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Epoxide hydrolase activities and epoxy fatty acids in the mosquito *Culex quinquefasciatus*

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Abstract

Culex mosquitoes have emerged as important model organisms for mosquito biology, and are disease vectors for multiple mosquito-borne pathogens, including West Nile virus. We characterized epoxide hydrolase activities in the mosquito *Culex quinquefasciatus*, which suggested multiple forms of epoxide hydrolases were present. We found EH activities on epoxy eicosatrienoic acids (EETs). EETs and other eicosanoids are well-established lipid signaling molecules in vertebrates. We showed EETs can be synthesized *in vitro* from arachidonic acids by mosquito lysate, and EETs were also detected *in vivo* both in larvae and adult mosquitoes by LC-MS/MS. The EH activities on EETs can be induced by blood feeding, and the highest activity was observed in the midgut of female mosquitoes. The enzyme activities on EETs can be inhibited by urea-based inhibitors designed for mammalian soluble epoxide hydrolases (sEH). The sEH inhibitors have been shown to play diverse biological roles in mammalian systems, and they can be useful tools to study the function of EETs in mosquitoes. Besides juvenile hormone metabolism and detoxification, insect epoxide hydrolases may also play a role in regulating lipid signaling molecules, such as EETs and other epoxy fatty acids, synthesized *in vivo* or obtained from blood feeding by female mosquitoes.

1. Introduction

Epoxide hydrolases (EHs) are enzymes that convert a variety of epoxides into their corresponding diols (Morisseau and Hammock, 2005). In insects, epoxide hydrolases are mainly studied as detoxification enzymes (Dauterman, 1982; Mullin, 1988; Taniai et al., 2003), and enzymes that are involved in the metabolism of juvenile hormones (Anspaugh and Roe, 2005; Casas et al., 1991; Keiser et al., 2002; Khalil et al., 2006; Seino et al., 2010; Severson et al., 2002; Tsubota et al., 2010; Zhang et al., 2005). It is not known whether insect epoxide hydrolases play other important roles in insect physiology, and what other substrates can be involved.

In mammals, epoxides of fatty acids such as epoxyeicosatrienoic acids (EETs) are a group of eicosanoids that are lipid signaling molecules. EETs are derived from arachidonic acids, and are mainly hydrolyzed by the soluble epoxide hydrolase (Yu et al., 2000; Zeldin et al., 1993). Inhibition of soluble epoxide hydrolase revealed therapeutic effects in several mammalian models, indicating EETs are biologically functional (Morisseau and Hammock, 2013). In invertebrates including insects, eicosanoids are also known to play physiological roles such as ion transport, immunity, reproduction and host-vector interactions, although most studies had focused on prostaglandins (Stanley, 2006; Stanley and Kim, 2014; Stanley and Miller, 2006). It remains unknown whether insects produce EETs that are metabolized by epoxide hydrolases, and what the biological roles are.

Culex mosquitoes are widely distributed around the world, both in tropical and subtropical areas (Diaz-Badillo et al., 2011). They feed on a variety of hosts and are vectors of many important mosquito-borne diseases, such as West Nile virus (Bartholomay et al., 2010). Mosquitoes need arachidonic acids as the essential fatty acids, and replacement of arachidonic acids with prostaglandins cannot rescue the mosquitoes, indicating other metabolites of arachidonic acids may be important (Dadd, 1980; Dadd and Kleinjan, 1984). Mosquitoes may oxidize arachidonic acids to form EETs by monooxygenases, such as the cytochrome P450 in mammals (Capdevila et al., 1992; Zeldin, 2001), and female mosquitos will also ingest xenobiotic EETs during the process of blood feeding, because EETs and other epoxy fatty acids are regular components in the blood (Jiang et al., 2012; Jiang et al., 2005). Many blood-derived molecules have been found and studied. When ingested by mosquitoes, some are still relatively stable, and can affect mosquitoes' capacity as disease vectors (Pakpour et al., 2013). As a result, EETs potentially may be among these molecules that have impacts on mosquito physiology and host-vector interactions.

Here we characterized the EH activities in the mosquito *Culex quinquefasciatus*, and demonstrated epoxy fatty acids are endogenous substrates. We showed that mosquitoes can produce epoxy fatty acids *in vivo*, and female mosquitoes will obtain xenobiotic epoxy fatty acids during blood feeding. The highest EH activities on EETs in adults were found in female midgut, which is the center of many physiological processes, including detoxification, digestion and molecular signaling. We also found urea-based sEH inhibitors can inhibit EH activities on EETs in mosquitoes. These inhibitors have been demonstrated to play diverse biological roles in mammalian systems (Shen and Hammock, 2011), which may be useful tools to evaluate epoxy fatty acid functions in mosquitoes.

2. Materials and Methods

2.1. Mosquito rearing

The mosquito *C. quinquefasciatus* were reared in an insectary incubator at a constant temperature of $28 \pm 1°C$ and $80 \pm 5\%$ relative humidity. Eggs were hatched in plastic water cups, and larvae were fed twice a day with grounded fish food (TetraMin, Germany) and cat food (Purina, MO) until pupation. Emerged adults were transferred to mosquito cages (30 cm × 30 cm ×30 cm) and fed 10% sucrose *ad libitum* soaked in cotton balls daily. Three or four days after eclosion, mosquitoes were fed with defribrinated sheep blood (Quad Five, MT) at 37°C for 30 minutes. Parafilm® M (Sigma-Aldrich, MO) was used as the artificial

membrane for blood feeding. After blood feeding, mosquitoes were provided with 10% sucrose daily, and water cups were provided for egg laying two days after blood feeding.

2.2. Enzyme preparation

4th instar larvae (8–9 days old after hatch) and adult mosquitoes (4–7 days female after eclosion) were homogenized by ceramic pestle and mortar in cold homogenization buffer (pH 8, 50 mM Tris-HCl buffer containing 1 mM phenylmethylsulfonyl fluoride and 1mM ethylenediaminetetraacetic acids). Because we were specifically interested in the EH activities in female mosquitoes, only female adults were selected. The whole mosquito extract was subjected to 100×g centrifugation for 5 minutes to remove debris. The supernatant was collected as the crude lysate. The mitochondria fraction was obtained by centrifuging the lysate at 18,000×g for 20 minutes, and the resulting pellets were resuspended in 50 mM, pH 8 Tris-HCl buffer. The resulting supernatant was centrifuged again at 100,000×g for 1 hour. The supernatant was collected as the cytosolic fraction, and the pellet was resuspended in Tris-HCl buffer as the microsomal fraction. The pellets in each step were washed once by homogenization buffer before any further processing. All differential centrifugations were processed at 4 °C. Protein concentration was measured by BCA assay (Pierce, Rockford, IL) with BSA (Pierce, Rockford, IL) as the standard throughout this study.

2.3. Measurement of epoxide hydrolase activity

Epoxide hydrolase activities on *c*-SO, *t*-SO, *t*-DPPO, *c*-DPPO, JH III, 14,15-EET and 9,10-EpOME were measured as previously described (Morisseau, 2007). To inhibit GST activity, diethyl maleate was added to the buffer at 1 mM final concentration. In the assays on JH III, 1 μl of 1 mM OTFP (3-octylthio-1, 1, 1-trifluoropropan-2-one) in ethanol was added into 100 μl enzyme solution (10 μM final concentration of OTFP) to inhibit JH esterase activity (Abdel-Aal and Hammock, 1985). Assays were done in triplicate, and all enzyme activities were corrected for non-enzymatic hydration. If inhibitors were added, different concentrations of inhibitors in DMSO were prepared, and 1 μl of inhibitor solution was added into 100 μl of lysate. 1 μl of DMSO was added in control assays although inhibition by DMSO was not observed at 1% (v:v) concentration.

2.4. Separation of epoxide hydrolase activities by ion exchange chromatography

A preliminary small-scale batch adsorption was used to define the loading and eluting conditions. DEAE Sepharose ion exchanger (1 ml) (GE Healthcare, UK) was added into a 10 ×75 mm borosilicate glass tube. The DEAE ion exchanger was equilibrated with pH 8, 20 mM, Tris-HCl containing 0.02% CHAPS by washing the gel five times with 10 ml of buffer. A known amount of enzyme solution solubilized by 0.02% CHAPS was added to the pre-equilibrated exchanger. The solution and gel was gently mixed, and the gel was allowed to settle. Proteins were eluted by step-wise elution by adding 1ml of pH 8, 20 mM Tris-HCl buffer containing increasing concentration of NaCl, ranging from 0.05M, 0.1M, 0.2M, 0.5M and 1.0M NaCl. Every fraction was collected, and assayed for epoxide hydrolase activities with *t*-DPPO, JH III and 14,15-EET as the substrates. To achieve better separation, a column and gradient elution was used. A batch of DEAE ion exchanger was equilibrated as

previously described, and a known amount of enzyme solution was added into the exchanger. The whole solution was poured into the column. The outlet valve was opened to allow the gel to pack. The column was washed by 5 column volumes of pH 8, 20 mM Tris-HCl buffer containing 0.02% CHAPS to elute unbound materials. Bound proteins were eluted by 30 column volumes of NaCl gradient (200 mM to 1M). At last 5 column volumes of 1M NaCl was used to elute any remaining materials on the column. Every 10 ml elutant was collected for enzyme activity assays.

2.5. Synthesis of EETs by mosquito crude lysate

The mosquito 4th instar (8–9 days old after hatch) crude lysate (2 mg/mL) may be purged with CO for 2 minutes (one bubble per second) or added EH inhibitor AUDA to a final concentration of 1 μM. Then 1 μl of 10 mM arachidonic acid in ethanol (100 μM final concentration) was added into 95 μl mosquito crude lysate (2 mg/mL) in pH 8, 50 mM Tris-HCl buffer with or without premixed 5 μl of NADPH generating system (100 mM glucose-6-phosphate, 20 mM $NADP^+$, 20 units glucose-6-phosphate dehydrogenase). Borosilicate culture tubes (ThermoFisher Scientific, MA) were used for the assay. The resulting solution was incubated at 30 °C in a shaking water bath, and the reaction was stopped by adding 400 μl methanol at 5, 10, 20 and 30 minutes respectively. CUDA (1 μl of 100 μM in methanol) was added into the solution as the internal standard (200 nM CUDA in a total volume of 500 μl solution). The tubes were vortexed for 5 seconds, and centrifuged at 2,500×g for 5 minutes. The supernatant was collected for LC-MS/MS analysis.

2.6. Mosquito dissection

Adult mosquitoes (4–7 days after eclosion) were anesthetized in a 4°C freezer, and transferred to a Petri dish on ice to keep immobilized. Mosquito legs were pulled off by hand. A drop of cold phosphate buffered saline was placed onto a glass slide mounted under the microscope, and mosquitoes were transferred to the slide. To get midguts and Malpighian tubules, a needle-tip probe was used to hold down the thorax, and a fine-tipped forcep was used to grasp the end of abdomen to gently pull off the abdomen in a slow motion. The midgut remained intact and attached to the thorax segment. The Malpighian tubules are long and slender tubules that are located between midgut and hindgut. To obtain salivary glands, a probe was used to hold down the thorax, and another probe was used to gently push down the anterior part of thorax, where the salivary gland is located. The dissected mosquito tissues were homogenized by a Pyrex® 1 ml glass tissue grinder (Sigma-Aldrich, MO) in pH 8, 50 mM Tris-HCl buffer.

2.7. Sampling, lipid extraction and LC-MS/MS

Mosquito 4th instar larvae (8–9 days old after hatch) or 4–7 days old adults (female only) were weighed and put in a 1.5 mL eppendorf tube. 10 μl of anti-oxidant solution (0.2 mg/ml of butylated hydroxytoluene and EDTA) and 10 μl of deuterated standards were added. 400 μl of ice-cold methanol with 0.1% of acetic acid and 0.1% of butylated hydroxytoluene were also added. Samples were stored in a −80°C freezer for 30 minutes. After freezing, samples were homogenized with a plastic pestle and stored in a -80°C freezer overnight. The next day the samples were centrifuged at 10,000×g for 10 minutes. The supernatant was collected, and the remaining pellets were washed with 100 μl of ice-cold methanol with

0.1% of acetic acid and 0.1% of butylated hydroxytoluene. The tubes were centrifuged again. The supernatant was combined and diluted with 1.5 ml of water. The resulting solution was loaded onto 60 mg Oasis HLB cartridges (Waters, MA).

The solid phase extraction (SPE) and LC-MS/MS analysis was followed as previously described (Yang et al., 2009). The eluted samples from SPE were evaporated by vacuum (SpeedVac) and reconstituted with 50 μl of 200 nM CUDA in methanol, which was used as the internal standard. Agilent 1200 SL liquid chromatography series (Agilent Corporation, CA) was used for separation with an Agilent Eclipse Plus C-18 reversed-phase column (2.1 × 150 mm, 1.8 μM particle size). Water with 0.1% glacial acetic acid was used as mobile phase A. Acetonitrile : methanol (84:16) with 0.1% glacial acetic acids was used as mobile phase B. The detection was carried out by monitoring the selected-reaction transitions using a 4000 QTrap tandem mass spectrometer (Applied Biosystems Instrument Corporation, CA) equipped with an electrospray source (Turbo V®). More detailed information of oxylipin analysis can be found somewhere else (Yang et al., 2009).

3. Results and Discussion

3.1. Biochemical characterization of EH activities

Among the substrates evaluated in the study, *c*-SO, *t*-SO, *t*-DPPO and *c*-DPPO are tritium-labeled compounds, which were surrogate substrates for EH activities. JH III is an endogenous substrate for EHs in insects. 14,15-EET and 9,10-EpOME are epoxides of arachidonic acid and linoleic acid respectively. The structure of the substrates is shown in Table 1. No EH activity on *c*-SO, *t*-SO and *c*-DPPO was found in 4th instar larvae (8–9 days old) (Table 1), while activities on JH III, 14,15-EET and 9,10-EpOME were detected. EH activity on *t*-DPPO was only detected in 4th instar larvae (8–9 days old after hatch), not in pupa (10–12 days old after hatch) and female adults (4–7 days old after eclosion) (Fig. 1). Activities on JH III, 14,15-EET and 9,10-EpOME were detected in larvae, pupa and adults (Fig. 1). The highest specific activity was found in the 4th instar larvae (Fig. 1), although the physiological significance of the difference is not known. In lysate of the 4th instar larvae, different levels of EH activities on four substrates were found in mitochondrial, cytosolic and microsomal fractions (Fig. 2), indicating distribution of EHs in multiple subcellular fractions. Solubilized EH activities on *t*-DPPO, JH III and 14,15-EET can all bind to the DEAE-Sepharose (GE Healthcare, UK) equilibrated by pH 8, 20 mM Tris-HCl buffer, and the activities were all eluted by 0.5M and 1.0M NaCl gradient by step elution (Fig. S1). When a NaCl gradient was used for elution, EH activities with different chromatographic characteristics were detected (Fig .3). The balance sheet for the purification is shown in Table S1. The activities on *t*-DPPO, JH III and 14,15-EET overlapped, but the peak for each substrate did not. Activities on four substrates in different developmental stages, subcellular locations and separation by ion exchange chromatography suggest the presence of multiple forms of epoxide hydrolases. They may have overlapping and complementary substrate selectivity, while sharing the same subcellular location.

Recently we have expressed and characterized an epoxide hydrolase from *Anopheles gambiae* in insect cells (AgEH) that has a high activity on epoxy fatty acids (Xu et al., 2014). When the protein sequence of AgEH was used as a query for blast in the genome of

C. quinquefasciatus, we found five protein coding sequences with epoxide hydrolase signature elements (Arand et al., 1996; Hopmann and Himo, 2006; Morisseau and Hammock, 2005). We included the five sequences in a phylogeny analysis with reported mammalian and insect epoxide hydrolases. In the phylogeny analysis (Fig. 4), the five sequences were evolutionarily more close to mammalian EH 3, EH 4 and sEHs, a family of EHs that are involved in a variety of mammalian physiology by regulating the metabolism of epoxy fatty acids (Decker et al., 2012; Yu et al., 2000). They are remotely homologous to mammalian and insect microsomal EHs, including reported insect JHEHs (Seino et al., 2010; Severson et al., 2002; Taniai et al., 2003; Touhara et al., 1994; Zhang et al., 2005). Three putative JHEH sequences from *C. quinquefasciatus* were also clustered with insect JHEHs, but had a different evolutionary history with the five sequences mentioned previously. Interestingly, putative epoxide hydrolase sequences homologous to the mosquito AgEH and EHs from *C.quinquefasciatus* were also found in the genomes of *Bombyx mori* (BmEH), *Apis mellifera* (AmEH) and *Tribolium castaneum* (TcEH) (Fig. 4), suggesting mammalian EH 3, EH 4 and sEH homologs may be common in insects. Besides juvenile hormone epoxide hydrolases, there are other epoxide hydrolases in insects that may regulate insect physiology by metabolizing other important substrates (such as epoxy fatty acids) other than juvenile hormones. It is widely accepted that insects can synthesize fatty acids or obtain them from food resources (Dadd, 1981). Insects not only have the capacity to structurally alter the fatty acids, but also these fatty acid derived molecules may have different biological functions from their parent molecules (Stanley-Samuelson et al., 1988). These functions include components of insect exoskeleton, synthesis of pheromones (Stanley-Samuelson et al., 1988), reproduction and immunity (Stanley and Kim, 2014). Epoxides of fatty acids, as lipid signaling molecules, have been demonstrated to play a variety of roles in mammalian physiology and medicinal biology (Morisseau and Hammock, 2013), which are regulated by epoxide hydrolases (Morisseau, 2013). It is fundamental to express and characterize the putative epoxide hydrolases in *B.mori, A. mellifera* and *T.castaneum*, and determine whether insects and mammals share another conserved signaling pathway regulated by epoxide hydrolases.

The phylogeny analysis is consistent with our biochemical characterization that multiple forms of epoxide hydrolases exist in *C. quinquefasciatus*. The repertoire of protein-coding genes in *C. quinquefasciatus* was reported to be 22% and 52% larger than that of *Aedes aegypti* and *Anopheles gambiae*, respectively (Arensburger et al., 2010). One of the gene-family expansions is the expansion of genes associated with xenobiotic detoxification (Arensburger et al., 2010). Epoxide hydrolases historically were studied as detoxification enzymes, and enzymes that hydrolyze juvenile hormones in insects (Morisseau and Hammock, 2008). Multiple EHs expressed in *C. quinquefasciatus* can be a reflection of *Culex*-specific characteristics (Reddy et al., 2012), and EHs may play a role in metabolizing epoxy fatty acids, as their mammalian counterparts do.

3.2. EET and EpOME are endogenous substrates for mosquito epoxide hydrolases

We were particularly interested in the activities on epoxy fatty acids such as EpOMEs and EETs. EETs are epoxides of arachidonic acid, and have not been reported so far in insects to our knowledge. In mammals, EETs are lipid signaling molecules mainly hydrolyzed by

soluble epoxide hydrolases (sEHs). The inhibition of sEH was shown to have therapeutic effects in several disease models, indicating EETs are anti-inflammatory, vasodilatory, angiogenic and analgesic (Morisseau and Hammock, 2013).

When arachidonic acids were added into the 4th instar (8–9 days old after hatch) crude lysate containing the premixed NADPH generating system, 8,9-EET, 11,12-EET, 14,15-EET and their corresponding diols were all detected by LC-MS/MS (Fig. 5A). The regioisomer 5,6-EET is not chemically stable (Fulton et al., 1998), and it is not reported here. The diols were also detected probably because of the epoxide hydrolase activities in the lysate. When 1 μM EH inhibitor AUDA was added into the reaction, 14,15-DHET (diols of 14,15-EET) detected decreased nine fold, and the amount of 14,15-EET detected was increased two fold (Fig. 5B). Using the regioisomer 14,15-EET as a representative of EETs, the largest amount of 14,15-EET was detected when NADPH generating system was added into the lysate (Fig. 5C). Interestingly, when the lysate was purged with CO or the NADPH generating system was not added, a lower amount of EET and diols was detected, but this amount was still significantly higher than the control groups (Fig. 5C). These data indicate cytochrome P450s are involved in the synthesis of EETs *in vitro*, but they are probably not the only source of EET production. During the 30-minute time course, the conversion rate from arachidonic acid to 14,15-EET and diols was 0.01%. Reasons for the low conversion may be arachidonic acid is the substrate for a variety of reactions, including β-oxidation, chain elongation and production of eicosanoids, leading to a small portion of arachidonic acid had been converted to EETs. Another possibility is that the EETs and their diols had been converted to other metabolites for which we have no standards for analysis.

When mosquito 4th instar larvae (8–9 days after hatch) and adult samples (female only, 4–7 days after eclosion) were extracted for oxylipin analysis, all epoxides of linoleic acid, arachidonic acid and their corresponding diols were detected (Table 2). The concentration of EpOMEs was 45 fold and 25 fold higher than EETs in larvae and adult respectively. The concentration of DiHOMEs was 42 fold and 315 fold higher than DHETs in larvae and female adult.

By *in vitro* EET synthesis and oxylipin analysis (Fig. 5 and Table 2), we demonstrated EETs, as well as EpOMEs, are the endogenous substrates for mosquito epoxide hydrolases. EETs and other eicosanoids have been extensively studied in terms of human health and drug development (Morisseau and Hammock, 2013; Tapiero et al., 2002). EETs have not been identified in insects, and their biology has not been reported to our knowledge. Although present in low concentrations, EETs and the corresponding diols clearly are endogenous molecules in mosquitoes. EETs and other epoxy fatty acids may also play physiological roles in mosquitoes as they do in mammals, which are regulated by epoxide hydrolases.

3.3. EH activities on 14,15-EET are highest in female midgut

To investigate the distribution of EH activities on EETs in mosquitoes, we dissected and homogenized a representative set of organs from 50 male or female (4–7 days old) adult mosquitoes. EH activities were measured with 14,15-EET as the substrate. There are sex differences in epoxide hydrolase activities on 14,15-EET in male and female mosquitoes

(Fig. 6). The highest specific activities were found in the midgut of female mosquitoes. Intermediate enzyme activities were found in Malpighian tubules. The activities in the salivary gland, head and whole fly were relatively low in both sexes.

The sexual difference and tissue distribution suggest that the epoxide hydrolase activities on epoxy fatty acids may serve varying physiological roles in different tissues and sexes. Since only female mosquitoes need blood meals, and the midgut is the center for digestion, detoxification and pathogenic infection, it is tempting to hypothesize that the EH activities in the midgut on epoxy fatty acids are involved in midgut function, and may be important in terms of disease transmission.

3.4. EH activities on 14,15-EET can be induced by blood feeding

It was reported that blood feeding could modulate the expression of a variety genes (Dana et al., 2005; Sanders et al., 2003) in mosquitoes. To test whether the EH activities on epoxy fatty acids can be affected by blood feeding, we blood-fed the mosquitoes and sampled 10 mosquitoes every 3 hours for the measurement of EH activities. The epoxy fatty acid composition and EH activities of the sheep blood on epoxy fatty acids are shown in supplementary information (Table S2 and Fig. S2). Because 10 mosquitoes were homogenized in 1 mL pH 8, 50 mM Tris-HCl buffer, and one mosquito usually takes 1–2 μl of blood, the fatty acid components and EH activity from the blood (Fig. S2) would not interfere the EH activities measured in our experiments.

Because blood in the gut will interfere with protein concentration determination, the activity was measured as pmol diol formed per mosquito per minute. Non-ingested female mosquitoes were also sampled at every time point as controls. When not blood fed, the EH activities on 14,15-EET remained consistent (Fig. 7) during the course of sampling. After blood feeding, EH activities on 14,15-EET were induced 2 fold at 12 hours post blood feeding, and reduced to the same level as in the non-ingested females at 18 hours post blood feeding.

3.5. EH activities on epoxy fatty acids are inhibited by urea-derived compounds designed as mammalian sEH inhibitors

In mammalian systems, the soluble epoxide hydrolase dominates the metabolism of epoxy fatty acids. Although EETs are relatively stable, establishing their roles *in vivo* as signaling molecules was delayed due to their rapid hydrolysis by the sEH. Because we are interested in the biology of EHs and epoxy fatty acids, we tested a representative of EH inhibitors at relevant concentrations with 14,15-EET as the substrate. The structure of the inhibitors is shown in Table 3. At 1 μM concentration (Table 3), a potent mEH inhibitor (elaidamide) and most sEH inhibitors showed inhibition no greater than 30%, expressed by assigning the activities in control sample (only DMSO was added at 1% final concentration) as 0% inhibition. The inhibitor *t*-AUCB inhibited 65% activity, while AUDA showed a 97% inhibition. Both compounds were designed as EET mimics to inhibit the mammalian sEH. AUDA is the most close mimics of the EETs and has structural similarity on only one side of the urea central pharmacophore (Shen and Hammock, 2011). We selected AUDA and determined its IC_{50}, which was around 86 nM (Fig. 8). We thus propose AUDA as an

inhibitor for the epoxide hydrolases from *C. quinquefasciatus* to facilitate the investigation of the biological roles of epoxy fatty acids. It can also serve as a lead molecule for the synthesis of more powerful inhibitors of the EHs acting on epoxy fatty acids in insects.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgement

Abbreviations

EH	epoxide hydrolase
JH	juvenile hormone
sEH	soluble epoxide hydrolase
JHEH	juvenile hormone epoxide hydrolase
AgEH	epoxide hydrolase from *A. gambiae*
HsEH	sEH from *H. sapiens*
MsEH	sEH from *M. musculus*
RsEH	sEH from *R. norvegicus*
EH 3	epoxide hydrolase 3 from *H. sapiens*
EH 4	epoxide hydrolase 4 from *H. sapiens*
CeEH1	sEH1 from *C. elegans*
HmEH	mEH from *H. sapiens*
RmEH	mEH from *R. norvegicus*
DmEH	mEH from *D. melanogaster*
BmJHEH-r1	JHEH-r1 from *Bombyx mori*
BmEH	epoxide hydrolase 4-like from *Bombyx mori*
AmEH	epxoide hydrolase 4-like from *A.mellifera*
TcEH	epoxide hydrolase 4 from *T. castaneum*
***c*-SO**	*cis*-stilbene oxide
***t*-SO**	*trans*-stilbene oxide
***t*-DPPO**	*trans*-diphenylpropene oxide
EET	epoxyeicosatrienoic acid

EpOME	epoxy octadecenoic acid
AUDA	12-(3-adamantan-1-yl-ureido) dodecanoic acid
***t*-TUCB**	*trans*-4-{4-[3-(trifluoromethoxy-phenyl)-ureido]-cyclohexyloxy}-benzoic acid
***c*-TUCB**	*cis*-4-{4-[3-(trifluoromethoxyphenyl)-ureido]-cyclohexyloxy}-benzoic acid
TPPU	1-trifluoromethoxyphenyl-3-(1-propionylpiperidin-4-yl) urea
***t*-AUCB**	*trans*-4-[4-(3-adamantan-1-yl-ureido)-cyclohexyloxy]-benzoic acid
***c*-AUCB**	*cis*-4-[4-(3-adamantan-1-yl-ureido)-cyclohexyloxy]-benzoic acid
TPAU	1-(1-acetylpiperidin-4-yl)-3-[(4-trifluoromethoxy)phenyl]urea
AEPU	1-adamantanyl-3-{5-[2-(2-ethoxyethoxy)ethoxy]pentyl]}urea
APAU	1-(1-acetypiperidin-4-yl)-3-adamantanylurea
TPAU	1-trifluoromethoxyphenyl-3-(1-acetylpiperidin-4-yl)urea
Elaidamide	(*E*)-octadec-9-enamide

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- Multiple EH activities were characterized in the mosquito *Culex quinquefasciatus*.

- Epoxy fatty acids are endogenous and xenobiotic substrates for EHs from mosquitoes.

- AUDA is a useful inhibitor to investigate the biological roles of epoxy fatty acids.
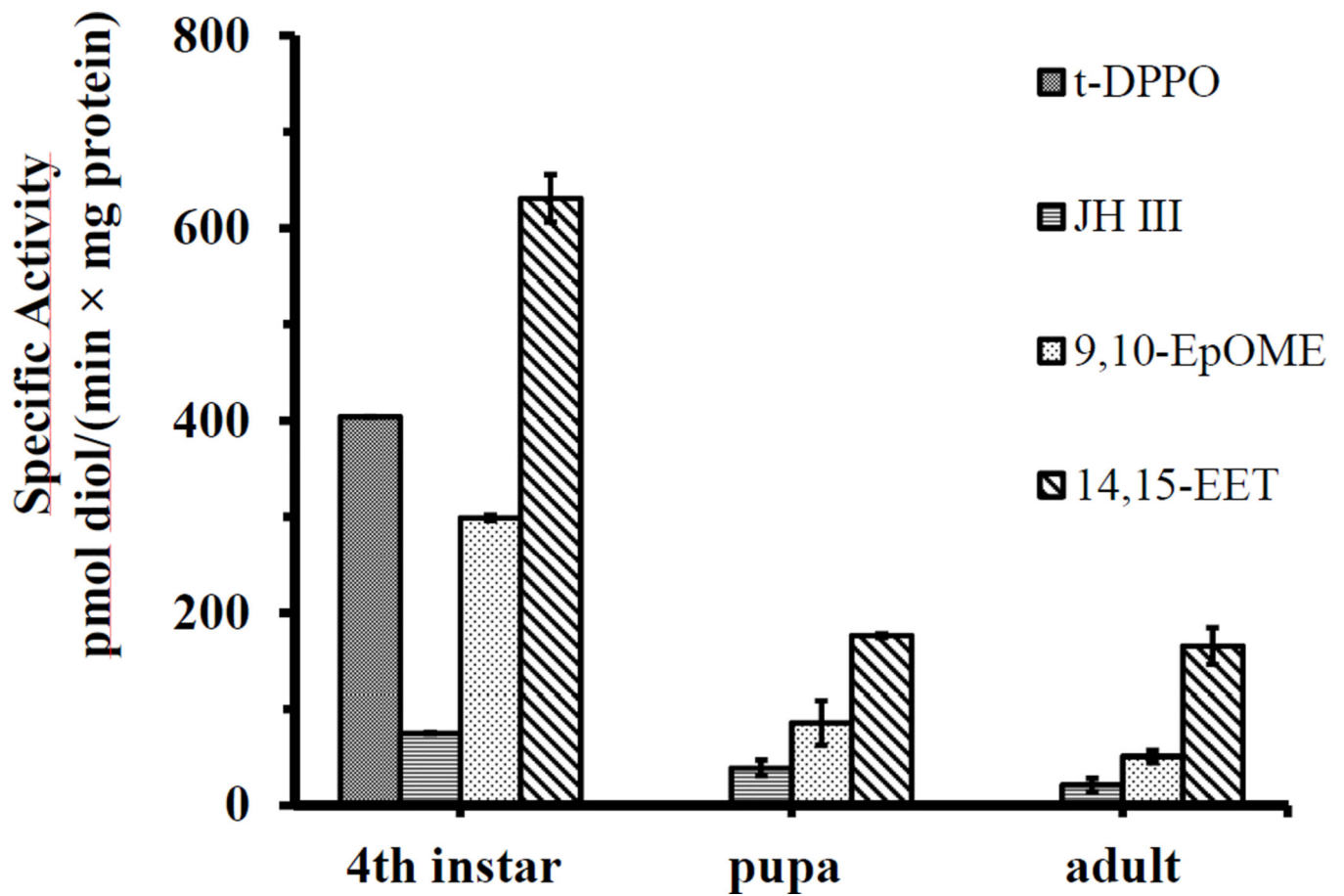
Fig. 1.
Epoxide hydrolase activities in different developmental stages of *Culex quinquefasciatus*. Enzyme activities were measured with triplicate assays. Values are mean activity ± SD based on three independent preparations. 4th instar (8–9 days old after hatch), pupa (10–12 days old after hatch) and female adults (4–7 days old after eclosion) were collected and homogenized for EH activity assays.
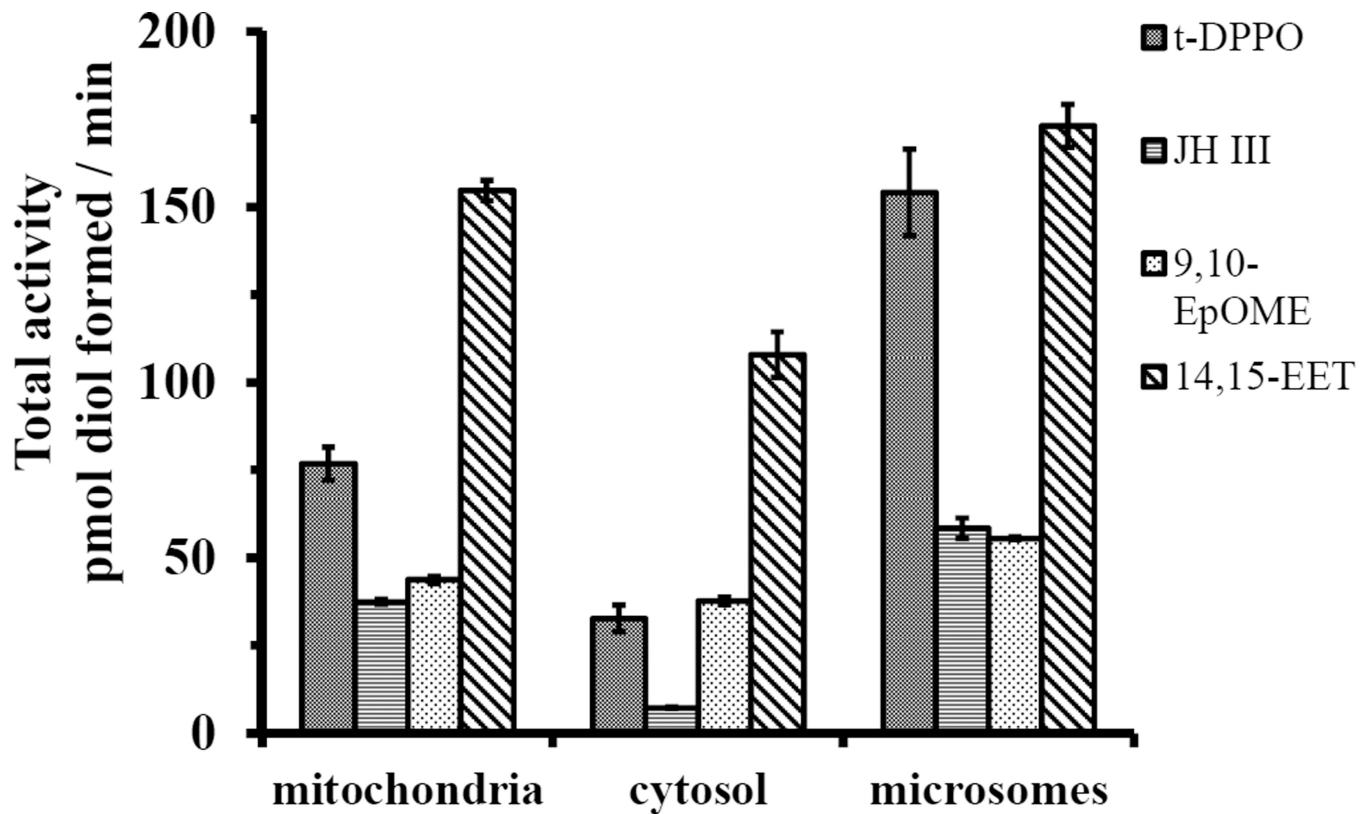
Fig. 2.
Subcellular distribution of EH activities in 4th instar larvae (8–9 days old after hatch) on four substrates. Values are mean activity ± SD based on three independent preparations. The mitochondria fraction was obtained by centrifuging the lysate at 18,000×g for 20 minutes, and the resulting pellets were resuspended in 50 mM, pH 8 Tris-HCl buffer. The resulting supernatant was centrifuged again at 100,000×g for 1 hour. The supernatant was collected as the cytosolic fraction, and the pellet was resuspended in Tris-HCl buffer as the microsomal fraction.
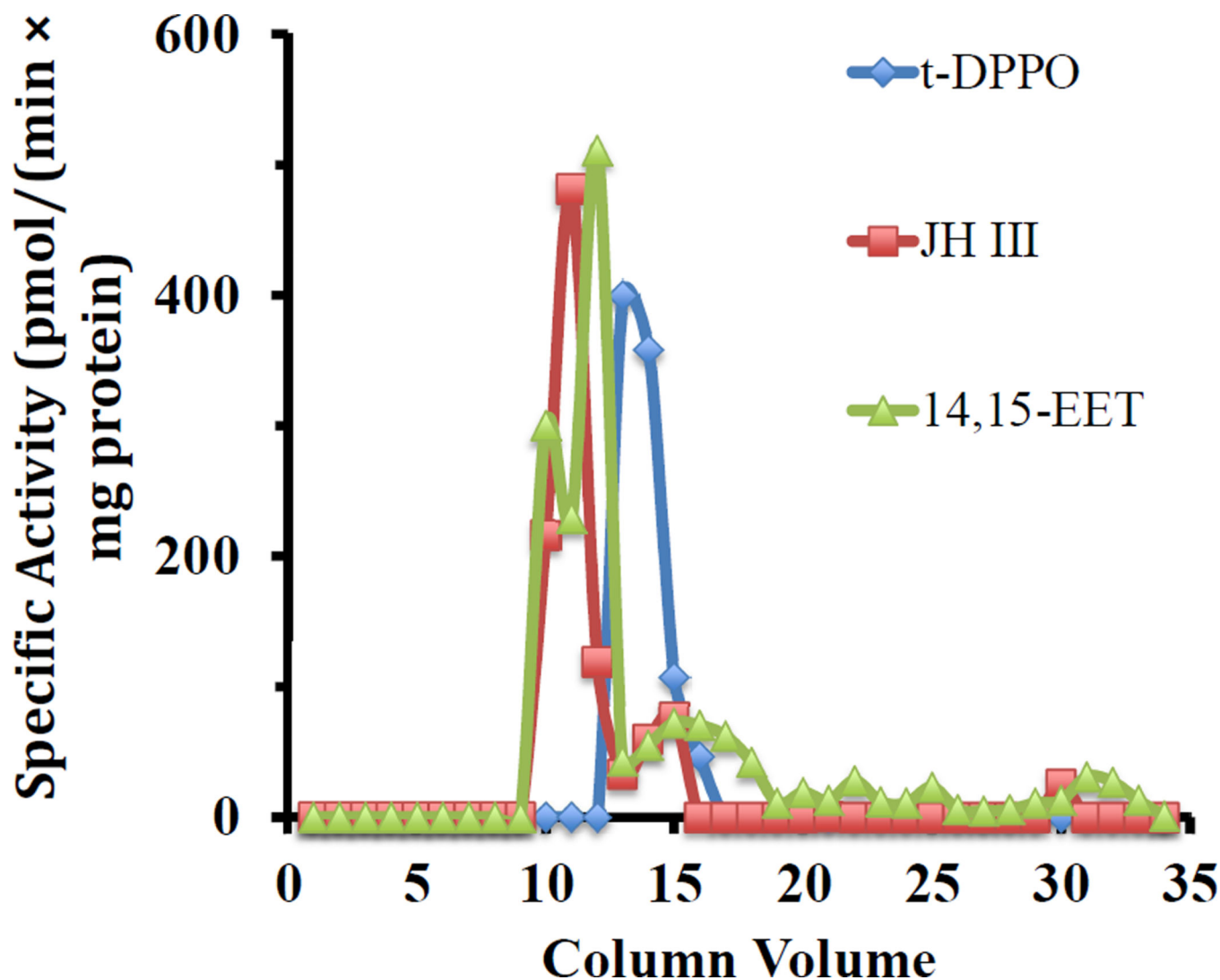
Fig. 3.
Epoxide hydrolase activities with different chromatographic characteristics. Each datum point is the mean of three independent chromatographic runs. The standard deviations are within 10% of the mean values and are not shown here. A column of 10 ml DEAE ion exchanger was used and 1 column volume is 10 ml of eluting buffer. The NaCl gradient was made by mixing 200 mM and 1 M NaCl in Tris-HCl buffer in a gradient maker. Gradient elution began at 5th column volume and ended at 35th column volume. EH activities in the first five column volumes (unbound enzyme fractions) were not detected. Protein concentration was measured by A280 with BSA as the standard. The binding and eluting conditions are shown in Fig. S1. The balance sheet for the purification is shown in Table S1.
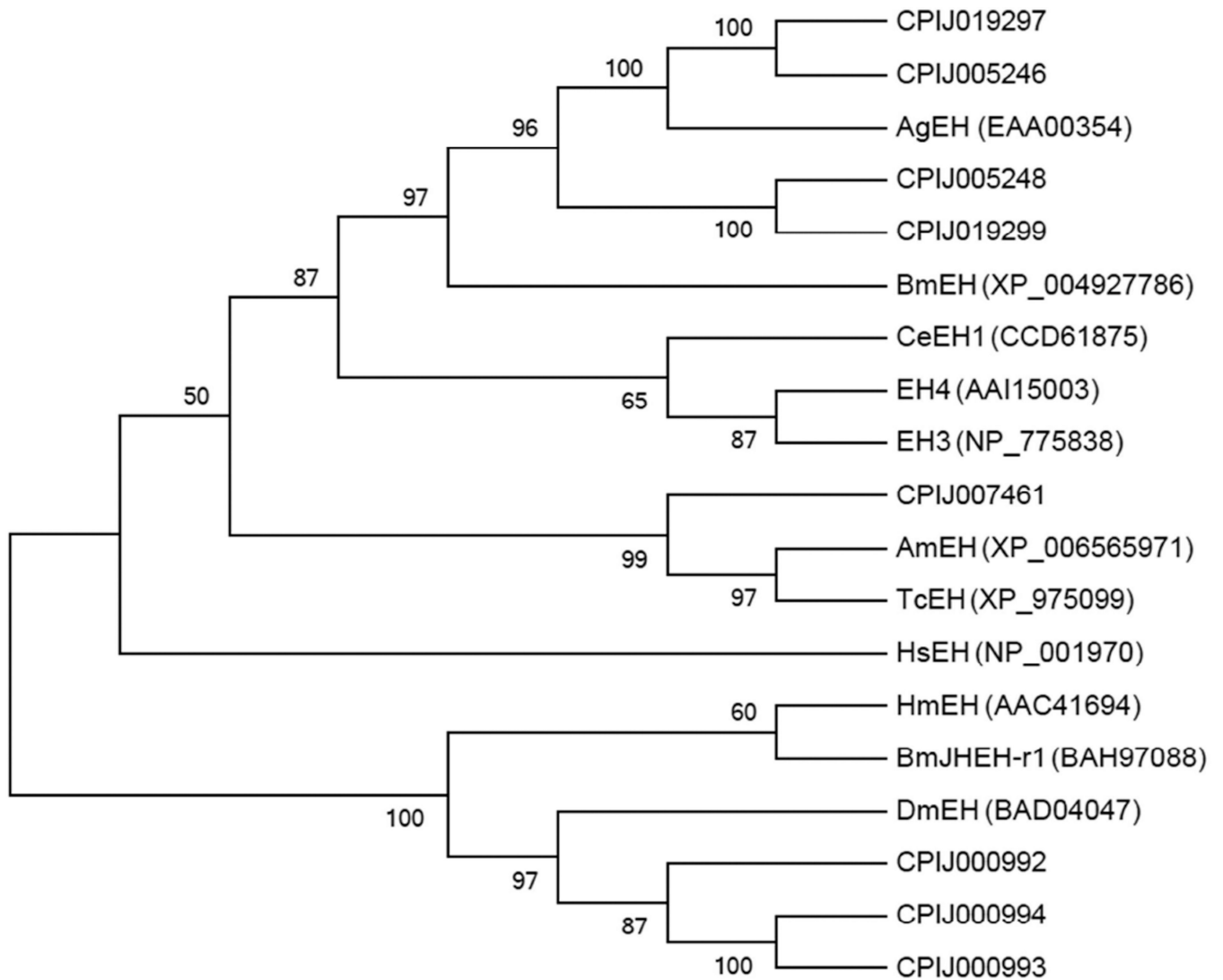
Fig. 4.
Phylogeny analysis of EH sequences in *Culex quinquefasciatus* and other reported EHs. The full names of abbreviations and corresponding references are: AgEH, epoxide hydrolase from *A. gambiae* (Xu et al., 2014). HsEH, sEH from *H. sapiens* (Beetham et al., 1993); MsEH, sEH from *M. musculus* (Grant et al., 1993); RsEH, sEH from *R. norvegicus* (Knehr et al., 1993); EH 3, epoxide hydrolase 3 from *H,sapiens* (Decker et al., 2012); EH 4, epoxide hydrolase 4 from *H.sapiens* (Decker et al., 2012); CeEH1, sEH1 from *C. elegans* (Harris et al., 2008); HmEH, mEH from *H. sapiens* (Skoda et al., 1988); RmEH, mEH from *R. norvegicus* (Falany et al., 1987); DmEH, mEH from *D. melanogaster* (Taniai et al., 2003) ; BmJHEH-r1, JHEH-r1 from *Bombyx mori* (Seino et al., 2010); BmEH, putative epoxide hydrolase 4-like from *Bombyx mori*; AmEH, putative epxoide hydrolase 4-like from *A.mellifera*; TcEH, putative epoxide hydrolase 4 from *T. castaneum*. The accession number of amino acid sequences is shown in the parenthesis. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches.
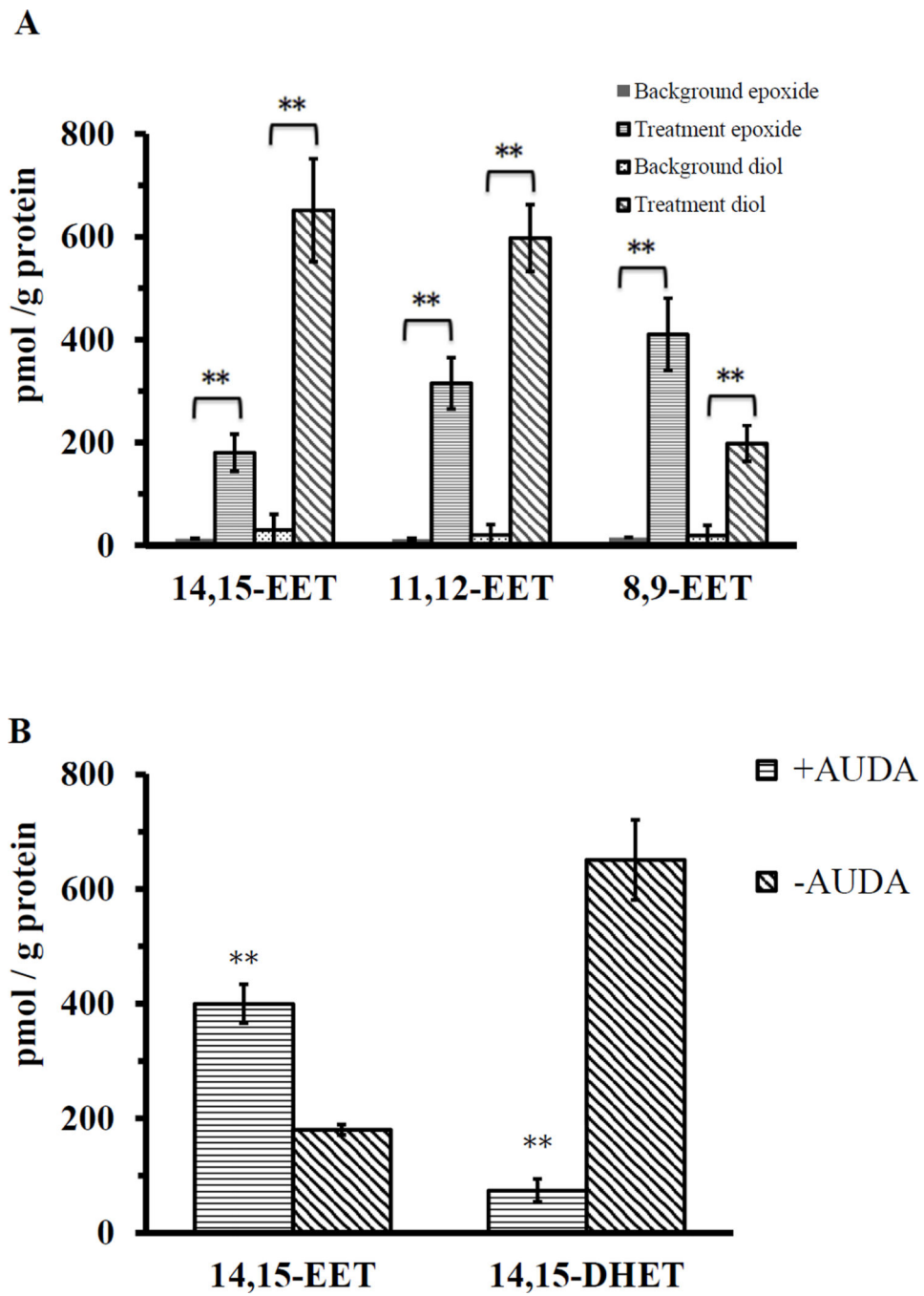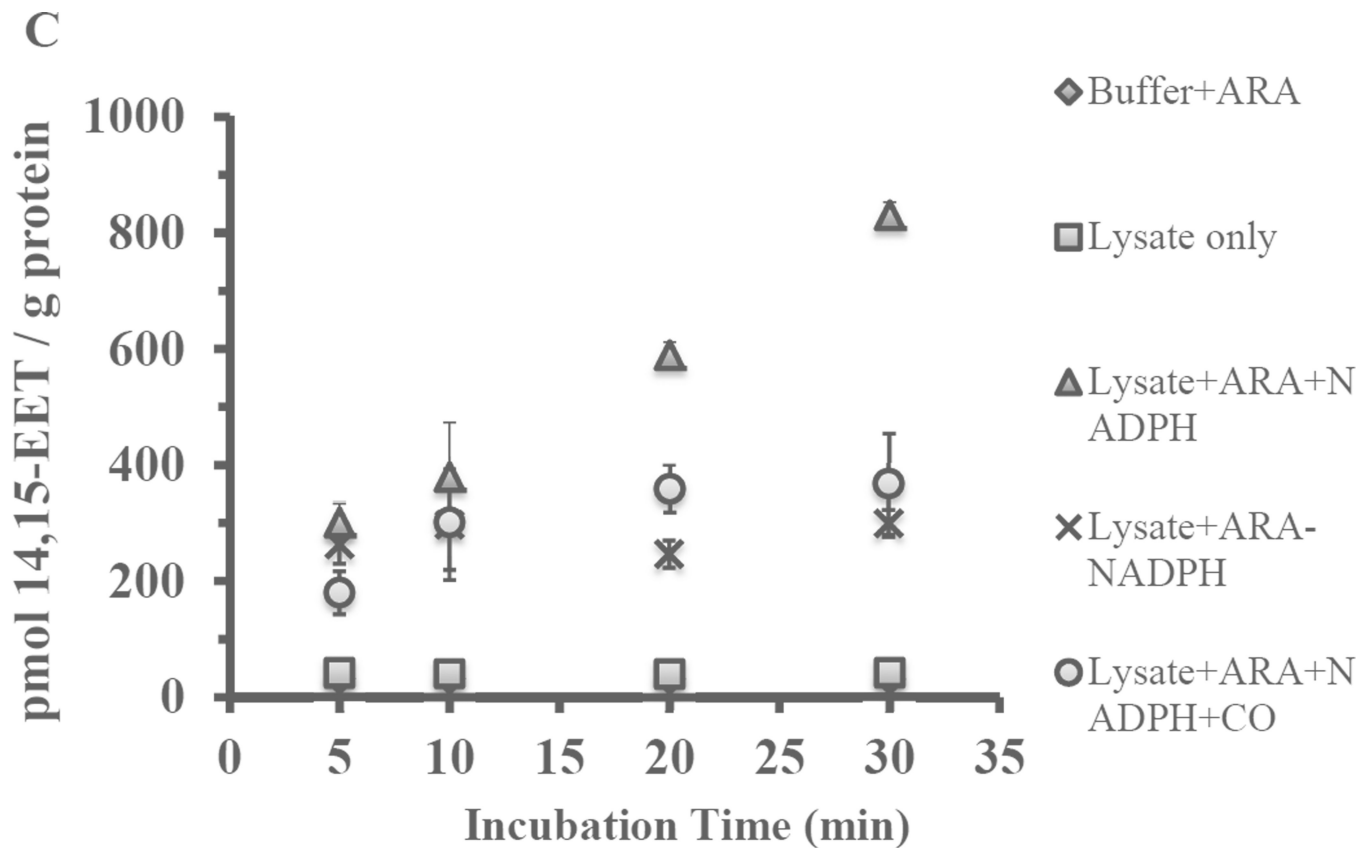
Fig. 5.
Synthesis of EETs by mosquito 4th instar (8–9 days old after hatch) lysates *in vitro*.
Student's t test was used to evaluate statistical significance (** indicates p<0.01). Error bars
represent the standard deviations of the means. A: Different regioisomers of EETs and
corresponding diols detected by LC-MS/MS in 30 minutes of incubation. 5,6-EET was not
included because its chemical instability. EETs and diols detected in the arachidonic acid
solution and mosquito 4th instar (8–9 days old larvae) lysate only were regarded as
background. In the treatment groups, arachidonic acid and NADPH generating system was
added into mosquito 4th instar (8–9 days old larvae) lysate. B. Effects of a sEH inhibitor
(AUDA) on the *in vitro* synthesis of 14,15-EET at 30 minutes of incubation. C: Synthesis of
14,15-EET with or without NADPH or CO in 30 minutes of incubation. The data of two
control treatments 'Buffer+ARA' and ' Lysate only' overlap and therefore only the data of
'Lysate only' is shown.
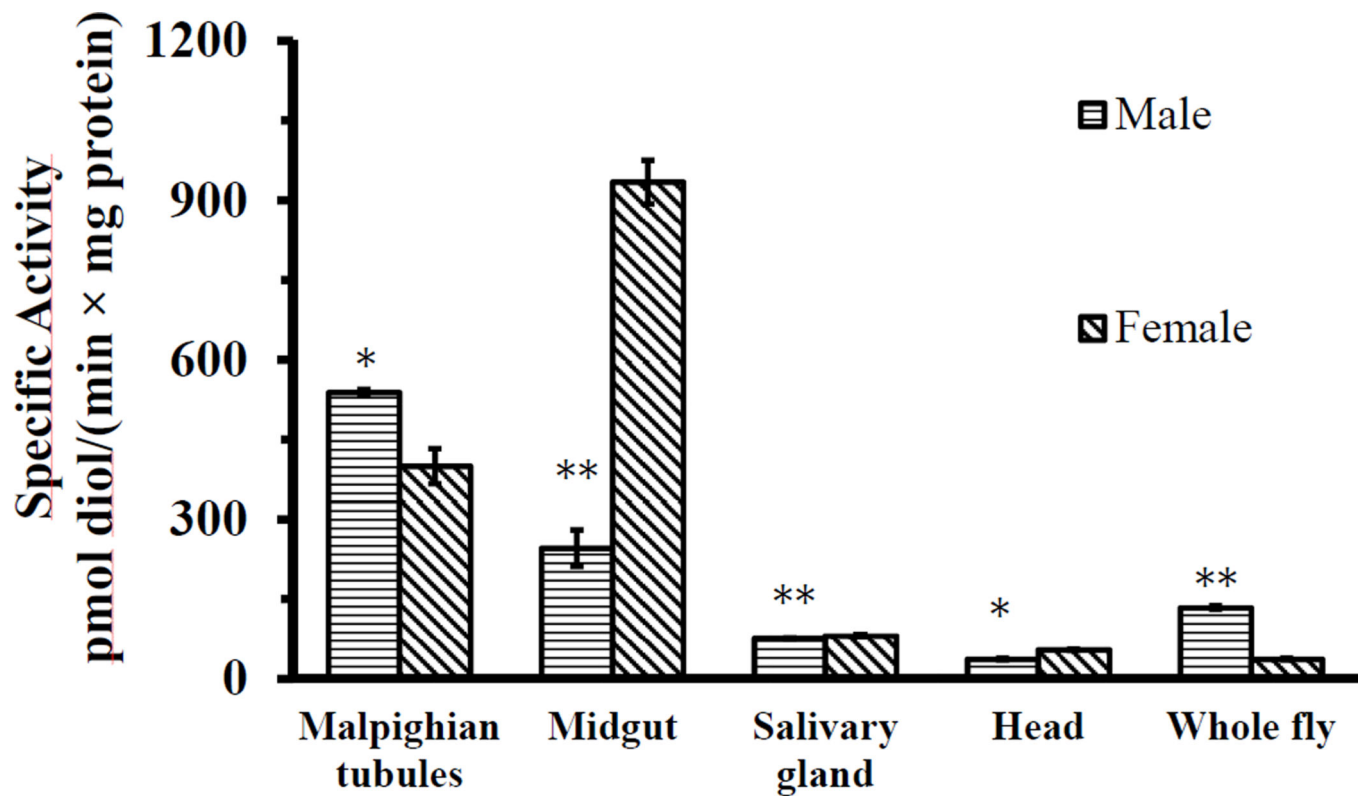
Fig. 6.
Epoxide hydrolase activities on 14,15-EET in different tissues of adult mosquitoes. Mosquitoes aged 4–7 days after eclosion were dissected under a microscope. Student's t test was used to evaluate statistical significance (*p<0.01, **p<0.001) between tissues of males and females. Error bars represent the standard deviations of the means based on three independent measurements.
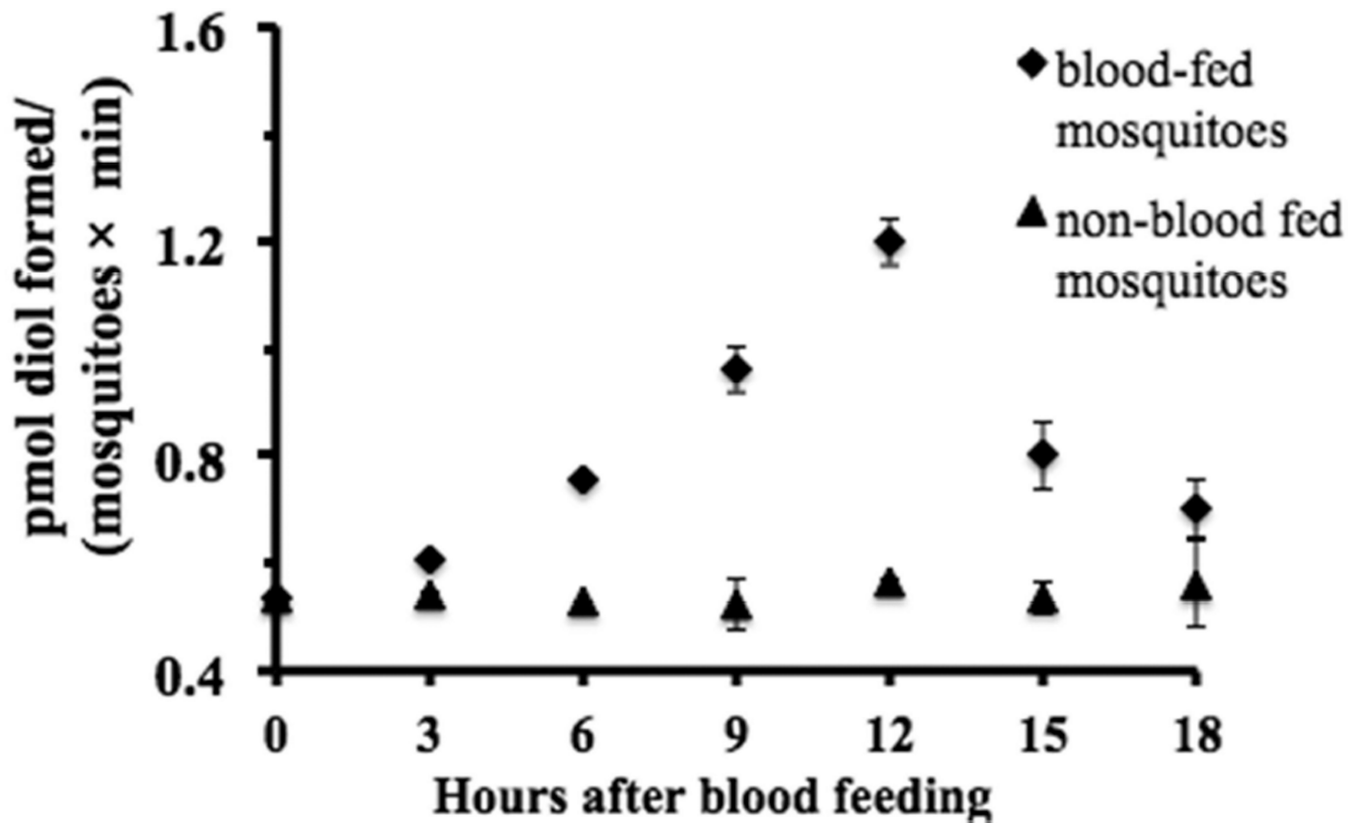
Fig. 7.
Induction of epoxide hydrolase activities on 14,15-EET after blood feeding. Each datum point represents means ± SD unless the SD is smaller than the datum point. Ten blood-ingested females were sampled every three hours after feeding, Non-ingested females were also sampled as controls. At 6, 9,12,15 hours post blood feeding, the differences were statistically significant (p<0.01 by Student's t test) from the controls.
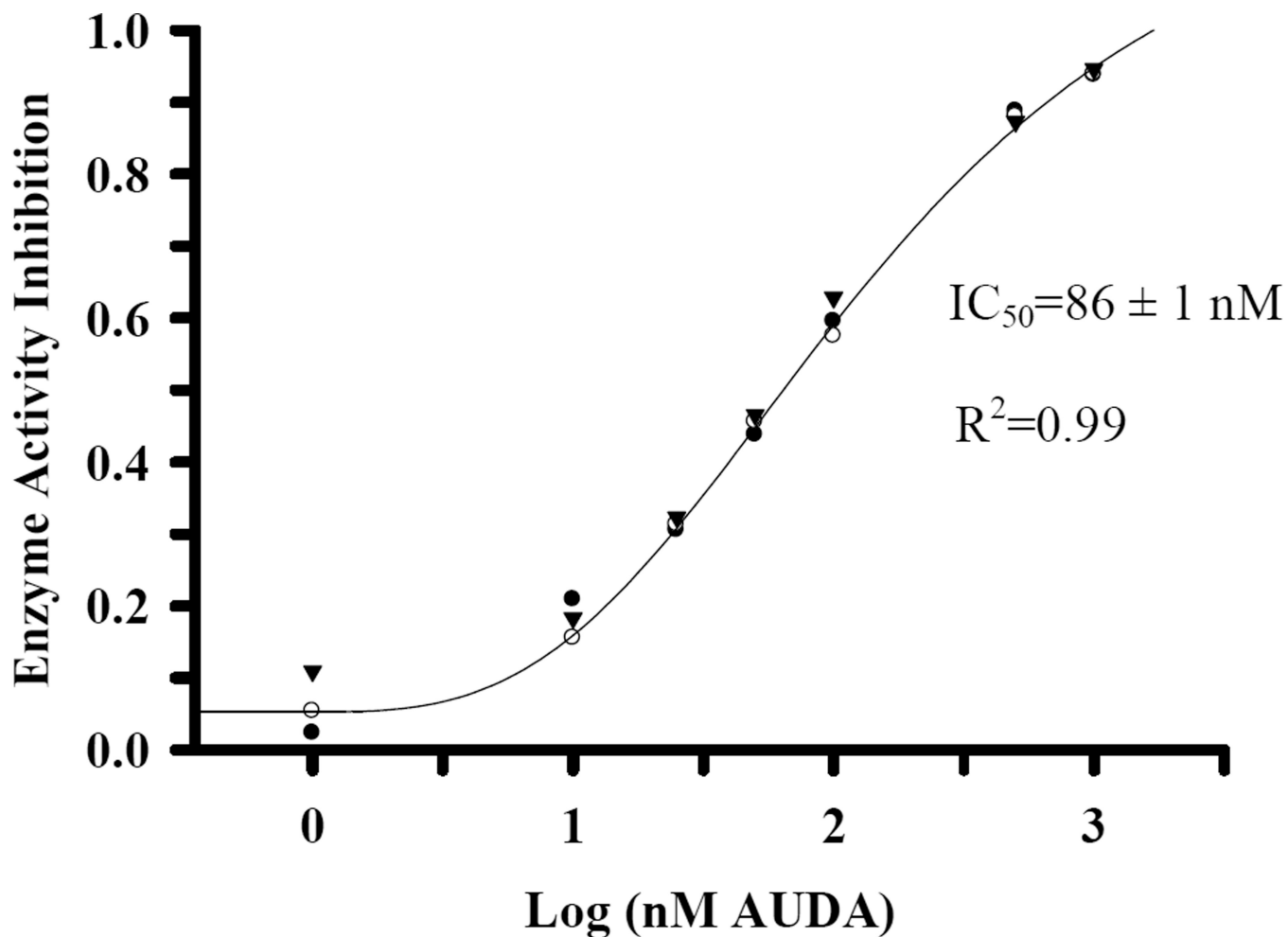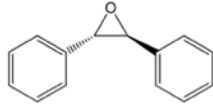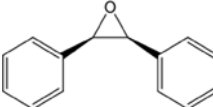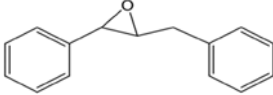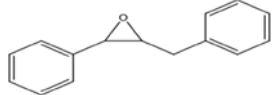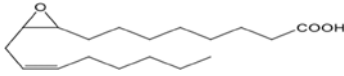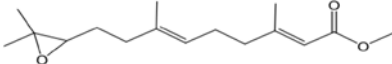
Fig. 8.
IC_{50} of AUDA on 14,15-EET. 4th instar (8–9 days old after hatch) lysate was incubated with different concentrations of AUDA, the most potent inhibitor identified in Table 3 for five minutes on ice. The symbols represent the mean of enzyme activity inhibition in three independent measurements. The IC_{50} is presented as mean ± SD based on three independent measurements. 1 l of 5 mM 14,15 EET in DMSO was added (50 M final concentration) for measuring enzyme activity. The 4 parameter logistic model describes the sigmoid-shaped response was used to calculate IC_{50} by the software SigmaPlot.

Table 1

Substrate selectivity of epoxide hydrolase activities from *Culex quinquefasciatus*.

Substrates	Structure of substrates	Specific Activity (pmol diols formed/(min × mg protein))
t-SO		<30
c-SO		<30
t-DPPO		404±23 (<30)
c-DPPO		<30
14,15-EET		631±25 (<0.4)
9,10-EpOME		299±3 (<0.4)
JH III		75±1 (<2)

Table 2

Detection of epoxy fatty acids *in vivo* by LC-MS/MS. Mosquito 4th instar larvae (8–9 days old larva after hatch) or adults (female, 4–7 days old after eclosion) were weighted before extraction of lipid by solid phase extraction. Concentrations of C-18 and C-20 epoxy fatty acids and corresponding diols are presented as mean ± SD based on three independent measurements.

Parent Fatty Acids	Oxylipin		pmol/g larva	pmol/g adult
Linoleic acid	Epoxy fatty acids	9,10-EpOME	9±2	11±1
		12,13-EpOME	6±2	10±1
	Dihydroxy fatty acids	9,10-DiHOME	12±4	150±30
		12,13-DiHOME	16±3	90±20
Arachidonic acid	Epoxy fatty acids	8,9-EET	0.20±0.04	0.25±0.08
		11,12-EET	0.07±0.02	0.35±0.09
		14,15-EET	0.08±0.02	0.30±0.05
	Dihydroxy fatty acids	8,9-DHET	0.18±0.05	0.10±0.04
		11,12-DHET	0.15±0.04	0.12±0.05
		14,15-DHET	0.30±0.10	0.5±0.1

Table 3
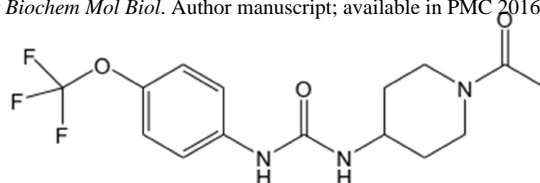
Inhibition of epoxide hydrolase activities on 14,15-EET by EH inhibitors. Enzymes were extracted from 4th instar larvae (8–9 days old).

values are mean

t-

c-

t-

c-