Quantitative analysis of gene amplification in insecticide-resistant *Culex* mosquitoes

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The amplification of carboxylesterase structural genes followed by their overexpression is the most common mechanism of resistance to organophosphorus insecticides in *Culex* mosquitoes. Most resistant *Culex quinquefasciatus* mosquitoes have co-amplified *est*$\alpha 2^1$ and *est*$\beta 2^1$ genes. Recently, Southern, DNA dot-blot analysis and phosphorimaging technology were used to quantify the *est* gene copy number in aphids and mosquitoes. Although more accurate than autoradiography, this method relies on probe hybridization, which can be variable. We have directly measured gene and mRNA copy number by using real-time quantitative PCRs in mosquitoes. The acquisition of fluorescence from incorporation of the double-strand-specific dye SYBR GreenI into a PCR product once per cycle is used to provide an absolute quantification of the initial template copy number. Thus it has been possible to show that *est*$\alpha 2^1$ and *est*$\beta 2^1$ are co-amplified approx. 80-fold in the genome of the resistant PelRR strain of *C. quinquefasciatus*. The two genes, although co-amplified in a 1:1 ratio, are differentially transcribed: the *est*$\beta 2^1$ gene from this amplicon has greater transcription than *est*$\alpha 2^1$ in all individual mosquito larvae tested, with an average ratio of 10:1. Purified esterases from mosquito homogenates were found in a ratio of 3:1, which, combined with the quantitative mRNA data, suggests the operation of both transcriptional and translational control mechanisms to regulate the expression of the amplified genes in *C. quinquefasciatus* insecticide-resistant mosquitoes.

Key words: carboxylesterase, gene amplification, LightCycler, quantitative PCR.

INTRODUCTION

The most frequently observed organophosphate-selected resistance mechanism in the common house mosquito *Culex quinquefasciatus* is the amplification of non-specific esterases [1–3]. These esterases confer insecticide resistance by rapid binding and slow turnover (i.e. sequestration), thus preventing the organophosphate insecticide from reaching its target site, acetylcholinesterase [3,4]. Owing to the 1:1 stoichiometry of the reaction between insecticide and esterase, for this resistance mechanism to be effective, large amounts of these enzymes must be produced. Approx. 7.7 pmol of Est$\alpha 2^1$ and Est$\beta 2^1$ elevated esterases (0.4% of total soluble protein) occurs in organophosphate-resistant fourth-instar larvae of the PelRR strain of *C. quinquefasciatus* [3], whereas in resistant aphids the E4 esterase accounts for up to 3% of the total protein [5].

The molecular mechanism underlying the overproduction of esterases in *Culex* is gene amplification [6–12] and regulation of gene expression [10,11,13]. The most prevalent amplified esterase genes are *est*$\alpha 2^1$ and *est*$\beta 2^1$, which occur worldwide [1,14,15]. Some insects have variants of *est*$\beta 1$ (alleles of *est*$\beta 2^1$) that might or might not have a co-amplified *est*$\alpha 3$ [16]. These rarer esterases, when co-amplified, are on a single amplicon but are at least 10 kb apart. Truncated copies of the amplicon can also occur that contain only the *est*$\beta 1$ gene [6,16–18]. The *est*$\alpha 2^1$ and *est*$\beta 2^1$ esterase genes are co-amplified on the same DNA unit (amplicon) in a head-to-head arrangement 2.7 kb apart [10,12].

Original estimates of *est*$\beta 1$ gene copy number in the TEMR strain of *C. quinquefasciatus* were reported as 250 copies per cell in resistant mosquitoes [6] and later revised to 150 copies [7]. These estimates were obtained by using Southern and DNA dot-blots and semi-quantified by autoradiography, which was later shown to overestimate amplification levels [10,11,19]. Phosphorimaging technology reassessed the amplification level of *est*$\beta 1^1$ in the TEMR strain to approx. 20 copies [11], whereas *est*$\alpha 2^1$ and *est*$\beta 2^1$ amplified esterase genes were estimated, by using this technique, at 20–30 or 30–40 copies in the SELAX strain and 40–60 copies in the CYPRUS strain of *C. quinquefasciatus* [10,11,20]. This study was designed to employ a technique that could accurately resolve the disparity in the reported *est* gene copy numbers and provide a means of quantifying the level of expression of these genes in individual mosquitoes. Fluorescent monitoring of rapid-temperature-cycle DNA amplification was used to achieve these objectives. The amplification of specific DNA sequences over 30 temperature cycles can be completed routinely in 10–15 min when using small volumes in glass capillaries and forced air heating with minimal denaturation and annealing times, to improve specificity and yield [21–23]. Incorporation of the double-strand-specific fluorescent dye, SYBR GreenI, allows PCR product formation and accumulation to be measured either continuously for melting curve analysis or once per cycle after product extension, for quantification of initial template copy number. Detection of PCR product above background fluorescence occurs at a cycle number dependent on the initial concentration of template. This method detects as few as 100 initial template copies [23,24].

The sensitivity of this technique allowed us to determine accurately not only initial genomic template copy number but also expression levels and the specificity of *est* genes in *Culex* mosquitoes. To complement this quantification, an estimate of

Abbreviations used: gDNA, genomic DNA.

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the relative abundance of the Est$\alpha 2^1$ and Est$\beta 2^1$ elevated esterases was undertaken.

EXPERIMENTAL

Mosquito strains

C. quinquefasciatus strains were obtained as follows: the insecticide-resistant PelRR, containing *est$\alpha 2^1$*/*est$\beta 2^1$*, and susceptible PelSS strains were established from Sri Lanka in 1986 [25,26]. cDNA encoding the *Ctrest$\beta 1^1$* gene was cloned from adult *C. tritaeniorhynchus* mosquitoes, which were collected from piggeries in Anuradhapura, Sri Lanka, as detailed previously ([9,27]; M. G. Paton, S. H. P. P. Karunaratne and J. Hemingway, unpublished work).

Enzyme purification and biochemical assays

Est$\alpha 2^1$ and an Est$\beta 2^1$ were purified from crude homogenate of PelRR by sequential column chromatography on Q-Sepharose, phenyl Sepharose, hydroxyapatite, *p*-chloromercuriobenzoate and preparative electrophoresis, as described previously [3,4]. Enzyme activities were assayed with the substrates *p*-nitrophenyl acetate or α-naphthyl acetate and β-naphthyl acetate [4,27]. Native PAGE was performed in 7.5% (w/v) polyacrylamide gels as detailed previously [4].

Extractions of mosquito RNA and genomic DNA

Genomic DNA and RNA were extracted with TRI reagent (guanidine thiocyanate/phenol in a monophase solution) (Sigma) in accordance with the manufacturer's instructions. In brief, genomic DNA was extracted from groups of 10 adult mosquitoes which were homogenized in 150 μl of TRI reagent with a motorized pestle and microcentrifuge tube. The homogenate was centrifuged at 12000 ***g*** for 10 min at 4 °C. The pellet was washed twice in 300 mM sodium citrate/10% (v/v) ethanol for 30 min each wash, then centrifuged at 12000 ***g*** for 10 min. The DNA pellet was washed in 75% (v/v) ethanol for 20 min, then centrifuged at 12000 ***g*** for 5 min. The pellet was air-dried and resuspended gently in 20 μl of 8 mM NaOH. The suspension was finally centrifuged at 12000 ***g*** for 10 min and the DNA solution was removed. The isolated DNA was treated with 10 μg/ml RNase A (Sigma) for 15 min at 37 °C, then extracted with phenol/chloroform/3-methylbutan-1-ol (25:24:1, by vol.) and then chloroform/3-methylbutan-1-ol (24:1, v/v) before precipitation with ethanol. The DNA concentration was adjusted to 200 ng/μl with TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 7.6) and subsequently used for PCR analysis. DNA concentrations were measured on a Cecil 6000 spectrophotometer.

Individual mosquitoes were used for RNA extraction in 150 μl of TRI reagent by using a motorized pestle and microcentrifuge tube. After centrifugation at 12000 ***g*** for 10 min at 4 °C, the TRI reagent containing RNA was transferred to another centrifuge tube and homogenized for 15 s with chloroform and left at room temperature for 2–15 min. The mixture was centrifuged at 12000 ***g*** for 15 min at 4 °C. The upper aqueous phase was transferred to another centrifuge tube and the RNA was precipitated with propan-2-ol and centrifuged at 12000 ***g*** for 10 min at 4 °C. The pellet was washed in 75% (v/v) ethanol and resuspended in 20 μl of water. The RNA was treated with 0.4 unit/μl DNaseRQI (Promega) for 15 min at 37 °C. The RNA was then re-extracted in 150 μl of TRI reagent as before.

Synthesis of mosquito cDNA

Mosquito RNA (10 μl) was combined with 500 ng of $(dT)_{12-18}$ (Promega) in a 12 μl volume, then heated to 70 °C for 10 min and immediately transferred to ice. The first-strand synthesis reaction was performed in 50 mM Tris/HCl (pH 8.3)/75 mM KCl/3 mM $MgCl_2$/10 mM dithiothreitol (Gibco BRL) containing each dNTP (Gibco BRL) at 0.25 mM and 200 units of SuperScript II Rnase H^- reverse transcriptase (Gibco BRL) at 42 °C for 50 min. The reaction was stopped by heating at 70 °C for 15 min. The samples were stored at −20 °C before use in PCR. Analysis by reverse-transcriptase-mediated PCR was used to confirm the absence of genomic contamination in the cDNA and RNA before use on the LightCycler.

Real-time PCR

The *estβ* standard plasmid pBF14 contained *C. tritaeniorhynchus est$\beta 1^1$* coding region (1600 bp) inserted into the TA cloning vector pCR2 (Invitrogen) (M. G. Paton, S. H. P. P. Karunaratne and J. Hemingway, unpublished work). The *estα* standard plasmid pEG8 contained a *C. quinquefasciatus* 540 bp genomic DNA PCR product from *est$\alpha 2^1$* flanked by primers LCA1F and LCA1R inserted into the pGEM Teasy vector (Promega). The *estα* standard plasmid pEG15 contained a *C. quinquefasciatus* 500 bp cDNA PCR product from *est$\alpha 2^1$* flanked by primers LCA1F and LCA1R inserted into the pGEM Teasy vector. The actin standard plasmid pAgAc contained a 350 bp cDNA PCR product from the *Anopheles gambiae* cytoskeletal actin gene [29] flanked by primers AcF and AcR inserted into the pGEM Teasy vector. These plasmids were used as template DNA at concentrations ranging from 1 ng to 10 fg to produce standard curves. The genomic DNA was used as template at 100 ng. For the cDNA, 0.5 μl of the synthesis reaction was used as template. The reactions were performed in 50 mM Tris/HCl, pH 8.5, containing 3 mM $MgCl_2$, each dNTP at 0.2 mM, 0.5 μM primers (Genosys) and 0.2 unit of *Taq* polymerase (Promega) per 5 μl reaction. SYBR GreenI (Molecular Probes) was used at 1:20000 dilution. The reactions were performed in glass capillary tubes in a LightCycler real-time PCR machine (Idaho Technology, Idaho Falls, ID, U.S.A.) with the parameters specified in Table 1.

PCR was conducted for 40 cycles. After the cycling protocol, a final step was applied to all reactions by continuously monitoring fluorescence though the dissociation temperature of the PCR product at a temperature transition rate of 0.1 °C/s to generate a melting curve. The reaction products were collected and run on a 1% (w/v) agarose/TAE (40 mM Tris/acetate, 2 mM EDTA, pH 8.0) gel to confirm product size. The product was ligated into pGEM Teasy vector and used to transform XL-1 blue *Escherichia coli* cells (Stratagene) by heat shock. The purified plasmid was then sequenced with an ABI 377 automated sequencer.

The results from the LightCycler were processed into melting peaks and quantified with Labview software (National Instruments, Austin, TX, U.S.A.).

RESULTS

Quantification of template copy number was dependent on the accurate production of standard curves for the PCR products indicative of each gene to be quantified. These standard curves represent the initial template copy number for a given fluorescence signal and allow the extrapolation of fluorescence levels from test samples to determine their initial copy number. Figure 1 illustrates the raw acquisition of fluorescence data

Table 1 LightCycler PCR parameters and primers used to quantify gene and cDNA copy numbers

The same PCR parameters and primers were used for either cDNA or gDNA for a given target gene.

PCR target	Primers	LightCycler PCR parameters
Actin	AcF, 5′-GCGACGAGGCCCAGAGCA-3′	95 °C, 1 s; 63 °C, 3 s; 72 °C, 14 s
	AcR, 5′-TCCAGCGCGACGTAGCACAG-3′	(fluorescence acquisition)
estα	LCA1F, 5′-GACCTTTCGCGGGGATTGTTTCA-3′	95 °C, 1 s; 70 °C, 3 s; 72 °C, 14 s; 86 °C, 1 s
	LCA1R, 5′-TTCATCTGCTTGCCCACCTCTTTG-3′	(fluorescence acquisition)
estβ	009TF, 5′-CTTCCGGTGATGCTGTACAT-3′	95 °C, 1 s; 60 °C, 3 s; 72 °C, 14 s; 84 °C, 1 s
	009TR, 5′-CCTTCGCCAACTTCTCAACC-3′	(fluorescence acquisition)

profiles and the standard curves obtained for the serial dilution from 1 ng of plasmids pBF14 containing *est*β cDNA/genomic DNA (gDNA) (Figure 1A, lower panel), pAgAc containing actin gene cDNA/gDNA (Figure 1B, lower panel), pEG8 containing *est*α gDNA (Figure 1C, lower panel) and pEG15 containing *est*α cDNA (Figure 1D, lower panel). The fluorescence incorporated into the PCR products was measured once per cycle and plotted against cycle number.

The fluorescence curves were displaced horizontally according to the initial template concentration approximately three cycles for each 10-fold dilution. Each sample was analysed in duplicate; quantities and profiles of each duplicate coincided reproducibly. The point at which each sample reached an arbitrary fluorescence value (1.0), the threshold crossing point, was plotted against the logarithm of the calculated initial copy number of target template for that sample, to derive the standard curves in Figure 1. The initial target template copy number was calculated by determining the molecular masses of the plasmid and target sequence to be amplified on the basis of their nucleotide sequence and, proportionally, deriving the number of moles of target sequence per fg of DNA. The numbers of copies were then calculated by multiplying the numbers of moles by Avagadro's constant. The calculated initial target template copy numbers were as follows: pBF14, 154.3 copies/fg; pAgAc, 253.7 copies/fg; pEG8, 239 copies/fg; pEG15, 242.6 copies/fg. Because the actin primer set AcF/AcR amplified the same 350 bp sequence from within an exon in both gDNA and cDNA, the same standard curve could be used for either gDNA or cDNA samples. This was also true for *est*β primers 009TF/009TR, which amplified the same 418 bp sequence from within exon 3. The 009TF/009TR primers were designed from a region of 100 % identity between the *est*β genes of *C. quinquefasciatus*, *C. tarsalis* and *C. tritaeniorhynchus*. However, *est*α primer set LCA1F/LCA1R amplified different products from gDNA (550 bp) and cDNA (500 bp) (Figure 2A) owing to the presence of a 50 bp intron between exons 4 and 5 in the gDNA. Two separate standard curves were therefore used for *est*α gDNA and cDNA.

The use of SYBR GreenI to follow the accumulation of PCR product by incorporation into double-stranded DNA can lead to the detection of non-specific product such as primer dimers at low template concentration or excess primer concentration; this has a direct effect on the accuracy of the quantification procedure. Figure 2(B) shows the melting peaks obtained for pEG15 (*est*α cDNA) at different Mg^{2+} concentrations, with primers LCA1F/LCA1R. The specific 500 bp product was detected as a fluorescence peak at a melting temperature (T_m) of 88.6 °C, whereas a non-specific product was also detected as a peak at T_m 84.1 °C, although no contaminating product could be detected on an agarose gel (Figure 2A). To avoid the incorporation of this fluorescence into any quantitative measurement, the temperature at which fluorescence was acquired was adjusted to 86 °C, at which point the non-specific product had already melted. Similarly, the acquisition temperature was adjusted to 84 °C for *est*β to avoid a non-specific product with T_m 82 °C, whereas the specific product had a T_m of approx. 89 °C. This adjustment was not necessary for the actin measurements.

The T_m of a particular product is dependent not only on the size of the product but also on its nucleotide sequence. Therefore products of identical size can have different T_m values; this was demonstrated for the *est*β products. Figure 3 shows the melting peaks for amplified products of *C. quinquefasciatus* PelRR and PelSS strains and for *C. tritaeniorhynchus* genomic DNA with the *est*β 009TF/009TR primers. It can be observed that the T_m values of the melting peaks were almost identical for PelRR and PelSS (T_m 89.1 °C and 88.8 °C respectively) but different for *C. tritaeniorhynchus* (T_m 87.9 °C). The products were all 418 bp as determined by DNA sequencing, which also showed that the sequences differed by 1.9 % between PelSS and PelRR and 17.2 % or 16.5 % between *C. tritaeniorhynchus* and PelSS and PelRR respectively (Table 2). With this technique, sequences of high similarity can therefore be distinguished by their melting peaks.

By using the standard curves (Figures 1A–1C) it was possible to extrapolate the fluorescence values from the test PelRR and PelSS genomic DNA and derive an accurate initial copy number for the genes *est*α, *est*β and actin. The fluorescence profiles are shown in Figure 4(A), with each genomic DNA sample being run in triplicate and each replicate coinciding with minimal variation. The copy number of the actin gene was determined as 1.7×10^6 in 100 ng of starting material for both PelRR and PelSS. The numbers of copies of the *est*α and *est*β genes in PelRR were 1.09×10^6 and 1.03×10^6 respectively, yielding a mean amplicon copy number of 1.06×10^6 copies in 100 ng of starting material. The numbers of copies of *est*α and *est*β in PelSS were 8.0×10^3 and 1.9×10^4 respectively in 100 ng of DNA, containing the equivalent number of copies of the actin gene in both strains. These results indicate an approximate 1:2 ratio of the non-amplified *est*α to *est*β genes. In comparison, the *est*α and *est*β are co-amplified in PelRR, with approx. 80-fold (range 132–56-fold) more copies of the amplicon containing these genes in PelRR than PelSS. The comparison of the copy numbers determined for the different genomic DNA and genes is shown in Figure 4(B). These differences in gene copy number are reflected by a difference in *est*α and *est*β activity with α- and β-naphthyl acetate substrates in the two strains, with approx. 6-fold more esterase activity in PelRR than PelSS (Figure 4C).

To assess the expression levels of *est*α and *est*β in resistant mosquitoes, 12 larvae from the PelRR strain of *C. quinquefasciatus* were used individually for RNA extraction and cDNA synthesis. The number of mRNA copies transcribed from the respective genes, *est*α, *est*β and actin were determined with the LightCycler. The fluorescence profiles for these individual larvae in triplicate from strain PelRR are shown in Figure 5(A). In all larvae tested, the number of copies of the *est*β cDNA was greater than that of *est*α, although not all the differences were statistically significant. The relative abundance of *est*β over *est*α within an individual was variable (Figure 5B), ranging from 32 to 1.3 copies of *est*β cDNA per copy of *est*α. The mean ratio of *est*β to *est*α cDNA was 10:1.

To estimate protein levels, Estα2[1] and Estβ2[1] were separated after phenyl-Sepharose column chromatography. The recovered

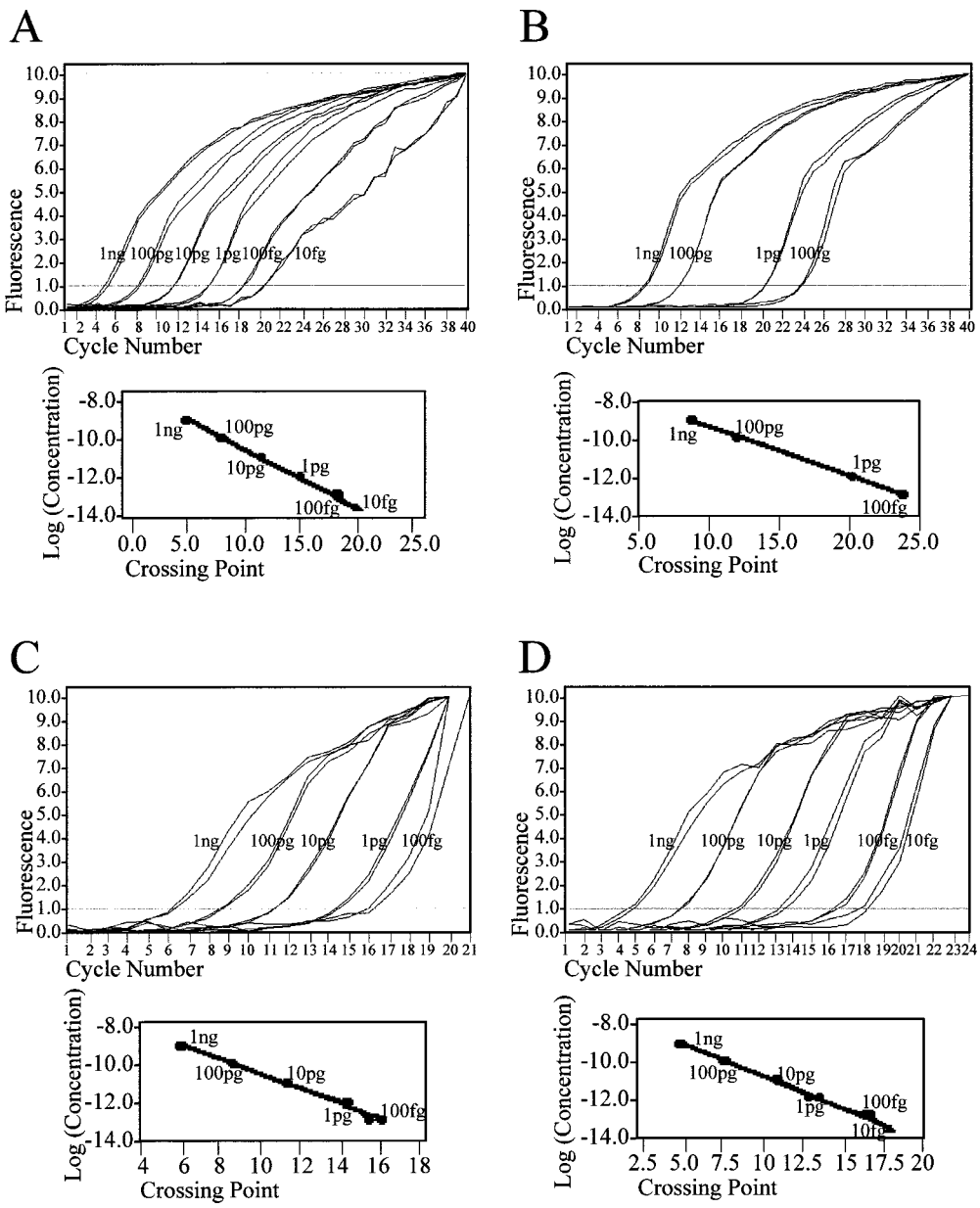

Figure 1 Standard curves used to quantify gene expression and copy number

(**A**–**D**, upper panels) SYBR GreenI fluorescence acquisition by PCR product from serially diluted (1 ng to 10fg) standard plasmids pBF14 (*est*β cDNA/gDNA; primers 009TF/009TR), pAgAc (actin cDNA/gDNA; primers AcF/AcR), pEG8 (*est*α gDNA; primers LCA1F/LCA1R) and pEG15 (*est*α cDNA; primers LCA1F/LCA1R) respectively against cycle number. The horizontal line in each panel is the noise band. (**A**–**D**, lower panels) Standard curves derived from plotting the crossing point against the logarithm of copy number for plasmids pBF14, pAgAc, pEG8 and pEG15 respectively.

enzymic protein ratio was approx. 60 % for Estα2^1 and 12 % for Estβ2^1. Because the V_{max} of Estβ2^1 is 7.5-fold less than that of Estα2^1 for *p*-nitrophenyl acetate [3], we estimate that 60 % of Estβ2^1 and 40 % of Estα2^1 ($12 \times 7.5 : 60$) are present as recovered enzymes. Because Estβ2^1 is more labile than Estα2, a higher proportion of Estβ2^1 is expected in the crude homogenate. A greater availability of Estβ2^1 in the crude homogenate was also demonstrated by using native PAGE gels stained for esterase activity. To obtain Estα2^1 and Estβ2^1 bands of similar intensities to those in crude insect homogenates, 3–4-fold more purified Estβ2^1 than Estα2^1 had to be applied. The relative amounts of Estα2^1 and Estβ2^1 in PelRR crude homogenate were further investigated by using the inhibitor eserine, which at 10 μM inhibits Estα2^1 but not Estβ2^1 [3]. When the crude homogenate was inhibited, 30 % of activity remained. After normalizing for different V_{max} values for the two enzymes, an estimate of 3-fold more Estβ2^1 than Estα2 ($30 \times 7.5 : 70$) was calculated.

DISCUSSION

Quantitative PCR with the LightCycler enables sequence specific detection and rapid quantification from 0 to 1000 initial template copies. The procedure has applications to the analysis of field samples, with particular emphasis on the identification and

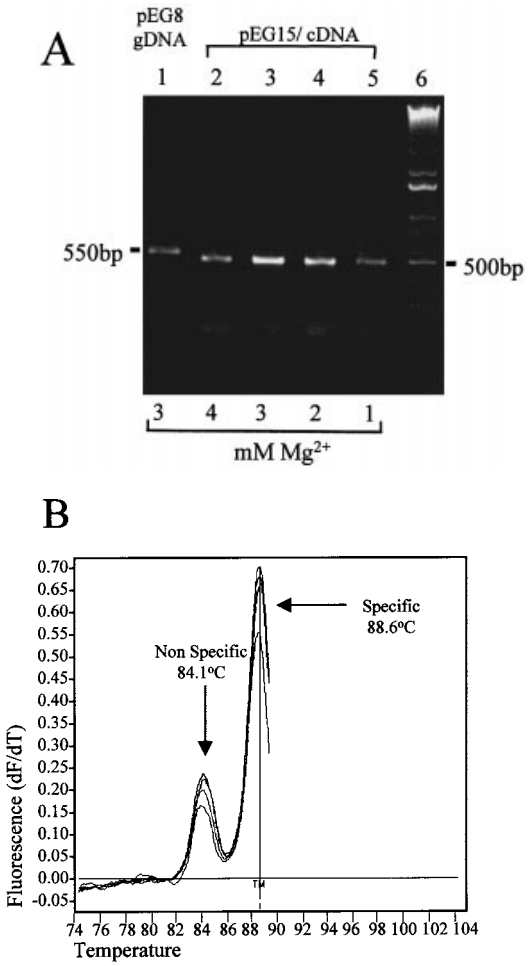

Figure 2 Optimization of LightCycler PCR conditions of plasmid pE15 for quantification of *est*α cDNA

(**A**) Agarose (0.75%)/1 × TAE gel with amplified *est*α gDNA (plasmid pE8, lane 1) and cDNA (plasmid pE15, lanes 2–5) PCR products by using primers LCA1F/LCA1R at different Mg^{2+} concentrations (4–1 mM, lanes 2–5 respectively). (**B**) Melting peaks of the pEG15 PCR products are shown, with their melting temperatures indicated.

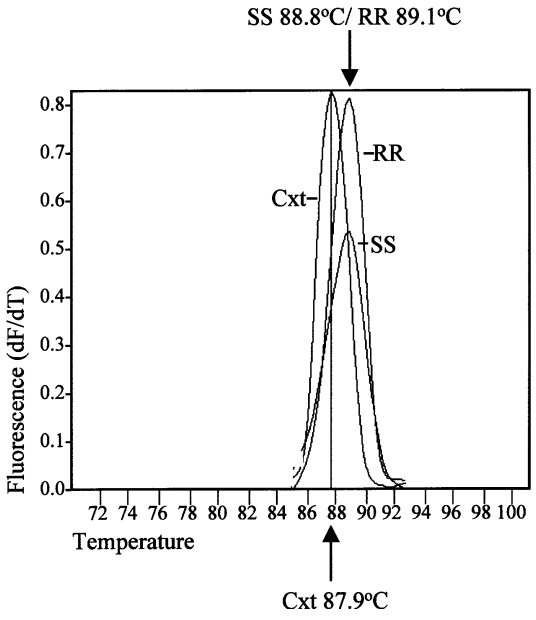

Figure 3 Differentiation of *est*β PCR products of *C. tritaeniorhynchus* and *C. quinquefasciatus* by melting-peak analysis

Melting peaks of the *est*β PCR products from *C. tritaeniorhynchus* (Cxt) and *C. quinquefasciatus* strains PelRR (RR) and PelSS (SS) are shown, with their melting temperatures indicated.

Table 2 Percentage similarity and divergence from the nucleotide sequence comparison of the same Est*β* PCR products: *C. tritaeniorhynchus* (Cxt), *C. quinquefasciatus* resistant (RR) and *C. quinquefasciatus* susceptible (SS)

The percentage similarity is shown in the upper triangle and the percentage divergence in the lower triangle.

	Similarity/divergence (%)		
Strain	Cxt	RR	SS
Cxt	–	83.0	81.8
RR	16.5	–	98.1
SS	17.2	1.9	–

measurement of different resistance alleles and their frequencies. Continuous monitoring of fluorescence through the dissociation temperature of the PCR product gives a DNA melting curve whose shape and position is dependent on the GC-to-AT ratio, the length of product and the sequence and can be used to distinguish between products of identical sizes that are separated by less than 2 °C in melting temperature [30]. Our studies demonstrate the ability to distinguish between isoenzymes of the *est*β genes in different *Culex* species. These studies have been extended to distinguish between alleles of the *est*β gene in field mosquito populations of *C. tritaeniorhynchus* (M. G. Paton, S. H. P. P. Karunaratne and J. Hemingway, unpublished work) and other genes of clinical interest [21].

Previous determinations of the extent of gene amplification in *Culex* mosquitoes have produced variable results, due in part to the insensitivity of techniques used to measure the gene copy number, such as dot-blots, and also in part to fluctuations in copy number due to the presence or absence of selection by insecticide. The limitations of previous techniques were demonstrated by the severalfold disparity observed between a recent competitive quantitative PCR technique and dot-blots that were used to measure the levels of esterase gene amplification in the aphid *Myzus persicae* [31]. The LightCycler offers a reproducibly accurate measurement of amplification levels through the direct recording of fluorescence once per cycle, thus avoiding the limitations of other PCR quantification methods that use end-point analysis, where the reaction has become stagnant [23]. Our results are consistent with previous indications of a 1:1 ratio of *est*α and *est*β genes located within the $est\alpha 2^1/est\beta 2^1$ amplicon of resistant mosquitoes [9,10]; however, we were unable to show a similar 1:1 organization in a susceptible strain in which there was an approximate 1:2 ratio of *est*α to *est*β. This could be explained by the presence of a second non-amplified *est*β gene, which would have a negligible contribution to overall gene copy number in PelRR but would double the *est*β gene copy number in PelSS. This hypothesis is consistent with previous observations of an intergenic spacer between the *est*α and *est*β genes in the TemR

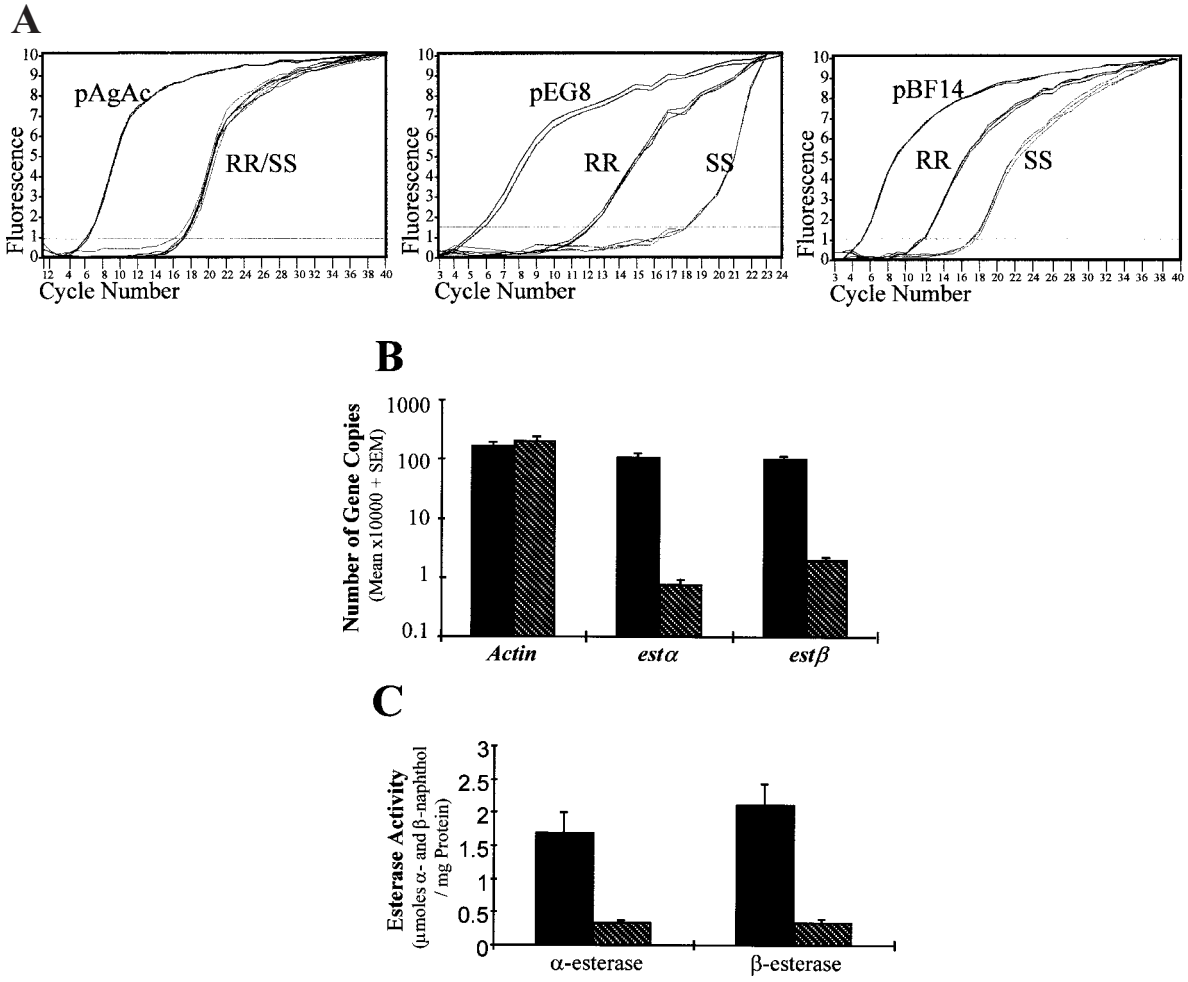

Figure 4 Quantification of *estα* and *estβ* gene copy number from pooled *C. quinquefasciatus* PelRR and PelSS larvae

(**A**) Graphs of PCR fluorescence profiles against cycle number with the LightCycler for plasmid (pAgAC, pEG15 or pBF14), PelRR strain (RR) and PelSS strain (SS) in triplicate for actin (AcF/AcR primers) (left panel), *estα* (LCA1F/LCA1R primers) (middle panel) and *estβ* (009TF/009TR primers) (right panel). The horizontal line in each panel is the noise band. (**B**) Gene copy numbers for actin, *estα* and *estβ* from triplicates (means ± S.E.M.) for *C. quinquefasciatus* PelRR strain (solid columns) and PelSS strain (hatched columns). (**C**) Esterase activities (means ± S.E.M.) with the substrates α-naphthyl acetate and β-naphthyl acetate from 12 individual mosquitoes of PelRR (solid columns) and PelSS (hatched columns).

strain of *C. quinquefasciatus*, which has an independently amplified *estβ1* gene [12]. This PCR product was indicative of a second non-amplified *estβ* gene. Furthermore, a partial clone of a second non-amplified *estβ* gene has been isolated from PelRR (A. Vaughan, personal communication).

The approx. 80-fold copy difference between PelSS and PelRR *estα* and *estβ* genes represents a level of gene amplification between those reported previously [6,7,10,11,20]. It is clear from population studies that amplification levels vary between individuals over time ([20], and M. G. Paton, S. H. P. P. Karunaratne and J. Hemingway, unpublished work), through variation in organophosphate selection pressure, promoting the loss or gain of gene copies, possibly through unequal sister-chromatid exchange as suggested for mosquitoes and aphids [20,32]. The approximate 80-fold copy difference of amplified genes between PelRR and PelSS does not directly translate into an equivalent difference at the protein level, predominantly because of the role of gene regulation and protein stability. The regulation of gene expression is dependent on physiological conditions, which can affect differential expression through promotor repression or activation at the transcriptional level and/or additional regulation to control the translation of the mRNA. Our results on levels of transcription of the $est\alpha2^1/est\beta2^1$ genes in PelRR indicate a 10:1 ratio of $est\beta2^1$ cDNA over $est\alpha2^1$ cDNA despite a 1:1 ratio of the genes. Such observed differential transcription of the $est\alpha2^1/est\beta2^1$ genes demonstrates that the promotors regulating their expression are independent and of different strengths. The identity and nature of these promotors are under current investigation (N. Hawkes, personal communication) [13]. Isolation of the enzymes from fourth-instar mosquito larvae revealed a 3-fold excess of *estβ* over *estα*, which is a significantly lower ratio than that of mRNA. This discrepancy can be attributed either to translational control, in which not all of the mRNA is translated into protein, or to differences in protein stability.

Translational control occurs in other organisms, such as the ookinete of *Plasmodium berghei* during its penetration of the mosquito midgut [33,34]. The accumulation of specific esterase transcripts by the mosquito might occur at times of low insecticide

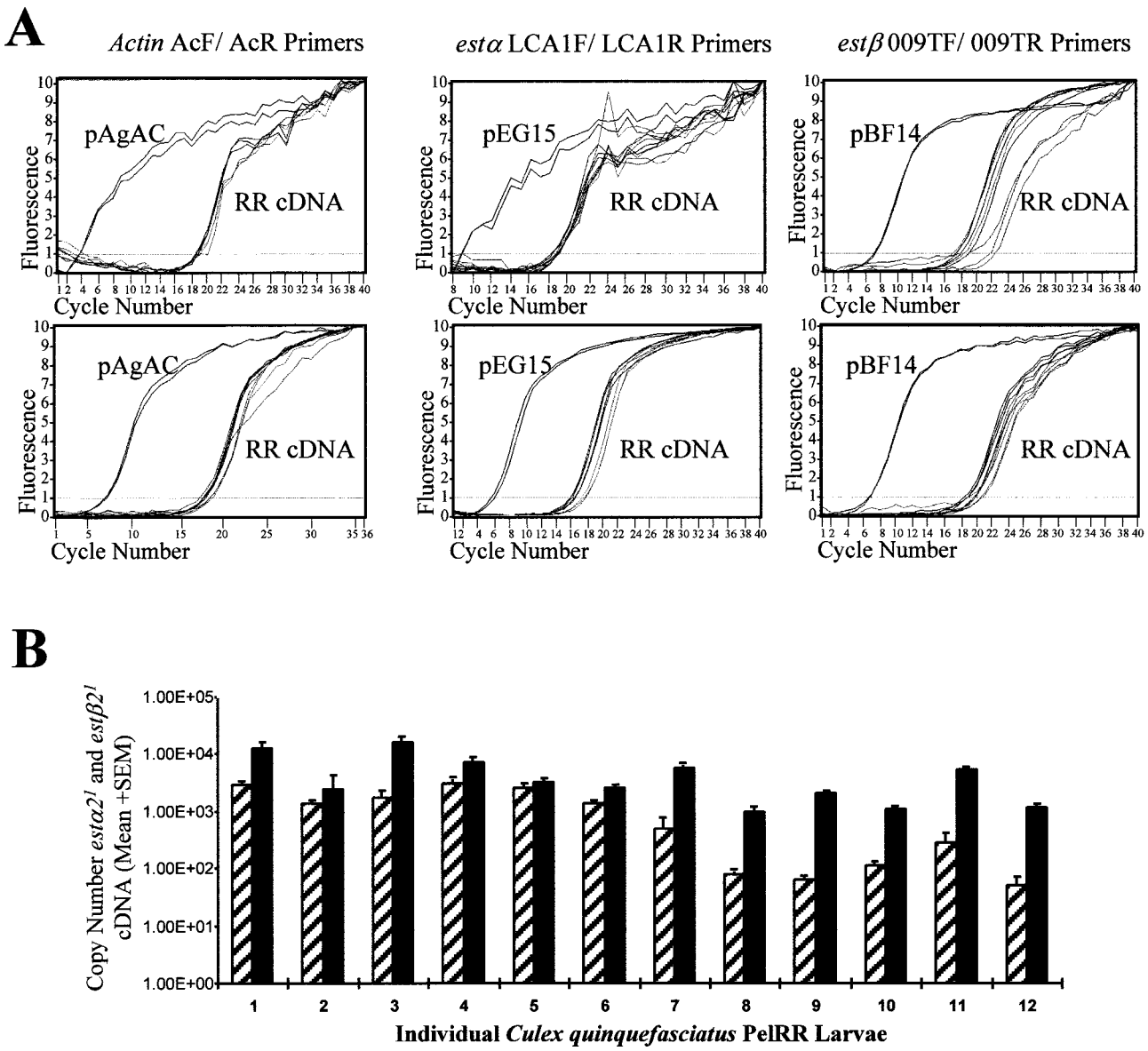

Figure 5 Quantification of *estα* and *estβ* mRNA expression levels in individual *C. quinquefasciatus* PelRR larvae

(**A**) Graphs of PCR fluorescence profiles against cycle number with the LightCycler for six individuals (upper panels, 1–3; lower panels, 4–6) in triplicate for actin (AcF/AcR primers) (left panels), *estα* (LCA1F/LCA1R primers) (middle panels) and *estβ* (009TF/009TR primers) (right panels). The horizontal line in each panel is the noise band. (**B**) mRNA copy numbers for *estα* (hatched columns) and *estβ* (solid columns); means ± S.E.M. from triplicates for 20 individual *C. quinquefasciatus* PelRR larvae.

selection to facilitate rapid mobilization and protein translation, in response to high selection pressure of insecticide. This possibility remains to be addressed. Previous biochemical results indicate an increased lability of purified Estβ compared with Estα protein [3]. If this is also true in the mosquito, then decreased stability might account for the differences in ratios observed at the mRNA and protein levels. A combined analysis of protein activity and amplification levels in individual mosquitoes was not possible for the complex *estα2*^{1}/*estβ2*^{1} amplicon owing to the overlapping substrate specificities of the two enzymes. However, such a study has been conducted on field material with the *Ctrestβ1*^{1} amplicon of *C. tritaeniorhynchus*, in which it was demonstrated that gene regulation, rather than amplification, was the determining factor in the amount of enzyme activity observed (M. G. Paton, S. H. P. P. Karunaratne and J. Hemingway, unpublished work).

This study shows the complex nature of the genetic control underlying the major organophosphorus resistance mechanism operating in the *Culex* mosquito and describes an accurate means by which to dissect this phenomenon.

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