlled.
- Factors known to affect fecundity of these mosquitoes are body size (Briegel, 1990a), teneral reserves (Briegel, 1990b), source of blood meal (Briegel, 1985), blood meal size (Lea *et al.* 1956) and the mosquito age and gonotrophic status (Hogg and Hurd, 1995a).

2.5.1 Body size

- Body size has a direct impact on fecundity
- Due to the small sample sizes obtained however, these data were more appropriately presented ungrouped and the overall trends analysed.

2.5.2 Teneral Reserves

- The larvae were reared in such a way that larval density did not permit overcrowding and that the larvae had ample food.
- The larvae were carefully monitored for growth at the different instar stages and transferred into larger bowls as they grew bigger, to avoid an overcrowded environment which would be detrimental to further life stages as described in section 1.2.1.

2.5.3 Source of blood meal, blood meal size and number

- Only the first gonotrophic cycles were looked at in subsequent experiments and the females would be given a maximum of two replete blood meals to synchronously mature their primary batch of oocytes.
- Adult females were allowed access to a controlled blood source (anaesthetised guinea pig) for 30 minutes, feeding to repletion within this time period.
- One blood meal was regarded as the minimum vital requirement for further experimentation on any given female to ensure that anautogenous oocyte development occurred.
- A second blood meal was offered three days later to ensure that all the females were satisfactorily engorged (full of blood) with enough blood to mature their primary oocytes.

2.5.4 Mosquito gonotrophic status and age

- The newly emerged females naturally, were nulliparous having not matured any egg batches prior to the experimentation.
- Performing all experiments on females in the first gonotrophic cycle meant that the number of blood meals were controlled for and the effect of dieldrin exposure was therefore tested on the fecundity only, during the first gonotrophic cycle.

2.5.5 Frequency of excretion

- Instead of measuring excretion frequency, the concentration of haematin within the faeces was measured (Briegel, 1980). This new data set provided more insight into the blood meal utilisation of the mosquitoes.

2.5.6 Ovary and oocyte development

- To observe the development of the oocytes within the ovaries of the females and also record any dissimilarity between the unexposed and exposed cohorts of females, ovarian dissections were performed.
- After a blood meal, the female mosquito rests while the oocytes develop. The development takes about 72 hours, during which time the female digests the blood meal (Clements, 1992).
- To observe this development at different stages of maturation it was decided that dissections performed at certain intervals after the blood meal, would be suitable.
- The dissections were performed after one or two blood meals to observe if any difference in development of the primary oocytes arose, between the number of blood meals after exposure to dieldrin.

2.5.7 *Insemination status*

- The insemination status of each individual female was confirmed after they died.
- To ensure that only data from inseminated females were used, each female was dissected after death to confirm mating status by looking for the presence of spermatozoa.
- Females that had not been inseminated were not included in any of the analyses relevant to fecundity or fertility.

Chapter 3

Sex-specific effects of dieldrin exposure in a resistant *Anopheles gambiae* laboratory colony

3.1 Introduction

Exposure to dieldrin has been shown to decrease the fertilisation efficiency of sea urchin sperm (Mwatibo and Green, 1997) as well as cause structural damage, specifically to the nucleus, in the sperm of earthworms (Reinecke *et al.* 1995). Picard *et al.* (2003) showed that exposure to dieldrin can alter oocyte maturation in mammals (mouse) and marine invertebrates (starfish). Dieldrin has also proven to have certain spermicidal activity and may constitute a serious hazard to octopus male fertility (Mann *et al.* 1988).

The exposure to dieldrin may therefore have negative affects on the male gametes in the testes and spermathecae of the mosquitoes, giving rise to other explanations of decreased fecundity observed after exposure, (i.e. if all the spermatozoa in the male testes were damaged by the exposure prior to mating or damaged within the female spermathecae after mating). To date there have been no studies that have investigated the effect of dieldrin exposure on spermatozoa of resistant male anophelines or any other insect species. This investigation is important due to the paucity of basic biological knowledge of these vector species and so that vector control methods can be revised and novel strategies assessed for future management.

The initial experimentation with the COGS F₁- progeny resulted in a new set of methods designed to gain as much information as possible from one cohort of mosquitoes. Additional experiments were designed to test the effect of exposure on mating success (spermatozoa present in the female spermathecae), spermatozoa viability and gender-

specific effects on fecundity. These additional experiments were performed individually with new cohorts of resistant laboratory reared *Anopheles gambiae* mosquitoes (GAH) for every experiment. Once all the experimental procedures were optimised, a single set of experiments were performed on one set of females from this resistant laboratory strain, resulting in a number of different data sets being obtained for each female.

3.1.1 Aims

The aim of the following chapter is to evaluate whether the decreased fecundity observed by Hunt (1984) and Norton (2008) is purely due to the dieldrin exposure affecting the male gametes or the ability for males to mate. The outcome of exposure on fecundity due to exposure of both males and females was also confirmed.

The following objectives were investigated:

1. The effect of dieldrin exposure on the spermatozoa will be tested by confirming their viability in the male testes before mating or in the female spermatheca after mating.
2. Investigate the effect of dieldrin exposure on the insemination status (presence of spermatozoa in the spermathecae) of the females.
3. Confirm whether or not gender-specific exposure of the mosquitoes has any effect on the recorded fecundity.

3.2 Materials and methods

Due to the loss of dieldrin resistance in COGS, a resistant strain from Ghana was used.

3.2.1 Dieldrin exposure bioassays

In order to determine whether exposure of one, or both of the sexes was affecting fecundity, a gender-specific exposure to dieldrin was performed. Males and females from the GAH strain (Resistant laboratory *Anopheles gambiae* strain) were separated after emergence and exposed separately following the protocol described in chapter two (Section 2.2.3).

3.2.2 Ovipositional fecundity

After exposure, each exposed cohort was combined with an unexposed cohort of the opposite sex (i.e. Exposed females combined with unexposed males and exposed males with unexposed females). These cohorts were given one week to mate and blood-feed to repletion twice, after which, the females were placed into oviposition vials. Blood feeding was assessed as described in chapter two (Section 2.2.5). Any eggs oviposited, were counted and transferred into larval-rearing bowls and the fecundity of each female was recorded. Two replicates of 20 females ($n = 40$) were performed.

3.2.3 Spermatozoa viability after exposure

The viability of the spermatozoa was also tested, to observe if exposure to dieldrin had any detrimental effect on these gametes when present in either the male testes or in the female spermathecae. After one week allowed for mating, the males and females were exposed to dieldrin. The testes of the males and the spermathecae of the females were dissected out 24 hours post exposure and the viability of the spermatozoa present was determined using motility as a defining characteristic. The mosquitoes were anaesthetised (unconscious, but

not dead), in groups of five, using ethyl acetate. The small sample size allowed sufficient time for the dissection of all individuals while the spermatozoa were still alive. Viability was characterised by movement of the spermatheca, observed under a dissecting microscope at 100 x magnification or by a wiggling movement when erupting from a ruptured testis observed under a compound light microscope at 400x magnification. All sperm viability dissections were performed in phosphate buffered saline (PBS) as rapidly as possible, before spermatozoan death and inaccurate results. Twelve replicates of five per gender (n = 60) were dissected.

3.2.4 Insemination success after exposure to 4 % dieldrin

In order to determine whether the exposure had any effect on mating ability or success, the insemination status of the females was determined. Males and females were separated after emergence and placed into cages. One cohort of males and females was exposed to 4 % dieldrin separately before being combined. Another cohort was left unexposed (as a control) but only combined in parallel with the exposed cohort. The insemination status of the females from the two groups was confirmed one week after having combined the mosquitoes. An anaesthetised female was placed into a drop of phosphate-buffered saline (PBS); the spermatheca was dissected out and placed into a fresh drop of PBS on a new slide with a cover-slip. The cover-slip was gently pushed down with a pin to rupture the spermatheca, releasing any spermatozoa into the saline. The presence or absence of spermatozoa was used as an indication of the success of insemination. Three replicates of 20 females per cohort (n= 60), were dissected.

3.2.5 Data analysis

The ovipositional fecundity was represented as a bar graph and analysed using a one-way ANOVA. Both the spermatozoa viability and the insemination success are indicated as percentages that were viable or successfully mated, respectively.

3.3 Results

The ovipositional fecundity of each group with a specifically exposed gender is given in Figure 3.1. Differential dieldrin exposure to the sexes was found to have no significant difference on ovipositional fecundity ($F_{2, 117} = 1.062$, $P > 0.05$). The ovipositional fecundity observed for cohort C was lower than the other two cohorts with cohort A, being the highest. However no significant difference was observed between any of the cohorts.

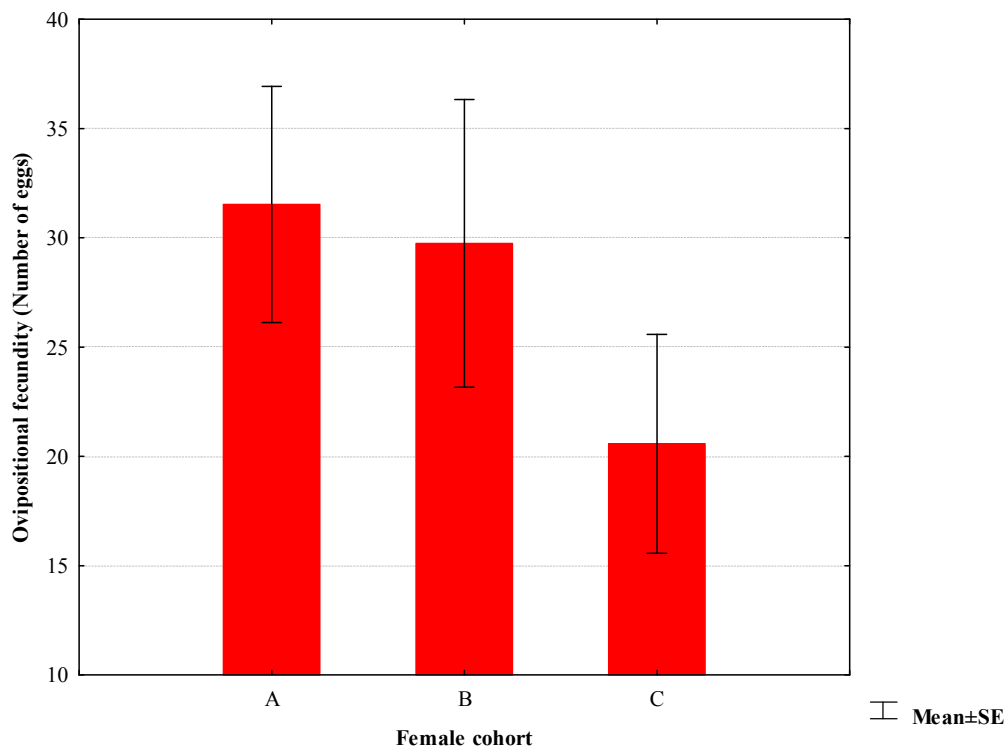


Figure 3.1. Ovipositional fecundity of two differentially exposed cohorts and one unexposed cohort. Cohort A; fecundity of exposed females combined with unexposed males, cohort B; fecundity of unexposed females combined with exposed males and cohort C; fecundity of unexposed females combined with unexposed males.

The dissections shown in Figure 3.2 indicated that the viability of the spermatozoa was unaffected by either exposure to dieldrin in the male testes or female spermathecae. There was 100 % spermatozoa viability ($n = 60$) and 100 % insemination success ($n = 60$).

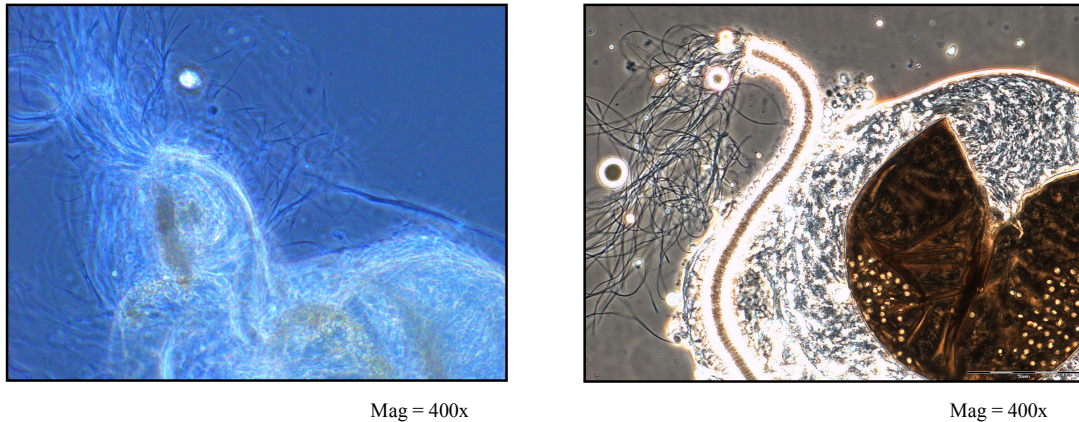


Figure 3.2. Male and female reproductive structures. The male testes releasing spermatozoa (Left). The female spermatheca and fertilisation tube (passage for spermatozoa to and from the spermatheca), filled with spermatozoa after being inseminated (Right).

3.4 Discussion

Based on the data collected, it was concluded that dieldrin exposure did not affect the spermatozoa viability in either the males or females. This suggests that this variable can be eliminated as a possible cause for decreased fecundity of *Anopheles gambiae* females. Reinecke et al. (1995) showed that dieldrin at relatively low concentrations, caused structural damage to spermatozoa specifically to the nucleus. There is a possibility that spermatozoa from the male mosquitoes were damaged, but this was not investigated here. The mating ability of the mosquitoes after exposure was also not affected, suggesting that spermatozoan transfer is unaffected by exposure to dieldrin. It was observed that exposure of both sexes to 4 % dieldrin did not cause any significant changes in fecundity when compared to the unexposed cohort. All the eggs that were oviposited by all of the cohorts

were fertile and hatched within days of being laid. It was decided that both the males and females would be exposed to dieldrin in all further investigations as exposure was shown not to have an effect on the subsequent fecundity but would keep the experiment uniform with respect to both sexes.

Chapter 4

Oocyte and ovarian development after exposure to dieldrin in resistant laboratory-reared *Anopheles gambiae* females

4.1 Introduction

Anopheles gambiae females are anautogenous requiring between one and two blood meals to develop their eggs to full size (Bertram, 1962). Laboratory strains of this species have been observed to develop their primary egg batch with one replete blood meal (Gillies and De Meillon 1968). Christopher (1911) divided the course of ovarian development into five stages, based on the appearance of the follicles under the microscope (Clements, 1992). These five stages have subsequently been characterised into phases of development and the descriptions elaborated upon (Clements, 1992). The growth and development of the oocytes occurs with two separate processes facilitating the maturation. These processes are oogenesis and vitellogenesis which are the formation of the oocyte and the synthesis of the yolk respectively. The effect of dieldrin exposure on the ovarian development in *An. gambiae* does not seem to have been investigated as there is currently no literature available. However, the effect of dieldrin and other organochlorines on ovarian function and rates of fertilisation has been observed in other organisms (Bretveld *et al.* 2006). It has been shown that dieldrin occurring in the environment can act as an artificial oestrogen that affects the ovarian function in women. The most common effects observed in human females after exposure to dieldrin were reduced fertility and developmental defects in the ovaries (Bretveld *et al.* 2006). Dieldrin has also been observed to reduce the fertilisation rate in the oocytes of the toad (*Bufo arenarum*) (De Schroeder and D' Angelo, 1995).

4.1.1 *General morphology of ovaries*

Anopheles gambiae females have a reproductive morphology that includes two fusiform ovaries that are situated dorsally in the abdomen at approximately the fifth abdominal segment (Figure 4.1) (Bertram, 1962). A lateral oviduct connects each ovary to a common oviduct which leads to the external genital opening between segments eight and nine. At the junction with the common oviduct, each lateral oviduct expands to form the ampulla. The fertilisation tube, situated dorsally, is located close to the genital opening, adjoining the common oviduct. Anopheline females store the spermatozoa from the males within a single spermatheca, after insemination, from where it will fertilize the oocytes (Bertram, 1962).

4.1.2 *Stages of ovarian development in *Anopheles gambiae**

The ovaries of a newly emerged *An. gambiae* female are generally found in the resting phase of development (Bertram, 1962 and Clements, 1992) and have been observed as translucent with an attained size at the end of this phase of around one millimetre in diameter. These ovaries will remain in this dormant stage until further nutrition allows for new growth. After a blood meal the development of the primary oocyte in the ovarioles proceeds as the meal is digested and the nutrients are assimilated. The ovaries grow in size, through the five stages of development, until the oocytes are fully mature and ready for oviposition which occurs after approximately 72 hours. Bertram, (1962) and Clements, (1992) have described extensive information on ovarian development which is summarised below.

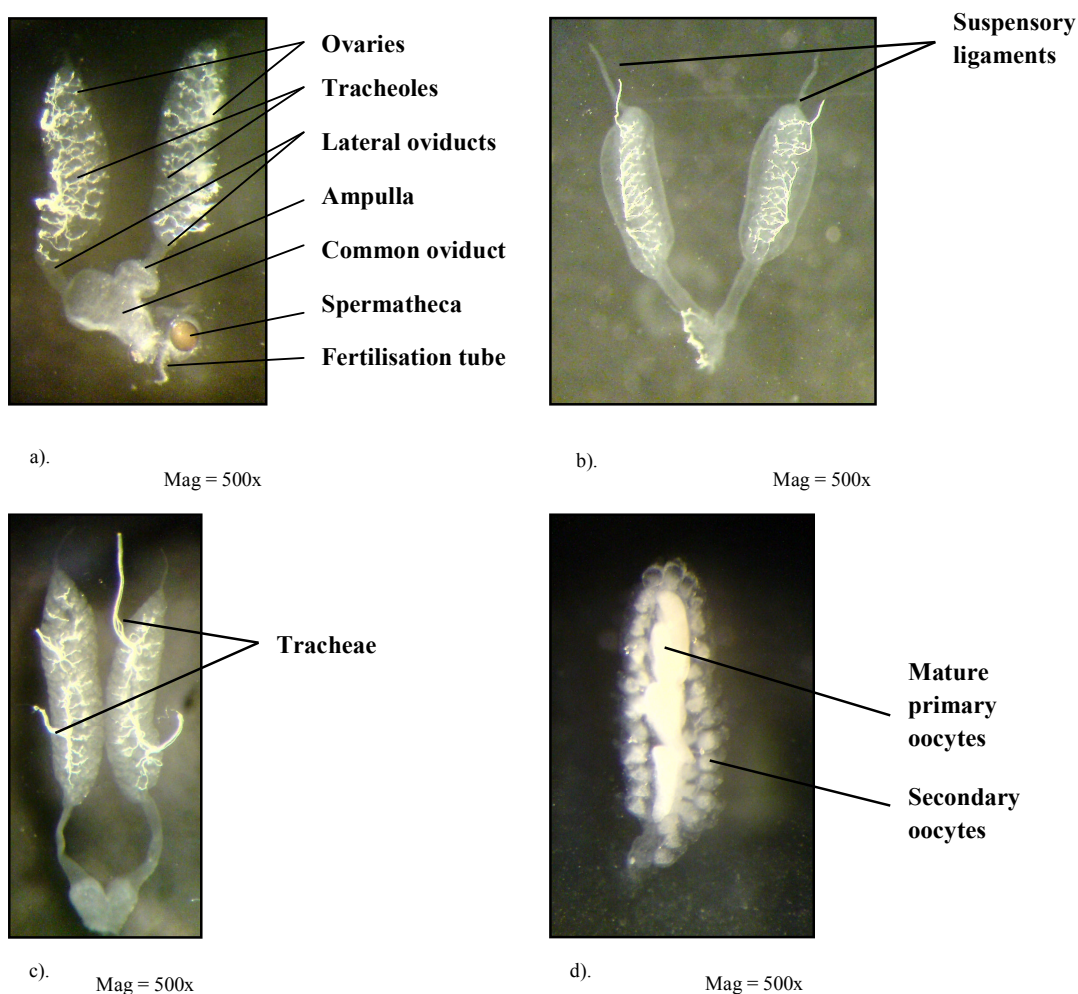


Figure 4.1. The general morphology of the ovaries as they are found within the female. **a)** This figure shows the two fusiform ovaries, the lateral oviducts, common oviduct, ampullae, fertilisation tube, spermatheca and tracheoles **b).** The ovaries from an *An. gambiae* female in the early previtellogenic phase of development. The suspensory ligaments can be observed at the anterior portion of each ovary, these are tapering prolongations of the ovarian sheath. The fine network of tracheoles is also observed as a white vein system in the photo. **c).** A photograph depicting the fine system of tracheoles and the two main tracheae of each ovary which can be observed protruding from the ovaries. **d).** One ovary from a female that has undergone the first gonotrophic cycle but still contains a few mature primary oocytes within the calyx of the ovary. The secondary oocytes are observed around the perimeter of the ovary are still in the early phases of development

4.1.2.1 Stage I

Only observed in newly emerged unfed females. The ovaries are 1mm long and the oocytes are about 60-80 μm long. The oocytes are characterised by the absence of yolk

granules in the ooplasm. Further development is initiated by the uptake of a blood meal (Bertram, 1962).

4.1.2.2 Stage II

As the digestion of the blood meal occurs, the ooplasm begins to become cloudy with the build up of yolk granules around the nucleus of the oocytes (Clements, 1992). The nucleus remains visible as the oocyte grows, reaching a length of about 100 μm (Bertram, 1962). The nurse cells, which contain the chromosomes, remain surrounded by a transparent area as the rest of the ooplasm becomes uniformly clouded by the presence of yolk. At this stage the entire oocyte attains an oval shape (Bertram, 1962).

4.1.2.3 Stage III

This stage is mainly characterised by the nucleus becoming obscured by the accumulating yolk granules within the ooplasm. The oocyte elongates into a broad oval, reaching 200 μm in length (Bertram, 1962).

4.1.2.4 Stage IV

The oocyte reaches the characteristic fusiform shape within the follicle (0.5-1mm). The oocytes are opaque from the yolk mass contained within and the nurse cells are restricted to a small transparent area of the anterior end of the follicle (Bertram, 1962 and Clements 1992).

4.1.2.5 Stage V

The final stage can be characterised by the initial presence of the striations of the egg floats (air sacs for buoyancy) and the completion of the chorion (egg shell) layer. This stage

precedes oviposition where the fully developed oocytes remain in the final resting phase until a suitable oviposition site is found (Bertram, 1962).

Throughout the entire development, the follicular epithelium becomes thinner as the oocyte grows. In the final stages, the epithelium is utilised in the formation of the chorion layer before it is eventually reduced to groups of degraded cells (Clements, 1992).

In each gonotrophic cycle, the oocytes develop synchronously forming large distinct bodies within the abdomen. In Figure 4.2a, the ovaries are observed as a distinct white area filling a large portion of the posterior part of the abdomen of the mosquito. The dark area viewed in front of the ovaries is the remains of an undigested blood meal indicating that this female is almost gravid. The ovaries have been described by Bertram (1962) as resembling, “bunches of tightly packed white bananas” which accurately represents these small taught packages (Figure 4.2b).

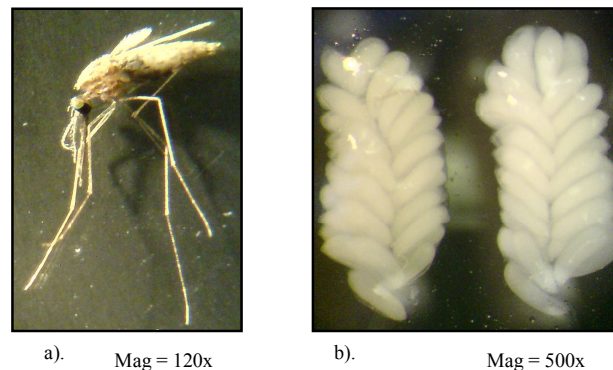


Figure 4.2. An *Anopheles gambiae* before dissection and the ovaries after dissection. **a)** A dead female *An. gambiae* before dissection, displaying her partially digested blood meal and ovaries with nearly mature oocytes contained within the abdomen. **b).** Banana bunch-like structure of the dissected ovary (Photographs by Ryan Norton).

4.1.3 Aims

The overall aim of this chapter was to compile a standard set of reference photographs that will be used in further investigation for this study. The photographs were obtained from females at different stages of ovarian development. Females that obtained both one or two blood meals were photographed. The reason for obtaining pictures of development after both one and two blood meals was to confirm how many blood meals were suitable for optimal oocyte development. The pictures simultaneously show comparisons in development of ovaries and oocytes between the unexposed and dieldrin exposed cohorts.

The following objectives were investigated:

1. The optimal number of blood meals required by female *An. gambiae* to mature their primary batch of oocytes in the first gonotrophic cycle and simultaneously observe the growth of the oocytes after one or two blood meals was determined. (A separate unpublished pilot study was performed to confirm the duration needed for the oocytes to reach maturity after a blood meal).
2. The stages of ovarian and oocyte development were determined. This was accomplished by using photographs that were subsequently used for identification and characterisation of these different developmental periods in subsequent dissections. The reference photographs were selected from the database according to literature descriptions by Bertram (1962) and Clements (1992).
3. The effect of dieldrin exposure on the developmental rate of oocytes and ovaries in *An. gambiae* females was determined. This was recorded by performing dissections at different time intervals post blood meal, after one or two blood meals on females that survive exposure to 4 % dieldrin and comparing images to the reference photographs obtained in the second objective.

4.2 Materials and methods

4.2.1 Mosquito strain

GAH was established in 2006 from wild caught *An. gambiae* S-form collected in Ghana. It is resistant to various insecticides, due to extensive use at the place of origin (Kaiser *et al.* 2010). This strain of *An. gambiae* has maintained its resistance to dieldrin without insecticide selection pressure. Dieldrin resistance in GAH is conferred by target site mutations and possibly metabolic mechanisms. The target site resistance to dieldrin (*Rdl*) first described by Ffrench-Constant *et al.* (1991) was found in GAH at a frequency of 83 % homozygous (RR) and 17 % heterozygous (RS) resistant individuals. A small number of homozygous susceptible (SS) individuals are occasionally found (Kaiser *et al.* 2010). After a standard one hour exposure to 4 % dieldrin all the SS individuals died, leaving only the RR and RS survivors. Therefore the dieldrin survivors used in all of the following experiments were a mixture of RR and RS with the majority of the individuals being RR.

4.2.2 Optimal number of blood meals for complete primary oocyte development

All females were allowed to feed to repletion after having a week for mating. To determine the optimal number of blood meals required for the synchronous maturation of the primary oocytes within the first gonotrophic cycle, one or two blood meals were provided. Females that had blood fed were separated from unfed females immediately after the first blood meal. These females were either dissected after a single blood meal or re-fed, after 72 hours. The females that fed to repletion a second time were used for a second set of dissections. Replete feeding was confirmed using the illumination technique described in chapter 2 (section 2.2.5).

4.2.3 Ovarian dissections: stages of development

The rate of growth of the oocytes within the ovaries of the female mosquito is relatively well documented (Christopher 1911; Detinova, 1959; Bertram, 1962; Clements, 1992). The time duration for a female to fully develop a primary batch of oocytes after the first blood meal is approximately 72 hours (Clements, 1992). An experiment involving dissections at set time periods post blood meal (PBM) was performed over the 72 hour period to confirm the developmental rate indicated by Clements (1992). The development of anopheline ovaries has not been extensively documented photographically. To visually record what happened during this developmental time frame, the ovaries of 240 females were dissected out at 12 hour intervals up to 72 hours PBM (40 females for 6 time intervals). The females were allowed to mate and fed either one or two blood meals respectively.

Only females that displayed the characteristics of having fed to repletion were used for dissection. Samples of 10 females were sacrificed at twelve hourly intervals PBM using ethyl acetate. The ovary dissections were performed according to Meadows (1968). The ovaries were placed into a drop of distilled water on a slide and photographs were taken using a Sony Ericsson K810i and W995 through the ocular lens of a dissecting microscope, following the procedure described by Frean (2007). Dissections were performed at 12, 24, 36, 48, 60 and 72 hours PBM. Since most of the references in literature are line drawings, a set of reference photographs had to be developed. A photograph of the ovaries and one oocyte at the developmental state most frequently recorded in a given 12 hour time-frame from a control group of unexposed females was used to represent the time frame. These photographs were then characterised into stages and phases of development according to descriptions given by Bertram (1962) and Clements (1992).

4.2.4 Effect of dieldrin exposure on ovarian development

The effect of dieldrin exposure on the rate of oocyte and ovarian development was monitored following similar methods described in section 4.2.3, above. The four-day-old males and females were allowed to mate after a standard one hour exposure to 4 % dieldrin following the method described in chapter 2 (Section 2.2.3). The females were subsequently fed one or two blood meals. Dissections of females that had fed to repletion were performed at 12, 24, 36, 48, 60 and 72 hours PBM. This included 120 females after one blood meal and 120 females after two blood meals for each unexposed and exposed cohort.

4.2.5 Data Analysis

The dissected pairs of ovaries were mounted on a slide in rows of 10 for each 12 hour time interval, each pair of ovaries was characterised into a specific Christopher's stage (Christopher 1911) and developmental phase using the reference photographs (obtained in section 4.2.3) and descriptions by Bertram (1962) and Clements (1992). The rate of development between the unexposed and exposed cohorts was compared using the Christopher's stages and associated developmental time.

4.3 Results

4.3.1 Optimal number of blood meals for complete oocyte development

Most females had developed their oocytes to maturity within 72 hours. One blood meal was sufficient for 72 % of the females to mature primary oocytes ($n = 36 / 50$) and 28 % of females ($n = 14 / 50$), contained immature oocytes after 72 hours. After two blood meals 94 % of females had mature primary oocytes ($n = 47 / 50$).

4.3.2 Ovarian dissections: stages of development

These photographs match the descriptions of oocyte development given by Bertram (1962) and Clements (1992). A representative pair of ovaries (**a**) and one oocyte (**b**) was chosen as a reference for comparison purposes of each stage.

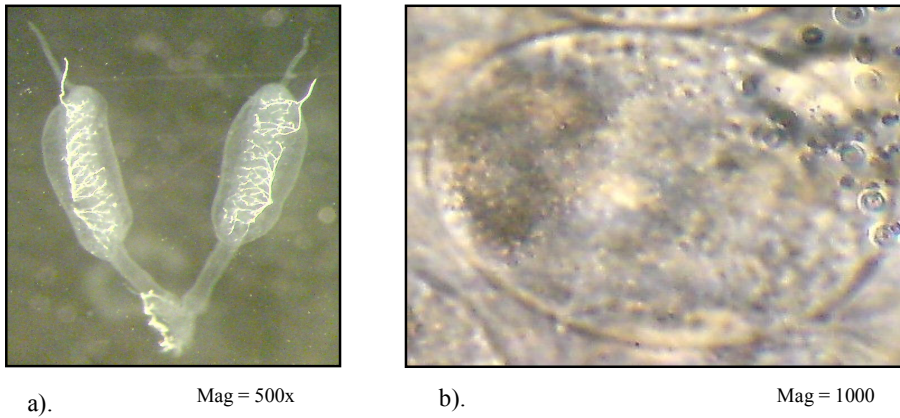


Figure 4.3. Stage G, Ia, Ib (*Previtellogenic phase at emergence*). The oocytes are not distinguishable as individual bodies within the ovaries (a). The oocyte is surrounded by epithelium (b).

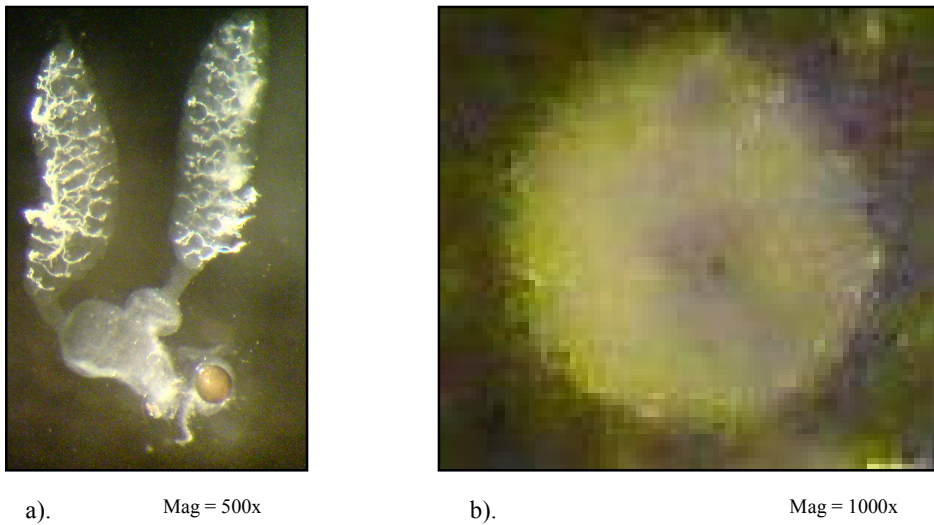
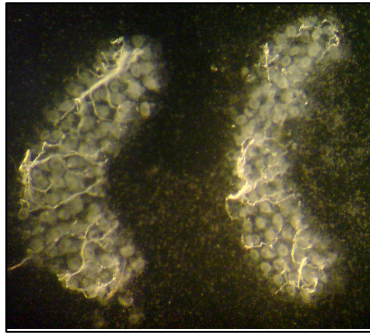


Figure 4.4. Stage IIa (*Previtellogenic phase, 24 hours post emergence*). The oocytes start to become visible (a). Small refractile lipid droplets are visible at 200x but not 50x magnification (b).



a). Mag = 500x



b). Mag = 600x

Figure 4.5. Stage IIb (*Previtellogenic phase, 48 hours post emergence*). The ooplasm contains lipid droplets that are visible from 50x to 20x, but not distinct at 10x (a and b)



a). Mag = 500x



b). Mag = 600x

Figure 4.6. Stage IIIa (*Initiation phase, 8-16 hours post blood meal*). The ooplasm becomes clouded with lipid inclusions and the large yolk spheres appear yellow (the nucleus is obscured by the yolk) (a and b).



a).

Mag = 500x



b).

Mag = 600x

Figure 4.7. *Stage IIIb* (Trophic phase, 16-20 hours post blood meal). The follicle increases in size without changing shape (a). The oocyte occupies 50-75 % of the length of the follicle (b).



a).

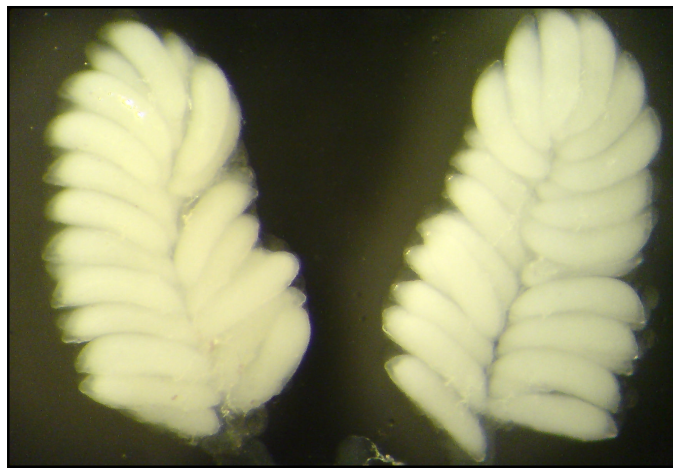
Mag = 500x



b).

Mag = 500x

Figure 4.8. *Stage IVa* (Trophic phase, 20-48 hours post blood meal). The follicle grows more, becoming narrower, assuming the shape of a mature oocyte (a). Oocyte occupies 90 % of the follicle (b)



a).

Mag = 500x



b).

Mag = 500x

Figure 4.9. Stage IVb (*Trophic phase, 40-60 hours post blood meal*). The Follicle assumes the shape of a mature oocyte (a and b) but does not reach full length. Chorionic structures such as floats, start appearing.



a).

Mag = 500x



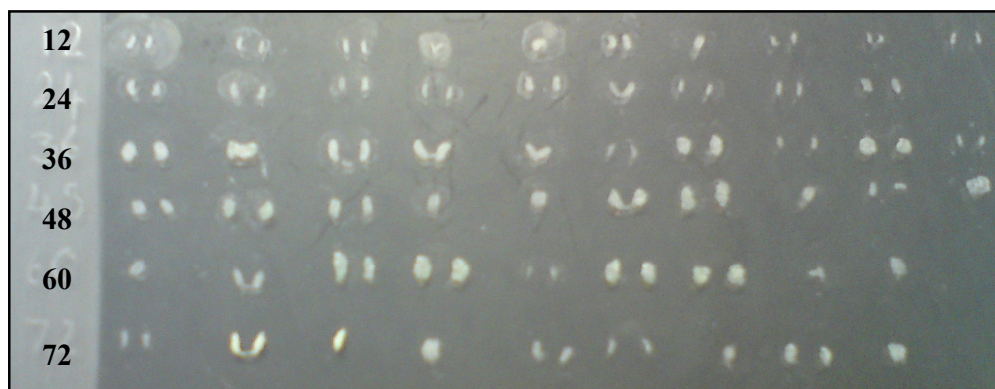
b).

Mag = 500x

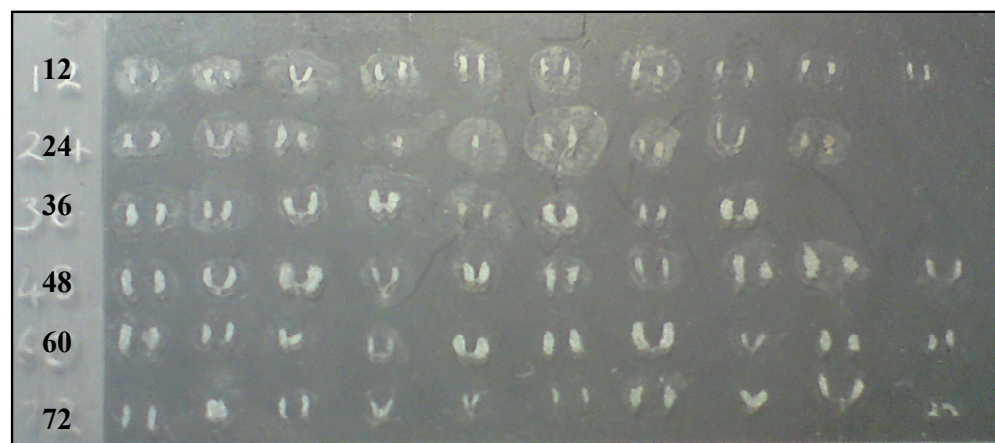
Figure 4.10. Stage V (*Post-trophic phase, 60-72 hours post blood meal*). The oocyte reaches its full length (a and b), the follicular epithelium degenerates and the chorionic structures (floats) become fully formed (b).

4.3.3 *Effect of dieldrin exposure on ovarian development*

The exposure to dieldrin was observed to have an adverse effect on the development of oocytes and ovaries of the females. The rate of growth was noted to be slightly slower in the exposed cohort than in the unexposed cohort. This trend continued for the entire maturation period and after 72 hours, there was a slight difference between the unexposed and exposed cohorts (Figure 4.11). The unexposed cohort developed oocytes to a higher phase of maturity than the exposed cohort. Table 4.1 represents the most common developmental phase and stage observed at each dissection interval.



a).



b).

Figure 4.11. Slides with mounted ovaries from six different developmental time periods. **a)** Shows the ovaries from unexposed females and **b)** shows the ovaries from exposed females. The development, as an increase in size going downwards from 12 hours to 72 hours, is observed through the time periods in both cohorts. These slides were then used to classify the stages of development and provided the data for Table 4.1.

Table 4.1. The number of unexposed and exposed female *An. gambiae* having oocytes in the different stages of development. The numbers in the brackets indicate the percentage of females that were successfully inseminated.

Unexposed									Exposed								
Hours PBM	Stage								Hours PBM	Stage							
	G,									G,							
	Ia,	IIa	IIb	IIIa	IIIb	IVa	IVb	V		Ia,	IIa	IIb	IIIa	IIIb	IVa	IVb	V
	Ib									Ib							
12 (80)	2	4	12	1			1		12 (60)	4	8	8					
24 (80)		3	8	8			1		24 (50)	2	2	4	12				
36 (90)			1	2	8	3	4	2	36 (70)		4	4	3	6		3	
48 (100)			2	5	7	2	4		48 (100)		4	5	2	1	4	4	
60 (70)			1	2	1	6	5	5	60 (80)		4	8	1	1		6	
72 (100)					1	1	7	11	72 (80)			6	1	3		4	6

The unexposed cohort of females was observed to mature at a more constant rate than the exposed cohort which had a lag phase where the oocytes were observed to stay in the previtellogenic phase up to 36 hours post blood meal after which the development commenced at a relatively normal rate. After the 72 hour time period, the exposed cohort was observed to comprise more females with immature oocytes and smaller ovaries than the unexposed cohort when characterised according to developmental stages.

4.4 Discussion

The determination of the optimal number of blood meals confirmed the developmental time described by Bertram (1962) and Clements (1992) however, there were still a few females that showed immature oocytes after only one blood meal. Therefore in all the following experiments to ensure that all females would mature primary oocytes, it was decided that the females would be fed one replete blood meal and allowed an opportunity to feed a second time 72 hours later. This would ensure that sufficient protein and nutrients for the maturation of the primary oocytes would be obtained by all females within the first gonotrophic cycle.

The ovary and oocyte reference photographs were chosen to match the descriptions by Bertram (1962) and Clements (1992), supplementing the existing descriptions with a pictorial reference for easy identification of the developmental stages. These will enable quick and effective characterisation of the stages of development for future studies.

Dieldrin exposure was recorded to have a subtle effect on oocyte development as seen in Table 4.1. The numbers of females present in each developmental stage at the successive 12 hour intervals indicated that females from the exposed GAH cohort developed their oocytes at a slower rate than the unexposed GAH females for the first 36 hours. After 36 hours however, similar growth to that of the unexposed cohort was observed in the exposed cohort. This lag in development may be due to hormesis, which is a favourable biological response to low exposures to toxins or other stressors. In this case, the females may be delaying their oocyte growth to recover from the dieldrin exposure, resuming development only when conditions become favourable again.

Chapter 5

The effect of dieldrin exposure on fecundity, fertility, longevity and haematin utilization in dieldrin resistant *Anopheles gambiae* females

5.1 Introduction

Male reproductive success in the red flower beetle (*Tribolium castaneum*) has been shown to increase in malathion resistant individuals indicating that reproductive fitness is not traded against malathion resistance (Arnaud and Haubruge, 2002). The oocyte development in *Aedes aegypti* and *Anopheles gambiae* has been shown to be disrupted by methoprene which modulates the ecdysteroid action in the process of vitellogenesis (Bai *et al.*, 2010). In humans, pesticides have been shown to disrupt hormonal function of the female reproductive system, particularly with the ovarian cycle (Bretveld *et al.* 2006). Dieldrin has been observed to cause numerous effects on the male and female reproductive systems in a number of different phyla (Reinecke *et al.* 1993; De Schroeder and D'Angelo, 1995; Mwatibo and Green, 1997; Bretveld *et al.* 2006). It is thus observed that pesticides have an effect on a wide variety of organisms and their reproductive potentials. It is also interesting to note that the change due to exposure is not always to the detriment of the organism with some even gaining enhanced characteristics such as improved reproductive success.

Effects of DDT exposure on longevity and fecundity in a micrometazoan (*Lepidodermella squammata*) indicated a reduction in both life processes, although no indication of a possible cause was conveyed (Hummon, 1974). Muturi *et. al.*, (2010) have shown variable effects on longevity when exposing *Culex pipiens* larvae to malathion and indicate that the

biotic conditions of the larval habitat can alter the impact of the insecticide exposure on mortality. There is only a small amount of available information regarding the effect of pesticide (specifically the organochlorine class) exposure on longevity in invertebrates, and the possible causes. Although dieldrin may not affect the resistant *Anopheles gambiae* females immediately, there may nevertheless be a shortening of the lifespan.

Anopheline mosquitoes have an ability to concentrate erythrocytes in the midgut whilst feeding (Briegel and Rezzonico, 1985) and it is therefore unfeasible to use a gravimetric analysis on bloodmeal size. An invasive method such as measuring the haemoglobin content of the midgut is also unsuitable for an experiment such as this and therefore Briegel's (1980) method of bloodmeal analysis by the measurement of haematin excretion was implemented. Haematin excretion has been used by Briegel (1990) as an indicator of the amount of haemoglobin in the blood meal, which can correlate with egg production of individual mosquitoes.

Factors known to affect fecundity such as body size, teneral reserves, blood meal size and mosquito age and gonotrophic status (Hogg and Hurd, 1995a) were controlled in the following chapter, isolating only the effect of the exposure to 4 % dieldrin on the fecundity or the reproductive processes leading up to oviposition.

5.1.1 Aims

The aim of this chapter was to investigate the effect of dieldrin exposure on fecundity, fertility and longevity by performing one experiment that includes multiple aspects of previously described methods including all revised recommendations dealt with in chapter 2. This investigation aims to obtain multiple data sets from two groups of resistant

laboratory-reared *Anopheles gambiae* females, one unexposed and the other exposed to 4% dieldrin. These data sets will be more valid than those obtained from multiple cohorts of females as the data will all be related (i.e. individual data on fecundity, fertility, body size, haematin utilisation and longevity will be obtained from each individual female).

The following objectives will be investigated:

1. Bioassay data from multiple replicates will create a comprehensive dieldrin resistance profile of the laboratory strain of *An. gambiae* (GAH), indicating how resistant the strain actually is.
2. The utilisation of the blood protein haemoglobin will be compared between unexposed and exposed females to test if this process was affected by the dieldrin exposure.
3. The effect of dieldrin exposure on fecundity and longevity will be measured.
4. Egg retention will be investigated.

Note: All of the data obtained are from experiments carried out on two cohorts of 40 female *An. gambiae*, one cohort will be unexposed and the other exposed to dieldrin.

5.2 Materials and methods

5.2.1 Dieldrin exposure bioassays

Cohorts of male and female *Anopheles gambiae* (GAH) mosquitoes were separated at emergence for separate sex exposures to dieldrin as previously described. The males were collected from laboratory strain material two days earlier than the females so that when combined, the males were two days older than the females but were still exposed to 4 % dieldrin at four days of age. The four-day-old mosquitoes were exposed to 4 % dieldrin for one hour following the methods described in chapter 2 (section 2.2.3). (29 replicates of 25 mosquitoes were used; n = 725).

5.2.2 Mating

After exposure, the males and females were combined into a cage at a ratio of two males for every one female. The males used were two days older than the females, as described above, to allow for optimal mating ability (Clements, 1999). The mosquitoes were allowed one week to mate, after which the first blood meal was offered.

5.2.3 Blood feeding

All females were allowed to feed to repletion within a half hour. Females that had blood fed once were separated from unfed females immediately after the first blood meal by using the method for confirming blood feeding and then aspirating the fed female into a new cage.

5.2.4 Haematin collection and measurement

The utilisation of the blood protein haemoglobin was determined by measuring the quantity of the digested product, haematin. This was performed to determine if the

exposure to dieldrin had any effect on the normal biological processes involving blood meal utilisation which would directly impact fecundity. After exposure to 4 % dieldrin and the first blood meal, each female was placed in a dry 15 ml Falcon tube with a gauze lid and provided with 10% sucrose solution. A second blood meal was offered 72 hours after the first. The females were kept in the tubes for four days after the second blood meal to ensure that all defecation was collected. After the four days they were transferred into oviposition vials to measure fecundity as described below. The dried haematin droplets collected at the bottom of the Falcon tubes were diluted in 4ml of 1% of lithium carbonate solution (Briegel, 1980). The optical densities of the samples were measured at an absorbance of 387 nm, following the protocol described by Briegel (1980), using a HELIOS- γ spectrophotometer (Thermo Scientific) and 1% lithium carbonate solution as the blank. A porcine haematin (Sigma) standard curve was produced from samples of known haematin concentration according to Briegel (1980) (Porcine was used as it is very similar to human haematin). The standard curve was produced using five samples per known concentration.

5.2.5 Wing length measurements

Wing length was measured as described in chapter two, section 2.2.8.

5.2.6 Oviposition and fecundity

Following one or two confirmed blood meals, each female was placed into a glass oviposition vial with a gauze lid (no further feeding took place after transfer into the oviposition vial). The vial contained an optimally shaped oval piece of moist filter paper onto which the eggs were oviposited (the paper was made so that part of it was submerged in approximately 1.5 mm of water while the rest was above the water line allowing the

female to rest). Sucrose solution (10 %) was made available *ad lib* and the filter paper was kept moist at all times with distilled water. After oviposition, the eggs were transferred to a plastic bowl containing distilled water where they were counted. The females were kept in the laying vials until they died. The eggs were left to hatch and reared through to adults and the proportion of adults that emerged from the egg batch was calculated. The dead females were dissected to measure insemination status and to count any retained eggs. Each female's total fecundity was counted as the sum of oviposited and retained eggs from the first gonotrophic cycle (Hogg and Hurd, 1995b).

5.2.7 *Longevity*

The longevity of the females was recorded as the number of days from emergence to death.

5.2.8 *Number of retained eggs*

The number of eggs remaining in the ovaries of the females after death were dissected out (see section 2.2.9 of chapter 2) and counted.

5.2.9 *Insemination status*

The insemination status of the females was determined by the presence or absence of spermatozoa contained within the spermathecae following the method described in chapter 3. Since the study required females to be mated, any females that were found to be unsuccessfully inseminated were removed from the experiment entirely and the data obtained for that individual female was not used in any of the body size, fecundity or fertility analyses.

5.2.10 Data analysis

The bioassay data are given in a line graph, indicating the difference in resistance levels between male and female *An. gambiae* from the GAH colony. Also represented on the graph are the susceptible control (SUA) showing what the mortalities should be with a susceptible strain and the non-exposed control (GAH) confirming that the exposure tubes were not contaminated with any other insecticide. The line graph represents the knock down data for the entire one hour exposure and the 24 hour post exposure recovery.

The haematin standard curve was represented as a linear correlation of optical density at 387 nm and the concentration of porcine haematin with an R^2 value and a corresponding line equation.

Ovipositional fecundity, retained egg numbers and total fecundity were represented in a combined bar graph and compared using ANOVA. Post-hoc tests were performed on the above data sets to confirm any significant differences between any of the unexposed and exposed cohorts.

The ovipositional and total fecundities of the unexposed and exposed cohorts were represented as regressions with wing length and the number of individuals recorded in each wing length group were shown.

Longevity was represented as a bar graph and unexposed and exposed cohorts were compared using a t-test for dependent variables. The insemination status was recorded as the percentage of females that were successfully inseminated, however only those females that were inseminated were used in the data analysis.

5.3 Results

The mean male and female 24 hour percentage mortality was very low ($17 \pm 7.3\%$ and $21 \pm 5.6\%$ respectively), indicating high frequencies of resistance (Figure 5.1).

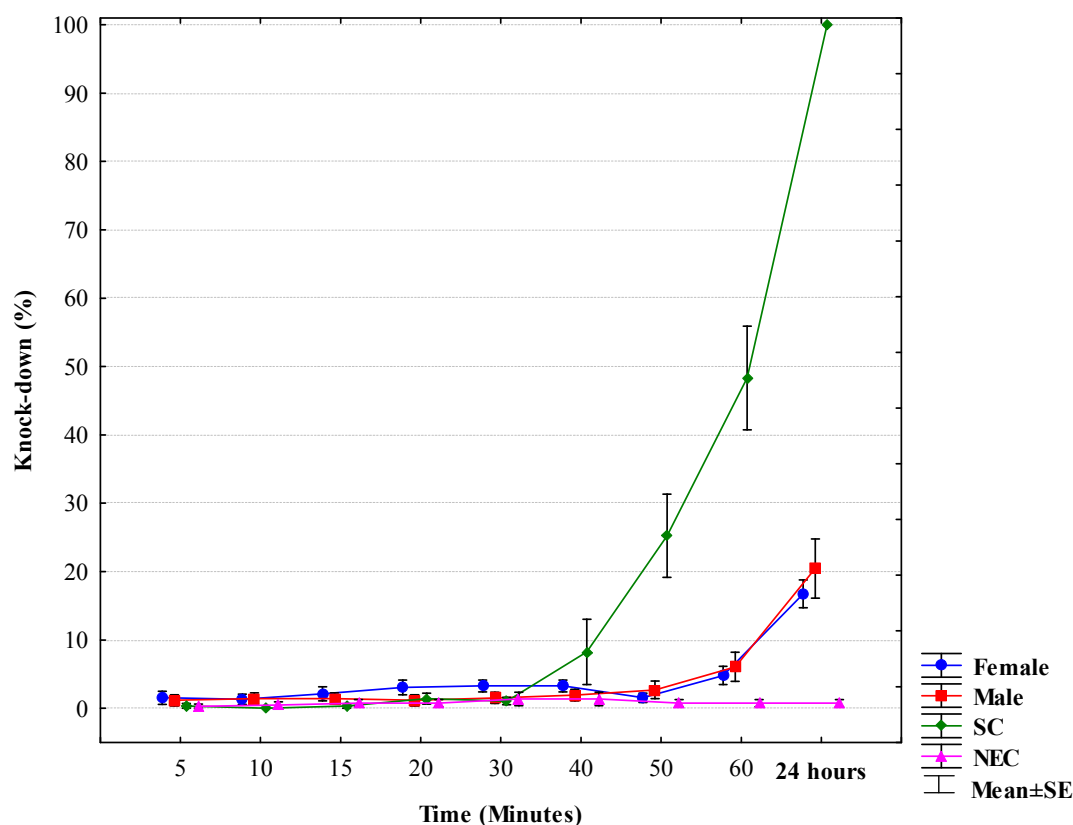


Figure 5.1. Bioassay knock-down data after a one hour exposure to 4 % dieldrin. Resistant GAH males and females were exposed separately to compare their resistance profiles. SC refers to the susceptible control (SUA) and NEC refers to the unexposed control GAH strain. The 24 hour mortality is shown as the last point on the plot.

The WHO recommends that mortality below 80% is regarded as resistant (WHO, 1998). The GAH strain therefore remained highly resistant, even in the absence of any insecticide selection pressures. The susceptible control (SUA) remained susceptible to the insecticide as indicated by the 100% mortality at 24 hours.

The non-exposed control was an indicator of any unwanted variable affecting the mortality of the mosquitoes and the 0 % mortality remained unchanged throughout the experimental period indicating a stable and controlled environment.

A haematin standard curve was produced, encompassing a range of concentrations of haematin that were above and below the required scope of study (Figure 5.2a). The known concentrations of haematin are represented on the x-axis and the corresponding measured absorbance on the y-axis.

The mean haematin concentrations for the unexposed and exposed GAH cohorts (Figure 5.2b) were similar, with no significant differences observed ($t_{39, 40} = 0.164$; $P = 0.87$).

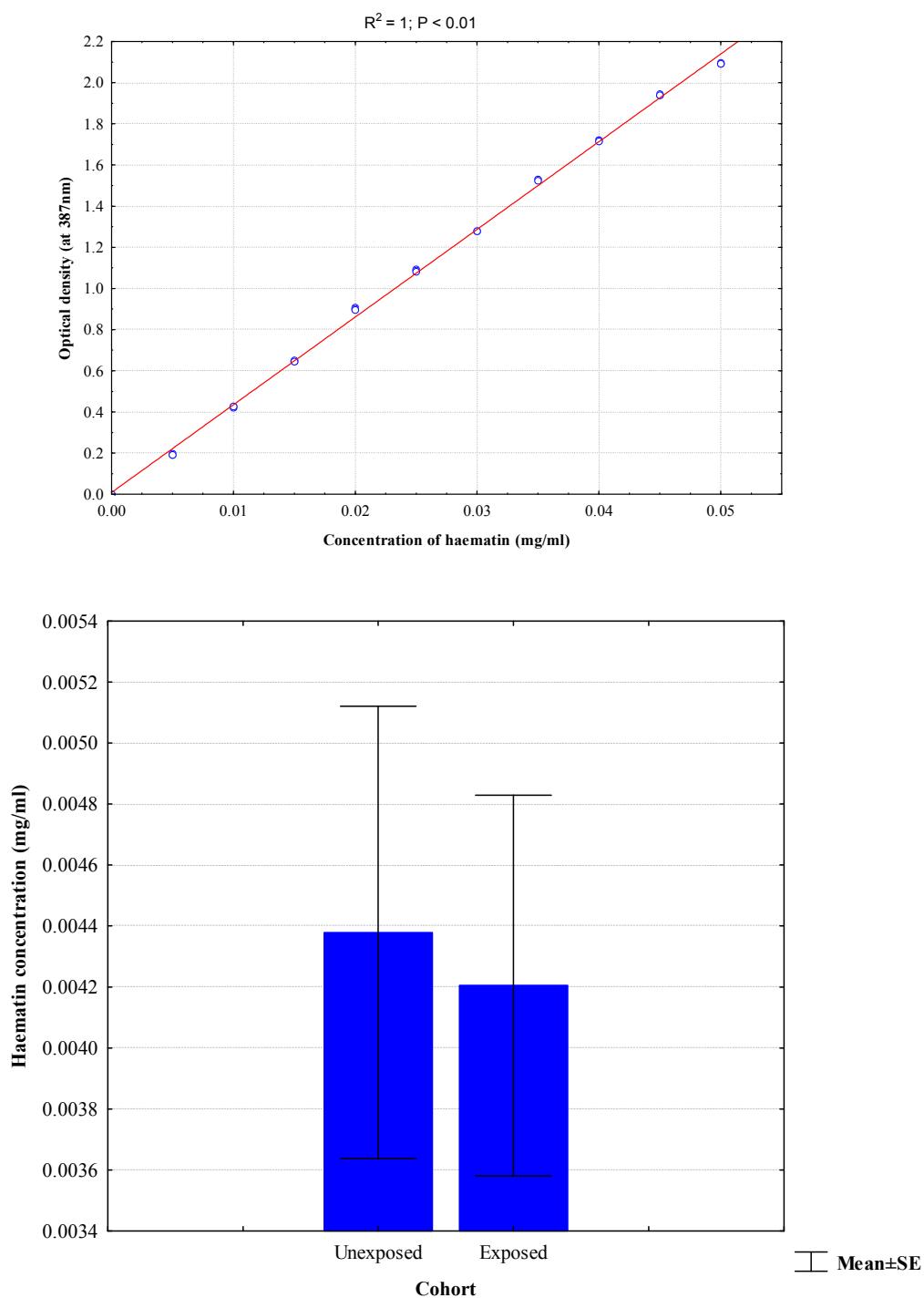


Figure 5.2. Haemoglobin utilisation in *An. gambiae* GAH. Porcine haematin standard curve (Top). The optical densities (OD) of the haematin were read at an absorbance of 387 nm and the concentrations of haematin were calculated using the linear formula, $OD = 0.0089 + 42.6097x$. Haematin concentrations of the unexposed and exposed cohorts representing the haemoglobin utilisation after the digestion of the blood meal (Bottom).

A bar graph representing the ovipositional and total fecundity for the unexposed and exposed cohorts is shown (Figure 5.3). The unexposed ovipositional fecundity (U1) was not significantly different to the unexposed total fecundity (U3). The ovipositional fecundity was much lower than total fecundity in the exposed cohort, but no significant difference was observed ($(F_{3; 28}) = 2.027$; $P > 0.05$). The ovipositional fecundity of the exposed cohort was lower than that of the unexposed cohort when compared, however the difference was not shown to be significant when using an LSD post-hoc test ($(F_{3; 28}) = 2.027$; $P > 0.05$). The total fecundity of the exposed cohort was also lower than the unexposed cohort but was not significant ($(F_{3; 28}) = 2.027$; $P > 0.05$). Overall the exposed cohort produced fewer eggs than the unexposed cohort.

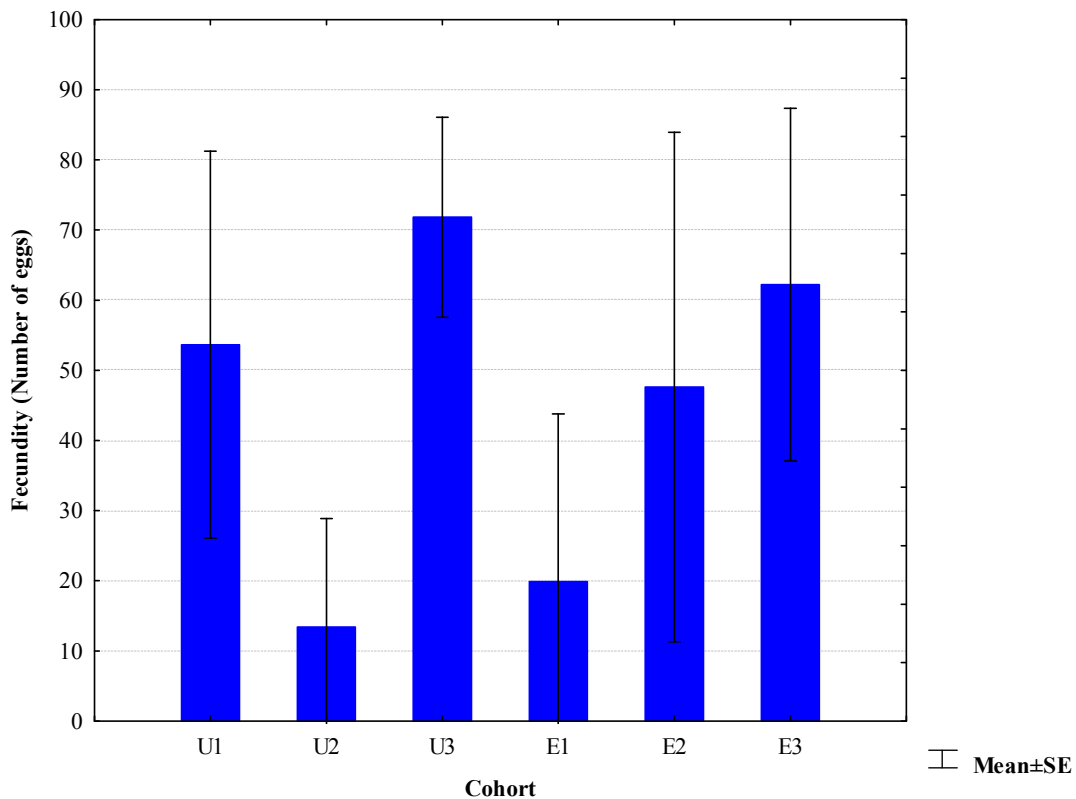


Figure 5.3. A combined fecundity graph of GAH, representing the ovipositional fecundity (1), number of eggs retained (2) and the total fecundity (3) of the unexposed (U) and exposed (E) cohorts. The oviposition fecundity refers to the number of eggs oviposited and the total fecundity refers to the combined number of eggs retained and oviposited.

Figure 5.4 represents the body sizes (in mm) of the unexposed and exposed females. The range of wing lengths only contained four groups that were significantly different from each other ($(F_{6; 20}) = 27.29$; $P = 0.000$).

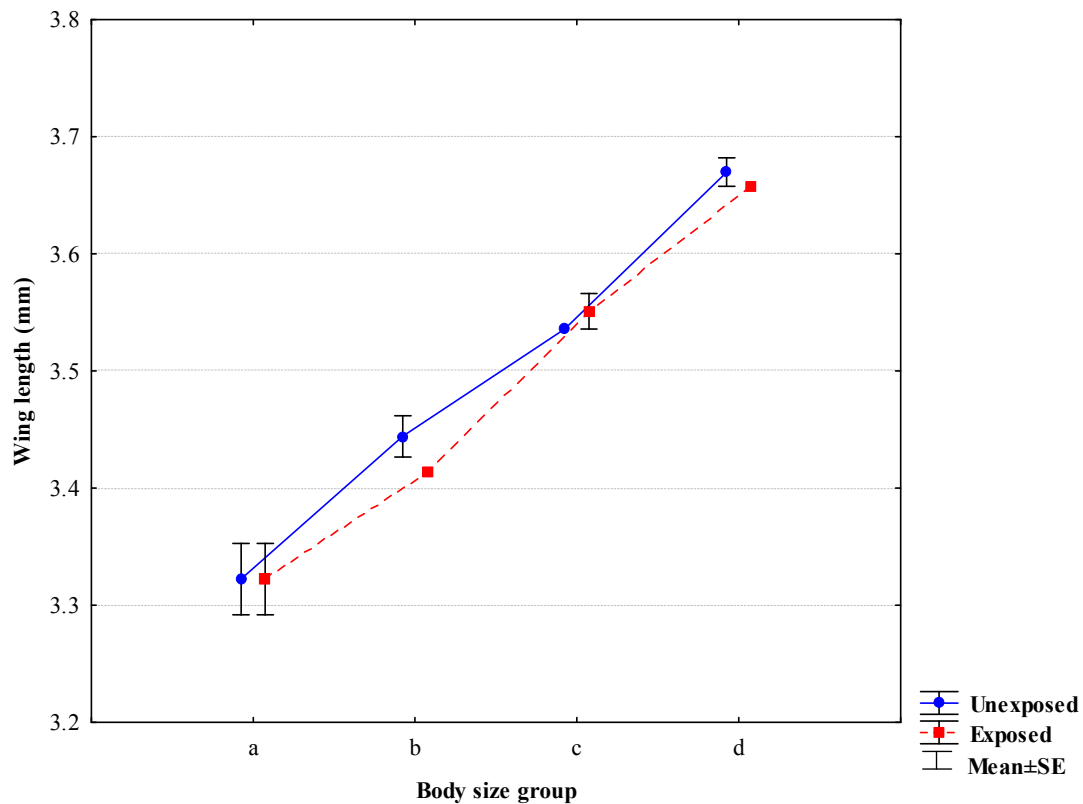


Figure 5.4. Body sizes of the female GAH mosquitoes that were significantly different to each other. The y-axis indicates wing length which is an indirect measure of body size and the x-axis represents the different body size groups. Body size group a = 3.3-3.4 mm; b = 3.4-3.5 mm; c = 3.5-3.6 mm and d = 3.6-3.7 mm.

The number of individuals present in each body size group of the unexposed GAH cohort (as indicated by wing length) is presented in Figure 5,5 alongside the ovipositional and total fecundity. The number of individuals can be observed to follow a normal distribution skewed to the right. Ovipositional fecundity showed a positive correlation with body size ($R^2 = 0.11$; $P > 0.05$) but with such small sample sizes it was difficult to make any solid

conclusions based on the data obtained. The total fecundity followed the same trend as the ovipositional fecundity with a positive correlation to body size ($R^2 = 0.01$; $P > 0.05$).

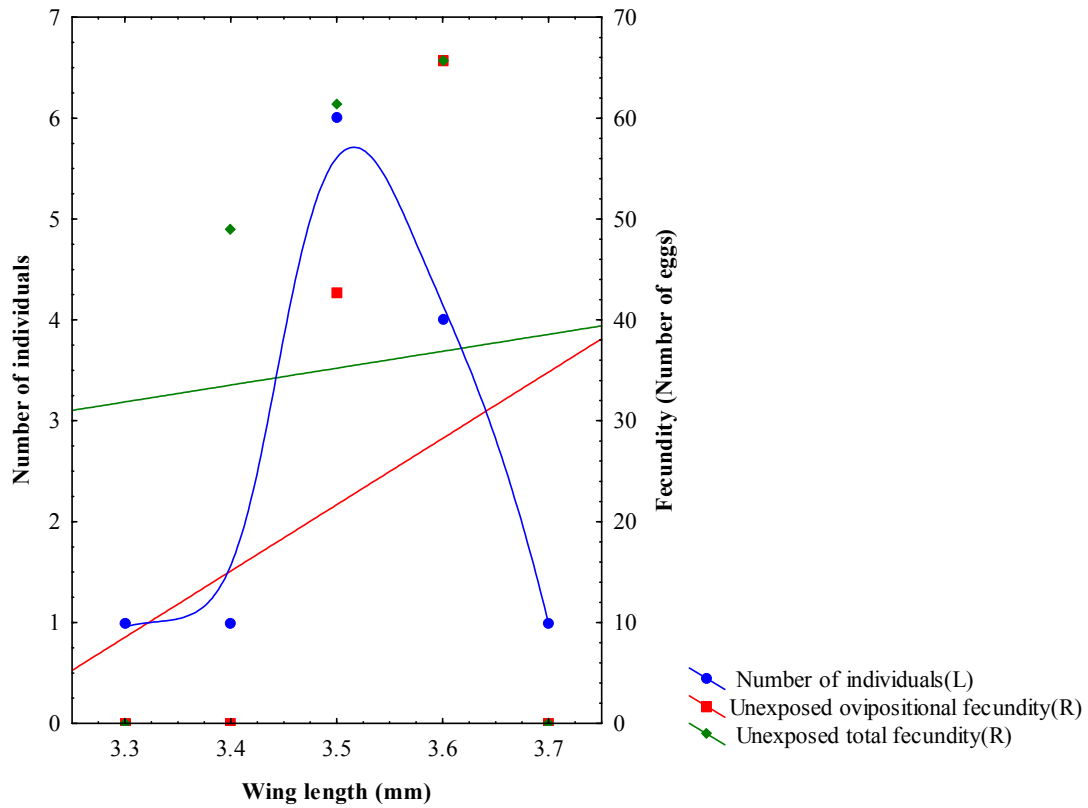


Figure 5.5. The number of individual females recorded in each wing length group and the corresponding ovipositional and total fecundity data for the unexposed cohort of *An. gambiae* GAH females.

Figure 5.6 depicts the number of individuals, ovipositional fecundity and total fecundity for the exposed GAH cohort. The number of individuals is observed to be much higher for the smaller wing lengths than the previous unexposed cohort. Ovipositional fecundity was observed to be negatively correlated with wing length ($R^2 = 0.1$; $P > 0.05$) however this is a weak correlation. Total fecundity was observed to be positively correlated with wing length ($R^2 = 0.14$; $P > 0.05$).

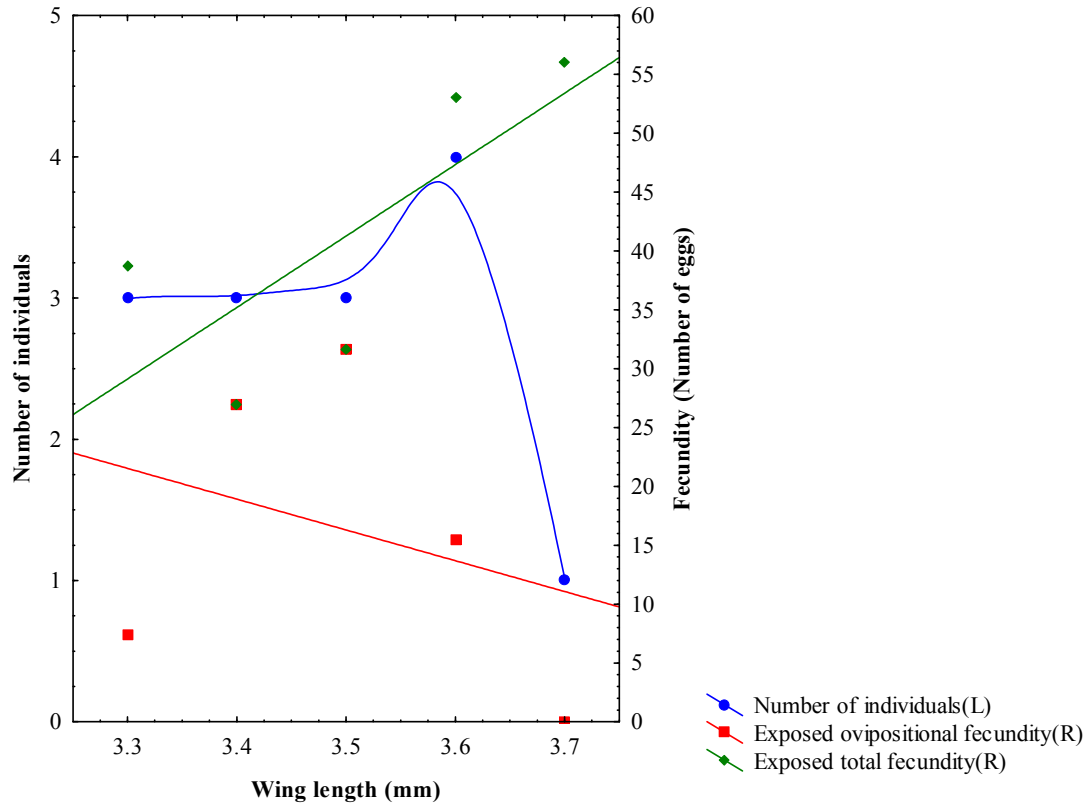


Figure 5.6. The number of individual females recorded in each wing length group and the corresponding ovipositional and total fecundity data for the exposed cohort of *An. gambiae* GAH females.

In this experiment the exposed cohort lived significantly longer than the unexposed cohort ($t_{39;40} = -2.397$, $P < 0.05$) (Figure 5.7).

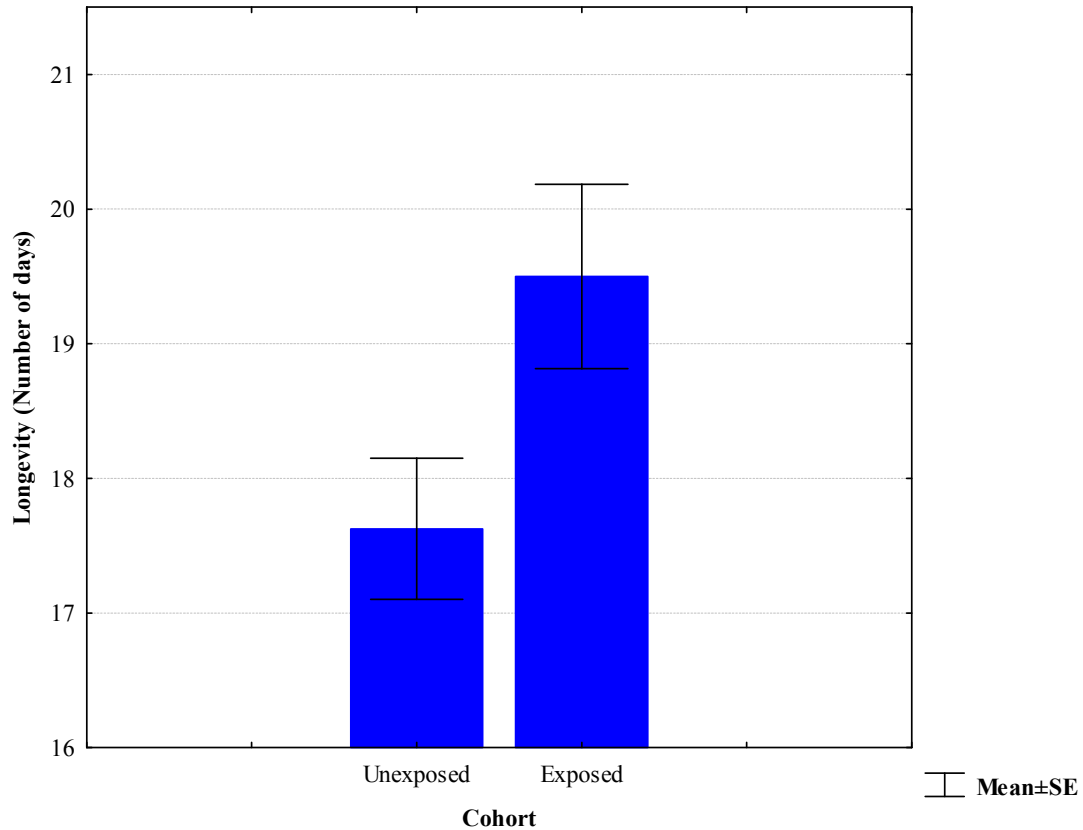


Figure 5.7. Longevity of the unexposed and exposed cohorts of *An. gambiae* GAH females.

Egg retention was observed in the exposed cohort, with numerous mature oocytes present in the ovaries of most females (Figure 5.8 is an example of the oocytes). Only 35% of the females were confirmed to be inseminated for both the unexposed and exposed cohorts. From the 35% that were inseminated 20% of the unexposed and 12.5% of the exposed were observed to oviposit. The percentage of larvae that survived to adulthood was 87.5% and 80% for the unexposed and exposed cohorts respectively. Figure 5.9a represents a successfully inseminated spermatheca and Figure 5.9b represents a spermatheca from a newly emerged virgin female which was used as a negative control image.



a).

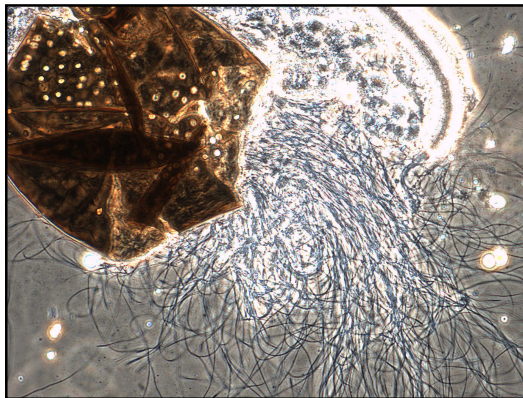
Mag = 120x



b).

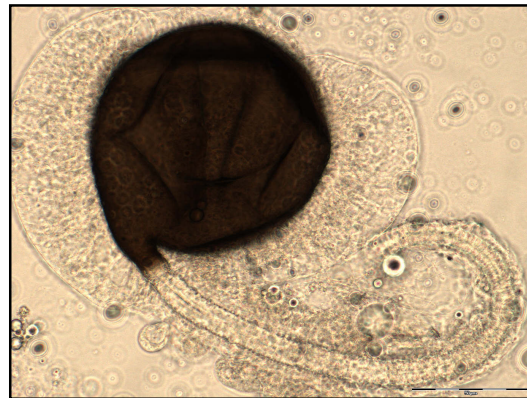
Mag = 120x

Figure 5.8. Eggs retained within the ovaries of GAH females after oviposition and only dissected out post mortem. **a)** The majority of the eggs are fully mature as indicated by the presence of egg floats. **b)** Eggs that were immature, not having developed floats.



a).

Mag = 600x



b).

Mag = 600x

Figure 5.9. Spermathecae from female *An. gambiae* **a)** Spermatheca from a successfully inseminated female showing the hundreds of spermatozoa being released from the capsule after being ruptured. **b)** A spermatheca from a virgin female, used as a negative control (no spermatozoa) to confirm the absence of spermatozoa.

5.4 Discussion

The many replicates of bioassay data indicated the persistent resistance present in GAH. The exposure data revealed how stable the inheritance of the resistance mechanism to dieldrin actually is as the mosquito strain has been reared in a laboratory environment for almost three years without any insecticide selection and yet the resistance still remains high. The resistance to dieldrin was very high and GAH more than meets the criteria for a resistant strain, (WHO, 1998). The main resistance mechanism in this strain has been shown to be target-site resistance (*Rdl*) which has been maintained without selection (Kaiser *et al.* 2010). It would appear that this mechanism does not carry major fitness costs to these mosquitoes, as selection against the resistance would have occurred in the laboratory environment (Mason *et al.* 1987). It is also possible that metabolic resistance mechanisms are present in this strain of *An. gambiae* but further testing would need to be performed to confirm this. Any changes in insecticide susceptibility would have been observed shortly after colonisation due to the short generation time of these mosquitoes in the laboratory environment.

Hunt (1984) found that *An. gambiae* females of the IANP20 strain would not feed after exposure to dieldrin and therefore fecundity was reduced. All females used in this experiment took at least one blood meal at the start of the experiment after exposure. Although a second blood meal was offered 72 hours after the first, only a small proportion of the females actually fed. This indicated that either the females did not require another blood meal or they were affected by the dieldrin exposure. The haematin concentrations collected for the unexposed and exposed cohorts were not significantly different, suggesting that similar biological function of digestive systems was taking place in both

cohorts of mosquitoes. Dieldrin exposure therefore may not have affected the biological pathways associated with blood feeding and digestion.

The fecundity results indicated that the exposed cohort had possibly been affected by the dieldrin exposure, as seen by the low ovipositional fecundity and a lower overall fecundity when compared to the unexposed cohort of females. The reduced fecundity in the exposed cohort was identified as egg retention. The post-mortem dissections indicated that large numbers of eggs had been retained within the ovaries of these females. Egg retention in *Anopheles* has been attributed to an artefact associated with the confined space of cages used for adult rearing (Chadee and Beier, 1996). The oviposition vials used were relatively small, however egg retention in the unexposed cohort was not observed to be as high as the exposed cohort. This indicates that dieldrin exposure may be negatively affecting the female's ability to oviposit.

The range in body size was not very large with only four significantly different wing length groups representing the entire GAH strain of mosquitoes. The positive correlation of fecundity with body size observed in the unexposed cohort correspond to findings described by Briegel (1990a) that describe a similar positive correlation in *An. gambiae* females. The ovipositional fecundity displays a much clearer difference between the unexposed and exposed cohorts. The negative correlation with body size in the exposed GAH cohort combined with very low overall ovipositional fecundity immediately indicates that there has been an affect by the dieldrin exposure, when compared with the unexposed GAH cohort. The unexposed cohort shows similar ovipositional fecundity and total fecundity indicating what would be expected as "normal". These differences were later correlated with egg retention as reported above but the actual cause still remains unknown.

A very small percentage of the females were successfully inseminated in this experiment. However, the percentage was the same for both unexposed and exposed cohorts indicating that it was not due to the exposure of the females to dieldrin. Possible explanations for the low insemination success have been attributed to problems occurring with the insectary climate control at the time of experimentation. Large fluctuations in the temperature and humidity were recorded at this time, with increases of as much as 10 °C. This was regarded as a possible reason for the decreased insemination as all previous experiments measuring mating success, indicated 100 % success rate as described in chapter three.

The longevity results from the GAH experiment were completely opposite to those obtained in chapter two for COGS. It was recorded that exposed GAH females lived significantly longer than unexposed females. The exposure to dieldrin could have affected the females in such a way that they had the ability to survive longer than their counterparts but at a cost of retaining their immature eggs for longer and dying without ovipositing. This, however, would need further investigation. The varying insectary conditions mentioned above may have also impacted on the survival of the females as unstable environmental conditions may negatively impact on biological functioning of the mosquitoes. Longevity has also been shown to be proportional to the number of blood meals taken by females (Briegel, 1990b). The longevity of these females was recorded after only one blood meal and therefore a reduced longevity for both cohorts was expected when compared to females that have had more than one blood meal.

Chapter 6

Summary and conclusion

6.1 Summary

Two experimental *An. gambiae* strains were used in the present study, a wild field population and a laboratory strain. The wild population (COGS) was used to establish a colony and subsequent experimentation showed a decrease in the resistance towards dieldrin (Koekemoer *et al.* 2011). Field testing indicated high frequencies of resistance to the insecticide but in the laboratory environment, the resistance decreased significantly by the 8th generation. This suggests that exposure to the insecticide in the field is more common than previously anticipated, allowing for the selection of resistant individuals in naturally existing populations. In the laboratory, no selective pressures were actively applied to this strain and the subsequent resistance levels decreased. It has been shown that if resistance is associated with a biological constraint, such as decreased fecundity, then the resistance gene(s) would decrease in frequency once the selection pressure for the resistance has been removed, (Campanhola *et al.* 1991). COGS and GAH are genetically significantly different due to inversion 2LA (Du *et al.* 2005; Brooke *et al.* 2006 and Koekemoer *et al.* 2011) and it is therefore possible that in contrast to GAH, the mutation in the COGS population is found elsewhere and this may have allowed the resistance mechanism to disappear in the absence of selection. There may also be other mechanisms involved, but this goes beyond the scope of this may be worth looking at in future.

It was discussed in chapter 2 that exposure to dieldrin induced egg retention in the wild exposed population, revealing that this negative impact on the anopheline reproductive biology is associated with dieldrin exposure. The longevity of these females was also

negatively affected by the exposure to dieldrin as exposed females showed decreased longevity.

Further experimentation discussed in chapter 3 elucidated that the male gametes, whether present in the male testes or in the spermathecae of inseminated females, remained apparently unaffected after exposure to dieldrin. In addition to this, it was observed that dieldrin exposure had no adverse effect on mating in either male or female mosquitoes.

Investigations into the processes leading up to oviposition, with an emphasis on blood meal number and oocyte developmental rates confirmed observations made by previous authors. An optimal number of two blood meals provided all females with enough energy to mature their primary egg batch of the first gonotrophic cycle. The growth stages of oocytes and ovaries were recorded, with the entire developmental process captured photographically.

The development of the oocytes and corresponding ovaries were observed to mature at a slower rate in dieldrin exposed females, with a large proportion not having matured eggs within the normal 72 hour period.

When a single cohort of GAH females was tested for multiple data sets per female (chapter five), a clearer picture was revealed. Egg retention was observed to be higher in exposed females. The utilization of haemoglobin remained unaffected after exposure to dieldrin. Most biological traits that could have affected fecundity after exposure to dieldrin were observed to remain unchanged. Blood feeding however was observed to be slightly affected as most GAH females only imbibed one blood meal after being offered two. This corresponds partially with observations by Hunt (1984).

Longevity was recorded to be higher in females that had been exposed to dieldrin. This result contradicts the results outlined in chapter two where the opposite occurred. Possible explanations for this are strongly related to the genetically different mosquito strains used in the different experiments. When wild females are used to form laboratory strains, immediate genetic bottle-necks are formed and the allelic variation within the new colony will be determined by the numbers used to initialise the strain. As laboratory strains grow older, the mosquitoes become more homogenous due to constant selection pressures presented in the laboratory. GAH is an older colony with stable resistance traits, therefore extended longevity in resistant individuals may be due to increased ability in adapting to adverse conditions as apposed to COGS.

The characteristics of GAH indicate a more genetically homogenous mosquito strain when compared to COGS. This could be observed in the smaller variation of body size as well as the resistance profile. The COGS strain, conversely, indicated a much higher frequency of variation in body size and the changing resistance profile.

Feeding was observed to be slightly affected by exposure to dieldrin but not to the extent observed by Hunt (1984). This result may be due to the fact that all females were allowed up to 30 minutes to feed, giving them adequate time to settle down and take a blood meal. Reproductive processes were observed to occur slower than normal indicating that exposure to dieldrin may have a negative effect on oocyte growth.

6.2 Conclusion

Overall, similar results to those observed by Hunt (1984) and Norton (2008) were found in the laboratory strain of *An. gambiae*. Further investigation showed that the decreased fecundity was due to the retention of eggs within the ovaries of exposed females. Dieldrin exposure was not observed to affect haematin utilisation, spermatozoa viability or the ability to mate in both males and females. This is an indication that the effect of exposure may be limited entirely to the ovarian and oocyte tissues of the females. A more in depth investigation into the exact mechanisms, however, is needed to confirm this.

The use of dieldrin has been banned worldwide. However stockpiles remain dispersed in African countries and the insecticide remains unregulated in certain countries due to the lack of infrastructure and little or no education in areas of vector control. The present study indicates that dieldrin is possibly still being used in areas in or around Pointe Noire in the Republic of the Congo as there is no information or evidence of the usage of fipronil in this area. Given the high frequencies of resistance in the F₁-progeny of wild females, and the rapid loss of resistance by the 8th generation of colonisation, one can only assume that this dieldrin resistance confers a fitness cost (unlike GAH) and that resistance in Point Noire is maintained through selection pressure of an otherwise unknown source. This study also indicates that dieldrin exposure may possibly affect fecundity and longevity to the extent that it may be detrimental to the mosquito's survival and fitness of progeny. More information however, is needed to confirm this possibility and new insights into the molecular effects of dieldrin exposure on ovarian tissues may help elucidate the mechanisms that may be involved.

It must also be remembered that when testing any vector control strategy in the laboratory, results should always be confirmed in the field before any sort of control measures can be used in the wild. The differences observed between a wild population and a laboratory population in this current study clearly indicate the importance of thorough investigation.

Appendices

References

- Ackerman, M. J. and Clapham, D. E. (1997). Ion channels--basic science and clinical disease. *New England Journal of Medicine*. **336** (22), 1575.
- Arnaud, L. and Haubruge, E. (2002). Insecticide resistance enhances male reproductive success in a beetle. *Evolution*. **56** (12), 2435-2444.
- Bai, H., Gelman, D. B. and Palli, S. R. (2010). Mode of action of methoprene in affecting female reproduction in the African malaria mosquito, *Anopheles gambiae*. *Pest Management Science*. **66**, 936-943.
- Bayoh, M. N. and Lindsay, S. W. (2003). Effect of temperature on the development of the aquatic stages of *Anopheles gambiae* sensu stricto (Diptera: Culicidae). *Bulletin of Entomological Research*. **93** (5), 375-381.
- Beklemishev, W. N., Detinova, T. S. and Polovodova, V. P. (1959). Determination of physiological age in anophelines and of age distribution in anopheline populations in the USSR. *Bulletin of the World Health Organization*. **21** (2), 223.
- Bergé, J. B., Feyereisen, R. and Amichot, M. (1998). Cytochrome P450 monooxygenases and insecticide resistance in insects. *Philosophical Transactions of the Royal Society B: Biological Sciences*. **353** (1376), 1701.
- Bermudez, I., Hawkins, C. A., Taylor, A. M. and Beadle, D. J. (1991). Actions of insecticides on the insect GABA receptor complex. *Journal of Receptor Research*. **11** (1-4), 221-232.
- Bertram, D. S. (1962). The ovary and ovarioles of mosquitoes. *Age-Grouping in Diptera of Medical Importance*. Geneva: World Health Organization.
- Bielza, P., Quinto, V., Gravalos, C., Abellan, J. Fernandez, E. (2008). Lack of fitness costs of insecticide resistance in the western flower thrips (Thysanoptera: Thripidae). *Journal of Economic Entomology*, **101**, (2), 499-503.
- Bloomquist, J. R. (1999). Insecticides: Chemistries and Characteristics. (<http://ipmworld.umn.edu/>), 1-17.

- Bock, M. E. and Milby, M. M. (1981). Seasonal variation of wing length and egg raft size in *Culex tarsalis*. *Proceedings and Papers of the Annual Conference-California Mosquito Vector Control Association*, **49**, 64-66.
- Bretveld, R. W., Thomas, C. M., Scheepers, P. T., Zielhuis, G. A. and Roeleveld, N. (2006). Pesticide exposure: the hormonal function of the female reproductive system disrupted. *Reproductive Biology and Endocrinology*. **4** (30), 1477-7827.
- Briegel, H. (1980). Determination of uric acid and hematin in a single sample of excreta from blood-fed insects. *Cellular and Molecular Life Sciences (CMLS)*. **36** (12), 1428-1428.
- Briegel, H. (1985). Mosquito reproduction: Incomplete utilization of the blood meal protein for oogenesis. *Journal of Insect Physiology*. **31** (1), 15-21.
- Briegel, H. (1990a). Fecundity, metabolism, and body size in *Anopheles* (Diptera: Culicidae), vectors of malaria. *Journal of Medical Entomology*. **27** (5), 839-850.
- Briegel, H. (1990b). Metabolic relationship between female body size, reserves, and fecundity of *Aedes aegypti*. *Journal of Insect Physiology*. **36** (3), 165-172.
- Briegel, H. and Rezzonico, L. (1985). Concentration of host blood protein during feeding by anopheline mosquitoes (Diptera: Culicidae). *Journal of Medical Entomology*. **22** (6), 612-618.
- Brooke, B. D., Hunt, R. H. and Coetzee, M. (2000). Resistance to dieldrin + fipronil assort with chromosome inversion 2La in the malaria vector *Anopheles gambiae*. *Medical and Veterinary Entomology*. **14** (2), 190-194.
- Brooke, B. D., Hunt, R. H., Matambo, T. S., Koekemoer, L. L., Van Wyk, P. and Coetzee, M. (2006). Dieldrin resistance in the malaria vector *Anopheles gambiae* in Ghana. *Medical and Veterinary Entomology*. **20** (3), 294-299.
- Brown, A. W. A. (1986). Insecticide Resistance in Mosquitoes: A Pragmatic Review. *Journal of the American Mosquito Control Association*. **2** 123-140.

- Campanhola, C., Mccutchen, B. F., Baehrecke, E. H., Plapp jr, F. W. (1991). Biological constraints associated with resistance to pyrethroids in the tobacco bud worm (Lepidoptera: Noctuidae). *Journal of economic Entomology*, **84** (5) 1404-1411.
- Chadee, D. D. and Beier, J. C. (1996). Natural variation in blood-feeding kinetics of four mosquito vectors. *Journal of Vector Ecology*. **21** (2), 150-155.
- Chalmers, A. E. (1993). Using the Techniques of Molecular Biology to Search for Novel Insecticides. *Environmental Health Perspectives*. **101** (7), 594-596.
- Chambers, G. M. and Klowden, M. J. (2001). Age of *Anopheles gambiae* Giles male mosquitoes at time of mating influences female oviposition. *Journal of Vector Ecology*. **26** (2), 196-201.
- Charlwood, J. D., Pinto, J., Sousa, C. A., Ferreira, C. and Rosário, V. E. (2002). Male size does not affect mating success of *Anopheles gambiae* in Sao Tome. *Medical and Veterinary Entomology*. **16** (1), 109-111.
- Christopher, S. R. (1911). The development of the egg follicle in anophelines. *Paludism*. **2** 73-88.
- Clements, A. N. (1992). Development, Nutrition and Reproduction. *In: The Biology of Mosquitoes*. Volume 1. Chapman and Hall, London.
- Clements, A. N. (1999). Sensory Reception and Behaviour. *In: The Biology of Mosquitoes: Volume 2*: CABI Publishing.
- Coetzee, M., Horne, D. W. K., Brooke, B. D. and Hunt, R. H. (1999). DDT, Dieldrin and Pyrethroid Insecticide Resistance in African Malaria Vector Mosquitoes: An Historical Review and Implications for Future Malaria Control in Southern Africa. *South African Journal of Science*. **95** (5), 215-218.
- David, J. P., Strode, C., Vontas, J., Nikou, D., Vaughan, A., Pignatelli, P. M., Louis, C., Hemingway, J. and Ranson, H. (2005). The *Anopheles gambiae* detoxification chip: a highly specific microarray to study metabolic-based insecticide resistance in malaria vectors. *Proceedings of the National Academy of Sciences of the United States of America*. **102** (11), 4080.

- Davidson, G. (1956). Insecticide resistance in *Anopheles gambiae* Giles: a case of simple Mendelian inheritance. *Nature*, **178**, 863.
- Davidson, G. and Hamon, J. (1962). A case of dominant dieldrin resistance in *Anopheles gambiae* Giles.
- De Kom, J. F. M. and Dewan, A. (2007). Dieldrin. 21. Draft revised poisons information. *Monograph for peer review*. National Institute for Occupational Health, India.
- De Meillon, B. (1936). The control of malaria in South Africa by measures directed against the adult mosquitoes in habitations. *The control of malaria in South Africa by measures directed against the adult mosquitoes in habitations*. 134-137.
- De Schroeder, T. M. F. and De D'Angelo, A. M. P. (1995). Dieldrin modifies the hydrolysis of PIP₂ and decreases the fertilization rate in *Bufo arenarum* oocytes. *Comparative Biochemistry and Physiology Part C: Comparative Pharmacology and Toxicology*. **112** (1), 61-67.
- Detinova, T. S. (1959). Age-grouping methods in Diptera of medical importance. *Course in advanced entomological techniques applied to malaria eradication*. Geneva: World Health Organization.
- Devonshire, A. L. (1977). The properties of a carboxylesterase from the peach-potato aphid, *Myzus persicae* (Sulz.), and its role in conferring insecticide resistance. *Biochemical Journal*. **167** (3), 675.
- Du, W., Awolola, T. S., Howell, P., Koekemoer, L. L., Brooke, B. D., Benedict, M. Q., Coetzee, M. and Zheng, L. (2005). Independent mutations in the *Rdl* locus confer dieldrin resistance to *Anopheles gambiae* and *An. arabiensis*. *Insect Molecular Biology*. **14** (2), 179-183.
- Enna, S. J. and Mohler, H. (2007). The GABA receptors. *The GABA receptors*. 1-21.
- Etang, J., Manga, L., Chandre, F., Guillet, P., Fondjo, E., Mimpfoundi, R., Toto, J. and Fontenille, D. (2003). Insecticide Susceptibility Status of *Anopheles gambiae* sl (Diptera: Culicidae) in the Republic of Cameroon. *Journal of Medical Entomology*. **40** (4), 491-497.

- Farenhorst, M., Farina, D., Scholte, E. J., Takken, W., Hunt, R. H., Coetzee, M. and Knols, B. G. J. (2008). African water storage pots for the delivery of the entomopathogenic fungus *Metarhizium anisopliae* to the malaria vectors *Anopheles gambiae* ss and *Anopheles funestus*. *The American Journal of Tropical Medicine and Hygiene*. **78** (6), 910.
- Favia, G., Della Torre, A., Bagayoko, M., Lanfrancotti, A., Sagnon, N. F., Toure, Y. T. and Coluzzi, M. (1997). Molecular identification of sympatric chromosomal forms of *Anopheles gambiae* and further evidence of their reproductive isolation. *Insect Molecular Biology*. **6** (4), 377-383.
- Feyereisen, R. (1995). Molecular biology of insecticide resistance. *Toxicology Letters*. **82** 83-90.
- Ffrench-Constant, R. H. (1994). The molecular and population genetics of cyclodiene insecticide resistance. *Insect Biochemistry and Molecular Biology*. **24** (4), 335-345.
- Ffrench-Constant, R. H., Mortlock, D. P., Shaffer, C. D., MacIntyre, R. J. and Roush, R. T. (1991). Molecular cloning and transformation of cyclodiene resistance in *Drosophila*: an invertebrate gamma-aminobutyric acid subtype A receptor locus. *Proceedings of the National Academy of Sciences of the United States of America*. **88** (16), 7209.
- Ffrench-Constant, R. H., Anthony, N., Aronstein, K., Rocheleau, T. and Stilwell, G. (2000). Cyclodiene insecticide resistance: from molecular to population genetics. *Annual Review of Entomology*. **45** (1), 449-466.
- Fiil, A. (1976). Oogenesis in the malaria mosquito *Anopheles gambiae*. *Cell and Tissue Research*. **167** (1), 23-35.
- Fillinger, U., Knols, B. G. J. and Becker, N. (2003). Efficacy and efficiency of new *Bacillus thuringiensis* var. *israelensis* and *Bacillus sphaericus* formulations against Afrotropical anophelines in Western Kenya. *Tropical Medicine & International Health*. **8** (1), 37-47.

- Frean, J. (2007). Microscopic images transmitted by mobile cameraphone. *Transactions of the Royal Society of Tropical Medicine and Hygiene*. **101** (10), 1053.
- Gallup, J. L. and Sachs, J. D. (2001). The economic burden of malaria. *The American Journal of Tropical Medicine and Hygiene*. **64** (1 suppl), 85.
- Gary Jr, R. E. and Foster, W. A. (2004). *Anopheles gambiae* feeding and survival on honeydew and extra-floral nectar of peridomestic plants . *Medical and Veterinary Entomology*. **18** (2), 102-107.
- Georghiou, G. P. (1969). Parasitological review. Genetics of resistance to insecticides in houseflies and mosquitoes. *Experimental Parasitology*. **26** (2), 224-255.
- Georghiou, G. P. and Metcalf, R. L. (1963). Dieldrin susceptibility: partial restoration in *Anopheles* selected with a carbamate. *Science (New York, N.Y.)*. **140** 301-302.
- Gillies, M. T. (1954). The recognition of age-groups within populations of *Anopheles gambiae* by the pre-gravid rate and the sporozoite rate. *Annals of Tropical Medicine and Parasitology*. **48** (1), 58-74.
- Gillies, M. T. and Coetzee, M. (1987). A Supplement to the Anophelinae of Africa South of the Sahara. *Publications of the South African Institute for Medical Research*. **55** 1-143.
- Gillies, M. T. and De Meillon, B. (1968). The Anophelinae of Africa South of the Sahara (Ethiopian Zoogeographical Region). The Anophelinae of Africa south of the Sahara (Ethiopian Zoogeographical Region). *Publications of the South African Institute for Medical Research*. **54**
- Gimnig, J. E., Ombok, M., Otieno, S., Kaufman, M. G., Vulule, J. M. and Walker, E. D. (2002). Density-dependent development of *Anopheles gambiae* (Diptera: Culicidae) larvae in artificial habitats. *Journal of Medical Entomology*. **39** (1), 162-172.

- Guerra, C. A., Gikandi, P. W., Tatem, A. J., Noor, A. M., Smith, D. L., Hay, S. I. and Snow, R. W. (2008). The Limits and Intensity of *Plasmodium falciparum* Transmission: Implications for Malaria Control and Elimination Worldwide. *PLoS Medicine*. **5** (2),
- Hayes, W. J. and Laws, E. R. (1991). Handbook of pesticide toxicology. Academic Press, New York. 612-613.
- Hemingway, J., Hawkes, N., Prapanthadara, L., Jayawardenal, K. G. I. and Ranson, H. (1998). The role of gene splicing, gene amplification and regulation in mosquito insecticide resistance. *Philosophical Transactions: Biological Sciences*. **353** (1376, Insecticide Resistance: From Mechanisms to Management), 1695-1699.
- Hemingway, J. and Ranson, H. (2000). Insecticide resistance in insect vectors of human disease. *Annual Review of Entomology*. **45** (1), 371-391.
- Hogg, J. C. and Hurd, H. (1995a). *Plasmodium yoelii nigeriensis*: the effect of high and low intensity of infection upon the egg production and bloodmeal size of *Anopheles stephensi* during three gonotrophic cycles. *Parasitology*. **111** (05), 555-562.
- Hogg, J. C. and Hurd, H. (1995b). Malaria-induced reduction of fecundity during the first gonotrophic cycle of *Anopheles Stephensi* mosquitoes. *Medical and Veterinary Entomology*. **9** (2), 176-180.
- Hummon, W. D. (1974). Effects of DDT on Longevity and Reproductive Rate in *Lepidodermella squammata* (Gastroticha, Chaetonotida). *American Midland Naturalist* **92** (2), 327-339.
- Hunt, R. H. (1984). Natural and induced chromosomal rearrangements in three members of the *Anopheles gambiae* complex. *Masters Dissertation*. University of the Witwatersrand, Johannesburg.
- Hunt, R. H., Coetzee, M. and Fettene, M. (1998). The *Anopheles gambiae* complex: a new species from Ethiopia. *Transactions of the Royal Society of Tropical Medicine and Hygiene*. **92** (2), 231-235.

- Hurd, H., Hogg, J. C. and Renshaw, M. (1995). Interactions between bloodfeeding, fecundity and infection in mosquitoes. *Parasitology Today*. **11** (11), 411-416.
- Insecticide Resistance Action Committee (IRAC) (www.iraconline.org).
- Kadous, A. A., Ghiasuddin, S. M., Matsumura, F., Scott, J. G. and Tanaka, K. (1983). Difference in the picrotoxinin receptor between the cyclodiene-resistant and susceptible strains of the German cockroach. *Pesticide Biochemistry and Physiology*. **19** (2), 157-166.
- Kaiser, M. L., Koekemoer, L. L., Coetzee, M., Hunt, R. H. and Brooke, B. D. (2010). Staggered larval time-to-hatch and insecticide resistance in the major malaria vector *Anopheles gambiae* S form. *Malaria Journal*. **9** (1), 360.
- Karch, S., Manzambi, Z. A. and Salaun, J. J. (1991). Field trials with Vectolex (*Bacillus sphaericus*) and Vectobac (*Bacillus thuringiensis* (H-14)) against *Anopheles gambiae* and *Culex quinquefasciatus* breeding in Zaire. *Journal of the American Mosquito Control Association*. **7** (2), 176-179.
- Kikankie, C. K., Brooke, B. D., Knols, B. G., Koekemoer, L. L., Farenhorst, M., Hunt, R. H., Thomas, M. B. and Coetzee, M. (2010). The infectivity of the entomopathogenic fungus *Beauveria bassiana* to insecticide-resistant and susceptible *Anopheles arabiensis* mosquitoes at two different temperatures. *Malaria journal*. **9** 71.
- Koekemoer, L. L., Spellings, B. L., Christian, R., Lo, T. M., Kaiser, M. L. Norton, R. A. I., Oliver, S., Choi, K. S., Brooke, B. D., Hunt, R. H. and Coetzee, M. (2011). Multiple insecticide resistance in *Anopheles gambiae* (Diptera: Culicidae) from Pointe Noire, Republic of the Congo. *Vector-Borne and Zoonotic Diseases*. (In press).
- Koenraadt, C. J. M., Majambere, S., Hemerik, L. and Takken, W. (2004). The effects of food and space on the occurrence of cannibalism and predation among larvae of *Anopheles gambiae* sl. *Entomologia Experimentalis et Applicata*. **112** (2), 125-134.

- Koenraadt, C. J. M. and Takken, W. (2003). Cannibalism and predation among larvae of the *Anopheles gambiae* complex. *Medical and Veterinary Entomology*. **17** (1), 61-66.
- Lea, A. O., Dimond, J. B. and DeLong, D. M. (1956). Role of diet in egg development by mosquitoes (*Aedes aegypti*). *Science*. **123** (3203), 890-891.
- Lewontin, R. C. and Hubby, J. L. (1966). A molecular approach to the study of genic heterozygosity in natural populations. II. Amount of variation and degree of heterozygosity in natural populations of *Drosophila pseudoobscura*. *Genetics*. **54** (2), 595.
- Lyimo, E. O. and Takken, W. (1993). Effects of adult body size on fecundity and the pre-gravid rate of *Anopheles gambiae* females in Tanzania. *Medical and Veterinary Entomology*. **7** (4), 328-332.
- Majori, G., Ali, A. and Sabatinelli, G. (1987). Laboratory and field efficacy of *Bacillus thuringiensis* var. israelensis and *Bacillus sphaericus* against *Anopheles gambiae* S. L. and *Culex quinquefasciatus* in Ouagadougou, Burkina Faso. *Journal of the American Mosquito Control Association*. **3** (1), 20-25.
- Mann, T., Martin, A. W. and French, M. (1988). Entry of pollutants from seawater into the spermatophore and spermatozoa of the giant octopus of the North Pacific. *Marine Pollution Bulletin*. **19** (12), 669-671.
- Martin, I. L., Bowery, N. G. and Dunn, S. M. (2002). GABA receptors. *Tocris Review*. **20**, 1-16.
- Mason, L. J., Pashley, D. P. and Johnson, S. J. (1987). The laboratory as an altered habitat: phenotypic and genetic consequences of colonization. *Florida Entomologist*. **70** (1), 49-58.
- Meadows, K. E. (1968). A Simple Method of Mosquito Ovary Dissection. *Florida Entomologist*. **51** (1), 31-35.

- Muturi, E. J., Costanzo, K., Kesavaraju, B., Lampman, R. and Alto, B. W. (2010). Interactions of a pesticide and larval competition on life history traits of *Culex pipiens*. *Acta Tropica*. **16** (2), 141-146.
- Mwatibo, J. M. and Green, J. D. (1997). Effects of methoxychlor pre-exposure on sea urchin gametes. *Bulletin of Environmental Contamination and Toxicology*. **58** (4), 589-595.
- Norton, R. A. I. (2008). The effect of dieldrin exposure on egg production and fecundity in *Anopheles gambiae* and *Anopheles arabiensis*. *Honours Project*. University of the Witwatersrand, Johannesburg.
- Ortelli, F., Rossiter, L. C., Vontas, J., Ranson, H. and Hemingway, J. (2003). Heterologous expression of four glutathione transferase genes genetically linked to a major insecticide-resistance locus from the malaria vector *Anopheles gambiae*. *Biochemical Journal*. **373** (3), 957.
- Ottea, J. A. and Plapp, F. W. (1984). Glutathione S-transferase in the house fly: biochemical and genetic changes associated with induction and insecticide resistance. *Pesticide Biochemistry and Physiology*. **22** (2), 203-208.
- Packer, M. J. and Corbet, P. S. (1989). Size variation and reproductive success of female *Aedes punctator* (Diptera: Culicidae). *Ecological Entomology*. **14** (3), 297-309.
- Patel, T. B. and Rao, V. N. (1958). Dieldrin Poisoning in Man. *British Medical Journal*. **1** (5076), 919.
- Payne, P. A., Dryden, M. W., Smith, V. and Ridley, R. K. (2001). Effect of 0.29% w/w fipronil spray on adult flea mortality and egg production of three different cat flea, *Ctenocephalides felis* (Bouche), strains infesting cats. *Veterinary Parasitology*. **102** (4), 331-340.
- Pennetier, C., Warren, B., Dabire, K. R., Russell, I. J. and Gibson, G. (2010). "Singing on the Wing" as a Mechanism for Species Recognition in the Malarial Mosquito *Anopheles gambiae*. *Current biology*. **20** (2), 131-136.

- Picard, A., Palavan, G., Robert, S., Pesando, D. and Ciapa, B. (2003). Effect of organochlorine pesticides on maturation of starfish and mouse oocytes. *Toxicological Sciences*. **73** (1), 141.
- Pinto, J., Lynd, A., Vicente, J. L., Santolamazza, F., Randle, N. P., Gentile, G., Moreno, M., Simard, F., Charlwood, J. D. and Do Rosário, V. E. (2007). Multiple origins of knockdown resistance mutations in the Afrotropical mosquito vector *Anopheles gambiae*. *PLoS One*. **2** (11), 1243.
- Prapanthadara, L. A., Koottathep, S., Promtet, N., Hemingway, J. and Ketterman, A. J. (1996). Purification and characterization of a major glutathione S-transferase from the mosquito *Anopheles dirus* (species B). *Insect Biochemistry and Molecular Biology*. **26** (3), 277-285.
- Ramakrishna, V. and Elliott, R. (1959). Insecticide resistance in *Anopheles gambiae* in Sokoto Province. *Transactions of the Royal Society of Tropical Medicine and Hygiene*. **53** (1), 102-109.
- Ranson, H., Rossiter, L., Ortelli, F., Jensen, B., Wang, X., Roth, C. W., Collins, F. H. and Hemingway, J. (2001). Identification of a novel class of insect glutathione S-transferases involved in resistance to DDT in the malaria vector *Anopheles gambiae*. *Biochemical Journal*. **359** (2), 295.
- Reinecke, S. A., Reinecke, A. J. and Froneman, M. L. (1995). The effects of dieldrin on the sperm ultrastructure of the earthworm *Eudrilus eugeniae* (Oligochaeta). *Environmental Toxicology and Chemistry*. **14** (6), 961-965.
- Sachs, J. and Malaney, P. (2002). The economic and social burden of malaria. *Nature*. **415** (6872), 680-685.
- Scholte, E. J., Knols, B. G. J. and Takken, W. (2006). Infection of the malaria mosquito *Anopheles gambiae* with the entomopathogenic fungus *Metarhizium anisopliae* reduces blood feeding and fecundity. *Journal of Invertebrate Pathology*. **91** (1), 43-49.

- Scott, T. G., Willis, Y. L. and Ellis, J. A. (1959). Some Effects of a Field Application of Dieldrin on Wildlife. *The Journal of Wildlife Management*. **23** (4), 409-427.
- Shahabuddin, M., Toyoshima, T., Aikawa, M. and Kaslow, D. C. (1993). Transmission-blocking activity of a chitinase inhibitor and activation of malarial parasite chitinase by mosquito protease. *Proceedings of the National Academy of Sciences of the United States of America*. **90** (9), 4266.
- Shen, Z. and Jacobs-Lorena, M. (1997). Characterization of a novel gut-specific chitinase gene from the human malaria vector *Anopheles gambiae*. *Journal of Biological Chemistry*. **272** (46), 28895.
- Sinka, M. E., Bangs, M. J., Manguin, S., Coetzee, M., Mbogo, C. M., Hemingway, J., Patil, A. P., Temperley, W. H., Gething, P. W., Cabaria, C. W., Okara, R. M., Van Boeckel, T., Godfray, H. C. J., Harbach, R. E., Hay, S. I. (2010). The dominant *Anopheles* vectors of human malaria in Africa, Europe and the Middle East: occurrence data, distribution maps and bionomic précis. *Parasites and Vectors*. **3** (1), 117-132.
- Skovmand, O. and Sanogo, E. (1999). Experimental formulations of *Bacillus sphaericus* and *B. thuringiensis israelensis* against *Culex quinquefasciatus* and *Anopheles gambiae* (Diptera: Culicidae) in Burkina Faso. *Journal of Medical Entomology*. **36** (1), 62-67.
- Slotman, M. A., Tripet, F., Cornel, A. J., Meneses, C. R., Lee, Y., Reimer, L. J., Thiemann, T. C., Fondjo, E., Fofana, A. and Traore, S. F. (2007). Evidence for subdivision within the M molecular form of *Anopheles gambiae*. *Molecular ecology*. **16** (3), 639-649.
- Steinwascher, K. (1982). Relationship between pupal mass and adult survivorship and fecundity for *Aedes aegypti*. *Environmental Entomology*. **11** (1), 150-153.
- Takken, W. and Costantini, C. (2006). The Genetics of Vector-Host Interactions: Alternative Strategies for Genetic Engineering for Malaria Control. In: *Genetically Modified Mosquitoes for Vector Control*. Christophe Boëte, Institut de Recherche pour le Développement.

- Takken, W. and Knols, B. G. J. (1999). Odor-mediated behavior of Afrotropical malaria mosquitoes. *Annual Review of Entomology*. **44** (1), 131-157.
- Tanaka, K. and Matsumura, F. (1986). Altered picrotoxinin receptor as a cause for cyclodiene resistance in *Musca domestica*, *Aedes aegypti* and *Blattella germanica*. In: Membrane Receptors and Enzymes as Targets of Insecticidal Action. Plenum Press, New York.
- Tanaka, K., Scott, J. G. and Matsumura, F. (1984). Picrotoxinin receptor in the central nervous system of the American cockroach: Its role in the action of cyclodiene-type insecticides. *Pesticide Biochemistry and Physiology*. **22** (1), 117-127.
- Tempelis, C. H. (1975). Host-feeding patterns of mosquitoes, with a review of advances in analysis of blood meals by serology. *Journal of Medical Entomology*. **11** (6), 635-653.
- Thompson, M. and Steichen, J. C. (1993). Conservation of cyclodiene insecticide resistance-associated mutations in insects. *Insect Molecular Biology*. **2** (3), 149-154.
- Toure, Y. T. (1984). Sensitivity of *Anopheles gambiae* s.l. to insecticides in the Selingue dam area. *Parassitologia*. **26** (3), 311-318.
- Tren, R., Bate, R. and Liberty Institute (New Delhi, India). (2000). When politics kills: malaria and the DDT story. (<http://cei.org/PDFs/malaria.pdf>).
- Trigg, P. I. and Kondrachine, A. V. (1998). Commentary: malaria control in the 1990s. *Bulletin of the World Health Organization*. **76** (1), 11.
- Vontas, J., Blass, C., Koutsos, A. C., David, J. P., Kafatos, F. C., Louis, C., Hemingway, J., Christophides, G. K. and Ranson, H. (2005). Gene expression in insecticide resistant and susceptible *Anopheles gambiae* strains constitutively or after insecticide exposure. *Insect Molecular Biology*. **14** (5), 509-521.
- Walker, K. and Lynch, M. (2007). Contributions of *Anopheles* larval control to malaria suppression in tropical Africa: review of achievements and potential. *Medical and Veterinary Entomology*. **21** (1), 2-21.

- Wandiga, S. O. (2001). Use and distribution of organochlorine pesticides. The future in Africa. *Pure and Applied Chemistry*. **73** (7), 1147-1156.
- World Health Organisation. (1975). Food and agriculture organisation: Data sheets on pesticides No. 17: Dieldrin. VBC/DS/75.17.
- World Health Organization. (1998). Test procedures for insecticide resistance monitoring in malaria vectors, bio-efficacy and persistence of insecticides on treated surfaces. Document WHO/CDS/CPC/MAL/98 12. WHO, Geneva.
- World Health Organisation. (2002). Supplies for monitoring insecticide resistance in disease vectors: Procedures and conditions. WHO/CDS/CPE/PVC/2001.2
- World Health Organisation. (2006). Pesticides and their application: For the control of vectors and pests of public health importance.
- World Health Organisation. (2008). World malaria report. WHO/HTM/GMP/2008.1.
- World Health Organisation. (2010). World malaria report. WHO.HTM/GMP.2010.1.
- Yapabandara, A., Curtis, C. F., Wickramasinghe, M. B. and Fernando, W. P. (2001). Control of malaria vectors with the insect growth regulator pyriproxyfen in a gem-mining area in Sri Lanka. *Acta Tropica*. **80** (3), 265-276.
- Yu, S. J. (1996). Insect glutathione S-transferases. *Zoological Studies*. **35** (1), 9-19.
- Zimmermann, G. (1993). The entomopathogenic fungus *Metarhizium anisopliae* and its potential as a biocontrol agent. *Pesticide Science*. **37** 375-379.