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Insecticide resistance in malaria vectors in central Sudan

Abdalla, Hiba Mohammed Abu Bakr

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APPENDIX I

SUSCEPTIBILITY TESTS FORM

Date:

Area information

Country: Province:

Sample information

Species tested: Species control: Sex: Age (days) :

Collection method :

Human Landing Indoor	☐	Resting night Indoor	☐
Human Landing Outdoor	☐	Resting night Outdoor	☐
Cattle Collection	☐	Larval collection	☐
Resting morning indoor	☐	Colony	☐

Physiological stage Non-blood fed ☐ Blood fed ☐ Semi-gravid ☐ Gravid ☐

Insecticide information

Insecticide tested: Concentration:

Impregnated papers prepared by: Date of impregnation:

Date of expiry: Date paper removed from pack ☐☐☐☐☐☐

Number of times the paper is used:

Storage conditions: Room temperature ☐ Refrigerated ☐

Test conditions

	Temperature °C	Relative humidity
Exposure period: Start		
Holding period: after 12 hours		
Holding period: End		

Test results: **Period of exposure (minutes)**

	Replicate 1		Replicate 2		Replicate 3		Replicate 4		Replicate 5		Control	
No.exposed												
Number of knocked down mosquitoes after exposure for minutes :												
	Replicate 1		Replicate 2		Replicate 3		Replicate 4		Replicate 5		Control	
	Time	No.	Time	No.	Time	No.	Time	No.	Time	No.	Time	No.
START												
10’												
15’												
20’												
30’												
40’												
50’												
60’												
Number of killed mosquitoes at the End of holding period												
	Replicate 1		Replicate 2		Replicate 3		Replicate 4		Replicate 5		Control	
After 24 hours:												

APPENDIX II

LABORATORY METHODS

a) Preparation of solutions for PCR (Sambrook *et al*., 1989)

- **TAE buffer 50X (pH 8). (Tris Acetic EDTA)**

  242 g Tris

  37.2 g Na_2 EDTA.$2H_2O$

  57.1M glacial acetic acid

  Make up volume to 1L.

- **Agarose gel**

  10g agarose in 400ml 1XTAE for a 2.5% agarose gel or

  6g agarose in 400ml 1XTAE for a 1.5% agarose gel

- **Ficoll dye**

  50% sucrose

  1ml of 0.05M EDTA (pH 7.0)

  0.1% Bromophenol blue

  10% ficoll

- **Ethidum bromide**

  Dissolve 10mg crystallized EtBr in 1ml distilled H_2O

b) DNA extraction (Collins *et al*., 1987)

- **Grinding buffer**

  0.08M NaCl

  0.16M Sucrose

  0.06M EDTA

  0.5% SDS

  0.1M Tris-HCl

- TE Buffer (Tris EDTA) (Sambrook *et al*., 1989)

  100ml 1M tris (pH 8)

  20ml 0.5M EDTA

  Make up volume to 1 L

c) Preparation of solutions for ELISA

Blood meal ELISA (Beier *et al*., 1988) ; sporozoite ELISA (Wirtz *et al*., 1987)

- **Blocking buffer (BB)**

2.5% Casein

50ml, 0.1N NaOH

450ml, o.01M PBS pH 7.4

0.002% Phenol red

- **Boiled casein**

5% casein

100ml 0.1N NaOH

900ml 0.01M PBS

0.01% Thimerosal

0.002% Phenol red

- Phosphate Buffer Saline (PBS) 10X

- Phosphate Buffer Saline Tween 20 (PBS-Tween 20)

500µl Tween 20 to 1 litter 1XPBS

- Grinding Buffer (BB-NP40).

50ml BB + 250µl NP-40

d) Preparation of the buffers used in the biochemical analysis

ANALYSIS Penilla *et al*. (1998) and Hemingway *et al*. (1997)

- **0.1M Na_2HPO_4 pH 7.4***

  Na_2HPO_4 14.2g/1 litre dH_2O – dibasic.

  Na_2PO_4 12g/1 litre dH_2O – monobasic.

  Add monobasic to dibasic to adjust pH.

- **0.05M Na_2HPO_4 pH 7.4**

  350ml 0.1M buffer + 350ml dH_2O. Monobasic and dibasic solutions.

  Add monobasic to dibasic to adjust pH.

- **0.02M Na_2HPO_4 pH 7.2**

  100ml 0.1M buffer + 400ml dH_2O. Monobasic and dibasic solutions.

  Add monobasic to dibasic to adjust pH.

- **0.1M Na_2HPO_4 pH 6.5**

  As * but to adjust pH

- **0.1M Na_2HPO_4 5%SDS pH 7.0**

  As * but to adjust pH + 25g SDS per 500ml buffer

  SDS (Sodium dodecyl sulphate)

- **0.1M Na_2HPO_4 1% Triton pH 7.8**

  As * but to adjust pH + 1ml Triton per 100ml buffer

  Triton X-100.

- **0.25M Sodium acetate buffer $NaC_2H_3O_2$ pH 5.0**

  10.2537g/500ml dH_2O

  Add HCl to adjust pH

- **0.0625M K_2HPO_4 pH 7.2**

  2.7219g K_2HPO_4/250ml dH_2O. Dibasic

  2.1265g/ KH_2PO_4/250ml dH_2O. Monobasic

  Add monobasic to dibasic to adjust pH

- **30M α- Naphthyl acetate and β- Naphthyl acetate**

  0.055g α or β NA in 10ml acetone

  α or β Naphthol used for std curve from Sigma

- **0.1M propoxur**

  0.292g propoxur in 10ml acetone

- **0.1M p-nitrophenyl acetate (PnPa)**

  0.724g in 25ml acetonitrile

APPENDIX III

Matambo *et al*. (2007) paper

Medical and Veterinary Entomology, 21, 97–102

I contributed to the *kdr* molecular analysis for SENN base and the subsequent writing of the manuscript.

Insecticide resistance in the malarial mosquito *Anopheles arabiensis* and association with the *kdr* mutation

T.S. MATAMBO[1,3], H. ABDALLA[1,3,5], B.D. BROOKE[1,2], L.L. KOEKEMOER[1,2], A. MNZAVA[4], R.H. HUNT[1,3] and M. COETZEE[1,2,*]

[1]Vector Control Reference Unit, National Institute for Communicable Diseases, NHLS, Sandringham, Johannesburg, South Africa, [2]Division of Virology & Communicable Diseases Surveillance, School of Pathology of the National Health Laboratory Service and the University of the Witwatersrand, Johannesburg, South Africa, [3]School of Animal, Plant and Environmental Sciences, University of the Witwatersrand, Johannesburg, South Africa, [4]World Health Organization, Eastern Mediterranean Region, Cairo, Egypt, [5]Blue Nile Research and Training Institute, Wad Medani, Gezira, Sudan

Correspondence: Prof M. Coetzee, Vector Control Reference Unit, NICD, Private Bag X4, Sandringham 2131, South Africa. E-mail: **maureenc@nicd.ac.za**

Abstract. A colony of *Anopheles arabiensis* Patton (Diptera: Culicidae) from the Sennar region of Sudan was selected for resistance to DDT. Adults from the F-16 generation of the resistant strain were exposed to all four classes of insecticides approved for use in malaria vector control and showed high levels of resistance to them all (24 h mortalities: malathion 16.7%, bendiocarb 33.3%, DDT 12.1%, dieldrin 0%, deltamethrin 24.0%, permethrin 0%). Comparisons between the unselected base colony and the DDT resistant strain showed elevated glutathione S-transferase ($P<0.05$) in both sexes and elevated esterases ($P<0.05$) in males only. The Leu-Phe mutation in the sodium channel gene was detected by PCR and sequencing, but showed no correlation with the resistant phenotype. These results do not provide any explanation for why this colony exhibits such widespread resistance and further studies are needed to determine the precise mechanisms involved. The implications for malaria vector control in central Sudan are serious and resistance management, for example through the rotational use of different classes of insecticides, is recommended.

Key words: *Anopheles arabiensis*, carbamates, DDT, insecticide resistance, *kdr* mutation, organophosphates, pyrethroids, Sudan

Introduction

Anopheles arabiensis Patton is a member of the *Anopheles gambiae* Giles complex and the third most important malaria vector mosquito in Africa (Gillies & Coetzee, 1987). Insecticide resistance in this species based on the WHO susceptibility tests, has been reported from West Africa (DDT, dieldrin, Brown 1986), southern Africa (dieldrin/BHC, Green 1982; DDT, Hargreaves *et al.* 2003), Sudan (malathion, DDT, dieldrin, permethrin, Hemingway 1983) and the Indian Ocean islands of Madagascar (dieldrin) and Mauritius (DDT) (Brown, 1986).

Two primary mechanisms of DDT resistance have been identified in malaria vector mosquitoes: (1) significant metabolic detoxification based on increased activity of glutathione-S-transferase (GST) described in *Anopheles gambiae* Giles *s.s.* (Ranson *et al.*, 2000a) and apparently operational in southern African *An. arabiensis* (Hargreaves *et al.*, 2003); (2) target site insensitivity, referred to as knockdown resistance (*kdr*), shown to confer significant resistance to DDT and pyrethroid insecticides in *An. gambiae s.s.* (henceforth referred to here as *An. gambiae*) in West Africa (Martinez-Torres *et al.*, 1998). DDT and pyrethroids share the same mode of action on the nervous system, targeting the neuronal voltage-gated sodium ion channels. Molecular characterizations have revealed that various mutations in the S1-S6 transmembrane segments of domain II of the sodium ion channel give rise to DDT and pyrethroid resistance in a number of insect species (Knipple *et al.*, 1994; Williamson *et al.*, 1996; Martinez-Torres *et al.*, 1997; 1998). In West African *An. gambiae s.s.*, a mutation resulting in an amino acid change from Leucine to Phenylalanine (Leu → Phe) within the S6 hydrophobic

transmembrane segment has been associated with DDT/pyrethroid resistance (Martinez-Torres *et al.*, 1998). A resistance associated mutation in the same codon resulting in an amino acid change from Leucine to Serine (Leu → Ser) was found in an East African population of *An. gambiae* (Ranson *et al.*, 2000b). In recent reports it was shown that the *kdr* Leu → Phe mutation was present in a single specimen of *An. arabiensis* from Burkina Faso (Diabaté *et al.*, 2004) and two specimens from Tanzania (Kulkarni *et al.*, 2006). The Leu → Ser mutation has been found in two specimens from Uganda (Verhaeghen *et al.*, 2006) and one specimen from Kenya (Stump *et al.*, 2004). All the above specimens were heterozygous for the *kdr* alleles and none of the reports correlate the *kdr* alleles with resistance phenotypes.

Anopheles arabiensis is the principal vector of malaria in most regions of Sudan. Indoor residual house spraying for malaria vector control in the Sennar/Gezira region began with BHC in the 1950s (El Gadal *et al.,* 1985). DDT replaced BHC when resistance to the latter was detected in 1965 but lasted only four years until resistance to DDT too was detected (Haridi, 1972). Extensive use of the organophosphate insecticide malathion followed and resistance to this insecticide was reported in *An. arabiensis* in the region in 1978 (El Gadal *et al.,* 1985). Studies on a laboratory colony from Gezira indicated that a carboxylesterase-based resistance mechanism was present, but only in adult mosquitoes (Hemingway, 1983). Current surveys of *An. arabiensis* in Sudan show high levels of DDT and permethrin resistance in Gezira and Central States but low or no resistance in most other areas of Sudan (unpublished data). The Ministry of Health malaria vector control programme relies mostly on insecticide treated bed nets (ITNs).

In the present study we investigated the importance of the *kdr* mutation and potential enzyme-related resistance mechanisms in a DDT resistant strain of *An. arabiensis* from Sennar, Sudan.

Material and methods

Mosquito samples

The *An. arabiensis* colony used in the present study (SENN) was obtained from the Malaria Training Centre, Sennar, in south-central Sudan where it is under no insecticide selection pressure. It was stabilized in the insectary of the NICD, Johannesburg, and reared through five generations prior to selection of a sub-colony for resistance to DDT.

Selection for DDT resistance

Newly emerged mosquitoes were separated by gender and kept for a minimum of 2 days on 10% sugar solution. Cohorts of 25 mosquitoes per experiment were exposed to 4% DDT treated papers for 1 h according to the standard WHO susceptibility test protocol (WHO, 1998). Knockdown was recorded after 1 h and final mortality was recorded 24 h post exposure, during which time a 10% sugar solution was made available to survivors. Male and female survivors were pooled and maintained under standard insectary conditions (25°C, 80% relative humidity) to produce the next

generation. This process has been repeated through 16 generations. At the F-16 generation, cohorts of the DDT resistant strain (SENN-DDT) and the unselected base colony (SENN-U) were exposed to insecticides from all four classes approved for use in malaria vector control: 5% malathion (organophosphate), 0.1% Bendiocarb (carbamate), 4% dieldrin (cyclodiene), 0.05% deltamethrin and 0.75% permethrin (pyrethroids).

Biochemical analysis

Male and female samples from the SENN-U and SENN-DDT F16 strains were assayed to determine 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. These assays were performed using a kinetic multiskan microtitre plate reader according to the method of Penilla *et al.* (1998). Samples from the unselected and selected strains were assayed on the same 96 well microtitre plate in order to allow for direct comparison of optical density values between unselected and selected samples following adjustment for total protein content.

Molecular analysis

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 for the *kdr* mutation. DNA was extracted using the

method of Collins *et al.* (1987). The *kdr* genotype for each mosquito was determined using a two-step PCR procedure and primer sequences from Martinez-Torres *et al.* (1998) and Ranson *et al.* (2000b). In the first reaction, primers Agd1 and Agd3 were used to amplify the West African resistance allele, while primers Agd1 and Agd5 were used to detect the East African resistance allele. Both of these reactions amplify a 195 bp fragment. In each of the above multiplex PCR reactions, the susceptible allele was assayed using primers Agd2 and Agd4 which yields a 137 bp fragment (Martinez-Torres *et al.*, 1998).

Sequence analysis of the IIS6 domain

A 293 bp fragment of the IIS6 segment containing the *kdr* mutation was amplified from the 20 SENN-DDT mosquitoes described above and 10 randomly selected adults from the SENN-U colony using primers Agd1 and Agd2. This fragment was sent to Inqaba Biotechnical Industries, South Africa, for sequencing in order to cross-check the PCR results.

Results

Successive selections for DDT resistance showed a steady decline in mortality through 16 generations (Table 1) resulting in the current mortality rate of 12.1%. Different age cohorts tested at the F-16 generation showed increased mortality with age (Fig. 1). Exposures of F-16 generation adults to other insecticides (Table 2) showed a high level

of resistance to all four classes of insecticides used for malaria vector control, while the base colony exhibited a high level of resistance to dieldrin only.

Direct comparisons of optical density values between the unselected and SENN-DDT F-16 males and females are summarized in Table 3. Two-sample t tests revealed significantly higher levels of GST activity in both male and female DDT selected samples ($P<0.05$), while a significantly higher esterase level in the DDT selected strain was seen in male mosquitoes only ($P<0.05$). Both unselected and selected samples of both sexes showed >55% inhibition of acetylcholinesterase activity when challenged with propoxur (Table 4) with the exception of one male in the unselected sample that showed only 5.6% inhibition (not included in Table 4). The selected female sample showed a significantly lower mean percentage inhibition compared with the unselected female sample.

The PCR-based methods used to ascertain the *kdr* genotype of each specimen showed that all 20 specimens from the SENN-DDT selected strain, regardless of insecticide susceptibility phenotype, were homozygous (RR) (Fig. 2) for the West African resistance allele (Table 5). The East African Leu – Ser resistance allele was not detected in any of the specimens. These results were confirmed by sequence analysis of all specimens with the exception of two of the male DDT-exposed survivors for which good sequence data could not be obtained (Fig. 3). A random sample of 10 females from the unselected SENN-U strain was sequenced and all specimens were SS genotype. During 2004, 53 specimens from the SENN-DDT colony were screened using the kdr-PCR assay and the susceptible specimens showed SS (n=7) and RS (n=3) genotypes,

while the resistant specimens showed SS (n=38), RS (n=3) and RR (n=2) (data not shown). However, none of these specimens were sequenced to confirm the PCR results.

Discussion

Selection for a comparatively high level of DDT resistance in the *An. arabiensis* SENN colony was achieved within four generations (Table 1) and by the sixteenth generation mortality was 12.1%. This mortality at the F-16 generation showed some increase with age of the mosquitoes particularly in males (Fig. 1). Lines & Nassor (1991) demonstrate a similar decline in DDT resistance with age in the closely related malaria vector *An. gambiae*. However, it has been shown that blood meals and possibly mating, impact on the ability of females to survive exposure to insecticides (Hunt *et al.*, 2005). The females used in the present study were not blood fed and were unmated and this could have affected the results presented here.

The susceptibility results for malathion and dieldrin are comparable with those of Hemingway (1983) who showed high levels of resistance in colonies from the Gezira region. Results for DDT and permethrin exposures were in the range of 80% mortality each, but this was using samples from a malathion-selected resistant strain (Hemingway, 1983). When a DDT-selected or permethrin-selected strain was used, mortality for both these insecticides dropped to around 40% for DDT and <20% for permethrin (Hemingway, unpublished data). These results are in line with those found in the present study, although our resistance levels at the F-16 generation in the DDT-selected strain are higher (DDT 12.1% mortality, permethrin 0% and deltamethrin 24%). The combined

results clearly indicate some association between DDT and pyrethroid resistance. Together with organophosphate and carbamate resistance, this strain of *An. arabiensis* is the most resistant member of the *An. gambiae* complex documented to date.

Recent surveys of natural populations of *An. arabiensis* throughout Sudan show high levels of insecticide resistance to all classes of insecticides except carbamates in Geriza and Central States (which includes Sennar) (unpublished data). The fact that the SENN laboratory colony developed resistance to all four classes of insecticides when selected on DDT alone, mitigates against the use of DDT for indoor residual house spraying, which is currently being considered for implementation by the Ministry of Health.

Biochemical analysis of Sudan *An. arabiensis* by Hemingway (1983) did not confirm the bioassay and synergist results implicating carboxylesterases as the mechanism responsible for the resistance. The results of the biochemical assays in the present study also do not support the role of esterases in conferring resistance, although some elevation was seen in male mosquitoes from the selected line (Table 3). Elevated GST enzyme levels were found in both males and females, but not to any great extent. Although there is a small indication of an altered acetylcholinesterase, the generally high level of acetylcholinesterase inhibition by propoxur in the selected and unselected strains precludes this mode of resistance as an explanation for the high levels of carbamate and organophosphate resistance recorded in the DDT selected strain. The possibility of esterase mediated carbamate and organophosphate detoxification based on mutations that serve to enhance the enzyme's ability to hydrolyse insecticides needs to be investigated (Heidari *et al*., 2005).

Rapid selection for resistance is usually associated with phenotypically recessive mechanisms in which most exposure survivors are homozygous for the resistant loci. In this way resistance alleles are driven to a high frequency very quickly. The *kdr* mutation described by Martinez-Torres *et al.* (1998) in *An. gambiae* has been shown to be recessive in expression. However, the data presented here for *An. arabiensis* showed no correlation between the resistant phenotype as ascertained by bioassay and the presence of the *kdr* mutation, with all SENN-DDT individuals tested being homozygous RR for the West African *kdr* allele whether they succumbed to a 1 h exposure or not. Presumably, the *kdr* mutation was present, most likely at a very low frequency, in the material initially used to establish the SENN laboratory colony over 25 years ago. The kdr-PCR screening performed in 2004 revealed the presence of all three genotypes (SS, RS and RR) suggesting that with selection for resistance to DDT, the RR genotype was also selected for, although this genotype alone does not explain fully the expression of the resistance phenotype. If the independent appearance of the *kdr* mutation in *An. arabiensis* occurred without the simultaneous development of a thus far unidentified co-factor necessary to produce the resistance phenotype, this would explain the lack of association between the resistance phenotype and presence of the *kdr* mutation in SENN.

A recent study on West African *An. gambiae* suggested that a large proportion of homozygous *kdr* RR females were killed by prolonged exposure to insecticides (Chandre *et al.*, 2000). This explanation cannot be invoked for the results presented here since all mosquitoes were exposed to the standard WHO 1-hour exposure.

The *kdr* mutation has been shown to be closely associated with DDT and pyrethroid resistance in several *An. gambiae* populations (particularly the molecular S

form) (Awolola *et al.*, 2003; Fanello *et al.*, 2003; Diabate *et al.*, 2002; Chandre *et al.*, 1999; Brooke *et al.*, 1999; Martinez-Torres *et al.*, 1998). However, neither of the *kdr* mutations reported in *An. arabiensis* from four different African countries (Diabate *et al.*, 2004; Stump *et al.*, 2004; Verhaeghen *et al.*, 2006; Kulkarni *et al.*, 2006) has been positively correlated with the DDT/pyrethroid resistance phenotype as detected by insecticide bioassay. In the light of the results presented here, the role of *kdr* in conferring resistance in *An. arabiensis* requires further investigation.

Diabate *et al.* (2004) identified three nucleotides upstream of the Leu → Phe *kdr* mutation which differentiates *An. gambiae* from *An. arabiensis* and which associates with the *kdr* mutation in *An. arabiensis*. They propose that this mutation has arisen independently in *An. arabiensis* as opposed to inheritance through introgression with *An. gambiae*. Stump *et al.* (2004) on the other hand, suggest introgression as one of the explanations for the occurrence of the *kdr* allele in a single individual in Kenya. Since *An. gambiae* has never been recorded from Sudan other than in the far south regions, the likelihood of *kdr* having arisen in *An. arabiensis* in central Sudan through introgression is highly improbable. Unpublished data from southern Sudan show no evidence of any insecticide resistance in these populations of *An. arabiensis*.

In summary, this Sudan colony of *An. arabiensis* exhibits the most widespread resistance in terms of classes of insecticides of any member of the *An. gambiae* complex to date. Our knowledge of the mechanisms responsible for the resistance has not advanced in the 23 years that have elapsed since the first studies were reported. Elevated GST and esterase levels may play a role in conferring *An. arabiensis* resistance to some of the insecticides tested in this study but not all, while the *kdr* mutation apparently does

not play an important role. It is obvious that further investigations into the mechanisms involved in resistance in the SENN strain are needed and these are underway.

The implications for malaria control in this area of central Sudan are serious. While the base colony showed >90% mortality on all insecticides except dieldrin, the rapidity with which high levels of resistance to DDT were selected, suggests that this insecticide should be avoided for vector control interventions and underlines the need for rotational use of the other classes of insecticides to manage the situation. Presumably rapid selection for similar levels of resistance could occur with the use of pyrethroids, carbamates or organophosphates. Unfortunately, there are no other classes of insecticides approved by the World Health Organization for malaria vector control. This study also highlights the burning need for baseline surveys in any country contemplating vector control in order to make informed decisions on choice of insecticide to be used in control operations.

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References

Awolola, T.S., Brooke, B.D., Koekemoer, L.L. and Coetzee, M. (2003) Absence of the kdr mutation in the molecular 'M' form suggests different pyrethroid resistance mechanisms in the malaria vector mosquito *Anopheles gambiae s.s. Tropical Medicine and International Health,* **8,** 420-422.

Brooke, B.D., Hunt, R.H., Koekemoer, L.L., Dossou-Yovo, J. and Coetzee, M. (1999) Evaluation of a polymerase chain reaction assay for detection of pyrethroid insecticide resistance in the malaria vector species of the *Anopheles gambiae* complex. *Journal of the American Mosquito Control Association,* **15,** 565-568.

Brown, A.W.A. (1986) Insecticide resistance in mosquitoes: a pragmatic review. *Journal of the American Mosquito Control Association,* **2,** 123-140.

Chandre, F., Darriet, F., Duchon, S., Finot, L., Manguin, S., Carnevale, P. and Guillet, P. (2000) Modifications of pyrethroid effects associated with kdr mutation in *Anopheles gambiae*. *Medical and Veterinary Entomology* **14,** 81-88.

Chandre, F., Manguin, S., Brengues, C., Dossou Yovo, J., Darriet, F., Diabate, A., Carnevale, P. and Guillet, P. (1999) Current distribution of a pyrethroid resistance gene (kdr) in *Anopheles gambiae* complex from West Africa and further evidence for reproductive isolation of the Mopti form. *Parassitologia,* **41,** 319-322.

Collins, F.H., Mendez, M.A., Rasmussn, P.C., Mahaffey, H.J., Besansky, N.J. and Finnerty, V. (1987) A ribosomal RNA gene probe differentiates member species

of the *Anopheles gambiae* complex. *American Journal of Tropical Medicine and Hygiene,* **37,** 37-41.

Diabaté, A., Baldet, T., Chandre, F., Akogbeto, M., Guiguemde, T.R., Darriet, F., Brengues, C., Guillet, P., Hemingway, J., Small, G.J., and Hougard, J.M. (2002) The role of agricultural use of insecticides in resistance to pyrethroids in *Anopheles gambiae s.l.* in Burkina Faso. *American Journal of Tropical Medicine and Hygiene,* **67,** 617-622.

Diabaté, A., Baldet, T., Chandre, F., Dabire, K.R., Simard, F., Ouedraogo, J.B., Guillet, P., and Hougard, J.M. (2004) First report of *kdr* mutation in *Anopheles arabiensis* from Burkina Faso, West Africa. *Journal of the American Mosquito Control Association,* **20,** 195-196.

El Gadal, A.A., Haridi, A.M., Hassan, F.T. and Hussein, H. (1985) Malaria in the Gezira Managil Irrigated Scheme of the Sudan. *Journal of Tropical Medicine and Hygiene,* **88,** 153-159.

Fanello, C., Petrarca, V., della Torre, A., Santolamazza, F., Dolo, G., Coulibaly, M., Alloueche, A., Curtis, C.F., Toure, Y.T. and Coluzzi, M. (2003) The pyrethroid knock-down resistance gene in the *Anopheles gambiae* complex in Mali and further indication of incipient speciation within *An. gambiae s.s. Insect Molelcular Biology,* **12,** 241-245.

Gillies, M.T. and Coetzee, M. (1987) *A supplement to the Anophelinae of Africa South of the Sahara (Afrotropical Region).* Publications of the South African Institute for Medical Research. no. **55**.

Green, C.A. (1982) Malaria epidemiology and anopheline cytogenetics. *In:* R. Pal, J.B. Kitzmiller and T. Kanda, eds. *Cytogenetics and Genetics of Vectors.* Amsterdam: Elsevier Biomedical, pp 21-29.

Hargreaves, K., Hunt, R.H., Brooke, B.D., Mthembu, J., Weeto, M.M., Awolola, T.S. and Coetzee, M. (2003) *Anopheles arabiensis* and *An. quadriannulatus* resistance to DDT in South Africa. *Medical and Veterinary Entomology,* **17,** 417-422.

Haridi, A.M. (1972) Inheritance of DDT resistance in species A and B of the *Anopheles gambiae* complex. *Bulletin of the World Health Organization,* **47,** 619-626.

Heidari, R., Devonshire, A.L., Campbell, B.E., Dorrian, S.J., Oakeshott, J.G. and Russell, R.J. (2005) Hydrolysis of pyrethroids by carboxylesterases from *Lucilia cuprina* and *Drosophila melanogaster* with active sites modified by in vitro mutagenesis. *Insect Biochemistry and Molecular Biology,* **35**, 597-609.

Hemingway, J. (1983) Biochemical studies on malathion resistance in *Anopheles arabiensis* from Sudan. *Transactions of the Royal Society of Tropical Medicine and Hygiene,* **77,** 477-480.

Hunt, R.H., Brooke, B.D., Pillay, C., Koekemoer, L.L. and Coetzee, M. (2005) Laboratory selection for and characteristics of pyrethroid resistance in the malaria vector *Anopheles funestus*. *Medical and Veterinary Entomology,* 19: 271-275.

Knipple, D., Doyle, K.E., Marsella-Herrick, P.A. and Soderlund, D.M. (1994) Tight genetic linkage between the *kdr* insecticide resistance trait and a voltage-sensitive sodium channel gene in the housefly. *Proceedings of the National Academy of Sciences, USA,* **91,** 483-2487.

Kulkarni, M.A., Rowland, M., Alifrangis, M., Mosha, F.W., Matowo, J., Malima, R., Peter, J., Kweka, E., Lyimo, I., Magesa, S., Salanti, A., Rau, M.E. and Drakeley, C. (2006) Occurrence of the leucine-to-phenylalanine knockdown resistance (*kdr*) mutation in *Anopheles arabiensis* populations in Tanzania, detected by a simplified high-throughput SSOP-ELISA method. *Malaria Journal* **5,** 56 (7 pp).

Lines, J.D. and Nassor, N.S. (1991) DDT resistance in *Anopheles gambiae* declines with mosquito age. *Medical and Veterinary Entomology,* **5**, 261-265.

Martinez-Torres, D., Devonshire, A.L. and Williamson, M.S. (1997) Molecular studies of knockdown resistance to pyrethroids: cloning of domain II sodium channel gene sequences from insects. *Pesticide Science,* **51,** 265-270.

Martinez-Torres, D., Chandre, F., Williamson, M.S., Darriet, F., Berge, J.B., Devonshire, A.L., Guillet, P., Pasteur, N. and Pauron, D. (1998) Molecular characterization of pyrethroid knockdown resistance (*kdr*) in the major malaria vector *Anopheles gambiae s.s. Insect Molecular Biology,* **7,** 179-184.

Penilla, R.P., Rodriguez, A.D., Hemingway, J., Torres, J.L., Arrendo-Jimenez, J.I. and Rodriguez, M.H. (1998) Resistance management strategies in malaria vector mosquito control. Baseline data for a large scale field trial against *Anopheles albimanus* in Mexico. *Medical and Veterinary Entomology,* **12,** 217-233.

Ranson, H., Jensen, B., Wang, X., Prapanthadara, L., Hemingway, J., and Collins, F.H. (2000a) Genetic mapping of two loci affecting DDT resistance in the malaria vector *Anopheles gambiae*. *Insect Molecular Biology,* **9,** 499-507.

Ranson, H., Jensen, B., Vulule, J.M., Wang, X., Hemingway, J. and Collins, F.H. (2000b) Identification of a point mutation in the voltage-gated sodium channel

gene of Kenyan *Anopheles gambiae* associated with resistance to DDT and pyrethroids. *Insect Molecular Biology,* **9,** 491-497.

Stump, A.D., Atieli, F.K., Vulule, J.M. and Besansky, N.J. (2004) Dynamics of the pyrethroid knockdown resistance allele in western Kenyan populations of *Anopheles gambiae* in response to insecticide-treated bed net trials. *American Journal of Tropical Medicine and Hygiene* **70,** 591-596.

Verhaeghen, K., Van Bortel, W., Roelants P., Backeljau, T. and Coosemans, M. (2006) Detection of the East and West African kdr mutation in *Anopheles gambiae* and *Anopheles arabiensis* from Uganda using a new assay based on FRET/Melt Curve analysis. *Malaria Journal* **5,** 16 (9 pp).

WHO (1998) Test procedures for insecticide resistance monitoring in malaria vectors. Bio-efficacy and persistence of insecticides on treated surfaces. Document WHO/CDS/CPC/MAL/98.12. World Health Organization, Geneva.

Williamson, M.S., Martinez-Torres, D., Hick, C.A. and Devonshire, A.L. (1996) Identification of mutations in the housefly *Para*-type sodium channel gene associated with knockdown resistance (*kdr*) to pyrethroid insecticides. *Molecular Gen. Genetics,* **252,** 51-60.

Table 1: Selection of *Anopheles arabiensis* (SENN) from Sudan exposed to 4% DDT for 1 hour. Results show knockdown after 1 h exposure (60 min) and final mortality 24 h post exposure. n = overall sample size.

Generation	60 min	24 hrs dead	24 hrs alive	n	% mortality
F1	5050	5215	544	5759	90.6
F2	964	960	994	1954	49.1
F3	439	446	1067	1513	29.5
F4	529	457	1428	1885	24.2
F5	794	732	2032	2764	26.5
F16	65	147	1063	1210	12.1

Table 2. Exposures of unselected and SENN-DDT F16 selected strains to all four classes of insecticides approved for use in malaria control. Results show final mortality 24 h post exposure. n = sample size.

Insecticides	**SENN unselected**		**SENN DDT F-16 selected**	
	n	**% Mortality**	**n**	**% Mortality**
Malathion	25	100	24	16.7
Bendiocarb	25	96.0	24	33.3
Dieldrin	26	42.3	25	0
Deltamethrin	25	100	25	24.0
Permethrin	26	92.3	22	0

Table 3. Mean optical density values of *An. arabiensis* SENN strains for glutathione S-transferase (GST), monooxygenase (Oxy) and esterase (Est) enzymes. "n" = sample size; figures in parentheses are the standard deviations for each sample; p indicates significance in difference between unselected and selected samples by enzyme system following 2-sample t tests.

		n	GST	Oxy	Est
Males	Unselected	47	0.0036	0.259	0.137
			(0.0011)	(0.098)	(0.039)
	DDT	43	0.006	0.262	0.169
	Selected		(0.0018)	(0.108)	(0.034)
	p		<0.05	0.88	<0.05
Females	Unselected	44	0.0037	0.554	0.171
			(0.0018)	(0.152)	(0.045)
	DDT	48	0.0074	0.607	0.178
	Selected		(0.0025)	(0.146)	(0.047)
	p		<0.05	0.09	0.42

Table 4. 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 2-sample t tests.

		n	% inhibition
Males	Unselected	22	75.74 (60.04-81.15)
	DDT selected	23	72.39 (56.35-80.24)
	p		0.11
Females	Unselected	24	79.43 (74.62-82.56)
	DDT selected	24	72.61 (60.59-84.5)
	p		0.0001

Table 5: Association of the *kdr* genotype with DDT susceptible and resistant specimens of the *An. arabiensis* SENN-DDT strain following 1 hr exposure to 4% DDT. R = Resistance allele. Two phenotypically resistant males were not successfully sequenced.

Phenotype	**N**	***kdr* genotype by PCR**	***kdr* genotype by sequence analysis**
Susceptible	5 ♀; 5 ♂	all RR	all RR (n=10)
Resistant	5 ♀; 5 ♂	all RR	all RR (n=8)

Figure 1. Susceptibility of *An. arabiensis* SENN-DDT F-16 male (M) and female (F) adults to 4% DDT at various ages.

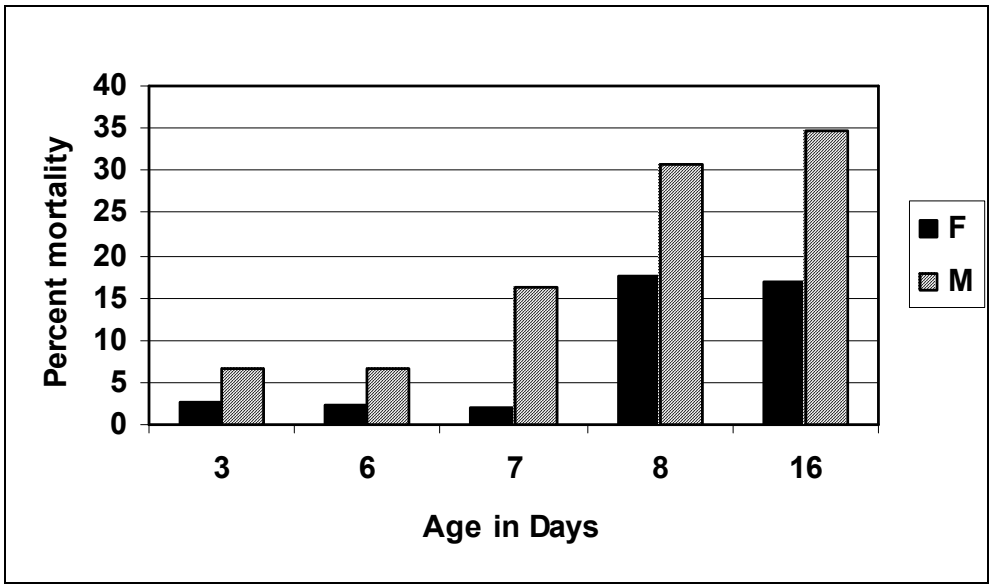


Figure 2. PCR-based detection of the *kdr* mutation. Primers Agd1, Agd2, Agd3 and Agd4 were used to detect the presence of the susceptible allele (TTA) and the resistant allele (TTT) from the SENN-DDT strain. The PCR samples were separated on a 1 % agarose gel. Lane M: 100 bp ladder; lane 0: negative control (no DNA); lanes 1-5: susceptible males; lanes 6-10 susceptible females; lanes 11-15: resistant males; lanes 16-20: resistant females.

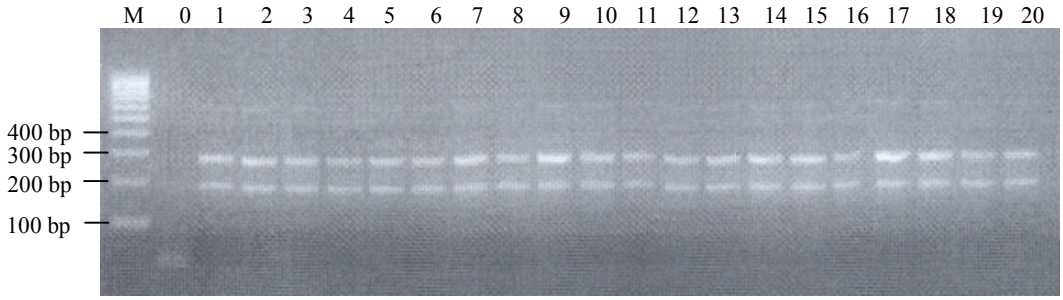
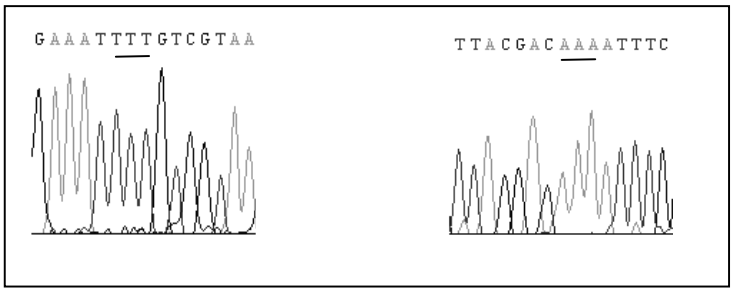
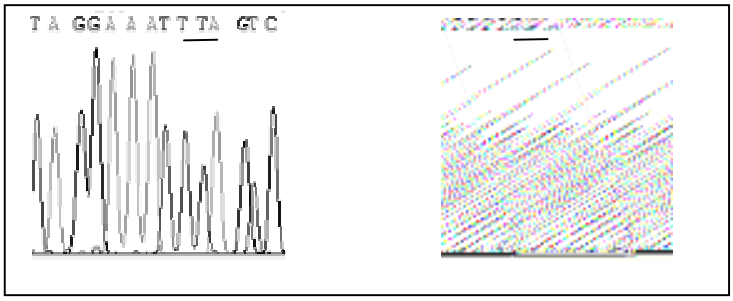


Figure 3. Sequencing images (scf-files) showing the forward and reverse strand position of the *kdr*-genotype for a resistant (RR) specimen and a susceptible (SS) specimen. A) Forward primer (Agd1) showing the R-allele, and B) reverse primer (Agd2) also showing R-allele. This individual was genotyped as RR. C) Sequence type S-allele when Agd1 was used, and D) S-allele when Agd2 was used on the same specimen. This specimen was then genotyped as SS.



A **B**



C **D**

APPENDIX IV

Abdalla *et al*. (2006) poster