# CHAPTER THREE

# RESULTS

## 3.1 STUDIES ON FIELD MATERIAL

### 3.1.1 *Anopheles gambiae* complex species-specific polymerase chain reaction (PCR)

The PCR method of Scott *et al*., (1993) identifies 5 species of the *An. gambiae* complex with the following diagnostic fragments: *An. quadriannulatus* (153 bp), *An. arabiensis* (315 bp), *An. gambiae* (390 bp) and *An. merus*/*melas* at (464 bp).

This method was used to identify a total of 960 members of *An. gambiae* complex from Gezira and Sennar states in central Sudan. The majority, 89.5% (n=859) were successfully identified as *An. arabiensis* (Figure 3.1), while the remaining 10.5% (n=101) did not amplify even after two attempts. Table 3.1 summarizes the results per locality and the sample size analyzed.

**Table 3.1:** *Anopheles gambiae* complex species-specific PCR carried out on adults obtained from larval collections (LC) and indoor resting collections (IRC).

| Locality | PCR identification results | | | | |
|---|---|---|---|---|---|
| | No. of Samples identified | | Not identified | | Species and total identified |
| | LC | IRC | LC | IRC | |
| El Masalamia | 50 | - | - | - | *An. arabiensis* |
| El Hoosh | 39 | 55 | 11 | - | *An. arabiensis* |
| El Khoor | 38 | 60 | 12 | - | *An. arabiensis* |
| El Managil | 35 | 90 | 15 | - | *An. arabiensis* |
| Mobi | 38 | - | 12 | - | *An. arabiensis* |
| Rofaa | 35 | - | 15 | - | *An. arabiensis* |
| Tabat | 43 | 55 | 7 | - | *An. arabiensis* |
| Wad Medani | 43 | - | 7 | - | *An. arabiensis* |
| Wad Rawa | 48 | - | 2 | - | *An. arabiensis* |
| El Boster | 43 | 50 | 7 | - | *An. arabiensis* |
| El Suki | 50 | - | - | - | *An. arabiensis* |
| Sennar | 40 | - | 10 | - | *An. arabiensis* |
| Singa | 47 | - | 3 | - | *An. arabiensis* |
| **Total** | **549** | **310** | **101** | - | **859** |

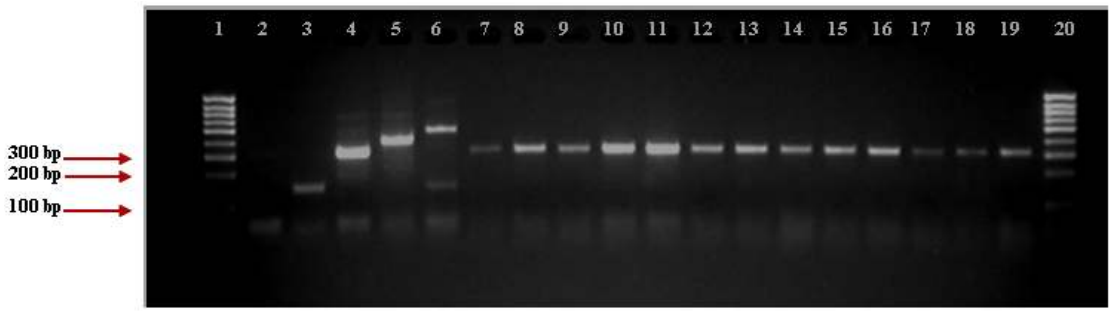



**Figure 3.1:** Amplified fragments using the species-specific PCR assay for the identification of members of the *An. gambiae* complex. Lane 1 and 20: 1 kb molecular markers; lane 2: negative control; lanes 3 to 6 positive controls (*An. quadriannulatus*, *An. arabiensis*, *An. gambiae* and *An. merus* respectively); Lanes 7 to 19: wild caught *An. arabiensis*. The double band appeared in lane 6 (*An. merus* standard) was reported also by Paskewitz *et al.*, (1993) and Scott *et al*., (1993), but it was noted that the specific band was always brighter than the nonspecific (Van Rensburg *et al.*, 1996).

### 3.1.2 ELISA for blood meal analysis

All 310 blood-fed indoor resting *An. arabiensis* were used in the blood meal analysis. The majority 273 (89.2%) were positive for human blood while 33 (10.8%) were positive for bovine blood. Four specimens (1.3%) were not identified as having either human or bovine blood. Details on blood meal analysis per locality are shown in Figure 3.2. Figure 3.3 shows a typical example of a microtitre plate with positive samples for the human blood

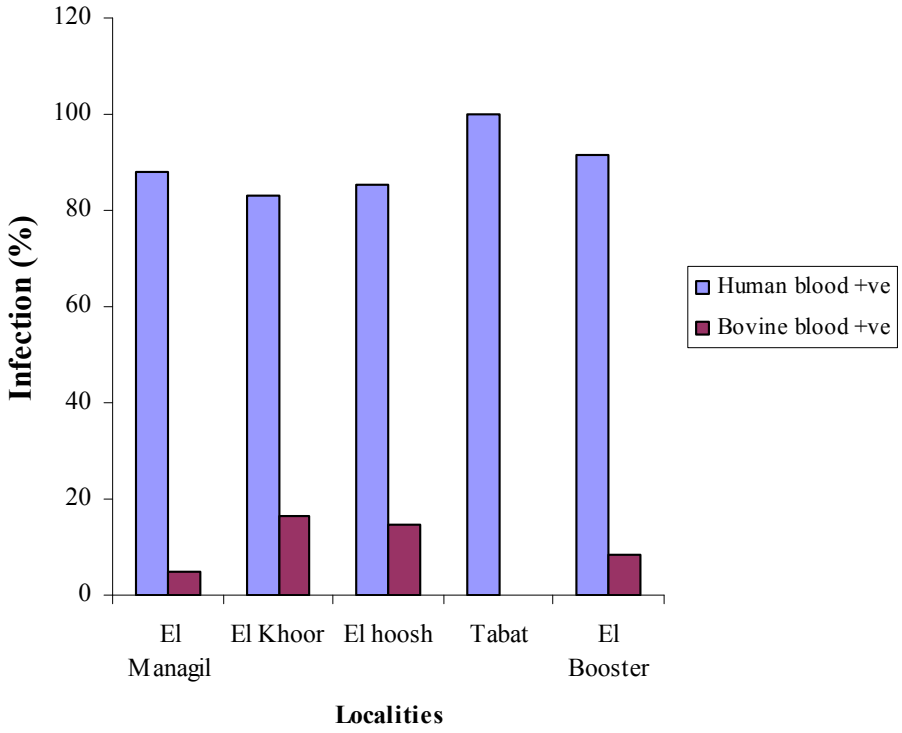



**Figure 3.2:** Blood meal analysis for *An. arabiensis* from the five sentinel sites.

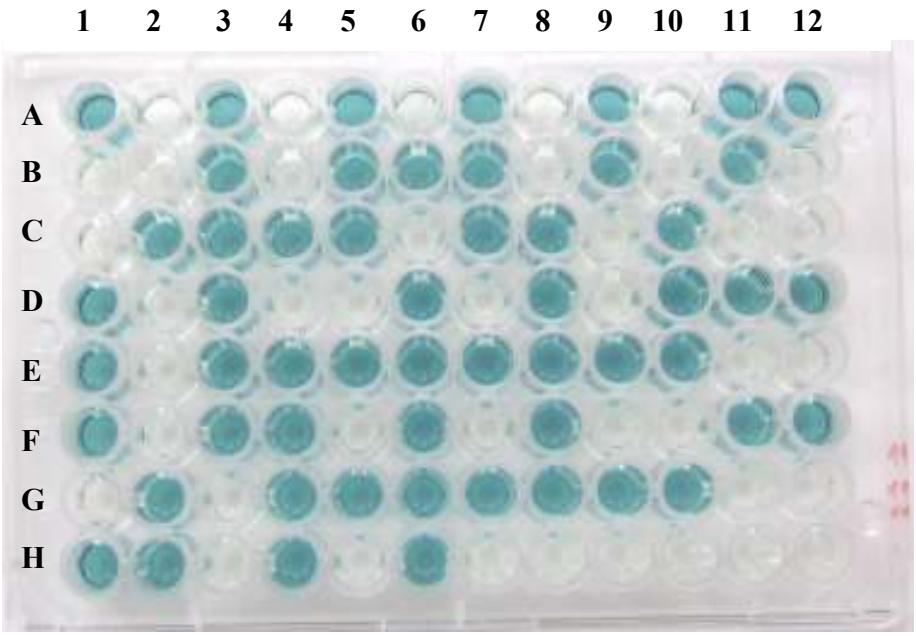



**Figure 3.3:** Microtitre plate showing results of ELISA after blood meal identification in *An. arabiensis*. The green color indicates positive results for human blood meal. Well A1 is a positive control and H9 to H12 are negative controls.

### 3.1.3 Sporozoite ELISA

*Plasmodium falciparum* sporozoite infections were determined in the 310 indoor collected *Anopheles arabiensis* mosquitoes using ELISA technique. The highest sporozoite rate of *P. falciparum* was found in Tabat where three out of fifty five samples were found to be infective, giving a sporozoite rate of 5.5%. In El Hoosh the infection rate was 1.8% (1/55), in El Khoor at 1.6% (1/60) and in El Booster 4% (2/50). None of the 90 mosquitoes examined from El Managil were found to be positive.

The overall infection rate for *An. arabiensis* in central Sudan was therefore 2.3 % (7/310).

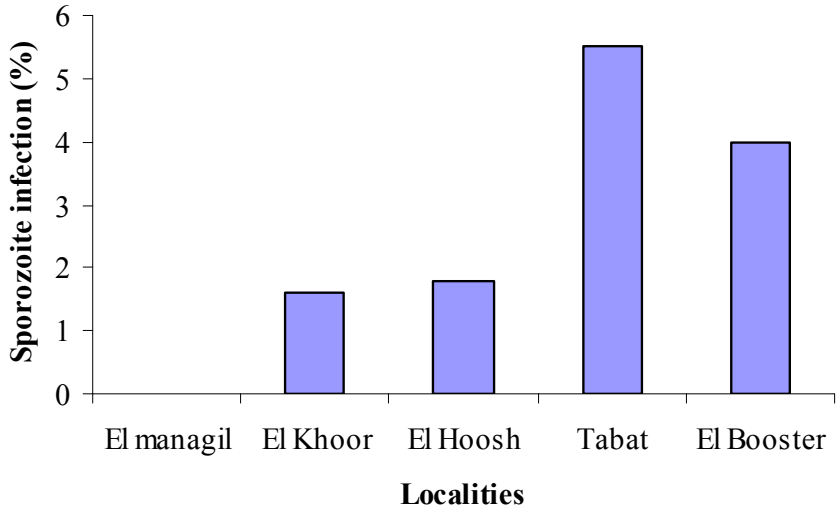



**Figure 3.4:** Results of *Plasmodium falciparum* sporozoite infection rate in *An. arabiensis* per site.

### 3.1.4 WHO susceptibility tests

Mosquito collections were made at 13 localities in Gezira and Sennar states of central Sudan. In total, 5,749 female mosquitoes were reared from larvae collections and identified as members of *An. gambiae* complex using the keys of Gilles and Coetzee (1987). Once adults were 1-3 days old they were used in the susceptibility tests. Table 3.2 shows the susceptibility tests results from these two states. Sub samples of specimens were later identified as *An. arabiensis* (section 3.1.1).

The WHO criteria for determining resistance or susceptibility were applied throughout. Mortality between 98-100% mortality indicates susceptibility, <80% mortality suggests resistance and 80-97% mortality requires confirmation of resistance (WHO, 1998b). Mortalities in control groups were less than 5%, thus, no corrections were required.

**Permethrin (0.75%)**

Permethrin resistance was recorded from both states. The highest levels of resistance were found in Gezira state from El Masalamia, El Hoosh, El Khoor, Tabat, Mobi, Wad Rawa and Wad Medani ranging from 54.6% to 78.1% (Table 3.2). In Sennar state resistance was recorded only in Singa city in the south with a mortality rate of 72% (Table 3.2). The mortality rates from the other five sites within the two states were between 84% – 94.2%, which suggest the possibility of resistance and needs further investigation (Table 3.2).

**DDT (4%)**

Mosquitoes were susceptible to DDT in El Booster locality in Sennar state, however, high levels of DDT resistance were observed in Singa city with 64.8% mortality rate (Table 3.2). In Gezira state, resistance was reported from Masalamia, El Hoosh, Wad Rawa and Wad Medani localities where the percentage mortality rates were 59.2%, 55.4%, 60.8% and 66.4% respectively (Table 3.2). Resistance needs further investigation at the other seven sites.

**Malathion (5%)**

Mosquitoes from El Booster locality and Sennar city in Sennar state were susceptible to malathion insecticide with 100% and 99% mortality rates respectively (Table 3.2). Resistance was only observed in El Hoosh and Tabat localities in Gezira state (Table 3.2). At the other nine sites, the mortality rates were between 80.2% - 97.3% indicating

the possibility of resistance that needs further investigation according to WHO criteria (Table 3.2).

**Bendiocarb (0.1%)**

Mosquitoes from all 13 localities were fully susceptible to bendiocarb where 100% mortality rates were reported after 24 hr post-exposure period (Table 3.2)

**Table 3.2:** WHO standard susceptibility results on 1-3 days old female *An. arabiensis* from13 localities in Central Sudan

| State | Locality | Permethrin 0.75% | | DDT 4% | | Malathion 5% | | Bendiocarb 0.1% | |
|---|---|---|---|---|---|---|---|---|---|
| | | number | %mortality | number | %mortality | number | %mortality | number | %mortality |
| **Gezira State** | **El Masalamia** | 121 | 72.7* | 120 | 59.2* | 111 | 89.2 | 121 | 100 |
| | **El Hoosh** | 121 | 54.6* | 112 | 55.4* | 114 | 78.1 | 136 | 100 |
| | **El Khoor** | 114 | 78.1 | 116 | 90.5 | 110 | 97.3 | 114 | 100 |
| | **El Managil** | 109 | 89.9 | 82 | 95.1 | 98 | 90.8 | 123 | 100 |
| | **Mobi** | 119 | 72.3* | 146 | 83.6* | 80 | 86.3 | 127 | 100 |
| | **Rofaa** | 105 | 85.7 | 88 | 83 | 100 | 97 | 126 | 100 |
| | **Tabat** | 121 | 76.3 | 82 | 84.1 | 82 | 76.8 | 125 | 100 |
| | **Wad Medani** | 113 | 66.4* | 113 | 66.4* | 99 | 86.7 | 100 | 100 |
| | **Wad Rawa** | 116 | 58.6* | 120 | 60.8* | 126 | 89.7 | 129 | 100 |
| **Sennar State** | **El Booster** | 103 | 94.2 | 111 | 99.1 | 100 | 99 | 100 | 100 |
| | **El Suki** | 102 | 84* | 103 | 95.2 | 116 | 80.2 | 126 | 100 |
| | **Sennar** | 102 | 92.2 | 107 | 93.5 | 97 | 100 | 107 | 100 |
| | **Singa** | 111 | 72* | 108 | 64.8* | 108 | 91.7 | 109 | 100 |
| **Total samples tested** | | **1,457** | | **1,408** | | **1,341** | | **1,543** | |

- Numbers in red indicate resistance, * used in molecular *kdr* mutation studies

### 3.1.5 *kdr* mutations detection

#### 3.1.5.1 Polymerase chain reaction (PCR)

A total of 60 permethrin resistant, 60 DDT resistant, 60 permethrin susceptible and 60 DDT susceptible specimens of *An. arabiensis* were analysed for the presence of the West and East African *kdr* mutations. Samples were chosen from six sites within the two states where high levels of permethrin and DDT resistance were detected (Table 3.2). Ten random specimens from El Booster locality were assayed as controls. Five DDT resistant samples could not be amplified and no results were obtained.

The results revealed the presence of the West African *kdr* mutation only. The *kdr* genotypes obtained are given in Tables (3.3-3.4). These frequencies were compared to the expected Hardy-Weinberg proportions using chi-square analysis. Overall, there were no differences between the genotypes observed and Hardy-Weinberg proportions using permethrin and DDT (survivors/dead) (Tables 3.3 and 3.4). All the ten random samples used as control were found to be heterozygous (RS) for the *kdr* mutation. The observed numbers of genotypes from permethrin resistant specimens compared with the observed numbers from susceptible permethrin specimens showed no significant differences ($P>0.05$) (Table 3.3). When the same comparisons were made using DDT, however they showed significant differences ($P<0.05$) (Table 3.4).

**Table 3.3**: The observed and expected genotype numbers (Hardy-Weinberg, H-W) and allele frequencies for the West African *kdr* mutation for permethrin survivor and dead samples are given.

| *Permethrin survivors* | **Genotypes** | | | | **allele frequencies** | |
|---|---|---|---|---|---|---|
| | **RR** | **RS** | **SS** | **total** | **R** | **S** |
| Number observed | 19 | 31 | 10 | 60 | 0.57 | 0.43 |
| Number expected H-W | 19.5 | 29.4 | 11.1 | 60 | | |
| $\chi^2$= 0.11, ***P* =0.9484** | | | | | | |

| *Permethrin Dead* | **Genotypes** | | | | **allele frequencies** | |
|---|---|---|---|---|---|---|
| | **RR** | **RS** | **SS** | **total** | **R** | **S** |
| Number observed | 11 | 35 | 14 | 60 | 0.47 | 0.53 |
| Number expected H-W | 13.3 | 29.9 | 16.8 | 60 | | |
| $\chi^2$= 0.83, ***P*= 0.6607** | | | | | | |

$\chi^2$ for permethrin survivors vs permethrin dead= 2.00, ***P*=0.1570**

**Table 3.4**: The observed and expected genotype numbers (Hardy-Weinberg, H-W) and allele frequencies for the West African *kdr* mutation for DDT survivor and dead samples are given.

| *DDT survivors* | **Genotypes** | | | | **allele frequencies** | |
|---|---|---|---|---|---|---|
| | **RR** | **RS** | **SS** | **total** | **R** | **S** |
| Number observed | 25 | 25 | 5 | 55 | 0.68 | 0.32 |
| Number expected H-W | 25.4 | 24.0 | 5.6 | 55 | | |
| $\chi^2$=, 0.01, ***P* = 0.9944** | | | | | | |

| *DDT Dead* | **Genotypes** | | | | **allele frequencies** | |
|---|---|---|---|---|---|---|
| | **RR** | **RS** | **SS** | **total** | **R** | **S** |
| Number observed | 7 | 42 | 11 | 60 | 0.47 | 0.53 |
| Number expected H-W | 13.2 | 30.0 | 16.8 | 60 | | |
| $\chi^2$=4.70, ***P*=0.0945** | | | | | | |

$\chi^2$ for DDT survivors vs DDT dead= 9.02, ***P*=0.0027**

### 3.1.5.2 Sequencing results

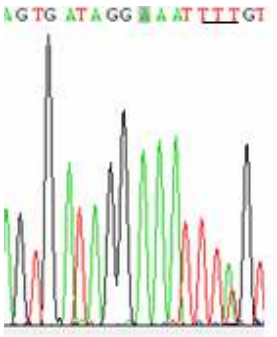
Eight susceptible and resistant random samples were sequenced once. The results confirmed the presence of the West African *kdr* mutation. Some samples showed no correlation with the PCR results. Sequence results obtained are shown in Table 3.5 and examples of *kdr* genotype chromatograms obtained by sequence analysis are shown in Figure 3.5.

**Table 3.5:** Association of the *kdr* genotypes with DDT/permethrin susceptible and resistant phenotypes of the *An. arabiensis* samples from central Sudan following 1 hr exposure to 4% DDT and 0.75% permethrin. R = Resistance allele. S= susceptible allele.

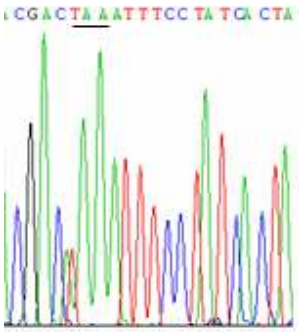
| **Bioassay phenotype** | **n** | ***kdr* genotype by PCR** | ***kdr* genotype by sequence analysis** |
|---|---|---|---|
| Susceptible | 6 | 3 SS<br>1 RS (East Africa)<br>2 RS | 2 SS and 1 RS<br>1 RS west Africa<br>2 RS |
| Resistant | 8 | 2 RS<br>6 RR | 1 RS and 1 RR<br>4 RS, 1 SS and 1 RR |

**1. Heterozygous (RS)**

Forward



Reverse



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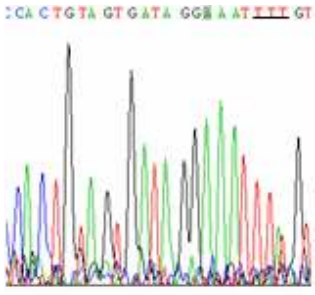
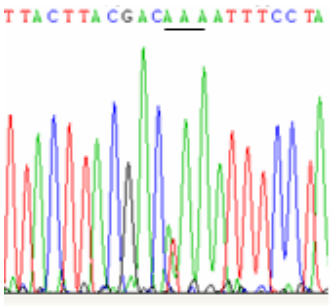
**2. Resistant homozygous (RR)**

Forward



Reverse



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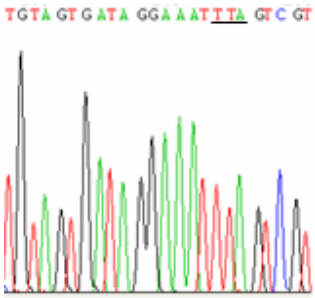
**3. homozygous susceptible (SS)**

Forward



Reverse

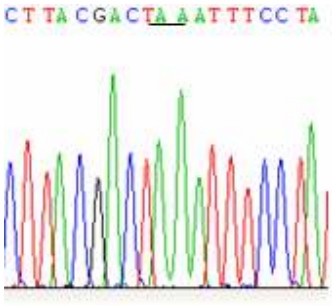


**Figure 3.5**: Chromatogram examples of the sequence results obtained

## 3.2 STUDIES ON COLONY MATERIAL

### 3.2.1 Selection of DDT resistance

Due to difficulties found in transferring wild mosquitoes from Sudan to the laboratory in South Africa, a laboratory colony (SENN-U) originating from Sennar city in central Sudan (one of the sentinel sites) and maintained at the NICD insectary was used. A sub-colony was selected for DDT resistance (SENN-DDT). These two colonies were used in the biochemical analysis.

Bioassay data obtained by generation and gender ($F_1$ to $F_{17}$) based on selection for resistance to 4% DDT are shown in Figure 3.6. By the second generation of selection, resistance was successfully achieved in both sexes with 49.5% mortality rate in the males and 48.3% in the females. There was no significant difference in the mortalities between the males and females (two samples *t*-test: $P = 0.8676$).

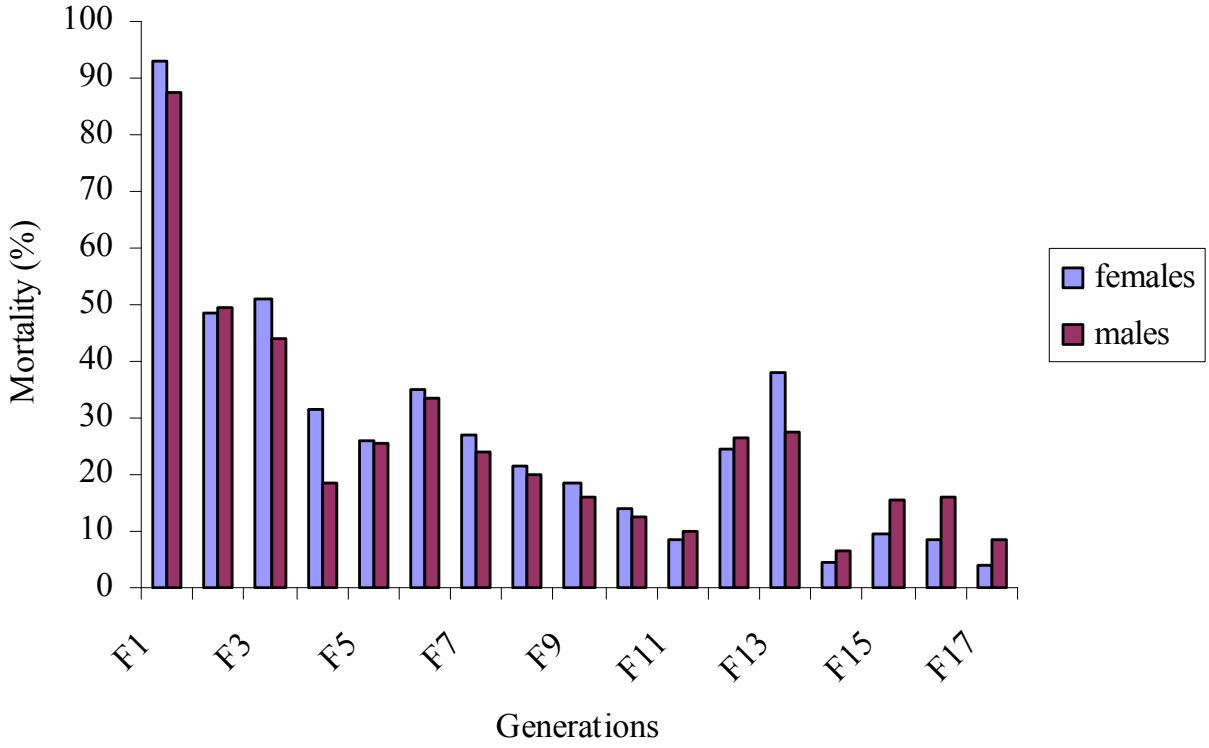



**Figure 3.6:** Susceptibility results using males and females of *An. arabiensis* (SENN-U) colony from central Sudan exposed to DDT 4% for 1 hour. Results show final mortality 24 hour post exposure.

### 3.2.3 Molecular analysis of *Kdr* detection

Ten random samples from SENN-U were assayed for the presence of *kdr* mutation. A sample from SENN-DDT of 10 resistant (5 males and 5 females) and 10 susceptible (5 males and 5 females) mosquitoes, based on their responses to 1 h exposure to 4% DDT, were assayed also for the *kdr* mutation (Matambo *et al*., 2007, Appendix III).

Results obtained by PCR and sequencing showed that all the 10 samples from SENN-U were SS while all the samples from SENN-DDT regardless of insecticide susceptibility phenotype were RR (for details see Matambo *et al*., 2007 in Appendix III)

### 3.2.5 Biochemical studies of metabolic resistance

Biochemical studies were carried out using female samples from SENN-U and SENN-DDT strains to assays the levels of activity of a range of detoxifying enzyme systems including monooxygenases, non-specific esterases and glutathione S-transferases (GST). Assays were also performed to determine the inhibitory effect of the carbamate insecticide propoxur on acetylcholinesterase activity.

Mean optical density values obtained for each enzyme are shown in Table 3.6. Two-sample *t*-tests revealed no significant differences ($P>0.05$) in the esterases and the oxigenases detoxifying enzyme levels between the selected line (SENN-DDT) and the unselected strains (SENN- U). The selected strain showed significantly higher levels of GSTs than the unselected line (two sample *t*- test: $P= 0.003$). Both unselected and selected samples showed >54% inhibition of acetylcholinesterase activity when

challenged with propoxur (Table 3.7) with the exception of one female in the selected line that showed 44.4% inhibition (included in Table 3.7).

**Table 3.6:** Mean optical density values for glutathione S-transferase (GSTs), monooxygenase (Oxy) and esterase enzymes (EST) using α and β naphthyl acetate as substrates. n = sample size; figures in parentheses are the standard errors for each sample; *p* indicates significance in difference between SENN-U and SENN-DDT samples by enzyme system by following two sample *t* test.

| **Colony** | **Mean values/standard errors** | | | |
|---|---|---|---|---|
| | **GSTs** | **Oxy** | **ESTα** | **ESTβ** |
| **SENN-U (n=48)** | 0.00695 (0.000556) | 0.7191 (0.0312) | 0.5814 (0.0258) | 0.3040 (0.0145) |
| **SENN-DDT (n=46)** | 0.0105 (0.000764) | 0.7930 (0.0468) | 0.6486 (0.0374) | 0.2960 (0.0186) |
| ***P*** | 0.0003 | 0.1925 | 0.1434 | 0.7341 |

**Table 3.7:** Mean percentage inhibition of acetylcholinesterase activity by the carbamate insecticide propoxur. "n"=sample size; figures in parentheses are the data range for each sample. *P* indicates significance in difference between unselected and selected samples following two sample *t* tests.

| | **n** | **% inhibition** |
|---|---|---|
| **SENN-U** | 47 | 82.167 (53.33 - 98.97) |
| **SENN-DDT** | 46 | 85.475 (44.44 - 95.46) |
| ***P*** | | 0.0970 |