**Functional and immunohistochemical characterization of CCEae3a, a carboxylesterase associated with temephos resistance in the major arbovirus vectors *Aedes aegypti* and *Ae. albopictus.***

Linda Grigoraki1,2, Vassileia Balabanidou1,2, Christos Meristoudis3, Antonis Miridakis4, Hilary Ranson5, Luc Swevers3, John Vontas 2,6\*

1 Department of Biology, University of Crete, Vassilika Vouton, 73100, Heraklion, Greece

2 Institute of Molecular Biology and Biotechnology, Foundation for Research and Technology-Hellas, 73100 Heraklion, Greece

3 Insect Molecular Genetics and Biotechnology, Institute of Biosciences and Applications, National Centre for Scientific Research ‘Demokritos’, Athens, Greece

4 Environmental Chemical Processes Laboratory (ECPL), Department of Chemistry, University of Crete, 71003 Heraklion, Greece

5 Department of Vector Biology, Liverpool School of Tropical Medicine, Pembroke Place, Liverpool, UK

6 Pesticide Science Laboratory, Department of Crop Science, Agricultural University of Athens, 11855 Athens, Greece

\*Correspondance: J. Vontas ([vontas@imbb.forth.gr](mailto:vontas@imbb.forth.gr))

**Abstract**

Temephos is a major organophosphate (OP) larvicide that has been used extensively for the control of *Aedes albopictus* and *Aedes aegypti*, the major vectors for viral diseases, such as dengue fever, zika and chikungunya. Resistance to temephos has been recently detected and associated with the upregulation of carboxylesterases (CCEs) through gene amplification, in both species. Here, we expressed the *CCEae3a* genes which showed the most striking up-regulation in resistant *Aedes* strains, using the baculovirus system. All *CCEae3a* variants encoded functional enzymes, with high activity and preference for p-nitrophenyl butyrate, a substrate that was shown capable to differentiate temephos resistant from susceptible *Aedes* larvae. Enzyme kinetic studies showed that CCEae3as from both *Ae. aegypti* and *Ae. albopictus* (CCEae3a\_aeg and CCEae3a\_alb, respectively) strongly interact with temephos oxon and slowly released the OP molecule, indicating a sequestration resistance mechanism. No difference was detected between resistant and susceptible CCEae3a\_aeg variants (CCEae3a\_aegR and CCEae3a\_aegS, respectively), indicating that previously reported polymorphism is unlikely to play a role in temephos resistance. HPLC/MS showed that CCEae3as were able to metabolize temephos oxon to the temephos monoester [(4-hydroxyphenyl) sulfanyl] phenyl O,O-dimethyl  
phosphorothioate. Western blot and immunolocalization studies, based on a specific antibody raised against the CCEae3a\_alb showed that the enzyme is expressed at higher levels in resistant insects, primarily in malpighian tubules (MT) and nerve tissues.

**Introduction**

*Aedes albopictus* and *Aedes aegypti* are the main vectors for a variety of arboviral diseases, such as dengue fever, yellow fever, zika and chikungunya, which have a large socio-economical impact and threaten about half of the world’s population ([WHO, 2012](#_ENREF_30)). Both species are found in Asia, Oceania and the Americas, while climate changes and trade have facilitated the introduction and spread of *Ae. albopictus* also in Europe ([Kraemer et al., 2015](#_ENREF_17)).

The control of *Aedes* vectorsrelies on clean-up campaigns that reduce the larval breeding sites, repellents (spatial or personal), and insecticides (larvicides and adulticides) ([WHO, 2009](#_ENREF_29)). Temephos, a low toxicity highly effective organophosphate (OP) larvicide, has been widely used in Asia and Central America against both *Aedes* vectors ([Grisales et al., 2013](#_ENREF_10); [Lima et al., 2003](#_ENREF_18)). Temephos has been also used in Europe for several decades, although its use is currently banned in most countries; nevertheless, it remains an important backup solution, in case of failure of the limited alternative larvicides, or emergencies.

The heavy use of temephos resulted in selection of insecticide resistance in both *Ae. albopictus* and *Ae. aegypti* populations from several geographical regions ([Ranson et al., 2010](#_ENREF_24); [Vontas et al., 2012](#_ENREF_27)). In a previous study we showed that temephos resistance in *Ae. albopictus* is associated with elevated carboxylesterases (CCEs): two CCE genes, the *CCEae3a* and the *CCEae6a* had the most striking up-regulation (27- and 12-folds respectively, compared to the reference susceptible strain), which was at least partially due to gene amplification, an event further linked to temephos resistance via genetic crosses ([Grigoraki et al., 2015](#_ENREF_9)). Interestingly, up-regulation and amplification of the orthologous CCEs(*CCEae3a* and *CCEae6a*) was also associated with temephos resistance in *Ae. aegypti* ([Poupardin et al., 2014](#_ENREF_23)). In this case it was additionally hypothesized that amino acid polymorphisms in the sequence of CCEae3a from the resistant *Ae. aegypti* mosquitoes might contribute to resistance, by influencing the conformation of the Organophosphate (OP) binding site ([Poupardin et al., 2014](#_ENREF_23)). Polymorphisms in the sequence of CCEae3a have recently also been related to pyrethroid resistance in an *Ae.aegypti* strain from Thailand ([Faucon et al., 2015](#_ENREF_8)).

With the exception of a few cases where CCEs quickly metabolize (hydrolyze) OPs, like the *Lucilia cuprina* E3 aliesterase ([Devonshire et al., 2003](#_ENREF_4)), CCEs usually confer resistance by sequestration: they rapidly bind OP molecules with higher affinity than the target site Acetylcholinesterase (AChE) and slowly hydrolyze the phosphoester bond formed ([Hemingway et al., 2004](#_ENREF_12)). The specific kinetics of CCE - OP interaction have been primarily studied in isoenzymes isolated from crude mosquito homogenates ([Karunaratne et al., 1993](#_ENREF_15); [Ketterman et al., 1992](#_ENREF_16)), however functional characterization of specific recombinant CCEs associated with insecticide resistance has been achieved in other insects ([Newcomb et al., 1997](#_ENREF_21); [Teese et al., 2013](#_ENREF_25)). Little is known about the physiology of this interaction, i.e. where insecticide sequestration/detoxification occurs and thus which tissues are primarily responsible for the resistance phenotype. Previous studies using *Culex quinquefasciatus* and *Culex pipiens* mosquitoes have shown the localization of CCE enzymes associated with resistance in the alimentary canal and malpighian tubules (MT), but also in neurons, the subcuticular layer and salivary glands ([McCarroll and Hemingway, 2002](#_ENREF_19); [Pasteur et al., 2001](#_ENREF_22)). The localization of cytochrome P450s (CYPs) - insecticide metabolizers, in MT was recently thoroughly investigated in *Anopheles gambiae* adult mosquitoes ([Ingham et al., 2014](#_ENREF_13)). However, the tissue localization of detoxification enzymes and CCEs in particular has not been explored in *Aedes* mosquito larvae.

Here, we have functionally expressed the CCEae3as from *Ae. albopictus* and *Ae. aegypti*, using a baculovirus expression system, and analyzed the interaction of the recombinant enzymes with temephos, by using enzyme kinetic assays and HPLC/MS. A specific antibody was raised and used to study the tissue localization of CCEae3a in *Ae. albopictus* larvae through western blots and immunohistochemistry approaches.

**Materials and methods**

*Mosquito strains*

The *Ae. albopictus* strains “Lab”, a reference susceptible strain originating from Malaysia, kindly provided by Dr Charles Wondjii (Liverpool School Tropical Medicine, UK) and “Tem-GR”, a temephos resistant strain originated from Greece ([Grigoraki et al., 2015](#_ENREF_9)), as well as the *Ae. aegypti* strains “Platthalung” ([Poupardin et al., 2014](#_ENREF_23)), a susceptible strain originating from Thailand and the “Nakhon Sawan” ([Poupardin et al., 2014](#_ENREF_23)), a temephos resistant strain originated from Thailand, were used in this study.

*Functional expression of recombinant CCEs*

*CCEs* (CCEae3a\_aegR from Nakhon Sawan, CCEae3a\_aegS from Platthalung, [Poupardin et al., 2014](#_ENREF_23) and CCEae3a\_alb from TemGR, [Grigoraki et al., 2015](#_ENREF_9)) were cloned and N-terminally Myc tagged, in a pEA expression vector ([Douris et al., 2006](#_ENREF_7)) using the primers listed in Table S1, and then transferred to a pFastBac1 vector (Invitrogen) as SmaI-NotI fragments. Generation of recombinant baculovirus was carried out using the Bac-to-Bac Baculovirus expression System (Invitrogen), according to manufacturer’s instructions. pFastBac1 vectors were transformed into DH10Bac *E. coli* and colonies with recombinant bacmids were selected on kanamycin/tetracycline/gentamycin plates by blue-white selection. DNA from positive colonies was used to transfect Sf21 insect cells and recombinant baculovirus was collected at 5-7 days after transfection. Recombinant baculovirus that expresses Yellow Fluorescent Protein (YFP) was generated from pFatBac1 vector containing YFP and was used as a negative control in all experiments. To check for esterase expression, fresh Sf21 cells at 300.000 cells/mL were infected with baculovirus stock at m.o.i (multiplicity of infection) of 5. Three days after infection, cells were collected by centrifugation (2.500 rpm, 5 min) and cell pellets were analyzed by Western. For Western blot, pellets were re-suspended in phosphate-buffered saline (PBS), frozen at -70°C for 30 min and centrifuged to separate the soluble protein fraction from the insoluble fraction. Detection of recombinant protein was done using an anti-Myc antibody (Cell Signalling) at a dilution of 1:1.000. For esterase activity assays infected Sf21 cell pellets from 1,5-2 mL culture, were re-suspended in 150μL 0,1M Sodium Phosphate buffer pH 7 containing 0,05% Triton and freeze thawed three times. Subsequently samples were centrifuged at 11.000rpm, 4oC for 3min and supernatant transferred to a new tube.

*Biochemical assays*

Recombinant esterase activity towards p-nitrophenyl acetate (Sigma), p-nitrophenyl butyrate (Sigma), α-naphthyl acetate and β-naphthyl acetate (Sigma) was measured in 0,1M sodium phosphate pH 7, as previously described ([Grigoraki et al., 2015](#_ENREF_9); [Van Leeuwen et al., 2005](#_ENREF_26)), while esterase activity in *Ae.albopictus* larvae was measured according to WHO protocols ([WHO, 1998](#_ENREF_28)). All reactions were carried out in 96-well plates (Nunc MaxiSorp) and absorbance was measured using a Spectra Max M2e multimode microplate reader (Molecular Devices, Berkshire, UK). The protein concentration was determined according to Bradford (1976). Control reactions were included in all experiments consisting of supernatants from Sf21 cells infected with virus expressing YFP.

Oxidation of temephos was achieved using the Abraxis Organophosphate/Carbamate plate kit (96T). Temephos (96% Fluka, Pestanal Sigma) was dissolved in methanol at a concentration of 300μΜ and mixed with equal volume of oxidant. Reaction proceeded for 15min and was stopped with reducing agent. Τhe volume ratio of insecticide: oxidant: reducing agent was 1:1:1.

Kinetic constants were estimated, as described in ([Ketterman et al., 1992](#_ENREF_16)). Briefly, recombinant CCE - temephos oxon inhibition reactions were performed in 96-well plates (Nunc MaxiSorp) by incubating the Sf21 cell extract (containing CCE; initial activity towards 100μΜ p-nitrophenyl acetate over 60mOD/min) with a series of temephos-oxon concentrations (0,0625-5μΜ). Remaining esterase activity, compared to control reactions lacking temephos, was tested using p-nitrophenyl acetate. The bimolecular rate constant (*ka*) was calculated according to ([Aldridge and Reiner, 1972](#_ENREF_1)). To estimate the reactivation rate (*k3*), Sf21cell extract containing recombinant CCE was incubated with temephos oxon until a 90% inhibition in esterase activity towards p-nitrophenyl acetate was observed. Unbound insecticide was removed by passing the reaction through a pre-equilibrated with 0,1M Sodium phosphate buffer pH 7, Q-sepharose column (GE Healthcare) and reactivation was measured over several hours by withdrawing aliquots from the eluted sample and testing the esterase activity towards 100μΜ p-nitrophenyl acetate. Values obtained were plotted over time in a logarithmic scale and the slope of the produced straight line gave the *k3* constant ([Aldridge and Reiner, 1972](#_ENREF_1)). For all experiments control reactions without temephos were included.

For the analysis of temephos oxon metabolites, temephos oxon (25μΜ) was incubated with Sf21 cell extract containing recombinant CCE in 0.1M Sodium phosphate buffer pH 7, final volume 1mL, at 30oC, 500rpm stirring for 30 min. Two different control reactions were included: (a) cell extracts from Sf21 cells expressing the YFP, to test for endogenous esterase activity and (b) no temephos oxon reactions.

*HPLC-MS/MS analysis of insecticide metabolism*

Prior to HPLC-MS/MS analysis, samples (500μL) were prepared and cleaned-up with liquid-liquid extraction as follows: samples were spiked with 100 μl isotopically labeled internal standard solution (d6-di-methyl-thio-phosphate and d6-di-methyl-phosphate in water; 2 mg/mL), 0,5 g NaCl were added and two extraction steps were performed with 2 mL ethyl acetate and 4 mL dichloromethane, respectively. Extracts were combined, evaporated to dryness with a rotational vacuum concentrator RVC2-25 (Martin Christ, Germany), resuspended to 1 mL 20% water in acetonitrile and transferred to HPLC autosampler vials. Sample injections (20 μL loop) were performed via a Surveyor Autosampler. Chromatographic separation was achieved using a Surveyor LC system and mass detection was accomplished with a TSQ Quantum triple quadrupole (Thermo Finnigan, USA).Chromatographic analysis of temephos, its oxygenated analogues and metabolites 1-3 was performed with a reversed phase Gemini C18 (3 μm, 100 mm × 2 mm) analytical column (Phenomenex, USA). A gradient elution was applied with acetonitrile (A) – water (B), both containing 0,1% formic acid (0-2,5 min, 60% A; 5-9 min, 100%A; 9,1-10,5 min 60% A) and flow rate was set at 200 μL/min. As ionisation source, positive electrospray (ESI+) was used and mass spectrometer parameters were set as follows: spray voltage at 5000 V, sheath gas pressure at 20 arbitrary units, auxiliary gas pressure at 10 arbitrary units, capillary temperature at 300 °C and source collision induced dissociation at -10 eV. Metabolites 4-5 (di-methyl-thio-phosphate and di-methyl-phosphate; Figure 1A) were chromatographically separated with a hydrophilic interaction (HILIC) Kinetex (2,6 μm, 150 mm × 2.1 mm) analytical column (Phenomenex, USA). An isocratic elution was applied with 7,5 mM ammonium acetate in 85% acetonitrile-15% water for 7 min and flow rate was set at 250 μL/min. As ionisation source, negative electrospray (ESI-) was used and mass spectrometer parameters were set as follows: spray voltage at -5.000 V, sheath gas pressure at 19 arbitrary units, auxiliary gas pressure at 5 arbitrary units, capillary temperature at 350°C and source collision induced dissociation at 16 eV. Mass spectrometer was operated in full scan, single ion monitoring, product ion scan and selected reaction monitoring modes. Single ion and selected reaction monitoring analyses were applied by monitoring only for the molecular ions [MH]+ of Temephos (m/z: +467), its oxygenated forms (mono and di-oxygenated, m/z +451; +435), metabolites 1-3 (m/z: +327; +343; +219 respectively) and through selected reactions for metabolites 4-5 (-125🡪-79 m/z, 39 eV and -141🡪-126 m/z, 17 eV ). The system was controlled by the Xcalibur software, also used for the data acquisition and analysis. The Sheath/auxiliary gases were high purity nitrogen and collision gas was high purity argon.

*Raising of antibodies*

The sequence of CCEae3a\_alb encoding the following peptide YETLMRASPDNLIARSEECVTDQDRAVFRIFAFTPVVEPLESDDPFITKMYLDLLSDPNMTNIPLILGLTSNEAICFIENLSMDLFANDVKMFAPPQLAVPE was cloned in a pET16b vector (Novagen) using the primers listed in Table S1. JM109 cells were transformed and expressed the recombinant peptide N-terminally 6xHis-tagged, upon addition of 0,4mM Isopropyl β-D-1-thiogalactopyranoside (IPTG). The peptide was affinity purified to homogeneity by Ni-NTA2+ chromatography and sent to  Davids Biotechnologie GmbH (Germany) where rabbit polyclonal antibodies were raised and affinity purified.

*Western blots, Immunofluorescence and confocal microscopy*

For western blots MT, heads and carcass tissues were isolated from 15-17 individuals of the Lab (Susceptible) or Tem-GR (Resistant) strains, pooled together and homogenized in 50μL ice cold 1X PBS supplemented with 1mM EDTA, 3mM PMSF and 1X protease cocktail inhibitors (Thermo scientific). Protein concentration was measured with Bradford and equal amount of protein was used for SDS polyacrylamide gel electrophoresis. Proteins were electro-blotted onto a nitrocellulose membrane, which was blocked with 5% milk for 1hour and subsequently cut into two pieces. The upper part hybridized overnight at 4oC with 1/5.000 CCEae3a antibody (Davids Biotechnology) and the lower part with 1/2.500 anti-actin (A5060 SIGMA, Sigma-Aldrich), which served as a control for equal loading. Antibody binding was detected with 1/10.000 goat anti-rabbit IgG coupled to horse-radish peroxidase (Invitrogen).

For immunohistochemical experiments on paraffin sections late third to early fourth instar larvae were fixed for 24h, 4oC, in 1X Phosphate-buffered saline (PBS) containing 4% methanol free formaldehyde (Thermo Scientific). Samples were subsequently washed 3 times, 5min each with 1X PBS containing 0,1% Triton X-100 then washed 2 times 30min each with 0,8% NaCl and dehydrated (2 washes 30min each with 70% ethanol, 2 washes 30min each with 96% ethanol, 2 washes 20min each with 100% ethanol, 2 washes 1hour each with chloroform). Larvae were embedded in paraffin (Tyco Health care) and 10μm sections were obtained on SuperFrost Plus slides (O.Kindler GmbH). Samples were de-paraffinised as follows: 2 washes 10 min each with xylene, 1 wash 5min with ethanol 100%, 1 wash 5min with ethanol 96%, 1 wash 5min 70% ethanol, 2 washes 5min each with water, 1 wash 10min with1X PBS containing 0,1% Triton X-100. Slides were boiled for 5min in a solution of 23,5% citric acid and 5% sodium citrate and stored at 4oC before further use. Staining of the samples included washing of the slides three times 5min each with 0,1% Tween in PBS, blocking for 1 h in blocking solution (1% Fetal Bovine Serum, biosera, in 0,1% Triton X-100) and incubation with the rabbit anti-CCEae3a in a dilution of 1/500 over night at 4oC. The next day slides were washed and incubated with the secondary antibody (anti-rabbit Alexa Fluor 488, Molecular Probes) at a dilution of 1/1.000 for 1h in the dark. Incubation with To-PRO 3-Iodide (Molecular Probes) at dilution 1/1.000 for 5min was also done after treatment with RNAse A. Images were obtained on a confocal microscope SP1 LEICA, using the 20x or 40x-objective.

For staining of whole mounts, late third to early fourth instar larvae were dissected. Malpigian tubules, heads and carcasses were cut lateral or at the dorsal site and fixed for 30min using 4% formaldehyde (methanol free, Thermo scientific) in 1X phosphate-buffered saline (PBS), supplemented with 2 mM MgSO4 and 1 mM EGTA. After fixation samples were washed for 5 min with PBS, followed by a wash with methanol, strictly for 2 min. Subsequently tissues were washed three times, 5min each with PBS and then blocked for 1h in blocking solution (1% BSA, 0.1 % Triton X-100 in PBS). Incubation of samples with anti-CCEae3a at a dilution of 1/2.500 was performed over night. The next day tissues were washed and incubated with the secondary antibody (anti-rabbit Alexa Fluor 488, Molecular Probes) at a dilution of 1/1.000 for 2h in the dark. Finally tissues were stained with To-PRO 3-Iodide (Molecular Probes) at dilution 1/1.000 for 5min and observed on a fluorescence stereoscope (Leica M250 FA).

**Results**

*Production of catalytically active CCEs*

Expression of CCEae3a\_alb, as well as of both *Ae. aegypti* variants (CCEae3a\_aegR and CCEae3a\_aegS) in baculovirus infected SF21 insect cells was evaluated by western blots using α-Myc antibodies which confirmed products at the expected size (Figure S1). All three enzyme variants were highly active towards the substrate p-nitrophenyl butyrate, but considerable activity was also measured for the substrates p-nitrophenyl acetate, α-naphthyl acetate and β-naphthyl acetate (Table 1). Relatively small differences observed in the activities of the three recombinant CCEae3a variants (Table 1)might be associated with their expression efficiency and not their actual specific activity, as they were not used as purified enzymes. The use of p-nitrophenyl butyrate in detecting *Ae. albopictus* larvae over-expressing CCEae3a was subsequently tested using larvae homogenates from resistant to temephos (Tem-GR) and susceptible (Lab) larvae: TemR larvae showed an activity of 1±0,025 μmol/min/mgr (n=10), five times higher than the activity measured for the susceptible Lab colony (0,2±0,018 μmol/min/mgr, n=10).

*Inhibition kinetics of recombinant CCEae3as with temephos oxon*

In order to examine the interaction of CCEae3as with temephos we first converted it to its oxon form, as this is the actual toxic product *in vivo*. The oxidation of temephos was validated by HPLC-MS analysis (Figure S2). An aliquot of the oxidized mixture (the same mixture was used subsequently for the metabolism assays) was analyzed in full scan mode. Despite the fact that absolute quantification of the oxidized products is not possible without standard compounds, the peak areas of the three forms of temephos indicate that ~80% is in its di-oxidized (di-oxygenated) form, ~20% is in its mono-oxidized (mono-oxygenated) form and the residual temephos is <1%.

Subsequently the bimolecular rate constant, *ka* and the dissociation constant, *k3* were estimated (Table 2): *ka* for CCEae3a-alb was 0,76(±0,085)x105 M-1 min-1; *ka* for CCEae3a-aegR was 0,95(±0,24)x105 M-1 min-1 and *ka* for the CCEae3a-aegS 1,6 (±0,23)x105 M-1 min-1. The *k3* for CCEae3a-alb was 18x10-4(min-1), for CCEae3a-aegR 3x10-4(min-1) and for CCEae3a-aegS 5x10-4(min-1). Thus, all three recombinant proteins had a strong binding affinity for temephos oxon, and slow reactivation rates, in line with previously characterized CCEs in other insects (Table 2). The affinity for temephos oxon indicated by the ka of the CCEae3a\_aegR was not higher than the one indicated by the the respective ka of the CCEae3a\_aegS recombinant proteins.

*Temephos oxon metabolism by recombinant CCEs*

HPLC/MS analysis showed that CCEae3as are able to metabolize temephos oxon. Among possible metabolites (Figure 1A), the ion chromatogram of +343 m/z revealed a peak at 1,5 min (Figure 1B, up) in reactions containing CCEae3as, compared to control reactions. In addition, no peak was observed in control reactions lacking temephos-oxon, confirming that the produced metabolite represents a part of the insecticide. Subsequently, we proceeded in characterizing this metabolite with MS/MS analysis. Product ion scan mode, 55 eV showed three peaks at 202.9, 278.6 and 325.8 m/z (Figure 1B, down) which correspond to the proposed structure of [(4-hydroxyphenyl)sulfanyl]phenyl O,O-dimethyl phosphorothioate (“metabolite 2”) (Figure 1A).

*Tissue localization of* CCEae3a

The tissue localization of CCEae3a\_alb was initially investigated through western blots using homogenates of dissected tissues from both resistant and susceptible *Ae. albopictus* larvae. Results revealed the presence of CCEae3a\_alb in the head, carcass and MT, as well as showed an over-production of this esterase in tissues of the resistant strain (Figure 2).Midguts in western blots showed a very low signal.

For a more detailed analysis of CCEae3a tissue localization we conducted immunohistochemical experiments, where whole tissues (Figure 3A) and paraffin sections (Figure 3B) of *Ae. albopictus* larvae were stained with the specific CCEae3a antibody. Both approaches showed expression of this esterase in MT and the nerve cord. Although no staining could be observed in the head under experimental conditions, the specific and intense signal obtained in the western blots (Figure 2) further supports localization of CCEae3a\_alb in the central nervous system. Both whole mounts and paraffin sections experiments did not show any signal in the midgut or the fat body.

**Discussion**

We expressed the *Ae.aegypti* and *Ae.albopictus* CCEae3a carboxylesterases, which have been strongly associated with temephos resistance in these major arbovirus vectors. CCEae3as showed strong affinity for temephos oxon, with *ka* constants similar to those previously determined for other mosquito esterase isoenzymes, like the *Culex quinquefasciatus* B2 esterase with malaoxon ([Karunaratne et al., 1993](#_ENREF_15)), but lower than the *ka* estimated in other cases, like the *Myzus persicae* E4 esterase with paraoxon ([Devonshire, 1977](#_ENREF_3)) (Table 2). A slow turnover in the interaction of CCEae3as with temephos-oxon was shown from the estimated deacylation rate constants, which are also in the same range with those estimated for the interaction of *Culex quinquefasciatus* B2 esterase and the CtrEstβ1 from *Cx. tritaeniorhynchus* with a range of OPs (Table 2) ([Karunaratne et al., 1993](#_ENREF_15)) ([Karunaratne and Hemingway, 2000](#_ENREF_14)). No major difference was observed in the comparison of the kinetic constants of CCEae3a\_aegS and CCEae3a\_aegR, indicating that the amino acid differences of those enzyme variants seems unlikely to play a role in temephos sequestration/resistance, as previously hypothesized ([Poupardin et al., 2014](#_ENREF_23)).. Although a detailed kinetic interaction of temephos oxon with the *Aedes* Acetylcholinestares (AChE), the molecular target of OPs, has not been determined and thus a direct comparison between the relative affinities of CCEs *vs* AChE for the temephos oxon is not possible, our results show that *Aedes* CCEae3a has a strong affinity to temephos-oxon. Thus it might be possible that they bind temephos oxon quicker than the AChE thereby protecting the molecular insecticide target and conferring resistance, in line to previous studies ([Chen et al., 2001](#_ENREF_2)). The very slow turn-over (*k3*), is typical for esterases playing a role in insecticide resistance via sequestration ([Karunaratne et al., 1993](#_ENREF_15)).

Subsequent HPLC/MS analysis showed that CCEae3as were capable to metabolize temephos oxon to its temephos monoester (“metabolite 2”). As CCEae3a seems to attack the oxygenated phosphoester bond of temephos we would expect to find also metabolite 1, (4-[(4-hydroxyphenyl)sulfanyl]phenyl dimethyl phosphate) (Figure 1A). The reason we did not detect this metabolite might indicate a preference of CCEae3as for the mono-oxygenated form of temephos oxon (possibly produced by the *in vivo* temephos oxidation). Regarding the toxicity of the produced metabolite, as the remaining phosphoester bond is of the “ thion’’ form and not the “oxon” form it will probably be less toxic, although although actual toxicity *in vivo* tests have not been performed.

We subsequently raised a specific antibody for the CCEae3a\_alb, which was used in western blots revealing a significant difference in the expression levels between resistant to temephos and susceptible *Ae. albopictus* larvae. This antibody could be used in developing ELISA or field applicable immune-strip diagnostic methods to detect larvae over-expressing CCEae3a and thereby resistant to temephos, as developed previously in *Myzus persicae* (detection of E4-mediated OP resistance ([Devonshire et al., 1986](#_ENREF_6)) and *Bemisia tabaci* (detection of CYP6CM1-mediated neonicotinoid resistance ([Nauen et al., 2015](#_ENREF_20)).

The antibody was subsequently used to investigate the tissue localization of the CCEae3a\_alb in *Ae.albopictus* larvae. Western blot analysis indicated its expression in MT, the carcass and the head. Immunohistochemical experiments showed expression in MT and the nerve cord. Thus, CCEae3as might be capable to provide protection right at the target tissue, sequestering the insecticide molecules and keeping them away from the AChE. Additionally, it could reduce the overall amount of temephos oxon reaching the central nervous system by sequestering insecticide molecules in MT, an organ of insects with excretory function. Our results are in agreement with a study in *Culex* larvae where esterases associated with organophosphate resistance have also been found in MT and the nervous system ([Pasteur et al., 2001](#_ENREF_22)). MT have been associated with detoxification of pyrethroid insecticides in *An. gambiae* adult mosquitoes, as cytochrome P450s associated with pyrethroid resistance have been found expressed in this tissue ([Ingham et al., 2014](#_ENREF_13)). However, the expression of CYP6BQ9, a P450 shown to metabolize deltamethrin in *T. castaneum* was detected primarily in the head ([Zhu et al., 2010](#_ENREF_31)). Further work is needed to address the tissue localization of detoxification enzymes in mosquitoes, which can improve our understanding of the insecticide resistance phenomenon and the physiological barriers which influence insecticide toxicity.

In conclusion, we showed that the *Aedes* carboxylesterases CCEae3as previously associated with temephos resistance are primarily expressed in MT and nervous system and encode proteins that strongly interact and metabolize temephos oxon, and thus it is likely that they confer resistance via sequestration.

**Acknowledgements:**

We thank Prof Kostas Iatrou (NCSR “Demokritos”) for kindly providing the expression vectors used in this study. We also thank Prof Evripidis Stefanou (University of Crete) for his help with the HPLC/MS, Dr Rodolphe Poupardin (University of Veterinary Medicine, Austria) for providing his help in CCE cloning, and Anastasia Kampouraki, Stella Kounadi and Dimitra Tsakirelli (University of Crete) for their help with the immunolocalisation and protein work. The research received funding from the European Union FP7 (2013) under grant agreement no 228421 to INFRAVEC (<http://www.infravec.eu/>) and a bilateral Greece–France Cooperation Program (no 1641) (www.gsrt.gr).

Aldridge, W.N., Reiner, E., 1972. Enzyme Inhibitors as Substrates:

Frontiers of Biology (Neuberger, A. & Tatum, E. L., eds.), vol. 26,

North-Holland, Amsterdam.

Chen, Z.Z., Newcomb, R., Forbes, E., McKenzie, J., Batterham, P., 2001. The acetylcholinesterase gene and organophosphorus resistance in the Australian sheep blowfly, Lucilia cuprina. Insect Biochem Molec 31, 805-816.

Devonshire, A.L., 1977. Properties of a Carboxylesterase from Peach-Potato Aphid, Myzus-Persicae-(Sulz), and Its Role in Conferring Insecticide Resistance. Biochem J 167, 675-683.

Devonshire, A.L., Heidari, R., Bell, K.L., Campbell, P.M., Campbell, B.E., Odgers, W.A., Oakeshott, J.G., Russell, R.J., 2003. Kinetic efficiency of mutant carboxylesterases implicated in organophosphate insecticide resistance. Pestic Biochem Phys 76, 1-13.

Devonshire, A.L., Heidari, R., Huang, H.Z., Hammock, B.D., Russell, R.J., Oakeshott, J.G., 2007. Hydrolysis of individual isomers of fluorogenic pyrethroid analogs by mutant carboxylesterases from Lucilia cuprina. Insect Biochem Mol Biol 37, 891-902.

Devonshire, A.L., Moores, G.D., Ffrench-Constant, R.H., 1986. Detection of insecticide resistance by immunological estimation of carboxylesterase activity in Myzus persicae (Sulzer) and cross reaction of the antiserum with Phorodon humuli (Schrank) (Hemiptera: Aphididae). Bulletin of Entomological Research 76, 97-107.

Douris, V., Swevers, L., Labropoulou, V., Andronopoulou, E., Georgoussi, Z., Iatrou, K., 2006. Stably transformed insect cell lines: Tools for expression of secreted and membrane-anchored proteins and high-throughput screening platforms for drug and insecticide discovery. Adv Virus Res 68, 113-+.

Faucon, F., Dusfour, I., Gaude, T., Navratil, V., Boyer, F., Chandre, F., Sirisopa, P., Thanispong, K., Juntarajumnong, W., Poupardin, R., Chareonviriyaphap, T., Girod, R., Corbel, V., Reynaud, S., David, J.P., 2015. Identifying genomic changes associated with insecticide resistance in the dengue mosquito Aedes aegypti by deep targeted sequencing. Genome Res 25, 1347-1359.

Grigoraki, L., Lagnel, J., Kioulos, I., Kampouraki, A., Morou, E., Labbe, P., Weill, M., Vontas, J., 2015. Transcriptome Profiling and Genetic Study Reveal Amplified Carboxylesterase Genes Implicated in Temephos Resistance, in the Asian Tiger Mosquito Aedes albopictus. PLoS Negl Trop Dis 9, e0003771.

Grisales, N., Poupardin, R., Gomez, S., Fonseca-Gonzalez, I., Ranson, H., Lenhart, A., 2013. Temephos resistance in Aedes aegypti in Colombia compromises dengue vector control. PLoS Negl Trop Dis 7, e2438.

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

Hemingway, J., Hawkes, N.J., McCarroll, L., Ranson, H., 2004. The molecular basis of insecticide resistance in mosquitoes. Insect Biochem Molec 34, 653-665.

Ingham, V.A., Jones, C.M., Pignatelli, P., Balabanidou, V., Vontas, J., Wagstaff, S.C., Moore, J.D., Ranson, H., 2014. Dissecting the organ specificity of insecticide resistance candidate genes in Anopheles gambiae: known and novel candidate genes. Bmc Genomics 15.

Karunaratne, S.H.P.P., Hemingway, J., 2000. Insecticide resistance spectra and resistance mechanisms in populations of Japanese encephalitis vector mosquitoes, Culex tritaeniorhynchus and Cx. gelidus, in Sri Lanka. Med Vet Entomol 14, 430-436.

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.

Ketterman, A.J., Jayawardena, K.G.I., Hemingway, J., 1992. Purification and Characterization of a Carboxylesterase Involved in Insecticide Resistance from the Mosquito Culex-Quinquefasciatus. Biochem J 287, 355-360.

Kraemer, M.U., Sinka, M.E., Duda, K.A., Mylne, A.Q., Shearer, F.M., Barker, C.M., Moore, C.G., Carvalho, R.G., Coelho, G.E., Bortel, W.V., Hendrickx, G., Schaffner, F., Elyazar, I.R., Teng, H.-J., Brady , O.J., Messina, J.P., Pigott, D.M., Scott, T.W., Smith, D.L., Wint, G.W., Golding, N., Hay, S.I., 2015. The global distribution of the arbovirus vectors Aedes aegypti and Ae. albopictus. eLife.

Lima, J., Da-Cunha, M., Da Silva, R., Galardo, A., Soares Sda, S., Braga, I., RP, R., D, V., 2003. Resistance of Aedes aegypti to organophosphates in several municipalities in the State of Rio de Janeiro and Espírito Santo, Brazil. The American journal of tropical medicine and hygiene 68, 329-333.

McCarroll, L., Hemingway, J., 2002. Can insecticide resistance status affect parasite transmission in mosquitoes? Insect Biochem Molec 32, 1345-1351.

Nauen, R., Wolfel, K., Lueke, B., Myridakis, A., Tsakireli, D., Roditakis, E., Tsagkarakou, A., Stephanou, E., Vontas, J., 2015. Development of a lateral flow test to detect metabolic resistance in Bemisia tabaci mediated by CYP6CM1, a cytochrome P450 with broad spectrum catalytic efficiency. Pestic Biochem Phys 121, 3-11.

Newcomb, R.D., Campbell, P.M., Russell, R.J., Oakeshott, J.G., 1997. cDNA cloning, baculovirus-expression and kinetic properties of the esterase, E3, involved in organophosphorus resistance in Lucilia cuprina. Insect Biochem Molec 27, 15-25.

Pasteur, N., Nance, E., Bons, N., 2001. Tissue localization of overproduced esterases in the mosquito Culex pipiens (Diptera : Culicidae). J Med Entomol 38, 791-801.

Poupardin, R., Srisukontarat, W., Yunta, C., Ranson, H., 2014. Identification of Carboxylesterase Genes Implicated in Temephos Resistance in the Dengue Vector Aedes Aegypti. Plos Neglect Trop D 8.

Ranson, H., Burhani, J., Lumjuan, N., Black, W., 2010. Insecticide resistance in dengue vectors. TropIKA.net.

Teese, M.G., Farnsworth, C.A., Li, Y., Coppin, C.W., Devonshire, A.L., Scott, C., East, P., Russell, R.J., Oakeshott, J.G., 2013. Heterologous expression and biochemical characterisation of fourteen esterases from Helicoverpa armigera. Plos One 8, e65951.

Van Leeuwen, T., Van Pottelberge, S., Tirry, L., 2005. Comparative acaricide susceptibility and detoxifying enzyme activities in field-collected resistant and susceptible strains of Tetranychus urticae. Pest Manag Sci 61, 499-507.

Vontas, J., Kioulos, E., Pavlidi, N., Morou, E., della Torre, A., Ranson, H., 2012. Insecticide resistance in the major dengue vectors Aedes albopictus and Aedes aegypti. Pestic Biochem Phys 104, 126-131.

WHO, 1998. Techniques to detect insecticide resistance mechanisms (field and laboratory manual). WHO reference number: WHO/CDS/CPC/MAL/98.6.

WHO, 2009. Dengue: guidelines for diagnosis, treatment, prevention and control.

WHO, 2012. Dengue and severe dengue in: WHO (Ed.), World Health Organization, Geneva.

Zhu, F., Parthasarathy, R., Bai, H., Woithe, K., Kaussmann, M., Nauen, R., Harrison, D.A., Palli, S.R., 2010. A brain-specific cytochrome P450 responsible for the majority of deltamethrin resistance in the QTC279 strain of Tribolium castaneum. P Natl Acad Sci USA 107, 8557-8562.

**FIGURE LEGENTS**

**Figure 1: HPLC/MS analysis of the CCEae3a interaction with temephos-oxon**. A) Chemical structure of temephos, its oxygenated forms and of all possible metabolites. B) UP: HPLC Ion Chromatogram of +343 m/z revealing a peak at 1,5 min in reactions containing recombinant CCEae3a esterases. DOWN: Electrospray ionization mass spectrum of the identified temephos oxon metabolite.

**Figure 2: Western blot analysis of CCEae3a in *Ae. albopictus* larval tissues.** Homogenates from dissected malpigian tubules (A), carcasses (B) and heads (C) from resistant (R) and susceptible (S) *Ae. albopictus* larvae separated on a 10% SDS acrylamide gel and immunoblotted with α-CCEae3a  and α-actin,  serving as a loading control. Sf21 cell extracts expressing recombinant CCEae3a (Bacul +) or Yellow fluorescent protein (Bacul -) were included to test for signal specificity.

**Figure 3: Immunolocalization of CCEae3a in *Ae.albopictus* larvae.** A) Whole mount staining of malpigian tubules and abdominal carcasses of 4th instar resistant *Ae. albopictus* larvae. Left panel shows the tissues in bright field depiction, middle panel shows staining of nuclei with TOPRO (red color) and right panel shows staining of tissues with α-CCEae3a. Pictures were obtained using fluorescent stereoscope. B) Staining of paraffin sections of 4th instar resistant *Ae. albopictus* larvae. Left panel shows sections stained with α-CCEae3a (green color), middle panel shows sections stained with TOPRO (red) and right panel represents the merge of the other two panels. Scale bar: 10μm. Pictures were obtained using confocal microscopy.

**Figure S1: Expression of recombinant CCEae3a, using the baculovirus system.**

Expression of CCEae3a esterases in infected Sf21 cells (+CCEae3a) was tested through western blots using cell extracts and a Myc antibody.Sf21 cells infected with baculovirus expressing the YFP (-CCEae3a) were used as control.

**Figure S2: HPLC-MS analysis of oxidized temephos mixture**. Molecular ion chromatograms of Temephos (+466 m/z), mono-oxygenated Temephos (+450 m/z) and di-oxygenated Temephos (+434 m/z). PA, peak area; RT, retention time.