UNIVERSITY OF LIVERPOOL

Genetic Components of Insecticide Resistance in *Anopheles arabiensis*

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Abstract

Currently 3.3 billion people are at risk of becoming infected with malaria worldwide with over 90% living in sub-Saharan Africa. Recently it was estimated that approximately 131,000 fewer deaths and over 650,000 cases of malaria were averted during the last 15 years. These changes are attributed to the widespread implementation of long-lasting insecticide-treated nets, indoor residual spraying with insecticides, and artemisinin-combined therapeutics across Africa. However, current challenges have resulted in a stalling of progress with insecticide resistance as the most prominent and potentially dangerous.

Although there has been recent interest in insecticide resistance in *Anopheles arabiensis*, much is still unknown. Compared to the other main vectors, its exophilic nature and more catholic diet results in opportunistic feeding thereby circumventing home-based insecticide-treated interventions. Therefore, the goal of this dissertation is to examine insecticide resistance within the under-studied West Africa to better understand the origin and spread of anthropogenic-associated resistance in *An. arabiensis* by 1) using quantitative trait loci (QTL) analysis, to determine the genetic basis of dieldrin resistance in *An. arabiensis* from the Sudan, 2) determine the origin and spread of *Gaba*-296S in the Sahel region and 3) investigate novel bendiocarb resistance in Senegal using a paired QTL/microarray analysis.

Using QTL analysis, a single, strong peak was estimated in both the F2 and advanced intercross line families at a similar position on the 2L chromosomal arm. In neither instance did the peak contain with the *Gaba*-296S mutation, typically implicated in dieldrin resistance, but was found 4Mb upstream towards the telomere near an ATP-binding cassette (ABCB-4) transporter. Second, an approximately 2kb region of gamma-amino butyric acid (GABA) was sequenced in individuals

collected from the wild. In resistant individuals, the same base-pair polymorphisms were seen within 10 bases after the *Gaba*-296S mutation in samples from Senegal, Burkina Faso, and the Sudan. Based on this, it was concluded that a single origin of *Gaba*-296S had occurred with a subsequent sweep across the Sahel. This is the first broad geographical spread of resistance alleles through reported for this species. Last I investigated bendiocarb resistance in a population from Rufisque, Senegal. While QTL analysis was inconclusive, microarray analysis found the cuticular protein genes CPLCX2 and CPLCG4 overexpressed in the resistant population.

Across West Africa, urbanisation and agriculture have had a major impact on the evolution of resistance in *An. arabiensis*. This dissertation highlights several important attributes that make *An. arabiensis* a particularly difficult species to control. My hope is that this body of work will stimulate research into the effects of urbanisation and resistance in *An. arabiensis*, especially in areas where urban and peri-urban agriculture is present.

Chapter 1

1.1 Introduction

It is estimated that 3.3 billion people are at risk of becoming infected with malaria worldwide (World Health Organization, 2015). Although mortality from malaria has decreased an estimated 47% since 2004, malaria was still responsible for 367,000 to 755,000 deaths globally in 2014 (World Health Organization, 2015; Murray, et al., 2012). Although this disease is found mainly in the tropic and sub-tropic regions of the world, 90% of malaria deaths occurred in Africa and of those 78% were in children under the age of five (World Health Organization, 2015). The burden in Africa is uniquely different. Widespread poverty coupled with highly favourable climatic conditions that promote rapid parasite and vector growth and reproduction, the presence of several highly competent vectors that are primarily anthropophagic, disease being caused mainly by the highly virulent parasite *Plasmodium falciparum*, and a lack of accessible health care makes the burden of malaria the most intense in sub-Saharan Africa (Eisele et al. 2000, Gallup & Sachs 2001). The burden of malaria is most profound on impoverished peoples as they are less likely to be able to afford medical care or anti-mosquito tools to protect themselves. Overall, malarious nations had lower gross domestic product per capita than non-malarious nations and over a 25-year period had a 5-fold lower economic growth overall (Gallup & Sachs, 2001).

Malaria is a protozoan disease transmitted via the bite from infected female anopheline mosquitoes (**Figure 1.1**). Human malaria is caused by one of five species of *Plasmodium*: *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae* and *P. knowlesi* (World Health Organization, 2015). Of the five, *P. falciparum* and *P. vivax* have widespread distributions in the tropic and sub-tropic regions and are responsible for much of malaria worldwide (Breman, Alilio, & White, 2007) with *P. falciparum* responsible for most of the deaths reported annually. In areas of moderate to high transmission, exposures start from birth and result in high

mortality mainly in children under the age of five (Murray et al. 2012). Constant infections in young children typically result in high rates of anaemia and delayed or stunted growth. Multiple exposures will lead to partial immunity, therefore subsequent infections often lead to less severe disease in individuals who survive past the age of five. In areas where malaria is less frequent or seasonal, infections often have more severe sequelae in all age groups (WHO 2015b).

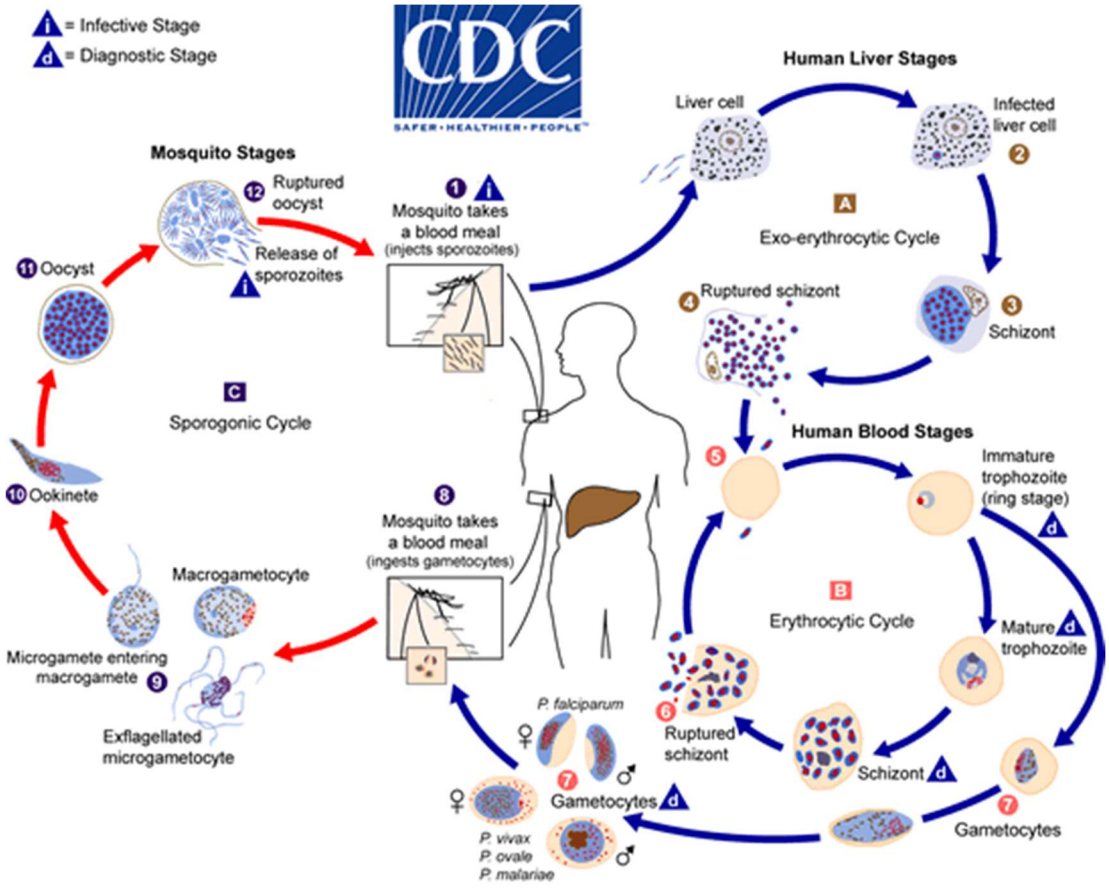


Figure 1.1: The life-cycle of the malaria parasite (courtesy of CDC).

1.2 Tools for Combating Malaria

1.2.1 Vector Control

Most malaria control programmes in Africa focus on community-level protection utilizing long-lasting insecticide-impregnated bed-nets (LLINs), also known as insecticide treated nets (ITNs), indoor-residual spraying (IRS) or a combination thereof (Kleinschmidt, et al., 2009; World Health Organization, 2012). Implementation of both IRS and LLINs have been found to have differing results on malaria prevalence rates in children under 5 (WHO 2017), however it is hypothesized that the benefits would be greatest if interventions utilising compounds with different modes of action were employed with a high coverage rate (Okumu and Moore 2011). Current estimates show that IRS and LLINs have resulted in a 70% decrease in the burden of malaria (Bhatt et al. 2015).

IRS involves covering the walls of a home with residual, or long-lasting, insecticides that target indoor-resting mosquitoes. IRS functions via two known modes of action: excito-repellency, whereby vectors are deterred from entering homes as seen with pyrethroids and organochlorines, and latent-killing in which vectors absorb toxic compounds, such as carbamates and organophosphates, while resting after a blood meal reducing their life spans. Used since the 1930's IRS has been shown to be effective in reducing malaria transmission in many trials, albeit at variable rates (Guyatt, 2002; Mabaso, Sharp, & and Lengeler, 2004; Sharp, 2007; World Health Organization, 2007; Bukirwa, et al., 2009; Kim, Fedak, & and Kramer, 2012; Hamusse, Balcha, & Belachew, 2012). Currently there are 15 approved compounds in IRS spraying in four classes: pyrethroids, carbamates, organophosphates, and organochlorines (Takken, 2002). Although there are more classes of compounds available for IRS, its deployment is not without issues. First, the bioefficacy of any insecticide is dependent on both the compound used and the substrate to which it is applied (Etang et al. 2011, Okumu et al. 2012). Second, most insecticides have been found to be effective for 2-6 months meaning that reapplication is necessary in areas of stable transmission to control endophilic mosquitoes (Guyatt, 2002; Bradley J. M., 2012). Third,

IRS campaigns have also been hampered in some areas by a lack of properly trained sprayers resulting in either inadequate protection or waste (WHO 2007b). Lastly, with widespread insecticide resistance to commonly used, inexpensive pyrethroids, operational costs are increasing due to the need to employ more expensive compounds leading to some programmes ending IRS spraying (Conteh et al. 2004, Oxborough 2016). Although IRS is more expensive, it is an effective intervention compared to LLINs (Guyatt et al. 2002). Originally, IRS was thought to be only beneficial in areas of unstable malaria transmission, however the reductions in parasitemia rates has led to the expansion of IRS into areas of stable transmission (World Health Organziation, 2006). In 2014 an estimated 6%, 50 million, of people in SSA were protected by IRS down from 11% in 2010 (Dengela et al. 2018). Even with a reduction in IRS spraying being reported, it has contributed to the decline in malaria prevalence especially in West Africa and the southern portion of South Africa (Bhatt et al. 2015).

LLINs, or bed-nets, are physical barriers that are treated with long-lasting insecticides targeting crepuscular vectors. Traditionally they are constructed from polyester or polyethylene and impregnated with long-lasting pyrethroid formulations, either alpha-cypermethrin, deltamethrin, or permethrin, due to their low toxicity in humans (WHO 2016). LLINs work through two different mechanisms: first as a primary barrier preventing female mosquitoes from feeding and second through latent-killing via the insecticide impregnated into the fibres of the net. Even though the number of insecticide classes employed to treat LLINs is limited, they have been found to be very effective in reducing malaria when used properly even when resistance to pyrethroids is present. In Benin and Kenya, where vectors are uniformly resistant to pyrethroids, LLINs were shown to reduce the incidence or prevalence of malaria in children (Bradley et al. 2017, Ochomo et al. 2017). Unlike IRS that requires frequent reapplication, modern LLINs are designed to last from three to five years before needing replacement. Although LLINs have only been used for the last 30 years to control malaria vectors in Africa, they have been found to be equally protective in various malaria transmission settings (Lindsay, 1989; Gimnig, et al., 2003).

Initial issues with levels of insecticide concentrations on LLINs (Lindblade et al. 2005, Kilian et al 2008, Kilian et al. 2011), net durability (**Table 1.1)** (Kilian et al. 2008, Kilian et al. 2011, Asidi et al. 2012), loss of insecticide over time (Lindblade et al. 2005, Kilian et al. 2008), community uptake (Toe, 2009; Macintyre, 2012; Adjei, 2012), and the presence of insecticide resistance in local vector populations (Asidi, 2012) has resulted in varying rates of malaria reduction across sub-Saharan Africa. Universal coverage is recommended by the World Health Organization (WHO) for people living in areas at risk for malaria, however preference is given to children under the age of five and pregnant women (World Health Organization, 2007). In 2016 it was estimated that 54% of people living in sub-Saharan Africa (SSA) slept under a LLIN, an increase of 24% since 2010 (WHO 2017). Implementation of LLINs alone is estimated to be responsible for a 68% reduction in the prevalence of malaria across SSA (Bhatt et al. 2015).

Table 1. 1: Net durability as determined by the presence of holes in various LLINs

Country	Brand	Percent with holes- 1 yr follow up	Average no. holes/net at 1 yr.	Percent with holes- 2 yr follow up	Percent with holes- 3 yr follow up	Reference
Benin	---	nd	9.5-10.2	nd	nd	Asidi 2012
Ethiopia	Permanet 2.0	54.5%	12	85.5%	92.5%	Wills 2013
Liberia	Interceptor	24%	2.5	nd	nd	Banek 2010
Uganda	Permanet I	70%	4.4	85%	nd	Kilian 2008
Uganda	Interceptor	25.7%	nd	46.9%	63.1%	Kilian 2011

*nd = data not presented

Larviciding was one of the primary tools used to eliminate immature mosquitoes in early vector control programmes but its large-scale use was dismissed after the introduction of inexpensive adulticides (Walker & and Lynch, 2007). Historically, the use of oils and poisons, like Paris green, were very effective at eliminating breeding sites however they were extremely toxic and highly persistent in nature, so they are no longer recommended for use (Walker & and Lynch, 2007). Unlike the insecticides used for LLINs or IRS, larvicides are relatively short lived and need to be reapplied on a frequent basis to maintain their effectiveness. Based on this, larviciding is recommended when larval sites are few, fixed, and findable (WHO 2012b). Compounds like temephos and insect-growth regulators are available, but only the bacterium *Bacillus thuringiensis* var. *israeliensis* (Bti) has been employed often in Africa. Several trials in Africa have found that Bti is effective at controlling anopheline vectors, however the duration of efficacy varies (Karch et al. 1991, Shililu et al. 2003, Geissbuhler 2009). It has been found to be effective in semi-arid regions or during the dry season where larval sites adhere to the WHO prerequisites (Maheu-Girox and Castro 2013, Mpofu et al. 2016). Currently larviciding is considered less effective than IRS or LLINs in completely controlling malaria vectors and should be considered a complimentary program that would enhance the efficacy of either or efficacious in carefully selected, amenable areas (Tusting et al. 2013).

Vector control interventions have had the largest effect towards eliminating malaria (Bhatt et al. 2015, WHO 2017). However, as LLIN distribution and IRS spraying take up a sizeable portion of spending, preserving their effectiveness is paramount to maintain efforts in eliminating malaria (Guyatt, 2002). Therefore, future interventions should be tailored to be both safe for public usage but also not based on current chemistries. Current IRS trials with new chemistries involve the organophosphate pirimiphos-methyl (Actellic) and the neonicotinoid clothianidin (SumiShield®). Although Actellic was not long-lasting, there was a 94% reduction in human-feeding compared to an untreated village (Aïkpon et al. 2014). Although there was no difference in biting-rates between the various treatment groups, approximately 90% of laboratory-reared mosquitoes assayed perished in huts

sprayed with SumiShield® up to eight months post application (Agossa et al. 2018). As these insecticides involved novel pathways, they were found to be effective against highly resistant anophelines *in natura*. Similarly, next-generation LLINs will employ multiple insecticides to increase their efficacy against resistant vectors. The addition of piperonyl butoxide (PBO) on a portion of the net increases mortality rates in mosquitoes possessing metabolic resistance by inhibiting over-expressed oxidases used to detoxify pyrethroids (Pennetier et al. 2013).

Additionally, several non-insecticide-based alternatives have been suggested which include biopesticidal fungi (Scholte, 2004; Hancock, 2009), population suppression using either sterile males through the sterile insect technique (SIT) (Alphey L, 2010) or genetically modified (GMM) vectors (McGraw EA, 2013), attractive toxic sugar baits (Mueller GC, 2010), or population replacement with genetically modified mosquitoes refractory to parasites (Alphey L, 2010). These, however, are not realistically ready for deployment into the field due to issues regarding longevity, feasibility, or regulations for use as in SIT and GMM.

1.2.2 Chemoprophylaxis in Humans

Treatment of malaria in Africa is centred on the use of inexpensive chemoprophylactic agents that target different stages of parasite growth, traditionally chloroquine or sulfadoxine/pyrimethamine (SP) (Guerin et al. 2002). However, the long-term use of these compounds, and others from the same class, as well as their use as monotherapies has resulted in the development of and subsequent rapid spread of resistance jeopardizing people across the continent. Resistance has led to the discontinuance of chloroquine while SP is still effective in some parts of Africa (Coldiron et al. 2017). The choice of a primary chemoprophylactic compound, such as SP or primaquine, is now supplemented by the addition of an artemisinin derivative to enhance the effectiveness of the treatment (Nosten and White 2007, WHO 2015b). The artemisinin combined therapies (ACTs) help preserve primary chemoprophylactics by preventing the emergence and spread of resistance while

possessing strong gametocidal properties thus interrupting parasite transmission (Nosten and Brasseur 2002). Proper diagnosis and treatment of malaria cases depends on several variables including properly trained health workers, the availability of materials to perform parasite detection assays, and the presence of a local health facility that can provide these services (Guerin, et al., 2002). Rapid diagnostic tests (RDTs) eliminate the need for highly skilled laboratorians, however in non-endemic areas they are more expensive compared to purchasing preventative therapeutics based on presumption of malaria infection (Hanson et al. 2017). The use of RDTs to diagnose children under the age of five has increased from 31% to 87% between 2011 and 2016 leading to more appropriate treatments and outcomes (WHO 2017). Timely diagnosis and treatment, therefore, are necessary to accurately identify human infections, provide prompt and appropriate treatment regimens to reduce mortality from severe disease and concomitantly reduce the parasite reservoir, thereby lowering transmission rates by mosquitoes and reducing the burden of disease in at-risk populations.

Large-scale chemoprophylaxis, using quinine then later chloroquine, was one of the initial tools to control malaria (Butler et al. 2010). However, concerns of increasing resistance to antimalarial drugs due to their proposed continual use and that their use could limit a child's ability to acquire antibodies that proffers immunity to later malarial infections led to a halt to this (Alexander, 2007; Greenwood, 2010). In lieu of mass drug administration against malaria, the WHO recommends selectively targeting and treating vulnerable populations, regardless of their infection status, with regular doses of antimalarial agents referred to as intermittent preventive treatment (IPT) or seasonal malaria chemoprevention (SMC) (WHO 2015b). The two main populations targeted by these interventions are infants and pregnant women (Bardaji 2012, WHO 2015b). As most malaria cases occur in areas of intense, seasonal transmission (Cairns, 2012), SMC is being implemented in areas targeting older children who are at a higher risk from malaria compared to infants (Greenwood, 2010). In two different meta-analyses, it was shown that using SMC could reduce clinical and severe malaria in children by approximately 75-83% (Wilson, 2011; Meremikwu,

2012). SMC in infants has led to a 30-50% reduction in clinical malaria in infants with no detrimental increase in malaria after stoppage (Greenwood, 2010). Additionally, the delivery of these chemoprophylactic agents via community health care workers ensures a large population can be covered inexpensively (Cisse, 2009; Cairns, 2012).

Although historically chemoprophylaxis has been the most common method for combating malaria infections, the current arsenal of effective agents is dwindling due to resistance while few new drugs are being brought onto the market (Guerin et al. 2002). The development of a low-cost, efficacious, long-lasting vaccine is desirable to complement the current interventions to control malaria. The main difficulty in developing a reliable vaccine is related to a lack of understanding in how the human immune system responds to a natural malaria infection (Good and Doolan 2010). Due to this knowledge gap, only one vaccine has been licensed for use against malaria however several are in various trial stages throughout Africa. Of those developed, only one has been found to have some protective effect in clinical trials in Africa (Bairwa, 2012); The pre-erythrocytic vaccine RTS,S/AS01. Currently this vaccine is in phase III and IV testing in Africa, has been found to reduce malaria in children by approximately 36.3% (Agnandji et al. 2012, Han et al 2017). Continuation of these trials is guaranteed however there are no immediate plans for widespread use of these at this time. Instead, current reductions in malaria incidence in SSA are attributable in part to accurate diagnosis and prompt chemoprophylaxis that includes artemisinin (Bhatt et al 2015).

Finally, there are intrinsic factors that affect malaria that cannot be controlled by any intervention. Worldwide economic downturn and donor fatigue, coupled with the weakening of regional control programmes, has reduced the level of monetary commitments for malaria elimination from various countries and led to resurgences in malaria (Cohen 2012, Pindolia et al. 2013). More than likely, resistance to any of interventions alone could jeopardize malaria control. The impact of each on parasitaemia rates in populations can be enhanced by the addition of other interventions (Barnes 2005, Nyarango 2006, Otten 2009, Konate 2011). Most common is the addition of LLINs to

seasonal malaria chemoprevention. Although the cost of the net is not negligible, the costs associated with delivery can be next to nil as, for example, nets can be distributed during routine vaccination campaigns in villages or through clinics where new mothers or infants are routinely seen (Sexton 2011). Although not mutually exclusive of one another, the effects of combining IRS and LLINs are being investigated to determine if implementation of both simultaneously is more effective at reducing malaria than separately or at disparate times (Corbel 2012, WHO 2014, West et al. 2015). As such, current WHO guidelines recommend that multiple interventions be implemented for an expanded period to have the greatest chance at eliminating malaria. However, even when considering the rise in resistance to interventions by both parasites and vectors, there has been a reported 20% reduction in the incidence of malaria in SSA between 2010 and 2016 resulting in approximately 131,000 fewer deaths (WHO 2017) and over 650,000 cases of malaria averted in 2015 (Bhatt et al. 2015). These steady reductions are due to the widespread implementation of LLINs, IRS, and ACT chemotherapeutics across Africa (Bhatt et al. 2015, Eckert et al. 2017).

1.3 The Vectors

Of the nearly 460 named species of *Anopheles* mosquitoes in the world, approximately 70 are known to transmit malaria and 52 are considered dominant vectors (Hay, 2010). Although many vectors have been found naturally infected across sub-Saharan Africa (Antonio-Nkondjio, 2006; Moffett, 2007), only eight are implicated in regular transmission of malaria (Moffett, 2007; Hay, 2010; Sinka, 2010) and 95% of malaria transmission attributed to only four species: *An. gambiae*, *An. coluzzii*, *An. arabiensis* and *An. funestus* (Coluzzi 1984). While *An. coluzzii*, *An. gambiae*, and *An. funestus* are considered the most important malaria vectors, vectors such as *An. arabiensis, An. moucheti, An. pharoensis* and *An. nili* are often understudied despite their importance in many parts of Africa (Kiszewski et al 2004, Antonio-Nkondjio et al. 2006). These vectors are traditionally considered either geographically limited, more exophilic or opportunistic feeders but exist and transmit malaria in niches where primary vectors are less abundant (Moffett et al 2007,

Ayala et al. 2009). Additionally, these vectors may be more abundant in different seasons when primary vector populations are smaller (Fontenille and Simard 2004). While implementation of IRS and LLINs in malaria control programmes has proven successful in reducing populations of *An. gambiae*, *An. coluzzii* and *An. funestus* due to their extremely anthropophilic and endophilic nature, in many areas malaria transmission has been sustained by these species even in the presence of interventions (Lindblade 2006, Sharp 2007, Sougoufara et al. 2016).

1.3.1 The Anopheles gambiae complex

Anopheles arabiensis is one of eight members of the cryptic *An. gambiae* complex (White et al. 1974, Fanello et al. 2002, Coetzee et al. 2013). The species vary based on their importance in malaria transmission with *An. gambiae*, *An. coluzzii* and *An. arabiensis* considered primary vectors across most of SSA, *An. melas* and *An. merus* locally important along the coasts of SSA, while *An. bwambe*, *An. quadriannulatus*, and *An. amharicus* are considered unimportant in transmission (White et al. 2011). Some of the various species also occupy specific niches such as the halophilic East African *An. merus* and West African *An. melas*, the zoophilic North African *An. amharicus* or South African *An. quadriannulatus*, or the thermophilic *An. bwambe* which is limited to a single region in Uganda (Lanzaro and Lee 2012, Coetzee et al. 2013). The members of this complex are also morphologically indistinguishable, instead they are readily identified by species-specific chromosomal inversions or more commonly by PCR which identifies species based on fixed mutations within their mitochondrial DNA (Coluzzi et al. 1979, Scott et al. 1993, Fanello et al. 2002, Coetzee et al. 2013).

1.3.2 Chromosomal inversions in the An. gambiae complex

Chromosomal inversions are important in identifying the various species (**Figure 1.2**) with an estimated ten inversions fixed within specific members of the *An. gambiae* complex with *An. gambiae* and *An. arabiensis* having the most (Coluzzi et al. 2002). Primary discrimination of species is based on X chromosome arrangements (**Figure 1.2**) with *An. gambiae, An. coluzzii* and *An. merus* uniquely possessing the Xag inversion and *An.*

arabiensis the Xbcd. Inversions on the X chromosome are hypothesized to contain genes associated with reproductive isolation resulting in speciation, as seen in drosophilids (Noor et al. 2001). Indeed, most genetic divergence between *An. gambiae*, *An. coluzzii*, and *An. arabiensis* is found within the inverted regions of the X chromosome compared to the autosomes (Lee et al. 2013, O'Loughlin et al. 2014, Crawford et al. 2015). As *An. gambiae*, *An. coluzzii* and *An. arabiensis* are widely distributed across several biomes, inversions may also act as carriers of beneficial alleles that accelerate adaptation via introgression when hybridisation does occur (Kirkpatrick and Barrett 2015). The 2La inversion, which is polymorphic in *An. coluzzii* and *An. gambiae*, is associated with xeric tolerance and is believed to have introgressed from *An. arabiensis* (Fouet et al. 2012).

In *An. arabiensis*, inversions have been associated with specific behaviours and niches. The aforementioned inverted 2La and West African specific $2Rd^1$ inversions are strongly associated with xeric tolerance (Petrarca et al. 2000, Fouet et al. 2012, Ayala et al. 2017). The inverted form of 2Rb is found in more humid, forest-savanna biomes and more often in East Africa (Petrarca et al. 2000, Ayala et al. 2014, Ayala et al. 2017) while the standard form (2R+b) is more commonly found in West Africa and associated with endophily and anthropophagy (Coluzzi et al. 1977, Petrarca and Beier 1992, Petrarca et al. 2000). Conversely, the inverted 3Ra is also commonly associated with anthropophagy and more humid biomes in West Africa while the standard 3R+a is associated with zoophagy and more common in the East and South (Petrarca et al. 2000, Main et al. 2016). Therefore, biotic and abiotic pressures select for inverted regions, and alleles contained within, allowing for improved survival, which are subsequently selected and spread either through underdominance or by a reduction in recombination (Hoffman and Rieseberg 2008, Stevison et al. 2011). While beneficial to the mosquito, the reduced recombination results in low linkage disequilibrium (LD) making association studies difficult unless except where there is a large allelic effect or sample size (Marsden et al. 2014).

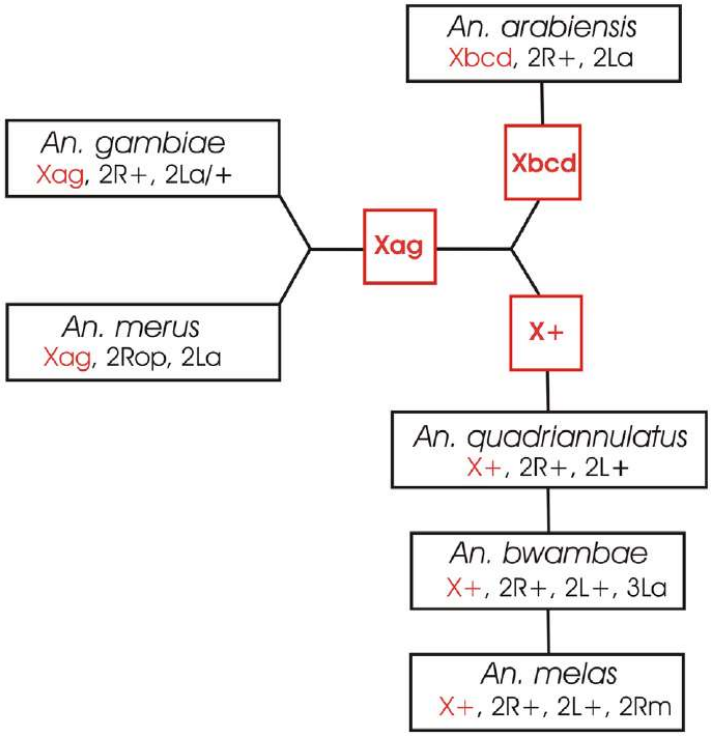


Figure 1.2: Chromosomal inversions specific for the various members of the *An. gambiae* complex (from Kamali et al. 2012).

1.3.3 Anopheles arabiensis

While originally considered to be a single, continent-wide population, *An. arabiensis* is now not believed to be panmictic across Africa. Population stratification within this species has been observed over long distances potentially revealing two or more different sub-populations (Donnelly et al. 1999, Petrarca et al. 2000, Nyanjom et al. 2003). There is also some evidence of additional stratification between mainland and island populations as seen in Tanzania (Maliti et al. 2014). Petrarca et al. (2000) found three predominant groups of *An. arabiensis* across Africa based on cytotaxonomy. This stratification is more distinct when comparing the distribution and behaviour of *An. arabiensis* populations across Africa both when they are the predominant vector or in the presence of a sympatric sibling species.

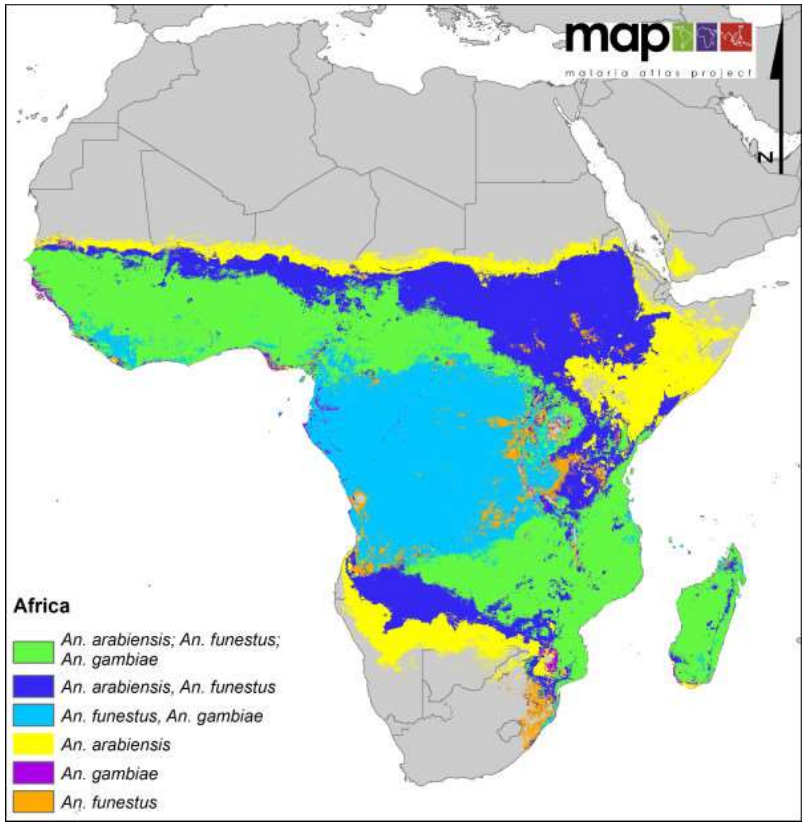


Figure 1.3: The geographical distribution of the primary vectors of malaria in SSA (Sinka et al. 2010).

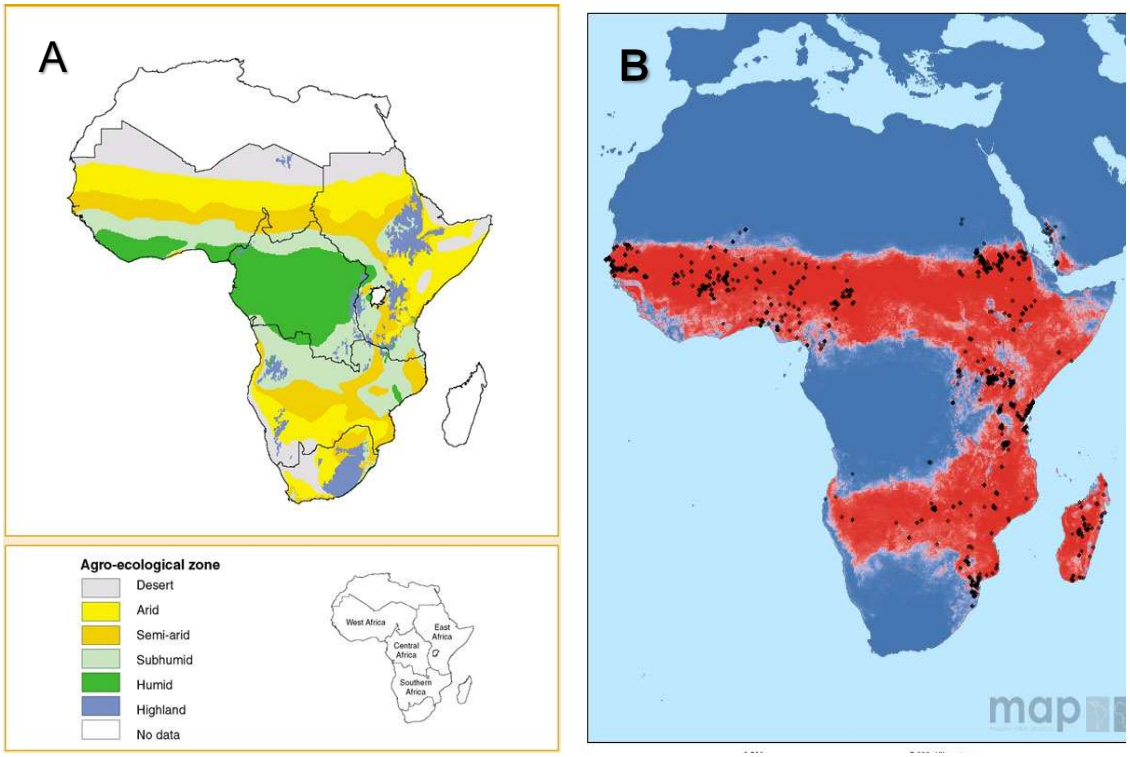


Figure 1.4: The primary ecological zones in Africa (A) courtesy of fao.org (accessed 11/2016) and the estimated distribution of *An. arabiensis* (B) shown in red (Lee et al. 2013).

1.3.4 Ecology of An. arabiensis

Anopheles arabiensis, An. gambiae and *An. coluzzii* are found in varying levels of sympatry across sub-Saharan Africa (Sinka et al. 2010) (**Figure 1.3**), however *An. arabiensis* is more likely to predominate in xeric areas (Coluzzi et al. 1979, Sinka et al. 2010) (**Figure 1.4**). While *An. gambiae* populations often peak in size during the rainy season, *An. arabiensis* are more commonly collected shortly afterward. In the West and North regions of Africa – henceforth referred to as West Africa (**Figure 1.5**) - where three or more members of the complex may occur, *An. arabiensis* larvae are often found alongside *An. gambiae* in more ephemeral, sun-lit sites such as puddles or recently flooded areas and typically do not predominate in collections while *An. coluzzii* is often found in more permanent water

sources (Faye et al. 1997, Caputo et al. 2008, Djegbe et al. 2011, Foster et al. 2016). As the biome becomes more arid into the Sahel region the presence of *An. gambiae* and *An. coluzzii* decreases allowing *An. arabiensis* to dominate. Aestivation by this species allows for small populations to remain year-round while other species must reinvade thereby placing them at a disadvantage (Taylor et al. 1993, Simard et al. 2000).

Figure 1.5: Graphical representation of the main regions across sub-Saharan Africa (www.dreamstime.com accessed May 2018).

In the more arid East African region, *An. arabiensis* is the predominant vector encountered while *An. pharoensis* and *An. funestus* are regionally important. Larvae are often found in lowland areas and associated with sun-lit permanent water sources or man-made water catchments (Shililu et al. 2003b, Bayoh et al. 2011, Gone et al. 2014). Adults peak during

the rainy season but are collected year-round mainly due to the continual presence of larval habitats associated with agricultural activity and water impoundment.

Last, in the South African region *An. arabiensis* is typically found in sympatry with but often secondary to *An. gambiae* or *An. funestus*. Larvae reside in more permanent water sources while adults are predominantly collected in lowland areas and more abundant in the dry season akin to populations from East Africa (Mnzava and Kilama 1986, Mzilahowa et al. 2012). *An. arabiensis* is often found sympatrically in larval sites with *An. gambiae* in West Africa, however as populations of *An. gambiae* have decreased or disappeared, there has not been a subsequent increase in *An. arabiensis* (Bayoh et al. 2010). Therefore, in the South *An. arabiensis* appears to occupy a limited and separate niche.

1.3.5 Feeding behaviour of An. arabiensis

Compared to *An. gambiae* and *An. coluzzii*, *An. arabiensis* is more catholic in choices for blood-feeding however the rates of anthropophagy vary across their range based on reported Human Blood Indices (HBI). In West Africa, HBI rates in blood-fed *An. arabiensis* average 55-98% however HBI rates decrease in the East and increase as you move south into the South often explained by the presence of livestock on which they readily feed (**Table 1.2**). While *An. arabiensis* is commonly believed to be more zoophagic than *An. gambiae* and *An. coluzzii*, the true rate of anthropophagy is probably unknown as HBI rates differ amongst regions in Africa and between indoor and outdoor collections (Githeko et al. 1994, Adugna and Petros 1996, Mahande et al. 2007). Populations of *An. arabiensis* may prefer anthropophily and endophagy in the West and South however some zoophagy is seen, while in the East *An. arabiensis* primarily is zoophagic and tends to be more exophilic (Tirados et al. 2006, Degefa et al 2017). These variances in anthropophagy are best explained by the presence of specific inversions reported from within each region.

Table 1.2: Human blood-feeding index rates (HBI) in the three primary domains in which *An. arabiensis* predominates. In the West and South *An. arabiensis* is predominantly anthropophagic while in the East it is zoophagic.

			Human only	Animal only	Reference
West	Mauritania	urban	58.70%	0.00%	Lekweiry et al. 2016
		rural	11.10%	55.50%	
	Gambia		82.00%	26.00%	Bøgh et al. 2001
	Nigeria		98.00%	nd	Samdi 2012
	Senegal	Sahel	68.00%	28.00%	Lemasson et al. 1997
		savanna	73.8%*	28.3%*	Fontenille et al. 1997
North	Sudan		60-92%	nd	Himeidan et al. 2011
			88.10%	10.60%	Abdalla et al. 2008
East	Eritrea		16.90%	67.00%	Okbaldet et al. 2006
			22.00%	41.00%	Waka et al. 2004
	Ethiopia		<10.00%	37.00%	Massebo et al. 2015
			34.00%	66.00%	Gone et al. 2014
	Kenya		23.00%	77.00%	Githeko et al. 1994
			<10.00%	39.00%	Muturi et al. 2013
		outdoors	6.90%	41.30%	Muriu et al. 2008
		indoors	7.90%	71.80%	
		2007	14.00%	75.00%	Mwangangi et al. 2013
		2008	16.00%	72.00%	
	Tanzania		66.7-73.4%	nd	Kulkarni et al. 2006
		savanna indoor	66.40%	nd	Ijumba et al. 2002
		savanna outdoor	4.10%	nd	
		rice indoor	47.90%	nd	
		rice outdoor	4.60%	nd	
		sugar indoor	68.40%	nd	
		sugar outdoor	25.80%	nd	
		Lupiro	24.00%	41%	Main et al. 2016
		Minepa	7.00%	74%	
		Sagamanganga	11.00%	62%	
South	Malawi		85.00%	10.90%	Mzilahowa et al. 2012
	Botswana		27.30%	46.80%	Chirebvu et al. 2016
	Zambia		92.30%	nd	Kent et al. 2007

1.4 Anthropogenic changes and their impact on *An. arabiensis*

During the last few decades there has been an increase in anthropogenic changes resulting from unplanned urbanisation, agriculture and water impoundment projects across Africa changing the landscape of many countries. Anthropogenic changes to the environment have been shown to impact local vector populations. Although man-made perturbations occur across many continents, the response by local vectors to the presence of the numerous new breeding sites is varied (Manga et al. 1995, Vittor et al. 2006, Saxena et al. 2014) and lead to a decrease in local vector species richness (Shochat et al. 2006, Ferraguti et al. 2016). The lack of interspecific competition can result in changes in blood feeding and pathogen transmission rates due to the dominance of a few select vectors capable of adapting to the novel environment (Faeth et al. 2005).

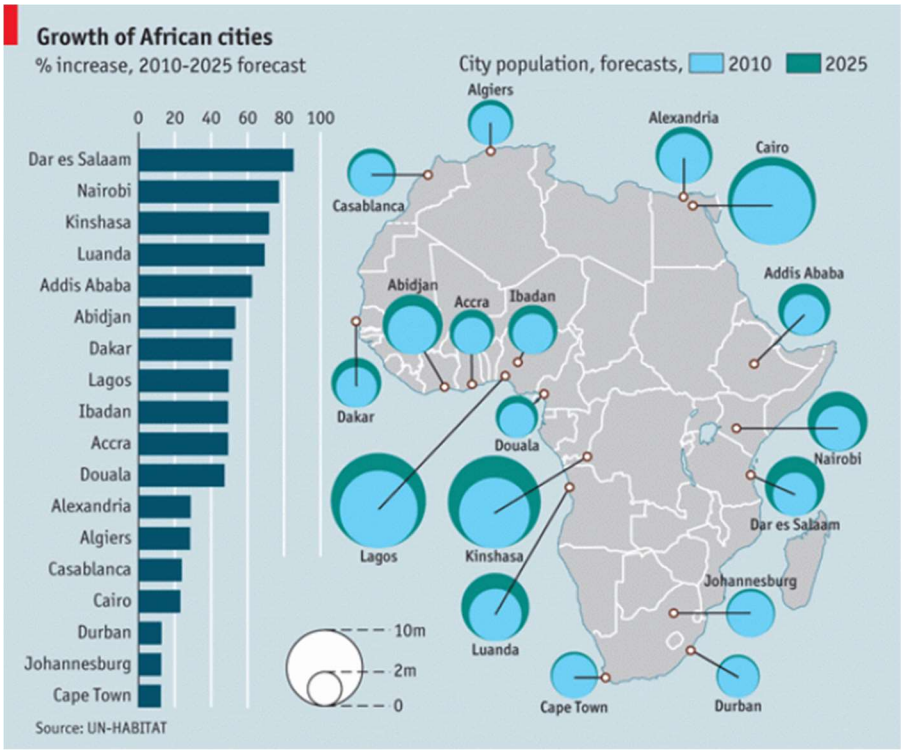


Figure 1.6: A map of the major urban areas across Africa with their projected growth through 2025 (www.economist.com/blogs/dailychart/2010/12/urbanisation_africa).

1.4.1 Urbanisation and An. arabiensis

Urbanisation in Africa is occurring at a rate of 2-6% per annum with an anticipated 55% of the continents inhabitants living in an urban area by 2050 (United Nations 2014). Primary centres of urbanisation are typically located along the coasts of Africa, and in west and central Africa (**Figure 1.6**). Due to increased human migration from rural areas, unplanned communities have multiplied around urban centres and are often associated with increases in vectors due to the presence of more suitable sites for mosquito larvae resulting from inadequate city planning (Keiser et al. 2004). Additionally, rapid urbanisation has also resulted in an increase in man-made habitats include such as canals, wells, and cisterns to meet water needs of the burgeoning population (Impoinvil et al. 2008, Machault et al. 2009).

Urban centres across Africa have historically been found to be ill-suited for anopheline mosquitoes resulting in lower vector abundances and malaria rates often due to paucity of suitable larval habitats (Trape and Zoulani 1987, Robert et al. 1998, Afrane et al. 2004). When present, urban anopheline larvae are associated with natural water sources such as ponds, river beds, streams, pools, and puddles akin to sites they occupy in the wild (Sattler et al. 2005, Diedhiou et al. 2016, Mattah et al. 2017). In West Africa, however, *An. coluzzi*, *An. gambiae* and *An. arabiensis* have begun to adapt and expand into new urban areas previously thought to be unsuitable. In these areas, larvae are found in non-natural sites such as water traditionally considered too polluted and even tree holes (Sattler et al. 2005, Awolola et al. 2007). Recently *An. arabiensis* has been found displacing *An. gambiae* in several urban areas (Djogbenou et al. 2010, Dabire et al. 2012). Even though replacement has been shown, it is not known if these trends are due to actual competitive displacement by *An. arabiensis,* normal temporal variations in the population size of other species, or due to domestic protective measures which select against highly endophilic species like *An. gambiae*. In Lagos, however, *An. gambiae s.s.* was found in more polluted urban sites compared to *An. arabiensis* therefore the latter species may be less adapted to survival in highly polluted environments here (Awolola et al. 2007).In East and South of Africa, larvae

are more likely found in man-made sites compared to natural, temporary pools. In Malindi and Nairobi, *An. arabiensis* or *An. gambiae* s.l. larvae were more often found in wells, drainage troughs, ditches, canals and unused swimming pools (Impoinvil et al. 2008, Kasili et al. 2009). In Dar es Salaam it was estimated that 80% of urban larval sites identified were not in agricultural areas (Dongus et al. 2009, Castro et al. 2010).

1.4.2 Agriculture and An. arabiensis

Agriculture has had a dramatic effect on the abundance of malaria vectors across Africa and throughout the world. Rice is an important crop in SSA with a high imbalance between domestic consumption and production (Nakano et al. 2011) therefore its production has increased dramatically over the last 30 years (Balasubramanian et al. 2007). Rice is grown in four primary ecosystems with >80% dependent on rain and primarily cultivated in West and South Africa (Balasubramanian et al. 2007) (**Figure 1. 7**). However, expansion in semi-arid and sub-humid regions of Africa is occurring primarily due to advances in and expansion of irrigation. In West Africa *An. coluzzii* predominates in such agricultural settings (Faye et al. 1995, Edillo et al. 2002, Klinkenberg et al. 2003, Doannio et al. 2006, Caputo et al. 2008, Gimonneau et al. 2012,) though in the semiarid northern Sahel parts, *An. arabiensis* dominates with *An. funestus* and *An. pharoensis* also found in these irrigated agricultural settings (Edillo et al. 2002, Abdalla et al. 2008, Caputo et al. 2008, Himeidan and El Rayah 2008, Kerah-Hinzoumbe et al. 2009, Ndiath et al. 2012). In the semi-humid Savanna, *An. arabiensis* was found year-round in rice farms however it was more abundant in the rainy season (Robert et al. 1992, Antonio-Nkodjio et al. 2008). While in the more arid Sahel *An. arabiensis* was predictably more abundant from August through October however in Senegal *An. arabiensis* was found year-round in a rice-growing village (Faye et al. 1993).

Anopheles arabiensis, however, has benefited the most from increased irrigated rice production in East Africa (Ijumba et al. 2002, Mboera et al. 2010). Vector populations have been shown to increase after the introduction of irrigation schemes as part of agricultural activities around the world (Ijumba and Lindsay 2001, Boccolini et al. 2012, Diakite et al.

2015). Mboera et al. (2010) found a seven-fold increase in the number of larvae found in rice growing areas of which 74% were *An. arabiensis*. Similarly, 85% of potential breeding sites within the irrigated areas of Mwea, Kenya were positive for larvae compared to 15% in non-irrigated sites (Jacob et al. 2007). As most of these rice fields are in semiarid regions, 75.6% of sites positive for *An. arabiensis* were associated with broken irrigation pipes, leakages, or poor flooding and draining associated with crop production (Kibret et al. 2014). In this region there are two growing seasons associated with the rainy seasons elongating the period in which *An. arabiensis* can breed in large numbers potentially transforming this region from sporadic malaria into more stable malaria transmission (Muturi et al. 2008, Mboera et al. 2010). Mwangangi et al. (2010) found that although there were fewer positive breeding sites within the rice-growing areas, they were more likely to be positive for larvae year-round compared to the highly productive, but often dry, habitats found in the non-irrigated village. Although the presence of rice-growing regions may lead to increases in local vector abundance, it doesn't always equate to a similar increase in malaria transmission in many areas, referred to as the "paddy paradox" (Ijumba and Lindsay 2001, Keiser et al. 2004, Diuk Wasser et al. 2007, Diakate et al. 2015). These reductions may also be associated with lower anthropophagy in vectors typically associated with rice growing areas compared to those collected in non-agricultural areas (Muturi et al. 2008). Additionally, rice farming is associated with more socio-economic growth resulting in residents of these villages being able to afford personal protective tools (Ijumba and Lindsay 2001).

1.4.3. The intersection of urbanisation and agriculture

A newer phenomenon is the role of agriculture and its effect on mosquito populations in urban areas traditionally considered less suitable for vectors. There is a growing body of evidence showing vector expansion associated with an increase in urban and peri-urban agriculture (UPA) which is necessary to meet the food demands of the rapidly growing cities across Africa (Cofie et al. 2003). Approximately 80% of farming activity across sub-Saharan Africa is done on small, family-run farms which are rain-dependent.

Consequently, in UPA areas larval habitats are frequently associated with wells, shallow pools and canals created to irrigate crops year-round (Afrane et al. 2004, Shililu et al. 2007, Dongus et al. 2009). As UPA provides more than 70% of foodstuffs needed in some urban areas (Cofie et al. 2003, Afrane et al. 2004), continued agricultural expansion could result in more larval habitats and a subsequent increase in local populations of competent vectors to feed on an ever-expanding pool of naïve hosts.

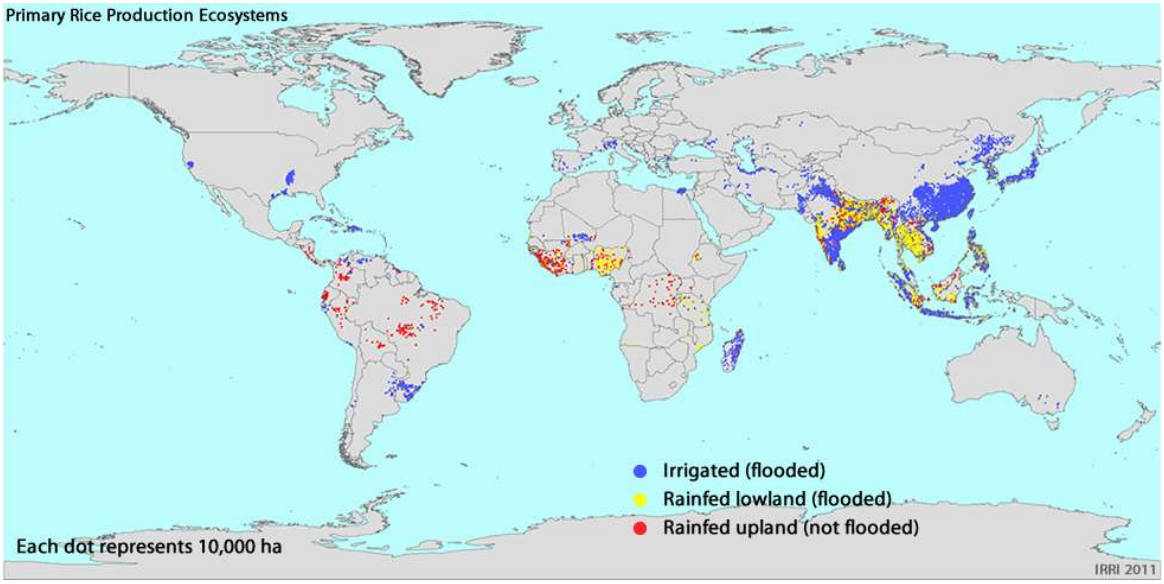


Figure 1.7: Map showing the primary areas where rice is grown in Africa (http://www.knowledgebank.irri.org/submergedsoils/index.php/rice-growing-environments/lesson-1, Nov, 2017).

The extent of the effect of UPA on vector abundance or epidemiological importance has not yet been fully measured. Regarding *An. gambiae*, more adult mosquitoes were collected in urban agricultural sites compared to non-agricultural areas in the more humid parts of West Africa with no mention of *An. arabiensis* (Afrane et al. 2004, Matthys et al. 2006, Klinkenberg et al. 2008, Akono et al. 2015). Similar abundances of *An. arabiensis* were attributed to urban agricultural activity have been reported in Tanzania (Dongus et al. 2009,

Mboera et al. 2015). Therefore, the effects of UPA on *An. arabiensis* may be limited in West Africa where *An. gambiae* and *An. coluzzii* dominate as the amount of fertile crop land is limited in many countries (Martellozzo et al. 2014). However, in the East and South the levels of urbanization are lower resulting in more land available and a subsequent increase in the growth of the agricultural sector between 2000-2009 while in West Africa it has declined (NEPAD 2013). In these areas UPA, and its associated anthropogenic changes, have resulted in an increase in *An. arabiensis* abundance in these regions.

1.5 Insecticide Resistance

Of the various challenges hampering vector control, insecticide resistance is the most prominent and potentially dangerous. Historically, resistance has emerged during the implementation of malaria control programmes and with its current rise across Sub-Saharan Africa, resistance could challenge progress made towards controlling this disease to date (Ranson and Lissenden 2016). During the initial Global Malaria Eradication Programme (1955-1969), behavioural and insecticide resistance to dieldrin and DDT coupled with financial woes led to the stoppage of several national anti-malaria IRS campaigns and a rebound in malaria incidence (Coetzee et al. 1999). A similar trend was seen later in South Africa in which pyrethroid resistance in *An. funestus* led to a dramatic increase in malaria incidence and a subsequent return to DDT to control this vector (Hargreaves et al. 2000).

Insecticide resistance is defined by IRAC as "a heritable change in the sensitivity of a pest population that is reflected in the repeated failure of a product to achieve the expected level of control when used according to the label recommendation for that pest species" (IRAC 2011). Resistance occurs in a population when rare mutants are positively selected for when exposed to insecticides (Hammond et al. 2017) (**Figure 1.8**). Although these individuals are rare initially, their numbers grow either due to multiple origins of the same resistance allele in geographically distinct populations (Pinto et al. 2007, Etang et al. 2009) or one origin with a rapid expansion due to migration or introgression (Weill et al. 2000,

Diabate et al. 2004, Djogbenou et al. 2008). It is hypothesized that once insecticide pressure is removed, the proportion of those possessing resistance mutations will decrease due to fitness effects associated with them, however resistance may never fully disappear from a population but instead exist as rarer heterozygotes (Keiding 1967).

Susceptibility within populations is typically investigated by using one of two assays: the CDC bottle bioassay or the WHO tube assay. Both assays are designed to expose vectors to a pre-established concentration of insecticide for one hour. The main difference between the two is that the CDC bottle bioassay estimates susceptibility one-hour post exposure while the WHO tube assay requires 24 hours. Permutations of these assays utilise either pre-exposure with a synergist to determine if metabolic mechanisms are responsible for the phenotype or a suite of discriminating doses to determine the intensity of resistance (Matowo et al. 2010, Bagi et al. 2015).

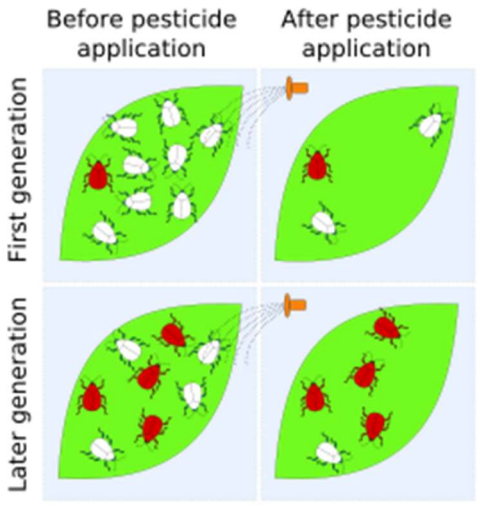


Figure 1.8: A simple diagram showing the selection of resistance in an insect population (https://en.wikipedia.org/wiki/Pesticide_resistance). Susceptible individuals (white) are selected against during pesticide application allowing resistant individuals (red) to increase.

At the heart of the current insecticide resistance problem is the lack of options approved for use in protecting vulnerable populations. Currently there are only four classes of insecticides approved by the WHO for use in combating malaria (World Health

Organization, 2012): pyrethroids, carbamates, organochlorides, and organophosphates (**Figure 1.9**). Unfortunately, resistance to these compounds is spreading quickly in Sub-Saharan Africa especially in the three primary vectors, *An. coluzzii*, *An. gambiae*, and *An. funestus*. There are four well documented pathways of resistance reported in anophelines: target-site modification, metabolic resistance, behavioural resistance, and physiological modification (Corbel and N'Guessan 2013).

Figure 1.9: A current list of insecticides approved for IRS by the WHO for use in controlling malaria vectors (www.who/int/whopes/Insecticides_IRS_Malaria_09.pdf)

Insecticide compounds and formulations[a]	Class group[b]	Dosage (g a.i./m^2)	Mode of action	Duration of effective action (months)
DDT WP	OC	1–2	contact	>6
Malathion WP	OP	2	contact	2–3
Fenitrothion WP	OP	2	contact and airborne	3–6
Pirimiphos–methyl WP & EC	OP	1–2	contact and airborne	2–3
Bendiocarb WP	C	0.1–0.4	contact and airborne	2–6
Propoxur WP	C	1–2	contact and airborne	3–6
Alpha–cypermethrin WP & SC	PY	0.02–0.03	contact	4–6
Bifenthrin WP	PY	0.025–0.05	contact	3–6
Cyfluthrin WP	PY	0.02–0.05	contact	3–6
Deltamethrin WP, WG	PY	0.02–0.025	contact	3–6
Etofenprox WP	PY	0.1–0.3	contact	3–6
Lambda–cyhalothrin WP, CS	PY	0.02–0.03	contact	3–6

[a] CS: capsule suspension; EC= emulsifiable concentrate; SC= suspension concentrate; WG= water dispersible granule; WP= wettable powder.
[b] OC Organochlorines; OP= Organophosphates; C= Carbamates; PY= Pyrethroids.
Note: WHO recommendations on the use of pesticides in public health are valid ONLY if linked to WHO specifications for their quality control. WHO specifications for public health pesticides are available on the WHP home page at http:/www.who.int/whopes/quality/en/.
Source: WHOPES (http:/www.who.int/whopes/Insectisides_IRS_Malaria_09.pdf

1.5.1 Target-site modification

Target-site mutations and their effects on insecticide resistance are the best understood, easiest to detect and have multiple tools developed to identify them in nature (Corbel and N'Guessan 2013). These arise from selection of rare individuals that possess a mutation that prohibits normal binding by the target protein. As most current insecticides approved by the WHO target the insect nervous system, these mutations often occur in their associated tissues.

1.5.1.1 Voltage-gated sodium ion channels

Voltage-gated sodium ion channels (*Vgsc* or *kdr*) rely on changes in electrical impulses for enervation. Pyrethroid and organochlorine insecticides interact with the transmembrane proteins thereby slowing their activation or deactivation resulting in the classic "knock-down" effect of paralysis followed by death (Hemingway et al. 2004). *Vgsc*-1014F (West African *kdr*) was originally described in *An. gambiae* s.l. in which a single point mutation changing a leucine to phenylalanine in domain II of the *para*-type sodium channel led to increased resistance to permethrin and DDT (Martinez-Torres et al. 1998). Ranson et al. (2000) found a second mutation in *An. gambiae* (*Vgsc*-1014S or East African *kdr*) with similar resistance patterns however the leucine was replaced with serine. Originally these two mutations were geographically limited to either West or East Africa, however *Vgsc*-1014S has been found across the continent while *Vgsc*-1014F is slowly moving towards the East. A third, more limited form, *Vgsc*-1575Y, is emerging and spreading in tandem with *Vgsc*-1014F in which it is hypothesized that *Vgsc*-1575Y ameliorates fitness effects associated with *Vgsc*-1014F while adding to the resistance levels to DDT and permethrin (Jones et al 2012).

1.5.1.2 Acetylcholinesterase-1

Mutations in acetylcholinesterase (AChE), work by terminating signals within the mosquito nervous system thereby making them "insensitive". Resistance is conferred by

SNPs that alter the active gorge site preventing carbamate access. Based on which SNP is present, the population can display high carbamate and low OP resistance or high OP and varying carbamate resistance (Hemingway et al. 2004). Weill et al. (2004) found the single point mutation *Ace*-119S, in which a glycine is replaced by a serine, was strongly associated with bendiocarb resistance in *An. gambiae* and *Culex pipiens*.

1.5.1.3 Ligand-gated ion channels

These channels, much like *Vgsc*, receive chemical messengers such as neurotransmitters, that regulate channels along the synaptic ends of the nervous system. When a mutation occurs, the level of neurotransmitter received is diminished preventing the neuron from desensitizing (ffrench-Constant et al. 2000). *Gaba*-296 has been found to be highly conserved across several insect genera with alanine replaced with either glycine or serine (ffrench-Constant et al. 2000, Du et al. 2005). The mutations typically occur in the second trans-membrane region of the gamma–amino butyric acid (GABA) receptor making it less sensitive to binding by cyclodienes as well as phenylpyrazoles and potentially imidacloprid and deltamethrin (ffrench-Constant et al. 2004, Taylor-Wells et al. 2015).

1.5.2 Metabolic resistance

Metabolic resistance typically arises from gene duplications or alterations to *cis* or *trans* regulatory factors (Hemingway et al. 2004). Several different gene families have been implicated in metabolic resistance including cytochrome P450 monooxygenases, glutathione-s-transferases, and esterases (ffrench-Constant et al. 2004, Hemingway et al. 2004).

1.5.2.1 Cytochrome P-450

Cytochrome P450 monooxygenases (CYPs) are a diverse family of genes responsible for metabolising several endogenous and exogenous compounds. Resistance to organophosphates and pyrethroids is achieved by detoxification of the insecticide resulting from enhanced metabolic activity. Several P450s have been implicated in pyrethroid and

DDT resistance including CYPM2 (Mitchell et al. 2012, Edi et al. 2014), CYP6P3 (Mueller et al. 2008, Kwiatkowska et al. 2013, Edi et al. 2014), CYP6Z2 (Kwiatkowska et al. 2013), and CYP9K1 (Main et al. 2015) however CYP6P3 has also been shown to provide cross resistance to bendiocarb.

1.5.2.2 Glutathione-s-transferase

Glutathione-s-transferases (GSTs) resistance is achieved by binding unknown compounds to glutathione to increase its solubility and eventual expulsion or secondarily by catalysing metabolites produced by CYPs. Most commonly, GSTs are implicated in organophosphate resistance but have been implicated in resistance to all four classes in other insect genera (Hemingway et al. 2004). The most commonly detected class associated with DDT resistance is the epsilon class (Ding et al. 2005). Within this family, GSTe2 is unique as resistance is conferred by an amino acid change and not an over-expression of the allele. This unique form has been reported across Africa in both *An. gambiae* (I114T) and *An. funestus* (L119F) (Mitchell et al. 2014, Riveron et al. 2014). Other GSTs have also been found to have catalytic properties against DDT including AgGST3-2 (Ranson et al. 2001) and GSTZ1 (Kwiatkowska et al. 2013).

1.5.2.3 Esterases

Esterases function by either hydrolysing ester bonds or sequestering insecticides typically in the carbamate or organophosphate families. While resistance associated with non-specific esterases is reported, rarely is a single mechanism implicated. Recently, however, COeae1d was found to be associated with pyrethroid resistance in East Africa (Weetman et al. 2018).

Although singly, many of these mechanisms can provide high levels of resistance, more concerning is that in many parts of Africa populations of vectors are being detected with multiple resistance mechanisms. Although there is some debate as to any genetic linkage between point mutations and metabolic resistance genes, there is a growing body of

evidence showing that they can have an additive effect (Mitchell et al. 2014, Edi et al. 2014).

1.5.3 Other pathways of resistance

Although there are four predominant resistance mechanisms in insect vectors, there has been an increase in evidence showing alternative resistance pathways may be emerging. These mechanisms seem to be adaptive in nature and based on mostly unknown allelic factors. The most studied of these is behavioural resistance. Behavioural resistance is defined as alterations in behaviours to avoid insecticide interventions and continue host seeking (Chareonviriyaphap et al. 2013). Plasticity in anophelines is high and responds quickly to man-made evolutionary pressures exerted (Crispo et al. 2010). It is hypothesized that the application of an excito-repellent insecticide on indoor walls or the implementation of an LLIN would force highly anthropophagic and endophilic vectors to alter their behaviours to survive. In West Africa shifts in peak biting times have been reported in *An. gambiae s.l.* and *An. funestus* after the implementation of LLINs (Reddy et al. 2011, Moiroux et al. 2014, Sougoufara et al. 2014). *An. arabiensis* from East Africa, however, showed no discernible shift in either biting time or endophagy rates (Geissbuehler et al. 2007, Russell et al. 2011, Yohannes and Boelee 2012). Whether these changes are directly related to LLIN implementation is difficult to assess, but daytime feeding is rarely reported in anophelines and is resulting in an increase in exophily in normally endophilic vectors. Another possibility is that the diverted vectors are potentially susceptible to insecticides and avoiding contact with the excito-repellent chemicals. This highlights the difficulty to detect behavioural resistance as extrinsic factors may be exerting unequal forces not detected in study design.

A less studied response potentially implicated in insecticide resistance is cuticle thickening. Insects with thicker cuticles will have a lower absorption rate of insecticides allowing for weak detoxification genes to metabolize insecticides more thoroughly. Although the cuticle thickens naturally over time, it has been experimentally shown that resistant *An.*

funestus females had significantly thicker cuticles than matched susceptible controls (Wood et al. 2010). There is some limited microarray data that shows enhanced up-regulation or over-amplification of insect cuticle genes in resistant populations (Awolola et al. 2009, Bonizzoni et al. 2012). Recent data has shown study cuticle thickness (Wood et al. 2010) and the upregulation of CYP4G16 associated with cuticular hydrocarbon (CHC) production (Balabanidou et al. 2016) lead to a decreased penetrance of insecticides in anophelines.

1.5.4 Spread of resistance

It is widely reported that resistant populations of anophelines are quickly spreading across SSA jeopardizing current gains against malaria prevalence (Ranson and Lissenden 2016). Sequencing of several hundred *An. gambiae* and *An. coluzzii* revealed high levels of introgression and spread between the species and distant populations as well as multiple origins for many insecticide resistance mechanisms (Weill et al. 2000, Diabate et al. 2003, Diabate et al. 2004, Djobenou et al. 2008, Kawada et al. 2011, *Anopheles* 1000 Genomes 2017). Hybrids between *An. gambiae* and *An. coluzzii* are found regularly (Lee et al. 2013) and as speciation is not complete, interbreeding is possible allowing for the introgression of resistance alleles between the two (Clarkson et al. 2014, *Anopheles* 1000 Genomes 2017). Less common would be *An. gambiae* and *An. arabiensis* hybrids that, under Haldane's rule, are sexually inviable and any resulting progeny would suffer high fitness costs. However, recent evidence has shown extensive introgression between *An. coluzzii, An. gambiae* and *An. arabiensis* including the sharing of resistance alleles (Kawada et al. 2011, Crawford et al. 2014, Weetman et al. 2014). Hybridisation between *An. gambiae* and *An. arabiensis* has been shown to favour the retention of 2L and 2R chromosomal arms, both of which contain several known resistance alleles including *Vgsc* (*kdr*) (Slotman et al. 2005). Regarding *Vgsc*, there is evidence that adaptive introgression between *An. gambiae* and *An. arabiensis* is responsible for the appearance and spread of resistance in response to LLIN pressure in East Africa (Kawada et al. 2011, Ochomo et al. 2015). However, in West Africa less is known about the spread of resistance with Diabate et al. (2004) finding

Vgsc-1014F arising naturally in *An. arabiensis* but introgression from *An. gambiae* into *An. coluzzii.* Pinto et al. (2007) found at least four independent origins of *Vgsc* resistance in *An. gambiae* based on intronic haplotype variation. The spread of metabolic resistance in *An. arabiensis* in the East was found mainly to arise uniquely in a population and spread rapidly in response to vector control efforts (Kawada et al. 2011, Jones et al. 2013). In these instances, the rise and spread of the resistance allele was found to have occurred within 3-5 years. Therefore, insecticide pressures – especially from pyrethroids - are resulting in adaptive introgression of target-site mutation-based resistance in *An. arabiensis* from other members of the *An. gambiae* complex at an evolutionarily rapid pace. This is more worrisome from a public health view as *An. arabiensis* has proven to be difficult to control with current vector control methodologies and increases in malaria have been reported in regions where *An. gambiae* has disappeared (Mwangangi et al. 2013).

1.5.5 Insecticide resistance in An. arabiensis

Much research has been focused on insecticide resistance in the primary vectors of malaria in SSA, *Anopheles gambiae, An. coluzzii* and *An. funestus* (Coetzee et al. 2006, Casimiro et al. 2007, Nwane et al. 2009, Ranson et al. 2009). These species are considered the most important malaria vectors due to their high levels of anthropophagy and endophilic nature while *An. arabiensis* was often overlooked despite its importance in eastern and southern Africa (Kizsewki et al. 2004). Insecticide resistance in *An. arabiensis* has been reported from several countries across SSA (**Figure 1.11**). However, mosquitoes are not uniformly exposed to insecticides in Africa. When compared to resistance patterns in *An. funestus* (**Figure 1.10**), *An. arabiensis* is less likely to be found resistant to carbamates but more likely to be resistant to organochlorines and organophosphates. However, there are large areas of SSA where resistance in this vector is unknown (**Figure. 1.11**).

However, resistance studies in *An. arabiensis* can be difficult, which is often related to their bionomics associated with a geographic region. In West Africa *An. arabiensis* is often in the minority of collected species. Resistance studies in this region tend to either report specifically on *An. gambiae* or *An. coluzzii* or will combine all three cryptic siblings into a

general *An. gambiae s.l.* category and provide general resistance trends disregarding the actual contribution of each individual species as testing is often done *a priori* (eg. Cisse et al. 2015, Gnangnuenon et al. 2015). In the more humid parts of this region exposure to insecticides by *An. arabiensis* is probably like that of *An. gambiae* as they both occupy similar ephemeral breeding sites and their ranges overlap. In this domain *Vgsc*-1014F and 1014S are often found but resistance does not always correlate with possession of either of these alleles (Jones et al. 2012, Dabire et al. 2014, Ibrahim et al. 2016). Pyrethroid resistance, as estimated from a limited number of samples, has been associated with elevated metabolic mechanisms like GST, over expressed superoxide dismutase SOD2, and CYP4G16 (Mueller et al. 2008, Jones et al. 2012).

However, further north towards in the arid Sahel region, *An. arabiensis* tends to dominate and resistance can be directly assessed. In many of these nations the increase in developed, arable land with an associated increase in the agricultural use of pesticides as well as area-wide vector control efforts are the primary routes of exposure. In the western part of this range, susceptibility is variable ranging from tolerant to resistant. Both *Vgsc*-1014F and 1014S are found, often with limited corroborating insecticide exposure data (Lekweiry et al. 2016). In the Sudan, permethrin and DDT resistance has been reported as has the presence of *Vgsc*-1014F and 1014S, in both instances no correlation between *Vgsc* and resistance is seen (Abdalla et al. 2008, Himeidan et al. 2007). Conversely, in Chad resistance to permethrin and DDT has been shown to be attributed to metabolic mechanisms either through synergist pre-exposure or QTL analysis with no reports of *kdr* mutations (Witzig et al. 2013, Foster et al. 2016). Instead, it was shown that CYP6P4 was responsible for metabolizing many Type I and II pyrethroids (Ibrahim et al. 2016).

In East Africa *An. arabiensis* is predominantly zoophagic nonetheless exposure to insecticides is not primarily limited to the agricultural and food-security arenas but also includes the peridomestic environment with varying levels of exposure to pesticides used for either LLINs or IRS. For opportunistic-feeding species such as *An. arabiensis* selective pressure in this domain is associated with the insecticide treatment of cattle, referred to as

zooprophylaxis (Habtewold et al. 2004). As the efficacy of these treatments is often short lived they may result in sub-lethal exposures to adult vectors (Habtewold et al. 2004) resulting in small, temporal increases in metabolic resistance in local populations (Diabate et al. 2002). Resistance is primarily to pyrethroids and organophosphates and conferred by metabolic mechanisms. In Uganda and Ethiopia *Vgsc*-1014F was found in *An. arabiensis* however susceptibility to pyrethroids was recovered after pre-exposing wild-caught vectors to PBO suggesting that *kdr* alone was not responsible for the resistance phenotype (Mawejje et al. 2013, Messenger et al. 2017). In the more southern countries of Kenya, Tanzania, and Zanzibar *Vgsc*-1014S frequency is sporadic and pyrethroid resistance was found primarily associated with CYP4G16 (Matowo et al. 2014, Kiuru et al. 2018). Although exophily is high in *An. arabiensis* in this domain, the use of pyrethroids to control malaria vectors, either via LLINs or IRS, is the most probable cause of resistance in this region with novel metabolic resistance arising in response to insecticidal pressure (Flaxman et al. 2010).

Last, in South Africa, where *An. arabiensis* is more anthropophilic, exposure to insecticides occurs in both the peridomestic environment and in agricultural settings. Reported resistance is mainly to pyrethroids as well as organophosphates in some areas. As with *An. arabiensis* in the East, *Vgsc*-1014S has been reported and its role in resistance is equally as clouded. In Rwanda, where approximately one third of *An. arabiensis* tested possessed at least one copy of the *Vgsc*-1014S allele, susceptibility to pyrethroids was restored after pre-exposure to PBO suggesting that metabolic mechanisms are also responsible for the resistance phenotype (Hakizimana et al. 2016). In Zimbabwe, monooxygenases were determined to be potentially responsible for pyrethroid resistance based on microplate bioassays (Munhenga et al. 2008).

Although there has been recent interest in insecticide resistance in *An. arabiensis*, much is still unknown, especially in West Africa (**Figure 1.11**). Compared to the other main vectors, the exophilic nature and more catholic diet of *An. arabiensis* means that they opportunistically feed thereby circumventing current household-based interventions such

as LLINs and IRS (Mathenge et al. 2001). Using limited continent-wide data, a meta-analysis showed that both interventions affect *An. arabiensis* the least potentially providing this species a competitive advantage (Sinka et al. 2016). The spread and intensity of resistance, therefore, becomes more important as its vectoral role is expanding in this region resulting from expanded urbanisation and agriculture.

Figure 1.10: Map showing the distribution of resistance in *An. funestus* reported between 2005 and 2018 (courtesy of IR Mapper, anopheles.irmapper.com, Jan 2018). Resistance patterns are shown for carbamates (top left), organochlorines (top right), organophosphates (bottom left), and pyrethroids (bottom right).

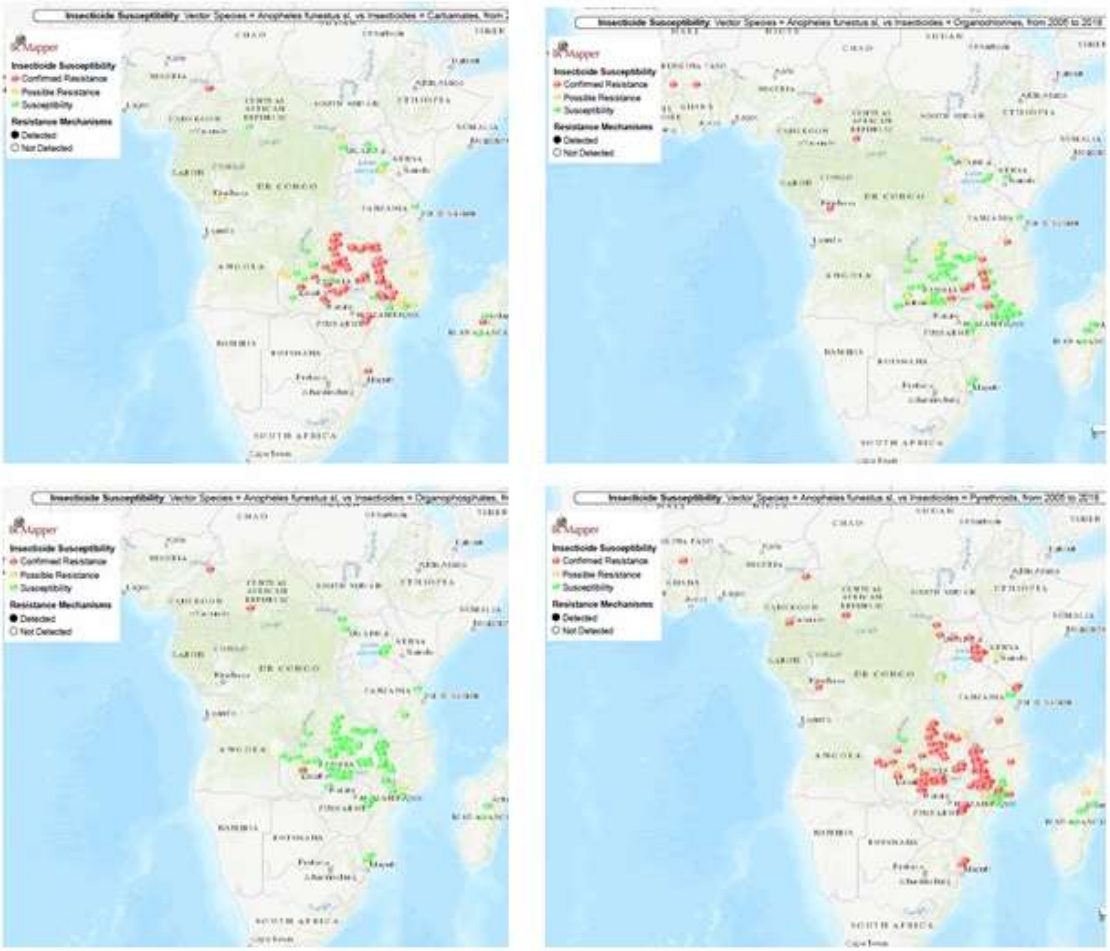
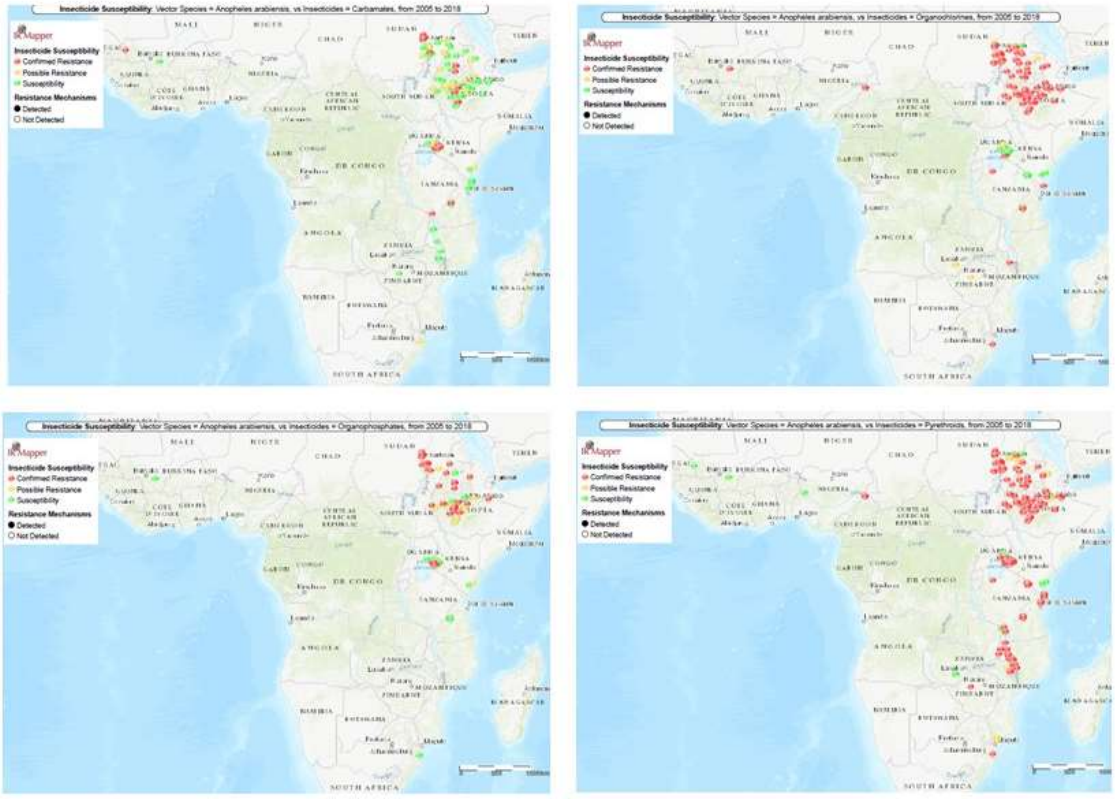


Figure 1.11: Map showing the distribution of resistance in *An. arabiensis* reported between 2005 and 2018 (courtesy of IR Mapper, anopheles.irmapper.com, Jan 2018). Resistance patterns are shown for carbamates (top left), organochlorines (top right), organophosphates (bottom left), and pyrethroids (bottom right).



1.6 Quantitative trait locus (QTL) mapping

Associating phenotypes with specific genotypes can be accomplished through a variety of methods including microarrays, genome-wide association studies (GWAS) or QTL mapping. Microarrays are biased towards known genes included in the set up while GWAS and QTL look for genetic differences associated with a phenotype of interest. Both microarrays and GWAS are often hindered by low levels of linkage disequilibrium or high

levels of population sub-structuring (Weetman et al. 2010). QTL mapping, however, provides for genome-wide coverage and has been used extensively to discern genotype/phenotype associations in mosquitoes at a lower cost. QTL is simply the association between a phenotype and genotype. QTL accomplishes this by discerning the location of a region which is exerting a magnitude of effect associated with a trait of interest (Paterson 2002, Collard et al. 2005). This is accomplished by using a series of genetic markers (in this instance microsatellites) in non-coding regions that are different between the two group. One method is to use the F2 or inbred offspring from a cross between the two populations (Zhang 2012). Between generations, recombination results in the segregation of alleles that are associated with the trait of interest. Phenotyped F2 offspring are genotyped and a linkage map is generated for which relatedness between the trait and the position along the map is determined. QTL has been used to determine salt water tolerance in *An. melas* (Smith et al. 2015), malaria parasite immunity (Rottschaefer et al. 2011), and melanotic encapsulation of parasites in *An. gambiae* (Zheng et al. 2003). Finally, QTL has been used extensively to associate novel resistance mechanisms to insecticide resistance in anophelines across SSA (Ranson et al. 2004, Wondji et al. 2007, Irving et al. 2012, Witzig et al. 2013, Wondji et al. 2013, Ibrahim et al. 2016).

1.7 Goals of this study

A great deal has been published examining the emergence and spread of resistance in *An. gambiae*, *An. coluzzii* and *An. funestus*, however until recently little was reported on *An. arabiensis*. The importance of *An. arabiensis* in sustaining malaria transmission in SSA has increased due to the inability to effectively control this species across most of its range using current interventions (Sinka et al. 2016). Additionally, this species has recently benefitted from anthropogenic changes within West and South Africa, many of which have been shown to result in increases in xenobiotic pressures and subsequent insecticide resistance. As little is known about insecticide resistance in *An. arabiensis* in West Africa (**Figure 1.11**), it is critical to determine current levels as the ability to control this species will only get more difficult. Therefore, the goal of this dissertation is to examine insecticide

resistance in this species within West Africa to better understand the origin and spread of anthropogenic-associated resistance in *An. arabiensis*.

1. The first part of this study will be done as a proof of concept to validate the tools needed. Using a known phenotype (*rdl* resistance), QTL was employed to validate the use of either F2 or AIL lines as well as the utility of the markers designed. Although a resistance-associated variant has been mapped to the 2L region in *An. gambiae* (Brooke, Hunt, and Coetzee 2000), and SNPs within the GABA gene (found within 2L) are associated with dieldrin resistance (ffrench-Constant et al. 1993, Du et al. 2005), no study has employed advanced molecular techniques to determine if resistance is monogenic. Additionally, this primary study will be used as a model system for further resistance candidate validation.
    a. I will develop a panel of microsatellites that provide 5-10 cM coverage across the entire genome specifically for *An. arabiensis* using the recently published genome.
    b. Using those microsatellites, I will use QTL mapping to determine the genetic basis of dieldrin resistance in a colony of *An. arabiensis* using two different crossing schemes between a resistant colony, SENN, and the genome reference strain DONGOLA
        i. F2 inter-cross hybrids from single pair matings
        ii. F8 inter-cross hybrids, or advanced inter-cross lines (AILs), between the original F2 hybrids.
    c. Finally, I will investigate, in a limited manner, fitness costs associated with and cross-resistance to other antagonistic GABA compounds which have been used more recently in agricultural applications across sub-Saharan Africa associated with *Gaba*-296S.
2. As the need to develop and employ alternative insecticides due to the increases of resistance to many of the currently approved compounds, it is important to

discern if resistance mechanisms are present in populations which could confer cross-resistance. Therefore, I will investigate the persistence of *Gaba*-296S in wild populations from West Africa and any associated signals of selection.

3. Last, after previously proving the usefulness of QTL, I will employ the previously developed microsatellite panel and a well-established microarray tool designed for studying the sibling species *An. gambiae* to investigate novel bendiocarb resistance in a population from Rufisque, Dakar, Senegal compared to several susceptible laboratory colonies. This will allow for validating the QTL markers for discerning resistance in field populations of *An. arabiensis*.
    a. QTL analysis using microsatellite data generated from the previously developed panel on phenotyped samples will be performed on F2 inter-cross hybrids.
    b. PCR and sequencing will be performed to investigate if novel SNPs are associated with the resistance phenotype.
    c. Last, an 8 X 15K microarray, designed for *An. gambiae s.s.*, will be employed to look for additional differentially expressed genes associated with the resistance phenotype.

Chapter 2

Quantitative trait analysis of dieldrin resistance in a laboratory colony of *Anopheles arabiensis* from Sudan

2.1 Introduction

Insecticide resistance is considered a threat to malaria control programmes in many parts of the world (Hargreaves et al. 2000; Kigozi et al. 2012) and the current rise of resistance across Sub-Saharan Africa, challenges the progress that has been made towards controlling this disease (WHO 2012; Ranson and Lissenden 2016). Currently there are three primary interventions for controlling / eliminating malaria: prompt diagnosis and appropriate treatment of cases, chemoprevention in vulnerable populations and vector control (WHO 2017). The most common tools used by vector control programmes in Africa to curtail malaria are the employment of insecticide-impregnated bed-nets (LLINs), indoor-residual spraying (IRS) or a combination thereof (Kleinschmidt et al. 2009; WHO 2017). Currently there are only four classes of compounds approved for controlling malaria vectors and resistance has been observed to all four (Edi et al. 2012; Namountougou et al. 2012). Whilst great emphasis has been focused on cross-resistance from target-site mutations, resistance between classes has also been shown to be mediated by other mechanisms (Ranson and Lissenden 2016). Metabolic resistance in *Anopheles gambiae*, in which there is differential expression of insecticide detoxification or sequestration enzymes, has been shown to provide cross-resistance to different classes of pyrethroids as well as bendiocarb (Mitchell et al. 2012; Edi et al. 2014). Further, there is a growing body of evidence supporting cuticular resistance in which the cuticle becomes thicker slowing the rate of insecticide absorption allowing for increases in tolerance, potentially to multiple insecticides (Vannini et al. 2014; Toe et al. 2015, Balabanidou et al. 2016).

Current strategies for resistance management are based on determining local resistance patterns in vectors and, where possible, associated resistance mechanisms and employing insecticides that would be the most effective (Brogdon and McAllister 1998a). Underlying this strategy is the assumption that resistance carries a fitness cost and once selection pressure is removed resistance

will diminish, as the survival advantage proffered by the mechanism will outweighed by costs and be detrimental relative to wild type individuals (Keiding 1967; McKenzie et al. 1982).

However, resistance is often encountered in vectors with no known current insecticide exposure or in areas where a particular pesticide use has long been stopped, an observation termed "persistence of resistance" (Aronstein et al. 1994). Persistence of resistance in the absence of selection is a little-studied phenomenon. As previously stated, insecticide resistance is assumed to come with fitness costs due to various pleiotropic effects. These effects were seen by Rowland who found that *An. gambiae* and *An. stephensi* that possessed γ-hexachlorohexane resistance were less fecund, produced fewer eggs per female, and were less active than either heterozygous or susceptible controls (Rowland 2001). In *Aedes aegypti*, increases in resistance to pyrethroids and organophosphates was found to be correlated with diminished reproduction and adult longevity (Martins et al. 2012). Platt et al. (2015) found that multiply resistant *An. gambiae* males were less competitive in mating than their heterozygous counterparts. However, Okoye et al. (2007) and Plernsub et al. (2013) showed that pyrethroid resistant *An. gambiae* and *Aedes aegypti,* respectively, suffered little or no apparent fitness costs compared to susceptible populations. Decreases in fitness can also be ameliorated by gene duplication in which an individual possesses a susceptible and resistant copy of the resistance gene. In *An. gambiae* and *Culex pipiens*, a duplicated *Ace*-1 susceptible copy reduces the fitness costs associated with single copy *Ace1*-119S (Labbe et al. 2007; Assogba et al. 2015). Even if there are few fitness effects associated with a mechanism, duplication is assumed to allow for "evolutionary flexibility" allowing mutations to arise in one copy while homeostasis is preserved in the susceptible duplicate resulting in permanent heterozygosity as seen in *Drosophila* (Remnant et al. 2013). Therefore, the fitness costs associated with resistance may differ based on the mechanism responsible as well as the presence of balancing alleles which may counteract any pleiotropic effects.

Dieldrin, along with DDT, was one of the first organochlorine insecticides used in global malaria elimination campaigns in the 1950s. However, the use of dieldrin was restricted due to its high human toxicity and the rapid emergence and spread of resistance (Hayes 1959; Brown et al. 1976). Dieldrin resistance is commonly due to one of two target-site alterations conferred by single point mutations within the M2 transmembrane domain of the gamma aminobutyric acid (GABA) receptor termed *resistance to dieldrin* or *rdl* (Ffrench-Constant et al. 2000; Du et al. 2005). In *An. gambiae* resistance is conferred by a *Gaba*-296G substitution while in *An. arabiensis* and *An.*

coluzzii it is conferred by *Gaba*-296S (Du et al. 2005; Kwiatkowska et al. 2014; Platt et al. 2015). The mutation prevents insecticides from interacting within the active site within the chloride channel thereby abolishing the normal nerve potential within the insect neuron (Casida and Durkin 2013).

Even though dieldrin has not been employed for several decades, resistance to this compound is still found in anopheline populations even in the apparent absence of insecticide pressure (Brooke et al. 2006; Corbel et al. 2007; Koekemoer et al. 2011; Kwiatkowska et al. 2014). In Benin, the presence of the *rdl* genotypes were estimated as 55.3% r/r, 19% r/s, and 25.7% s/s (n=400) (Corbel et al. 2007) while in Burkina Faso to be 73/94 r/r, 18/94 r/s, and 3/94 s/s by pyrosequencing (Kwiatkowska et al. 2014). Although the presence of *rdl* in many anthropophagic species would not have an immediate public health impact, where *An. arabiensis*, a species known to be opportunistically zoophagic, is a major vector it could be highly detrimental as there is increasing interest in employing phenylpyrazoles such as fipronil as an endectocide in cattle (Poche et al. 2015). In addition, there is some evidence that *rdl* confers cross-resistance to deltamethrin and neonicotinoids which may impact the deployment of novel active ingredients into the public health arena (Brooke et al. 2000; Kolaczinski and Curtis 2001; Taylor-Wells et al. 2015).

As resistance to dieldrin is still found around the world in the absence of apparent selection, its persistence could be due to: an absence of deleterious pleiotropic effects associated with it; compensatory mechanisms which counteract any negative pleiotropic effects; the persistence of dieldrin in the environment; or from exposures to alternative compounds which interact with the GABA subunit such as fipronil.

This study was undertaken to investigate the following:

1. Although a resistance-associated variant has been mapped to the 2L region in *An. gambiae* (Brooke, Hunt, and Coetzee 2000), and SNPs within the GABA gene (found within 2L) are associated with dieldrin resistance (Du et al. 2005; Ffrench-Constant et al. 1993), no study has employed advanced molecular techniques to determine if resistance is truly monogenic. Microsatellites have been used previously in Quantitative Trait Loci (QTL) approaches to map insecticide resistance in several anophelines (Ranson et al. 2000; Wondji et al. 2007; Witzig et al. 2013). I will use QTL mapping to determine the genetic

basis of dieldrin resistance in a colony of *An. arabiensis* isolated from the Gezira State, Sudan in the 1980's with known dieldrin resistance.

2. Insecticide resistance in commonly thought to have detrimental effects that may become apparent in life table comparisons. Most commonly seen are differences in larval maturation, egg production, and adult longevity with resistant individuals most likely to suffer. Both colonies were assayed to determine the levels of resistance to 4 compounds. DONGOLA was found to be susceptible to all compounds tested while SENN was found to be resistant to dieldrin and partially resistant to DDT (**Figure 2.1**). I will examine potential pleiotropic effects associated with *Gaba*-296S in immature maturation rates and adult longevity by comparing the resistant colony SENN, originally from the southern Gezira, Sudan, with DONGOLA, a dieldrin susceptible colony originally from the North of Sudan.

3. As dieldrin was withdrawn from use over 40 years ago, the persistence of this mutation is worrisome, however its presence may be related to the more recent agricultural use of phenylpyrazoles or cyclodienes. GABA mutations have been shown to proffer cross-resistance between these classes. Finally, I will determine if *Gaba*-296S confers resistance to other antagonistic GABA compounds which have been used more recently in agricultural applications across sub-Saharan Africa.

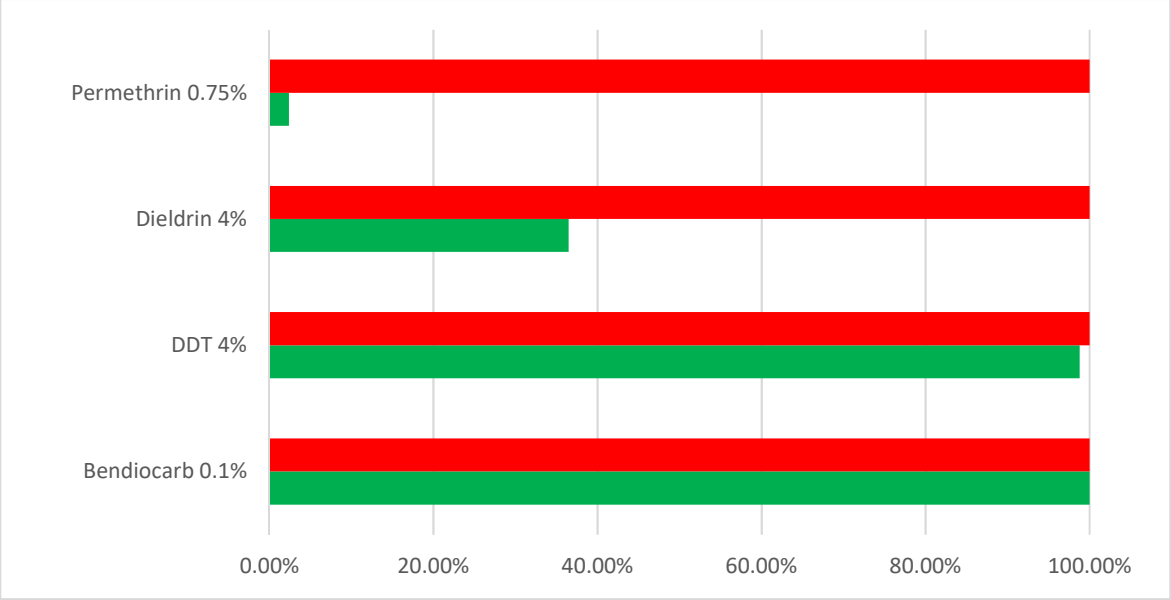
2.2 Methods

2.2.1 Iso-female lines

The mosquitoes used in this study are available from BEI Resources (www.beiresources.org) and maintained at the CDC in Atlanta, GA. The *An. arabiensis* strains used were the dieldrin resistant SENN (MRA-763) and susceptible DONGOLA-2Ra2Rb3R (MRA-1235). Resistance in SENN is maintained by exposing fourth instar larvae to 1ppm dieldrin solution for 1 hour every 5 generations. Resistance, defined as >97% survival 24 hours post exposure, is periodically confirmed by exposing adults to either a CDC bottle bioassay or WHO tube assay. DONGOLA was selected to be homozygous for 2Ra and 3Ra chromosomal inversions in the standard form and 2Rb in the inverted form while SENN is reported to be polymorphic for both the 2Ra and 3Ra

inversions (pers comm Olga Grushko and Nora Besansky). All mosquitoes were reared at 27°C ± 1°C and 80% ± 2% humidity using standard protocols (Benedict 1997) except that larvae were fed with Drs. Foster and Smith® Staple Diet Koi food (www.drsfosterandsmith.com) and adults were fed a diet of 10% sugar water with 0.2% methylparaben *ad libitum* (Benedict et al. 2009).

Figure 2.1: Average percentage of mortality of SENN (green) and Dongola (red) when exposed to different insecticides when exposed for 1-hour employing the WHO tube bioassay.



Reciprocal crosses between the 2 lines were done by combining single male and female pupae from each strain in a 200 ml Qorpack cup (Bridgeville, PA. No. 3891) for 10 days (Benedict and Rafferty 2002). Blood meals were offered on day 10 post-eclosion; those that did not feed were offered a blood meal daily until they did so or until they perished. Individual females, 3 days post blood feeding, were left to oviposit in a new Qorpack cup containing 50ml water and a strip of filter paper. F1 progeny were mated with kin to generate the F2 material that was subsequently used to generate new iso-female lines and material for the advanced intercross lines (AILs). Additional pairings were set up utilizing the induced copulation method as described by Ow Yang et al. (Ow Yang, Sta Maria, and Wharton 1963) as needed.

2.2.2 Advanced intercross lines (AIL)

An AIL methodology was chosen because it increases the probability of recombination between markers thereby narrowing the width of the quantitative trait loci (QTL) mapped (Gonzales and Palmer 2014). Unless otherwise stated, all crosses show the female first. AIL families were generated from 11 F2 families, 4 from the DONGOLA x SENN (DxS) crosses and 7 from SENN x DONGOLA (SxD). Five SxD families were excluded, as their F2 progeny were 100% resistant or susceptible in insecticide bioassays (below). These families were continually inbred for 6 generations before final bioassay selection. All F8 progeny exposed in this study were separated by phenotype and kept at -20°C for molecular genotyping (see below).

2.2.3 Insecticide bioassays

Two to three-day old mosquitoes were exposed to 4% dieldrin impregnated papers for 1 hour employing the WHO tube assay (WHO 2013). After exposure, all adults were aspirated into 500ml holding cups and held at 27°C, 80% humidity and provided 10% sucrose *ad libitum*. Mortality was recorded for each family 24 hours post exposure and siblings were separated based on resistant (alive) or susceptible (dead) phenotypes. All phenotyped specimens were transferred individually to 1.5ml cryotubes and stored at -20°C until DNA extraction.

2.2.4 Microsatellites

Total mosquito DNA was extracted using a previously published method (Livak 1984). Although Zheng (Zheng et al. 1996) developed a large suite of microsatellite markers for *An. gambiae*, many have been found to be uninformative or fail to amplify for other members of the *An. gambiae* complex (Deitz et al. 2012). Therefore, new primers were developed by searching for di- and tri-nucleotide repeats within theAaraD1.3 gene set published at VectorBase (https://www.vectorbase.org/). Microsatellites were identified using WebSat http://wsmartins.net/websat/ (Martins et al. 2009) and checked for polymorphism within *An. gambiae s.l.* using the BLASTn feature of VectorBase (Lawson et al. 2009). All primers were designed using Primer 3 (Rozen and Skaletsky 2000) with forward primers labeled with either of two fluorescent markers (HEX or FAM). Primers, both previously published and newly developed, included in this study are listed in Table 2.1. Multiplexed PCR reactions consisted of 1.5 units of Promega Go*Taq*™ polymerase, 1.5 mM dNTPs, and an additional 0.75 mM $MgCl_2$ in 20 µl total

volume (Promega, Madison, WI). PCR cycling consisted of denaturation at 95° C for 3 min followed by 30 amplification cycles of 95°C for 30 sec, 55°C for 28 sec, and 72°C for 30 sec, followed by 1 cycle of 72°C for 5 min (Lehmann et al. 1996). PCR products were visualised on 1.5% 0.5x tris-borate-EDTA agarose gels stained with ethidium bromide under UV transillumination (UVP LLC, Upland, CA) prior to genotyping. Genotyping was performed on a Hitachi ABI 3130 Genetic analyzer with 1µl of diluted PCR product, 0.4 µl ROX -250 ladder (ABI Biosystems, part no. 401734) and 10.6 µl Hi-Di formamide. Discrimination of band sizes was done using GeneMapper 4.1 (ABI Biosystems).

2.2.5 Real-Time PCR (RT-PCR) assay

For discrimination of the *Gaba*-296S allele in parental strains, F2, and AIL crosses, RT-PCR (Bass et al. 2010) was chosen due to the unreliable scoring of heterozygotes using an allele specific PCR method (Wilkins et al 2006). Initial trials on 54 individuals from SxD crosses and 76 from DxS crosses found that the allele-specific PCR assay failed to detect heterozygotes for the *Gaba*-296S mutation approximately 50% of the time. For each RT-PCR assay, a *Gaba*-296S positive, negative, and F1 hybrid heterozygote were used as controls. Both colonies employed were assayed to confirm their *Gaba*-296S status prior to the initiation of this study. All samples were processed on a Bio-Rad CFX96 RT-PCR machine and final genotypes for all samples were determined based on the relative fluorescence unit (RFU) measurement when compared to standardized resistant and susceptible controls from the parental lines.

2.2.6 QTL analysis

The software R/qtl was chosen as it was designed to work with inbred lines and can utilise incomplete genotype data (Broman et al. 2003). There are 3 common analytical methods that can be performed within R/qtl: marker regression, interval mapping, and multiple imputation with marker regression the most basic method. Marker regression simply considers the ratio of each phenotype to the known genotype markers and performs a t-test to determine the most likely position of the QTL (Kearsey and Hyne 1994). This method is not recommended for final analyses as it will exclude individuals that are missing genotype data and doesn't consider positions between markers as a location for a QTL. Instead, it is recommended that either interval mapping or multiple imputation be used (Broman & Sen 2011). Unlike marker regression, both techniques incorporate missing genotype data. In interval mapping, the program estimates the genotype at

each “step”, defined by the user in centimorgans, even between known markers for the entire genome. Next, the location of the QTL is estimated at the various “steps”, one at a time, and the most likely position is determined using a maximum likelihood estimation. One hinderance is that interval mapping will produce spurious results in regions with limited known genotype data and works best when detecting a single QTL (Jansen 1993). Lastly, multiple imputation is similar in nature to interval mapping except that it infers missing genotype data not only within known markers, but also between markers aside from defined steps (Bobb et al. 2011). Even though there are potentially more data points resulting from the inference, multiple imputation has been found to provide similar results to interval mapping when testing for a single QTL (Broman & Sen 2011).

Even though there was low coverage on chromosome 3, there was limited missing genotype data and good coverage on chromosome 2 therefore interval mapping, non-parametric analyses, and multiple imputation analyses were performed. Associations between dieldrin resistance or susceptibility and the genotype for each microsatellite were estimated using interval mapping by Haley-Knott (HK) regression and both non-parametric and multiple imputation analysis using an extension of the Kruskal-Wallis test statistic (NP, IMP) (R Development Core Team 2013). Logarithm of the odds (LOD) scores and Bayes credible intervals (a proxy for 95% confidence interval) were determined for both F2 and AIL crosses separately. The LOD threshold was estimated by calculating the 95% percentile LOD score on a chromosome with no QTL. Markers used in this study were pre-screened by PCR against 48 random individuals from both parental lines as well as against the P0 individuals used to establish the iso-female lines. Only microsatellites found to be unique to either colony were employed.

2.2.7 Cross-resistance

Additional testing was performed to determine if the dieldrin resistant SENN colony displays cross-resistance against fipronil and the organochlorine endosulfan. Two-to-three-day old females from both the SENN and DONGOLA colonies were exposed for 1 hour to Whatman qualitative grade 1 filter papers impregnated with 1% fipronil dissolved in acetone using the WHO tube assay protocol. A second cohort was then exposed to 50 µg and 100 µg of endosulfan dissolved in acetone using the CDC bottle bioassay (Brooke, Hunt, and Coetzee 2000; Brogdon and McAllister 1998b). The final concentration of endosulfan employed was determined after performing a series of serial dilutions as suggested by Dr. William Brogdon (personal communication) and based on

100% mortality of a susceptible colony after 30-minute exposure. After exposure, all adults were aspirated into 500ml holding cups and held at 27°C, 80% humidity and provided 10% sucrose *ad libitum*. Mortality for each bottle and strain was recorded 24 hours post exposure.

2.2.8 Bionomics

To determine if dieldrin resistance in SENN has any detrimental pleiotropic effects, several life cycle parameters were studied to see if there were any differences when compared to the susceptible DONGOLA strain. Common phenotypes studied to determine if there are negative effects associated with insecticide resistance include duration of the larval period, pupal emergence rates, adult longevity, fecundity and embryo production, and reproductive rate (Carriere et al. 1994; Rowland 2001; Liu and Han 2006; Martins et al. 2012). In this study, we used the following attributes: rate of larval development, time to pupation, time to emergence, percent emerging, and adult longevity.

2.2.8.1 Immature development

For all experiments, eggs from parental strains were hatched *en masse*. Twenty-four hours later, 200 first instar larvae were transferred into pans each containing 500ml of water and fed and reared as described above. Pupation was monitored twice daily, and pupae were removed, and the number and sex were recorded for each pan. There were 13 replicates of 200 larvae for each strain.

2.2.8.2 Adult attributes

Cohorts of 25 virgin male and female adults less than 48 hours post emergence were placed into six 8.75cm x 14.8cm *Drosophila* embryo collection cages (Genesee Scientific, #59-101) and provided sugar as described above. Cage location was changed daily to avoid potential positional bias. Daily mortality was recorded for cage, sex and strain for 30 days.

2.2.8.3 Bionomic analysis

Differences between the colonies in the average number of days to first pupae, survival to pupation, survival through eclosion, and sex ratios were tested using Student's t-tests. Adult longevity was estimated using the Cox Proportional Hazard regression function in R (within the "survival" package). Differences between sex and strain were estimated using a likelihood ratio test.

2.3 Results

2.3.1 F2 QTL analysis

Mapping QTLs is more effective when markers chosen are informative, i.e. different alleles are fixed in the parental strains. Offspring that have informative markers can be easily distinguished by having inherited different-sized alleles from each parent, whereas with semi-informative markers it is more difficult to ascertain the donor parent due to shared allele sizes. Due to reduced polymorphism and low rates of shared microsatellite sites between *An. gambiae* and *An. arabiensis,* new markers had to be created to obtain a high coverage rate. During this study, 122 microsatellites from across all three chromosomes were screened to determine informativeness. In total 35 previously published primers and an additional 59 novel microsatellites, designed from contig sequence data in the *An. arabiensis* genome (AaraD1.3), were found to be non-informative between the two populations tested. Of those, 2 failed to amplify in either strain, 2 did not amplify in Dongola alone, 52 shared one amplicon length, and 38 were monomorphic in both colonies. Due to restraints on equipment availability and reagents, semi-informative markers were not used in this study. In total, 27 informative microsatellites (10 previously published; 17 novel) plus 1 single-nucleotide polymorphism (SNP) marker were included in this study (**Table 2.1**). Coverage varied between chromosomes (**Figure 2.2**). For the X chromosome, 4 markers covered approximately 14.9 cM, for 2R 9 markers covered 56.16 cM and for 2L 8 markers covered 44.7 cM. Coverage on the third chromosome was lower due to many tested loci proving non-informative, mainly near the proximal region of the 3L chromosome arm. Five markers covered 44.1 cM on 3R, and for 3L, 2 markers - on the distal end of the chromosome arm - covered 15.7 cM (**Figure 2.3**). All markers were assessed for Hardy-Weinberg equilibrium (HWE) using GenePop v4.2 (http://genepop.curtin.edu.au/). No marker was found to be uniformly out of HWE, however CDC23 was for 4 of the 5 families (**Appendix Table 1**).

Haley-Knott regression and multiple imputation analyses were performed on 901 F2 offspring based on their ability to survive a one-hour exposure to 4% dieldrin (referred to as DLRES). For the F2 analysis, 94.2% (n=207) DxS and 96.4% (n=694) of individuals were included in the genotype model (**Table 2.2**). The LOD threshold for significance was estimated to be >5.0 with only markers scoring higher considered candidates. The threshold was estimated based on LOD values estimated for chromosome 3 which was considered neutral for this experiment. For Haley-

Knott regression the strongest association was near microsatellite CDC675 (**Figure 2.4**) with a LOD score of 54.476 (P<0.001) and the Bayes credible interval (95% CI) of the QTL residing between chr2: locus 90 (position 91.247) and chr2: locus 92 (pos. 93.248). QTL analysis of the F2 backcross generation found very little concordance between individual or combined families with only SD17 identifying marker CDC675 as the most likely candidate region (**Appendix Figure 1**). This discrepancy may be related to how the computer estimated the position of the markers, with each family showing strong skewing on chromosome 2 (**Appendix Figure 3**).

2.3.2 AIL QTL mapping

None of the DxS families survived past 3 generations of inbreeding so all AIL lines were generated from the SxD crosses alone. Losses were probably the result of severe inbreeding depression as each of the DxS AIL crosses started with less than 30 adults from an original single pair family. Five SxD families were inbred for an additional 6 generations resulting in 355 resistant and 221 susceptible individuals (**Table 2.3**).

In total, 94.3% (n=573) of AIL individuals were included in the genotype model. Unlike the F2 generation, 2 families in the AIL found a strong association between CDC675 and dieldrin resistance (**Appendix Figure 2**). As in the F2 analysis, Haley-Knott and multiple imputation method analyses were performed. For the 2 analyses, similar results were retrieved with the Bayes credible interval (95% CI) being located between chr2: locus 89 (pos 90.2, LOD = 68.92) and CDC675 (pos. 91.5, LOD = 66.2). This location is like the one reported from the F2 analyses and is not associated with the RDL marker, but instead is located near the microsatellite CDC675 nearly 4Mb apart (**Figure 2.5**). A χ^2 analysis on phenotype/genotype association for CDC675 confirmed that there is a strong association for this marker with dieldrin resistance (χ^2 =136.79, p <0.0001) with resistant individuals possessing at least one copy of *rdl* (**Appendix Figure 4**).

Figure 2.2: Genetic map estimated by R showing the position of microsatellites used in this study in centiMorgans (cM). Centromeres are located between markers CDC19 and CDC20 on chromosome 2 and 3E36D and CDC43 on chromosome 3.

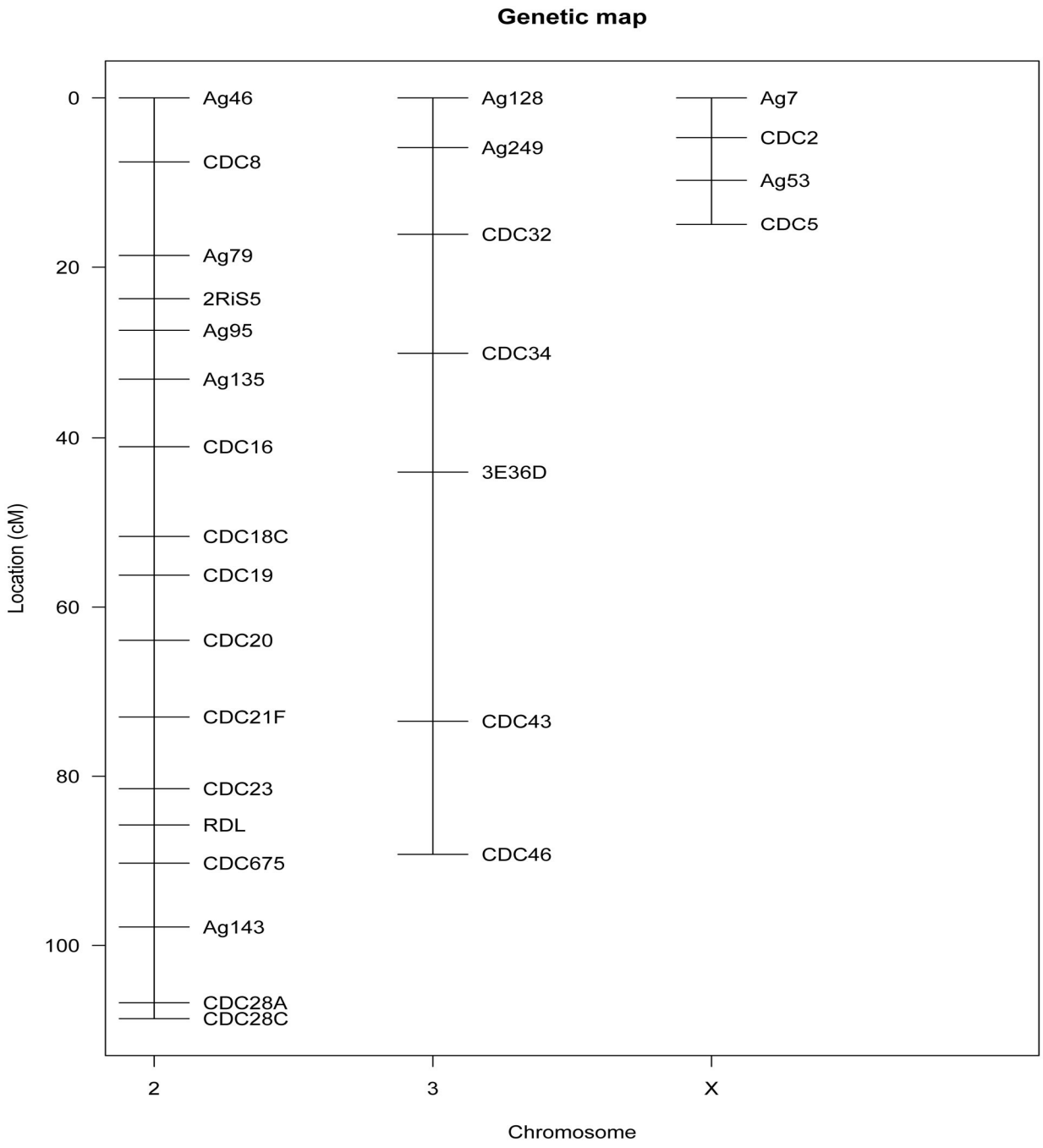


Table 2.1: Microsatellites, their position within the genome based on synteny in *An. gambiae*, PCR product size and oligonucleotide sequence information used to determine the genic location of dieldrin resistance in an F2 interbred experimental population.

Marker	Repeat	Size	Division	Primers	Reference
X chromosome					
AgX:7			1C		Zheng 1996
CDC2	TC(12) TG(8)	160	2C	F: CCG GAG CGT AGA TAT CAG	
				R: TCA AAC CCT TCG TCA CTC	
AgX:53			4A		Zheng 1996
CDC6	GA (6) AC (8)	240	6A	F: ACT CGT GAT GTA TGC AGT TA	
				R: CTT CCC GCT CTT TTT CTA A	
Chromosome 2					
Ag2:46			7A		Zheng 1996
CDC8	ATC (5) GA (7)	231	8E	F: AAA GAT CAC TCG GCA TCA	
				R: TAC TAC GCG ACG GTT TTC	
Ag2:79			11B		Zheng 1996
2RiS5			12C		Witzig 2013
Ag2:95			13D		Zheng 1996
Ag2:135			14D		Zheng 1996
CDC16	CA (5) CA (9)	355	16A	F: AGA GCA GGA ATT GTA GGA CA	
				R: GCC CCA TCA TTT ACT TGT TA	
CDC18	GT (10)	133, 139	18C	F: CAG GAA GCG ATG TGT AAA GT	
				R: GGA GTG TTG TCG TTC ATC TT	
CDC19	AC (23)	341, 344	19C	F: GCG ACC AGT TTT TCT GTA AT	
				R: TAT CCT CTG ACC CTT TTC AC	
CDC20	TA (7) TA (5) TAA (7)	261	20D	F: CGT CGT GTC CAT CAT AGT TC	
				R: AAG ATG CAA ACA GCA AAA CA	
CDC21	TC (6) CT (28)	204	21F	F: TTA AAT GGA TAG GGC AGC TC	
				R: GAA TTC GCA TCC GTC TTA TC	
CDC23	CA (9) GT (5)	280	23A	F: AAT CGG AAA AAT GCA AAT C	
				R: CGA CGA GGA AAA GAA AAA	
RDL*			23C		Bass 2010
CDC675	AC (10) AC (7)	183	24C	F: TCA AAC TCG AAC TCC TCA AC	
				R: TTT CCG TCG ATA GTT TTC TG	
Ag2:143			25D		Zheng 1996
CDC28A	GA (8)	93	28A	F: AGC AGG TAG TCT CCA AAG	
				R: TCG TTG TGT TGT ATT CGC A	
CDC28C	CA (8)	226	28C	F: TGT GCC GGT TGA GAG AGA	
				R: GGG CGA GAA CAT TAA CAA	
Chromosome 3					

Ag3:128			29C		Zheng 1996
Ag3:249			30B		Zheng 1996
CDC32	GGC (5) CT (3)(7)	176	32A	F: GTT TGC TTG CTT GTT GTT GT	
				R: GTG CTC AAC GCC TAC AAA T	
CDC34	CA (10) CT (9) TC (8)	178, 196	34B	F: AAA ACT TTT CCC TCC CAT TC	
				R: AAG TGC AGC AAT TGA CGA G	
3E36D			36C		Blandin 2009
CDC42ǂ	AC (30)	182	42C	F: TAT TTT CCC ATT TCA CGA TT	
				R: GTT TTG TTG CCT GTT TTT GT	
CDC46	GT (8) CA (6)	255	45C	F: GTG GTT GAC CGA TTT GTA AG	
				R: ATT TAT TCA CTC GCC AAG AA	

* RDL is a SNP marker

ǂ SENN has two insertions possible, 16bp and 153bp.

Figure 2.3: Location of microsatellites and the *rdl* SNP employed in this assay overlaid on a chromosomal map of *An. gambiae* (www.vectorbase.org).

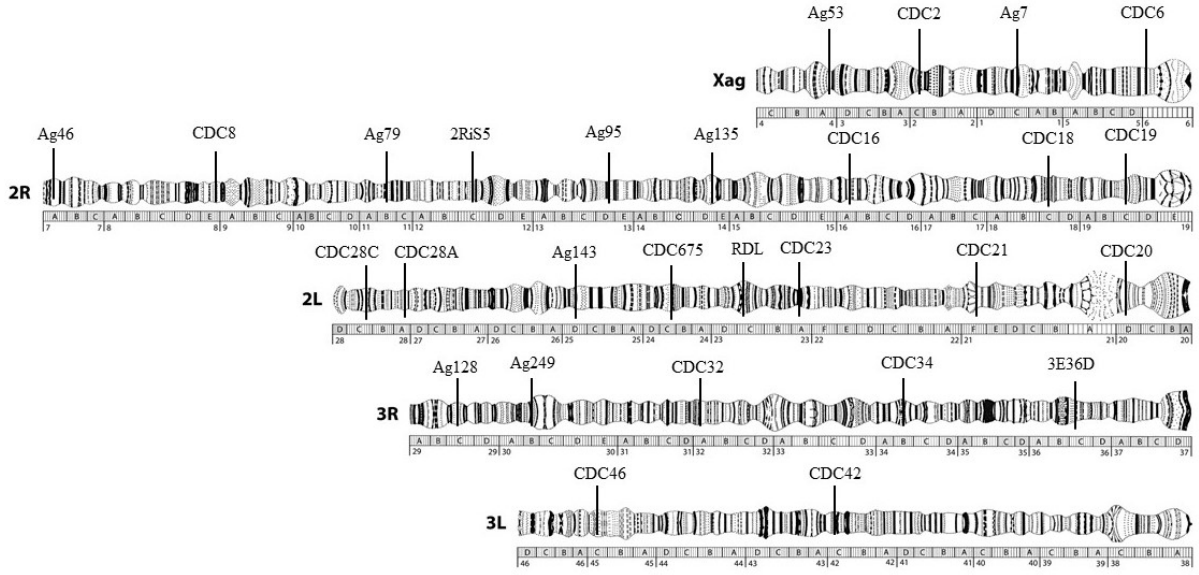


Figure 2.4: LOD scores for marker regression (blue), Haley-Knott (red) and multiple-imputation (black) QTL analyses of F2 intercross individuals based on 24-hour survivorship after 1-hour exposure to 4% dieldrin.

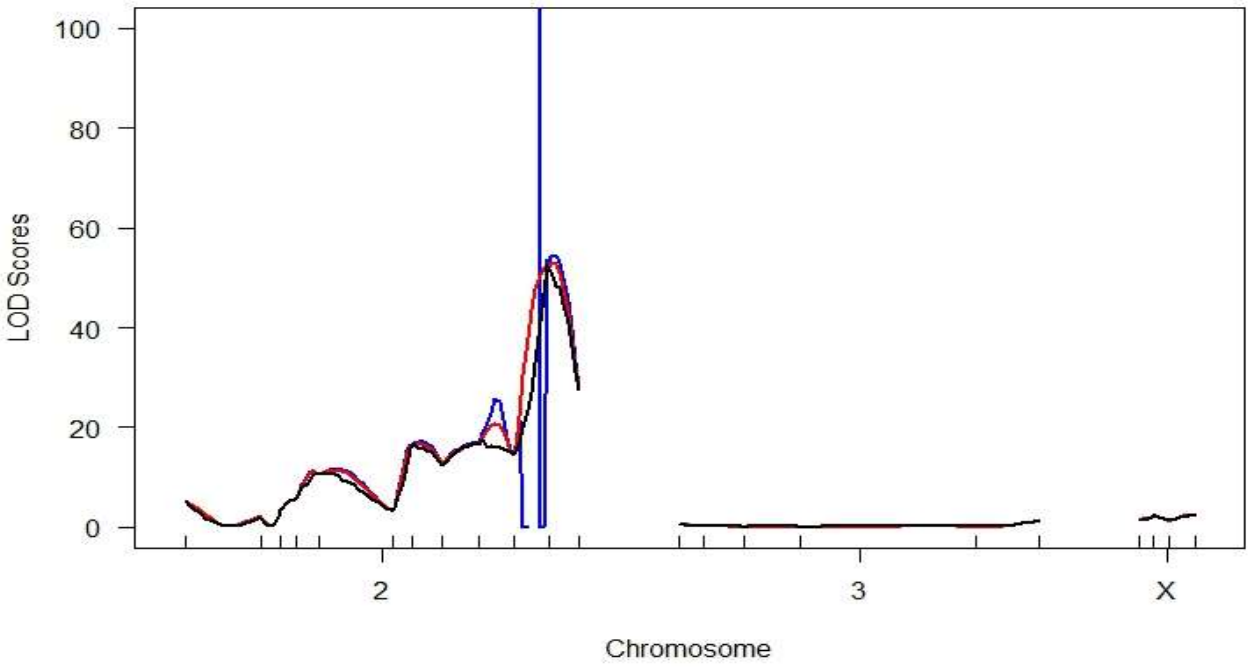


Table 2.2: Twenty-four-hour survivorship of F2 intercross adults exposed to 4% dieldrin for 1 hour using the WHO tube bioassay included in the QTL analysis.

Family	No. progeny	Resistant	(%)	Susceptible	(%)
SENN x DONG					
Fam O	67	32	47.76%	35	52.24%
Fam M	79	41	51.90%	38	48.10%
Fam D	5	2	40.00%	3	60.00%
Fam T	24	14	58.33%	10	41.67%
Fam C	113	52	46.02%	61	53.98%
Fam 1	407	302	74.20%	105	25.80%
DONG x SENN					
Fam Tau	132	55	41.67%	77	58.33%
Fam 5	35	26	74.29%	9	25.71%
Fam 6	18	11	61.11%	7	38.89%
Fam U	22	10	45.45%	12	54.55%

Figure 2.5: LOD scores for Haley-Knott (blue) and multiple-imputation (green) QTL analyses of F8 AIL individuals based on 24-hour survivorship after 1-hour exposure to 4% dieldrin.

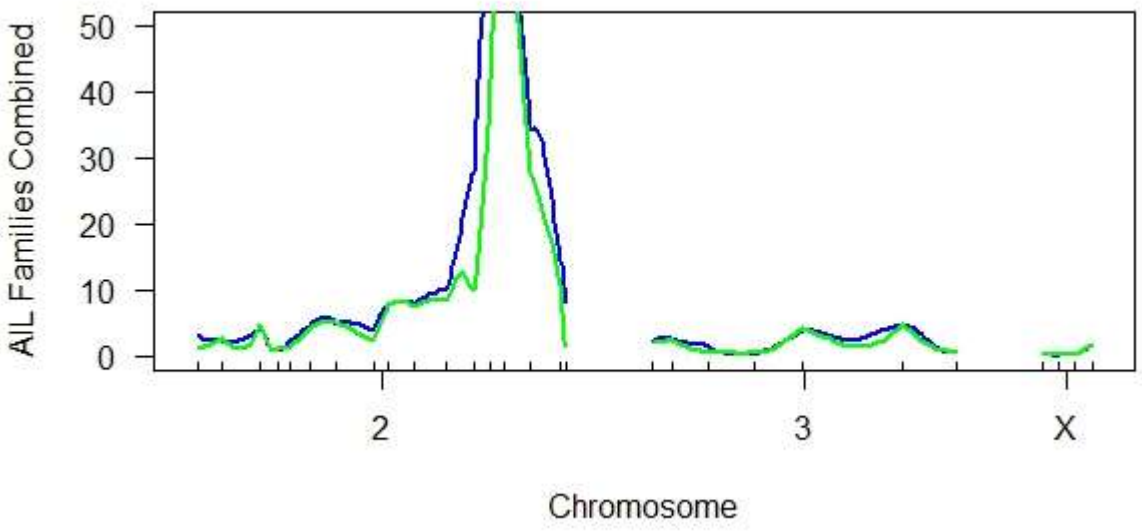


Table 2.3: Twenty-four-hour survivorship of F8 AIL interbred adults exposed to 4% dieldrin for 1 hour using the WHO tube bioassay included in the QTL analysis.

	Total no.	Resistant				Susceptible			
Family	genotyped	Female		Male		Female		Male	
SD1_A	174	58	33.33%	37	21.26%	39	22.41%	40	22.99%
SD1_2	68	25	36.76%	28	41.18%	6	8.82%	9	13.24%
SD1_8	24	8	33.33%	6	25.00%	5	20.83%	5	20.83%
SD1_16	264	88	33.33%	70	26.52%	52	19.70%	54	20.45%
SD1_17	46	13	28.26%	21	45.65%	7	15.22%	5	10.87%
Total	576	192		162		109		113	

2.3.3 Cross-resistance testing

Resistance to fipronil was seen in SENN but not in DONGOLA. Survivorship at 24 hours post exposure to 1% fipronil resulted in 70.3% (n = 118) of SENN surviving compared to 0.0% (n = 69) in DONGOLA. For endosulfan, 2 different concentrations were tested: 50 µg/bottle and 100 µg/bottle for 45 minutes. For the 50 µg/bottle trial, survivorship at 24 hours post exposure was 73.9% (n=46) and for 100 µg, 62.7% (n=67) for SENN. DONGOLA was uniformly susceptible for both compounds with mortality usually occurring in the first 15 minutes of exposure. However, the correlation between *Gaba*-296S and resistance was not determined.

2.3.4 Bionomics

In this study, the dieldrin resistant SENN colony was the more vigorous of the 2 colonies in the pre-adult stages. SENN larvae pupated earlier than DONGOLA and more survived both through pupation and emergence (**Table 2.4**). However, both pupal production and sex ratios were similar between the 2 colonies (**Table 2.4**, **Figure 2.6**). For adult longevity, there was no significant difference between the colonies for males (median 13 days compared to 11 days for SENN) however DONGOLA females were more likely to survive for longer than SENN females (median 20 days compared to 15 days) (**Figures 2.7 & 2.8**).

Table 2.4: Mean (± C.I.) and p-values from a two-tailed paired t-test for immature life table attributes for comparing the dieldrin resistant SENN and susceptible DONGOLA colonies (n=13 replicates, 200 larvae per replicate).

	Days to first pupae		Survivorship to pupation		Survivorship to emergence		Sex ratio (females/total)	
DONGOLA	6.692	± 0.462	0.864	± 0.035	0.846	± 0.038	0.4991	± 0.045
SENN	6.077	± 0.267	0.93	± 0.035	0.916	± 0.035	0.4764	± 0.036
	p = 0.005		p = 0.002		p = 0.001		p = 0.356	

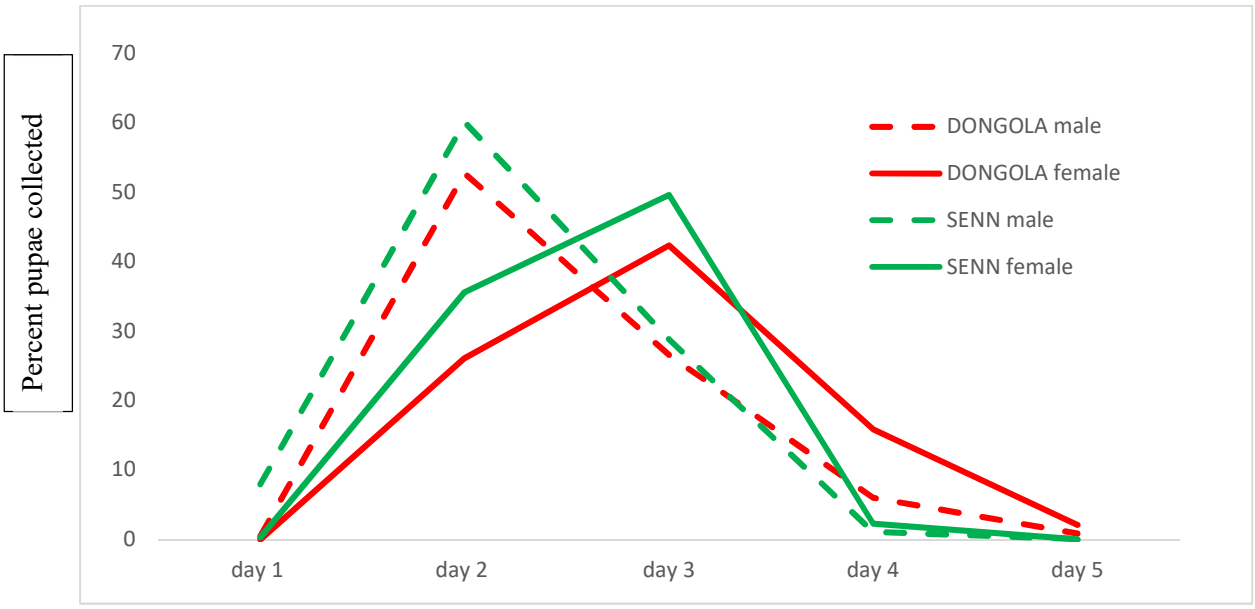


Figure 2.6: The mean number of pupae produced by day and sex for the dieldrin resistant SENN and susceptible DONGOLA colonies.

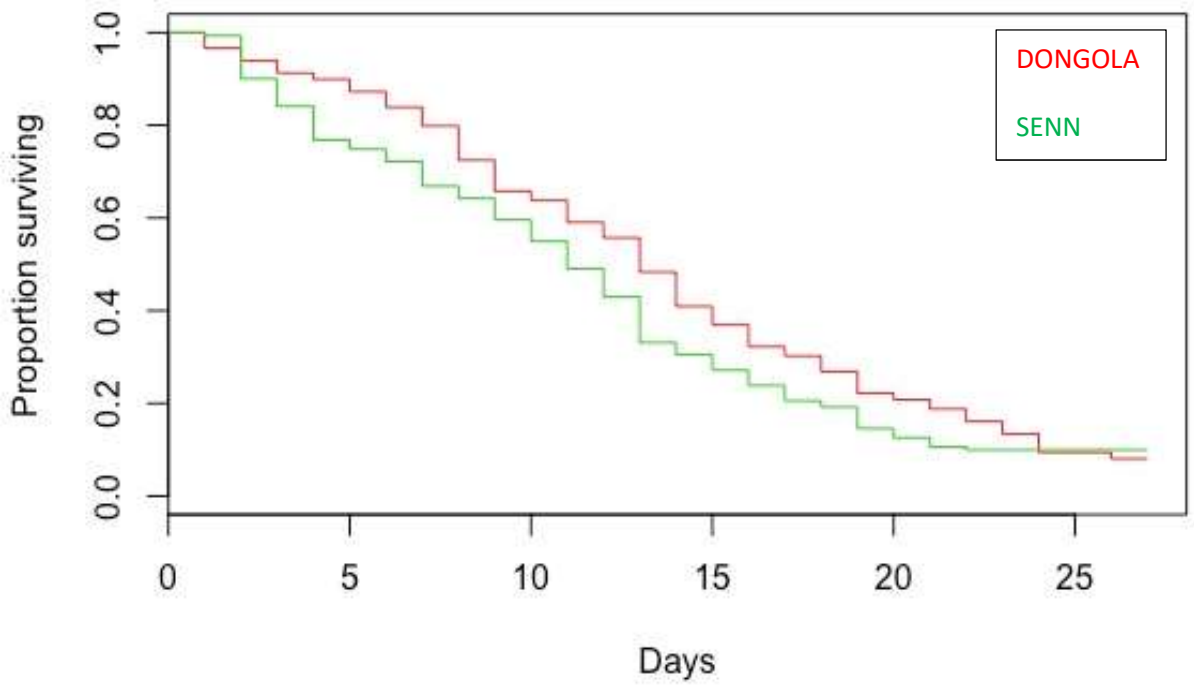


Figure 2.7: Cox proportional hazard regression models comparing adult longevity between dieldrin resistant SENN and susceptible DONGOLA males. (N=150, likelihood ratio test: p=0.106)

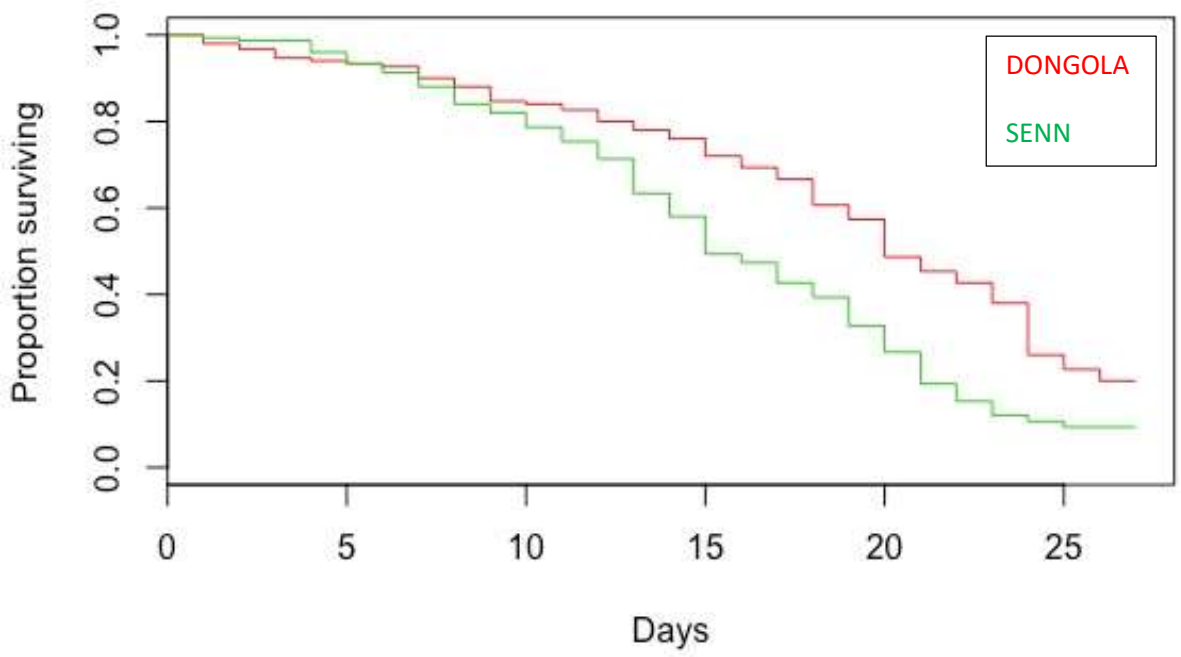


Figure 2.8: Cox proportional hazard regression models comparing adult longevity between dieldrin resistant SENN and susceptible DONGOLA females (N=150, likelihood ratio test: $p<0.0001$)

2.4 Discussion

The association between GABA mutations in the M2 transmembrane segment and dieldrin resistance has been well documented in several insect genera (Ffrench-Constant et al. 2000; Buckingham et al. 2005). The present study identified one strong QTL associated with dieldrin resistance in both the F2 and AIL crosses, but this was not associated with GABA; instead located 4Mb in the 5' direction on the same genomic contig: KB704125. It cannot be discounted that the deviation of the strong QTL signal from the *rdl* SNP site is an artefact caused by an inability to properly discriminate homozygous resistant (*Gaba*-296S/S) individuals from heterozygotes. Additional regions of interest could have been located within chromosome 3, however due to a limited number of markers coverage was low thereby increasing the odds of not detecting a QTL. Low coverage on this chromosome could be associated with the presence of inversions which are unknown in SENN.

Based on annotation of the genes within the QTL region, most code for protein-binding, ion-binding, or DNA/RNA binding, however a few of the genes have been previously highlighted in resistance candidate identification studies (**Table 2.5**). From microarray expression data from VectorBase, there are two genes within the interval which have been identified as being significantly upregulated in insecticide resistant mosquitoes, the 2 most likely candidates being Cuticle Protein 144 (CPR144) and ABC Transporter (ABCB-4). Although cuticular proteins have been implicated with resistance, CPR144 which is orthologous to *Drosophila melanogaster* Cpr73D has never been associated with resistance. Conversely, alleles in the ABCB family have been associated with increased insecticide transport out of cells or with resistance to xenobiotics and insecticides (Dermauw 2014). In *Culex pipiens*, ABCB activity was experimentally inhibited with verapamil resulting in an increased toxicity of endosulfan to a previously resistant strain (Buss 2002). Similarly, a splice variant of an ABCB gene 'multi-drug resistant 49 (MDR49)' was isolated from and incriminated in providing some protection from DDT in *D. melanogaster* (Seong 2016). Upregulated ABC transporters have been identified in previous transcriptomic studies of insecticide resistance in anophelines (Jones 2012, Tene 2013). Although ABC transporters are often detected in transcriptomic studies, they are often dismissed due to their low levels of upregulation. However, as seen in *Drosophila*, mutations or splice variants may alter functionality of ABC transporters allowing for increased excretion of insecticides, many of which are organochlorines. Low levels of DDT resistance in SENN have been previously detected in our laboratory (data not shown) without any associated glutathione-S transferase activity or *kdr* resistance ever detected. Reduced susceptibility to DDT and increased excretion of dieldrin therefore could be provided by ABCB-4 that, due to its location near GABA, may be overlooked in previous studies.

Table 2.5: Annotated list of genes found, and their purported function, within the Bayes credible interval from the AgamP4 building in VectorBase. Genes in bold are those that have been found to be upregulated in insecticide resistant populations in the wild, italicized genes are those that were found to be downregulated, and all others were found to have non-significant differentiation or with contradictory results relating to their regulation in the Expression Data from VectorBase.

Gene	Name	function
AGAP006327	*Leucine-rich immune protein LRIM6*	*protein binding*
AGAP006328	*Heparin sulfate N-deacetylase/N-sulfotransferase NDST2*	*sulfotransferase/hydrolase activity*
AGAP006330	Ccdc109a	calcium channel activity
AGAP006340	*NIK related kinase*	*protein kinase activity*
AGAP006342	Peptidoglycan recognition protein PGRPS3	zinc ion binding
AGAP006343	*Peptidoglycan recognition protein PGRPS2*	*zinc ion binding*
AGAP006344	RAG1 activating protein	carbohydrate transport
AGAP006346	Spinster homolog 2	transmembrane transport
AGAP006347	K+ voltage gated channel KQT-like	ion channel activity
AGAP006348	*Leucine-rich immune protein LRIM1*	*protein binding*
AGAP006349	Solute carrier family19	transmembrane transport
AGAP006353	Histidine triad nucleotide binding protein	catalytic activity
AGAP006354	*Rab-like protein 3*	*GTP binding*
AGAP006355	*Transcription initiation factor TFIIE subunit alpha*	*sequence-specific DNA binding*
AGAP006356	Proline rich protein	
AGAP006357	WD repeat-containing protein 42A	protein binding
AGAP006359	WD repeat-containing protein 92	protein binding
AGAP006360	Glutamate synthase	ammonia assimilation
AGAP006361	Myosin light chain 5	calcium ion binding
AGAP006363	**Vitamin K dependent gamma carboxylase**	**glutanyl carboxylase**
AGAP006364	**ATP-binding cassette transporter ABCB4**	**transmembrane transport**
AGAP006365	**RNA binding protein 8A**	**nucleotide binding**
AGAP006366	*2-oxoglutarate dehydrogenase E1*	*oxidoreductase activity*
AGAP006368	Odorant binding protein 70	olfaction
AGAP006369	**Cuticular protein RR-2 family 144 (CPR144)**	**cuticular protein**
AGAP006371	**Amyrel**	**catalytic activity**
AGAP006372	U3-IMP3	RNA binding

In *An. gambiae*, dieldrin resistance in both laboratory colonies and wild populations has been strongly correlated with the *Gaba*-296G mutation (Du et al. 2005; Kwiatkowska et al. 2014; Taylor-Wells et al. 2015). In other anophelines, including *An. arabiensis*, resistance is related to the alternate *Gaba*-296S mutation (Du et al. 2005; Wondji et al. 2011; Kwiatkowska et al. 2014; Platt et al. 2015) which shows a high affinity for resistance to many GABA-antagonistic compounds (Buckingham et al. 2005). Most studies have found dieldrin resistance in anophelines

to be monofactorial (Davidson 1956; Davidson and Hamon 1962; Haridi 1974) and later confirmed it to be associated with *Gaba*-296 SNPs (Du et al. 2005). Monofactorial resistance was seen in this study, however the association with *Gaba*-296S was not definitive. In this instance, resolution was fine enough to differentiate between the known *rdl* allele and a region 4Mb further downstream in conferring resistance. Similarly, resistance to dieldrin and fipronil in two laboratory populations of *An. gambiae,* both which possess *Gaba*-296G, was strongly linked to 2L+/+a heterozygotes and found to be located near the inversion breakpoint as seen in this study (Brooke et al. 2000). Although the position of *rdl* is also near the 2L breakpoint, it is possible that dominant dieldrin resistance may be conferred by another tightly linked allele acting in concordance with *Gaba*-296S. In *Ae. aegpyti* resistance to pyrethroids was not exclusively related to the presence of two *kdr* alleles alone but was due to the upregulation of several metabolic detoxification mechanisms including ABCB4 (Bariami et al 2012). The additive effect of a detoxification mechanism could explain the high levels of resistance seen in *Gaba*-296S heterozygotes. Validation by qRT-PCR could have been used to validate the QTL, unfortunately due to a lack of availability of equipment this step was not done.

The varying levels of dieldrin resistance in different anopheline species affects discriminating heterozygotes effectively by bioassay alone. *An. gambiae* s.l. exposed to 0.4% and 4% dieldrin resulted in similar mortality rates in F2 hybrids and homozygous resistant individuals (Davidson and Hamon 1962; Emeka-Ejiofor, Curtis, and Davidson 1983). Similarly, in this study AIL offspring genotyped as *Gaba*-296S homozygous or *Gaba*-296A homozygous were found to be resistant (n=103, 94.5%) or susceptible (n=154, 96.3%) respectively while most heterozygous individuals survived a 1-hour exposure at 4% (n=245, 82.2%) compared to those classified as susceptible (n=53, 17.8%) (**Figure 2.9**). Davidson (1956) and Hamon (1962) showed that dieldrin resistance in *An. gambiae s.l.* can be either semi-dominant or dominant. It could be hypothesized that *Gaba*-296S in *An. arabiensis* is the dominant form while *Gaba*-296G in *An. gambiae* is semi-dominant. Another potential explanation for the larger than anticipated number of heterozygotes scored as resistant may be caused by an inadequate exposure period. Rowland (Rowland 2001) found that 2 hour exposures were necessary to discriminate heterozygotes from homozygous dieldrin resistant individuals in *An. stephensi* and *An. gambiae*. However, even a 2 hour exposure has been reported as inadequate to separate heterozygotes in *An. arabiensis* (Emeka-Ejiofor, Curtis, and Davidson 1983). Expression of insecticide resistance can also be influenced by the

process of selection and colonization. It is possible that during colonization, alleles enhancing fitness, both for survival in the laboratory and for exposure to insecticides, were selected in tandem with resistance alleles allowing for a more robust expression of resistance than what would have normally been seen in the wild (Bourguet, Genissel, and Raymond 2000). An alternative is that dieldrin resistance in heterozygous *An. arabiensis* adults may be higher than in *An. gambiae* heterozygotes (Kwiatkowska et al. 2014). Therefore, these differences may be attributable to the nature of the *Gaba*-296S mutation or the presence of another mutation which may be providing an additive effect on fitness or resistance.

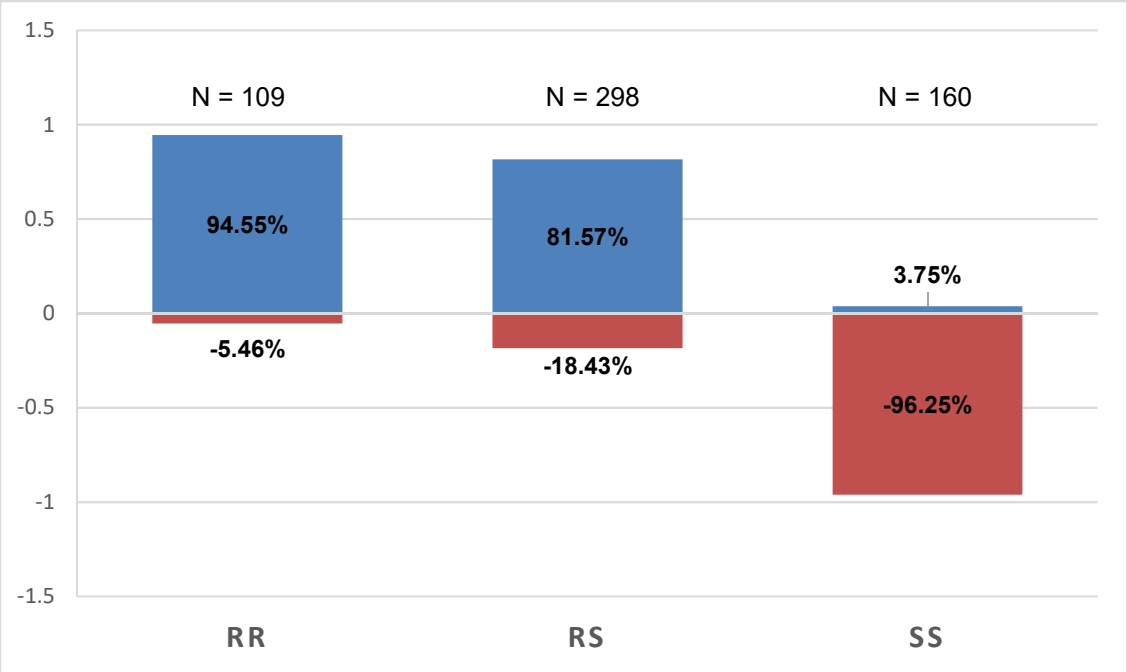


Figure 2.9: Percent of genotyped AIL individuals possessing the *Gaba*-296S (RR) and *Gaba*-296A (SS) SNP that survived a 1-hour exposure to 4% dieldrin.

In this study, very few consistent detrimental fitness costs were found to be associated with *Gaba*-296S resistance in the SENN colony. Indeed, SENN was found to pupate more quickly and was more likely to survive both through pupation and eclosion compared to the susceptible DONGOLA colony. Conversely, adult SENN females, though not males, were significantly shorter-lived than in the DONGOLA colony. Previous studies looking at *rdl* mutations and fitness-correlates provided differing results. Rowland (Rowland 2001) found no difference in larval growth or

eclosion rates between resistant and susceptible colonies of *An. gambiae* and *An. stephensi* however resistant females produced fewer eggs and were less active compared to their susceptible counterparts. These discrepancies may be because there are 2 mutations circulating in the wild, *Gaba*-296S and *Gaba*-296G, and studies were performed on both alleles. Between the resistant forms, *Gaba*-296G has been reported in fewer anophelines than *Gaba*-296S (Wondji et al. 2011; Asih et al. 2012; Kwiatkowska et al. 2014; Platt et al. 2015) but most fitness studies have been performed on species with *Gaba*-296G. However, potential fitness costs resulting in reduced fecundity or temperature stress were not tested in this study but have been shown to be affected by *rdl* in other taxa (ffrench-Constant et al. 1993b, Rowland 2001). Additionally, as these strains have been colonized for some time, it is possible that the effects reported are artefacts positively selected for during the colonization process and not indicative of what is seen in nature.

Traditionally, the appearance and spread of resistance is expected to be correlated initially with a loss of fitness due to alterations in the normal function of the target gene. Instead fitness effects associated with resistance mechanisms may disappear under constant insecticide exposure as seen in the laboratory possibly by the loss of deleterious alleles during inbreeding (Roush and McKenzie 1987; Carriere et al. 1994). The lack of additional QTLs associating with *Gaba*-296S in this study, therefore, may be due to an inability to detect them due to the scarcity of informative markers in certain regions. Additionally, it could be that compensatory genes have low, but combined effects which are not significant alone but together ameliorate fitness costs. Finally, as in *D. melanogaster* (Remnant et al. 2013), there may be few fitness costs associated with *Gaba*-296S.

Persistence of dieldrin resistance in African anophelines without obvious selection remains a mystery but may be caused by one or more intrinsic or extrinsic factors. Although dieldrin and many other organochlorine insecticides have not been used for malaria control in decades, many GABA antagonists such as endosulfan have been used in agricultural settings until recently (Wandiga 2001; Williamson, Ball, and Pretty 2008). Cross resistance to one of these compounds, fipronil, has been correlated with *rdl* mutations in *Drosophila* and *An. stephensi* and was confirmed in *An. arabiensis Gaba*-296S in this study (Hosie et al. 1995; Kolaczinski and Curtis 2001) while this is the first report of endosulfan resistance, another known GABA antagonist, in *An. arabiensis*. In *An. funestus*, it was hypothesized that agrochemical usage was most likely behind the persistence of resistance in nature (Wondji et al. 2011). Additionally, in West Africa, barrier

spraying with fipronil has also been used regularly for the control of locust plagues (Pesticide Referee Group 1997). Due to the persistent nature of many organochlorines they can still be found in measurable amounts in soil and water across Africa (Wandiga 2001; Ogunfowokan et al. 2012). Therefore, maintenance of resistance may be occurring due to persistent contact with GABA antagonistic compounds associated with current agricultural practices, however the varying levels of insecticide may not be enough to maintain resistance at appreciable levels.

Stability may also be related to the nature of the receptor itself. As the *Gaba*-296S mutation occurs in GABA receptors between neurons, the loss of functionality may not be as detrimental due to redundancies in signaling within an organism (Raymond and Sattelle 2002). The presence of non-target alternative splice variants of GABA and hetero-oligomers containing portions of the GABA allele without *Gaba*-296S would allow for normal signaling even if single GABA channels lost normal function due to the *Gaba*-296S mutation (Buckingham et al. 2005). This would allow for persistence of resistance in the absence of insecticide pressure even if the mutation was detrimental to the functionality of the channel. Instead, the population would plateau at a low level with a few individuals in a state of heterozygosity allowing for resistance to re-emerge quickly under renewed pressure (Keiding 1967).

Although modern technologies such as GWAS and microarray analyses have been used to detect potential resistance targets, they are often hindered by low levels of linkage disequilibrium or high levels of population sub-structuring making these economically unfeasible (Weetman et al. 2010). QTL mapping offers genome-wide coverage as well but at a lower cost and has been used extensively to identify targets associated with phenotypes such as insecticide resistance especially in laboratory bred populations (Wondji et al. 2007; Stinchcombe and Hoekstra 2008; Witzig et al. 2013). The laborious nature of locating and validating markers for a new species meant that this may initially take longer to perform, ultimately the overall cost is still lower than GWAS plus the creation of several *An. arabiensis* specific markers should be found useful as research into this vector expands.

Even though cyclodienes are no longer used in malaria control programmes, the persistence of the *Gaba*-296S mutation across West Africa and its noted cross resistance to other antagonistic GABA compounds could be costly if implemented without *a priori* knowledge of the presence of the resistance mutation in local populations. Therefore, the development of new compounds or the

employment of repurposed or reformulated insecticides to overcome current resistance issues must be carefully approached else failures may occur.

Chapter 3

Investigating the persistence of the *Gaba*-296S mutation in *Anopheles arabiensis* in the Sudano-Sahelian domain in the apparent absence of selection.

3.1 Introduction

Insecticide resistance alleles in absence of selective pressure, are often accompanied by a reduction in overall fitness, particularly when in a homozygous state (Carriere et al. 1994; Labbe et al. 2007; Hardstone, Lazzaro, and Scott 2009; Martins et al. 2012). While under insecticidal pressure selection for resistance may favour the mutant allele but when the insecticide is withdrawn or absent from an environment the relative importance of the deleterious fitness effects will increase and the allele should decrease in frequency and eventually disappear (Crow 1957; Melo-Santos et al. 2010). Modern insecticide resistance management is based on the concept that to mitigate resistance due to the continued exposure to a single compound, changes in insecticides employed should be changed in response to local resistance patterns (WHO 2012). However, there are instances in which resistance alleles have persisted in nature in the apparent absence of any selective pressure (Keiding 1967; Aronstein, Ode, and Ffrench-Constant 1994, 1995). Persistence of resistance could result from either a lack of pleiotropic effects (costs) associated with the resistance allele, the concomitant selection of balancing/compensatory alleles to mitigate any negative fitness effects, or the replacement of a more detrimental resistant allele with a less costly one (Eritja and Chevillon 1999; Arnaud et al. 2002). In malaria mosquitoes, persistence may also be associated with environmental pressures such as exposure pesticide residues in the soil or water (Lahr et al. 2000; Akogbeto, Djouaka, and Kinde-Gazard 2006) or inadvertent, secondary exposures to pesticides not used in malaria control programmes but resulting in cross resistance and maintenance of the resistance allele (Eritja and Chevillon 1999; Rodriguez et al. 2002).

Of unique interest is the persistence of *rdl* mutations within the γ-aminobutyric acid (GABA) receptor subunit conferring resistance to cyclodienes and phenylpyrazoles (Ffrench-Constant et al. 2000; Buckingham et al. 2005). Malaria control for most of the early 20th century relied on DDT, however during the 1950's insecticides that were equally long-lasting and effective, namely dieldrin and lindane (hexachlorocyclohexane), were employed (Bruce-Chwatt 1984). Although not widely used during initial trials in eight African nations, resistance to these compounds emerged within 2-3 years (Hamon & Garrett-Jones 1963). This coupled with issues of high human toxicity, environmental persistence, and the cost to employ them led to their quick withdrawal (Kouznetsov 1977). Cyclodienes have not been employed for over 40 years in a public health setting, however in sub-Saharan Africa, as well as globally, single-nucleotide polymorphisms (SNPs) associated with dieldrin resistance are still detected in various species (**Table 3.1**) (Aronstein, Ode, and Ffrench-Constant 1994; Hansen, Kristensen, and Jensen 2005; Wondji et al. 2011; Asih et al. 2012; Kwiatkowska et al. 2013; Platt et al. 2015). Although dieldrin resistance typically results from a single SNP at the *Gaba*-302 position within the M2 transmembrane segment in most insect genera (Ffrench-Constant et al. 2000), in *An. gambiae* s.l., resistance is conferred by one of two *Gaba*-296 SNPs, *Gaba*-296G in *An. gambiae* s.s. and *Gaba*-296S in *An. arabiensis* and *An. coluzzii* (Du et al. 2005, Lawniczak et al 2010) (**Figure 3.1**). Additional mutations in the M2 and M3 transmembrane segments have been found to offer some additive protection against phenylpyrazoles in *Drosophila simulans* and *Nilaparvata lugens*, typically in tandem with *Gaba*-302 (Le Goff et al. 2005; Taylor-Wells et al. 2015; Zhang et al. 2016).

Adaptations to environmental pressures, such as insecticide exposure, can result from rare mutations within populations becoming more prominent (selection from standing genetic variation) and/or the appearance of new alleles which confer protection (de novo mutation). As the resistant alleles increases in frequency in response to insecticide pressure, there is an accompanying reduction in observed genetic polymorphisms around the locus in the population termed a "selective sweep." The magnitude of region of reduced diversity is a function of the strength and recency of selection and the age of the mutation. These differences will gradually be eroded through recombination.

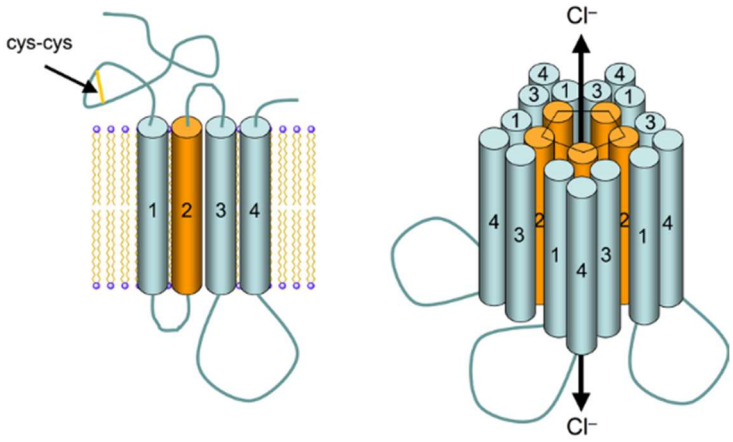
Table 3.1: The frequency of *rdl* as determined by PCR, in wild populations of various insects.

		Genotype frequency			
Species	**number**	**RR**	**RS**	**SS**	**Reference**
Blatella germanica	38	0.97	0.03	0.00	Hansen et al. 2003
	38	0.16	0.45	0.39	
Ctenocephalides felis	113	0.49	0.40	0.11	Bass et al. 2004
	30	0.30	0.50	0.20	
Culex quinquefasciatus	nd	0.02	0.15	0.83	Corbel et al. 2007
	nd	0.13	0.28	0.59	
	nd	0.01	0.28	0.71	
	nd	0.01	0.63	0.36	
Anopheles coluzzii	47	0.70	0.06	0.24	
	45	0.69	0.03	0.28	
Anopheles coluzzii	94	0.78	0.19	0.03	Kwiatkowska et al. 2013
Anopheles gambiae	nd	0.33	0.38	0.29	Mahande et al. 2012*
Anopheles gambiae	33	0.15	0.55	0.3	Koekemoer et al. 2010
Anopheles funestus	38	0.63	0.32	0.05	Riveron et al. 2014
	40	0.07	0.05	0.88	
Anopheles arabiensis	90	0.01	0.41	0.58	Sudan (this study)
	83	0.17	0.47	0.35	Senegal (this study)

* This is a 14-month average for rdl

nd = no data given

Figure 3.1. Diagram of the GABA protein including the four major transmembrane segments M1-M4. The *Gaba*-296S mutation is found in the M2 segment which lines the central chloride channel in the assembled homomer. Image courtesy of Wikiwand.



However, identifying these selective sweep patterns is often difficult due to the initial scarcity of the resistance allele in the population and the potential for recombination events, particularly when selecting from standing genetic variation, to occur obscuring linkage disequilibrium (LD) associated with the mutant allele as it increases in frequency in a population. Compounding these issues in accurate detection in *An. arabiensis* include a large population size over most parts of Africa (Donnelly et al. 1999; Marsden et al. 2014), low genetic diversity at the regional level (Donnelly, Licht, and Lehmann 2001; Donnelly et al. 2004), and short background linkage disequilibrium (Neafsey et al. 2010; Marsden et al. 2014). Several contemporary selective sweeps associated with insecticide resistance have been reported in *Anopheles gambiae* (Lynd et al. 2010; Jones et al. 2012; Mitchell et al. 2012; Weetman et al. 2015). To date, however, no study has examined selective sweeps associated with resistance that arose 50 years prior. Additionally, as this allele is found across West Africa, it is possible to ascertain if it has had a single or multiple origin as seen in *kdr* (Diabate et al. 2004, Kawada et al. 2011, Ochomo et al. 2015, *Anopheles* 1000 Genomes Consortium 2017). The unique persistence and stability of the *Gaba*-296S allele in the absence of obvious, purposeful selection allows for such.

3.1.1 Objectives of this study

As the potential need to employ alternative insecticides due to the increase in resistance to many prominent, WHOPES approved insecticides for controlling malaria in Africa, it is important to discern if SNPs associated with resistance are present in native populations prior to the introduction of a new compound. Although cyclodienes are not currently approved for use in IRS programmes in Africa, there have been efforts to develop pesticides that target GABA channels of late (Bloomquist 2003; Casida and Durkin 2015). The persistence of *Gaba*-296S in West Africa, as well as other parts of the world, could be worrisome if cross-resistance was seen to any novel GABAergic compounds developed. This study aims to determine why *Gaba*-296S in *An. arabiensis* persists in this region, even in the apparent absence of selection, by determining if this is the result of parallel evolution due to recent, non-specific pressures (i.e. agricultural exposure) or if it has remained over time either due to a stabilizing selection or potential exposure to persistent cyclodienes or other GABAergic compounds in larval habitats.

1. As it was recently shown that *kdr* has arisen multiple times in *An. gambiae* (*Anopheles* 1000 Genomes Consortium 2017), it is possible that multiple origins of *Gaba*-296S in *An. arabiensis* could have occurred in response to localized insecticide pressure as organochlorines were not used extensively for malaria control. Therefore, the first part will be to determine if the same mutation found across the extremes of this species range in West Africa.
2. Unlike *Vgsc* which is centromeric and *Ace*1 which is in a freely recombining autosomal region, *Gaba*-296S is uniquely positioned adjacent to and within the 2La inversion and thus may experience different levels of LD decay due to its location. If the same mutation is found across West Africa, then the second phase will estimate extended haplotype homozygosity (EHH) based on decay in LD around *Gaba* in wild-type and resistant individuals to determine how recent the sweep event occurred and if there was a single origin.

3.2 Methods

Mosquitoes used in this study were from wild populations from Senegal and Sudan and from a long-established resistant colony of Sudanese origin: MRA-764 SENN which is homozygous for *Gaba*-296S (available at www.beiresources.org). Additional limited sequence data for *An. arabiensis* and *An. coluzzii* collected in Burkina Faso were also available for genomic comparisons (Crawford et al. 2015).

Wild-caught individuals used in this study were obtained from sites where the *Gaba*-296S allele is present but not fixed. Whole genomic DNA from mosquitoes was provided from collections made in the Sudan originating from 4 cities: New Halfa (Kassala State), Galabat (Gadaref State) El Hoosh and Hag Abdallah (South Gezira State), as part of a national programme to determine baseline resistance levels in local vector populations (**Figure 3.2**). Whole genomic DNA aliquots for 96 individuals from Sudan were graciously provided by Hmooda Kafy and Bashir Adam (Federal Institute of Health, Sudan) and kept at -20°C.

For Senegal, wild individuals were sub-sampled from routine collections made by staff from the Cheikh Anta Diop University in 2015 around Rufisque which is the eastern-most and most rural of the 4 local government departments around Dakar (**Figure 3.3**). WHO

insecticide bioassays were conducted on 2-3-day old adults to determine their susceptibility to 4.0% dieldrin for one hour. Individuals were then scored as resistant or susceptible 24-hours post exposure. DNA from phenotyped specimens was extracted using a Quanta Extracta DNA Prep kit (Quanta Bio, cat no. 95091) according to manufacturer's instructions and kept at -20°C.

Discrimination of homozygous *Gaba*-296S individuals for the Sudanese samples was accomplished using a previously published TaqMan™ assay (Bass et al. 2010). The Senegalese samples were genotyped using a modified allele-specific PCR assay designed to detect the *Gaba*-296S mutation specifically in *An. arabiensis* (Wilkins, Howell, and Benedict 2006). As this assay was previously found to be not specific for detecting heterozygotes accurately (see Chapter 2), a new reverse primer (RDLRv2: GTG ACA ACG GAT TAC TTA GAG C) was designed which facilitated discrimination, with the susceptible allele producing a smaller (139 bp) amplicon than the resistant allele (242 bp) (**Figure 3.4**). To ensure correct genotyping in the Senegalese samples, 48 individuals previously genotyped by TaqMan were re-tested with the allele-specific PCR. Concordance in the results were seen in all 48 including 15 heterozygotes (data not shown).

The *An. arabiensis* reference sequence for the GABA gene, which is known to be susceptible to dieldrin, was obtained from Vectorbase (www.vectorbase.org) and localized on contig KB704125. From this, a portion of the GABA allele was chosen which contained the *Gaba*-296 site, which resides in exon 7, for genotyping. Several overlapping oligonucleotides were used in the sequencing of this 4,210 bp region (**Table 3.2**). PCR was performed with 0.5 µl of DNA template using Accustart II Supermix (Quanta Bio, cat no. 95137) with the following cycling conditions: 94°C -3m [94°C-30s, 58°C-30s, 72°C-90s] x 35- 72°C-10m. PCR products were visualized using ethidium bromide in a 1.5% agarose gel and then cleaned with ExoSAP-IT (Affymetrix cat no. 78200) according to manufacturer's instructions, with 3 µl of cleaned PCR product diluted in 10 µl of distilled water. Cycle sequencing was performed on 1µl of the diluted product with BigDye Terminator (Thermo Fisher cat no. 4337457) and then cleaned using BigDye XTerminator Purification kit (Thermo Fisher cat no. 4376486). All sequencing was performed on a Hitachi™ ABI 3530 Genetic Analyzer.

Figure 3.2: Map of the northeast area of Sudan bordering Egypt and Ethiopia (A). Collection sites are marked with yellow triangles and the capitol, Khartoum, is marked with a red star. http://www.mdpi.com/2076-3298/4/1/15 (accessed 12/2017). The region of interest is highlighted in the country map below in a red (B).

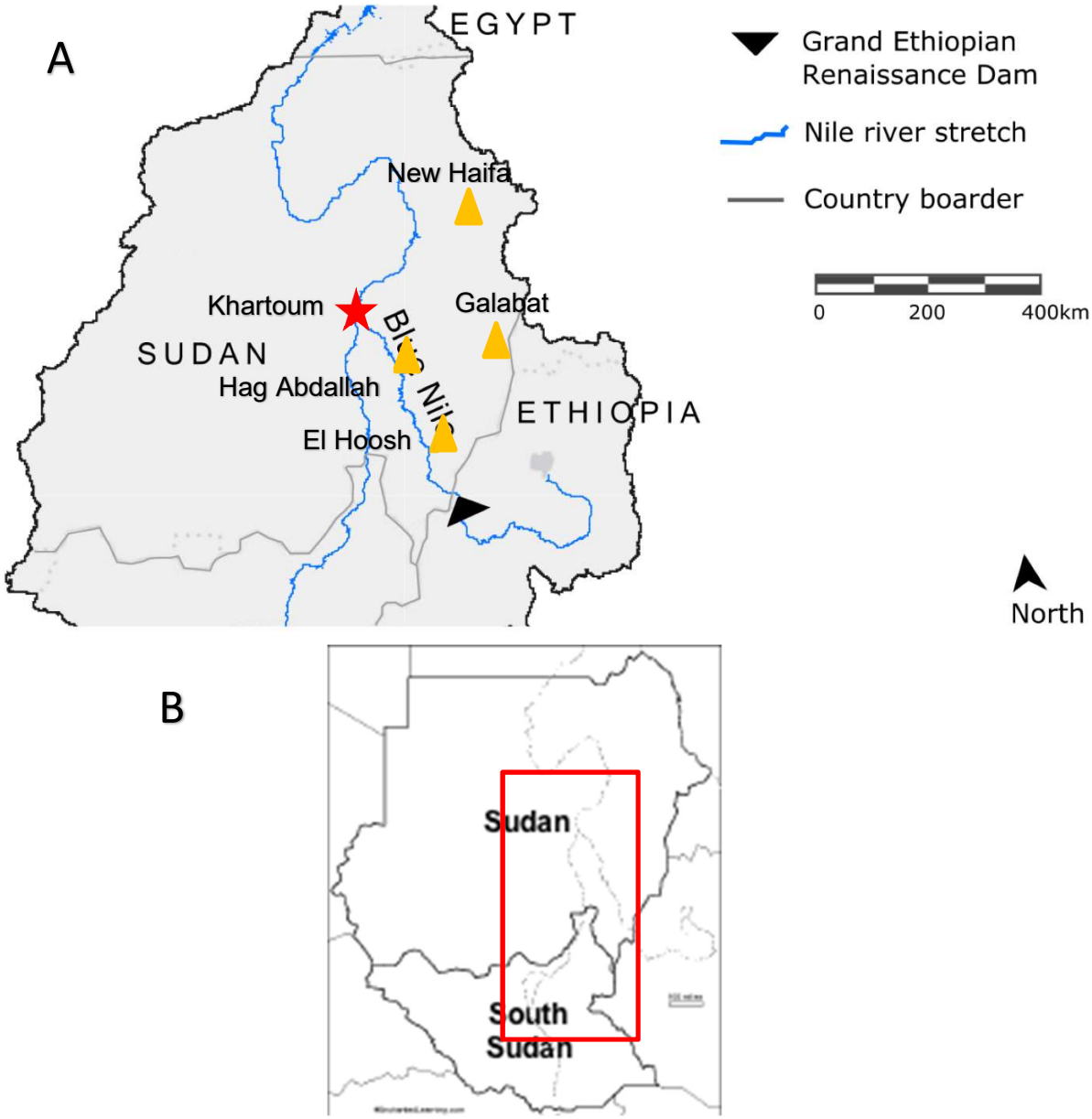


Figure 3.3: The Cap Vert peninsula showing the location of the collection of the adults used in this study. The location of the collection sites is approximated by the triangle.

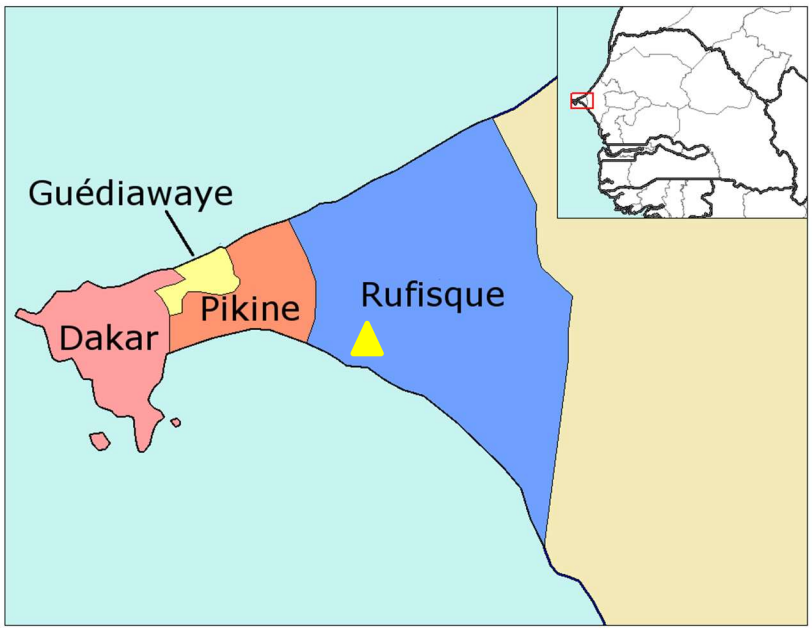
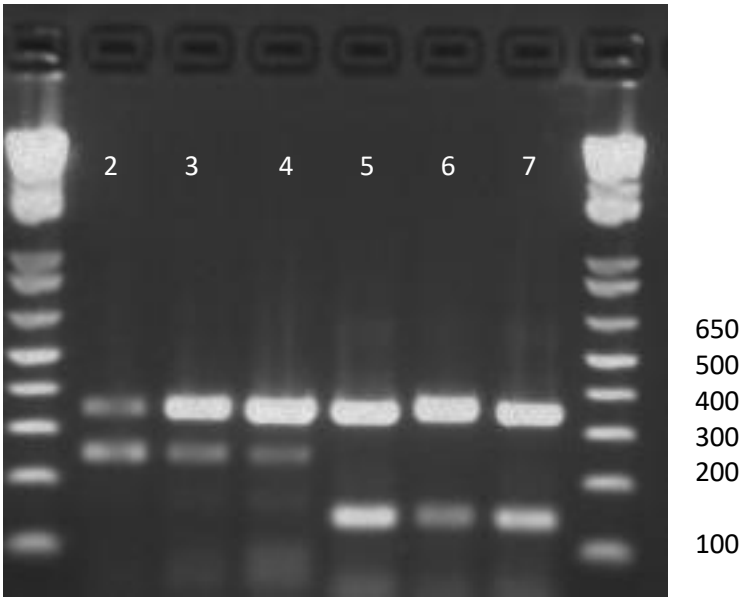


Table 3.2: Oligonucleotides used to sequence a portion of the GABA allele including exon 7 which contains the *Gaba*-296 mutation site.

Pair name	Primer name	Sequence
A	1830F	CAA AGG TGG TTA GAG AAG TAG
	2920R	ACC CAT CGT GAA ACC ACC AC
B	P2V3F	GAG CTA GAA CTA ACA CGA CAA G
	3430R	CTT CCT AAC GCG ATG G
C	3300F	TCT GCG TGT AAT TTC GGC AG
	4370R	CCA AAC GTT TCC TTT TTC TTG ATA C
D	P3V3F	CGT CCT TGC ACT CTG GC
	5290R	GTT CCA ACC AAT TTC TTC TGA TA
E	4930F	AAG AAA GCA GAC AGT AGG AA
	EX7R	CTC ATT CAA ATC TAT ATA CCA TCC GG
F	EX7F	CCA GCA GAC TGG CAA ATA CCA
	1000R	TCT TTT TAT CTT ACT AGC CCT TAA GCC

As phenotype/genotype data was collected, an allelic association test was performed on the Senegalese samples using a Fisher's exact test. Next, sequences were initially assembled and aligned using BioEdit (Hall 1999) to create a single consensus sequence for each sample before exporting to MEGA for molecular evolutionary analyses (Tamura et al. 2013). Parsimony and neighbor-joining tree analyses were performed in MEGA7 (Kumar et al. 2016). A neighbor-joining tree was inferred using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. Last, a haplotype genealogy was estimated used HapView (Barrett et al. 2005). There was a total of 623 positions in the final dataset centered around exon 7. Nucleotide diversity, Tajima's D and Fu and Li's D and F statistics were estimated using DnaSPv5 employing a sliding window length of 100 bases with a step size of 25 (Librado and Rozas 2009). Last, extended haplotype homozygosity (EHH) analysis was estimated in SWEEP and REHH (Gautier et al. 2017) to determine the patterns of LD decay between wild-type and *Gaba*-296S alleles together (Sabeti et al. 2002). EHH analysis is more robust in this scenario due to small sample sizes and problems with Hardy-Weinberg equilibrium in the Sudan samples (χ^2 = 4.018, $P < 0.05$) (Lynd et al. 2010). EHH values were estimated across all SNPs centered on the core *Gaba*-296 SNP for both wild-type and resistant separately and together.

Figure 3.4. Polyacrylamide gel image showing the resulting products of a modified PCR assay for discriminating between wild type *Gaba*-296A (lanes 2-4) and resistant *Gaba*-296S (lanes 5-7) in *An. arabiensis*. Lanes 1 and 8 contain a 100bp ladder.

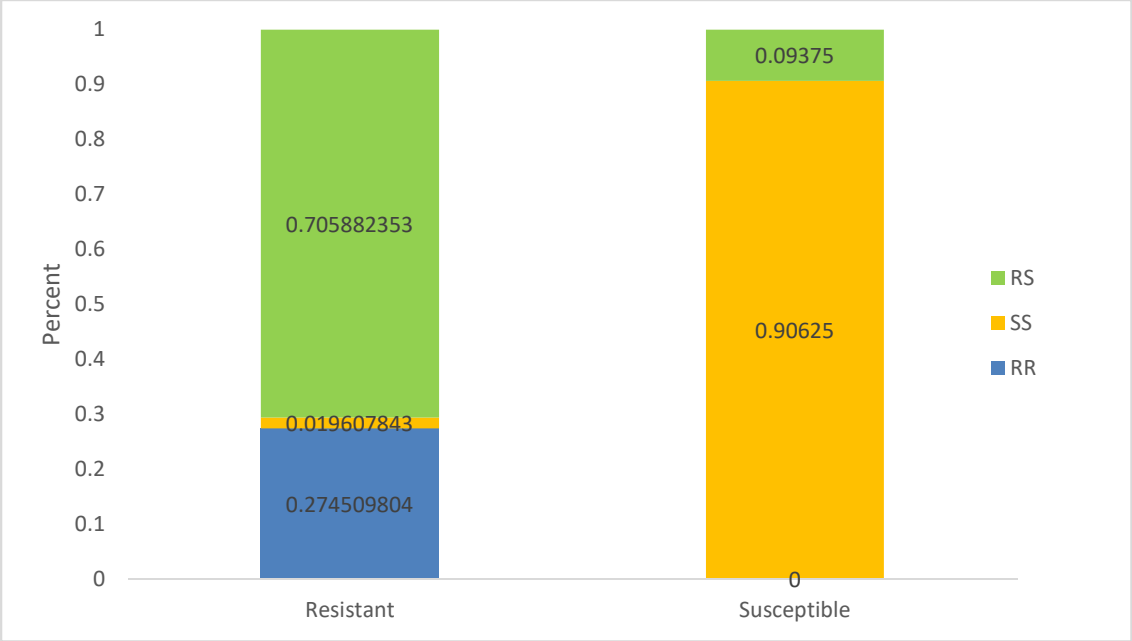


3.3 Results

Employing either the TaqMan™ assay or the modified single-step PCR, a total of 178 individuals were tested to determine their *Gaba*-296 genotype. Of 96 non-phenotyped genomic DNA samples from the Sudan tested using the TaqMan™ assay, 1 was homozygous for *Gaba*-296S, 37 were heterozygous, 52 were homozygous *Gaba*-296A (susceptible), and 1 failed to amplify any product. In the Senegalese collection, a total of 83 previously exposed adult mosquitoes were genotyped. Of the 51 individuals determined to be resistant by exposure to dieldrin, 14 were homozygous for *Gaba*-296S, 36 were heterozygous and 1 was homozygous *Gaba*-296A. Those that were scored as susceptible mainly possessed the wild-type *Gaba*-296A (90.6%, n=29) with only 3 heterozygotes and none homozygous for *Gaba*-296S ($P < 0.0001$) (**Figure 3.5**). Therefore, a significant association of *Gaba*-296S with dieldrin resistance is seen like populations from the Republic of the Congo (Koekemoer et al. 2011). Selected individuals were randomly

chosen from either homozygous resistant or homozygous susceptible for further sequencing and EHH analysis.

Figure 3.5: Percent of genotyped Senegalese samples that possess 1 or more copies of *Gaba*-296S based on their resistance to 4% dieldrin as determined by WHO bioassay.



Direct sequencing of the selected portion of the GABA allele was difficult due to the presence of several large tandem repeats interspersed within the intronic regions therefore a smaller 632 bp region including exon 7 was chosen for final analysis. In total, 24 screened samples were included for final phylogenetic analyses. For Tajima's neutrality test, π was estimated at 0.00733 ($P > 0.1$). When comparing the average number of segregating sites between the samples, Fu and Li's estimated a $D = 1.56213$ ($P < 0.02$) and $F = 1.26328$ ($P > 0.1$) with Tajima's $D = -0.0213$ ($P > 0.1$). Nucleotide diversity (π) in a population that has experienced a bottleneck is expected to be low while negative Tajima's D values typically indicate either population size expansion after a selective sweep or a purifying selection event. Based on both Tajima's D and π it can be presumed that an extensive bottleneck followed by a selective sweep is associated with *Gaba*-296S in *An. arabiensis* in West Africa. Evidence of a sweep is further bolstered when comparing SNP differences between resistant samples from Senegal, Sudan and Burkina Faso (**Table 3.3**) additionally supporting the hypothesis for a large interbreeding population in this region as proposed in

the first Chapter. Last, based on the neighbor-joining tree, a single homozygous haplotype associated with *Gaba*-296S is seen in all 3 West African populations (**Appendix Figure 5**), while there is some decay seen in susceptible individuals from Senegal and Sudan unique to each population.

EHH was estimated using REHH to be 0.7142857 (**Figure 3.6**), which is considered above the threshold for showing a definitive hard sweep (Walsh & Lynch 2018). The value, however, is indicative of a sweep event which may have occurred in the recent past and is becoming obscured by recombination.

Figure 3.6: REHH estimation for *rdl*, centered at position 154 on the X axis, in resistant (ancestral) and susceptible (derived) populations from across West Africa.

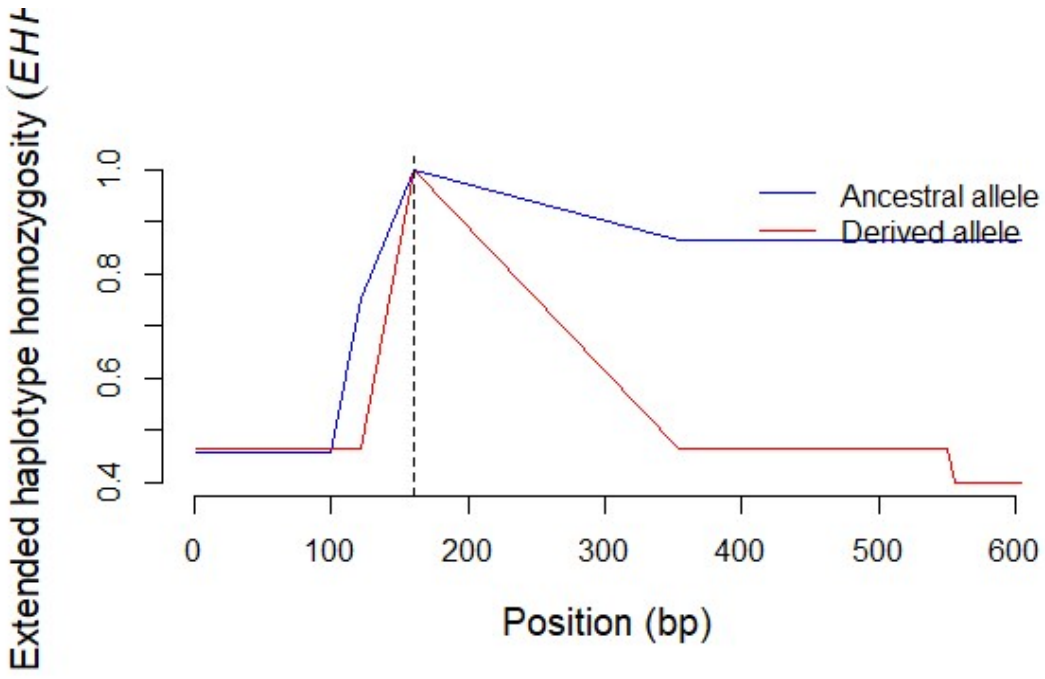


Table 3.3: Position of SNPs within the intronic region surrounding exon 7 in the GABA gene containing the *rdl* locus (in bold). Positioning is based on numbering of the 2L in the PEST genome available from VectorBase.

	Sequence position on the 2L																					
	2542923	2542943	2542945	2542946	2542947	2542948	2542950	2542951	2542953	2542953	2542954	2542954	2542962	2542962	2542963	2542964	2542965	2542965	2542967	2542967	2542967	2542968
SENNref	T	A	C	T	T	T	C	C	A	T	C	C	T	A	T	C	C	C	C	A	C	
SudR1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
Sud91S	G	.	.	.	.	.	A	.	G	.	A	A	.	.	C	.	.	.	.	.	.	
Sud89S	G	.	.	.	.	.	.	.	.	.	.	.	.	.	C	T	A	.	.	.	.	
Sud95S	G	.	.	.	.	.	.	.	.	.	.	.	.	.	C	T	.	.	.	.	.	
Sud90S	G	.	.	.	.	.	A	.	G	.	A	A	.	.	C	.	A	.	.	.	.	
RufR1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
RufR2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
RufR3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
RufR4	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
Thies	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
RufS11	G	C	A	G	G	G	.	G	G	A	A	A	C	C	C	T	.	T	T	C	A	
RufS13	G	C	A	G	G	G	.	G	G	A	A	A	C	C	C	T	.	.	T	C	A	
RufS12	G	C	A	G	G	G	.	G	G	A	A	A	C	C	C	T	.	T	.	.	.	
RufS14	G	C	A	G	G	G	.	G	G	A	A	A	C	C	C	T	.	.	T	C	A	
BF1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
BF2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
BF3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
BF4	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	
BF5	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
BF6	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
BF7	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
BF8	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
BF9	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	

3.4 Discussion

The persistent maintenance of resistance alleles in a population is expected to be correlated with a loss of fitness, the often-reported severity of the fitness costs outweighs their lack of benefit when insecticidal pressure is released (Rowland 2001; Martins et al. 2012; Platt et al. 2015). However, it has been shown in the laboratory that fitness effects associated with resistance may disappear even under constant insecticide exposure due to the loss of deleterious alleles during inbreeding which could occur during bottlenecking (Roush and McKenzie 1987; Carriere et al. 1994). Conversely, in *D. melanogaster* it was hypothesized that the persistence of the mutation in the population, and the concomitant lack of fitness costs, was due to the natural occurrence of *rdl* prior to cyclodiene pressure (Thompson 1993). Similar results were reported in *Lucilia cuprina* in which malathion resistance was detected in populations collected before use of this compound in Australasia (Harley et al. 2006). In these instances, it is hypothesized that these species were preadapted at the selected loci and that the mutation did not potentially affect the function of the allele thereby having negligible fitness costs. Lastly, resistance mechanisms may not truly disappear but instead most likely revert to a low level of heterozygosity in the population which, once challenged again, can revert (Keiding 1963).

The primary goal of this study was to determine if the presence of *Gaba*-296S in *An. arabiensis* in West Africa was the result of historical or recent selection. Based on sequence data from 24 individuals from 3 countries, there is evidence for a "hard" sweep associated with *Gaba*-296S (**Figure 3.6**) like *Vgsc*-1014F in *An. gambiae* (Lynd et al. 2010, Jones et al. 2012). Decay around recent target-site mutations have been shown to be rapid in one or both directions (Hartley et al. 2006, Pinto et al. 2007, Jones et al. 2012) as seen here with rapid decay in the telomeric direction. This level of LD decay is like that reported by Marsden et al. (2014) occurring within 200bp in *An. arabiensis*. However, unlike in *Vgsc*, there were a limited number of haplotypes detected (**Appendix Figure 5**). This lack of haplotype detection is most likely due to sampling bias from a small population. Discerning multiple haplotypes, although, is possible even when examining less than 500 base pairs (Hartley et al. 2006, Pinto et al. 2007). When compared to Weetman et al. (2015) who found over 124 SNPs within an approximately 2000 bp region of *Ace*1, only 23 SNPs were

detected in the similarly sized region sequenced in this study. A recent study found rates of nucleotide diversity within the 2La inversion in *An. arabiensis* estimated at 0.00202 with SNPs approximately every 318bp (Marsden et al. 2014). Therefore, the lack of diversity within GABA may be indicative of positional protection from recombination occurring due to GABA's proximity to the 2La inversion breakpoint. There is evidence that proximity to chromosomal inversions reduce the amount of recombination to potentially protect positive epistatic allele combinations (Santos et al. 2009). Nucleotide diversity between Sudanese resistant and susceptible populations may have also been confounded by a lack of homozygous *Gaba*-296S individuals. By estimating Hardy-Weinberg equilibrium (HWE), it was found that the Sudan population was not in equilibrium ($\chi^2 = 4.0118$, $P < 0.05$) while the Senegal population was ($\chi^2 = 0.047$, $P > 0.05$). The lack of HWE in Sudan may be due to either the aforementioned sampling bias or possibly gene duplication (Hosking et al. 2004, Plengvidhya et al. 2015). Weetman et al. (2015) determined that copy-number variants (CNVs) were responsible for deviations in HWE in *An. gambiae* as evidenced by an excess of heterozygotes for *Ace1*-119S. This is further potentially supported by data presented in Chapter 2 showing both a lack of fitness effects associated with *Gaba*-296S and an excess of heterozygous, resistant individuals. Research with several genera have shown that fitness costs associated with resistance alleles can be ameliorated via gene duplication (Remnant et al. 2013, Assogba et al. 2015).

As this mutation assumedly arose over 50 years ago, it appears that either there is either positional protection from recombination limiting LD decay or *Gaba*-296S has arisen recently due to agricultural pressures and swept across the entirety of the Sahel. From the limited amount of data gathered in this phase, determining either is likely not possible. To determine which scenario is most likely, a larger region would need to be examined as in *Ace*-1 (Weetman et al 2014). Additionally, samples of *An. arabiensis* from several other parts of the Sahel would help to estimate if this was a single event or if multiple origins have occurred. Further research should also be done to confirm the presence of CNVs of *Gaba*-296S in *An. arabiensis* from the Sudan. This would provide additional information as to whether the allele carries any fitness costs.

Although the detection of a resistance mechanism resulting from long-retired compound may not seem initially worrisome, evidence has shown that these same mechanisms may proffer resistance to other compounds being considered for current malaria control interventions (Kolaczinski and Curtis 2001; Taylor-Wells et al. 2015). The detection of resistance alleles affected by CNV and their role in maintaining resistance in populations should be prioritized as their presence could further hinder future efforts. Arthropod GABA is an attractive target as it is dissimilar to vertebrate GABA receptors affording selective toxicity (Casida et al 2015). The rise and spread of insecticide resistance across sub-Saharan Africa to the limited arsenal of pesticides approved for public health use may result in the deployment of repurposed or novel GABAergic compounds which could interact with GABA receptors (Turner et al 2016). Therefore, research into causes of persistence of resistance alleles is important, not only to catalogue the current distribution of these, but to allow for improved deployment of future insecticides.

Chapter 4

Quantitative trait loci and whole genome microarray analysis of bendiocarb resistance in *Anopheles arabiensis* associated with peri-urban agriculture in Rufisque, Dakar, Senegal

4.1 Introduction

An estimated 40% of Africans live within an urban area and whilst malaria transmission is generally lower in these settings (UN 2014), 200 million of these residents are at risk of contracting malaria, approximately 6 to 28% of the global malaria burden (Keiser et al. 2004). The number of people in urban areas at risk of malaria will increase as urbanization expands across sub-Saharan Africa with an estimated annual growth rate of 2-6% and 55% of individuals projected will be living in urban areas by 2050 (Donnelly et al. 2005; UN 2014).

Rapid urbanization and declining economies across sub-Saharan Africa have hindered many governments from providing basic infrastructure resulting in informal developments characterized by poor housing and a lack of healthcare and sanitation (Keiser et al. 2004; Warren et al. 1999). Rapid urbanization has also been met with a parallel decrease in food security, either from a lack of locally available foodstuffs or an inability to purchase what is available (Cofie et al. 2003). To address this disparity, the FAO, and other agencies, began to promote urban and peri-urban agriculture (UPA) as a means to meet the food demands of local populations (SPFS 2001; Cofie et al. 2003). UPA is a primary resource in many parts of Africa where it is estimated to provide approximately 70% of local vegetable needs in Dar es Salaam, Tanzania and closer to 90% in Kumasi, Ghana (Afrane et al. 2004; Ba et al. 2016; Cofie et al. 2003).

Whilst food needs may be being better met, there are possible deleterious effects associated with UPA. First, many of the unplanned communities and cultivated areas are located in lowlands or near permanent bodies of water (Antonio-Nkondjio et al. 2011); anthropogenic

changes to these landscapes can lead to increased seasonal flooding, more permanent and semi-permanent bodies of water, and an accompanying increase in potential mosquito larval habitats (Yadouleton et al. 2010; Antonio-Nkondjio et al. 2011; Diallo et al. 2012). Another consequence of these changes is the inadvertent contamination of local water sources due to runoff from cultivated areas (Mzilahowa et al. 2008; Nkya et al. 2013). As steady food production is required to meet the needs of the adjacent urban areas, farms are often operated year-round. This continual production is commonly accompanied by increases in fertilizer and pesticide use (Ngowi et al. 2007).

In several countries UPA has been associated anecdotally with an increase in insecticide resistance in *Anopheles gambiae* s.l. however direct linkage between agricultural practices and the emergence of resistance has never been shown experimentally or with rigorous controls (Afrane et al. 2004; Antonio-Nkondjio et al. 2011; Abuelmaali et al. 2013). Moreover, many of the published studies that purport to show an increase in the levels of the pyrethroid resistance mutation *Vgsc*-1014F (*kdr*) in areas of agriculture also show increases in adjacent areas apparently under low insecticide pressure (Kristan et al. 2003; Djouaka et al. 2008; Yadouleton et al. 2011; Chouaibou et al. 2016). Although definitive associations have not been shown, multivariate analyses have shown that increases in resistance are thought to arise from a combination of factors including the use and improper disposal of pesticides as well as their repurposing for personal protection in and around the home (Ngowi et al. 2007; Yadouleton et al. 2009; Abuelmaali et al. 2013 Reid and McKenzie 2016). In Senegal, many pesticides sold to farmers are without appropriate instructions resulting in misuse by farmers (Williamson 2003). Appreciable levels of agricultural pesticides have been detected in local water sources, suspended solids and soil samples in comparative studies within agricultural and non-agricultural areas (Lahr et al. 2000; Akogbeto et al. 2006). Experimentally, *An. gambiae* larvae reared exposed to soils or water from agricultural sites have been shown to exhibit increasing levels of resistance (Nkya et al. 2014) as well as preferential survivorship of larvae possessing known resistance mechanisms (Akogbeto et al. 2006; Yadouleton et al. 2009; Yadouleton et al. 2010; Luc et al. 2016).

Farmers use pesticides from all classes many of which overlap with those used in malaria control (Sereda and Meinhardt 2005; Seidahmed et al. 2012; Sy et al. 2014). Therefore, it is difficult to partition the roles of public health and agriculture as drivers of resistance. However, in those instances where resistance to compounds has been observed prior to their public health use for malaria control there is stronger evidence for agricultural-use driven selection (N'Guessan et al. 2003; Antonio-Nkondjio et al. 2011; Abuelmaali et al. 2013).

Although urbanization is often associated with a decrease in disease incidence (Hay et al. 2005), malaria is still transmitted in urban areas and is considered a growing threat (Donnelly et al. 2005; Klinkenberg et al. 2005). In Dakar, and the department of Rufisque, the malaria prevalence rate is estimated at approximately 4% along the coast but as high as 12.5% in centre of the department in children under 5 (Diallo et al. 2012; Giardina et al. 2012). Future increases in rural to urban migration, estimated at 2.8% annually in Dakar (Sy et al. 2014), coupled with expanding unplanned urbanization are likely to increase the current malaria burden. Currently malaria control in Dakar is achieved primarily through long lasting insecticide treated nets (LLINs) and artemisinin-combination therapy (ACTs) (PATH MACEPA 2015). Pyrethroid resistance is found at varying rates Dakar with mortality rates between 23.0-89.4% reported in 2014 for permethrin, deltamethrin, lambdacyhalothrin, and alpha-cypermethrin with susceptibility only shown to pirimiphos methyl (Presidents Malaria Initiative 2016; Ndiath et al. 2015). Bendiocarb resistance has also been reported in the Dakar region with the highest levels seen in the northern district of Guédiawaye (Figure 4.1) (Presidents Malaria Initiative 2016). Although there are no current plans to implement widespread IRS in Dakar, reported malaria incidence rates in Guédiawaye and Rufisque (5-15/1000) are approaching the current threshold value for initiating spraying (15-30/1000). Targeted IRS is being introduced into areas around Dakar, using pirimiphos-methyl because of widespread pyrethroid resistance and to provide a contrasting class to LLIN insecticides. During initial IRS campaigns in the south of Senegal in 2011, bendiocarb was employed to counteract pyrethroid resistance but was discontinued due to inadequate residual life on sprayed walls with mortality in cone bioassays on sprayed surfaces dropping from 100% at spraying to 52-65% in 2 months after application (Presidents Malaria Initiative 2016). It is possible that resistance could

emerge due to inadequate coverage even though resistance assays showed 100% susceptibility to bendiocarb in 2015 (Presidents Malaria Initiative 2016).

This study was initiated to characterize the insecticide resistance levels of a population of *An. arabiensis* from Rufisque, Dakar. Unexpectedly, >95% of individuals tested were resistant to a one-hour exposure to 0.1% bendiocarb. Although bendiocarb resistance is known to occur at varying levels within the Dakar Region, this is the first time it has been reported in Rufisque (Ndiath et al. 2015; Presidents Malaria Initiative 2016). Further research into the mechanisms underlying bendiocarb resistance was undertaken using quantitative trait loci analysis and whole genome microarrays.

4.2 Methods

4.2.1 Sampling

Rufisque is the eastern-most and most rural of the 4 local government departments in the Dakar Region. Together with the city of Dakar, Pikine, and Guédiawaye departments it is located on the Cap Vert peninsula on the western coast of Senegal (**Figure 4.1**). Of the 2.45 million residents of the greater Dakar area, 10.1% live in the Rufisque region (Sy et al. 2014) with an estimated 83.6% of residents residing in unplanned communities dispersed throughout the three arrondissements of Rufisque-Bargny, Sébikotane and Diamnadio (Gaye and Diallo 1997). A majority of land is dedicated to UPA in the form of medium to large sized market gardens (sized 1-50 ha) but there is an increase in the development of low-cost housing centres, or habitacions à loyer modéré, on reclaimed agricultural areas (Sy et al. 2014).

For this study, anopheline larvae were collected by El Hadji Diouf, Abjoulaye Kan Dia, and Babacar Ndiouck (Cheikh Anta Diop University) from flooded ruts along a dirt road between houses during routine mosquito monitoring at the end of the rainy season in September 2015. The collection sites were in an urban area within Cité Asecna, Rufisque at N 14°43.598, W 17°16.929 and N 14°43.580, W 17°16.974. Malaria prevalence in asymptomatic individuals from this area was previously estimated around 4% in 2008 (Diallo et al. 2012). The larval site was informally characterized as being temporary, polluted, containing organic matter and household trash, and populated with both

anopheline and *Culex* larvae. Immatures were transported to the CDC insectary in Atlanta and a colony was established for further studies. In addition to species determination, PCR assays were performed to detect known SNPs associated with insecticide resistance including *Vgsc*-1014F, *Vgsc*-1014S, *Ace1*-119S, and *Gaba*-296S.

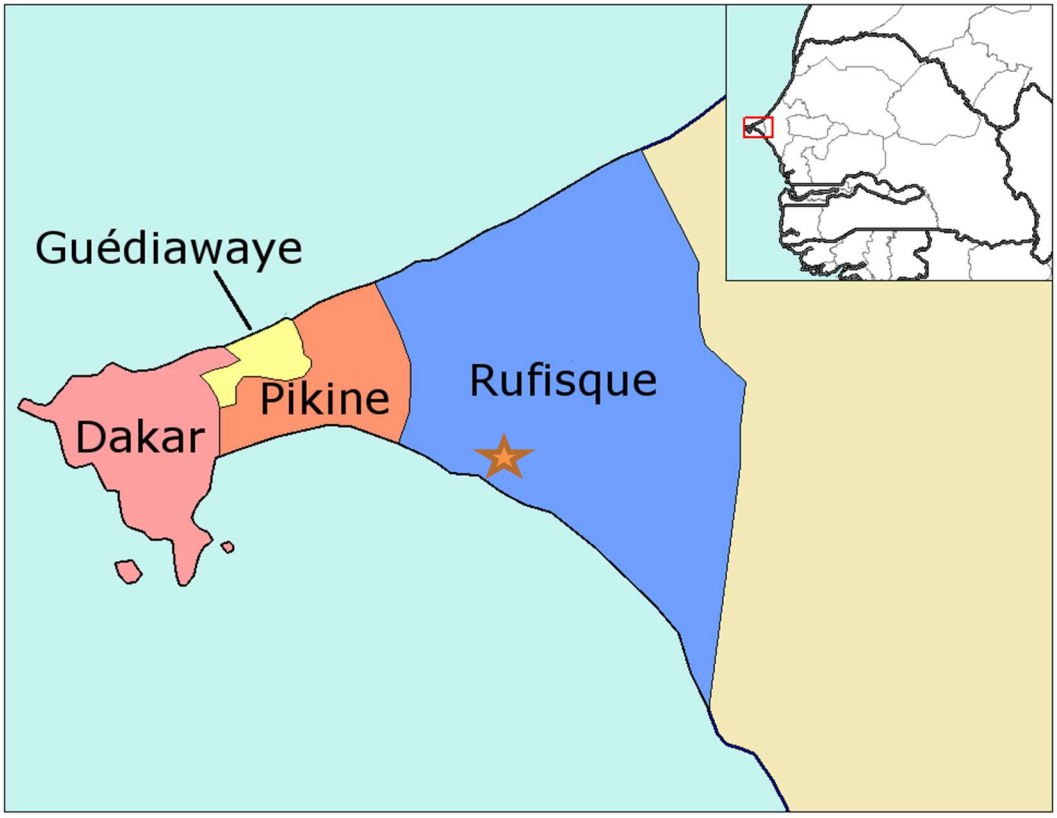


Figure 4.1: The Cap Vert peninsula showing the location of the collection of larvae used in this study. The location of the collection sites is approximated by the star.

4.2.2 Insecticide bioassays

Two to three day old female mosquitoes from the G2 generation were exposed to insecticide impregnated papers employing the WHO tube assay (WHO 2013). Insecticides used were 4.0% DDT (n=100), 0.75% permethrin (n=109), 0.05% deltamethrin (n=99), 0.5% etofenprox (n=104), 0.25% pirimiphos-methyl (n=101), 4.0% dieldrin (n=100), 5.0% malathion (n=86), and 0.1% bendiocarb (n=118). For each trial both a negative control (WHO control paper, n=25) and a susceptible control (Dongola strain, n=25) were

included. After exposure, all adults were aspirated into 500ml holding cups and held at 27°C, 80% humidity and provided 10% sucrose *ad libitum*. Mortality was recorded for each cohort 24 hours post exposure. To determine if cytochrome P450s were implicated in the bendiocarb resistance phenotype a second round of exposures was done on G9 material. Exposures were performed as before except all adults (n=160) were exposed to the synergist PBO (4%) for 1 hour, then allowed to rest for 15 minutes before being exposed to 0.1% bendiocarb for one hour. Mean mortality and confidence intervals were estimated and plotted using the z.test and ggplot functions in R.

4.2.3 Determining the mechanism of bendiocarb resistance

Bendiocarb resistance was an unexpected observation given that there was no history of use in the area and, therefore follow up assays were performed.

1. As both sexes were assayed separately, survivors of bendiocarb exposure were pooled and inbred for one generation. Candidate screens of known bendiocarb resistance associated SNPs were conducted on a proportion of phenotyped specimens which were transferred individually to 1.5ml cryotubes and stored at -20°C prior to molecular analysis. PCR was performed on these specimens to determine if the carbamate resistance associated *Ace*1-119S allele was present (Weill et al. 2004).
2. QTL study: A second set of phenotyped mosquitoes were retained for inclusion in an F2 interbred QTL study. These were crossed to a laboratory colony originating from the Sudan (SENN) that is known to be susceptible to bendiocarb.
3. Whole genome microarray study: 24 hours post bendiocarb exposure, a third set of phenotyped inbred G3 female mosquitoes were quickly rinsed in methanol and transferred in pools of five to 1.5 ml cryotubes contained 1ml RNAlater and stored at -80°C prior to RNA extraction. Paired, unselected individual females from the parent colony of Rufisque as well as samples from SENN (dieldrin resistant but bendiocarb susceptible strain, from south Gezira state, Sudan), Mozambique (insecticide susceptible strain) and Dongola (bendiocarb susceptible but tolerant to OPs, from Dongola, Sudan) were also prepared and stored in the same manner for use as controls.

4.2.4 Genotyping for single nucleotide polymorphisms

Gentoyping for SNPs in 3 known target-sites associated with resistance was done on a sample of the G3 generation: *kdr* (*Vgsc*-1014S and *Vgsc*-1014F), *rdl* (*Gaba*-296S), and *Ace*-1 (*Ace1*-119S). For *kdr* and *rdl*, previously published assays with internal-mismatched primers (IMP) were employed without modification except for the addition of the susceptible-specific primer for *rdl* as discussed in Chapter 3 (Wilkins et al. 2006). The presence of *Ace*-1 was determined using with modification to the published protocol (Weill et al. 2004) by replacement of the RFLP step with IMP primers specific for either wild-type susceptible or resistant SNPs (**Table 4.1**). Reactions were performed with 2 U Taq DNA polymerase (Promega), 0.12 mM dNTPs, 1 µl each primer and polymerase buffer according to the manufacturers suggestion in a 19 µl final volume. Thermal cycling was performed in a BioRad T100 thermocycler with the following conditions: initial melting at 95°C for 5 min, followed by 35 cycles of 95°C for 30 sec, 60°C for 1 min, and 72°C for 45 sec, and a final elongation step of 72°C for 5 minutes. PCR products were visualised on 1.5% 0.5x tris-borate-EDTA agarose gels stained with ethidium bromide under UV transillumination (UVP LLC, Upland, CA).

Table 4.1: Primer sequences and concentrations use to determine the presence of *Ace*1-G119S in *An. arabiensis*. Mutation specific bases are denoted in bold while internal-mismatch primers are in lower case.

Primer name	Sequence	Concentration
CDCACEF	GGT GGA CGT GTG TGG CTC	8 pmol/µl
CDCACER	CTA CCG TAG CGC AAG GTT C	8 pmol/µl
CDCWT	TGT GGA TCT TCG GCG tC**G**	10 pmol/µl
CDCG119SR	CGG TGC CGG AGT AGA AtC **T**	25 pmol/µl

Next, amplicon sequencing was performed to determine if F290V or N485I, both recently reported novel SNPs associated with bendiocarb resistance, were present in this population (Liebman et al. 2015; Ibrahim et al. 2016). Amplicons from a sample of 8 known resistant individuals from the parental colony were selected for sequencing with excess primers and

dNTPs removed using ExoSAP-IT (Affymetrix, Santa Clara, CA) per manufacturer's instructions. Samples were the processed with BigDye® Terminator v3.1 (Life Technologies, Grand Island, NY), purified with BigDye® XTerminator, and sequenced on an ABI3500 sequencer (Applied Biosystems, Foster City, CA). Orthologous sequences for *An. coluzzii*, *An. gambiae*, and *An. arabiensis* were obtained from VectorBase (www.vectorbase.org) and aligned in BioEdit (www.mbio.ncsu.edu/bioedit/bioedit.html).

4.2.5 QTL study

Individuals from the G3 generation of the Rufisque colony were crossed to the bendiocarb-susceptible strain SENN (MRA-763) available from BEI Resources (www.beiresources.org). Colonies were reared at 27°C ± 1°C and 80% ± 2% humidity using standard protocols (Benedict 1997) except that larvae were fed with Drs. Foster and Smith® Staple Diet Koi food (www.drsfosterandsmith.com) and adults were fed a diet of 10% sugar water with 0.2% methylparaben *ad libitum* (Benedict et al. 2009).

Reciprocal crosses between the 2 lines were performed by combining single male and female pupae from each strain in a 200 ml Qorpack cup (Bridgeville, PA. No. 3891) in which they were allowed to emerge and comingle for 10 days (Benedict and Rafferty 2002). Blood meals were offered on the tenth day; females that did not feed were offered a blood meal daily until they did so or until they perished. Individual females, 3 days post blood feeding, were left to oviposit in a new Qorpack cup containing 50ml water and a strip of filter paper. F1 progeny were allowed to inter-mate to generate F2 material that was subsequently used to generate new iso-female lines. As natural mating between Rufisque males and SENN females was rare in the Qorpack cups, 9 mated-pairs were established utilizing induced copulation (Ow Yang et al. 1963).

4.2.6 Microsatellites

Mosquito DNA was extracted in 30 µl Quanta Extracta DNA Prep (Quanta Bio, cat no. 95091) with 30 µl of stabilization solution added to enable long-term storage. New microsatellite loci were developed employing WebSat (Martins et al. 2009) and compared for polymorphisms against *An. gambiae s.l.* using the BLASTn feature of VectorBase

(Lawson et al. 2009) and primers designed using Primer 3 (Rozen and Skaletsky 2000). All newly developed primers are listed in **Table 4.2**. Although a suite of new *An. arabiensis* specific microsatellite primers were designed for elucidating a QTL responsible for dieldrin resistance (Chapter 2), due to high levels of microsatellite polymorphism in the recently established Rufisque strain 11 were eliminated due to shared insertion lengths with the SENN strain. Retesting 78 primers from the previous study resulted in identifying 6 primers which were discriminatory between the 2 colonies while the remainder had shared length polymorphisms. Forty-five additional primer pairs were tested with 7 showing no polymorphism between the two colonies, 23 having shared polymorphism and 15 with novel polymorphisms and determined to be new discriminatory markers. All markers used in this study were pre-screened by PCR against 48 random individuals from both lines as well as P0 individuals used to establish the iso-female lines. Multiplexed PCR reactions consisted of 1.5 units of Promega Go*Taq*™ polymerase, 1.5 mM dNTPs, and an additional 0.75 mM $MgCl_2$ in 20 µl total volume (Promega, Madison, WI). All triplexed assays contained primers at the following concentrations: 3 pmol/µl, 9 pmol/µl, and 18 pmol/µl from smallest to largest product respectively. PCR cycling was performed as described in (Lehmann et al. 1996). PCR products were visualised on 1.5% 0.5x tris-borate-EDTA agarose gels stained with ethidium bromide under UV transillumination (UVP LLC, Upland, CA) prior to genotyping. Genotyping was performed on a Hitachi ABI 3130XL Genetic analyzer with 1µl of diluted PCR product, 0.4 µl ROX -250 ladder (ABI Biosystems, part no. 401734) and 10.6 µl Hi-Di formamide. Discrimination of band sizes was done using GeneMapper 4.1 (ABI Biosystems).

Table 4.2: Microsatellites, their position within the genome based on synteny in *An. gambiae*, PCR product size and oligonucleotide sequence information used to determine the genic location of bendiocarb resistance in an F2 interbred experimental population. Markers without motif, size, or primer sequences have either been previously published or reported in Chapter 2 of this dissertation.

Marker	Repeat	Size	Division	Primers	Reference
X chromosome					
AgX:7			1C		Zheng 1996
AgX:711w			2B		Zheng 1996
AgX:503			4B		Zheng 1996
CDC6			6A		Chapter 2
Chromosome 2					
CDC7.3	GT(16)	144, 150	7B	F: CTG CCG CAC ATA AAA GAC	
				R: CTC CAT CGC GAT AAC AAC	
CDC9	AGA (13), AAG (6), GAA (19)	370, 380	9B	F: AAA GAA CTT GGA AAG CCT CT	
				R: ATG ACA TAG GCT GAC TGA CC	
2R11A	AC (13)	88	11A	F: CTG CTG GCG CAT AAT AGT	
				R: ATT TAC GCT CGC AAA CAC	
2RiS5			12C		Witzig 2013
Ag2:95			13D		Zheng 1996
2R14D	CT (38)	279, 325, 327	14D	F: GAC GGT GGA GTT TTG CTA	
				R: CGA TTG TGA TTG CCT TGT	
2R16B	GT (16)	217, 224	16A	F: AGG GAT GTC CGA TTG TG	
				R: TGT GAA AAC CAA TCA ATG TG	
R27ǂ	AG (15) CG (5)	187, 200	18A	F: GCA GAT AAC GAG CGA GAG	
				R: ACC TAC GTG CGA CCT ACA	
CDC19			19C		Chapter 2
CDC20D	CT (5) CA (18)	196, 215	20D	F: GAG TCA AAA CCC GTC CA	
				R: GCT GTA CCG TCG CTT TT	
CDC21	CA (11)	109	21D	F: GGT AAT TGG CGT CCA TC	
				R: TGC CAG AAA TAC TCG GAC	
CDC22	GT (12)	268	22D	F: GGG CAA AGA GAA AGC AA	
				R: AGC TGT GTG GCA GGT TT	
ARGB1	AG (22)	90	23C	F: GAA GAG CCA GCG AGA GAA	
				R: GCG GTC AAG AAA ATG GAA	
Ag2:787			24A		Zheng 1996
ND36P1			25C		Stump 2007
CDC27B	TG(19)+(5)	341, 352	27B	F: CCG CTG GTA TAG CAA GAA	
				R: GGG GAA GGG ATG AAA GA	
CDC28C			28C		Chapter 2
Chromosome 3					
3R29B	CA (31)	256	29B	F: CAC CAC CAT CAC AGT CCT	
				R: CTT TCA CGC TCG TTT TGT	
Ag3:249			30B		Zheng 1996
CDC32			32A		Chapter 2

Ag3:555			32C		Zheng 1996
33C1			33C		Wang 1999
3L38C	TG (17)	154	38C	F: ACC CTC ATT TGC ATT CAC TT	
				R: GCT GGA TAC AGA CAA CAA AGC	
CDC40B	TG (11)	218	40B	F: ATG CAT GCA AAT CGG TAT	
				R: TAT CGA GGC AAA TCG GTA	
CDC42			42C		Chapter 2
CDC44B	CA(18)	119	44B	F: CAA CTT CAA TCC CTG TGC AA	
				R: TGG ATG CAA CCA AAA CGA TA	
45C			45C		Wang 1999

4.2.7 QTL analysis

Associations between bendiocarb resistance or susceptibility and the genotype at each microsatellite locus were estimated using standard interval mapping (EM), Haley-Knott regression (HK), and multiple imputation (MIM) analyses. Although considered less reliable when there are few markers present, an additional analysis was done utilizing marker regression (MR) as it was found to provide comparable results in a previous QTL analysis (Chapter 2). Construction of linkage maps was performed using the R/qtl package in the R statistical platform version 3.3.1 (R Development Team 2013). Logarithm of the odds (LOD) scores were determined with Bayes credible 95% intervals for EM, HK, and MIM. The LOD cut-off was based on the 95% LOD score on a chromosome with no QTL.

4.2.8 Microarray design, hybridization and analysis

Although bendiocarb resistance has been detected in several anophelines across Sub-Saharan Africa, very little has been done to elucidate mechanisms behind such resistance aside from testing for the *Ace1*-G119S mutation. While *Ace1*-G119S may not explain the totality of resistance in most instances, mechanisms of resistance have been inferred based on microplate or synergist assays including CYPs (Aikpon et al. 2013; Aizoun et al. 2013; Kwiatkowska et al. 2013; Edi et al. 2014). While QTL analysis is most effective when looking for a relatively few loci with large effects such as target-site mediated resistance, resistance conferred by alternate pathways, such as metabolic or cuticular resistance, may be induced by several loci with small, but additive effects that may be difficult to discern. Since an identified QTL may contain dozens to hundreds of genes, pairing QTL with whole

genome microarray permits the identification of differentially expressed genes within QTLs to prioritise research efforts (Fisher, Hedler, et al. 2007; Jouffe et al. 2009). In this study, populations resistant and susceptible to bendiocarb, as well as insecticide-exposed and unexposed, were compared. The former pairing allowed detection of differentially expressed genes associated with resistance while the latter pairing enabled us to control for genes that are differentially expressed between the colonies of diverse geographic origin.

The Agilent 8 x 15 K *An. gambiae* s.s. microarray with 14,071 probes was employed (Mitchell 2012) (**Figure 4.2**). Details of the microarray design and candidate genes included can be obtained from ArrayExpress (www.ebi.ac.uk/arrayexpress/). RNA extractions were performed on 30 female mosquitoes per treatment group, pooled into groups of 5. Extractions were performed by Emily Rippon and Patricia Pignatelli (Liverpool School of Tropical Medicine) using TRI Reagent (Ambion) and DNase treated (TURBO-DNase free, Ambion). RNA pool quality and quantity were assessed using a Bioanalyzer and NanoDrop spectrophotometer (NanoDrop Technologies). All RNA pools include for hybridization analysis were labelled with either Cy3 or Cy5 dyes using the Low Input Quick Amp Labelling Kit (Agilent Technologies). Array hybridization, washing, and extraction were performed per manufacturer's protocol. Microarray normalization was performed in R (www.r-project.org) using the Limma package (https://bioconductor.org/packages/release/bioc/html/limma.html) per previous protocol (Muller et al. 2007). In brief, values were normalized within arrays using the Loess method prior to MAANOVA analysis (Workman et al. 2002). As the ratio of Cy3 to Cy5 was similar, no further between-array normalization was done. Normalised intensities were analysed using the MAANOVA package (https://bioconductor.org/packages/release/bioc/html/maanova.html). Last, fold-change and P-values were estimated comparing Rufisque singly to either Mozambique, Dongola, or SENN colonies. Annotated genes that were considered differentially expressed were those meeting the following criteria: FDR corrected Q value <0.05 and a two or greater log-fold change compared to the 3 bendiocarb-susceptible colonies.

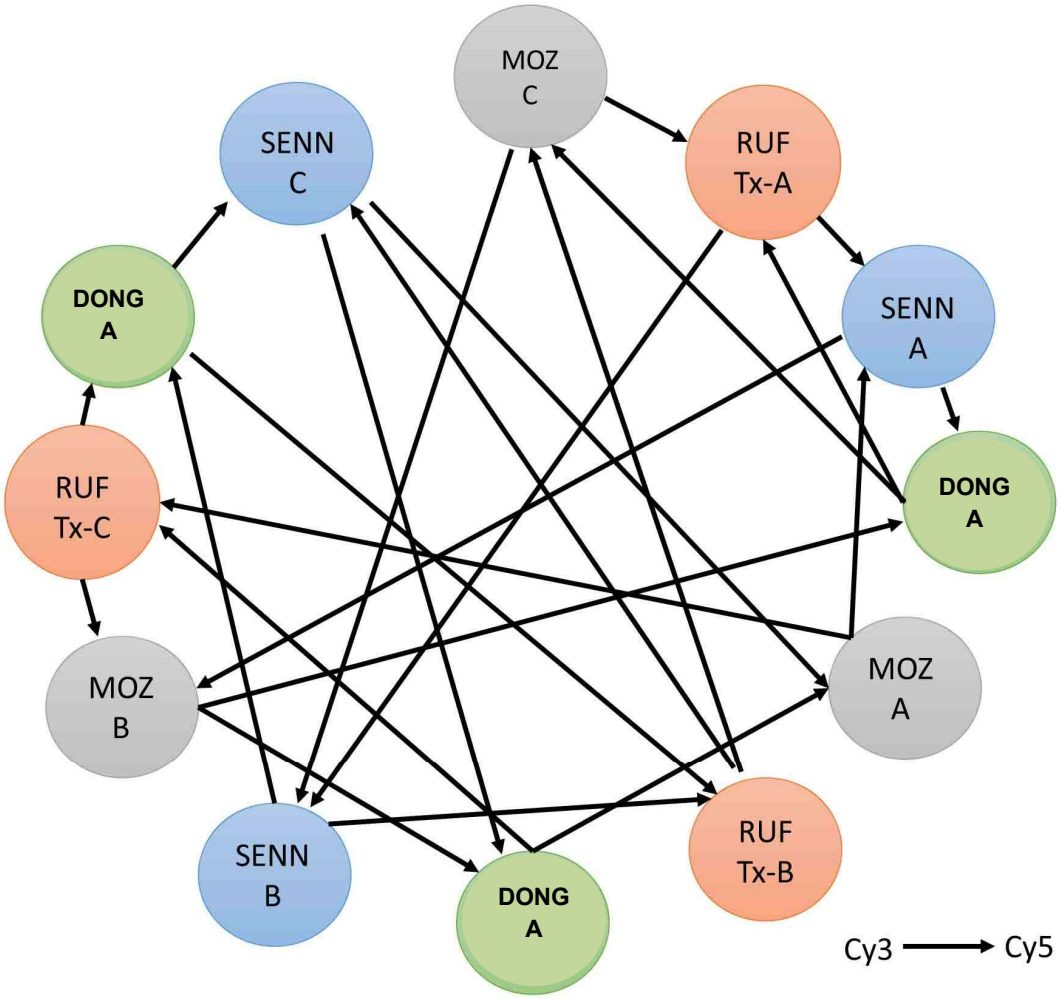


Figure 4.2: Interwoven microarray experimental loop design for comparing bendiocarb resistant *An. arabiensis* from Rufisque, Senegal to three laboratory colonies. MOZ is an insecticide susceptible strain that originates from Mozambique, DONG is an insecticide susceptible strain from the Sudan, and SENN is a dieldrin resistant strain originating from the Sudan. All three comparison strains are susceptible to bendiocarb (data not shown). Pools, indicated by circles, represent five 2-3-day old females from which RNA was extracted. Arrows indicate individual microarrays with the direction representing the Cy dye labeling.

4.3 Results

4.3.1 Insecticide bioassays

Pronounced resistance to eight of nine compounds was seen in *An. arabiensis* originating from Rufisque. As expected resistance levels were high for pyrethroids (mortality = 0.0-1.8%) and organochlorines (3.4-8.2%), high levels of resistance to bendiocarb (1.7%) was surprising as appreciable resistance to this compound has only been reported in Pikine or Guédiawaye (Ndiath et al. 2015; Presidents Malaria Initiative 2016) while Rufisque was completely susceptible to pirimphos methyl (100%) (**Figure 4.3**). Previously it was shown that carbamate/pyrethroid resistance in *An. funestus* resulted from an overexpressed CYP6Z1 (Ibrahim et al. 2016). Therefore, as resistance to pyrethroids was high and no *Ace*1-119S allele was found, a second cohort from the G9 generation was exposed to piperonyl butoxide (PBO) for 1 hour then 0.1% bendiocarb for 1 hour to determine if resistance was likely to be related to the presence of over-expressed cytochrome P450s (CYPs). Based on a Fisher's exact test comparing the mortality rates there was no difference between the PBO-exposed cohort and a cohort solely exposed to bendiocarb (2.5% vs. 3.75% respectively, P = 0.589, df=1) therefore there is an absence of evidence that CYPs are affecting resistance in this strain.

4.3.2 Screen for known candidate resistance loci

All genotyping was performed on individuals from the G3 generation. As anticipated, all individuals tested were homozygous resistant by PCR for *Vgsc*-1014F while none possessed *Vgsc*-1014S (n=48). Surprisingly all individuals were homozygous resistant for *Gaba*-296S even though there in the lack of insecticide pressure. Of 96 individuals phenotyped as bendiocarb resistant after exposure in a WHO tube assay, none possessed the *Ace1*-119S allele. Sequencing for recently reported rare novel *Ace*-1 SNPs associated with resistance in a sample of adults found that neither F290V nor N485I were present in this population however there was a SNP, T307P, found only in the Rufisque population when compared to the SENN and Dongola colonies (**Appendix Figures 6 and 7**). While this mutation is near F290V, which has been shown to affect bendiocarb resistance in *Culex pipiens*, its association with resistance in *An. arabiensis* is unknown.

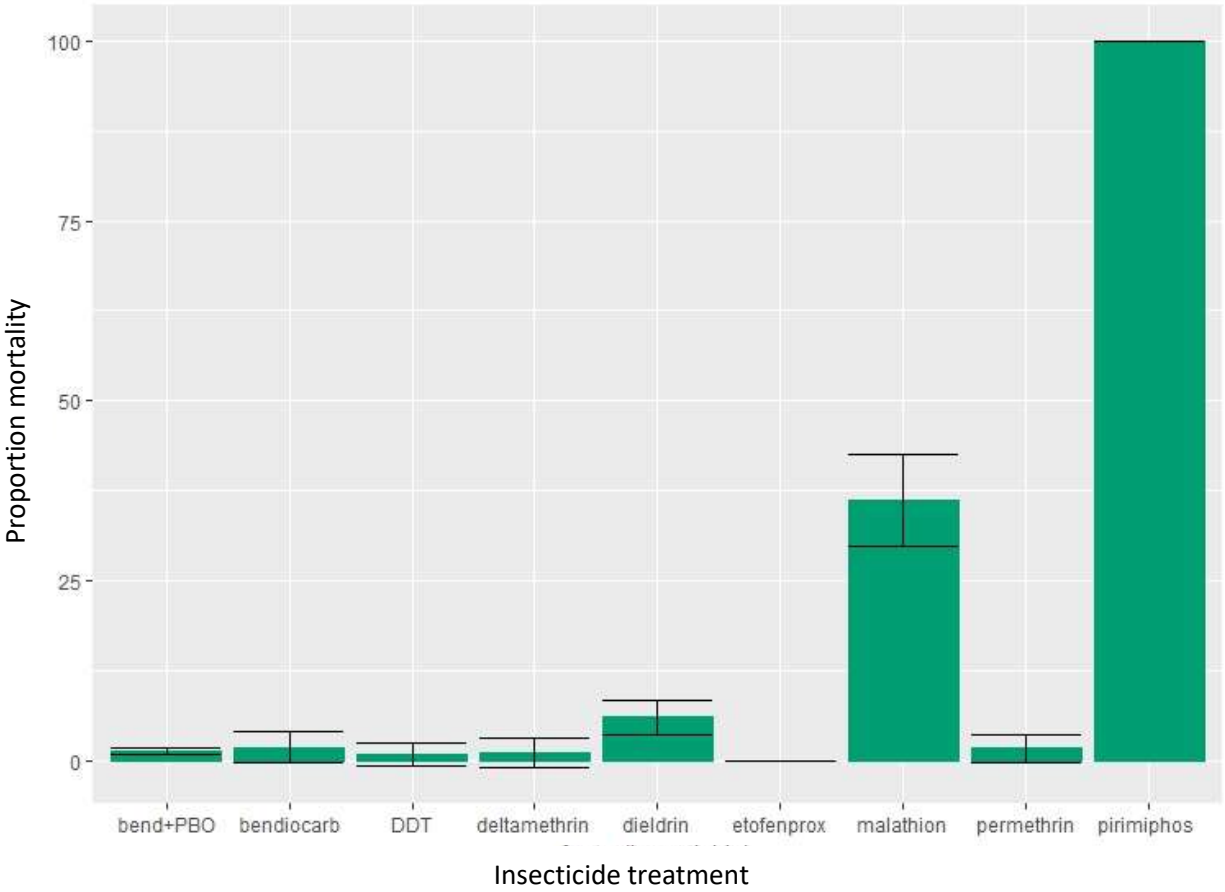


Figure 4.3: Proportion and 95% confidence intervals for mortality 24 hours post exposure to select insecticides employing the WHO tube assay for a population of *An. arabiensis* from Rufisque, Dakar, Senegal. All values were calculated using the z.test in R.

4.3.3 QTL analysis

For the Rufisque ♀ x SENN ♂ cross (RxS), three parental lines produced 15 F2 isofemale families while for SENN ♀ x Rufisque ♂ cross (SxR) 19 F2 isofemale families were generated from five parental lines originally established by induced copulation (**Table 4.3**). F2 offspring (n=941) were classified based on their ability to survive a two-hour exposure to 0.1% bendiocarb (referred to as BENRES). Exposure time to bendiocarb was lengthened to ensure all susceptible individuals would perish as initial testing found high levels of survivorship after one-hour exposures (data not shown). F2 phenotyped individuals were

genotyped at a total of 31 fully informative microsatellites in this study of which 15 came from previous published studies and 16 were newly designed (**Table 4.2**). Coverage varied among chromosomes with the X chromosome having four markers covering 15.55 cM; 2R a total of nine markers covering 55.15 cM; 2L eight markers covering 45.07 cM; 3R five markers covering 35.01 cM, and 3L five markers covering 32.43 cM (**Figure 4.4**). As there were no excessively large LOD values seen on chromosome 3, the LOD value was set at 4.9 with results above this considered significant. All markers were assessed for Hardy-Weinberg equilibrium (HWE) using GenePop v4.2 (http://genepop.curtin.edu.au/). No marker was found to be uniformly out of HWE, however several families did have an excess of alleles considered to be outside of HWE (**Appendix Table 2**). Their lack of HWE was most likely due to skewing as they were small families and their effect would be minimalised when assessing all families together.

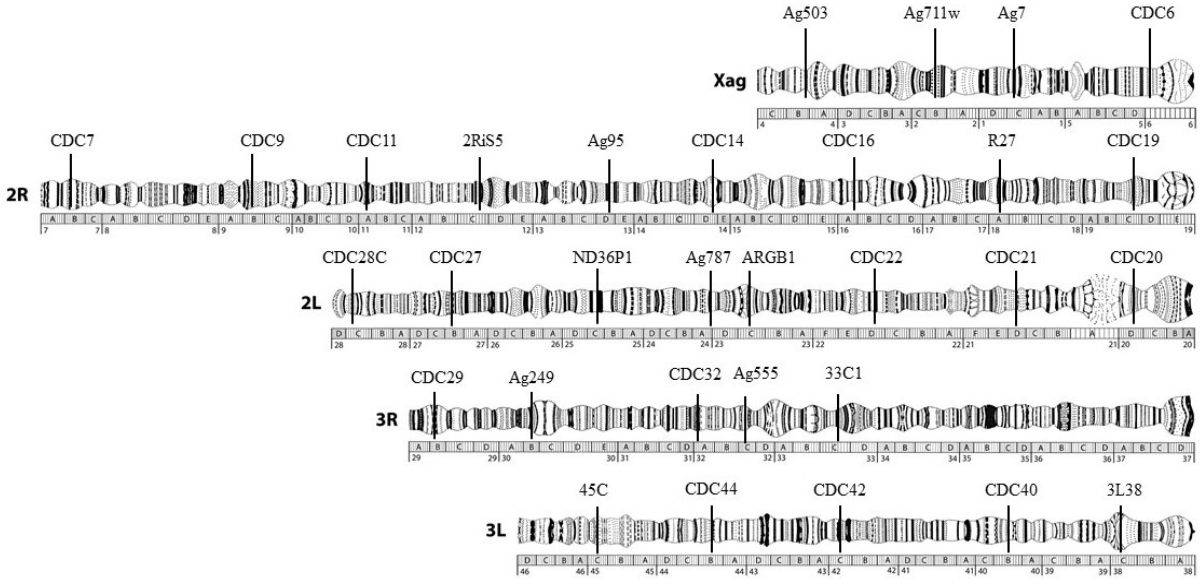
Table 4.3: F2 interbred families based on WHO tube bioassay outcome included in QTL analysis to determine bendiocarb resistance in *An. arabiensis* from Rufisque, Dakar, Senegal.

Family	No. progeny	Resistant		Susceptible	
SENN x RUFISQUE					
SRFM1	68	25	36.76%	43	63.24%
SRFM6	177	64	36.16%	113	63.84%
SRFM8	10	5	50.00%	5	50.00%
SRFM9	80	42	52.50%	38	47.50%
SRFM15	82	18	21.95%	64	78.05%
Totals	417	154		263	
RUFISQUE x SENN					
E10	275	161	58.55%	114	41.45%
E11	44	11	25.00%	33	75.00%
F4	205	57	27.80%	148	72.20%
Totals	524	229		295	

Standard interval mapping, Haley-Knott, and multiple imputation analyses were performed on 941 F2 offspring based on their resistance to a two-hour exposure to 0.1% bendiocarb (referred to as BENDRES). In total, 97.5% (n=926) individuals were included in the final

analyses associated with the genetic map estimated by R (**Appendix Figure 8**). As bias between the two crosses could not be discounted due to mating differences, analyses were performed for each cross separately. For the RxS intercross, the BCI was centered between loci 29 and 30 (31.341 – 32.341, LOD=20.32) and locus 50 (52.341, LOD=19.07) on the 2R. For the 2L, the peak was centered between loci 2 and 3 (5.23 – 6.23, LOD=22.79) and on locus 9 (12.23, LOD=21.55). Lastly, on 3R the BCI was between locus 17 and microsatellite 33C1 (18.689 – 25.55, LOD=6.78). For HK analysis, the most prominent positions were: Locus 46 (2R: 48.34), locus 3 (2L: 6.23), 33C1 (3R: 25.56), and Ag711w (X: 9.89) (**Figure 4.5**). For the SxR intercross, the BCI was centered between loci 40 and 46 (42.341 – 48.341, LOD=15.23) for the 2R. For the 2L, the most likely interval was between CDC27 and locus 41 (44.039-44.228, LOD = 10.83). On the 3R, a small region was identified at locus 5 (6.689, LOD = 1.44) with a larger region between CDC 32 and 33C1 (19.092 – 25.556, LOD = 3.013). Finally, on the 3L the BCI was between CDC44 and locus 29 (32.24 – 34.44, LOD=10.37). Analysis using the HK method found the most likely positions were: locus 43 (2R: 45.30), CDC27 (2L: 44.04), CDC32 (3R: 19.092), locus 27 (3L: 32.4) and CDC5 (X: 17.4) (**Figure 4.6**).

Figure 4.4: Location of microsatellites employed in this assay overlaid on a chromosomal map of *An. gambiae* (www.vectorbase.org).



As marker regression (MR) was previously found to provide comparable results when estimating dieldrin resistance in *An. arabiensis* in Chapter 2, a final analysis was performed to estimate the most likely candidate regions. When both intercrosses are combined, the strongest associations were seen at R27 (2R: 50.3719, LOD 24.458), CDC21 (2L: 9.98095, LOD 28.782), CDC32 (3R: 19.092, LOD 5.023), and CDC44 (3L: 32.241, LOD 7.931).

4.3.4 Microarray analysis

Microarray comparisons were performed between bendiocarb-exposed Rufisque and one of three control groups: Mozambique, SENN, and Dongola. Cut-off values were established with a log10 fold-change > 2.0 and a corrected $P < 0.05$ to filter results and provide a candidate list of the most likely genes associated with bendiocarb resistance.

4.3.4.1 Genes over-expressed in Rufisque compared to two susceptible colonies

When comparing Rufisque to the three bendiocarb susceptible colonies no single gene was over-expressed in all pairwise comparisons. Given OP resistance in SENN, and the possibility of OP and carbamate cross resistance, microarray analyses were focused on examining differential expression levels between Rufisque and the two completely insecticide susceptible colonies: Mozambique and Dongola. After filtering as before, 8 genes were significantly over-expressed in both susceptible comparison groups. Shared over-expression was found in two cuticle genes (CPLCG4, AGAP008447 and CPLCX2, AGAP003334) as well as one detoxification gene (GSTD11, AGAP004378) (**Table 4.4**). The most over-expressed genes unrelated to detoxification in Rufisque were phosphoglucomutase (AGAP005860, PGM1), alanyl-aminopeptidase (AGAP004809, APN1), chymotrypsin 1 (AGAP006709, CHYM1), a protease zinc m1 metalloprotease (AGAP012984), and a hypothetical conserved protein with CHK kinase-like properties (AGAP003757).

Values for the Rufsique x SENN comparison, on which the QTL analysis was performed, found few genes over-expressed, and none previously associated with insecticide resistance (**Appendix Table 3**).

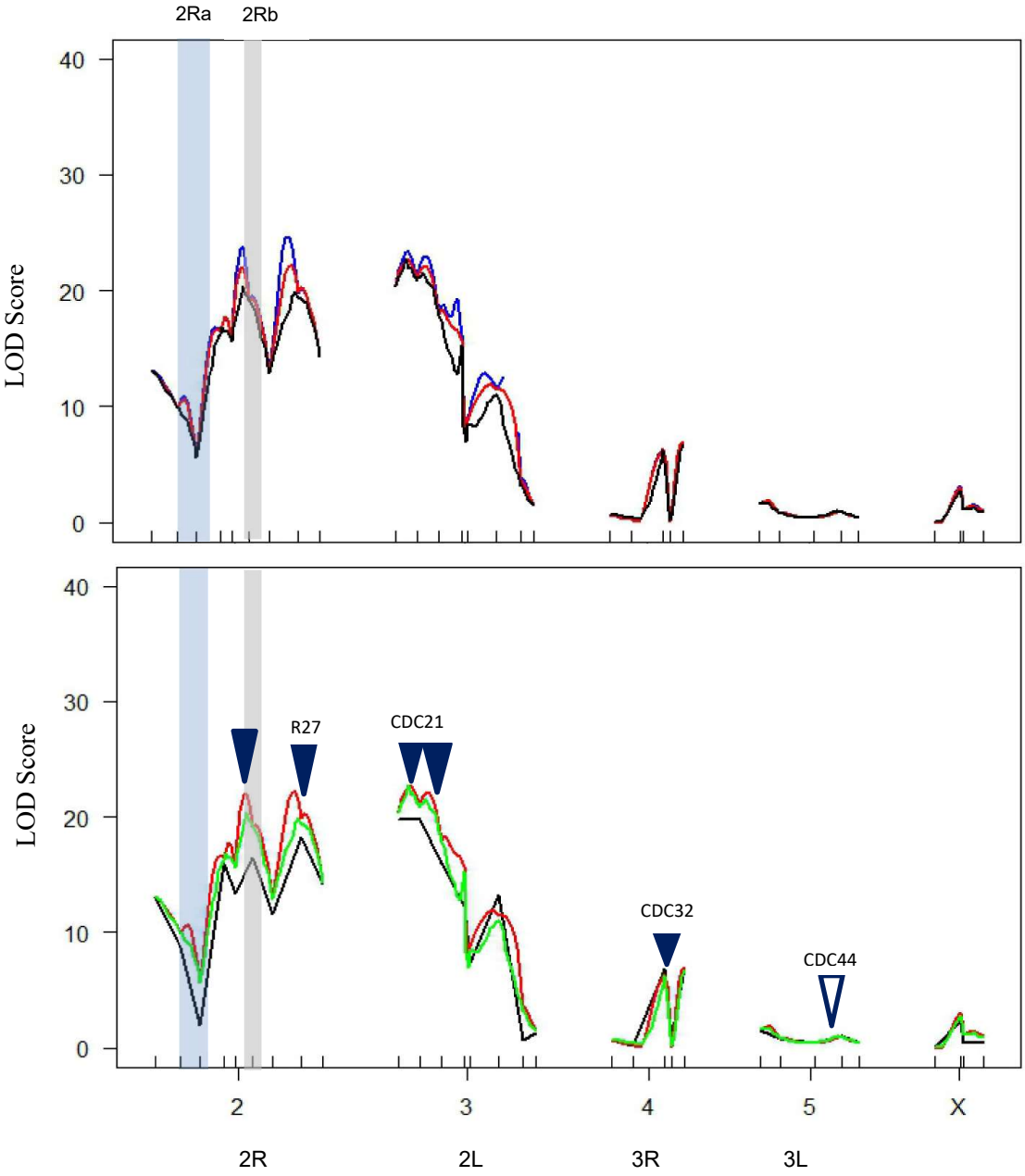


Figure 4.5. LOD scores for the QTL analyses of individuals based on 24-hour survivorship after 2-hour exposure to 0.1% bendiocarb in *An. arabiensis* from the Rufisque X Senn F2 intercross. Methods being compared are: in the upper graph, standard interval mapping (blue), Haley-Knott (red) and multiple-imputation (black) are shown while marker regression (black), Haley-Knott (red), and multiple-imputation (green) are represented on the lower graph. The approximate area covered by the 2Ra and 2Rb inversions are

represented by the light blue and gray boxes based on the position of the microsatellite markers employed. Dark arrows represent BCI regions identified by chromosome.

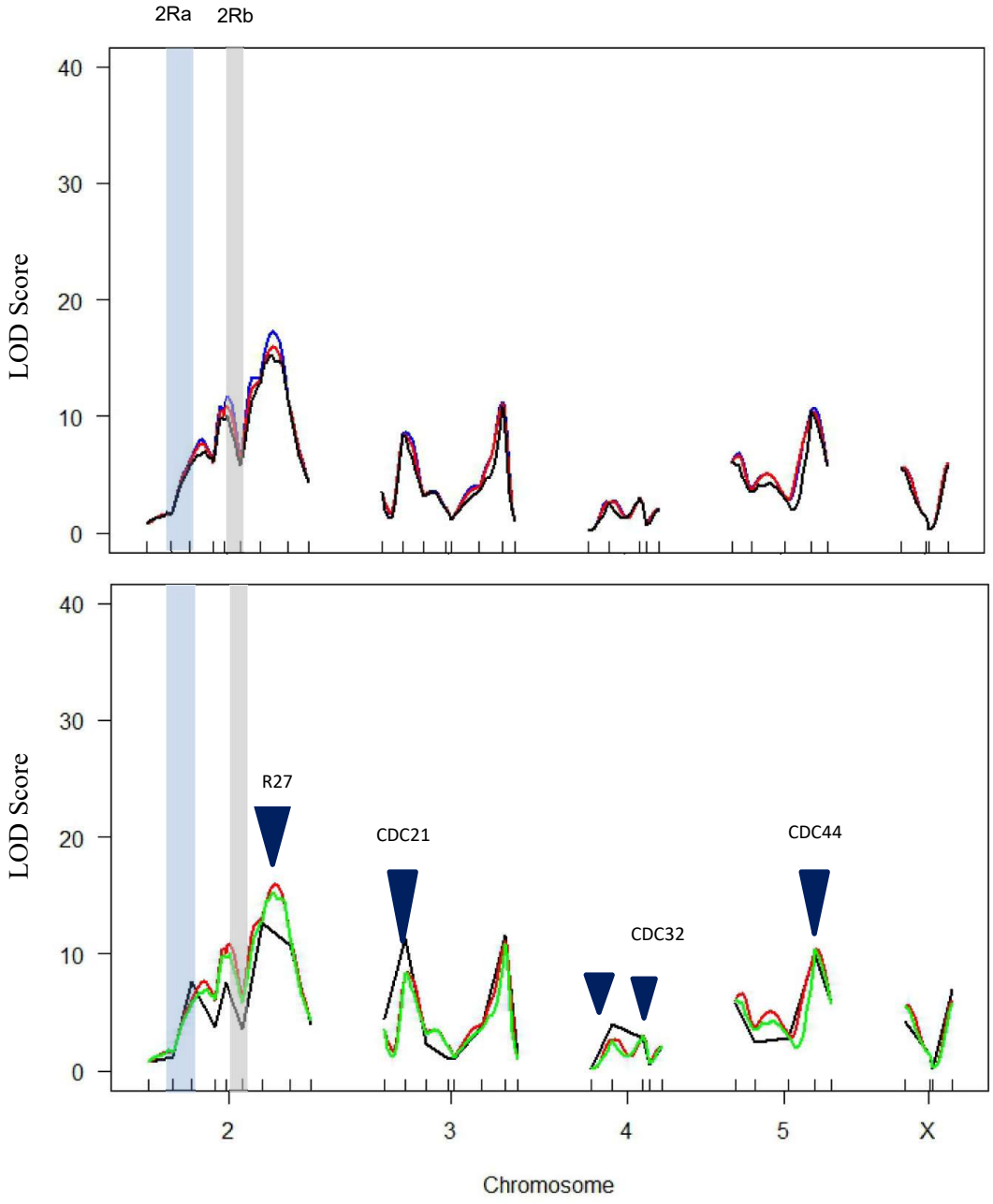


Figure 4.6. LOD scores for the QTL analyses of individuals based on 24-hour survivorship after 2-hour exposure to 0.1% bendiocarb in *An. arabiensis* from the Senn X Rufisque F2 intercross. Methods being compared are: in the upper graph, standard interval mapping (blue), Haley-Knott (red) and multiple-imputation (black) are shown while marker

regression (black), Haley-Knott (red), and multiple-imputation (green) are represented on the lower graph. The approximate area covered by the 2Ra and 2Rb inversions are represented by the light blue and gray boxes based on the position of the microsatellite markers employed. Dark arrows represent BCI regions identified by chromosome.

Table 4.4: List of genes displaying the highest over-expression (log fold change) between the bendiocarb resistant Rufisque compared to the susceptible colonies Mozambique (MOZ), Dongola (DONG), and Sennar (SENN). All displayed logFC values are significant at the P<.05 value with a cutoff logFC of 2-fold expression. Values for SENN were included in this table even though they did not reach the threshold. Italicized genes are those that have been previously found to be significantly downregulated in microarray studies of insecticide resistance (source: VectorBase).

		Log Fold Change		
Description	Systematic Name	MOZ	DONG	SENN
Phosphoglucomutase PGM	AGAP005860-RA	4.249	5.629	
hypothetical conserved protein	AGAP003757-RA	4.093	3.772	
chymotrypsin 1	AGAP006709-RA	3.389	3.586	1.611
protease m1 zinc metalloprotease	AGAP012984-RA	2.530	2.903	1.854
alanyl aminopeptidase N1	AGAP004809-RA	2.445	2.388	1.059
GSTD11 - Glutathione S-transferase	*AGAP004378-RA*	2.356	2.238	0.534
CPLCX2	AGAP003334-RA	2.222	2.982	
CPLCG4	*AGAP008447*	2.106	6.920	

4.3.4.2 Under-expressed genes in the Rufisque comparisons

Most intriguing was that several previously detected detoxification enzymes were significantly under-expressed in all three comparison groups (**Table 4.5**). Of 13 shared genes, CYP6Z1 (AGAP008219) and CYP6M2 (AGAP008212) were under-expressed detoxification enzymes in all three experiments while CYP9J5 (AGAP012296), CYP6Z2 (AGAP008218), CYP12F2 (AGAP008021), CYP6P3 (AGAP002865), and xanthine dehydrogenase (XD24352) were significantly under-expressed in the Dongola and Mozambique comparisons (**Table 4.5**).

4.3.5 Combined QTL/Microarray

When combining the results of both experiments, of the previously identified QTL BCI regions only three putative peaks were associated with any over-expressed genes identified by microarray analysis: the hypothetical conserved protein AGAP003757 (no orthologue identified in *An. arabiensis* but a putative protein match is found within KB704407 near microsatellite CDC16), hypothetical conserved protein AGAP011850 (orthologue found in KB704348 near microsatellite CDC44) and the gene with the highest fold-change PGM (AGAP005860: orthologue found in KB704125 near microsatellite CDC27) (**Figure 4.7**). Extraordinarily, one QTL peak (3R) was associated with five CYPs, including CYP6Z1 and CYP6M2, which were significantly under-expressed in the microarray analysis. The absence of an association between bendiocarb resistance and P450 expression corroborates the PBO synergist assay. Interestingly, within the Bayes Credible Interval and near AGAP003757 is CPAP1-C, a chitin metabolism protein, which may work synergistically with CPLCG4 (**Appendix Table 5**) as are several other alleles which have been associated with insecticide resistance in previous microarray studies.

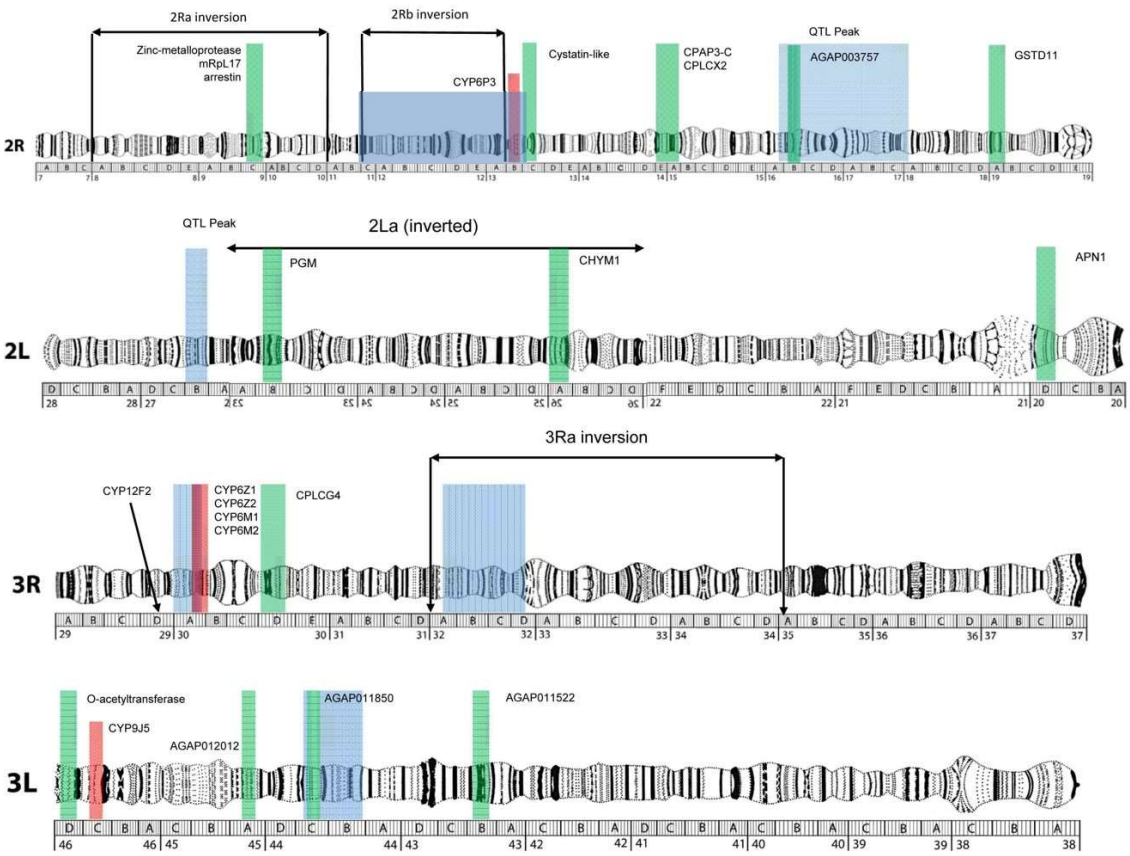


Figure 4.7: Chromosome map showing regions identified by QTL (blue rectangles) and microarray analysis (green rectangles: over-expressed genes, red rectangles: under-expressed genes). Additionally, chromosomal inversion regions are identified and inverted in one instance (2La is known to be fixed in the inverted position in *An. arabiensis*). Other inversions are not repositioned as they are known to be polymorphic in both SENN and Rufisque (2Ra, 2Rb, and 3Ra).

Table 4.5: A select list of genes displaying the highest under-expression (log fold change) between the bendiocarb resistant Rufisque compared to the susceptible colonies Mozambique (MOZ), Dongola (DONG), and SENN. All displayed logFC values are significant at the P<.05 value with a cutoff logFC of 2-fold expression. Genes in bold are those that have been previously found to be upregulated in insecticide resistant populations in the wild, italicized genes are those that were found to downregulated, and all others were found to have non-significant differentiation or with contradictory results regarding their role in regulation of expression.

		Log Fold Change		
Description	Systematic Name	MOZ	DONG	SENN
chymotrypsin like	AGAP001198-RA	-5.4516	-4.7776	-3.8314
dehydrogenase/reductase activity	**AGAP005645-RA**	-3.1101	-2.9036	-2.5319
troponin C	**AGAP006178-RA**	-2.8131	-2.2951	-2.9417
Sg3 protein	AGAP006507-RA	-2.9648	-3.4201	-2.3321
ester hydrolase C11orf54	*AGAP006729-RA*	-4.1318	-3.5202	-3.2663
Flightin	AGAP007249-RA	-2.8113	-2.2936	-2.2527
CYP6M2	AGAP008212	-2.3087	-2.1709	-2.0947
CYP6Z1	AGAP008219-RA	-3.2176	-2.3102	-2.7354
Trypsin 4	AGAP008292-RA	-7.555	-2.1618	-5.4597
Trypsin 7	*AGAP008293-RA*	-6.1611	-2.7621	-3.4389
farnesoic acid o-methyltransferase	AGAP009605-RA	-2.831	-3.2416	-3.0879
U11/12 small ribonucleoprotein	*AGAP010248-RA*	-2.363	-2.8471	-2.7725
cytoplasmic actin	*AGAP011515-RA*	-4.9689	-4.6709	-6.7822
CYP9J5	AGAP012296-RA	-3.2968	-2.8344	
CYP6Z2	AGAP008218	-2.8676	-4.2424	
CYP12F2	AGAP008021-RA	-2.5159	-2.1485	
XD24352	XD24352	-2.1711	-2.6447	
CYP6P3	AGAP002865	-2.0182	-2.3316	

4.4 Discussion

In Senegal carbamates are the insecticide least utilized (5%) by urban farmers (Ba 2016), however resistance has been reported within and around Dakar (Presidents Malaria Initiative 2016). Bendiocarb was employed for IRS in Senegal between 2011-2013, but it was mainly in the Kaffrine and Tambacounda regions 250km east of Dakar (Presidents Malaria Initiative 2016). Although the use of carbamates in urban and peri-urban agriculture (UPA) is low, resistance has emerged across Dakar and could be the result of selective pressures unintentionally exerted by urban farmers. As 60-70% of Dakar residents rely on UPA to meet daily dietary needs, year-round farming is necessary. Compounding this issue is the 306% increase in pesticide use by local farmers (de Bon et al. 2014). Vegetable farmers have been shown to use the greatest variety of insecticides in Senegal (20 or more compounds) and spray as frequently as every 3-10 days regardless of pest presence thereby adding selective xenobiotic pressure (Williamson et al. 2008; Chaudhuri 2009).

Most likely, contamination of ground water or soil in areas that flood and become larval habitats may have also contributed to resistance. Knowledge, attitude, and practice (KAP) surveys conducted with farmers found that they often rinsed spraying equipment or disposed of excess insecticide in or near local water sources (Ntow et al. 2006; Ngowi et al. 2007; Abuelmaali et al. 2013). Farmers have also been reported to spray more often in the rainy season increasing the amount flowing into local water sources (Williamson et al. 2008). Polluted water has been shown to exert a selective pressure on larvae resulting in increases in detoxification enzymes and a concomitant increase in resistance to insecticides in *Ae. aegypti* (Poupardin et al. 2008; David et al. 2010; Poupardin et al. 2012). Bendiocarb, as well as several other pesticides, have been shown to be extremely stable in run-off water sources (Lahr et al. 2000) and found in soil samples near agricultural sites (Yadouleton et al. 2010; Luc et al. 2016) long after they were employed. Thus, the frequent and often increasing use of pesticides and the concomitant contamination from run-off would result in highly focal or seasonal exposure levels exerting selective xenobiotic pressure on immatures residing therein.

While bendiocarb was not reported as being used for crop protection in Dakar, the carbamates carbofuran and carbosulfan are employed (Ba et al. 2016). Although primarily used in agriculture, carbosulfan has been considered for operational use in campaigns against malaria vectors in response to wide-spread pyrethroid resistance (Kolaczinski et al. 2000; Corbel et al. 2003). However, resistance to carbamates was seen to emerge quickly in *An. gambiae* when used alone (Corbel et al. 2003; Matowo et al. 2015). Resistance to carbosulfan in *An. gambiae* was found to be associated with an altered acetylcholinesterase target-site like what is seen in resistance to the oft-employed bendiocarb (N'Guessan et al. 2003). Carbamate resistance is typically associated with either metabolic resistance via esterases, broad-spectrum GSTs or through point-mutations within the target site of the acetylcholinesterase allele with the most commonly reported being *Ace*1-G119S (Hemingway 2000; Hemingway et al. 2004). Although SNPs in the *Ace1* gene have been shown to be associated with resistance to bendiocarb in many species (Alout and Weill 2008), the level of resistance to carbamates associated with *Ace*-119S differs between species (Corbel et al. 2007; Alou et al. 2010; Djogbenou et al. 2015). Previously, bendiocarb resistance has been associated with CYP over-expression in *An. gambiae* s.l. alongside a duplicated *Ace*1-119S allele (Edi et al. 2014; Antonio-Nkondjio et al. 2016), However their actual role in bendiocarb degradation has yet to be discerned with limited data showing some metabolism by CYP6Z1 in *An. funestus* (Ibrahim et al. 2016) and CYP6P3 in *An. gambiae* (Edi et al. 2014). Therefore, more specific genetic tools were needed to determine which mechanism or mechanisms are associated with bendiocarb resistance in this strain.

4.4.1 Quantitative trait loci

QTL mapping has been shown to be effective in detecting mechanisms of resistance in various anophelines (discussed in Chapter 2). Although several highly significant LOD regions were identified by QTL analysis, determining the genetic basis of bendiocarb resistance in the Rufisque population was inconclusive based on genes residing within the identified Bayes Credible regions of interest. This inability to detect specific genes associated with resistance may be due to one of several reasons. First, the presence of known inversions on the 2R chromosome present in the two colonies may have resulted in

differential segregation: 2Ra, less common except in *An. arabiensis* from the northern savanna regions from Senegal to the Sudan, and 2Rb which is common across the entirety of Africa (Coluzzi et al. 1979). Based on PCR data for the 2Rb inversion for which there is a molecular assay (Lobo et al. 2010) there was definite skewing as 100% of progeny from the Ruf x SENN stenogamous pairings were fixed for the inverted form while both forms were found in the SENN x Ruf force mated pairings (**Table 4.6**). Within each cross, however, there was no association of the 2Rb karyotype between resistant and susceptible individuals. Initial testing with both colonies found that 2Rb is fixed for the inverted form in Rufisque while it is heterozygous in SENN (data not shown). Inbreeding within the Ruf x SENN cross was apparently selective against the 2R+b/+b karyotype leading to the inverted form being fixed and potentially biasing towards genes within this region in the QTL analysis. While no assay has been developed to detect the 2Ra inversion, both strains are known to be heterozygous for this inversion (pers. comm. Nora Besansky and Igor Sharakhov). Additionally, of the 25 genes identified as uniquely over-expressed by microarray when comparing Rufisque to SENN alone, 16 (64.0%) were localized on the 2R chromosome with eight (32.0%) within the 2Ra and 2Rb inversions. Inversions, therefore, may have skewed QTL analyses by segregating relative to stenogamous mating by the parents or by minimizing recombination along the 2R between the two strains when cross-mated.

Table 4.6: Proportion of F2 individuals karyotyped by PCR for the 2Rb inversions in both the Rufisque x SENN cross (stenogamous) and the SENN x Rufisque cross (force mated).

	Phenotype	+b/+b	+b/b	b/b
SENN x RUF	Resistant	4	17	19
	Susceptible	8	21	20
RUF x SENN	Resistant	0	0	49
	Susceptible	0	0	47

Second, Rufisque was tested after only two generations had passed from the field. Based on initial microsatellite testing, this population had high genetic polymorphism at multiple loci and may have had unknown null alleles in a heterozygous state. Pairings between a heterozygote null and a homozygous parent could result in offspring with alleles artificially skewed towards the homozygous parent thereby confounding any real genetic association (Chapuis and Estoup 2007). Employing an advanced intercross strategy would have been more advantageous as it would have increased the probability of recombination between markers resulting in increased sensitivity to detect a true association however it still would have suffered from inaccuracy due to possible null alleles (Gonzales and Palmer 2014).

Third, there are 261 unknown, but hypothetically conserved genes located within the Bayes Credible regions estimated by QTL analysis. It is possible that one or more of these genes may regulate the resistance phenotype in a *cis*- or *trans*-acting manner or function down-stream regulating expression of detoxification genes such as microRNAs (mRNAs). In *Cx. pipiens pallens*, pyrethroid resistance associated with the cuticle gene *CpCPR4* was found to be controlled by the *trans*-regulatory miRNA miR-92A (Ma et al. 2016) while several miRNAs were implicated in regulation of CYPs and GSTs in the spider mite *Tetranychus cinnabarinus* (Zhang et al. 2016). Intriguingly, there are several miRNAs located within the identified BCI regions. This class of genes was not included in the microarray employed therefore their expression could not be evaluated however it is possible that one of these miRNAs could modulate one of the cuticle proteins or GSTd11 identified in the microarray analysis (Hong et al. 2014).

Fourth, the choice of comparison strain may have limited the ability to detect any meaningful QTL targets as SENN is known to be resistant to dieldrin, and partially resistant to deltamethrin and permethrin (Chapter 2). Resistance to dieldrin is maintained by periodic larval exposure which could have inadvertently led to increased levels of expression of one or more of the genes identified by microarray thereby diminishing their importance when compared to the two susceptible colonies. Over-expression of alleles associated with resistance in SENN could have reduced their significance in Rufisque

thereby resulting in an inability to detect them via QTL analysis however this is unlikely as there was no cross-resistance seen in SENN to organophosphates.

Last, in R/qtl most analyses assume that traits are monogenic in nature. Polygenic traits with small interactive effects between marker positions, therefore, may not be accurately identified by QTL analysis unless recombination rates between samples is high. Recombination rates in the Ruf x SENN cross may have been diminished as evidenced by potential non-random stenogamous mating selecting for 2Rb/b pairings. In Chapter 2, advanced intercross lines were employed to increase recombination rates thereby making QTL analysis more accurate however this was not done as it was hoped that QTL would help focus microarray results. Unfortunately, both potential candidates were found between peaks identified by QTL analysis: CPCLX2 on the 2R and CPCLG4 on the 3R (**Figure 4.7**), so the utility of QTL to detect regions of interest cannot be fully supported however specificity may have been increased if AIL crosses were employed.

4.4.2 Whole genome microarray

Unlike in QTL, several plausible candidate genes were identified when comparing the two susceptible populations to the Rufisque strain. The most intriguing result of the microarray phase was the primary identification of two cuticular proteins, CPLCG4 and CPLCX2, associated with bendiocarb resistance. Although not as commonly found associated with cuticular resistance as CPCLG3, CPLCG4 (previously referred to as CPLC#) has been shown to be over-expressed in *An. gambiae* from Nigeria resistant to pyrethroids (Awolola et al. 2009). This allele was also found to be over expressed via artificial induction by constant exposure of larvae to xenobiotics in the laboratory (Nkya et al. 2014). Although it not often associated with resistance, it is one of three CPLCG proteins (CPLCGs 3-5) expressed solely in the appendages in the adult stage and was experimentally shown to contribute to cuticle thickening in anophelines (Vannini et al. 2014). CPLCX2 is a member of a recently discovered novel low-complexity group of cuticle proteins with no known homologues in other insects and relatively few orthologues within anophelines (Cornman and Willis 2009). Although little is known about this group, expression for this gene was found to be higher in the pupal and pharate adult stages however it is mainly localized in

the insect head (Vannini et al. 2014) therefore it may not be involved in overall resistance unlike CPLCG4.

While several studies have shown over-expression of a few cuticular genes that may or may not be associated with resistance, there is limited data regarding the actual mechanism behind cuticular resistance with one study showing a link to cuticle thickness (Wood et al. 2010) and another to upregulation of CYP4G16 associated with cuticular hydrocarbon (CHC) production (Balabanidou et al. 2016) both resulting in a barrier that slowed the penetration rate of insecticides. Although neither cuticle thickness nor CHC content were determined, CHC over-production associated with CYP4G16 (AGAP001076) can possibly be eliminated as this gene was not found to be over-expressed in the microarray analysis nor was it detected as a QTL peak. Additionally, no other CYP was found to be consistently over-expressed however small, epistatic effects between CYPs and cuticular genes affecting resistance cannot be excluded.

Cuticle production presumably would require correlated over-expression of other alleles within the chitin synthesis pathway. Although expression of chitin synthesis proteins would be decreasing in pharate adults as cuticle formation is completed prior to eclosion, the over-expression of PGM1 may relate to the increased need of precursor elements for chitin synthesis, as well as other biological functions during metamorphosis.

Although cuticular thickening is the most likely cause of bendiocarb resistance in this strain, additional genes were identified which may be involved either in detoxification or enhancing it. Chymotrypsin 1 (CHYM1) was one of the three most over-expressed genes when comparing both susceptible colonies to Rufisque. Members of the chymotrypsin family are enzymes mainly associated with digestion in insects. Previously, weak metabolism of deltamethrin by chymotrypsin was reported in *Cx. pipiens pallens* resulting in an increase in resistance (Yang et al. 2008). Chymotrypsin, which can exhibit serine protease activity, has been shown to catalyze the hydrolysis of esters which could allow for it to affect the ester bond in bendiocarb resulting in detoxification. As these are primarily localized in the larval digestive tract, pesticide carryover could be ameliorated by these upregulated enzymes.

Although not definitively shown to be associated with resistance, the delta-class glutathione-s-transferase GSTd11 has been found to be differentially expressed regarding insecticide resistance in prior studies (Djouaka 2008, Jones 2012). If GSTd11 is involved in detoxification, then the over-expression of zinc-metalloprotease and APN1 might relate to their role in regulation of this gene. In one instance *Cx. pipiens pallens* was found with an over-expressed zinc-metalloprotease which strongly affected CYP6CP1 activity and expression resulting in deltamethrin resistance (Zou et al. 2016). Over-expression, however, was associated with susceptibility and resistance only being seen after the zinc metalloprotease was silenced. Therefore, this gene may not be directly regulating GSTd11, but another detoxification allele under-expressed and not estimated as significant by microarray. APN1 is a midgut localized digestive enzyme known to possess glutathione metabolism properties and is believed to be involved in the induction of detoxification enzymes associated with DDT resistance in *Musca domestica* (Ahmed et al. 1998). As with CHYM1, the localization of this enzyme in the larval digestive tract and its effects on xenobiotic detoxification that carryover through eclosion may be responsible for enhancing resistance.

Unusually, a majority of the previously identified over-expressed CYPs implicated in cross resistance, including CYP6Z1, CYP6P3 and CYP6M2, were significantly under-expressed in this study. Although the PBO synergist data is not definitive by itself, in previous studies partial or complete susceptibility to bendiocarb could be restored by pre-exposure to PBO (Oduola et al. 2012, Edi et al. 2014, Antonio-Nkondjio et al. 2016) while no difference in mortality was seen between exposed and un-exposed cohorts in this study. Thus, the microarray data combined with the PBO synergist data support the negligible role of CYPs conferring resistance in this study.

Paired QTL and microarray data have been used to accurately detect genes of interest associated with a particular phenotype, especially mutagenic traits (Fisher, Hedeler, et al. 2007; Jouffe et al. 2009). In these studies, QTL data are used to limit regions in which the most likely candidates are while microarray data are used to identify genes within these foci specifically associated with the phenotype. However, QTL/microarray studies have not always had concordant data. In determining organophosphate resistance in *Diabrotica*

virgifera virgifera, only one of four families used in the QTL analysis accurately detected a region in which a resistance allele identified by microarray resided (Coates et al. 2016). The inability to accurately detect QTL regions of interest in this earlier study were believed to be due to low recombination and poor marker coverage. Although marker coverage was relatively high in this study, the possibility of low levels of recombination along the 2R cannot be ruled out when considering the differential segregation of the 2Rb inversion in these experiments. Last, the Affymetrix chip was designed for *An. gambiae* and not *An. arabiensis*, therefore some alleles may not be accurately represented while the contribution of others may be artificially inflated. Additionally, qt-PCR of candidate genes would have been useful for confirming the role of the identified genes in resistance, however this was not possible as probes for these targets were difficult to validate.

4.5 Conclusion

UPA is necessary as urbanization, and the subsequent increase in local food insecurity, are expected to continue to rise over the next few decades across Africa (Cofie et al. 2003; UN 2014). Current estimates show that in some urban areas greater than 50-60% of all local foodstuffs are grown by UPA farmers. While larger, commercial farming operations are likely to use pesticides in a well-regulated manner, individual farmers are less likely to do so (Dinham 2003). This occasional, indiscriminate use of pesticides to protect food crops results in increased xenobiotic pressure on larvae due to run-off or point-of-use contamination. Constant sub-lethal xenobiotic exposures against mosquito larvae has resulted in an increase in expression of detoxification genes including several cuticular proteins in the laboratory (David et al. 2010, Nkya et al. 2014). In addition, unplanned urbanization results in an increase in potential larval habitats and local vector populations. Vectors, due to tolerances developed from various xenobiotic exposures, are thus capable of surviving in habitats once deemed unsuitable in many instances thereby exploiting more habitats within the urban area. These would thus represent a population that could maintain malaria transmission within the urban region and could be difficult to control via IRS or LLIN interventions.

The use of allopatric comparisons means that resistance in this strain is not definitively linked solely to carbamate exposure, however continued xenobiotic pressure from UPA in

and around Dakar has resulted in high levels of resistance to compounds in multiple classes which may have resulted in cuticular modification. Caution should be taken when choosing insecticides for controlling malaria vectors in urban areas with adjacent agricultural activities as novel resistance mechanisms may have arisen due to unknown xenobiotic pressures.

Chapter 5:

Summary and Conclusions

5.1 Introduction

It is undeniable that insecticide resistance in malaria vectors is increasing across SSA thereby reducing the number of licenced compounds operationally available and threatening the gains made in curtailing malaria transmission (Hemingway et al. 2016, Ranson et al 2016). Although adaptation to insecticides is occurring in vectors like *Anopheles gambiae* and *An. coluzzii*, their highly anthropophilic natures result in them constantly encountering current insecticide-based interventions (e.g. IRS, LLINs, insecticide-impregnated wall liners) therefore strategies focused on these areas will continue to be useful. For example, complete loss of function of LLINs due to insecticide resistance has not been reported. Instead, it has been shown that even in areas of high insecticide resistance, LLINs still provide some protection against malaria infection (Bradley et al. 2017, Ochomo et al 2017), however there is concern that the level of protection may not be as high due to heterogeneity in published data (Strode et al 2014). Moreover, reductions in local populations of *An. gambiae* and *An. funestus* attributed to LLINs have also been reported showing their effectiveness against anthropophagic vectors (Bayoh et al. 2010, Russell et al. 2011).

Conversely, while IRS and LLINs have had a suppressing effect on *An. gambiae, An. coluzzii* and *An. funestus*, they have been observed to have had a lesser effect on reducing populations of *An. arabiensis*. Pyrethroids typically do not possess any spatial-repellency attributes but are instead contact-killing or close-contact excito-repellent compounds that target endophilic vectors primarily (Achee et al. 2009). *An. arabiensis*'s exophilic nature and less stringent dietary requirements likely reduces time spent indoors, and thus time spent in contact with domestically applied insecticides, thereby circumventing many interventions. Mathenge et al (2001) found that although LLINs led to a reduction in human blood-feeding by *An. gambiae* and *An. funestus*, there was no noticeable reduction in human-biting by *An. arabiensis*. Additionally, even though local reductions in *An. gambiae* and *An. funestus* associated with LLIN usage have been shown it has not necessarily led to an increase in *An. arabiensis* populations. In Tanzania and Kenya, the number of *An. arabiensis* captured was unchanged compared *to An. gambiae* and *An. funestus* after

the introduction of LLINs (Bayoh et al. 2010, Mutuku et al. 2011, Russell et al. 2011). Therefore, the normally small populations of *An. arabiensis* became relatively more important in residual malaria transmission without increasing in numbers while the populations of *An. gambiae* were reduced to nil.

While insecticide resistance in *An. arabiensis* is relatively well documented in East and South Africa, resistance is less studied across West Africa (**Figure 5.1**). This heterogeneity in knowledge is compounded by a tendency for researchers to agglomerate members of the *An. gambiae* complex together precluding study of patterns between the different species (e.g. Ibrahim et al 2014, Cisse et al. 2015). Therefore, the actual extent of resistance and mechanisms involved is relatively unknown for this vector across most of its natural range.

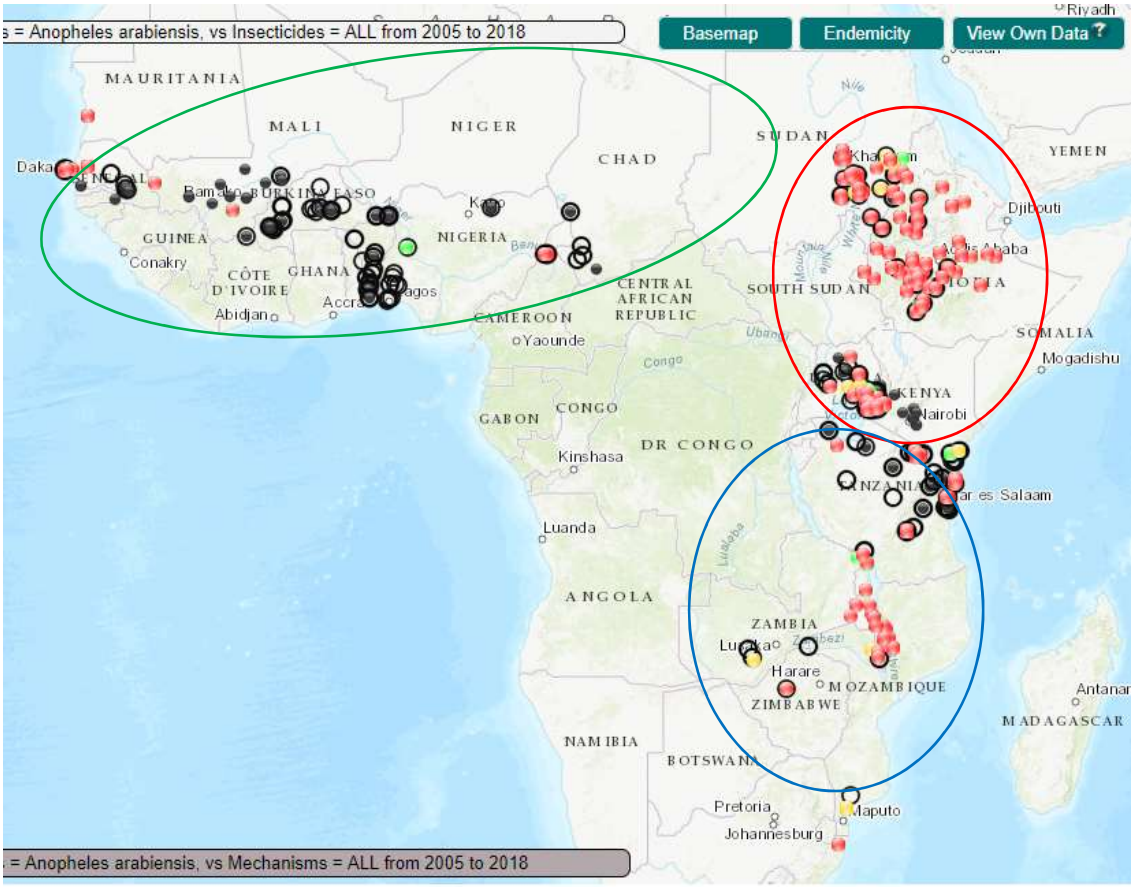


Figure 5.1: Insecticide resistance reported across sub-Saharan Africa for *An. arabiensis* with three of the largest regions identified (modified from Chapter 1). The green oval represents West Africa, East Africa is in red and South Africa is in blue. Black circles indicate the detection of a SNP associated with resistance while coloured circles indicate phenotypically associated insecticide resistance.

Second, the mechanisms underlying resistance are complex and to date there has been only limited use of tools such as QTL and microarrays. For example, microarray-based technologies have allowed for insecticide resistance-profiling in *An. gambiae* (David et al. 2005, Muller et al 2008) and QTL mapping has been used to identify regions associated with resistance in both *An. gambiae* and *An. funestus* (Ranson et al. 2000, Ranson et al. 2004, Wondji et al. 2007, Wondji et al. 2009). However, to date large-scale insecticide resistance profiling has rarely been done with *An. arabiensis* (Jones et al 2013, Witzig et al 2013, Matowo et al 2014). Additionally, although synteny is assumed to be high between *An. gambiae* and *An. arabiensis* (Wang et al 2001), caution should be taken when applying approaches developed in one species to another as significant differences in microsatellite diversity can exist even in closely related sibling species (Deitz et al 2012).

Further, the origin and spread of insecticide resistance in anopheline populations in SSA is known for only a few mechanisms (Pinto et al 2007, *Anopheles gambiae* 1000 Genomes Consortium 2017, Barnes et al. 2017). As discussed in Chapter 3, mechanisms such as *rdl* have been detected in various insect genera globally. The origin of *rdl* globally is mostly unknown as it is difficult to discern if it recently evolved due to insecticide pressure or if they have persisted either due to a lack of fitness costs or possibly to some continual, sublethal exposure. There is some evidence that persistence of certain alleles, such as *rdl*, may be due to "conditional-costs" intrinsically associated with their environment. In *Culex quinquefasciatus* and *Drosophila melanogaster*, pleiotropic effects associated with resistance were not seen unless resistant insects were exposed to a specific temperature (ffrench-Constant et al 1993, Hardstone et al 2009). Thus, populations not exposed to these conditions would be able to maintain resistance mechanisms with little to no fitness cost. Even when insecticide pressure is released, resistant populations have rarely completely reverted with appreciable numbers of individuals still found possessing the associated mechanism (Keiding 1963). Therefore, knowledge of potential detoxification mechanisms in populations of *An. arabiensis*, even when a specific insecticide is not being actively utilised, is necessary to best understand how to control this vector in the future.

With the increasing importance of *An. arabiensis* as a vector, particularly in urban settings, malaria in West Africa and the inability to reduce population sizes in East and South Africa, solutions for how best to control this species are vital as it will become relatively more important as populations

of *An. gambiae, An. coluzzii*, and *An. funestus* decline (Drake and Beier 2014). This PhD investigation was undertaken to determine the following:

1. Create a set of genetic tools (microsatellites and QTL) for investigating insecticide resistance in *An. arabiensis*
2. Apply a QTL proof-of-concept approach to a population of dieldrin resistant *An. arabiensis*; in part to validate tools on a phenotype expected to be mediated by one (*rdl*) or possibly a small number of loci
3. Determine if *rdl* in wild populations of *An. arabiensis* results from a single sweep event or if multiple origins have occurred and to look for compensatory mutations that may have resulted in persistence of this mutation.
4. Employ validated QTL tools and pair with microarray analysis to investigate novel carbamate resistance in a population from Dakar, Senegal.

5.2 Summary of results

As with previous research, several of the current microsatellite markers designed from *An. gambiae* were found to be non-informative in *An. arabiensis*. Poor amplification of primers originally designed for *An. gambiae* is often encountered when used in *An. arabiensis* (Temu et al 2005, Kent et al 2007) and can be attributed to variations in primer sites between the two species (Walton et al 1998). A similar experience was reported in *An. melas* (Deitz et al 2012) therefore caution should be used before employing these markers in any study aside from *An. gambiae*. During the initial QTL study, 58 microsatellites identified by Zheng et al (1996) were tested with only 10 found to be informative between the resistant and susceptible strains while the rest were non-informative or failed to amplify. Therefore, additional microsatellites were designed directly from the published *An. arabiensis* genome (Giraldo-Calderon et al 2015, Neafsey et al 2015). Based on these results an additional 74 microsatellites were identified with 16 found to be fully informative in the cross populations. In total 26 microsatellites and one SNP were employed providing extensive coverage across the genome except on the 3R (see Chapter 2 for discussion).

Although coverage was not as high as reported by Witzig et al (2013), a single, strong QTL was estimated in both the F2 and AIL families at a similar position on the 2L chromosomal arm. Surprisingly, in neither instance did the peak contain the *Gaba*-296S mutation, typically implicated

in dieldrin resistance, but was found 4Mb upstream towards the telomere. As stated in Chapter 2, the altered position could be related to an inability to accurately discern heterozygotes for *Gaba*-296S, however this many not be the only explanation. Some previous studies attempting to map dieldrin resistance have failed to associate resistance with *Gaba*-296S alone (Haridi 1974, Brooke et al 2006). Similarly, duplication of a part of the allele including *Gaba*-296S or the entire allele, as seen in *D. melanogaster* (Remnant et al 2013), could have also occurred which could explain the difficulty in discerning heterozygotes (Edi et al 2014, Weetman et al 2015). The preponderance of resistant heterozygotes at the 296-variant position (57/96) and lack of homozygous resistant individuals (1/96) falls outside of Hardy-Weinberg equilibrium ($\chi^2 = 4.01$) lends itself to the theory of heterozygote duplication as seen in *Ace*-1 (Edi et al. 2014, Weetman et al2015).

One interesting result was the determination of apparently limited impact of *Gaba*-296S on fitness of *An. arabiensis* in the laboratory. Rowland (1991) investigated several fitness parameters between resistant, heterozygous, and susceptible individuals including length of larval period, adult longevity, female fecundity, and circadian flight activity. While he found no difference in the duration of the immature stages between resistant and susceptible populations, in this study resistant individuals were more likely to pupate first and successfully eclose compared to susceptible ones providing a slight benefit. The only detrimental effect was that adult resistant females were not as long lived as their susceptible counterparts, a trend not seen in males, but was also observed in *An. gambiae* (Rowland 1991). Fecundity, which was lower in resistant *An. gambiae* mosquitoes for Rowland, was not tested in this study but would provide more evidence towards fitness costs of *Gaba*-296S.

Persistence of resistance is considered a deviation from the norm, as resistance mechanisms are hypothesized to be often associated with large fitness costs and expected to disappear when insecticide pressure is released. The presence of *Gaba*-296S in wild populations of *An. arabiensis* was investigated to see if the mutation was persisting in nature or if parallel evolution was occurring related to unknown, ongoing exposures, as well as for compensatory mutations that may have arisen to ameliorate fitness costs resulting in persistence (Maisnier-Patin and Andersson 2004). An approximately 2kb region covering exon 7 and spanning the intronic region before exon 8 was sequenced in individuals collected from the wild from the Sudan and Senegal and compared to the published sequence which is from a strain susceptible to dieldrin. In resistant individuals,

the same base-pair polymorphisms were seen within 10 bases after the *Gaba*-296S SNP regardless of the origin of the sample. Based on this evidence, it was concluded that a single origin of *Gaba*-296S had occurred with a subsequent sweep across the Sudano-Sahelian domain which encompasses both sites. A broad geographical spread of resistance alleles through a species is not unknown. In *Culex pipiens* an esterase associated with organophosphate resistance was determined to have a single origin with subsequent worldwide spread (Raymond et al 1991). Even within Africa, although several origins of *kdr* have been deduced, populations with the same haplotypes were found over large geographic areas (Pinto et al 2007, *Anopheles gambiae* 1000 Genomes Consortium 2017). This is the first study to look at the evolution and spread of *rdl* in anophelines, but it highlights an important point: the levels of any resistance mechanism may wane after the withdrawal of an insecticide however resistance may never truly disappear. Screening of local populations should be done to estimate the presence of these alleles as they may cause operational problems in the future due to unintended cross-resistance with insecticides like fipronil, deltamethrin, neonicotinoids or other GABAergic compounds (Taylor-Wells et al 2015).

The final phase of this research was to investigate bendiocarb resistance in a population originating from an urban area of Dakar, Senegal. Although the parental strain was highly resistant to several compounds, bendiocarb resistance was chosen as this compound has not been used in the Dakar area to control malaria vectors hence exposure was more than likely a result of urban agricultural practices. Of the original 26 microsatellites used in Chapter 2, only 13 were considered fully informative owing to high levels of variability within the Dakar population. Revisiting primers originally designed for *An. gambiae* (Zheng et al 1996) provided six new markers and an additional 15 were designed using the same strategy employed to detect markers in Chapter 2. When both intercrosses were combined for analysis, the strongest associations were seen at R27 (2R: 50.3719, LOD 24.458), CDC21 (2L: 9.98095, LOD 28.782), CDC32 (3R: 19.092, LOD 5.023), and CDC44 (3L: 32.241, LOD 7.931). To compliment the QTL analysis, additional samples were subjected to microarray analysis. Microarray analysis comparing the susceptible strains to Rufisque found the cuticular protein genes CPLCX2 and CPLCG4 as well as a glutathione-s-transferase, GSTd11, as over-expressed. It also identified phosphoglucomutase, chymotrypsin, alanyl aminopeptidase, and a zinc metalloprotease as highly over-expressed. When the results from the QTL and microarray were compared, only three BCI peaks were found to localized near an allele identified by microarray: hypothetical protein AGAP003767 (near CDC16), hypothetical protein AGAP011850

(near CDC44), and phosphoglucomutase AGAP005860 (near CDC27). Paired QTL/microarray analyses have been used before to determine genes of interest, however it is not always successful (Coates et al 2016). Potential low levels of recombination in the F2 intercrossed families may have hindered QTL from accurately identifying regions of interest. Interestingly, only the 2Rb/Rb inversion was found in the progeny resulting from the Rufisque♀ x Sennar♂ stenogamous cross while both inversion forms were found in the eurygamous reciprocal pairings. The lack of 2R+/b individuals in the stenogamous pairing could be the result of selective mortality in hybrids as well. Therefore, probable selective mating may have reduced high levels of recombination necessary for QTL analyses. The most interesting result of this phase was the incrimination of CPLCG4 as the likely primary mode of resistance. In most other studies in which cuticular genes are identified, other metabolic detoxification enzymes are often found over-expressed in tandem (Vontas et al 2007, Awolola et al. 2009, Yahouedo et al 2017), however in this study most of the oxidases and esterases were highly down-regulated. Thus, this study adds to the growing body of evidence that agricultural run-off can elicit resistance via novel detoxification pathways including cuticular resistance (David et al. 2010, Nkya et al. 2014).

5.3 Limitations

Although the initial phase of this study achieved it primary goals, there yet remains a possibility that the assumptions could have had been more robust. Regarding QTL analysis, synteny of genes between *An. gambiae* and *An. arabiensis* is assumed to be high except for the X chromosome, however a microsatellite map exists for the former species alone. Therefore, absolute synteny cannot be assumed and the QTL results discerned during this research may indeed be accurate with the position of *Gaba*-296S 4Mb further upstream. Additionally, as no copy-number variant (CNV) assay was performed, duplication of the *rdl* allele cannot be ruled out as a potential reason for the distance between the assumed position and the QTL LOD peak.

Second, due to low levels of homozygous *Gaba*-296S individuals detected in the wild by PCR, a more robust examination of this mutation across its geographic range could not be completely done. Sequencing a dieldrin resistant colony found that there were few polymorphisms between the two Sudanese populations even though they were separated by over a decade from the field.

Finally, regarding the QTL/microarray investigation of bendiocarb resistance in *An. arabiensis*. Using marker regression, Bayes Credible Intervals were identified near microsatellites R27 (2R chromosomal arm), CDC21 (2L), CDC32 (3R), and CDC44 (3L), none of which contained known resistance mechanisms. Although QTL has been shown repeatedly to detect resistance loci in several species successfully, the failure of it in this study is most likely due to the choosing of an inappropriate out-comparison group. The choice to attempt to discern two different resistance profiles from one cross led me to use Sennar, which has low levels of organochlorine resistance aside from its dominant dieldrin resistance. Furthermore, QTL may not be entirely suitable for polygenic resistance unless AIL crosses were employed to maximize recombination between the various alleles responsible for the phenotype or if there were a few localised regions of resistance as seen in *An. gambiae* (Ranson et al. 2004).

5.4 Future

Screening for mechanisms associated with insecticide detoxification should be a priority for the major malaria vectors in SSA but especially in *An. arabiensis* as this species is relatively understudied compared to *An. gambiae* and *An. funestus.* As current vector control insecticides appear to be failing due to resistance, this information could be used to develop novel insecticides or strategies that specifically target this species (Ranson and Lissenden 2016). Validation of these mechanisms could be explored through knock-out (KO) or targeted loss of function studies to determine their actual role in either detoxification, sequestration, or elimination of insecticides (McCart and ffrench-Constant 2008, Zimmer et al 2016).

Of primary interest would be to ascertain the actual role of CPLCG4 in insecticide resistance. Although it has been shown to be highly over-expressed in resistant *An. stephensi* and *An. gambiae* (Vontas et al. 2007, Awolola et al. 2009), it would be beneficial to extend this research to show definitively the role the cuticle plays in xenobiotic resistance. Previous validation studies of resistance mechanisms have been done by inserting the allele in *Xenopus* oocytes to determine their functionality (Meyers et al 2015, Taylor-Wells et al 2015). However, as CPLCG4 is involved in cuticle development in mature insects and primarily expressed during pupation, it would not express properly as there is no loss-of-function to test for. Alternatively, employing a ϕC31 docking strain would allow for inserting a copy of CPLCG4 to see if increased transcription of this allele produces cuticular resistance alone or if it is part of a more sophisticated resistance pathway.

Additionally, assaying CPCLG4 levels in wild populations using quantitative PCR (qPCR) would be beneficial as it is unknown if elevated expression levels are related resistance or if there are natural, varying levels or if over-expression is associated with aridity or another climatic conditions.

Another interesting result in which research should continue is to determine why pirimiphos methyl was the only compound tested that resulted in 100% mortality in exposed individuals. Major resistance to seven compounds representing the four major classes of insecticides used in public health was found in this population including organophosphates and carbamates. The presence of *Ace*-119S, which confers resistance to carbamates, was not detected in the Dakar population, therefore resistance was not associated with this SNP. Pirimiphos-methyl is an acetylcholinesterase inhibitor targeting AChE like bendiocarb, therefore, the mode of action of pirimiphos-methyl should be investigated as it may highlight a new pathway that could be exploited in future insecticide development.

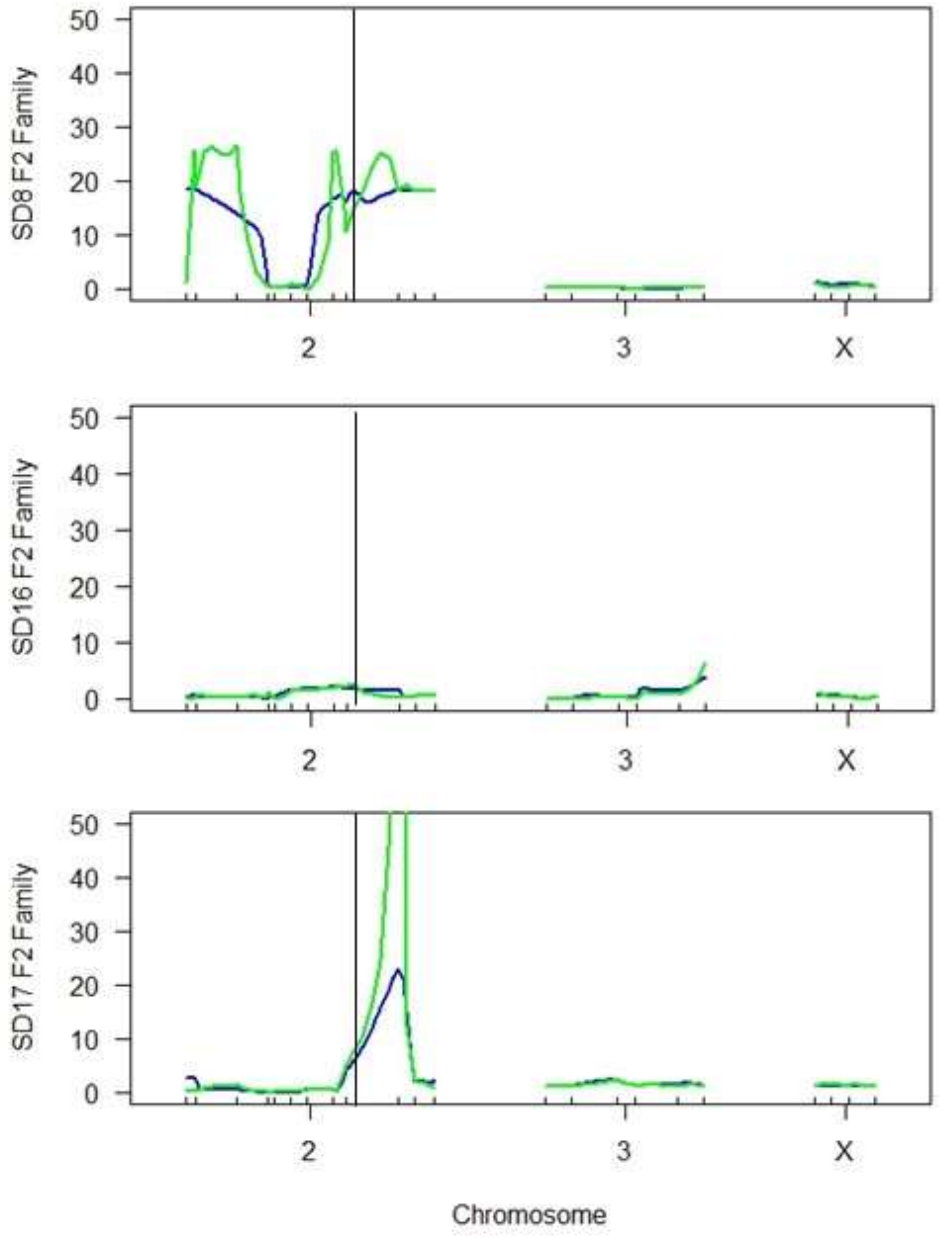
5.5 Conclusions

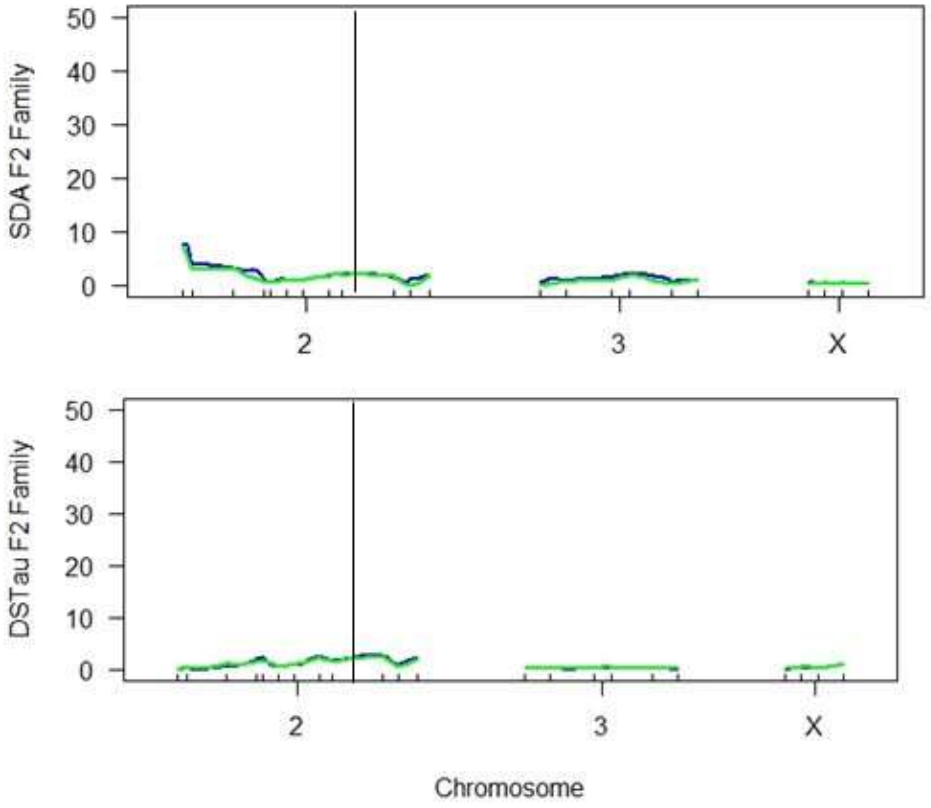
The previous chapters in this dissertation highlight several important attributes that make *An. arabiensis* a particularly difficult species to control. Vectors have responded time and time again to anthropogenic challenges with evolutionary changes including adapting novel behaviours, the ability to survive in polluted environments, as well as insecticide resistance. Across SSA, and especially in the Sudano-Sahelian domain, urbanisation has had a major impact on the evolution of these traits in *An. arabiensis*, especially insecticide resistance (Jones et al 2012). Phenotypic evolution is amplified by both high plasticity in the vector and multiple selective pressures acting at the same time (Alberti et al 2017). Urban and peri-urban agriculture (UPA) and the often-indiscriminate use of pesticides has only served to amplify adaptation in select vectors in many urban centres. UPA is necessary food insecurity is expected to continue to rise over the next few decades across Africa associated with urbanisation (Cofie et al. 2003). Therefore this, coupled with the ability for *An. arabiensis* to invade and survive in polluted waters associated with urban centres (Jones et al 2012), means that this species could become more important in malaria transmission in this region and potentially be difficult to control due to evolving resistance.

The inability to effectively control *An. arabiensis* is related to its plastic and primarily exophilic nature. Even when its behaviour is well documented, such as zoophagy in East Africa, control is often difficult as resistance is widespread to the compounds that can be used safely on livestock. Even when resistance is not an issue, insecticide coverage on animals is not uniform and biting by *An. arabiensis* has been shown to occur more readily on poorly treated parts of the animal (Habtewold et al. 2004). These continual, sublethal exposures likely contribute to the rise of insecticide resistance. In other parts of Africa, this species is more anthropophagic (see Chapter 1), however it is still primarily exophilic in nature thereby circumventing most interventions (Fornadel et al 2010). Resistance in these areas has been shown to consist primarily of target-site modifications and arises naturally through parallel evolution under insecticide pressure (Wilding et al 2015) or via introgression from a member of the *An. gambiae* complex (Kawada et al 2011). Tools to detect such mechanisms in the field are lacking as many are unknown, however studies like this help to elucidate novel mechanisms for which assays can be developed.

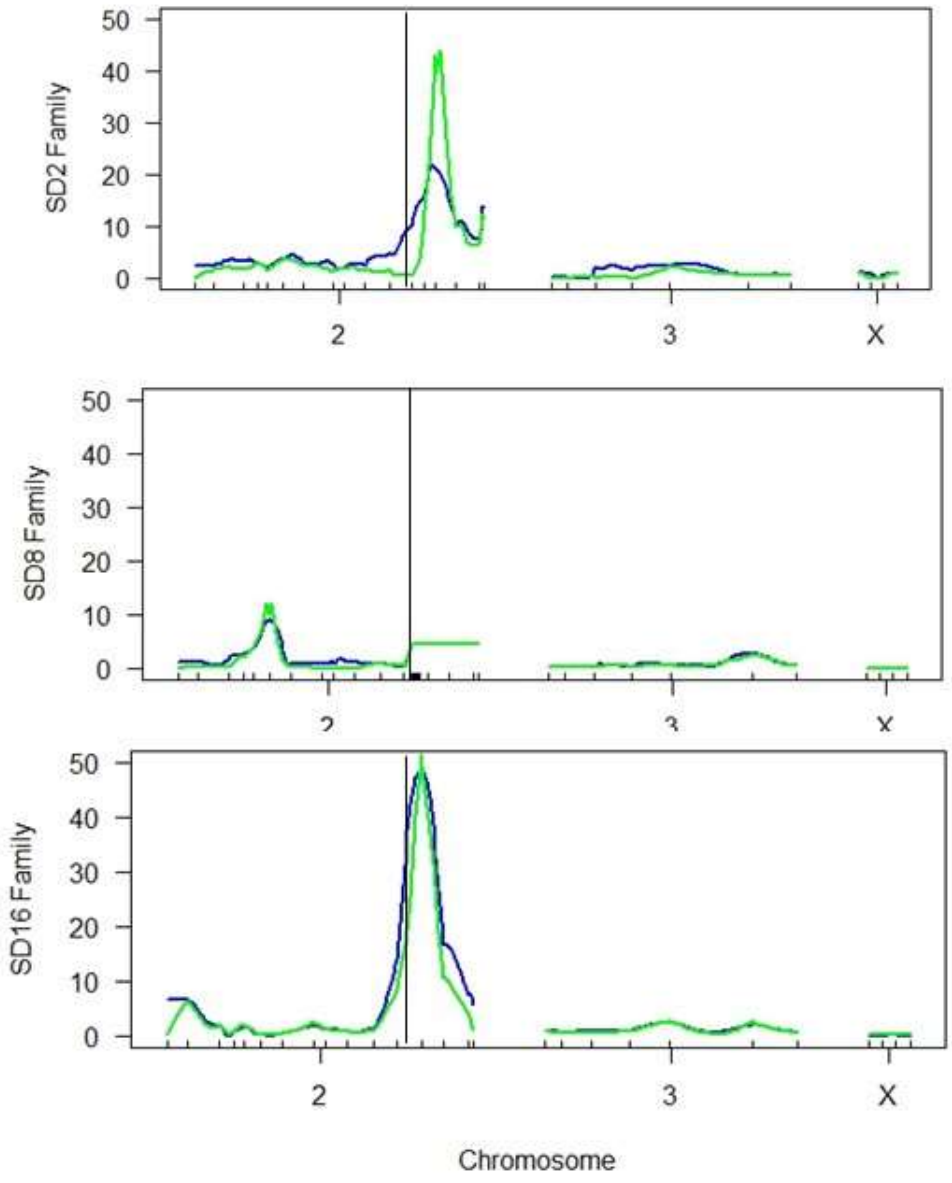
Atypical exposures, be they agricultural-associated or contact with polluted urban habitats, are becoming more important as xenobiotic pressures against *An. arabiensis*. The ability to quickly detect the underlying causes of resistance, either by QTL or microarray analysis, is paramount in effectively controlling this species and in developing assays to employ in the field. Last, the selection of insecticides to employ should be done with caution. As shown, persistence of resistance even 40 years after withdrawal of a compound is possible. However, as recent research has shown, these mechanisms arise and spread quickly and persist as they apparently carry little or no fitness cost to the vector and in some instances, provide protection to non-target xenobiotics encountered in the environment. My hope is that this body of work will stimulate research into the effects of urbanisation and resistance in *An. arabiensis*, especially in areas where UPA is present. The creation of a suite of new microsatellite markers which cover a large portion of the genome, paired with a readily available and sequenced colony, will allow for more robust analyses to be done with this species.

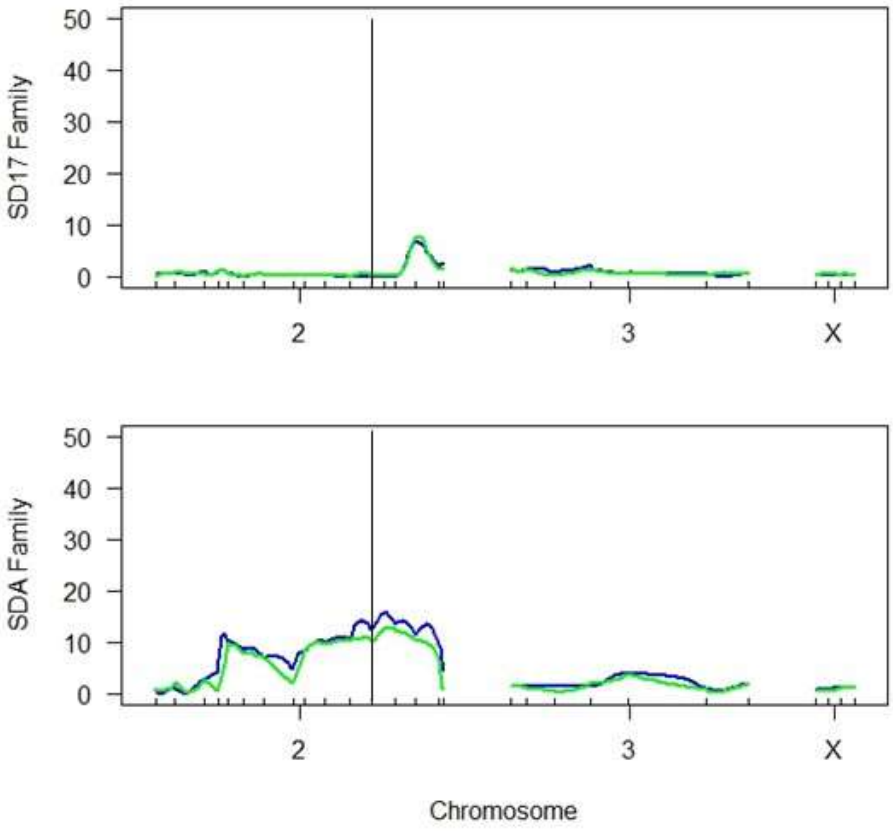
Appendix Figure 1: LOD scores for Haley-Knott regression (blue) and multiple-imputation (green) QTL analyses of individual families that comprise the F2 backcross based on 24-hour survivorship after 1-hour exposure to 4% dieldrin. The position of *rdl* is denoted by the black line. Note in families SDA, SD16 and DSTau, no marker reached the LOD threshold.



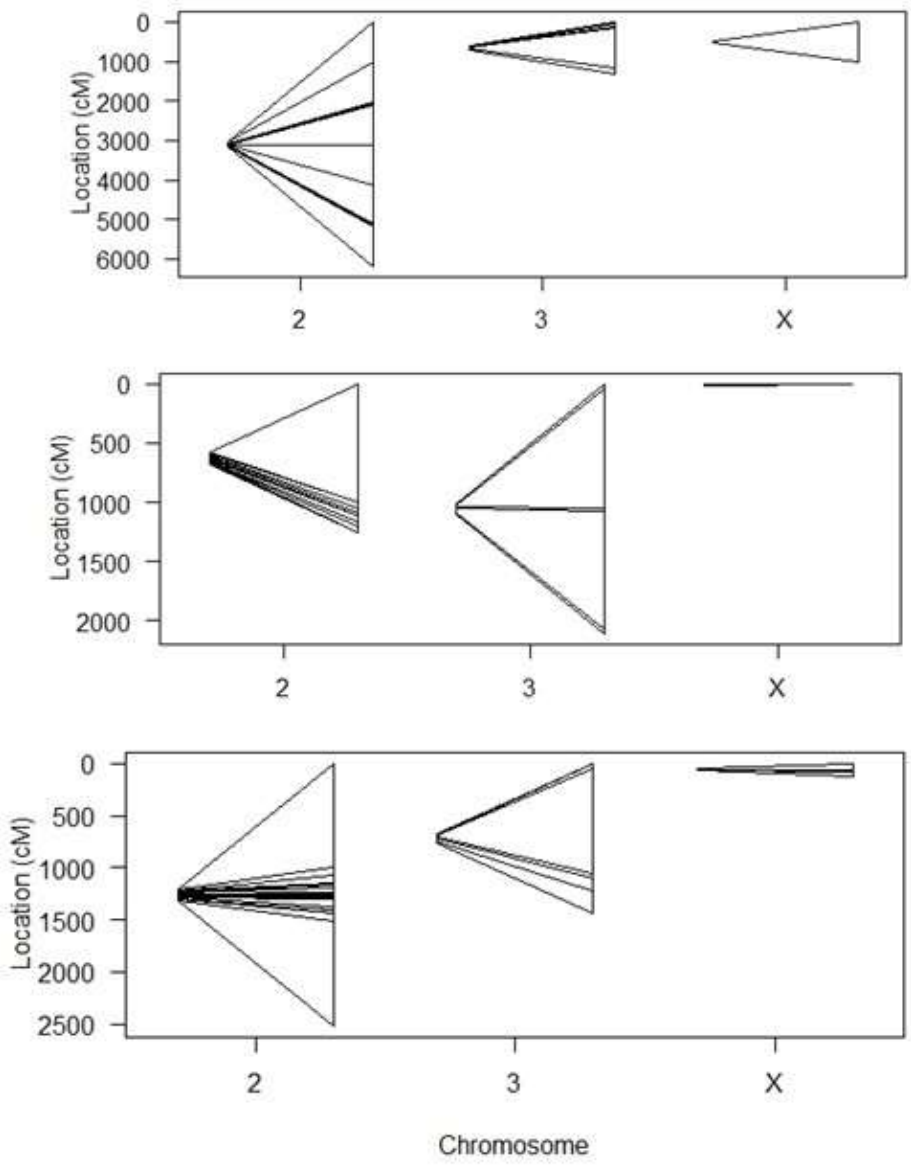

SDA F2 Family
50
40
30
20
10
0
2
3
X
DSTau F2 Family
50
40
30
20
10
0
2
3
X
Chromosome

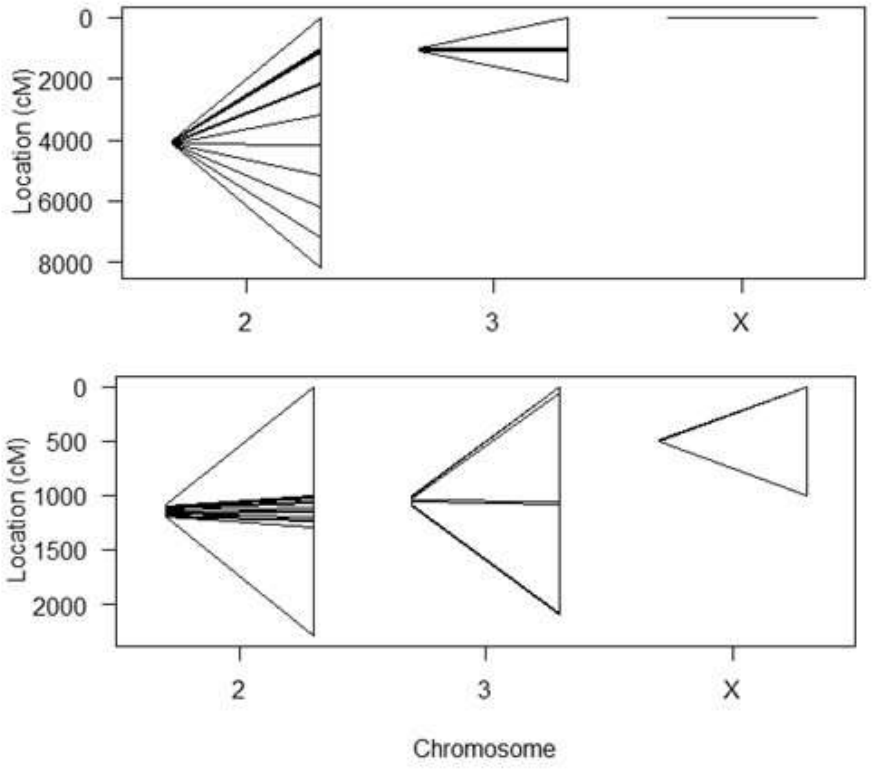
Appendix Figure 2: LOD scores for Haley-Knott regression (blue) and multiple-imputation (green) QTL analyses of individual families that comprise the F8 AIL intercross based on 24-hour survivorship after 1-hour exposure to 4% dieldrin. The position of *rdl* is denoted by the black line.



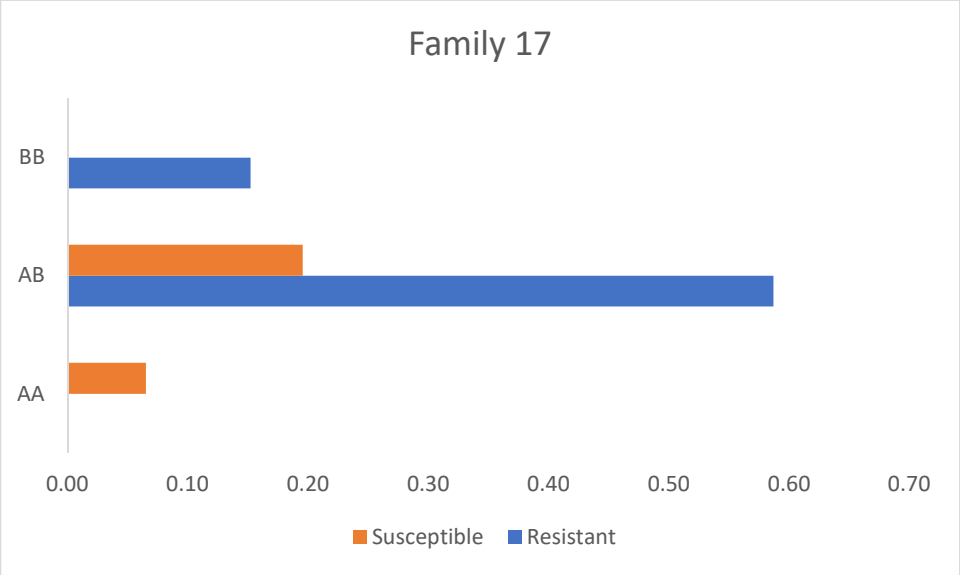

SD17 Family
50
40
30
20
10
0
2
3
X
SDA Family
50
40
30
20
10
0
2
3
X
Chromosome

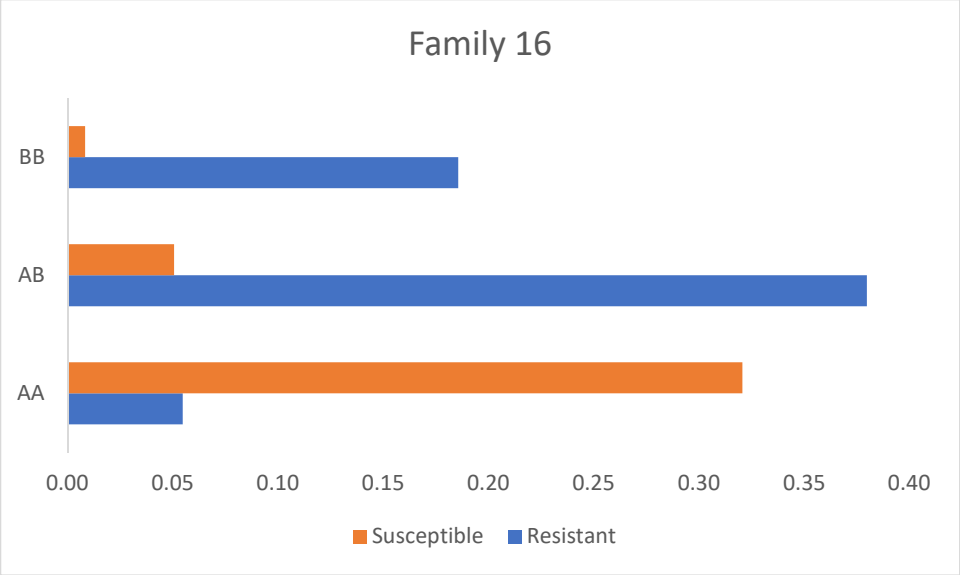
Appendix Figure 3: Comparison of genetic maps generated by r/qtl for individual families. These are presented in the same order as in Appendix Table 1.




Location (cM)
0
2000
4000
6000
8000
2
3
X
Location (cM)
0
500
1000
1500
2000
2
3
X
Chromosome

Appendix Figure 4: The mortality rate compared to the number of alleles inherited from their respective parents for 2 AIL families for marker CDC675.





Appendix Table 1: Hardy-Weinberg exact test for markers to determine if any had significant association with dieldrin resistance.

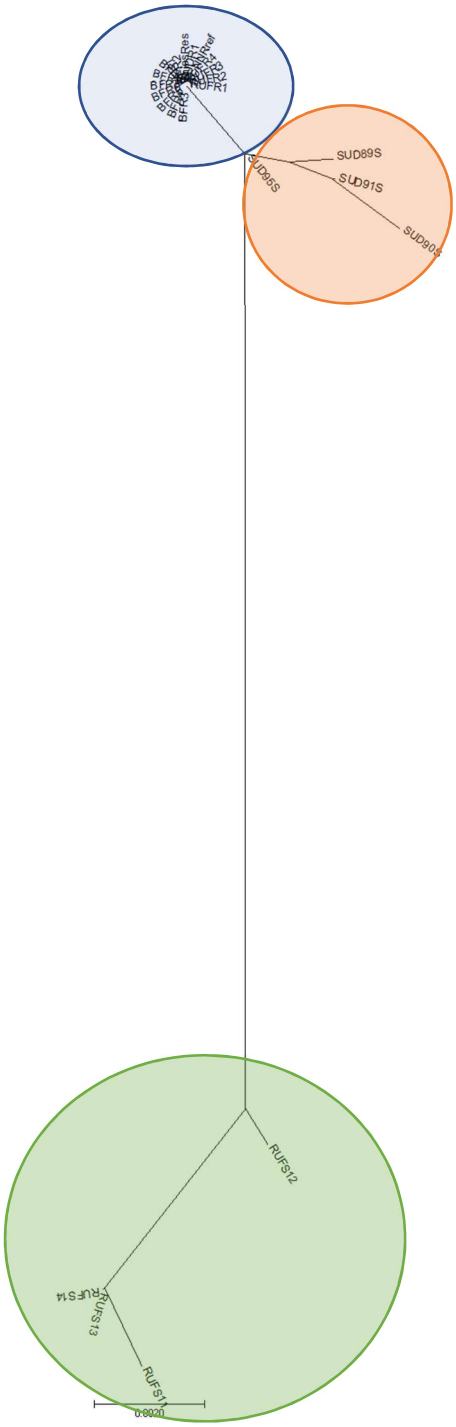
```
========================================
     Results by population
========================================


 Pop : SD17 Family
----------------------------------------
                             Fis estimates
                            ---------------
locus       P-val   S.E.    W&C     R&H     Steps
----------- ------- ------- ------- ------- ------
Ag7          No information.
CDC2         No information.
Ag53         No information.
CDC5        0.0184    -      0.3770  0.3827     16 matrices
CDC8        0.0000    -      0.7755  0.7914     13 matrices
Ag79        1.0000    -     -0.0714 -0.0722      4 matrices
2RiS5       0.2424    -     -0.2069 -0.2087     12 matrices
Ag95        0.2438    -     -0.2298 -0.2318     13 matrices
Ag135       0.2198    -     -0.2102 -0.2120     19 matrices
CDC16       0.0422    -     -0.3333 -0.3358     12 matrices
CDC18C      1.0000    -     -0.0714 -0.0722      4 matrices
CDC19       1.0000    -     -0.0602 -0.0609      4 matrices
CDC20       0.2941    -      0.1861  0.1887      5 matrices
CDC21F       No information.
CDC23       0.0000    -      0.8794  0.8977      5 matrices
RDL         0.0000    -     -0.6586 -0.6610     22 matrices
CDC675      0.0002    -     -0.5698 -0.5724     22 matrices
Ag143       0.0642    -     -0.3011 -0.3034     19 matrices
CDC28A      0.5540    -     -0.1019 -0.1029     20 matrices
CDC28C      0.2198    -     -0.2102 -0.2120     19 matrices
Ag128       0.3860    -      0.1396  0.1413     23 matrices
Ag249       0.0863    -     -0.2941 -0.2964     11 matrices
CDC34       0.0863    -     -0.2941 -0.2964     11 matrices
3E36D       0.0387    -      0.3223  0.3271     14 matrices
CDC43       0.1706    -     -0.2394 -0.2415     10 matrices
CDC46       1.0000    -      0.0070  0.0071     19 matrices

All (Fisher's method):
 Chi2 :    146.2012
 Df   :    44.0000
 Prob :    0.0000

 Pop : SDA Family
----------------------------------------
                             Fis estimates
                            ---------------
locus       P-val   S.E.    W&C     R&H     Steps
----------- ------- ------- ------- ------- ------
Ag7          No information.
CDC2        0.3764    -     -0.0816 -0.0818     39 matrices
Ag53         No information.
CDC5        0.0001    -      0.3260  0.3274     40 matrices
```

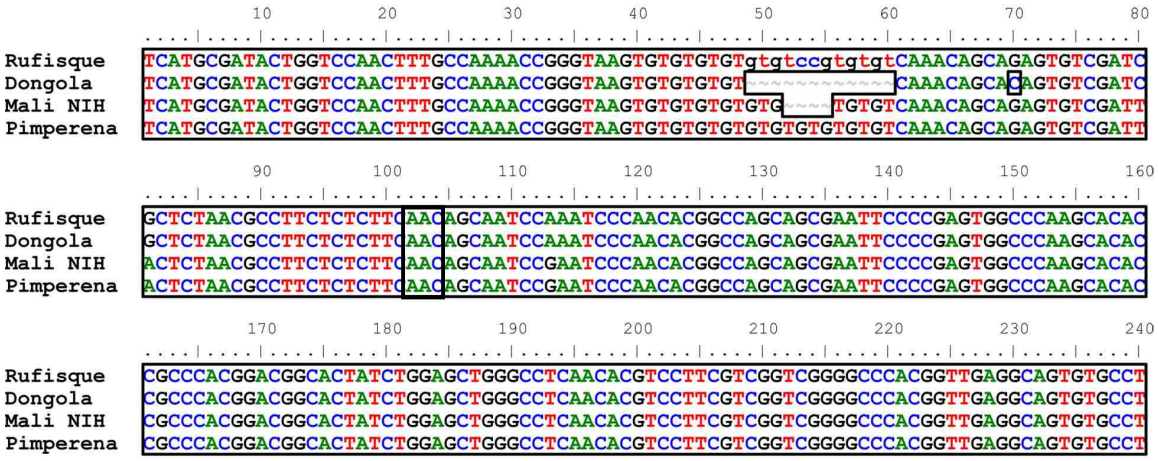
```
CDC8        0.0021    -      0.3223  0.3235     12 matrices
Ag79        1.0000    -     -0.0415 -0.0416     18 matrices
2RiS5        No information.
Ag95        0.0000    -     -0.3177 -0.3184     48 matrices
Ag135       0.0015    -     -0.2379 -0.2384     51 matrices
CDC16       0.0003    -     -0.2629 -0.2635     42 matrices
CDC18C      0.0001    -      0.3657  0.3671     17 matrices
CDC19       0.0000    -     -0.3358 -0.3365     45 matrices
CDC20       0.0493    -     -0.1581 -0.1586     46 matrices
CDC21F      0.0001    -     -0.2829 -0.2836     45 matrices
CDC23       0.0000    -      0.8746  0.8797     44 matrices
RDL         1.0000    -     -0.0031 -0.0031     84 matrices
CDC675      0.1785    -     -0.1065 -0.1067     60 matrices
Ag143       0.0000    -     -0.3040 -0.3046     51 matrices
CDC28A      0.2158    -     -0.0999 -0.1002     76 matrices
CDC28C      0.6369    -     -0.0414 -0.0416     72 matrices
Ag128       0.0000    -     -0.4315 -0.4322     57 matrices
Ag249       0.0000    -     -0.4678 -0.4685     56 matrices
CDC34       0.0156    -     -0.1761 -0.1765     26 matrices
3E36D       0.0000    -      0.5298  0.5325     53 matrices
CDC43       1.0000    -     -0.0301 -0.0302      6 matrices
CDC46       1.0000    -     -0.0241 -0.0241     21 matrices

All (Fisher's method):
 Chi2 :    Infinity
 Df   :    46.0000
 Prob :    High. sign.

 Pop : SD2 Family
-----------------------------------------
                            Fis estimates
                           ---------------
locus       P-val   S.E.    W&C     R&H     Steps
----------- ------- ------- ------- ------- ------
Ag7         0.0000    -      0.9115  0.9244     30 matrices
CDC2        0.5895    -     -0.1453 -0.1462     10 matrices
Ag53        0.5829    -     -0.1261 -0.1269      9 matrices
CDC5        0.0000    -      0.6152  0.6227     25 matrices
CDC8        0.0015    -      0.4335  0.4382     14 matrices
Ag79        0.2695    -      0.1316  0.1327      9 matrices
2RiS5       0.0851    -     -0.2331 -0.2345     22 matrices
Ag95        0.0000    -     -0.4667 -0.4685     22 matrices
Ag135       0.0032    -     -0.3553 -0.3570     21 matrices
CDC16       0.0008    -     -0.3814 -0.3832     20 matrices
CDC18C      0.0000    -     -0.5056 -0.5075     24 matrices
CDC19       0.0003    -     -0.4194 -0.4212     21 matrices
CDC20       0.0017    -     -0.3608 -0.3626     19 matrices
CDC21F       No information.
CDC23       0.0000    -      0.9515  0.9656     13 matrices
RDL         0.1521    -     -0.1785 -0.1796     32 matrices
CDC675      0.0917    -     -0.2117 -0.2130     31 matrices
Ag143       0.1331    -     -0.1955 -0.1967     28 matrices
CDC28A      0.0128    -     -0.3161 -0.3177     30 matrices
CDC28C      0.0001    -     -0.4565 -0.4584     22 matrices
Ag128       0.0000    -     -0.4889 -0.4907     23 matrices
Ag249       0.0000    -     -0.5349 -0.5367     24 matrices
CDC34       0.0000    -     -0.5172 -0.5191     24 matrices
```

```
3E36D       1.0000    -     -0.0160 -0.0161     25 matrices
CDC43       0.0027    -     -0.3819 -0.3837     31 matrices
CDC46       0.0068    -     -0.3393 -0.3410     21 matrices

All (Fisher's method):
 Chi2 :    Infinity
 Df   :    50.0000
 Prob :    High. sign.

 Pop : SD16 Family
----------------------------------------
                            Fis estimates
                           ---------------
locus       P-val   S.E.    W&C     R&H     Steps
----------- ------- ------- ------- ------- ------
Ag7         0.0000    -      0.5954  0.5972     55 matrices
CDC2        0.0000    -      0.3785  0.3795    129 matrices
Ag53        0.0000    -      0.5318  0.5333     56 matrices
CDC5        0.0000    -      0.4304  0.4317    120 matrices
CDC8        0.0000    -      0.8164  0.8193     25 matrices
Ag79        0.1937    -      0.0871  0.0873     82 matrices
2RiS5       0.0020    -      0.1945  0.1950     86 matrices
Ag95        0.4324    -     -0.0510 -0.0511    100 matrices
Ag135       0.2565    -      0.0750  0.0752    113 matrices
CDC16       1.0000    -      0.0065  0.0065     92 matrices
CDC18C      0.8864    -     -0.0136 -0.0137     83 matrices
CDC19       0.0003    -     -0.2264 -0.2268     99 matrices
CDC20       0.0536    -     -0.1281 -0.1283     90 matrices
CDC21F      0.0000    -      0.3012  0.3020    127 matrices
CDC23       0.0000    -      0.7377  0.7402     50 matrices
RDL         0.3749    -      0.0567  0.0568    110 matrices
CDC675      0.2053    -      0.0791  0.0793    109 matrices
Ag143       0.4566    -     -0.0530 -0.0531    123 matrices
CDC28A      0.0248    -     -0.1458 -0.1461     87 matrices
CDC28C      0.7994    -     -0.0183 -0.0183    109 matrices
Ag128       0.0000    -     -0.4440 -0.4445     88 matrices
Ag249       0.0000    -     -0.4700 -0.4705     89 matrices
CDC34       0.0007    -     -0.1864 -0.1867     42 matrices
3E36D       0.0000    -      0.3213  0.3223     86 matrices
CDC43       0.0000    -      0.3041  0.3048     87 matrices
CDC46       0.0133    -     -0.1558 -0.1561    128 matrices

All (Fisher's method):
 Chi2 :    Infinity
 Df   :    52.0000
 Prob :    High. sign.

 Pop : SD8 Family
----------------------------------------
                            Fis estimates
                           ---------------
locus       P-val   S.E.    W&C     R&H     Steps
----------- ------- ------- ------- ------- ------
Ag7          No information.
CDC2         No information.
Ag53         No information.
CDC5         No information.
```

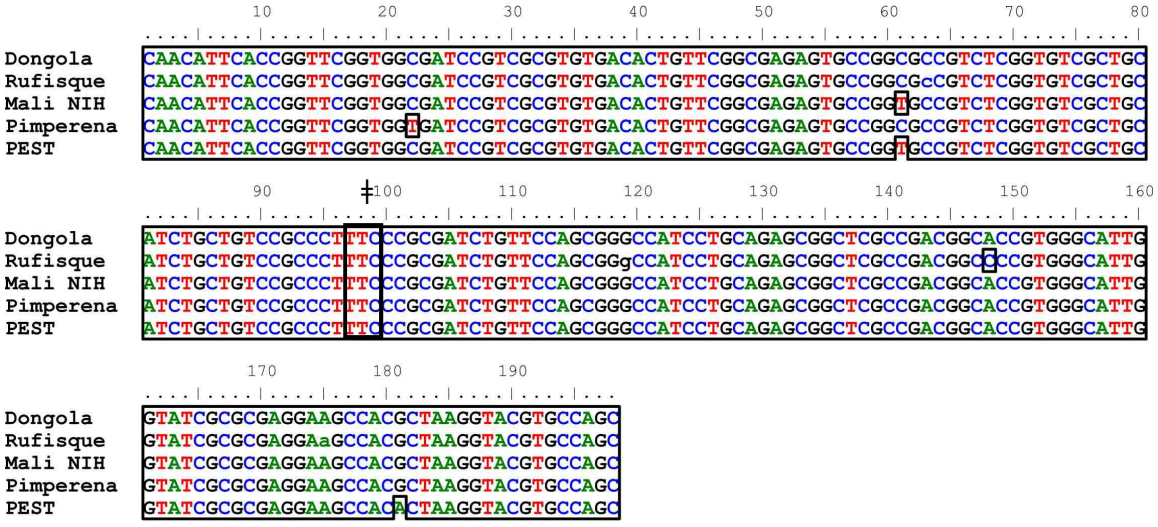
```
CDC8         No information.
Ag79         No information.
2RiS5       0.6198    -      0.1845  0.1896      8 matrices
Ag95        0.1509    -      0.3402  0.3499      8 matrices
Ag135       1.0000    -      0.0108  0.0110      6 matrices
CDC16        No information.
CDC18C       No information.
CDC19        No information.
CDC20        No information.
CDC21F      0.1269    -     -0.3939 -0.3990      8 matrices
CDC23       0.0638    -      0.6567  0.6802      2 matrices
RDL         0.0244    -     -0.4839 -0.4891      9 matrices
CDC675      0.0198    -     -0.5333 -0.5386      9 matrices
Ag143       0.0198    -     -0.5333 -0.5386      9 matrices
CDC28A      0.0198    -     -0.5333 -0.5386      9 matrices
CDC28C      0.0198    -     -0.5333 -0.5386      9 matrices
Ag128       0.0244    -     -0.4839 -0.4891      9 matrices
Ag249       0.0244    -     -0.4839 -0.4891      9 matrices
CDC34       1.0000    -     -0.1795 -0.1826      5 matrices
3E36D       0.0040    -      0.6303  0.6534      9 matrices
CDC43       0.0813    -     -0.4046 -0.4097     10 matrices
CDC46       0.0832    -      0.4081  0.4204     10 matrices

All (Fisher's method):
 Chi2 :    89.0551
 Df   :    32.0000
 Prob :    0.0000
========================================
 All locus, all populations
========================================
All (Fisher's method) :
 Chi2 :    Infinity
 Df   :    196.0000
 Prob :    High. sign.
```

Appendix Figure 5: The evolutionary history of *Gaba*-296S in the limited sample was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 0.01255012 is shown. Evolutionary analyses were conducted in MEGA7. All resistant individuals are clustered in blue while susceptible Sudan (orange) and Senegal (green) group separately.

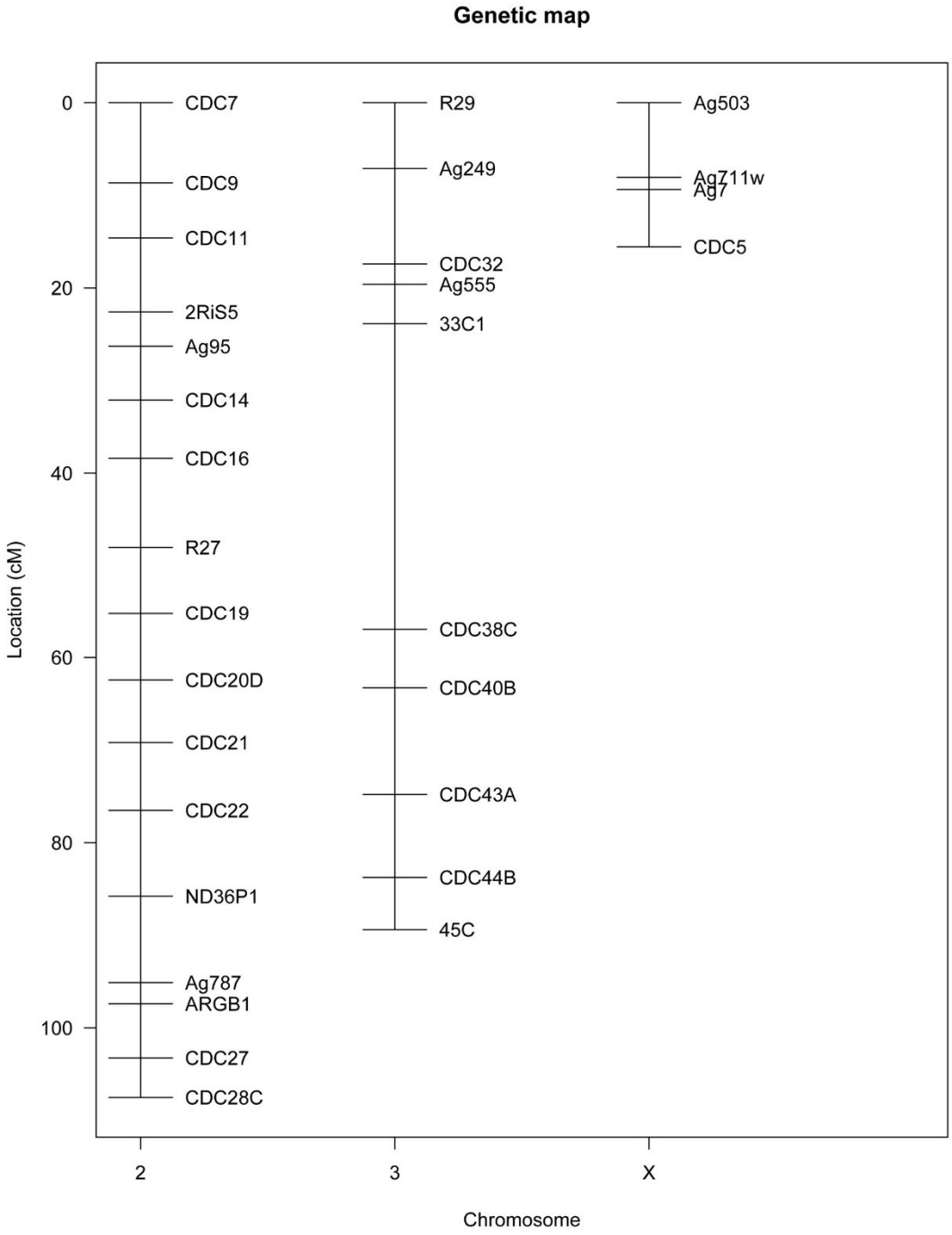




Appendix Figure 6. Sequence data for *Ace*-1 showing that Rufisque does not possess the N485I mutation (AAC asparagine between residues 103-105 using torpedo numbering and highlighted by a box) compared to three published sequences from VectorBase: *An. gambiae, An. coluzzii*, and *An. arabiensis,* all known to be susceptible to bendiocarb. The microsatellite region with the variable copy number is intronic and thus not related to the final gene product.



Appendix Figure 7. Sequence data for *Ace*-1 showing that Rufisque does not possess the F290V mutation (TTC asparagine between residues 97-99 using torpedo numbering and highlighted by a box) compared to four published sequences from VectorBase: *An. gambiae, An. coluzzii, An. arabiensis*, and the hybrid PEST. There is, however, a novel mutation present in all Rufisque samples at the 148-residue resulting in a threonine to proline switch within exon 6.



Appendix Figure 8: Genetic map estimated by R showing the position of microsatellites used in this study with a scale measuring genetic distance in centiMorgans (cM) on the left. The centromeres are located between markers CDC19 and CDC20D on chromosome 2 and 33C1 and 38C on chromosome 3. The map differs from *An. gambiae* in that the 2L is showed in the inverted La form which is the standard orientation for *An. arabiensis* (represented by the microsatellites ND36P1 – ARGB1).

Appendix Table 2: Hardy-Weinberg exact test for markers to determine if any had significant association with bendiocarb resistance.

```
=========================================
   Results by population
=========================================


 Pop : Family E11
-----------------------------------------
                      Fis estimates
                     ---------------
locus      P-val   S.E.    W&C     R&H     Steps
---------- ------- ------- ------- ------- ------
Ag503       0.0392  0.0013  -0.3559 -0.3587  69460 switches
Ag711w      0.0393  0.0012  -0.3443 -0.3470  69722 switches
CDC5        0.0470  0.0015   0.3367  0.3423  74291 switches
CDC7        0.2308  0.0025  -0.2420 -0.2444  69146 switches
CDC9        No information.
CDC11       No information.
2RiS5       0.0000  0.0000  -0.8636 -0.8650  75519 switches
Ag95        No information.
CDC14       0.0000  0.0000  -1.0000 -1.0000  76294 switches
CDC16       No information.
R27         No information.
CDC19       0.5495  0.0029  -0.0991 -0.1001  75377 switches
CDC20D      1.0000  0.0000   0.0238  0.0241  75150 switches
CDC21       0.5506  0.0026  -0.0991 -0.1001  75269 switches
CDC22       0.7606  0.0018  -0.0574 -0.0581  76109 switches
ARGB1       0.7589  0.0015  -0.0574 -0.0581  75937 switches

All (Fisher's method):
 Chi2 :    Infinity
 Df   :   22.0000
 Prob :    High. sign.

 Pop : Family E10
-----------------------------------------
                      Fis estimates
                     ---------------
locus      P-val   S.E.    W&C     R&H     Steps
---------- ------- ------- ------- ------- ------
Ag503       0.0000  0.0000   0.5129  0.5145  89905 switches
Ag711w      0.0000  0.0000   0.4836  0.4851  84521 switches
CDC5        0.0000  0.0000   0.6952  0.6976  81050 switches
CDC7        0.0000  0.0000   0.6559  0.6582  89674 switches
CDC9        0.4327  0.0066  -0.0562 -0.0563  89305 switches
CDC11       0.0518  0.0031   0.1311  0.1314  88970 switches
```

```
2RiS5      0.0000  0.0000  -0.2512 -0.2516  86586 switches
Ag95       0.5974  0.0063   0.0397  0.0398  89577 switches
CDC14      0.6899  0.0049  -0.0323 -0.0324  89374 switches
CDC16      0.0000  0.0000   0.4562  0.4576  88740 switches
R27        0.0000  0.0000   0.7697  0.7725  89362 switches
CDC19      0.0000  0.0000  -0.3483 -0.3487  89818 switches
CDC20D     0.1925  0.0060  -0.0869 -0.0871  89619 switches
CDC21      0.0046  0.0009  -0.1826 -0.1829  89664 switches
CDC22      0.0000  0.0000   0.3290  0.3299  86878 switches
ARGB1      0.0001  0.0001  -0.2496 -0.2500  89876 switches

All (Fisher's method):
 Chi2 :    Infinity
 Df   :   32.0000
 Prob :    High. sign.

 Pop : Family F4
----------------------------------------
                     Fis estimates
                    ---------------
locus      P-val   S.E.    W&C     R&H     Steps
---------- ------- ------- ------- ------- ------
Ag503      0.0000  0.0000   0.4272  0.4288  88274 switches
Ag711w     0.3111  0.0073   0.0739  0.0741  89127 switches
CDC5       0.0001  0.0001   0.2601  0.2609  88058 switches
CDC7       0.0000  0.0000   0.7641  0.7675  86440 switches
CDC9       0.7770  0.0036  -0.0229 -0.0230  88599 switches
CDC11      0.5643  0.0062   0.0488  0.0489  88663 switches
2RiS5      0.0008  0.0002  -0.2214 -0.2218  83941 switches
Ag95       0.5821  0.0065   0.0415  0.0416  88494 switches
CDC14      0.3184  0.0063   0.0703  0.0705  88969 switches
CDC16      1.0000  0.0000   0.0044  0.0044  87900 switches
R27        0.0000  0.0000   0.4078  0.4092  88383 switches
CDC19      0.0005  0.0002  -0.2396 -0.2401  88756 switches
CDC20D     0.0035  0.0007   0.2101  0.2107  88545 switches
CDC21      1.0000  0.0000   0.0035  0.0035  88714 switches
CDC22      0.8897  0.0018  -0.0148 -0.0149  88341 switches
ARGB1      0.8840  0.0022   0.0190  0.0191  88192 switches

All (Fisher's method):
 Chi2 :    Infinity
 Df   :   32.0000
 Prob :    High. sign.

 Pop : Family E13
----------------------------------------
                     Fis estimates
                    ---------------
```

locus P-val S.E. W&C R&H Steps
---------- ------- ------- ------- ------- ------
Ag503 0.5540 0.0017 0.2169 0.2267 55623 switches
Ag711w 0.1150 0.0017 0.6486 0.6892 11310 switches
CDC5 0.0044 0.0004 1.0000 1.0769 21670 switches
CDC7 0.5809 0.0015 0.1892 0.1983 57936 switches
CDC9 No information.
CDC11 No information.
2RiS5 No information.
Ag95 0.2098 0.0019 -0.5000 -0.5098 57705 switches
CDC14 1.0000 0.0000 0.0319 0.0331 58995 switches
CDC16 0.5125 0.0018 -0.3000 -0.3077 51190 switches
R27 0.3455 0.0023 0.3036 0.3184 34581 switches
CDC19 1.0000 0.0000 -0.1507 -0.1558 57927 switches
CDC20D 0.2219 0.0019 -0.4444 -0.4534 55258 switches
CDC21 0.2457 0.0022 -0.4118 -0.4213 50708 switches
CDC22 0.0395 0.0012 0.5938 0.6296 59170 switches
ARGB1 0.5046 0.0018 -0.3684 -0.3769 50496 switches

All (Fisher's method):
Chi2 : 37.6939
Df : 26.0000
Prob : 0.0646

Pop : Family 8

Fis estimates
---
locus P-val S.E. W&C R&H Steps
---------- ------- ------- ------- ------- ------
Ag503 0.0001 0.0000 1.0000 1.0714 59730 switches
Ag711w 0.0210 0.0008 0.6782 0.7183 50823 switches
CDC5 0.0218 0.0009 0.6782 0.7183 50926 switches
CDC7 0.0000 0.0000 1.0000 1.0714 60390 switches
CDC9 0.6132 0.0014 -0.1887 -0.1939 61496 switches
CDC11 No information.
2RiS5 1.0000 0.0000 0.1026 0.1065 52857 switches
Ag95 0.1223 0.0021 0.4684 0.4925 52885 switches
CDC14 1.0000 0.0000 -0.1667 -0.1714 61546 switches
CDC16 0.0318 0.0010 0.7895 0.8766 49336 switches
R27 0.1298 0.0019 -0.4667 -0.4751 61428 switches
CDC19 1.0000 0.0000 -0.0370 -0.0383 60365 switches
CDC20D 0.2913 0.0024 -0.3592 -0.3671 59816 switches
CDC21 0.4968 0.0015 -0.3333 -0.3421 49841 switches
CDC22 0.0093 0.0006 0.7172 0.7607 61559 switches
ARGB1 0.0207 0.0008 0.6782 0.7183 50976 switches

All (Fisher's method):

Chi2 : 91.1836
Df : 30.0000
Prob : 0.0000

Pop : Family 9_3

locus	P-val	S.E.	W&C	R&H	Steps
		Fis estimates			
Ag503	0.0000	0.0000	0.6740	0.6808	80635 switches
Ag711w	0.0000	0.0000	0.7280	0.7353	72693 switches
CDC5	0.0000	0.0000	0.5511	0.5560	82615 switches
CDC7	0.0000	0.0000	0.7191	0.7278	79419 switches
CDC9	0.2057	0.0041	-0.1414	-0.1421	82586 switches
CDC11	0.0609	0.0013	0.2342	0.2359	58942 switches
2RiS5	0.0000	0.0000	0.7747	0.7832	59728 switches
Ag95	0.0853	0.0026	-0.1981	-0.1991	81246 switches
CDC14	0.0000	0.0000	-1.0000	-1.0000	82991 switches
CDC16	0.0000	0.0000	0.5629	0.5681	82365 switches
R27	0.0000	0.0000	0.8772	0.8874	82591 switches
CDC19	1.0000	0.0000	-0.0450	-0.0452	75562 switches
CDC20D	0.0004	0.0002	0.4199	0.4234	73069 switches
CDC21	0.0020	0.0003	0.6577	0.6643	8730 switches
CDC22	0.0000	0.0000	0.5003	0.5047	82903 switches
ARGB1	0.0232	0.0011	0.2647	0.2666	75327 switches

All (Fisher's method):
Chi2 : Infinity
Df : 32.0000
Prob : High. sign.

Pop : Family 6_4

locus	P-val	S.E.	W&C	R&H	Steps
		Fis estimates			
Ag503	0.0000	0.0000	0.7221	0.7301	72139 switches
Ag711w	0.0000	0.0000	0.5007	0.5057	82286 switches
CDC5	0.0000	0.0000	0.6007	0.6069	76552 switches
CDC7	0.0000	0.0000	0.6899	0.6974	68763 switches
CDC9	0.0542	0.0023	0.2216	0.2233	81110 switches
CDC11	0.0000	0.0000	0.6259	0.6324	78022 switches
2RiS5	0.0000	0.0000	0.8101	0.8195	80838 switches
Ag95	0.0001	0.0000	0.5123	0.5174	68153 switches
CDC14	0.0000	0.0000	-0.6957	-0.6970	81686 switches
CDC16	0.5807	0.0029	0.0754	0.0759	77627 switches

```
R27        0.0000  0.0000   0.9744  0.9867  81539 switches
CDC19      0.0790  0.0026  -0.2148 -0.2158  79923 switches
CDC20D     0.4317  0.0037  -0.1078 -0.1084  79756 switches
CDC21      0.0000  0.0000  -1.0000 -1.0000  82397 switches
CDC22      0.0014  0.0003  -0.3724 -0.3739  82236 switches
ARGB1      0.0000  0.0000   0.7041  0.7118  58885 switches

All (Fisher's method):
 Chi2 :    Infinity
 Df   :   32.0000
 Prob :    High. sign.

 Pop : Family 6_9
-----------------------------------------
                      Fis estimates
                     ---------------
locus      P-val   S.E.    W&C     R&H     Steps
---------- ------- ------- ------- ------- ------
Ag503      0.2906  0.0023   0.2812  0.2890  63626 switches
Ag711w     0.2889  0.0020  -0.2778 -0.2820  55545 switches
CDC5       0.2881  0.0021  -0.2778 -0.2820  55750 switches
CDC7       0.5195  0.0021   0.1141  0.1169  52055 switches
CDC9       1.0000  0.0000  -0.0073 -0.0075  67178 switches
CDC11      0.0172  0.0008   0.5158  0.5326  67952 switches
2RiS5      No information.
Ag95       1.0000  0.0000  -0.0073 -0.0075  67210 switches
CDC14      0.2881  0.0022  -0.2778 -0.2820  55294 switches
CDC16      1.0000  0.0000   0.0630  0.0644  68423 switches
R27        0.0923  0.0017   0.4054  0.4182  68209 switches
CDC19      1.0000  0.0000  -0.0073 -0.0075  67406 switches
CDC20D     1.0000  0.0000  -0.0041 -0.0042  64959 switches
CDC21      0.0000  0.0000  -1.0000 -1.0000  68354 switches
CDC22      0.0122  0.0007   0.5607  0.5813  66730 switches
ARGB1      No information.

All (Fisher's method):
 Chi2 :    Infinity
 Df   :   28.0000
 Prob :    High. sign.

 Pop : Family 15
-----------------------------------------
                      Fis estimates
                     ---------------
locus      P-val   S.E.    W&C     R&H     Steps
---------- ------- ------- ------- ------- ------
Ag503      0.7228  0.0040   0.0396  0.0398  86427 switches
Ag711w     0.0058  0.0007   0.2410  0.2420  86380 switches
```

```
CDC5       0.0174  0.0019   0.2117  0.2126  86219 switches
CDC7       0.0001  0.0000   0.3308  0.3325  86438 switches
CDC9       0.5074  0.0059  -0.0625 -0.0627  86556 switches
CDC11      0.0105  0.0012   0.2263  0.2273  86160 switches
2RiS5      0.6010  0.0042   0.0596  0.0598  86104 switches
Ag95       0.3120  0.0051   0.0981  0.0985  86436 switches
CDC14      1.0000  0.0000   0.0088  0.0089  85637 switches
CDC16      0.0000  0.0000   0.7031  0.7083  83961 switches
R27        0.0005  0.0002   0.3103  0.3118  86263 switches
CDC19      0.0089  0.0009  -0.2258 -0.2265  85297 switches
CDC20D     0.1732  0.0042  -0.1234 -0.1238  84647 switches
CDC21      0.0007  0.0002   0.3068  0.3082  85733 switches
CDC22      0.0109  0.0011   0.2306  0.2316  86145 switches
ARGB1      0.8368  0.0019   0.0179  0.0180  84034 switches

All (Fisher's method):
 Chi2 :    Infinity
 Df   :   32.0000
 Prob :    High. sign.

 Pop : Family 6_7
-----------------------------------------
                      Fis estimates
                     ---------------
locus      P-val   S.E.    W&C     R&H     Steps
---------- ------- ------- ------- ------- ------
Ag503      0.3057  0.0015   0.4194  0.4514  30693 switches
Ag711w     0.0117  0.0006   0.8333  0.9091  57877 switches
CDC5       0.0110  0.0006   0.8333  0.9091  57804 switches
CDC7       1.0000  0.0000  -0.1250 -0.1307  15881 switches
CDC9       0.1911  0.0020  -0.5385 -0.5500  53676 switches
CDC11       No information.
2RiS5       No information.
Ag95       1.0000  0.0000  -0.1321 -0.1375  53718 switches
CDC14      0.1900  0.0017  -0.5385 -0.5500  53748 switches
CDC16      1.0000  0.0000  -0.1429 -0.1500  17521 switches
R27        0.2465  0.0024   0.4375  0.4714  51922 switches
CDC19      0.5043  0.0018  -0.3333 -0.3438  50563 switches
CDC20D     0.2224  0.0019  -0.5000 -0.5128  47817 switches
CDC21      0.4779  0.0019  -0.4286 -0.4400  47658 switches
CDC22      0.1937  0.0018  -0.5385 -0.5500  53685 switches
ARGB1       No information.

All (Fisher's method):
 Chi2 :    38.8509
 Df   :   26.0000
 Prob :    0.0504
```

```
 Pop : Family 6_2
----------------------------------------
                    Fis estimates
                   ---------------
locus      P-val   S.E.    W&C     R&H     Steps
---------- ------- ------- ------- ------- ------
Ag503       0.0000  0.0000   0.6759  0.6853  79972 switches
Ag711w       No information.
CDC5         No information.
CDC7        0.0002  0.0001   0.5143  0.5211  79037 switches
CDC9        0.4388  0.0039   0.1143  0.1153  79742 switches
CDC11        No information.
2RiS5        No information.
Ag95        0.0279  0.0008  -0.2766 -0.2782  71295 switches
CDC14       0.0257  0.0009  -0.2903 -0.2920  71553 switches
CDC16       0.0972  0.0018  -0.2473 -0.2489  69194 switches
R27         No information.
CDC19        No information.
CDC20D      0.1868  0.0023  -0.2211 -0.2225  66914 switches
CDC21       0.0006  0.0001   0.6446  0.6535  27280 switches
CDC22       1.0000  0.0000  -0.0909 -0.0916  38912 switches
ARGB1        No information.

All (Fisher's method):
 Chi2 :    Infinity
 Df   :    18.0000
 Prob :    High. sign.
==========================================
 All locus, all populations
==========================================
All (Fisher's method) :
 Chi2 :    Infinity
 Df   :    242.0000
 Prob :    High. sign.
==========================================
    Results by population
==========================================


 Pop : Family E11
----------------------------------------
                    Fis estimates
                   ---------------
locus      P-val   S.E.    W&C     R&H     Steps
---------- ------- ------- ------- ------- ------
ARGB1       1.0000  0.0000   0.0115  0.0116  75727 switches
Ag787       0.7661  0.0018   0.0575  0.0582  76224 switches
ND36P1      0.0569  0.0017   0.3302  0.3355  76000 switches
```

```
CDC27      0.0000  0.0000   0.8774  0.8991  40430 switches
CDC28C     0.7564  0.0016  -0.0889 -0.0899  75519 switches
R29        0.0000  0.0000  -1.0000 -1.0000  75545 switches
Ag249      No information.
CDC32      No information.
Ag555      0.0000  0.0000  -1.0000 -1.0000  76379 switches
33C1       0.0000  0.0000  -1.0000 -1.0000  76329 switches
CDC38C     0.0000  0.0000  -1.0000 -1.0000  75477 switches
CDC40B     0.7361  0.0018   0.0787  0.0797  73987 switches
CDC43A     0.0000  0.0000  -0.6735 -0.6761  75072 switches
CDC44B     0.0184  0.0009  -0.3898 -0.3927  70718 switches
45C        0.0072  0.0004  -0.4138 -0.4167  72681 switches

All (Fisher's method):
 Chi2 :    Infinity
 Df   :   26.0000
 Prob :    High. sign.

 Pop : Family E10
-----------------------------------------
                      Fis estimates
                     ---------------
locus      P-val   S.E.    W&C     R&H     Steps
---------- ------- ------- ------- ------- ------
ARGB1      0.6968  0.0030  -0.0299 -0.0299  84379 switches
Ag787      0.0000  0.0000   0.6541  0.6564  84841 switches
ND36P1     0.0000  0.0000  -0.4182 -0.4187  89555 switches
CDC27      No information.
CDC28C     0.0001  0.0001  -0.2479 -0.2483  87895 switches
R29        0.5802  0.0047  -0.0428 -0.0429  84673 switches
Ag249      No information.
CDC32      0.5001  0.0069   0.0463  0.0464  89277 switches
Ag555      0.0000  0.0000  -0.7351 -0.7355  89779 switches
33C1       0.0000  0.0000  -0.6000 -0.6005  89473 switches
CDC38C     0.0000  0.0000   0.3800  0.3811  86700 switches
CDC40B     0.3572  0.0052   0.0664  0.0666  85351 switches
CDC43A     0.0077  0.0009   0.1876  0.1880  77444 switches
CDC44B     0.2572  0.0055   0.0747  0.0749  86946 switches
45C        0.7417  0.0033   0.0274  0.0275  86408 switches

All (Fisher's method):
 Chi2 :    Infinity
 Df   :   26.0000
 Prob :    High. sign.

 Pop : Family F4
-----------------------------------------
                      Fis estimates
```

```
                    ---------------
locus      P-val   S.E.    W&C     R&H     Steps
---------- ------- ------- ------- ------- ------
ARGB1       0.0000  0.0000   1.0000  1.0050  87486 switches
Ag787       0.0674  0.0039   0.1354  0.1358  88638 switches
ND36P1      1.0000  0.0000   0.0089  0.0089  89250 switches
CDC27       No information.
CDC28C      0.0065  0.0008   0.1947  0.1953  88697 switches
R29         1.0000  0.0000   0.0086  0.0087  69652 switches
Ag249       0.2291  0.0021  -0.1014 -0.1016  68708 switches
CDC32       0.6102  0.0032  -0.0553 -0.0554  79968 switches
Ag555       0.0000  0.0000  -1.0000 -1.0000  88909 switches
33C1        0.0012  0.0002  -0.2073 -0.2077  80451 switches
CDC38C      1.0000  0.0000  -0.0061 -0.0061  83412 switches
CDC40B      0.0000  0.0000   0.7024  0.7054  55264 switches
CDC43A      0.8665  0.0017  -0.0212 -0.0212  86889 switches
CDC44B      0.0213  0.0011  -0.1577 -0.1581  79601 switches
45C         0.0000  0.0000  -0.3375 -0.3380  85630 switches

All (Fisher's method):
 Chi2 :    Infinity
 Df   :    28.0000
 Prob :    High. sign.

 Pop : Family E13
-----------------------------------------
                     Fis estimates
                    ---------------
locus      P-val   S.E.    W&C     R&H     Steps
---------- ------- ------- ------- ------- ------
ARGB1       1.0000  0.0000  -0.1818 -0.1873  34864 switches
Ag787       1.0000  0.0000  -0.1818 -0.1873  35087 switches
ND36P1      0.5908  0.0016  -0.2581 -0.2651  58923 switches
CDC27       No information.
CDC28C      1.0000  0.0000  -0.1818 -0.1873  34732 switches
R29         0.5140  0.0016  -0.3000 -0.3077  51600 switches
Ag249       0.2209  0.0021  -0.4444 -0.4534  55487 switches
CDC32       0.6237  0.0018   0.1613  0.1683  62038 switches
Ag555       1.0000  0.0000  -0.0400 -0.0414   3668 switches
33C1        0.2196  0.0021  -0.4444 -0.4534  55509 switches
CDC38C      0.2234  0.0020  -0.4444 -0.4534  55355 switches
CDC40B      0.2190  0.0019  -0.4444 -0.4534  55449 switches
CDC43A      0.6229  0.0020   0.1613  0.1683  62205 switches
CDC44B      0.0028  0.0003   0.8471  0.9069  55258 switches
45C         1.0000  0.0000  -0.0370 -0.0385  57807 switches

All (Fisher's method):
 Chi2 :    28.0960
```

Df : 28.0000
Prob : 0.4594

Pop : Family 8

Fis estimates

locus	P-val	S.E.	W&C	R&H	Steps
ARGB1	0.0096	0.0006	0.7172	0.7607	61839 switches
Ag787	0.2918	0.0023	0.3519	0.3685	61443 switches
ND36P1	1.0000	0.0000	-0.0370	-0.0383	60102 switches
CDC27	0.2957	0.0023	0.3137	0.3281	57845 switches
CDC28C	0.1118	0.0017	0.4717	0.4960	60062 switches
R29	1.0000	0.0000	0.1009	0.1048	62885 switches
Ag249	0.0000	0.0000	1.0000	1.0714	59974 switches
CDC32	1.0000	0.0000	-0.2174	-0.2232	46226 switches
Ag555	1.0000	0.0000	0.0841	0.0873	61680 switches
33C1	0.0834	0.0015	-0.5556	-0.5639	57827 switches
CDC38C	0.2987	0.0025	0.3137	0.3281	57779 switches
CDC40B	0.5294	0.0015	-0.2727	-0.2795	52556 switches
CDC43A	No information.				
CDC44B	0.5268	0.0016	-0.2727	-0.2795	52734 switches
45C	0.5082	0.0016	-0.3333	-0.3409	50534 switches

All (Fisher's method):
Chi2 : 49.6809
Df : 28.0000
Prob : 0.0070

Pop : Family 9

Fis estimates

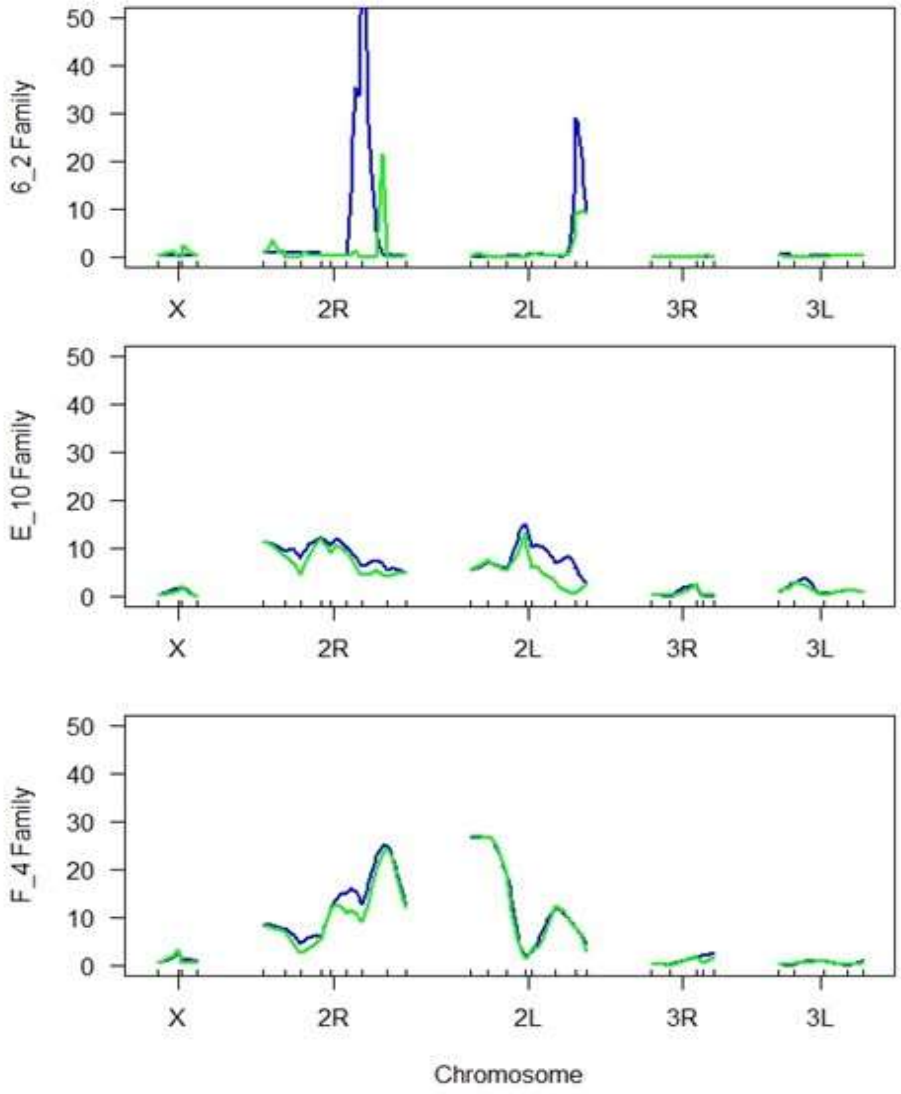
locus	P-val	S.E.	W&C	R&H	Steps
ARGB1	0.0000	0.0000	1.0000	1.0116	58924 switches
Ag787	0.1069	0.0026	0.2000	0.2014	75277 switches
ND36P1	0.0000	0.0000	0.7827	0.7907	81939 switches
CDC27	0.0000	0.0000	0.7090	0.7167	68163 switches
CDC28C	0.0001	0.0000	0.4505	0.4545	78198 switches
R29	0.6199	0.0019	0.0692	0.0696	61823 switches
Ag249	0.2032	0.0023	-0.1622	-0.1629	68131 switches
CDC32	0.0072	0.0005	0.4801	0.4843	15689 switches
Ag555	0.0000	0.0000	-0.9773	-0.9774	83398 switches
33C1	0.1967	0.0019	0.1849	0.1862	19773 switches
CDC38C	0.0073	0.0005	0.3835	0.3866	43438 switches
CDC40B	0.1185	0.0017	0.2612	0.2631	11965 switches

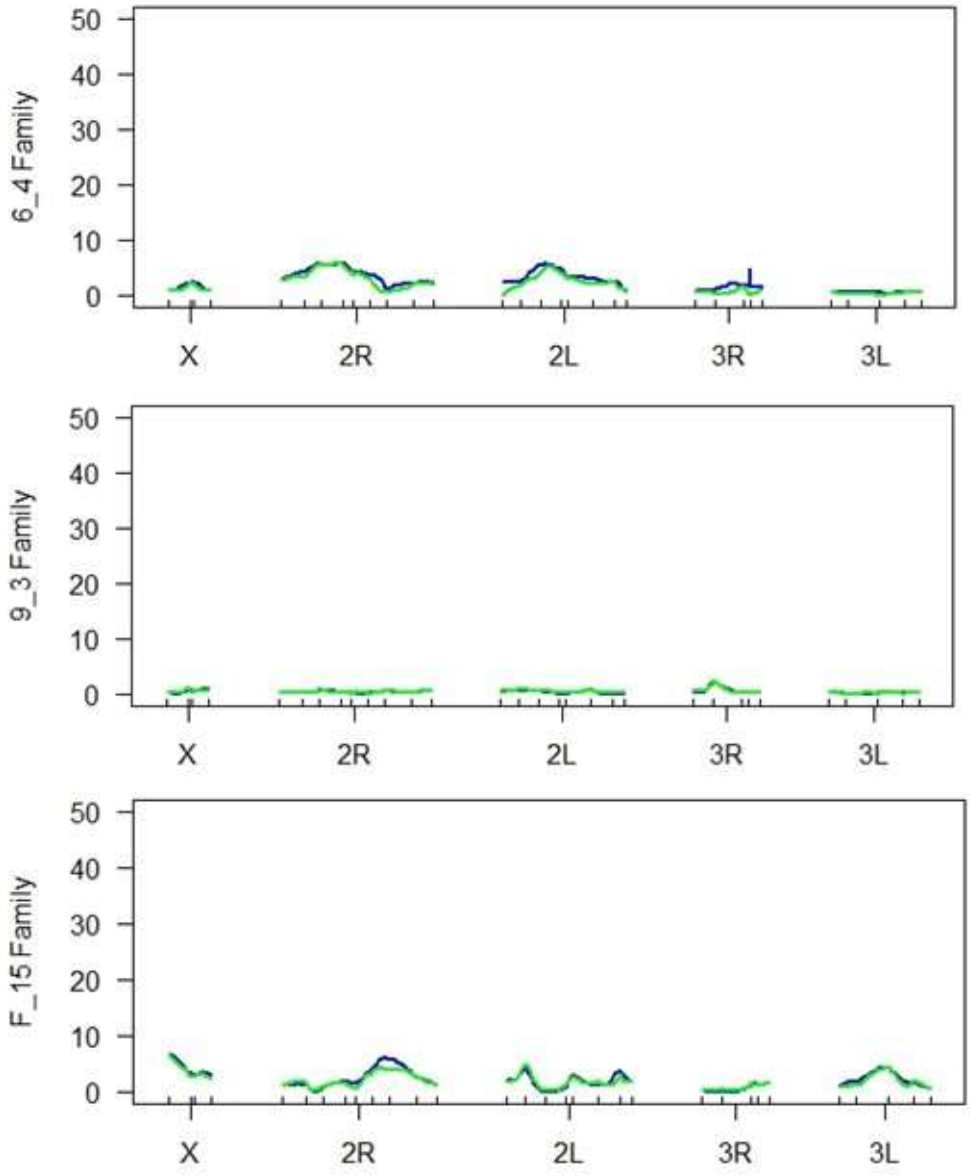
```
CDC43A      No information.
CDC44B     0.0000  0.0000   0.7133  0.7204  65629 switches
45C        0.0241  0.0010   0.2780  0.2800  61814 switches

All (Fisher's method):
 Chi2 :    Infinity
 Df   :   28.0000
 Prob :    High. sign.

 Pop : Family 6_9
-----------------------------------------
                      Fis estimates
                     ---------------
locus      P-val   S.E.    W&C     R&H     Steps
---------- ------- ------- ------- ------- ------
ARGB1      0.0000  0.0000   0.8992  0.9075  60013 switches
Ag787      0.1694  0.0043  -0.1409 -0.1414  84355 switches
ND36P1     0.6823  0.0032  -0.0497 -0.0499  83970 switches
CDC27      0.1590  0.0038  -0.1470 -0.1477  84311 switches
CDC28C     0.0012  0.0003   0.3324  0.3346  80759 switches
R29        0.8010  0.0019  -0.0529 -0.0531  79823 switches
Ag249       No information.
CDC32      0.3399  0.0036   0.1076  0.1082  81541 switches
Ag555      0.0000  0.0000  -1.0000 -1.0000  84580 switches
33C1       1.0000  0.0000   0.0108  0.0109  80024 switches
CDC38C     0.0002  0.0001   0.3768  0.3793  84552 switches
CDC40B     0.0000  0.0000  -0.7288 -0.7298  84156 switches
CDC43A     0.0001  0.0000   0.4804  0.4840  68669 switches
CDC44B     0.1126  0.0024  -0.1742 -0.1749  75800 switches
45C        0.0177  0.0009  -0.2402 -0.2411  79976 switches

All (Fisher's method):
 Chi2 :    Infinity
 Df   :   28.0000
 Prob :    High. sign.

 Pop : Family 15
-----------------------------------------
                      Fis estimates
                     ---------------
locus      P-val   S.E.    W&C     R&H     Steps
---------- ------- ------- ------- ------- ------
ARGB1      0.0000  0.0000   0.4504  0.4528  82684 switches
Ag787      0.0073  0.0009  -0.2472 -0.2478  86297 switches
ND36P1     0.3959  0.0060  -0.0790 -0.0793  85877 switches
CDC27      0.0000  0.0000   0.5457  0.5490  75104 switches
CDC28C     0.2956  0.0059  -0.1016 -0.1019  86628 switches
R29        0.8604  0.0023  -0.0271 -0.0272  86292 switches
```

```
Ag249      0.0000  0.0000   0.6397  0.6435  86662 switches
CDC32      0.2687  0.0047   0.1010  0.1014  85166 switches
Ag555      0.0000  0.0000  -0.9296 -0.9298  86458 switches
33C1       0.0022  0.0004  -0.2594 -0.2601  83630 switches
CDC38C     0.0001  0.0001  -0.3407 -0.3415  86611 switches
CDC40B     0.0031  0.0005   0.2542  0.2554  83915 switches
CDC43A     0.0000  0.0000   0.5858  0.5892  83183 switches
CDC44B     0.6864  0.0036  -0.0507 -0.0508  84096 switches
45C        0.0002  0.0001  -0.3398 -0.3406  86103 switches

All (Fisher's method):
 Chi2 :    Infinity
 Df   :    30.0000
 Prob :    High. sign.

 Pop : Family 6_7
-----------------------------------------
                      Fis estimates
                     ---------------
locus      P-val   S.E.    W&C     R&H     Steps
---------- ------- ------- ------- ------- ------
ARGB1       No information.
Ag787      0.4847  0.0019  -0.4286 -0.4400  48159 switches
ND36P1     1.0000  0.0000   0.1071  0.1128  55346 switches
CDC27      0.5386  0.0016   0.2593  0.2750  54117 switches
CDC28C     0.4813  0.0019   0.2500  0.2667  47776 switches
R29        1.0000  0.0000  -0.0526 -0.0550  54288 switches
Ag249       No information.
CDC32      0.5425  0.0016   0.3103  0.3300  54346 switches
Ag555      0.0025  0.0003  -1.0000 -1.0000  58070 switches
33C1       0.0155  0.0006   0.8077  0.8800  48219 switches
CDC38C     0.0640  0.0014  -0.6667 -0.6769  55555 switches
CDC40B     1.0000  0.0000   0.1071  0.1128  55416 switches
CDC43A     1.0000  0.0000   0.1379  0.1455  58079 switches
CDC44B     1.0000  0.0000   0.1379  0.1455  58310 switches
45C        0.5047  0.0015  -0.3333 -0.3438  50397 switches

All (Fisher's method):
 Chi2 :    32.5285
 Df   :    26.0000
 Prob :    0.1762

 Pop : Family 6_2
-----------------------------------------
                      Fis estimates
                     ---------------
locus      P-val   S.E.    W&C     R&H     Steps
---------- ------- ------- ------- ------- ------
```

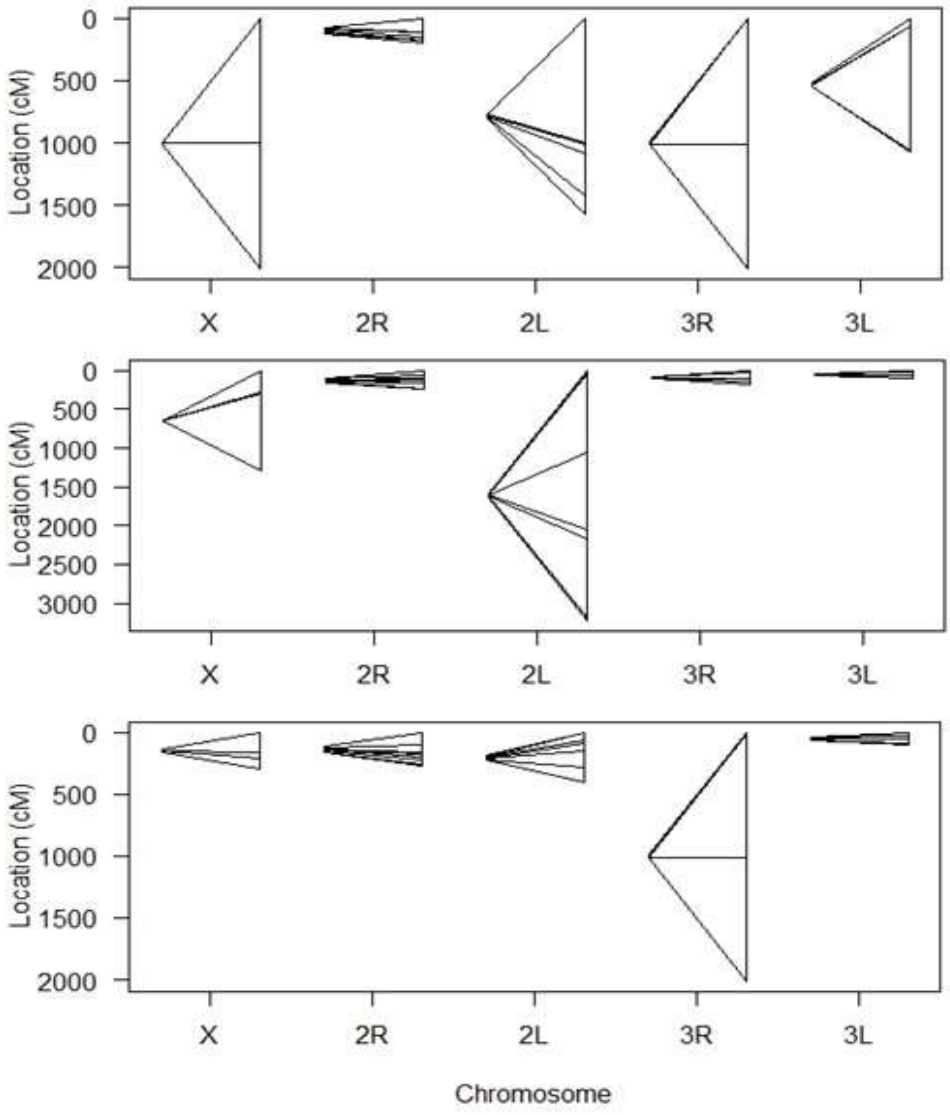
```
ARGB1       No information.
Ag787      0.0000  0.0000  -0.6216 -0.6236  78473 switches
ND36P1      0.0003  0.0002   0.4709  0.4767  79496 switches
CDC27       No information.
CDC28C      1.0000  0.0000  -0.0084 -0.0085    818 switches (low!)
R29        1.0000  0.0000  -0.0087 -0.0088    832 switches (low!)
Ag249       1.0000  0.0000  -0.0084 -0.0085    870 switches (low!)
CDC32       1.0000  0.0000  -0.0169 -0.0171   2414 switches
Ag555      0.0000  0.0000  -1.0000 -1.0000  79853 switches
33C1        No information.
CDC38C      0.0001  0.0001  -0.5000 -0.5021  77620 switches
CDC40B      0.0027  0.0003   0.6542  0.6632  12154 switches
CDC43A      No information.
CDC44B      0.0003  0.0001  -0.4458 -0.4478  76680 switches
45C        0.0002  0.0001  -0.4634 -0.4655  76704 switches

All (Fisher's method):
 Chi2 :    Infinity
 Df   :   22.0000
 Prob :    High. sign.
========================================
 All locus, all populations
========================================
All (Fisher's method) :
 Chi2 :    Infinity
 Df   :   212.0000
 Prob :    High. sign.
```

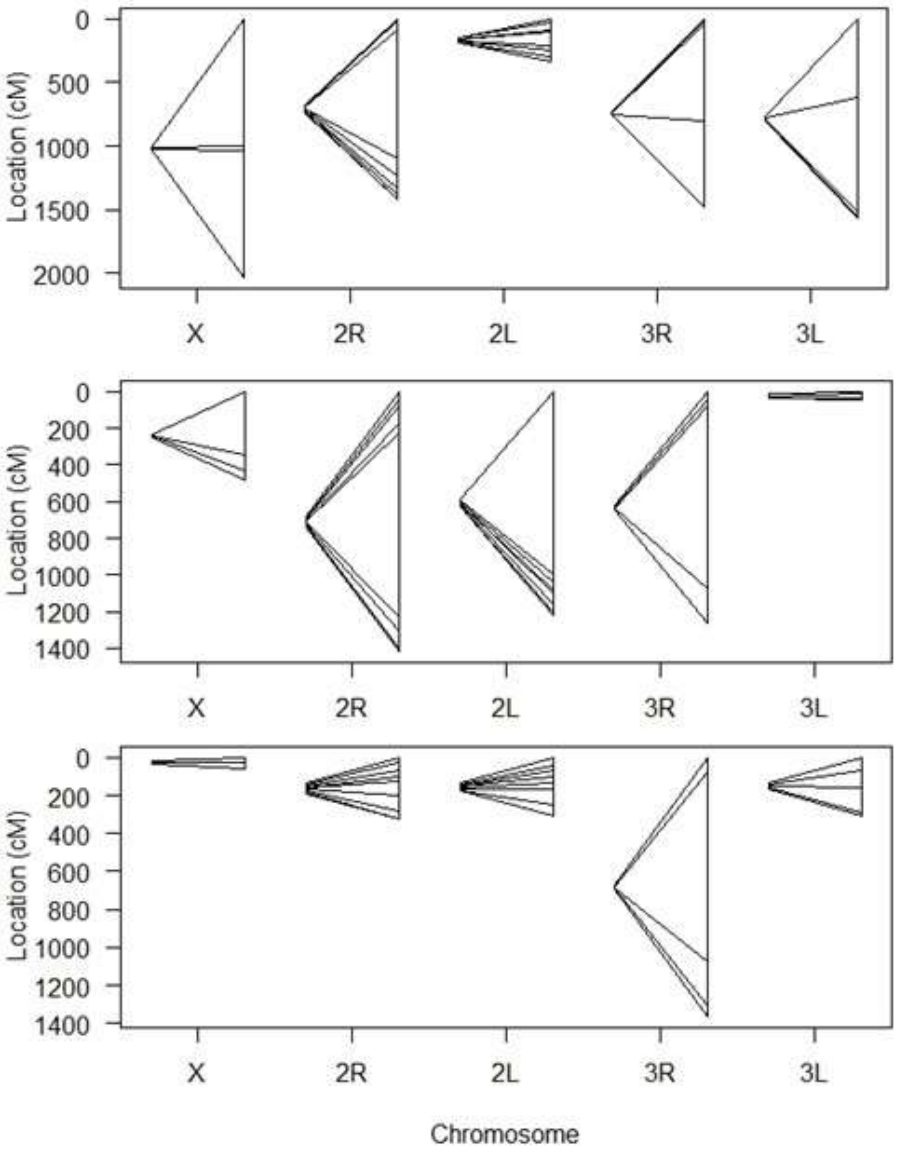
Appendix Figure 9: LOD scores for Haley-Knott regression (blue) and multiple-imputation (green) QTL analyses of individual families that comprise the F2 backcross based on 24-hour survivorship after 1-hour exposure to 0.1% bendiocarb. Note in families 6_4, 9_3, and F15 no marker reached the LOD threshold of 5.




6_4 Family
9_3 Family
F_15 Family
50
40
30
20
10
0
X
2R
2L
3R
3L

Appendix Figure 10: Comparison of genetic maps generated by r/qtl for individual families. These are presented in the same order as in Appendix Table 9.



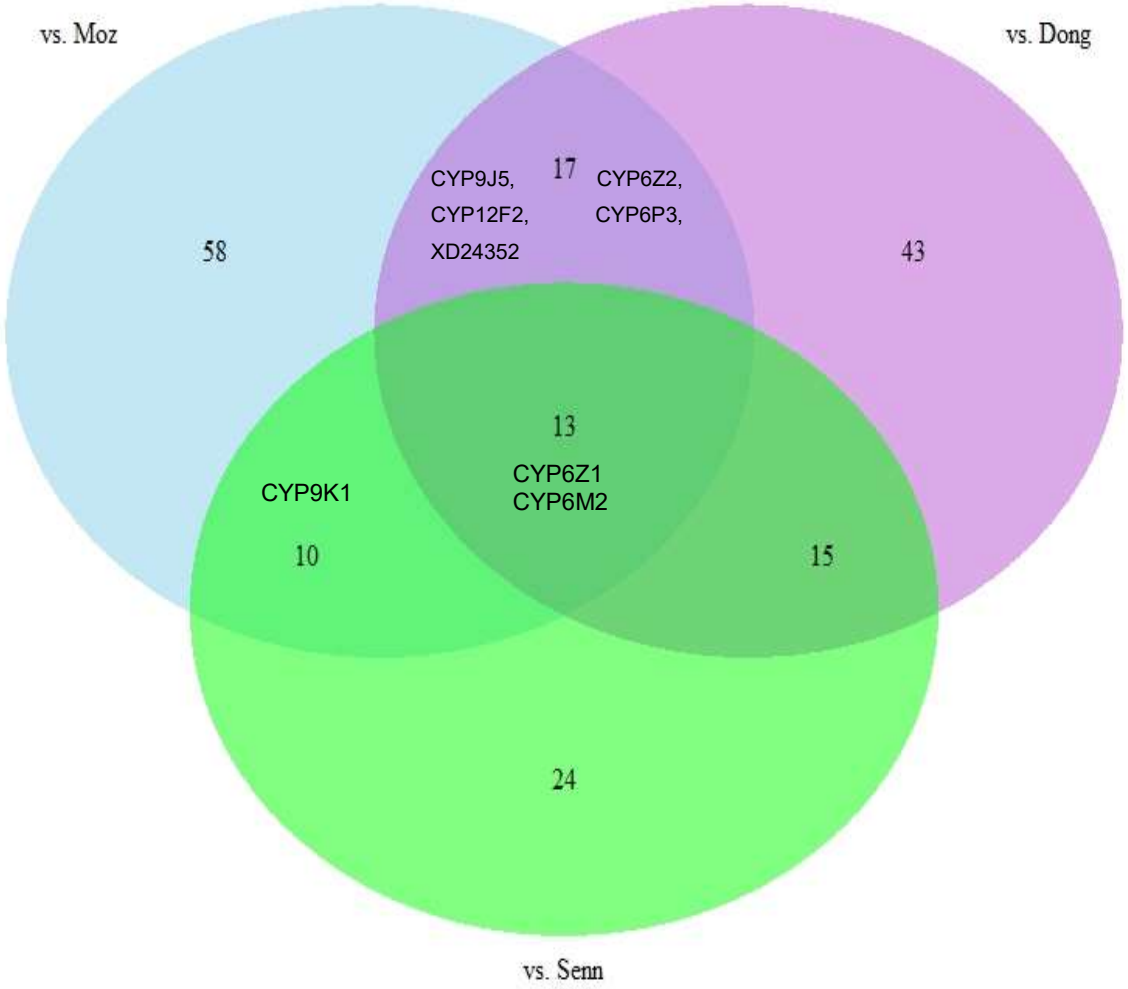

Location (cM)
0
500
1000
1500
2000
X
2R
2L
3R
3L
Location (cM)
0
200
400
600
800
1000
1200
1400
X
2R
2L
3R
3L
Location (cM)
0
200
400
600
800
1000
1200
1400
X
2R
2L
3R
3L
Chromosome

Appendix Table 3: List of genes displaying the highest over-expression (log fold change) between the bendiocarb resistant Rufisque compared to Sennar (SENN). All displayed logFC values are significant at the P<.05 value with a cutoff logFC of 2-fold expression. Genes in bold are those that have been previously found to be upregulated in insecticide resistant populations in the wild, italicized genes are those that were found to downregulated, and all others were found to have non-significant differentiation or with contradictory results regarding their role in regulation of expression.

Description	Systematic name	LogFC
solute carrier family 3 transmembrane	AGAP000586-RA	3.700
Stromal cell-derived factor 2	AGAP001749-RA	2.691
apolipoprotein d	AGAP002593-RA	2.064
translationally controlled tumor protein	AGAP002667-RA	2.423
solute carrier family 25 transmembrane	AGAP002704-RA	3.160
DUF4773	AGAP002733-RA	2.154
pyridoxal phosphate phosphatase phospho2	AGAP002841-RA	2.367
HEXIM	AGAP002875-RA	2.617
histone acetyltransferase type b catalytic subunit	AGAP003264-RA	2.207
inorganic diphosphatase activity	AGAP003398-RE	2.321
LIM and senescent cell antigen-like domains	AGAP003429-RA	3.088
BTB/POZ domain	AGAP003439-RG	2.278
Farnesoic acid O-methyltransferase activity	AGAP006199-RA	2.699
Integral component of membrane	AGAP007610-RA	2.183
Trypsin	AGAP008296-RA	3.529
CUB domain	AGAP009769-RA	2.809
Cytochrome b561/ferric reductase transmembrane	AGAP010004-RA	2.138
alkaline phosphatase	AGAP011305-RA	2.301
hypothetical conserved protein	*AGAP011923-RA*	3.500
Cytochrome b561/ferric reductase transmembrane	AGAP012644-RA	2.166
zinc finger protein 313	AGAP013215-RA	2.294
viral a-type inclusion protein	AGAP013358-RA	2.002
hypothetical conserved protein	**AGAP013359-RA**	2.426
hypothetical conserved protein	AGAP013481-RB	2.517
TPX2 - Thioredoxin dependent peroxidase	AGAP011054	2.241

Appendix Table 4: Genes of interest located within the 2Rb inversion (located approximately between 2R: 16.49 – 26.65). Boxes indicate levels of expression, either positive or negative, that are significant at the P<.05 level.

				RUF		RUF		RUF	
		Log FC	P value	MOZ	P value	DONG	P value	SENN	P value
2R	COEAE5O	0.4828036	0.000524	**0.6267**	0.000115	0.07906	0.412952	**0.228706**	0.037046
2R	CYP4D15	0.70912	0.392937	-0.320194	0.336303	-0.000702	0.998304	-0.487601	0.166352
2R	CYP4K2	0.3843517	0.129224	-0.027262	0.859138	0.31769	0.070008	-0.06043	0.701346
2R	CYP4AR1	2.8413755	0.000212	0.292341	0.261749	**1.0625**	0.002597	**-1.126456**	0.001839
2R	CYP4D17	1.2135348	0.002659	**-1.45448**	0.000571	**-1.151138**	0.002717	-0.554895	0.073451
2R	CYP4D22	3.8298993	1.74E-05	**-0.716873**	0.029829	**1.651842**	0.000327	**-1.664622**	0.000318
2R	CYP4D16	0.4531961	0.000521	**0.552265**	0.000177	-0.004896	0.955493	0.191336	0.055486
2R	CYP314A1	0.8248903	0.001169	0.229454	0.109418	**0.772053**	0.000345	-0.015482	0.90807
2R	CYP325K1	0.4641812	0.000569	**0.624018**	9.81E-05	0.141721	0.147148	**0.233391**	0.030135
2R	CPR7	0.5390692	6.65E-05	**0.611409**	2.92E-05	-0.041906	0.584982	**0.277641**	0.005475
2R	CPR8	0.6936907	0.000388	**0.731023**	0.000102	**0.56185**	0.000682	0.180418	0.123864
2R	CPR9	0.5963165	0.000353	**0.684717**	0.000117	0.024277	0.813647	**0.370152**	0.006031
2R	ABCH1	0.3873179	0.115794	0.285426	0.060569	0.236272	0.114882	-0.011793	0.931815
2R	ABCF1	0.3779564	0.227972	-0.512932	0.0517	-0.21623	0.374041	-0.301427	0.225801
2R	ABCB5	0.1879203	0.57963	-0.182445	0.335695	0.069907	0.711249	-0.058825	0.755112



Appendix Figure 11: Venn diagram showing under-expressed genes for each of the 3 susceptible strains compared to Rufisque. Genes with a FC value of -2.0 or less and significant at $P<0.05$ were considered differentially transcribed. Of the 13 shared genes, only 2 were known detoxification mechanisms: CYP6Z1 and CYP6M2.

Appendix Table 5: Annotated list of genes found, and their purported function, within the Bayes Credible interval from the AgamP4 build in VectorBase. Genes in bold are those that have been found to be upregulated in insecticide resistant populations in the wild, italicized genes are those that have been found to be downregulated, and all others were found to have non-significant differentiation or with contradictory results relating to their regulation in the Expression Data from VectorBase.

Gene	Name	Function
AGAP003662	prenylated Rab acceptor	membrane component
AGAP003663	ATP-dependent helicase DBP2	RNA unwinding
AGAP003664	Envelysin	proteolysis
AGAP003666	E3 ubiquitin RNF139	protein polyubiquitination
AGAP003669	**homeobox MSX**	transcription regulation
AGAP003670	homeobox nkX	transcription regulation
AGAP003671	Bap	transcription regulation
AGAP003674	T-cell leukemia homeobox	transcription regulation
AGAP003675	**CRZ**	neuropeptide signaling
AGAP003676	RAD50 DNA repair	DNA repair
AGAP003678	**LST2**	metal ion binding
AGAP003680	**ABCH2**	transmembrane transport
AGAP003681	huntingtin	binding
AGAP003684	vesicular amine transporter	cellular transport
AGAP003687	**glycerol-3-phosphate O-acyltransferase**	lipid metabolism
AGAP003688	threonine aldolase	amino acid metabolism
AGAP003689	CLIPC7	proteolysis
AGAP003692	*aminopeptidase*	proteolysis
AGAP003695	aminopeptidase N	proteolysis
AGAP003697	beta 1,4-N acetylglucosaminyltransferase	oligosaccharide biosynthesis
AGAP003698	zinc finger protein	ribosomal export
AGAP003699	*beta 1,4-N acetylglucosaminyltransferase*	oligosaccharide biosynthesis
AGAP003700	methionyl aminopeptidase	proteolysis
AGAP003701	*zinc finger protein*	ribosomal export
AGAP003702	tolkin	proteolysis
AGAP003703	*abnormal spindle-like protein*	spindle organization
AGAP003704	*oxygen-dependent protoporphyrinogen oxidase*	oxidase
AGAP003705	ASH2 histone methyltransferase	transcription regulation
AGAP003706	BolA protein	DNA binding
AGAP003707	transcription elongation factor 1	transcription regulation
AGAP003709	potassium large conductance calcium channel	ion transport
AGAP003711	V-type H+ transporting ATPase	ATP synthesis
AGAP003712	Ast1	neuropeptide signaling

AGAP003714	HPX3	oxidation-reduction process
AGAP003715	cyclin G-associated kinase	protein phosphorylation
AGAP003716	twf	actin filament polymerization
AGAP003717	twinfilin-1	actin filament polymerization
AGAP003718	**regulator of G-protein signaling**	signal transduction
AGAP003719	ferrochelatase	heme biosynthesis
AGAP003720	annexin A4	ion binding
AGAP003721	ANXB10B	ion binding
AGAP003722	ANXB10C	ion binding
AGAP003725	extended synaptotagmin-like protein	lipid transport
AGAP003727	protein unc-45	protein binding
AGAP003728	methyltransferase WBSCR22	RNA methylation
AGAP003730	neutral ceramidase	lipid metabolism
AGAP003731	vacuolar protein sorting-protein	protein transport
AGAP003733	alpha tocopherol transfer protein	
AGAP003737	ESF2/ABP1 family protein	ribosomal assembly
AGAP003742	regulator of chromosome condensation	DNA binding
AGAP003743	HAUS-augmin like complex	spindle assembly
AGAP003744	ring finger protein 220	ligase activity
AGAP003749	pancreatic triacylglyceral lipase	lipid metabolism
AGAP003750	glucose dehydrogenase	oxidation-reduction process
AGAP003751	CPAP1-C	chitin metabolism
AGAP003763	juvenile hormone inducible protein	
AGAP003764	juvenile hormone inducible protein	
AGAP003765	juvenile hormone inducible protein	
AGAP003766	**juvenile hormone inducible protein**	
AGAP003768	Rps2	ribosomal protein
AGAP003769	**importin beta-3**	nuclear translocation
AGAP003770	mRpL51	ribosomal protein
AGAP003772	NADPH oxidase	oxidation-reduction process
AGAP003779	serine/threonine protein phosphotase	hydrolase
AGAP003780	glucose dehydrogenase	oxidation-reduction process
AGAP003781	glucose dehydrogenase	oxidation-reduction process
AGAP003782	glucose dehydrogenase	oxidation-reduction process
AGAP003783	glucose dehydrogenase	oxidation-reduction process
AGAP003784	glucose dehydrogenase	oxidation-reduction process
AGAP003785	glucose dehydrogenase	oxidation-reduction process
AGAP003786	glucose dehydrogenase	oxidation-reduction process
AGAP003787	glucose dehydrogenase	oxidation-reduction process
AGAP003788	glucose dehydrogenase	oxidation-reduction process
AGAP003789	flotilin	membrane component
AGAP003790	ANXB9	ion binding

AGAP003791	RAD51-like protein	DNA repair
AGAP003792	*phosphatidylinositol glycan*	GPI biosynthesis process
AGAP003794	splicing factor	transcription regulation
AGAP003796	cyclin dependent kinase	cell division
AGAP003797	protein tipE	membrane component
AGAP003798	growth hormone regulated TBC protein	protein transport
AGAP003800	golgi SNAP receptor	protein transport
AGAP003803	anastral spindle 1	spindle organization
AGAP003805	EF hand domain	calcium binding
AGAP003806	pseudouridine-5 phosphate glycosidase	hydrolase
AGAP003807	serine-type endopeptidase	proteolysis
AGAP012979	glucose dehydrogenase	oxidation-reduction process
AGAP013001	aminopeptidase N	proteolysis
AGAP013016	glucose dehydrogenase	oxidation-reduction process
AGAP013123	glucose dehydrogenase	oxidation-reduction process
AGAP013146	APN5	proteolysis
AGAP013148	NAD dependent deacetylase	ADP ribosylation
AGAP013180	spindle B	DNA repair
AGAP013188	APN4	proteolysis
AGAP013245	IR101	membrane component
AGAP013255	APN3	proteolysis
AGAP013274	L-allo threonine aldolase	amino acid metabolism
AGAP013393	APN2	proteolysis
AGAP013457	CDC-like kinase	protein phosphorylation
AGAP013492	*glucose dehydrogenase*	oxidation-reduction process

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