CHAPTER FOUR

GENERAL DISCUSSION

4.1 FIELD MATERIAL

4.1.1 Species identifications

The most efficient vectors of human malaria in Africa are members of the *An. gambiae* complex and *An. funestus* group (Coluzzi *et al.*, 1979, Coetzee *et al*., 2000). In Sudan, there are 29 anopheline mosquito species that have so far been recorded (Lewis, 1958). They are widely distributed throughout the country, but more often are localized in the humid areas. *Anopheles arabiensis* and *An. gambiae* are the only species of the *An. gambiae* complex reported in Sudan (Zahar, 1985; Petrarca *et al*., 2000).

Information about the species composition, biology and behaviour are essential in understanding their epidemiological role in malaria transmission and therefore the correct planning for a malaria control programme (Hunt *et al*., 1998). Previous studies carried out in central Sudan based on morphological and cytogenetic experiments showed that *An. arabiensis* is the most predominant species in the area (El Gaddal *et al*., 1985; Petrarca *et al.*, 2000). The only other anopheline species that have been reported from the study area were *An. rufipes* Gough and *An. pharoensis* Theobald, but these two species play no role in malaria transmission because of their largely zoophilic behaviour (Lewis, 1958; El Gadal *et al.*, 1985). However, Lewis (1948) reported *An. rufipes* feeding on humans and resting indoors during the day in southern Sudan.

This study presents the first application of the PCR method of Scott *et al.* (1993) on field-collected specimens from Sudan. Out of 960 *An. gambiae* complex tested, 859 (89.5%) were identify as *An. arabiensis* and 101 (10.5%) were not identified, probably due to either misidentification during the morphological identification process or DNA degradation due to bad preservation.

These results confirmed that *An. arabiensis* is the only member of the *gambiae* complex found in the study area. The abundance of *An. arabiensis* in the area is governed by the rainfall, dams and the spread of the irrigation schemes with new irrigation practices which constitute favorable larval habitats (Mouchet *et al.*, 1998; El Gadal *et al.*, 1985). However, studies on *An. arabiensis* in Wadi Halfa area in northern Sudan showed that this species has the ability to survive the dry season (Dukeen and Omer, 1986).

The PCR has the advantages of using any life stage and either sex of mosquitoes for the identification. Small parts of individual mosquitoes fresh or preserved can also be used, which allows testing for blood meal sources and the presence of the sporozoites with the remaining samples (Paskewitz *et al*., 1993). Furthermore, the use of non extracted DNA makes the method easy and suitable for routine work as 100 samples can be identified in a single day (Van Rensburg, 1996). The only disadvantage of this method is the cost of the reagents and equipment.

4.1.2 Blood meal and sporozoite analysis

One of the most important parameters in understanding the vectorial capacity of malaria vectors is the identification of blood meal source (Rosenberg *et al.,* 1989, 1990). Blood meal identification is also important in understanding the transmission dynamics of malaria (Rosenberg *et al.,* 1989). The anthropophilic index (percentage feeding on humans) is a vital component of vectorial capacity, while knowledge of other hosts reveals the relative importance of reservoirs of vector borne zoonotic or enzootic infections (Boakye *et al.,* 1999*)*.

In this study, 89.2% *An. arabiensis* females were found fed on human while 10.8% fed on bovine. The highest human blood index (HBI) (100%) was found in Tabat locality where the mosquitoes were collected from a camp located in the middle of a cotton growing area. In El Booster locality in Sennar state, *An. arabiensis* also showed more than 91% anthropophilic behaviour. Degrees of zoophilic behaviour (5-16.6%) in *An. arabiensis* were found in El Khoor, El Hoosh, El Managil and El Booster. A small percentage (1.3%) could not be identified as being either human or bovine blood. These results showed that *An. arabiensis* in the study area is highly anthropophilic. These findings are in agreement with those of Mwangangi, *et al*., (2003) who found, *An. arabiensis* feed predominantly on humans along the Kenyan coast despite the availability of cattle and other domestic animals. However, these results are contrary to many other studies on *An. arabiensis*. Highton *et al*., (1979) found *An. arabiensis* on the Kano plain in Kenya, to be highly zoophilic with 59% bovine blood index. In Ethiopia, Boreham (1975) reported 5% HBI while Ameneshewa and Service (1997) found 33%

HBI. These variations in HBI levels could be attributed to the opportunistic feeding behaviour of *Anopheles arabiensis* (White, 1974; Gillies and Coetzee, 1987; Costantini *et al*., 1999).

Traditionally, people from all the sentinel sites prefer sleeping outdoors especially during summer, which increase the chances of mosquitoes feeding on humans. However, all *An. arabiensis* mosquitoes used in the blood meal analysis were collected indoors. This means that *An. arabiensis* from the study area prefers resting indoors even after feeding outdoors (on human or bovine). These findings suggest that malaria vector control in the area using indoor residual spraying and impregnated bed nets would be effective in reducing human-vector contact.

Sporozoite infection rate is another important factor in studying the vectorial capacity and the transmission of malaria. Overall, among the indoor resting *An. arabiensis* females, the sporozoite infection rate was 2.3%. This rate could be considered average, as Beier (1998) indicates that sporozoite rates between 1-5% for members of the *An. gambiae* complex are normal. In some neighboring countries, wide ranges of sporozoite rates have been reported in *An. arabiensis*. Fettene *et al*. (2004) reported a low sporozoite rate (0.24) from Ethiopia. In Eritrea, Waka *et al*. (2005) and Okbaldet *et al*. (2001) recorded 0.1% and 0.68% infection rates respectively. However, in Kenya, *An. arabiensis* showed variations in the sporozoite rates: 2.8-7.8% (Joshi *et al*., 1975), 0.5% (Service *et al*., 1978) and 2.8%-3.4% (Petrarca *et al*., 1991). Indoor resting females used in this analysis were collected during the rainy season (the peak of transmission). Previous studies showed that the sporozoite infection is high during the rainy season

(Molineaux and Gramiccia, 1980). Therefore, another study is needed to investigate the malaria transmission during the dry season as *An. arabiensis* has the ability to survive these conditions (Dukeen and Omer, 1986). The high infection rate in this study could be due to the high anthropophilic behaviour of *An. arabiensis* found in this study.

These findings confirm the efficacy of *An. arabiensis* as a malaria vector and its important role in the transmission of malaria in the study area.

4.1.3 Susceptibility tests

This study presents results of a large-scale insecticide resistance survey in Gezira and Sennar states of central Sudan where insecticides have been used intensively for both public health and agricultural purposes. Results showed multiple resistance of *An. arabiensis* to permethrin, DDT and malathion. The most resistant population was found in El Hoosh locality, one of the most important sections in the Gezira Agricultural Scheme (GAS), where resistance occurred to three insecticides out of four (permethrin, DDT and malathion).

DDT resistance in *An. arabiensis* in central Sudan was reported for the first time in a colony originating form Elgunied area in east Gezira in 1970. The average mortality rate was 34% (Haridi, 1972). Also Abdel Nour in 1972 (unpublished data) reported resistance to both DDT and dieldrin and attributed it to the extensive use of these insecticides in cotton spraying. A recent study from eastern Sudan showed evidence of resistance in *An. arabiensis* to DDT in a cotton growing area (unpublished data). Resistance in *An. arabiensis* to DDT was detected also in South Africa in 2003

(Hargreaves *et al*., 2003). DDT is currently not in use, however, high levels of resistance were still observed. Possible reasons maybe that these populations have been subjected to insecticide selection pressure during the past decades. In addition, other insecticides that target the same site, such as pyrethroids may confer cross-resistance to DDT. As a result, high levels of DDT resistance could be observed even in areas where DDT use had been disconnected for years.

Considering that only 34% mortality rate to DDT was recorded in east Gezira in 1970 (Haridi, 1972), results from the same area in this study (Rofaa and Wad Rawa) showed 71% mortality rate, thus only 32% revision rate of resistance has achieved over the past ±30. Suggesting that there is no fitness cost for DDT resistance in these populations. This observation reduces the feasibility of the re-introduction of DDT as a part of a resistance management strategy. The absence of fitness cost was noted before in *Anopheles culicifacies* and *An. subpictus* in Sir Lanka. The resistance was around 80% in 1970s compared to 50% in the 1990s (IRAC, 2006).

The use of malathion, for IRS in central Sudan was stopped in 1978 as a result of physiological resistance. Insecticides from the organophosphate group are still in use for agricultural purposes (GAS, unpublished data). This might be an indicator of the role of agriculture in selecting for resistance to this group. In Sennar state, evidence of malathion resistance was found in El Suki and Singa cities where the organophosphate group is still in use in small scale vegetable farming. The finding of malathion resistance in the present study is in agreement with initial insecticide resistance studies on a colony originated from Gezira area where high levels of malathion resistance (28% mortality

rate) in adult mosquitoes of *An. arabiensis* were found to be associated with the use of this insecticide for indoor residual house spraying. The mechanism involved was the esterase enzymes (Hemingway, 1983). If this mechanism is currently involved in the malathion resistance found in this study, it was expected to find cross-resistance between malathion and bendiocarb. The malathion mortality rate found in Gezira state in the present study is about 60% higher than the mortality rate obtained by Hemingway (1983) suggesting that malathion resistance has reverted faster than DDT resistance.

Pyrethroids are currently the only group of insecticides used in public health in central Sudan, while insecticides from both pyrethroid and organophosphate groups are extensively used for agricultural purposes (GAS, unpublished data). The highest levels of resistance in this study were found using permethrin with mortalities of under 98% at all the thirteen sites, suggesting that these populations had been submitted to strong insecticide selection pressure. However, a recent report from eastern Sudan demonstrated the complete susceptibility of *An. arabiensis* population to permethrin despite the use of pyrethroids for cotton pest control (unpublished data).

Resistance to this important group has been reported in all three major malaria vectors in Africa. In West Africa, resistance in *An. gambiae* has been recorded from Benin, Burkina Faso, Cote d'lvoire and Ghana (Elisa *et al*., 1993; Chandre *et al*., 1999a, b; Coetzee *et al*., 2006). The widely accepted reason for this resistance in these West African populations of *An. gambiae* was the indiscriminate use of insecticides for agricultural purposes and for the control of household pests (Curtis *et al*., 1998; Chandre *et al*., 1999a). In Kenya, resistance to pyrethroid insecticides was noted 1 year after the

introduction of a permethrin–impregnated bed net programme (Vulule *et al.,* 1994). In *An. arabiensis*, resistance to permethrin and deltamethrin were reported from Cameroon (Etang *et al*., 2003), and evidence of resistance detected recently from Mozambique (Casimiro *et al*., 2006). In *An. funestus*, pyrethroid resistance has been reported from South Africa (Hargreaves *et al*., 2000) and Mozambique (Casimiro *et al*., 2006).

Results obtained during the present study have also shown that there is full susceptibility to bendiocarb. This could be considered as a possible alternative to the existing pyrethroids for use in IRS vector control. Bendiocarb has the advantage that it has a different mode of action from the pyrethroids and so the possibility of cross-resistance to pyrethroids is low.

It is clear that the use of insecticides in public health and the extensive use in agriculture has led to the high prevalence of insecticide resistance in the study area making this population of *An. arabiensis* the most resistant population in the *An. gambiae* complex. However, the role of ITNs in selecting for resistance needs to be investigated.

4.1.4 The West African *kdr* mutation

Vector control through the use of insecticides for indoor house spraying (IRS) and impregnation of bed nets (ITNs) is an important component of the global malaria control strategy (Lengeler *et al*., 1996). Pyrethroids are currently the only available and recommended insecticides for use on ITNs, because of their quick knockdown effects, high insecticidal potency and relatively low mammalian hazard at operational doses (Elliot *et al.,* 1978; Martinez-Torres *et al*., 1997). The wide use of these insecticides has

led to the rapid development and spread of resistance in mosquitoes, which has implications on the sustainability of any vector control programme (Pinito *et al*., 2006).

Knockdown resistance (*kdr*) is a major mechanism of resistance to both DDT and permethrin. These insecticides share the same target site (sodium channel in the nervous system), and mutations in this site result in reduced sensitivity of the channel to these insecticides (Martinez-Torres *et al.,* 1998). Knockdown resistance was originally described in DDT-resistant houseflies, *Musca domestica* (Busvine, 1954). In mosquitoes, two types of *kdr* mutations are found in *An. gambiae*, the first one is the West African *kdr* mutation (*w-kdr*) which results in an amino acid substitution from Leucine to Phenylalanine (Leu→ Phe), and was first observed in *An. gambiae* from Ivory Coast and Burkina Faso in West Africa (Martinez-Torres *et al.,* 1998). The second mutation, found at the same amino acid position but is a change from Leucine to Serine (Leu → Ser), has been found in an East African population of *An. gambiae* (*e-kdr*) (Ranson *et al*., 2000). These mutations appear to be spreading rapidly and they have been detected in several African countries, especially in areas where DDT resistance also occurs (Akogbeto and Yakoubou, 1999; Chandre *et al*., 2000; Awolola *et al*., 2002; Weill *et al*., 2000; Diabate *et al*., 2002, 2003; Fanello *et al*., 2003; Coetzee *et al*., 2006).

In *An. arabiensis*, *w-kdr* mutation was reported for the first time in a single specimen collected from an agricultural area in Burkina Faso (Diabaté *et al.,* 2004). Stump *et al*., (2004) reported the *e-kdr* in the same species from Kenya also from a single specimen. Recently, the same mutation has been reported in two specimens from Uganda Verhaeghen *et al*., (2006). In Tanzania Kulkarni *et al*., (2006) reported the presence of

w-kdr also in a single specimen. All these studies were on wild specimens of *An. arabiensis* and were not correlated with resistance phenotypes.

In this study, results revealed the presence of the West African *kdr* mutation among (DDT/permethrin) survivor and dead specimens with the majority being heterozygous (RS). Comparing the observed *kdr* genotypic frequencies using permethrin and DDT (survivor/dead) specimens from all the sentinel sites with Hardy-Weinberg expectations, showed no significant differences ($P>0.05$), either between insecticides or the two phenotypes.

Due to the small sample size per locality ($n<30$), specimens were combined for the accuracy of the statistical analysis (Tables 3.4 and 3.5). Overall, no significant differences ($P>0.05$) were found between the allele frequencies obtained using permethrin survivors and those obtained using permethrin dead. If the *kdr* mutation was important in conferring the high levels of permethrin resistance that were detected, we would have expected to see an excess of the R allele amongst survivors. This suggests that other mechanism/s such as metabolic detoxifying enzymes are involved. For example, Vulule *et al*., (1999) showed the involvement of P450 enzymes in pyrethroid resistance in the Kenyan population of *An. gambiae*.

On the other hand, the allele frequencies were found to be significantly different between DDT survivor and dead specimens (***P*=0.0027**) with survivors showing a 15.8% higher frequency than the dead specimens. This indicates that the selection of DDT resistance had also led to the selection of high frequencies of the R allele among survivors. However, finding permethrin and DDT susceptible specimens carrying the

resistance alleles (RR), indicates that the *kdr* was not enough to protect the insect against the insecticide and it showed other mechanism stronger than the *kdr* is responsible of DDT resistance in these populations. In addition, finding 9% of DDT resistant mosquitoes did not possess the *kdr* mutation support the hypothesis that another mechanism such as GSTs might be involved. The majority of DDT resistance reports in *Anopheles* species indicate the involvement of GSTs enzymes (Herath *et al*., 1988; Prapanthadara *et al*., 1995; Hargreaves *et al*., 2003) and this could confer cross-resistance to pyrethroids.

The sequence analysis confirmed the presence of the *w-kdr* mutation but showed lack of correlations in some specimens with the PCR results. Furthermore, the misidentification found between *e-kdr* and *w-kdr* during the processing of *kdr* genotyping using PCR, undermines the use of the two-step PCR to determine susceptibility. A reliable detection method should therefore be used in future *kdr* surveys.

Findings from this study are inconsistent with those of Himeidan, (2007, unpublished report), who found susceptibility to permethrin and slight evidence of DDT resistance in *An. arabiensis* from eastern Sudan (about 200 kilometers from our study area). However, he reported both *w-kdr* and *e-kdr* mutations respectively. He found the high frequency for the *w-kdr*. Matambo *et al*., (2007, Appendix III) reported the presence of the *w-kdr* mutation with RR genotype from both DDT susceptible and resistant specimens of *An. arabiensis* from a colony originated from central Sudan. These variations in results underline the need for further studies on the role of *kdr* in DDT and permethrin resistance in these Sudanese *An. arabiensis* populations.

In Summary, these results showed lack of correlation between the resistant phenotypes and the *kdr* genotypes. Similar lack of correlation has been found in other species such as *Culex quinquefasciatus* (Xu *et al*., 2005), *C. pipiens (*McAbee *et al*., 2004), and *An. gambiae* (Yawson *et al*., 2004).

4.2 LABORATORY COLONY STUDIES

Selection for resistance to DDT showed a marked increase in survivors from the second generation. Finding no significant differences ($P>0.05$) in the mortality rates between males and females suggest that no sex-linked factor is associated with resistance to DDT in SENN colony.

Biochemical analysis showed significant differences ($P<0.05$) in GSTs enzymes between the selected and unselected strains, suggesting that this enzyme system is associated with DDT resistance. GSTs have been reported to play an important role in DDT resistance in *An. gambiae* (Ranson *et al*., 2001), *Anopheles dirus* species B (Prapanthadara *et al*., 1998) and *An. arabiensis* (Hargreaves *et al*., 2003). GSTs were also found to play a minor role in pyrethroid resistance in *An. funestus* from South Africa (Brooke *et al*., 2001).

Molecular analysis of the *kdr* mutations revealed the absence of *kdr* mutation from the ten random samples which were sequenced from the unselected line SENN and the presence of the *w-kdr* mutation in SENN-DDT samples with all the samples homozygous RR, regardless of their resistance phenotype.

These results clearly indicate the involvement of GSTs in DDT resistance. However, the role of *kdr* still needs to be investigated.