THE ROYAL SOCIETY

The role of gene splicing, gene amplification and regulation in mosquito insecticide resistance

Janet Hemingway[1], Nicola Hawkes[1], La-aied Prapanthadara[2], K. G. Indrananda Jayawardenal[1] and Hilary Ranson[3]

[1]*School of Pure and Applied Biology, University of Wales Cardiff, PO Box 913, Cardiff CF1 3TL, UK*
[2]*Research Institute of Health Sciences, Chiang Mai University, PO Box 80 CMU, Chiang Mai 50202, Thailand*
[3]*Department of Biological Sciences, University of Notre Dame, Notre Dame, IN 46556, USA*

The primary routes of insecticide resistance in all insects are alterations in the insecticide target sites or changes in the rate at which the insecticide is detoxified. Three enzyme systems, glutathione S-transferases, esterases and monooxygenases, are involved in the detoxification of the four major insecticide classes. These enzymes act by rapidly metabolizing the insecticide to non-toxic products, or by rapidly binding and very slowly turning over the insecticide (sequestration). In *Culex* mosquitoes, the most common organophosphate insecticide resistance mechanism is caused by co-amplification of two esterases. The amplified esterases are differentially regulated, with three times more Est$\beta 2^1$ being produced than Est$\alpha 2^1$. *Cis*-acting regulatory sequences associated with these esterases are under investigation. All the amplified esterases in different *Culex* species act through sequestration. The rates at which they bind with insecticides are more rapid than those for their non-amplified counterparts in the insecticide-susceptible insects. In contrast, esterase-based organophosphate resistance in *Anopheles* is invariably based on changes in substrate specificities and increased turnover rates of a small subset of insecticides. The up-regulation of both glutathione S-transferases and monooxygenases in resistant mosquitoes is due to the effects of a single major gene in each case. The products of these major genes up-regulate a broad range of enzymes. The diversity of glutathione S-transferases produced by *Anopheles* mosquitoes is increased by the splicing of different 5′ ends of genes, with a single 3′ end, within one class of this enzyme family. The *trans*-acting regulatory factors responsible for the up-regulation of both the monooxygenase and glutathione S-transferases still need to be identified, but the recent development of molecular tools for positional cloning in *Anopheles gambiae* now makes this possible.

Keywords: mosquitoes; insecticide; gene amplification; gene splicing; *Anopheles*; *Culex*

1. INTRODUCTION

Major mechanisms of insecticide resistance in insects involve either mutation within the target site of the insecticide, or an alteration in the rate of insecticide detoxification. The enzymes involved in this detoxification may be quantitatively and/or qualitatively altered. There are three enzyme groups, esterases, glutathione S-transferases and monooxygenases, involved in metabolic resistance to the four major groups of insecticides. Esterase-based resistance has been reported from more than 30 different medical, veterinary or agricultural insect pests (Hemingway & Karunaratne 1998). In mosquitoes it is the primary mechanism for organophosphorus (OP) insecticide resistance (Bisset *et al*. 1991; Herath *et al*. 1987; Karunaratne *et al*. 1993), and in some cases a secondary mechanism for carbamate resistance (Peiris & Hemingway 1993). Esterases produce a broad spectrum of resistance in many *Culex* species, but in *Anopheles* esterase-based resistance is usually specific to the OP malathion (Hemingway 1983, 1985; Herath *et al*. 1987).

Glutathione S-transferases in mosquitoes commonly confer resistance to the organochlorine insecticide DDT (Prapanthadara *et al*. 1993, 1996), and can act as a secondary OP resistance mechanism (Hemingway *et al*. 1991). In house flies, their role in OP resistance is more widely documented (Clark *et al*. 1984, 1986). DDT resistance in mosquitoes has generally been attributed to a single major-gene effect (Davidson 1963, 1956), although multigenic effects have been suggested in some instances (Lines & Nassor 1991). Reports of monooxygenase-based resistance are relatively rare in mosquitoes, and many of these are based on synergistic effects with piperonyl butoxide, which is not absolutely diagnostic. Pyrethroid resistance in *Anopheles gambiae* in East and West Africa appears to be linked to increased monooxygenase titres, in the latter case combined with an altered target-site mechanism (Brogdon *et al*. 1997). OP resistance in *A. subpictus*, a vector of malaria in Sri Lanka, is also linked to increased monooxygenase titres and higher insecticide metabolic rates (Hemingway *et al*. 1991; Martinez-Torres *et al*. 1998).

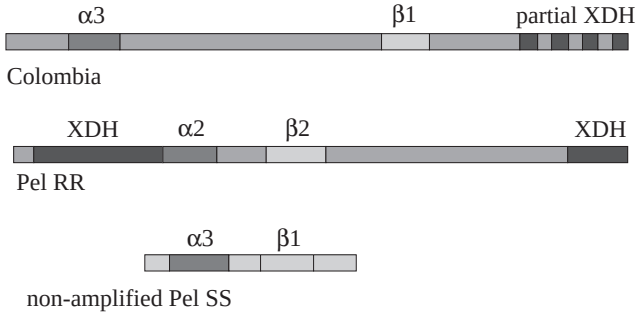

Figure 1. Structure of the amplicons associated with insecticide resistance in *Culex quinquefasciatus* strains from Colombia and Sri Lanka (PelRR) compared with the non-amplified esterase gene arrangement in the insecticide-susceptible strain PelSS. The Estα2[1]/β2[1] amplicon also has a complete gene with high homology to xanthine dehydrogenase (XDH) (Coleman & Hemingway 1997).

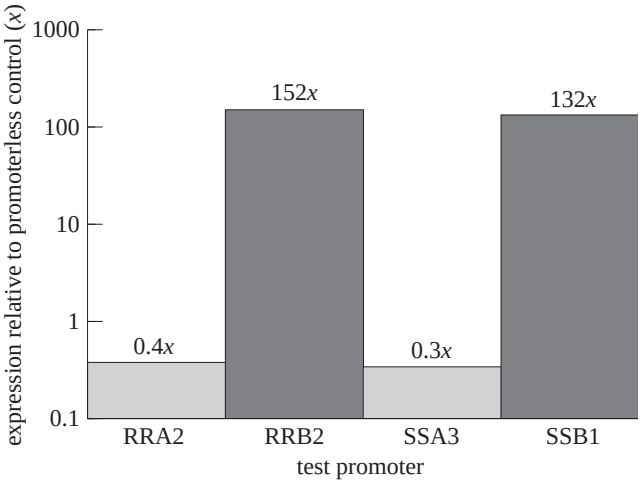

Figure 2. Expression rates in a luciferase reporter assay relative to a promoterless control when the intergenic spacer region from resistant (RR) and susceptible (SS) insects is cloned in the orientation of either the Estβ gene or the Estα gene (labelled A and B, respectively).

2. THE MOLECULAR BASIS OF METABOLIC RESISTANCE IN MOSQUITOES

(a) *Gene amplification*

The development of resistance to xenobiotics by amplification of the genes involved in their detoxication is common in several organisms. Gene amplification in the insecticide-resistant TEMR strain of the mosquito *Culex quinquefasciatus* was first shown for the Estβ1[1] esterase. It was originally estimated that there were up to 250 copies of this esterase gene per cell in resistant insects (Mouches *et al*. 1990), but this estimate has recently been revised to approximately 20 copies, i.e. similar to the estimates for *Myzus* esterases (Guillemaud *et al*. 1997). The Estβ1[1] genes are clustered between the centromere and the apex of chromosome II (Nance *et al*. 1990), and are inherited in a pseudo-monofactorial manner (Peiris & Hemingway 1993). The most common amplified esterases in *Culex* are Estα2[1] and Estβ2[1], which occur in *ca*. 90% of all the OP-insecticide-resistant *C. quinquefasciatus* strains analysed (Hemingway & Karunaratne 1998). The TEMR Estβ1[1] and common Estβ2[1] from numerous strains have 97% identity at the amino-acid level. The high identity suggests that the Estβs are an allelic series from a single locus. The Estα2[1] has approximately 47% deduced amino-acid homology with all the Estβs. This level of homology, along with conserved intron–exon boundaries and the close proximity (1.7 kb) of the two genes in a head-to-head arrangement in the susceptible insects (Vaughan *et al*. 1997), suggests that the two genes arose through an ancient duplication. In resistant insects Estα2[1] and Estβ2[1] are also in a head-to-head arrangement, but they are *ca*. 2.7 kb apart (Vaughan *et al*. 1997). The increase in the intergenic DNA between the two genes is accounted for by two large (*ca*. 500 bp) and one small insertion in the resistant insects. These insertions introduce DNA motifs that have high homologies to BARBIE boxes, ARE elements and Zeste elements (Vaughan *et al*. 1997; N. Hawkes, unpublished data). The structure of the Estα2[1]/ESTβ2[1] amplicon and the related Estβ1 amplicons compared with the esterase gene arrangements in susceptible insects are given in figure 1.

It has been suggested, on the basis of the identical nature of the amplified Estα2[1] and Estβ2[1] restriction digest patterns from resistant *Culex* populations worldwide, that amplification is a rare or unique event that occurs primarily through migration (Raymond *et al*. 1991). We now know that amplification of these genes has appeared independently at least five times (Hemingway & Karunaratne 1998), and that resistance is occurring through gene amplification and rapidly spreading by migration. Further evidence that the chromosomal region containing these esterases represents an amplification 'hot-spot' comes from other *Culex* species. In *C. tritaeniorhynchus* the homologous Estβ gene CtrEstβ1[1] has been amplified and there is no possibility of this having occurred through gene flow between the species (Karunaratne *et al*. 1998).

In *Anopheles stephensi* there are at least three enzymes that are able to metabolize the OP malathion in resistant insects. None of these esterase genes are amplified and they are all present in very low quantities in the resistant and susceptible insects, conferring resistance through efficient insecticide metabolism (K. G. I. Jayawardena and J. Hemingway, unpublished data).

(b) *Gene expression*

Increased gene expression, rather than gene amplification, is the primary molecular basis of glutathione S-transferase and monooxygenase-based resistance in mosquitoes. However, gene amplification and elevated expression are not mutually exclusive. The Estα2[1] and Estα2[1] genes from *Culex* appear to be both amplified and increased in expression. The two genes are present in a 1:1 stoichiometry, being co-amplified, but approximately three times more Estβ2[1] than Estα2[1] is obtained from protein purifications of resistant insect homogenates (Karunaratne 1994). This difference may reflect differential protein or mRNA stability, or result from variations in the efficiencies of the two promoters, which are both contained within the intergenic spacer (figure 1). We are currently characterizing the Estα2[1] and Estβ2[1] promoters, and have cloned the intergenic spacer in both orientations upstream of the reporter gene luciferase. The

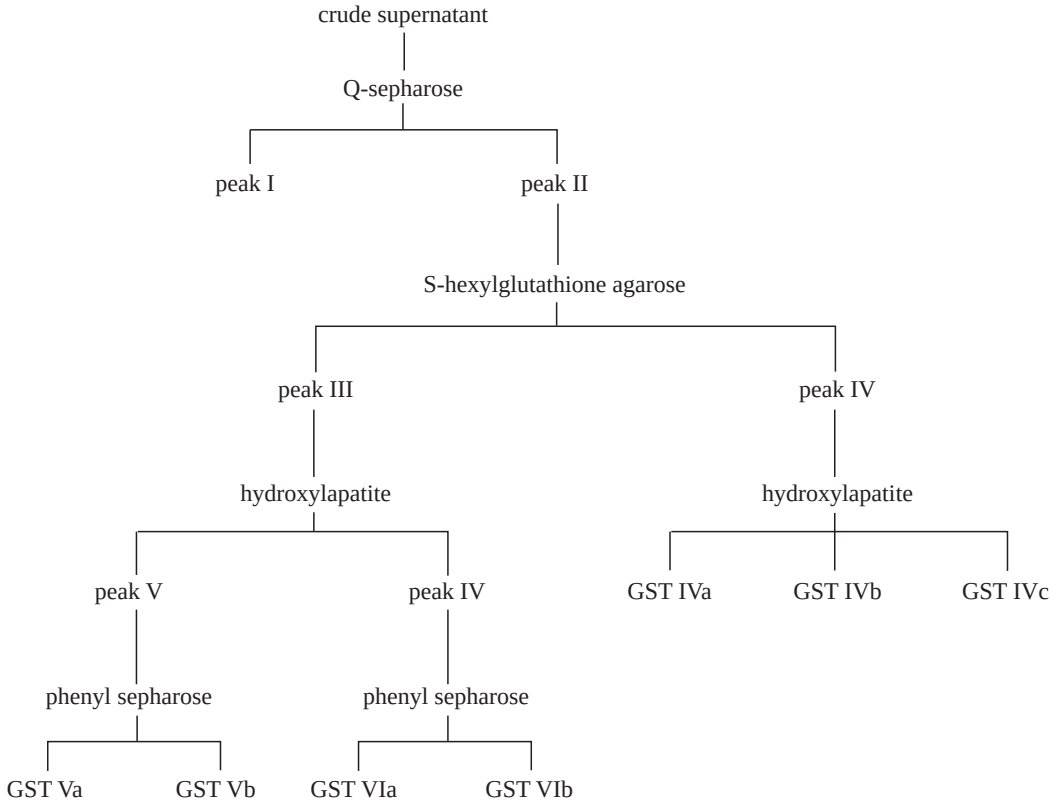

Figure 3. Schematic representation of the purification of glutathione S-transferases (GST) from *Anopheles gambiae*. The final peaks of GST activity still all contained multiple protein bands.

Table 1. *DDT dehydrochlorinase activity*[a] *exhibited by various peaks of glutathione S-tranferase activity partially purified from* A. gambiae, *as described in figure 3*

(S, susceptible strain enzymes; R, resistant strain enzymes.)

	nmole DDE mg^{-1}		nmole/unit GST[b] DDE activity		nmole DDE g^{-1} larvae	
GSTs	S	R	S	R	S	R
IVa	—	278.0	<0.3	6.1	—	14.6
IVb	—	912.5	<0.3	22.6	—	15.6
IVc	—	22.0	<0.3	2.2	—	1.5
Va	173.3	564.7	144.5	241.3	48.4	344.9
Vb	65.6	765.6	121.5	243.1	7.6	68.1
VIa	27.4	50.5	94.6	112.2	4.2	39.6
VIb	9.8	178.6	98.3	235.1	0.9	52.4

[a] DDT-dehydrochlorinase activity is defined as nmole DDE formation per two hours.
[b] A unit of GST activity is defined as μmole min^{-1} mg^{-1} with CDNB as the substrate.

resultant constructs have been transfected into a range of insect and mammalian cell lines. Inserting the spacer at the same site, but in different orientations, reproducibly generates luciferase expression from the Est$\beta 2^1$ promoter many times greater than from the Est$\alpha 2^1$ orientation. This is true from both the resistant (2.7 kb) and susceptible (1.7 kb) spacers (figure 2). The differences in promoter strength may reflect differences in the relative locations of significant regulatory elements. Further studies are in progress.

In the malaria vector, *Anopheles gambiae*, resistance to DDT in both larvae and adults is conferred by increased levels of many glutathione S-transferases (GSTs). Resistance in the two life stages is conferred by different genes, although the end result of both is a measurable increase in GST activity and DDT dehydrochlorination. Resistance has been studied in greatest detail in larvae. Resistant insects have increased levels of DDT dehydrochlorinase activity associated with seven partially purified peaks of GST activity (figure 3; and table 1) (Prapanthadara *et al*. 1993, 1995). There is an enormous diversity of GSTs found in both resistant and susceptible insects.

At present two broad classes of GSTs have been cloned from insects. Representatives from both classes have been cloned from *A. gambiae* (although the single insect class II GST cloned from *A. gambiae* is not expressed in larvae). All three class I GSTs cloned are able to use DDT as a

substrate (Ranson *et al*. 1997*a*,*b*). Antisera raised to these expressed GSTs indicates that all of them belong to the peak IV GSTs represented in figure 3. We do not have, as yet, have any molecular data on the GSTs from peaks V and VI, but our current information suggests that they belong to GST classes that have not so far been characterized from any insect. The simplest hypothesis for the molecular basis of this GST-based resistance and for the similar organization of the monooxygenase-based pyrethroid resistance is that *trans*-acting regulators are involved in the up-regulation of these enzyme families. We are currently employing a positional cloning approach, in collaboration with Professor F. Collins, USA, to identify these regulator genes. The positional cloning takes advantage of the high-density microsatellite marker genetic map, *in situ* hybridization, polytene chromosome microdissection, and a BAC library, which have all been recently developed for *A. gambiae*.

(c) *Gene splicing*

Initial biochemical work on GSTs from *A. gambiae* demonstrated the diversity of GST enzymes present in this insect. The molecular work undertaken subsequent to this showed that the class I GSTs were all at a single chromosome location. Extensive sequencing of stretches of DNA at this location revealed a full-length intron-less gene, as occurs in *Drosophila*, and numerous apparent 5′ truncated pseudogenes. Reverse transcriptase-polymerase chain reaction and Southern blot analysis has demonstrated that these are not pseudogenes, but are actively transcribed with the splicing of different 5′ exons to a single 3′ exon, occurring to produce a diversity of GSTs in both resistant and susceptible *A. gambiae* (Ranson *et al*. 1998).

The next decade should see extensive progress in our understanding of metabolically based insecticide resistance in insect pests, allowing for the development of new control methods to allow us to counteract this rapidly changing evolutionary phenomenon.

The unpublished work cited in this paper was carried out with project grant funding to J.H. from the Medical Research Council and a Wellcome Prize studentship to H.R.

REFERENCES

Bisset, J. A., Rodriguez, M. M., Hemingway, J., Diaz, C., Small, G. J. & Ortiz, E. 1991 Malathion and pyrethroid resistance in *Culex quinquefasciatus* from Cuba: efficacy of pirimiphos-methyl in the presence of at least three resistance mechanisms. *Med. Vet. Entomol.* **5**, 223–228.

Brogdon, W. G., McAllister, J. C. & Vulule, J. 1997 Heme peroxidase activity measured in single mosquitoes identifies individuals expressing the elevated oxidase mechanism for insecticide resistance. *J. Am. Mosq. Cont. Ass.* **13**, 233–237.

Clark, A. G., Shamaan, N. A., Dauterman, W. C. & Hayaoka, T. 1984 Characterization of multiple glutathione transferases from the housefly, *Musca domestica* (L). *Pestic. Biochem. Physiol.* **22**, 51–59.

Clark, A. G., Shamaan, N. A., Sinclair, M. D. & Dauterman, W. C. 1986 Insecticide metabolism by multiple glutathione S-transferases in two strains of the house fly, *Musca domestica* (L). *Pestic. Biochem. Physiol.* **25**, 169–175.

Coleman, M. & Hemingway, J. 1997 Amplification of a xanthine dehydrogenase gene is associated with insecticide resistance in the common house mosquito *Culex quinquefasciatus*. *Biochem. Soc. Trans.* **25**, 526.

Davidson, G. 1956 Insecticide resistance in *Anopheles gambiae* Giles a case of simple Mendelian inheritance. *Nature* **178**, 863.

Davidson, G. 1963 DDT-resistance and dieldrin-resistance in *Anopheles albimanus*. *Bull. Wld Hlth Org.* **28**, 25–33.

Guillemaud, T., Makate, N., Raymond, M., Hirst, B. & Callaghan, A. 1997 Esterase gene amplification in *Culex pipiens*. *Insect. Molec. Biol.* **6**, 319–327.

Hemingway, J. 1983 The genetics of malathion resistance in *Anopheles stephensi* from Pakistan. *Trans. R. Soc. Trop. Med. Hyg.* **77**, 106–108.

Hemingway, J. 1985 Malathion carboxylesterase enzymes in *Anopheles arabiensis* from Sudan. *Pestic. Biochem. Physiol.* **23**, 309–313.

Hemingway, J. & Karunaratne, S. H. P. P. 1998 Mosquito carboxylesterases: a review of the molecular biology and biochemistry of a major insecticide resistance mechanism. *Med. Vet. Entomol.* **12**, 1–12.

Hemingway, J., Miyamoto, J. & Herath, P. R. J. 1991 A possible novel link between organophosphorus and DDT insecticide resistance genes in *Anopheles*: supporting evidence from fenitrothion metabolism studies. *Pestic. Biochem. Physiol.* **39**, 49–56.

Herath, P. R. J., Hemingway, J., Weerasinghe, I. S. & Jayawardena, K. G. I. 1987 The detection and characterization of malathion resistance in field populations of *Anopheles culicifacies* B in Sri Lanka. *Pestic. Biochem. Physiol.* **29**, 157–162.

Karunaratne, S. H. P. P. 1994 Characterization of multiple variants of carboxylesterases which are involved in insecticide resistance in the mosquito *Culex quinquefasciatus*. PhD thesis, University of London.

Karunaratne, S. H. P. P., Jayawardena, K. G. I., Hemingway, J. & Ketterman, A. J. 1993 Characterization of a B-type esterase involved in insecticide resistance from the mosquito *Culex quinquefasciatus*. *Biochem. J.* **294**, 575–579.

Karunaratne, S. H. P. P., Vaughan, A., Paton, M. G. & Hemingway, J. 1998 Amplification of a serine esterase gene is involved in insecticide resistance in Sri Lankan *Culex tritaeniorhynchus*. *Insect. Molec. Biol.* **7**, 307–315.

Lines, J. D. & Nassor, N. S. 1991 DDT resistance in *Anopheles gambiae* declines with mosquito age. *Med. Vet. Entomol.* **5**, 261–265.

Martinez-Torres, D., Chandre, F., Williamson, M. S., Darriet, F., Berge, J. B., Devonshire, A. L., Guillet, P., Pasteur, N. & Pauron, D. 1998 Molecular characterisation of pyrethroid resistance in the malaria vector *Anopheles gambiae* s.s. *Insect. Molec. Biol.* **7**, 179–184.

Mouches, C. (and 10 others) 1990 Characterization of amplification core and esterase Bl gene responsible for insecticide resistance in *Culex*. *Proc. Natn. Acad. Sci. USA* **87**, 2574–2578.

Nance, E., Heyse, D., Britton-Davidian, J. & Pasteur, N. 1990 Chromosomal organisation of the amplified esterase Bl gene in organophosphate-resistant *Culex pipiens qinquefasciatus* Say (Diptera: Culicidae). *Genome* **33**, 148–152.

Peiris, H. T. R. & Hemingway, J. 1993 Characterization and inheritance of elevated esterases in organophosphorus and carbamate insecticide resistant *Culex quinquefasciatus* (Diptera:Culicidae) from Sri Lanka. *Bull. Entomol. Res.* **83**, 127–132.

Prapanthadara, L., Hemingway, J. & Ketterman, A. J. 1993 Partial purification and characterization of glutathione S-transferase involved in DDT resistance from the mosquito *Anopheles gambiae*. *Pestic. Biochem. Physiol.* **47**, 119–133.

Prapanthadara, L., Hemingway, J. & Ketterman, A. J. 1995 DDT-resistance in *Anopheles gambiae* Giles from Zanzibar Tanzania, based on increased DDT-dehydrochlorinase

activity of glutathione S-transferases. *Bull. Entomol. Res.* **85**, 267–274.

Prapanthadara, L., Koottathep, S., Promtet, N., Hemingway, J. & Ketterman, A. J. 1996 Purification and characterization of a major glutathione S-transferase from the mosquito *Anopheles dirus* (species B). *Insect. Biochem. Molec. Biol.* **26**, 277–285.

Ranson, H., Cornel, A. J., Fournier, D., Vaughan, A. & Hemingway, J. 1997*a* Cloning and localisation of a glutathione S-transferase class I gene from *Anopheles gambiae*. *J. Biol. Chem.* **272**, 5464–5468.

Ranson, H., Prapanthadara, L. & Hemingway, J. 1997*b* Cloning and characterisation of two glutathione S-transferases from a DDT resistant strain of *Anopheles gambiae*. *Biochem. J.* **324**, 97–102.

Ranson, H., Collins, F. H. & Hemingway, J. 1998 The role of mRNA splicing in generating heterogeneity within the *Anopheles gambiae* class I glutathione S-transferase gene family. *Proc. Natn. Acad. Sci. USA*. (In the press.)

Raymond, M., Callaghan, A., Fort, P. & Pasteur, N. 1991 Worldwide migration of amplified insecticide resistance genes in mosquitoes. *Nature* **350**, 151–153.

Vaughan, A., Hawkes, N. & Hemingway, J. 1997 Co-amplification explains linkage disequilibrium of two mosquito esterase genes in insecticide resistant *Culex quinquefasciatus*. *Biochem. J.* **325**, 359–365.