

Molecular analysis of olfactory perception in *Drosophila melanogaster*

Faisal Younus

CSIRO Land and Water, Black Mountain, Canberra, ACT, Australia

and

Research School of Chemistry, Australian National University, Canberra, ACT,
Australia

A thesis submitted for the degree of Doctor of Philosophy of The Australian National
University

September 2017

© Copyright by Faisal Younus 2017

Declaration

The research and results presented in this thesis are the original work of mine except where otherwise acknowledged. The papers published during this PhD are mentioned below along with the nature and extent of my contributions.

1. **Identification of candidate odorant degrading gene/enzyme systems in the antennal transcriptome of *Drosophila melanogaster***

Authors: Faisal Younus, Thomas Chertemps, Stephen L Pearce, Gunjan Pandey, Françoise Bozzolan, Christopher W Coppin, Robyn J Russell, Martine Maïbèche-Coisne, John G Oakeshott

Journal: Insect Biochemistry and Molecular Biology, 2014

Contribution: I did all of the empirical work, except the sequencing, which was contracted out. I did the analyses in collaboration with other authors. I drafted the manuscript. Overall contribution: 65%.

2. **An antennal carboxylesterase from *Drosophila melanogaster*, Esterase 6, is a candidate odorant-degrading enzyme toward food odorants**

Authors: Thomas Chertemps, Faisal Younus, Claudia Steiner, Nicolas Durand, Chris W Coppin, Gunjan Pandey, John G Oakeshott, Martine Maïbèche

Journal: Frontiers in Physiology, 2015

Contribution: I did all of the biochemistry and 40% of the EAG recordings. I drafted about half of the manuscript. Overall contribution: 40%.

3. **Molecular basis for the behavioral effects of the odorant degrading enzyme Esterase 6 in *Drosophila***

Authors: Faisal Younus, Nicholas J Fraser, Chris W Coppin, Jian-Wei Liu, Galen J Correy, Thomas Chertemps, Gunjan Pandey, Martine Maïbèche, Colin J Jackson, John G Oakeshott

Journal: Scientific Reports, 2017

Contribution: I did all of the enzyme assays and prepared the enzyme for crystallography. I drafted about half of the manuscript. Overall contribution: 65%.

4. **Structure and function of an insect α -carboxylesterase (α -Esterase7) associated with insecticide resistance**

Authors: Colin J Jackson, Jian-Wei Liu, Paul D Carr, Faisal Younus, Chris Coppin, Tamara Meirelles, Mathilde Lethier, Gunjan Pandey, David L Ollis, Robyn J Russell, Martin Weik, John G Oakeshott

Journal: Proceedings of the National Academy of Sciences, 2013

Contribution: I did the enzyme assays related to substrate specificity and drafted a part of the paper. Overall contribution: 15%.

Faisal Younus

John Oakeshott
(Senior Author)

Acknowledgements

I would first of all like to thank my supervisor Dr. John Oakeshott for his remarkable support over the years. His maddening attention to detail drove me to finally learn to punctuate prose. I am glad that I am finally able to submit this thesis in your lifetime. I would not have come to this stage without Dr. Chris Coppin, whose selfless time and care were sometimes all that kept me going. You have not only taught me how to do “science” but also how to do it with methodical precision and love. I am grateful to Dr. Jian Wei Liu who provided me with immense support while purifying enzymes from the flies. A task so difficult was made easier because of your help and support. I learnt a lot from you. I am eternally grateful to Dr. Gunjan Pandey who took me under his wings during my PhD and guided me through some very difficult times. I am extremely thankful for all that you have done for me and I am so glad to say that we have come a long way from where we started, trying to sort out technical issues with GCMS instrumentation. I want to thank Dr. Colin Jackson for his inputs and suggestions during my PhD. I was fortunate enough to tap into your intelligence and we have done some tremendous work together, for which I am grateful. I am also thankful to Dr. Matt Taylor, for all your help and support especially with the Metabolomics project. I am highly appreciative of Metabolomics Australia, particularly Dr. Dedreia Tull and Dr. Dave De Souza for their tremendous support over the years.

I am so much in debt to Prof. Martine Maibèche and Assoc. Prof. Thomas Chertemps from the UPMC/INRA, Paris. I am so grateful that you guys hosted me in your lab and I had an amazing time in Paris, not only learning about the neurobiology of flies but also learning how to appreciate arts and culture and to be precise how to live life to its fullest! The huge amount of time you guys spent training me and providing suggestions during these very challenging projects is something I will never forget. Also, what a wonderful bunch of people to work with in the lab at UPMC and INRA. I am so glad that I got to know all of you. I am absolutely delighted to see our labs are continuing with further collaborations on various fly related projects.

I want to thank every lab member past and present at CSIRO, Black Mountain, Canberra for your ongoing support, love and laughter that you have provided. As I am leaving, I can't help but notice how everyone's hair is turning grey and the process of “ageing

gracefully” has started to kick in. I will miss you guys. I am so thankful to Lyndall Briggs for being an absolutely amazing human being and the most supportive colleague and friend (at times “Mom”) a person can ever ask for. Also, I am thankful to Dr. Alisha Anderson for chasing me around the lab with a caterpillar, helping to get over my fear over flies and teaching me a lot about olfactory biology. Thanks for being my rock at Paris, although I am probably the best housemate you ever had. I will be damned if I did not say how appreciative I am to Dr. Olivia Leitch for being an extraordinary friend and keeping me sane over the last few years. All those time that you dealt with my childish behaviour has not gone unnoticed and I am thankful for all that you have done for me. Dr. Amy Paten, thank you for reading my thesis and giving me your feedback. I know how busy you are but now that you have the privilege to call me a “friend” I guess we are even.

I want to thank CSIRO for my OCE scholarship, the French-Australian Science and Technology Program for providing additional funding for my work and the ANU for providing me with a tuition fee scholarship.

Personally, I am in huge debt to a few close friends of mine without whom I would have never been able to finish my PhD studies. Firstly, I want to thank Steve Malesic, my adopted dad, who passed away during the course of this study and rest of the Malesic clan. Over the years, the tremendous love, support and encouragement that I have received from you guys makes me realise how fortunate I am to find a home away from home, in Geelong. To all my friends who became family in Geelong and still “appreciates” my weirdness, I am grateful for all your love over the years. As my scholarship ended and there was a need to find an alternative source of income I ended up working at First Choice Superstore where I was fortunate enough to come across two incredible human beings. Firstly, Digby Swan, who kept me motivated and always encouraged to push through all the obstacles in life and secondly, Sarah Burcher, who always believed in me, brought a smile to my life with her silliness and made sure that I never doubted my abilities. I am also eternally grateful to my dear friend Hayley Smith, who over the years has been so kind with her time and words and provided me with huge mental support. You asked the toughest questions of me when I was a mess last year and could not see a way through all my problems and made me believe that I have what it takes to finish what I started. I am also very thankful to Cassie Lyons and Tobias

Mitchell for providing me with endless laughter, love and support especially when Xavier was on his way “prematurely” and during all those difficult times at the hospital. Those few months would have been incredibly tough if you two were not a constant source of fun and amusement.

Finally, I would like to thank my family back home in Bangladesh, my parents and my brother, for all their prayers. Most importantly, I want to thank Shahana Afroze for all that she has done for me. Your love, care and sacrifices have not gone unnoticed and I am forever in your debt. Still feels like yesterday we both came to Canberra to commence our studies and here we are now!

I want to dedicate this work to my new born son, Xavier Younus. Your smile and hugs gave me the courage and determination that I needed to finish the rest of this PhD journey.

Abstract

Over the last decade the insect olfactory system has emerged as an important model system with which to investigate the biochemical basis of eukaryote signalling processes. It is believed that certain odorant degrading enzymes are required to maintain the ongoing sensitivity of an insect's olfactory neuronal system by repriming neurons. However, relatively few ODEs have been identified and characterized to date, especially in the model insect *Drosophila melanogaster*.

The study presented here takes biochemical, neurobiological and behavioural approaches to elucidate the role of ODEs in *D. melanogaster*. After a review of relevant literature in Chapter 1, Chapter 2 decodes the antennal transcriptome of *D. melanogaster* for the first time. Using high quality genome sequence and transcriptomic data for many other tissues of this species already available, I identified a few antennae-selective esterases, cytochrome P450s (P450s), glutathione S-transferases (GSTs) and UDP-glycosyltransferases (UGTs). Of these, the activity of one esterase JHEdup, against a range of volatile odorants was found to be comparable with other known ODEs from different species, mainly Lepidoptera. I also identified the presence of another esterase, EST6, at high levels in the antennae. It has previously been proposed that EST6 is a catalyst for the transformation of pheromonal and kairomonal esters to the corresponding alcohols and acids, thereby mediating various mating behaviours. I further examined the proposed effect of EST6 by comparing wild type and EST6 null flies at a neurobiological and behavioural level. The findings, presented in Chapter 3, show this enzyme is important for the flies to respond to incoming volatile odorants and affects their subsequent behaviours. Additionally, EST6 has previously been reported to hydrolyse *cis*-vaccenyl acetate (cVA), the major pheromone known in *Drosophila*, *in vitro* and recent electroantennogram (EAG) experiments with EST6 wild type and null flies exposed to cVA suggest that this might also be an *in vivo* function. I therefore conducted experiments to understand the biochemical activity of EST6 against cVA. I also measured its activity against 84 other bioactive esters. The results categorically show that EST6 has no activity against cVA but has very good activity against a wide range of fruit- and yeast-derived volatiles known to play a role in mediating female reproductive behaviour. These results are presented in Chapter 4, along with a crystal structure of EST6.

The final chapter of this thesis then discusses the overall findings of these studies and offers a broader perspective on future directions. The three major conclusions from the work are as follows. Firstly, JHEdup and EST6 are broad range ODEs active against a wide range of food odorants. Secondly, EST6 may also have a role in cVA processing but not actually as an ODE against this substrate. Thirdly, ODEs may be a fruitful system to develop biocontrol systems for pest insects based on disrupting their olfactory system.

Table of Contents

Declaration	i
Acknowledgements.....	iii
Abstract	vi
Chapter 1: Introduction and literature review	1
1.1. Insect olfaction.....	1
1.2. Chemical ecology of <i>Drosophila melanogaster</i>	2
1.3. Olfactory organs.....	3
1.4. Olfactory proteins	4
1.4.1. Odorant receptors.....	4
1.4.2. Odorant binding proteins	6
1.4.3. Ionotropic receptors.....	6
1.4.4. Gustatory receptors.....	7
1.4.5. Sensory neuron membrane proteins.....	8
1.4.6. Odorant degrading enzymes.....	8
1.5. β -esterases in <i>D. melanogaster</i>	10
1.6. JHE and JHEdup	12
1.7. Scope and goals of this thesis	12
1.8. Papers published.....	13
Chapter 2: Identification of candidate odorant degrading gene/enzyme systems in the antennal transcriptome of <i>Drosophila melanogaster</i>	15
Chapter 3: An antennal carboxylesterase from <i>Drosophila melanogaster</i> , Esterase 6, is a candidate odorant-degrading enzyme toward food odorants.....	30
Chapter 4: Molecular basis for the behavioral effects of the odorant degrading enzyme Esterase 6 in <i>Drosophila</i>	40
Chapter 5: General discussion.....	53
5.1. EST6 and JHEdup as ODEs.....	53
5.2. Some broader perspectives	56
References.....	59
Appendix: Structure and function of an insect α -carboxylesterase (α Esterase7) associated with insecticide resistance	66

Chapter 1: Introduction and literature review

1.1. Insect olfaction

An animal olfactory system possesses remarkable sensitivity and ability to discriminate between thousands of different odour molecules, thus allowing appropriate responses to a wide variety of volatile stimuli ¹. Insects in particular have proven a valuable model for investigating how the chemosensory machinery of animals functions at a molecular level ². The findings from these works have also proven useful in the development of management tools for socially and economically important insects. For example, mating disruptants and chemical lures have been developed for agricultural pests such as the cotton bollworm, *Helicoverpa armigera* ³, and disease vectors such as the mosquito *Anopheles gambiae* ⁴. Conversely, the management of beneficial insects such as the honeybee, *Apis mellifera*, is also improved by the in-depth understanding of their chemosensory behaviour ⁵.

Insect olfaction is coordinated at various levels and involves a range of olfactory organs, neurons, proteins and other molecules. The process initiates when an external signal (volatile semiochemical) is intercepted by peripheral olfactory organs, followed by its processing in, for example, the antennal lobes, and then the integration of olfactory and other sensory response modalities in the higher processing centre of the brain. The signal may then be translated into behaviours such as locating food or reproduction. Importantly, to retain the sensitivities of the olfactory system, the insect requires the ability to rapidly inactivate minute amounts of odorants once they have conveyed their information, so as to maintain the ongoing responsiveness of the system to new inputs ⁶.

The research described in this thesis specifically investigates the enzymes involved in this latter inactivation process in the model insect *Drosophila melanogaster*. This literature review first outlines the chemical ecology of the species and then the organs and processes so far known to be involved in its chemoreception. It then reviews previous work on two particular esterases which emerged as key players in the signal inactivation process mentioned above. Finally, it outlines the content of the following chapters. This includes three chapters in the form of published papers, which describe

my research and a final discussion chapter that synthesises my findings and proposes areas for further study.

1.2. Chemical ecology of *Drosophila melanogaster*

D. melanogaster preferentially feeds on yeasts growing on sugar-rich over-ripe fruit ⁷. The yeasts generate a range of ester volatiles such as ethyl acetate, isoamyl acetate and amyl acetate which are highly attractive to flies and may be specifically produced by the yeasts to help them secure dispersal ⁸. Interestingly, brewer's yeast, which is unable to produce acetate esters, is dispersed poorly as it is much less attractive to the flies ⁸. Another group of important yeast volatiles that elicit attraction are phenolics such as 2-phenyl ethanol and 2-phenethyl acetate ⁹. *Brettanomyces* yeasts produce 4-ethylphenol and 4-ethylguaiacol which are antioxidants and have a positive effect on the vigour of the flies. They are also known to induce feeding and oviposition in female flies ¹⁰.

Fermenting fruits also contain a diverse population of bacteria. Amines such as putrescine and spermidine generated by the bacterial degradation of arginine are highly attractive to flies ^{11,12}. Other volatiles produced by bacteria, such as trimethylamine (derived from choline) and phenyl ethylamine (from the decarboxylation of phenylalanine), also serve as attractants and activate various sensory receptors in the fly brain ¹².

Flies also respond to odours emitted directly by fruit. Fruit odours such as ethyl hexanoate and related esters (e.g. hexyl acetate, ethyl butyrate, butyl butyrate and pentyl acetate) and aromatic esters such as ethyl benzoate and methyl salicylate have been shown to induce strong attractive behaviours in *D. melanogaster* ^{8,13,14}.

Apart from kairomones produced by other organisms in its environment, another key feature of the *D. melanogaster* chemosensory system is its major pheromone *cis*-vaccenyl acetate (cVA), which behaves as a modifier of the species' aggregation ¹⁵ and reproductive behaviour ¹⁶. It is an adult male-specific fatty acid derivative which is not only present on the body cuticle ¹⁷ but also at very high levels in the sperm ejaculatory bulb ¹⁸. A thorough understanding of the molecular and biochemical basis of

cVA function is absolutely essential to unravel its various roles in *D. melanogaster*, and several significant findings have already been made, as outlined below:

- a. Acute exposure to cVA promotes aggression among males¹⁹. This effect is mediated primarily through activation of the odorant receptor, Or67d²⁰. High densities of male flies are reported to promote aggression by the release of volatile cVA, which in turn may mediate negative-feedback control of male population density²⁰. Paradoxically however, chronic exposure to cVA has also been found to reduce aggression among males through its interaction with the odorant receptor, Or65a¹⁹. cVA therefore acts in a temporally differential manner to elicit different behaviours through two specific olfactory receptor neurons (ORNs).
- b. cVA gets deposited by males and mated females onto food, where it acts as an aggregation pheromone attracting flies to a potential breeding ground^{15,21}. Studies by Bartlett *et al.*¹⁵ show that flies of both sexes would preferentially land at sites where cVA was being emitted, leading to aggregations and ultimately mating, even when abundant other food sources existed.
- c. cVA enhances the receptivity of virgin females to courtship by acting as an aphrodisiac, encouraging them to mate more rapidly with males when sensing this pheromone²².
- d. After being transferred (approximately 200-300 ng) during copulation to the female, cVA acts as an anti-aphrodisiac and suppresses their response to courtship by males in subsequent encounters²³. The males find the odour aversive and avoid females that have already been courted. This reduces the potential loss of progeny sired by the female's first mate and also ensures that reproductive energy, at least for the male, is conserved²⁴.

1.3. Olfactory organs

D. melanogaster primarily detects odorant molecules via two types of olfactory sensory organs on the head, namely the antennae and maxillary palps. These organs are covered with numerous sensory hairs, called sensilla, which house and protect the underlying olfactory receptor neurons (ORNs) that are specialized to detect various odorant

molecules. Scanning electron and light microscopy studies have revealed that these sensory hairs can be classified on the basis of position, size, shape, and pore densities into three main morphological classes: basiconic, trichoid, and coeloconic sensilla. There are approximately 1200 ORNs in each antenna across the three sensillar categories²⁵. The maxillary palps contain approximately 120 ORNs, all of which are housed in basiconic sensilla¹³.

Each sensillum usually houses between one to four ORNs which are bathed in a sensillar lymph and have different odorant response profiles²⁶. ORNs of basiconic sensilla respond to food odours, including many esters and alcohols; those of trichoid sensilla respond to fly odours; and those found in coeloconic sensilla respond to many amines and carboxylic acids from rotting fruits^{11,13,27,28}. Trichoid sensilla are further subdivided according to the number of ORNs that innervate them into sensilla with one (T1 sensilla), two (T2 sensilla) or three (T3 sensilla) ORNs²⁵.

Most ORNs express only one kind of odorant sensing (tuning) Odorant Receptor (OrX) in combination with the universal Olfactory Co-Receptor Orco²⁹. More information on the OrXs and Orco is provided in the next section.

1.4. Olfactory proteins

A plethora of biochemical, biophysical, structural biology and kinetic studies have identified six peripheral olfactory protein families that play significant roles in chemoreception and are found in the sensillar fluid. These are the odorant receptors (ORs), odorant binding proteins (OBPs), ionotropic receptors (IRs), gustatory receptors (GRs), sensory neuron membrane proteins (SNMPs) and odorant degrading enzymes (ODEs)⁴.

1.4.1. *Odorant receptors*

ORs were first identified in *D. melanogaster* over a decade ago using a bioinformatics-driven approach³⁰. These ORs were then independently verified by Gao and Chess³¹ and Voshall *et al.*³² using experimental approaches. With about 400 amino acid residues, ORs are seven transmembrane domain proteins that belong to the superfamily of G-protein coupled receptors (GPCRs)³³. Recently, Benton *et al.*³⁴ reported that ORs

may adopt an unconventional inverted topology compared to other GPCRs, with an intracellular N-terminus and an extracellular C-terminus. *D. melanogaster* has a relatively small *Or* gene repertoire, with 60 *Or* genes encoding 62 gene products through alternative splicing, compared to insects such as the red flour beetle, *Tribolium castaneum*, which has 341 predicted ORs^{35,36}. The corresponding number in vertebrates such as mice and rats is about 1000³⁷.

Each OR except Orco is expressed within a restricted subpopulation of ORNs^{30-32,38}. Orco, originally identified as Or83b, also appears to function as an universal co-receptor^{29-31,38} in other insect species such as moths³⁹ and mosquitoes⁴⁰. Orco is highly conserved among insect species and orthologues from different species can be functional substitutes for one another^{29,41,42}. Orco forms heteromeric complexes with other ORs²⁹ and is involved in the localization of ORs to ORN dendrites, where it enhances their responsiveness to odorants without altering their co-expressed ligand specificity^{34,43}.

With the exception of a handful of ORNs, such as Or67d and Or65a on the trichoid sensilla, which are specially tuned to the sex and aggregation pheromone cVA^{19,20}, the majority of sensilla on the antennae and maxillary palps of *D. melanogaster* house ORNs that appear to respond to various food-derived odorants^{2,9}. The acetate esters from yeasts activate broadly tuned receptors such as Or43b, Or47b and Or85b^{2,8,9} while the phenolics from yeasts are detected primarily via Or67b and Or85d⁹. Fruit-derived volatiles, such as ethyl hexanoate, hexyl acetate, ethyl and butyl butyrate and related esters, are detected via a specially tuned receptor, Or22a in the basiconic sensilla^{13,14,44}, whereas aromatic esters, such as ethyl benzoate and methyl salicylate, which are found in ripe fruit, activate mainly Or10a and Or98a^{8,13}. Notably, Or56a (which detects geosmin)⁴⁵ and Or33b (which detects 2, 5-dimethyl pyrazine)⁴⁶ have evolved to detect the presence of harmful microbes. Once activated these latter receptors induce avoidance behaviours in flies.

The cVA receptors, Or65a and Or67d, head two distinctive olfactory circuits. The attractive properties of cVA are sensed by Or67d, which is solely expressed in the sensilla T1²⁰. The function of inhibiting courtship and acting as an aversive cue for mating is mediated by Or65a, expressed in the trichoid sensilla T3¹⁹.

1.4.2. *Odorant binding proteins*

OBPs) are present at very high concentrations (10 mM) in the sensillar fluid^{47,48}. Their physiological function is yet to be confirmed but they are thought to solubilize normally hydrophobic odorants in the aqueous sensillum lymph and efficiently present these ligands to the ORs^{6,49}. They are also hypothesised to assist in terminating the signal generated by the odour by removing the respective ligands from their cognate receptor⁵⁰. There are at least 51 OBP genes in the *D. melanogaster* genome, some of which are also highly conserved among other insect species^{4,51}.

Although a direct role for OBPs in odour recognition has been discussed over the years, it was only recently that the first functional evidence that OBPs indeed participate in olfactory responses was brought forward. This was from a study on *D. melanogaster* mutants that lacked an OBP named LUSH⁵². The LUSH protein is encoded by the *lush* gene, and the phenotype of LUSH null mutants is a failure to be repelled by high concentrations of ethanol⁵³. This suggests that this OBP may function in the transport of the odorant to its receptor and its recognition by the latter, perhaps via a ligand-OBP complex⁵⁴. Interestingly, however, studies in vertebrates and nematodes indicate that some of their receptors can be activated by ligands in the absence of OBP^{28,55}.

1.4.3. *Ionotropic receptors*

IRs are a recently characterized family of olfactory receptors in *D. melanogaster*⁵⁶. IRs are not related to ORs phylogenetically, but rather have evolved from ionotropic glutamate receptors, a conserved family of synaptic ligand-gated ion channels⁵⁷. Genomic analysis has identified 66 *IR* genes (including nine putative pseudogenes) in *D. melanogaster*⁵⁶. Comprehensive expression analysis of these genes by RT-PCR, fluorescence RNA *in situ* hybridization and/or using transgenic reporters has shown that 16 of these are expressed in the antenna⁵⁷. IRs are expressed in ORNs and located only in the coeloconic sensilla of *D. melanogaster*, a sensillum class that does not normally express ORs²⁷.

IRs mediate olfactory responses to a variety of odours, including acids and aldehydes and perhaps humidity⁵⁷. Studies suggest that mutations in *IR84a*, *IR64a*, *IR8a*, and *IR25a* of *D. melanogaster* inhibit odour-evoked neuronal responses^{16,58}. The specificity of

ligand recognition by IRs, however, still remains to be completely elucidated ⁵⁷. Intriguingly, it is reported that some members of the IR superfamily are also expressed in taste neurons and IR94b has also now been implicated in auditory system functions ⁵⁹. Recent comparative electrophysiological analysis of ORs and IRs in the adult antenna highlighted some significant differences in ligand specificity; i.e. carboxylic acids and some aldehydes activate IR8a, whereas IR25 was shown to be preferentially activated by amines and ORs are more tuned to detect esters, alcohols and ketones ¹¹.

1.4.4. *Gustatory receptors*

GRs are related to ORs structurally and are also likely to act as ligand-gated ion channels. However, some *in vivo* studies suggest the involvement of heterotrimeric GR proteins in taste transduction ^{60,61}. It is challenging to determine the role of individual GRs because they are expressed combinatorially in gustatory neurons ⁶². This is in direct contrast with the singular expression of ORs in most ORN populations. The expression profiles of over half of the GR repertoire have yet to be found ⁶³. This could possibly be because of their extremely low transcript levels in gustatory neurons or due to their presence in non-chemosensory tissues ⁶⁴. Comparative genome studies show GRs to be a rapidly evolving family with relatively few orthologues surviving across insect families ⁶⁵.

Some GRs have been shown to play a role in detecting compounds such as amino acids and bitter tastants. Some also help in sensing CO₂ and acidic pH conditions ⁶³. In addition, functional studies have suggested that GRs function in the detection of environmental chemicals and pheromones, and emerging data implicate particular family members in thermosensation and photoreception as well as in non-sensory roles ⁶⁶. At least 38 of the 68 GRs in *D. melanogaster* are reported to be expressed in the labellum (the primary taste organ on the head) ⁶⁷ and 28 of them were found to be present in the legs ⁶⁸, which enable the flies to sample potential foods without actually consuming them ⁶⁵. In the labellum Gr5a responds to sugar and elicits feeding behaviour ⁶⁹, whereas Gr66a and Gr33a respond to bitter compounds, triggering avoidance behaviour ⁷⁰.

1.4.5. *Sensory neuron membrane proteins*

SNMPs, a homologue of transmembrane CD36 receptors, are expressed in a population of ORNs implicated in pheromone detection ⁷¹. The CD36 receptors are found across metazoans where they are known to play a role in lipid binding and transport. In addition, several CD36 proteins acts as scavenger receptors for bacterial pathogens, aiding immune function ⁷². SNMPs are essential for the electrophysiological responses of ORNs expressing the cVA receptor Or67d ⁷³. It has been suggested that SNMPs acts in concert with ORs to capture pheromone molecules on the surface of olfactory dendrites ^{71,74}.

1.4.6. *Odorant degrading enzymes*

ODEs are believed to play a crucial role in re-priming sensory neurons, thus ensuring the insect's ongoing ability to locate food and potential mates. Although considerable research has been done on ODEs in lepidopteran species ⁷⁵⁻⁷⁸, little is known about the identity and role played by ODEs in *Drosophila* olfactory circuitry.

There are two criteria that must be met for an enzyme to be classified as an ODE. These are that the enzyme has to reside in the sensillar lymph of the insect and that it can degrade an odorant at physiologically relevant rates ⁷⁹. These criteria have been fulfilled by certain antennal esterases which can degrade pheromones in Lepidoptera, Coleoptera and Hymenoptera ⁸⁰.

The first insect ODE identified was by Vogt and Riddiford ⁷⁵. It was an antennae-specific esterase (ApoISE) from the sensillar fluid of male silkworm, *Antheraea polyphemus*. It was demonstrated that ApoISE could effectively degrade the acetate component of a pheromone blend. The half-life of the acetate in the presence of this esterase *in vivo* was conservatively estimated to be just 15 ms, thus making it potentially a highly effective ODE ⁷⁵. Studies on a purified preparation of ApoISE subsequently indicated considerably higher k_{cat} values (127 s^{-1} cf $\sim 0.033 \text{ s}^{-1}$) ⁷⁶.

Similarly high k_{cat} estimates for candidate pheromone esters have been reported for other purified antennal esterases from *A. polyphemus* and the Japanese beetle, *Popillia japonica* ^{76,81}. Other antennal esterases have since been identified in the moths

Mamestra brassicae, *Sesamia nonagrioides*, *Spodoptera littoralis*^{77,82}, *Spodoptera exigua*⁸³ and *Epiphyas postvittana*⁸⁴ and the honey bee *Apis mellifera*⁸⁵. Apart from the *S. littoralis* esterase SICXE7, where k_{cat} was reported to be 0.4 s^{-1} for two acetate pheromone components and 36 s^{-1} for (*Z*)-3-hexenyl acetate (a green leaf volatile), and the *S. exigua* esterase SexiCXE4, where the reported values were similar to that of *S. littoralis* but 25 s^{-1} against (*Z*)-3-hexenyl acetate, kinetic data are not yet available for the rest of these enzymes.

It is not surprising that esterases are implicated in insect ODE functions, as many insect species inhabit fruity environments where ester volatiles are abundant. Many insects also have esteratic components in their pheromone blends^{13,14}. Esterases are among the most genetically variable catabolic enzymes known in both plants and animals⁸⁶.

Eukaryotic esterases were originally classified on functional grounds into four different groups, namely acetyl esterases, arylesterases, carboxylesterases and cholinesterases. This classification relies mostly on their sensitivities *in vitro* to diagnostic concentrations of three groups of inhibitors, namely sulfhydryl reagents (e.g. p-chloromercuribenzoic acid, PCMB), organophosphates (typically paraoxon or di-isopropyl fluorophosphate, DFP) and eserine sulphate. Carboxylesterases, which are only inhibited by organophosphates, are the most numerous and best characterized in insects, including *D. melanogaster*⁸⁷⁻⁸⁹. Interestingly, the first biochemical polymorphism discovered in *D. melanogaster* was a carboxylesterase called Esterase 6 (EST6)⁹⁰. Genome sequencing work has now identified a gene family called the carboxyl/cholinesterase family which, in insects at least, includes all their known carboxyl and cholinesterases and also some arylesterases⁹¹.

The carboxyl/cholinesterase gene family is subdivided into three putatively functional groups, each of which is made up of several clades. The first group contains intracellular esterases often linked to xenobiotic metabolism while the second group is primarily composed of secreted esterases, which are mainly implicated in hormonal and pheromonal functions⁸⁶. The last group is mainly comprised of non-catalytic neuro/developmental esterases. Candidate ODEs might be expected to be in the second functional group of clades.

1.5. β -esterases in *D. melanogaster*

β -carboxylesterases are so called because they have a preference for β -naphthyl esters over α -naphthyl esters in *in vitro* assays⁹². In the melanogaster species group of the subgenus Sophophora, the genes encoding β -carboxylesterases form a tightly linked cluster of two tandemly arranged genes named *Est6* and *Est7* (the latter also referred to as *EstP*)⁹³. These genes encode secreted esterases belonging to clade E of the carboxyl/cholinesterase gene family⁸⁶.

EST6, the enzyme produced by the upstream gene *Est6*, is widely distributed across various tissues, e.g. cuticle, haemolymph, salivary gland etc., in many *Drosophila* species. Interestingly, in its ancestral state (as seen in *D. yakuba* and *D. erecta*) it exists as a homodimer expressed mainly in the haemolymph across various life stages⁹⁴. However, in the melanogaster subgroup (*D. melanogaster*, *D. simulans* and *D. mauritiana*) it has acquired an ejaculatory duct function and primarily exists as a monomer, while retaining its haemolymph activity⁹⁴. There is considerable debate as to whether *Est7* in *D. melanogaster* is becoming a functionless pseudogene because, at least in *D. melanogaster*, a high frequency of *Est7* null alleles are found⁹⁵. *Est7* lies 197 bp 3' of the *Est6* coding region and is also transcribed in the same direction, with an overall similarity of 64% and 60% at the DNA and protein levels, respectively, with *Est6*⁹⁵. *Est7*, encodes a homodimer whose expression is mainly confined to late larval integument⁹⁶ but also includes a brief pulse of expression in early embryos⁹⁷. Little is known about the function of EST7, however, its expression in the larval cuticle might imply some role in processing esters from the external environment.

The functional role of the ancestral haemolymph activity of EST6 still remains to be elucidated but it is known that the acquired ejaculatory duct expression of EST6 in the melanogaster subgroup plays a significant role in its reproductive biology^{98,99}. In these species the EST6 produced in the ejaculatory duct of the male¹⁸ is transferred to the female reproductive tract during mating, alongside cVA^{23,100}. It is then rapidly translocated to the female haemolymph, where traces of it can be detected for several days, although it is not known where it goes from there^{101,102}. Oakeshott *et al.*¹⁰³ raised the possibility that the evolutionary shift of EST6 to its monomeric structure in the melanogaster subgroup is to facilitate its transport of EST6 across the female vaginal

wall. It was noted above that there is also endogenously produced EST6 in the haemolymph ¹⁰⁴ and the antenna of the female before mating ^{105,106} and against this background it remains unclear how the additional male donated enzyme in the female haemolymph impacts her overall physiology, and leads to downstream behavioural effects. What is clear so far however, from comparison of females mated to *Est6* null and wild type males, is that male-donated EST6 affects female post-mating behaviour, specifically in stimulating egg laying and delaying receptivity to re-mating ⁹⁹.

Similar effects have also been reported for sex peptides present in the semen, but no evidence exists as to any direct interactions between the sex peptides and EST6 ^{107,108}. Although EST6 shows activity against a broad range of artificial substrates such as α/β -naphthyl acetate and various propionates *in vitro*, its *in vivo* substrate(s) still remains to be elucidated ⁸⁶. Early claims that it catalyses the hydrolysis of cVA ¹⁰⁹ were later withdrawn ¹⁰⁰. Although a *D. melanogaster* laboratory culture possessing a null allele of EST6 shows no apparent loss of fitness, it is noteworthy that no null alleles have been found in the field ^{99,110,111}.

Two recent studies suggest a role of EST6 in *Drosophila* olfaction. The first study was by Anholt and Williams ¹⁰⁵, where the soluble proteome of the antenna was examined. Their results show the presence of EST6 in the antenna of both males and females. The second study ¹¹², published by our French collaborators during the course of this thesis, shows that there is indeed a yet to be understood relationship between EST6 and antennal cVA biochemistry. This study produced electroantennograms (EAGs) of adult male flies exposed to cVA, allowing the average output of the antenna to the brain for that odour to be measured. The result shows that prolonged stimulation by cVA leads to adaptation (i.e. reduction of sensitivity and a prolongation of response kinetics) in wildtype *Est6* flies whereas *Est6* null flies show much less adaptation under the same conditions. This suggests the possibility of EST6 acting as an ODE, allowing repolarization of the membrane and ultimately adaptation. The anomaly here of course is the apparent lack of EST6 activity against cVA, at least *in vitro*, as outlined above.

The localization of EST7 activity primarily to the carcasses of late third instar larvae points towards a specific function during this major metamorphic transition ⁹⁶. However, as mentioned earlier, the high frequency of *Est7* null alleles in the field suggests that the

function may be dispensable and no significant effects on relative fitness have yet been found ⁹⁵. Orthologues of *Est7* have been identified in *D. erecta*, *D. yakuba*, *D. simulans* and *D. mauritiana* but their expression profile has not yet been documented ⁹⁴.

1.6. JHE and JHEdup

Another carboxylesterase that became increasingly relevant during the course of this study due to its antennal selective expression is JHEdup. The *Jhedup* gene is an adjacent duplication of the *Jhe* gene ¹¹³. JHE performs a specific function in the hydrolysis of the conjugated methyl ester juvenile hormone (JH) ¹¹⁴ and therefore plays a key role in insect development and reproduction. JH regulates the transition between larval moults and larval-pupal metamorphosis through its interaction with ecdysone. It also helps to regulate egg production, development of both male and female gonadotrophic organs ¹¹⁵ and, in other insects, caste determination ¹¹⁶ and diapause ¹¹⁷.

Little is known about the function of JHEdup although it has been shown to have good activity against artificial substrates such as short chain esters of 4-methyl umbelliferone (such as acetate and butyrate) *in vitro*, with K_M values in the low micromolar range ¹¹⁸. In this respect, it has comparable specificities among various 4-methyl umbelliferone substrates as JHE ¹¹⁸. Little was known about the expression of JHEdup across tissues until this thesis, but interestingly, its expression in the head of *D. melanogaster* has recently been shown to be regulated by ethanol ¹¹⁹. The expression of OBPs such as LUSH is also regulated by alcohol ¹²⁰, thus mediating chemosensory responses in *D. melanogaster* ⁵³. Perhaps alcohols produced in the sensillar lymph by the action of esterases such as JHEdup on volatile esters have a similar regulatory effect. JHE and JHEdup are secreted esterases belonging to clade F of the carboxyl/cholinesterase gene family ⁸⁶.

1.7. Scope and goals of this thesis

Surprisingly, little work has been done to date to investigate ODEs compared to the tremendous efforts on ORs and OBPs. This is unfortunate for two reasons. Firstly, the olfactory system cannot be understood comprehensively without understanding ODE functions. Secondly, ODEs may have potential as targets for insect pest control. If odours

are perceived as precise mixtures of structural isomers or other closely related compounds, as is often the case^{121,122}, then manipulation of, or interference in the ODE for particular components of the mixture could alter the blend ratio within a sensillum, resulting in misperception of the odour.

This thesis therefore focuses on the identification and characterisation of ODEs. In particular it attempts to address some key questions in the model species *D. melanogaster*:

- a. What candidate ODEs are present in this insect's primary olfactory organs, in particular the antenna? Are any of them antennae-specific?
- b. Are the candidate ODEs able to degrade food and pheromonal odorant molecules, both *in vitro* and *in vivo*? How do these activities relate to the behavioural effects of these odorants?
- c. Are individual ODEs capable of degrading a range of volatiles or are they odorant-specific?

These questions are addressed in the following three chapters, which are copies of three papers I have published during the course of my PhD studies. A final chapter then synthesises the results in a new view on the role of ODEs in maintaining the ongoing sensitivity of an insect's olfactory system. This also includes a commentary on the prospects of targeting ODEs in new strategies for insect pest management. Another paper on an insect esterase not involved in olfaction, for which I have provided enzyme activity data and am a co-author, is also attached (Appendix 1).

1.8. Papers published

The first paper attached is titled "**Identification of candidate odorant degrading gene/enzyme systems in the antennal transcriptome of *Drosophila melanogaster***". The paper describes the first comprehensive transcriptome analysis of *D. melanogaster* antennae. It highlights the identification of an antennal-selective enzyme, JHEdup, and compares its catalytic activity with known insect ODEs from other species. The second paper presented here is titled "**An antennal carboxylesterase from *Drosophila melanogaster*, Esterase 6, is a candidate odorant-degrading enzyme toward food**".

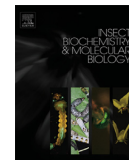
odorants". It presents biochemical, electrophysiological and behavioural studies on the possible role of EST6 in re-priming sensory neurons and shows how the lack of it, after exposure to food odours, may subsequently modulate the behavioural response of a fly. Finally, the last paper, titled "**Molecular basis for the behavioral effects of the odorant degrading enzyme Esterase 6 in *Drosophila***", showcases the range of food volatiles that EST6 can act on and addresses the key question of whether it is a general or odorant-specific ODE. It includes a crystallographic structure of EST6 and establishes beyond question that EST6 does not degrade cVA.

**Chapter 2: Identification of candidate odorant degrading
gene/enzyme systems in the antennal transcriptome of
*Drosophila melanogaster***



Contents lists available at ScienceDirect

Insect Biochemistry and Molecular Biology

journal homepage: www.elsevier.com/locate/ibmb

Identification of candidate odorant degrading gene/enzyme systems in the antennal transcriptome of *Drosophila melanogaster*



Faisal Younus^{a, b}, Thomas Chertemps^c, Stephen L. Pearce^{a, b}, Gunjan Pandey^a,
 Françoise Bozzolan^c, Christopher W. Coppin^a, Robyn J. Russell^a,
 Martine Maibèche-Coisne^c, John G. Oakeshott^{a, *}

^a CSIRO Ecosystems Sciences, Black Mountain, Clunies Ross Street, Canberra, ACT 0200, Australia

^b Research School of Chemistry, Australian National University, Canberra, ACT 0200, Australia

^c Université Pierre et Marie Curie, Institut d'Ecologie et des Sciences de l'Environnement de Paris, F-75252 Paris, France

ARTICLE INFO

Article history:

Received 2 July 2013

Received in revised form

3 July 2014

Accepted 7 July 2014

Available online 16 July 2014

Keywords:

Antenna

Transcriptome

Esterase

Cytochrome P450

Odorant degradation

ABSTRACT

The metabolism of volatile signal molecules by odorant degrading enzymes (ODEs) is crucial to the ongoing sensitivity and specificity of chemoreception in various insects, and a few specific esterases, cytochrome P450s, glutathione S-transferases (GSTs) and UDP-glycosyltransferases (UGTs) have previously been implicated in this process. Significant progress has been made in characterizing ODEs in Lepidoptera but very little is known about them in Diptera, including in *Drosophila melanogaster*, a major insect model. We have therefore carried out a transcriptomic analysis of the antennae of *D. melanogaster* in order to identify candidate ODEs. Virgin male and female and mated female antennal transcriptomes were determined by RNAseq. As with the Lepidoptera, we found that many esterases, cytochrome P450 enzymes, GSTs and UGTs are expressed in *D. melanogaster* antennae. As olfactory genes generally show selective expression in the antennae, a comparison to previously published transcriptomes for other tissues has been performed, showing preferential expression in the antennae for one esterase, JHEdup, one cytochrome P450, CYP308a1, and one GST, GSTE4. These largely uncharacterized enzymes are now prime candidates for ODE functions. JHEdup was expressed heterologously and found to have high catalytic activity against a chemically diverse group of known ester odorants for this species. This is a finding consistent with an ODE although it might suggest a general role in clearing several odorants rather than a specific role in clearing a particular odorant. Our findings do not preclude the possibility of odorant degrading functions for other antennally expressed esterases, P450s, GSTs and UGTs but, if so, they suggest that these enzymes also have additional functions in other tissues.

© 2014 CSIRO. Published by Elsevier Ltd. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/3.0/>).

1. Introduction

The rapid inactivation of signals by odorant degrading enzymes (ODEs) plays an integral role in insect chemoreception. This is because it prevents the accumulation of stimulants and subsequent sensory adaptation (Vogt and Riddiford, 1981), allowing the insect to rapidly respond to changes in the volatiles in its environment. However, relatively few ODEs have been characterized to date. They include certain esterases, cytochrome P450s, glutathione S-transferases (GSTs), UDP-glycosyltransferases (UGTs), aldehyde oxidases and alcohol dehydrogenases which show high specificity for known

odorants and occur in major chemosensory tissues such as the sensillar lumen, antennal integument, waxy cuticle and scales (reviewed in Vogt, 2005). Insect ODEs thus belong to well-studied detoxification enzyme classes, mostly known for their involvement in the metabolism of exogenous (xenobiotics, allelochemicals) and endogenous (hormones, vitamins) compounds (Li et al., 2007).

The first ODE to be identified was an antennae-specific esterase (ApoISE) isolated from the sensillar fluid of male silkworm, *Antheraea polyphemus* (Vogt and Riddiford, 1981). Vogt et al. (1985) demonstrated that ApoISE could effectively degrade the acetate component of the pheromone blend; in the presence of this esterase the half-life of the acetate *in vivo* was conservatively estimated to be just 15 ms. Furthermore, work on a purified preparation of the enzyme subsequently suggested considerably

* Corresponding author. Tel.: +61 262464157.

E-mail address: john.oakeshott@csiro.au (J.G. Oakeshott).

higher K_{cat} values than the original estimate (127 s^{-1} cf. $\sim 0.033 \text{ s}^{-1}$) (Ishida and Leal, 2005). Other studies using purified recombinant antennal esterases from *A. polyphemus* and Japanese beetle *Popillia japonica* (Ishida and Leal, 2005, 2008) have yielded similarly high K_{cat} estimates for candidate pheromone esters. A few more antennal esterases have since been identified in the moths *Mamestra brassicae*, *Sesamia nonagrioides*, *Spodoptera littoralis* (Maibèche-Coisne et al., 2004; Merlin et al., 2007), *Epiphyas postvittana* (Jordan et al., 2008), *Spodoptera exigua* (He et al., 2014) and the bee *Apis mellifera* (Kamikouchi et al., 2004). Apart from the *S. littoralis* esterase SICXE7 and *S. exigua* esterase SexiCXE4, kinetic data are not yet available for these latter enzymes. K_{cat} values for SICXE7 and SexiCXE4 were reported to be $0.3\text{--}0.4 \text{ s}^{-1}$ with their respective acetate pheromone components and 36 s^{-1} and 25 s^{-1} respectively with (Z)-3-hexenyl acetate, a green leaf volatile.

The next most studied group of detoxification enzymes linked to odorant degradation is the cytochrome P450s. While these enzymes are often associated with xenobiotic degradation (Feyereisen, 2006; Gilbert, 2004), it has also been suggested that they could protect insect olfactory receptor neurons (ORNs) against volatile natural toxins or insecticides (Ding and Kaminsky, 2003). Cytochrome P450s have also been implicated more directly in pheromone metabolism. For example, the degradation of an alkaloid pheromone by an antennae-specific P450 has been reported in pale brown chafer *Phyllopertha diversa* (Wojtasek and Leal, 1999). Significantly also, treatment of *P. diversa* sensilla with a P450 inhibitor induced anosmia in pheromone detecting olfactory receptor neurons (ORNs) (Maibèche-Coisne et al., 2004). Furthermore, two P450 genes (CYP4L4 and CYP4S4) in the moth *M. brassicae* have been shown to be strongly expressed in olfactory sensilla trichodea specifically tuned for detection of odorants and pheromones (Maibèche-Coisne et al., 2002).

Glutathione S-transferases are also key detoxification enzymes in various insect species. These enzymes catalyse the conjugation of glutathione to electrophilic molecules, increasing their solubility and facilitating their elimination (Enayati et al., 2005). Like the esterases and P450s, these enzymes have often been involved in insecticide (Li et al., 2007) and plant allelochemical metabolism (Despres et al., 2007) but one study reports that GSTs can also take part in signal termination. In the tobacco hornworm, *Manduca sexta*, a GST restricted to pheromone sensilla called GST-ms01f plays a significant role in sex pheromone detection by inactivating the aldehyde component of the sex pheromone blend (Rogers et al., 1999).

UGTs are another group of enzymes linked to odorant and xenobiotic degradation (Ahn et al., 2012). In insects UGTs are known to assist in enzymatic detoxification by catalyzing the glycosylation of lipophilic compounds (Despres et al., 2007). The role of UGTs in vertebrate olfaction is well established (Heydel et al., 2010; Lazard et al., 1991) but in insects the evidence is limited to the report of some UGT expression in antennae of *Drosophila melanogaster* (Wang et al., 1999), *Bombyx mori* (Huang et al., 2008) and *M. sexta* (Robertson et al., 1999). UGT activity has been shown to play a key role in detoxification of plant allelochemicals in *B. mori* (Luque et al., 2002).

Because of the diversity of detoxification enzymes expressed in each species and the variety of the possible functions that they can play, identification and characterization of individual members of these enzyme families which are specialized in odorant degradation within the antennae is still challenging, even in *D. melanogaster*. Up to date, only one esterase, (Esterase 6; EST6) and one P450 (Cyp6a20) have been implicated in odorant detection and perception in *D. melanogaster* (Chertemps et al., 2012; Wang et al., 2008). Thirty five esterases, 87 P450s, 37 GSTs and 19 UGTs have been annotated in the genome of this species, but the

repertoire of detoxification enzymes expressed in its antennae has not been established until now.

Here we report the antennal transcriptome of *D. melanogaster*. Prior availability of high quality genome sequence and transcriptomic data for many other tissues of this species then enables us to identify antennae-selective esterases, P450s, GSTs and UGTs as putative candidate ODEs. As seen above, one criterion commonly used to identify putative ODEs is their preferential expression in olfactory tissues and almost all the ODEs functionally characterized to date have proven to be antennal-specific, or at least antennal-enriched.

As a further insight into possible functions of candidate ODEs, we also compared the transcriptomes of the antennae of unmated males and mated and unmated females. Contrary to Lepidoptera, *D. melanogaster* males and females have very similar olfactory sensitivities. The studies available tend to show that the expression of odorant-receptors and ionotropic receptors are generally quite similar under different conditions (sex, development, reproductive state, and social environment), although expression of odorant binding proteins is more variable (Zhou et al., 2009). Very little is known about ODEs in this respect.

Finally, an esterase, JHEdup, identified as selectively expressed in the olfactory organs of both sexes, was expressed in the baculovirus system and shown to have high catalytic efficiency against some bioactive fruit volatiles, validating the strategy used to identify candidate ODEs.

2. Materials and methods

2.1. Insect rearing and tissue collection

Canton-S cultures were reared on a $24 \text{ }^\circ\text{C}$ 12 h light: 12 h dark cycle on standard yeast/cornmeal/agar food medium. The adults were removed after 72 h of egg laying. Virgins were collected on ice within 4 h of eclosion and aged in single sex groups of 20, on standard media. Two replicates of approximately 1000 antennae were dissected by hand from 4 to 5 day old virgins of each sex, placed promptly in ice-chilled TRIzol (Invitrogen, California), and snap frozen in liquid nitrogen until further treatment.

Antennae from mated females were obtained in a similar way. 50 cohorts of 25, 4–5 day old virgin females were each combined with 10 similarly aged virgin males in fresh vials and allowed to mate. Flies were observed to ensure that only females that had completed mating were selected for further work. These females were then removed from the vials and left for 1–3 h, after which their antennae were dissected as described above.

For qPCR experiments, male and female antennae and proboscides, legs, abdomens, and heads without chemosensory appendages were collected from adults aged 5 days since eclosion and stored at $-80 \text{ }^\circ\text{C}$ before RNA extraction.

2.2. RNA extraction, sequencing and cDNA synthesis

Tissues were homogenised in 1 ml of TRIzol (Invitrogen, California) using a motorized homogenization drill and total RNA was extracted as per the manufacturer's instructions. Quantification of the RNA was carried out using a Nanodrop spectrophotometer and the quality of the total extracted RNA was assessed using an Agilent Bioanalyzer (Agilent Technologies, California). For antennal tissues, 500 bp paired-end sequencing libraries were generated using the Truseq mRNA preparation kit (Illumina, California) as per the manufacturer's instructions and sequenced on an Illumina HiSeq2000.

For qPCR analysis, total RNAs (5 μg) from various tissues were treated with DNase I (Roche, Switzerland) before single-stranded

Table 1
Statistical summary of *D. melanogaster* antennal transcriptomes.

Sample	Replicate	No. of raw reads	No. of read pairs	No. of genes with	
				FPKM > 1	FPKM > 10
Virgin male	1	31779788	29603450	8932	4785
	2	24747081	22767056	9615	4239
Virgin female	1	31665940	29085595	8777	4711
	2	22296913	20202286	8717	4626
Mated female	1	21433041	18305488	8876	4615
	2	29343121	27132416	8710	4606

cDNA synthesis using Superscript II reverse transcriptase (Invitrogen, California) and oligo(dT)18 primer, according to the manufacturer's instructions.

2.3. Data processing and analysis

Reads were filtered and trimmed using the SolexaQA package (Cox et al., 2010), with a quality cut-off of 20 and a minimum read length of 25. The average read length calculated was 100 and the average quality of reads was determined to be Phred 37. These reads were mapped to the *D. melanogaster* genome using the Tophat aligner with default parameters (Trapnell et al., 2009). The quality control processes resulted in high quality reads, with 95–97% of reads in each sample having both paired reads aligned to the genome. Expression estimates for annotated genes (NCBI release 5.3) were generated with Cufflinks (Trapnell et al., 2010) (Table 1) using the upper quartile normalisation (-N), fragment bias correction (-b) and multi-read correction (-u) options. To identify candidate antennal-specific genes, the expression values determined in the antennal samples were compared with those obtained from other tissue-specific samples from the modENCODE database (Graveley, 2011). For comparison across different experiments, the FPKM values were binned into five categories (from low to extremely high expression) with cut off values selected to best cover the range and distribution of expression values from all samples. DESeq (Anders and Huber, 2010) was used to test for differential expression of transcripts between samples. A false discovery rate of 0.05 was used to detect genes with differential expression between samples. Functional analysis of all genes detected as expressed in the antennal samples, as well as those determined to be differentially expressed, was carried out using the PANTHER™ (Protein ANalysis THrough Evolutionary Relationships) Protein Classification System (Applied Biosystems, Foster City, CA). The expression of selected genes in protein families known or predicted to play a role in odorant degradation was validated by qRT-PCR.

Table 2

Major classes of antennal protein detected in the antenna of *D. melanogaster*. RNA-seq reads generated from dissected antennae were mapped to the genome and the level of expression was determined in terms of FPKM (Fragments per Kilobase per Million Mapped reads). Genes with FPKM > 1 were considered as detected. The total number of genes complementing the major classes of antennal proteins in the *D. melanogaster* genome was assembled from Flybase (Marygold et al., 2013). For each sample the number of transcripts identified as belonging to the respective antennal protein class is given along with its percentage (in brackets) of the total genome complement in that class.

Major classes of antennal protein	Genome complement	Detected in males (%)	Detected in virgin females (%)	Detected in mated females (%)
Odorant binding proteins (OBP)	52	30 (58)	28 (54)	30 (58)
Odorant receptors (Or)	61	39 (64)	39 (64)	38 (62)
Gustatory receptors (GR)	60	11 (18)	10 (17)	11 (18)
Ionotropic receptors (IR)	66	19 (29)	19 (29)	18 (27)
Chemosensory proteins (Che A/B)	20	4 (20)	5 (25)	5 (25)
Sensory neuron membrane proteins (SNMP)	2	2 (100)	2 (100)	2 (100)
Carboxylcholinesterases	35	25 (71)	26 (74)	26 (74)
Cytochrome P450s (P450)	87	57 (66)	56 (64)	57 (66)
Glutathione-S-transferases (GST)	37	31 (83)	30 (81)	31 (83)
UDP-glycosyltransferases (UGT)	19	9 (47)	9 (47)	9 (47)

2.4. Confirmation of candidate gene expression profiles by qRT-PCR

A qRT-PCR study was conducted on some key genes to address their tissue distribution in more detail and check any differences apparent between male and mated and unmated female antennae. All reactions were performed as previously described (Durand et al., 2011) on the LightCycler® 480 Real-Time PCR System (Roche, Switzerland). Each reaction was run in triplicate with at least three independent biological replicates. The *Pgk*, *RPS20* and *Rp49* genes were used as reference genes (Chertemps et al., 2012). Specific primers were designed using AMPLIFIX software (<http://www.brothersoft.com/amplifix-159421.html>) and are shown in Supplementary Table 1. Normalized expression was calculated using the standard ddCT method with Q-Gene software (Simon, 2003).

2.5. Assays of JHEdup activity

JHEdup (FlyBase Release 6 version, not the N-terminal truncated version in Crone et al., 2007) was heterologously expressed using the BacuVance™ Baculovirus Expression System in SF9 cells, using the service provided by Genscript (USA). Native *D. melanogaster* gene sequence (including its signal peptide) was used. Most of the protein was found to be expressed in the media, which was further concentrated (~10-fold) using a 30 K Amicon filter, to achieve the desired working concentration for the enzyme. The titre of the expressed enzymes was determined by testing for diethyl 4-methylumbelliferyl phosphate (dEUP, Sigma) degradation by the fluorometric methods of Coppin et al. (2012). The activity of JHEdup towards the test odorants (all purchased from Sigma Aldrich) was monitored in triplicate by gas chromatography/mass spectrometry (GC–MS) assays using methods modified from those described in Jackson et al. (2013). Briefly, each reaction mixture (200 µl) consisted of odorant substrate (200 µM), enzyme (0.21169 nM), BSA (5 µg, for enzyme stability) and ethanol (5% v/v) in 25 mM Tris–HCl buffer (pH 8.0). The reaction was carried out in a silanized vial (Agilent, USA) at 25 °C and quenched at specific time points with an equivalent amount of ice chilled hexane containing 250 µM of heptanone (Sigma Aldrich, Australia) as a standard. The tubes were then placed in a vortex shaker (MS1 minishaker, IKA, Germany) for 15 min at maximum speed. The upper (hexane) layer was then carefully transferred with a glass pipette to a deactivated glass insert (Agilent) for analysis with an Agilent 7890 series GC–MS. The compounds were separated on a J&W DB-WAX column (30m × 250µm × 0.25 µm, Agilent) with helium (2 ml/min) as the carrier gas. The oven temperature was initially set at 50 °C for 2 min and then subsequently increased with a gradient of 10°C/minute to 275 °C and held for 10 min. The injector and detector temperature was set at 250 °C and a 10:1 split ratio was used.

Due to the limitations of the above assay (e.g. sample processing time, consumables used and limited dynamic range in working substrate concentration) we were unable to determine Michaelis–Menten kinetics directly. As an alternative, we took advantage of the fact that each substrate in a reaction containing two competitive substrates can be treated as a competitive inhibitor of the other. Thus the inhibition constant, K_i , of each “inhibitor” is equivalent to its Michaelis constant, K_m (Cornish-Bowden, 1995; Eisenthal et al., 2007). Having determined K_m and activity at a

single known substrate concentration in the methods above, one can then use the Michaelis–Menten equation to derive K_{cat} . To this end we used the odorant substrates as inhibitors of the chromogenic substrate 4-nitrophenyl acetate, the hydrolysis of which can be monitored in a facile continuous microplate assay. Initially the full Michaelis–Menten kinetics of 4-nitrophenyl acetate were determined by monitoring the formation of 4-nitrophenol in a clear 96-well plate (Greiner Bio-one, Germany) using a SpectraMax 190 spectrophotometer (Molecular Devices, USA) set at 28 °C. Wells

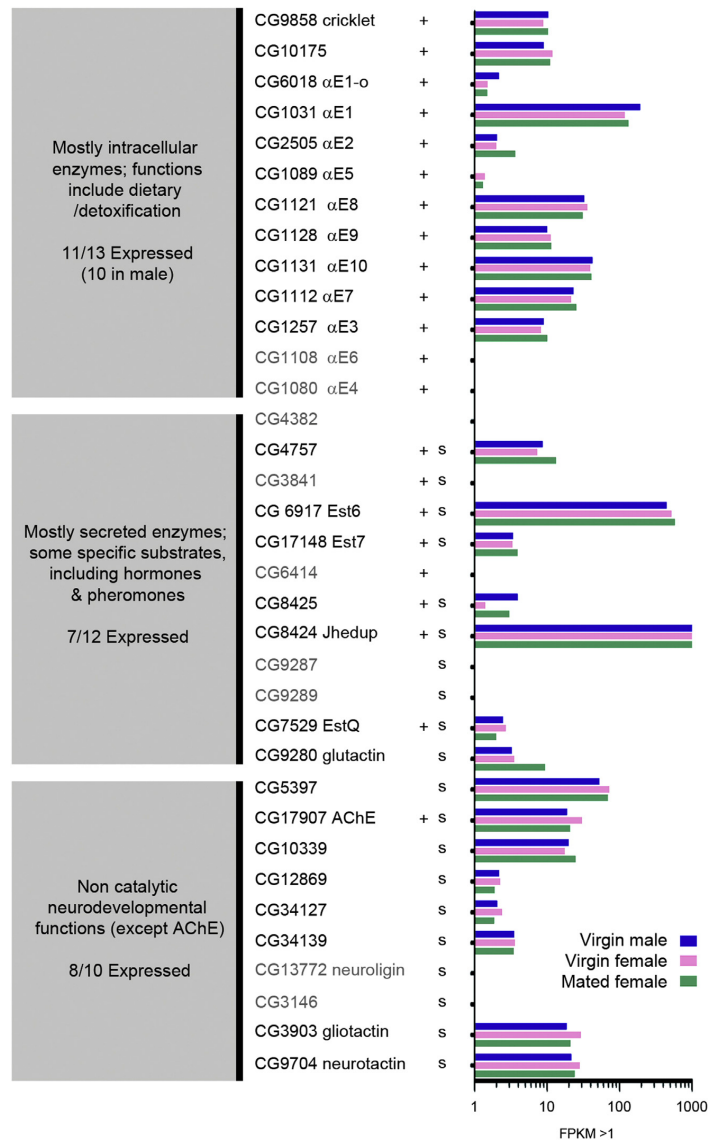


Fig. 1. Expression level of carboxyl/cholinesterases in the antennae of *D. melanogaster*. The classification system of esterases described in Oakeshott et al. (2005) was used to designate the clades. Broad functions associated with the *D. melanogaster* or related genes are described in the shaded box. The presence of a functional catalytic triad (Ser-His-Glu or Ser-His-Asp) is indicated by the symbol + and s signifies the presence of a secretion signal. Esterases not expressed in any of the three treatment samples are shown in grey. Expression values are means of the Cufflinks-determined FPKM values for the two samples in each treatment.

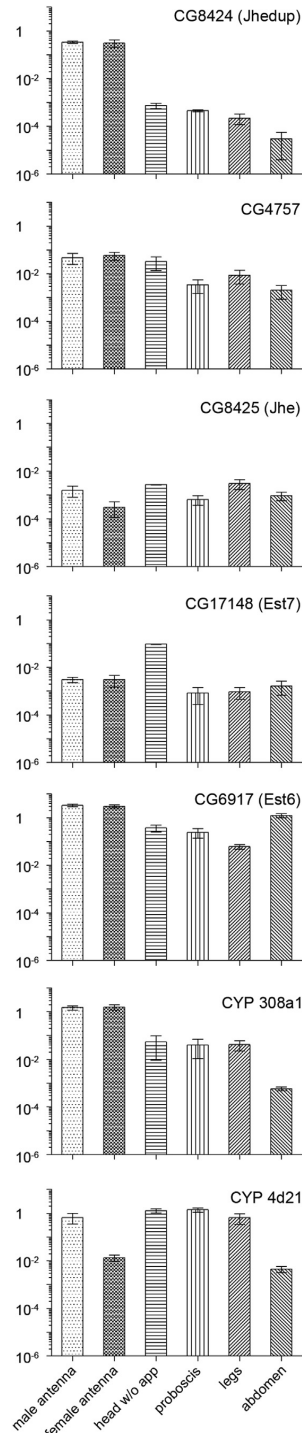


Fig. 2. Expression analysis of candidate genes by qRT-PCR across various tissues. Compared to other tissues *Jhedup* shows antennal selectivity. *Est6* is most abundantly

contained substrate (20–2000 μM), enzyme (2.09 nM), BSA (5 μg , for enzyme stability) and ethanol (5% v/v) in 25 mM Tris–HCl buffer (pH 8.0) in a 200 μl volume and reactions were monitored at 400 nm every 10 s over a 15 min time course. Michaelis–Menten constants were calculated using the GraphPad Statistical package (Prism, USA). To then determine the K_i of the odorant substrates, a similar assay was performed except that the 4-nitrophenyl acetate concentration was held constant (400 μM) and the odorant substrate was included at different concentrations (0–4000 μM). K_i was calculated from the formula:

$$K_i = \frac{\frac{v_0}{i} - [I]}{1 + \frac{[S]}{K_m}}$$

where $i = 1 - a$; $a = v_i/v_0 =$ relative activity; $v_i =$ the initial velocity at a given $[S]$ in the presence of inhibitor $[I]$; and $v_0 =$ the initial velocity at the same $[S]$ in the absence of inhibitor (Segel, 1993).

3. Results and discussion

3.1. Overview of the antennal transcriptome and identification of candidate olfactory genes

High quality 500 bp libraries were generated, with the number of filtered reads ranging between 18 and 30 million across the three types of antennae and the two replicates of each type analysed (Table 1). The number of genes with at least one read mapped varied from 11,305 to 13,640 across the six libraries. Using the commonly adopted arbitrary inclusion threshold of FPKM>1 (Graveley et al., 2011), we identified 8710 to 9615 genes in the antennae in one or more of the three types of samples (virgin males and mated and unmated females). This represents 55%–61% of the total of the 15,806 genes recognized in the species (NCBI build 5.3). As potential ODEs are hypothesized to be expressed at high level we also considered the application of a more stringent criterion of FPKM>10, that reduces these numbers to 4239 to 4785 genes (Table 1).

The only other dipteran antennal transcriptome assembled from RNA-seq data so far published (Pitts et al., 2011) found over 11,000 genes in *Anopheles gambiae* antennae, but used a less conservative inclusion criterion of FPKM>0. However, two other RNA-seq studies on individual *D. melanogaster* tissues using FPKM>1 also reported large numbers of genes expressed; Catalan et al. (2012) found over 10,000 with FPKM>1 in each of their eight adult brain libraries and Chang et al. (2011) found over 9000 with FPKM>1 in all six of their replicate libraries for at least one of the genotypes they analysed. In our case we suggest that the relatively large number of tissue types contained within the antennae (Wang and Sun, 2012) would contribute to the relatively high number of genes found to be expressed.

Given that a high proportion of all the genes identified in the *D. melanogaster* genome were detected in our transcriptomes, it is not surprising that the antennal protein sets that we have found contain relatively high proportions of all the broad categories of molecular function and biological process recognized by PantherTM (Mi et al., 2013) in the genome (Supplementary Fig. 1).

expressed in antenna when compared to genes encoding other secreted esterases such as *CG 4757*, *Jhe* and *Est7*. *CYP308a1* also shows antennal-selective expression when compared to other tissues. A strong male bias in the antennal expression of *CYP4d21* is indicated. All the data were obtained from triplicate experiments, using three reference genes and are plotted as means \pm SDs on a log scale.

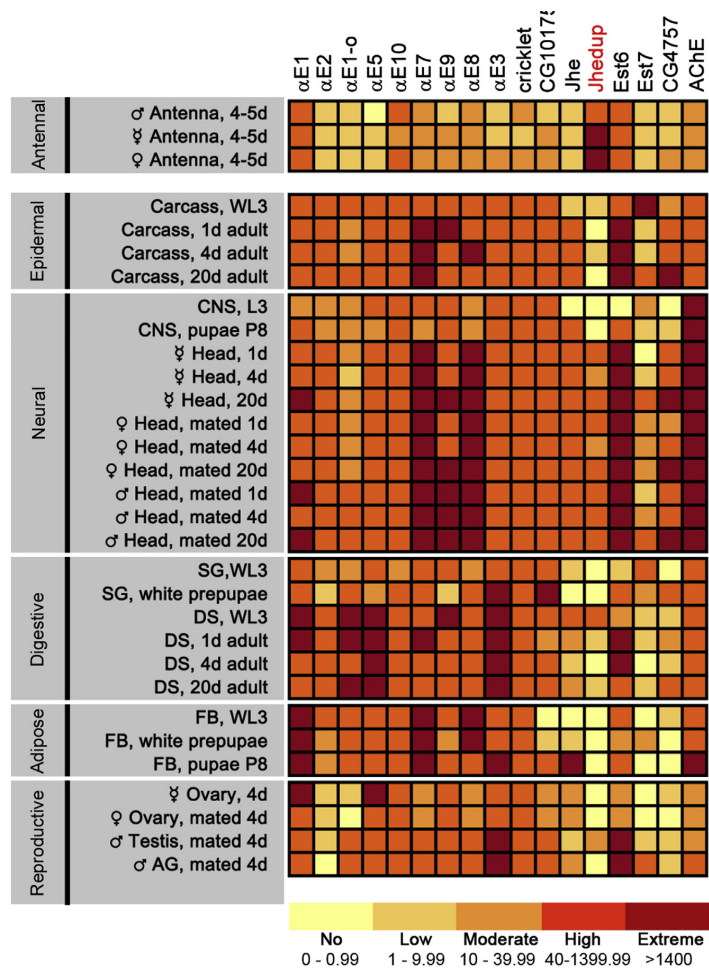


Fig. 3. Heat map illustrating the various degrees of expression of the 17 catalytically active esterases across different tissues. The tissues, i.e. antenna, carcass, central nervous system (CNS), head, salivary gland (SG), digestive system (DS), fat body (FB), ovary, testis and accessory gland (AG), were further classified into six groups (antennal, epidermal, neural, digestive, adipose and reproductive). The data (other than antennal tissues) for prepupae (P), wandering larvae (WL) and larvae (L), for various days (denoted by d), are obtained from modENCODE tissue expression data, available in Flybase (Marygold et al., 2013). The *Jhedup* gene highlighted in red appears to be antennae-selective. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.) ♀ and ♂ refer to virgin and mated females respectively.

Table 3

Comparison of kinetic properties of JHEdup against five fruit volatiles with known ODEs against their respective substrates.

ODEs	Ester	K_{cat} ($s^{-1} \pm SE$)	K_m ($\mu M \pm SE$)	Specificity constant ($M^{-1} s^{-1}$)
ApolPDE (<i>A. polyphemus</i>) ^a	E6Z11-16:acetate	127	1.27	1.00×10^8
Pjap-PDE (<i>P. japonica</i>) ^b	(R)-japonilure	1.36	680	2.00×10^3
SICXE7 (<i>S. littoralis</i>) ^c	(Z)-3-Hexenyl acetate	36	1500	2.40×10^4
	Z9E11-14:acetate	0.4	53	7.60×10^3
	Z9E12-14:acetate	0.4	37	1.08×10^4
SICXE10 (<i>S. littoralis</i>) ^d	(Z)-3-Hexenyl acetate	45	9574	4.70×10^3
SexiCXE4 (<i>S. exigua</i>) ^e	Hexyl acetate	26.18	5103 ± 1236	5.13×10^3
	(Z)-3-Hexenyl acetate	25.35	8545 ± 2480	2.97×10^3
	Z9E12-14:acetate	0.38	139 ± 50	2.75×10^3
	Z9-14:acetate	0.29	110 ± 39	2.64×10^3
JHEdup (<i>D.melanogaster</i>)	Butyl acetate	1456 ± 152	3902 ± 229	3.73×10^5
	E2-Hexenyl acetate	277 ± 24	3249 ± 98	8.54×10^4
	Hexyl acetate	3226 ± 461	7235 ± 863	4.46×10^5
	Isopentyl acetate	1080 ± 152	2849 ± 229	3.79×10^5
	Pentyl acetate	1490 ± 357	5676 ± 1206	2.62×10^5

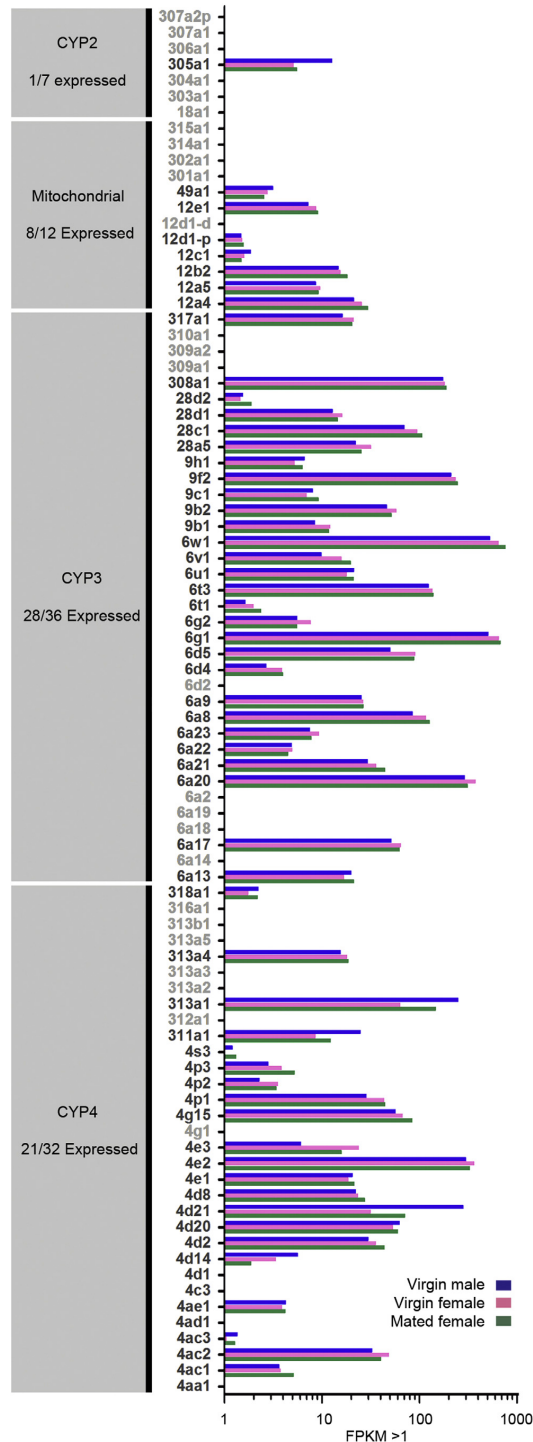
^a Ishida and Leal, 2005.

^b Ishida and Leal, 2008.

^c Durand et al., 2011.

^d Durand et al., 2010.

^e He et al., 2014.



Our study found very similar numbers of genes in the six libraries for each of the six major gene families associated with antennal chemosensory functions (odorant receptors [ORs], odorant binding proteins [OBPs], ionotropic receptors [IRs], sensory neuron membrane proteins [SNMPs], gustatory receptors [GRs], chemosensory proteins [CheAs and CheBs]) and the four major families of detoxification enzymes (esterases, P450s, GSTs and UGTs) that could include ODEs (Table 2). Overall, both of the SNMPs, nearly three quarters of the esterases, P450s, GSTs and UGTs, just over half the OBPs and ORs and about a quarter of the GRs, IRs and Che proteins annotated in the fly genome were shown to be expressed in the antennae at FPKM > 1. Interestingly, this includes seven more GRs than previously reported to be expressed in *D. melanogaster* antennae (Gr64f, Gr28b, Gr64b, Gr64d, Gr93a, Gr43a, Gr64a) (de Bruyne and Warr, 2006) plus three more IRs (IR94c, IR62a and IR60a) in addition to the 16 IRs previously described for this tissue (Rytz et al., 2013). We also report expression of four more Ches (CheA7a, CheA75a, CheB93b and CheB42c) than previously described in antennae (Starostina et al., 2009) (Supplementary Fig. 2).

The most heavily expressed genes across all ten families of interest here were OBPs (Supplementary Fig. 2); 11 of these averaged FPKM scores above 10,000, including LUSH, which is known to be required for sensitivity to the pheromone *cis*-vacenyl acetate (cVA) in *D. melanogaster* (Laughlin et al., 2008), and Obp19a, which has been previously linked to odorant binding and degradation in the signal transduction process (Shanbhag et al., 2001). The expression of the universal binding partner for ORs, Orco, which forms a heteromer with ORs to produce odorant-gated ion channels (Benton et al., 2006) was also very high in our data (Supplementary Fig. 2), which is consistent with other evidence that it is heavily expressed in most ORNs (Couto et al., 2005; Vosshall et al., 2000). Enzymes implicated in metabolism of alcohols such as alcohol dehydrogenase and formaldehyde dehydrogenase (David et al., 1981) are also well expressed in the antennae (average FPKM 475 and 61 respectively).

The numbers of members of most of the major odorant processing and detoxification families found in our *D. melanogaster* transcriptome are broadly similar to the numbers recovered from lepidopteran antennal transcriptomes, although fewer GRs and no Ches have yet been described in lepidopteran antennae.

3.2. Esterases: overview and in vitro characterization of JHEdup as a new ODE in *D. melanogaster*

We found 25 and 26 esterases expressed in *D. melanogaster* male and female antennae, respectively, at FPKM > 1 and 13 and 16 at FPKM > 10 (Fig. 1). Most members of two of the three major groups in the esterase gene family (Oakeshott et al., 2005) are represented in our transcriptomes. These are the intracellular esterases, which have often been linked to xenobiotic metabolism, and the non-catalytic neuro/developmental esterases. The expression of the intracellular group is consistent with the hypothesis that many antennally expressed esterases are performing general detoxification functions, while the expression of the neuro/developmental group presumably reflects the development of neural tissues in the antenna. The third major group of esterases, the secreted esterases, has most commonly been associated with specific hormonal and

Fig. 4. Expression level of cytochrome P450s in the antennae of *D. melanogaster*. The genes were divided into four groups, based on Tijet et al. (2001). As in Fig. 1, genes not expressed in any of the three treatments are highlighted in grey. Expression values are the mean of the Cufflinks-determined FPKM values for the two samples in each treatment.

protein in sensillar lymph, then JHEdup should be quite effective in processing these odorants. Significantly, all the substrates tested against JHEdup are short-medium chain acetates, but they contain a mix of primary and secondary and saturated and unsaturated acyl groups. While the unsaturation decreased K_{cat} by about tenfold, the data suggest that the range of potential substrates could still be broader than previously considered for ODEs. Further biochemical and electrophysiological analyses are needed to elucidate the physiological substrates for JHEdup in *D. melanogaster* antennae. Nevertheless the kinetic data herein validate the transcriptomic strategy we have used to identify particular detoxification enzymes that could play a function in olfaction as ODEs.

3.3. Cytochrome P450s

As in most other insect species, four phylogenetically distinct clades of P450s can be distinguished in *D. melanogaster*, namely the CYP2, CYP3, CYP4 and mitochondrial clades (Tijet et al., 2001). Our study reveals that only one out of seven CYP2 P450s was expressed in the antennae at FPKM > 1, whereas at least two thirds of each of the other clades were expressed in this tissue above this threshold, albeit expression levels were generally higher among the CYP3s and CYP4s (19 and 13 at > FPKM 10 respectively) than in the mitochondrial P450s (2 at FPKM > 10) (Fig. 4).

Several *D. melanogaster* CYPs in the CYP3 clade that have been linked to xenobiotic metabolism and insecticide resistance (Feyereisen, 2012) were found to be expressed in the antennae. CYP6g1, for example, has previously been associated with DDT and neonicotinoid resistance in *D. melanogaster* (Daborn et al., 2001, 2002) and our data show it to be one of the most highly expressed P450s in antennae. Cyp6a8, which is overexpressed in DDT resistant strains (Le Goff et al., 2003), is also found in antennae at a relatively high level. However, the CYP6a2 enzyme that metabolizes organochlorine and organophosphorus insecticides (Feyereisen, 2012) is not recovered in our transcriptome. Cyp6w1, also over expressed in DDT resistant strains (Pedra et al., 2004), is strongly expressed in our antennal transcriptome. The substrate for Cyp6w1 is yet unknown but our expression pattern is consistent with the findings of Wang et al. (1999).

CYP4s have been less commonly linked to xenobiotic metabolism but some have been associated with odorant or pheromone metabolism in other species (Feyereisen, 2012). Two antennally expressed *D. melanogaster* cytochrome P450s in this clade (CYP4c3 and CYP4d8) are closely related to CYP4s4 from *M. brassicae* (Maibèche-Coisne et al., 2002), which is expressed in olfactory sensilla trichodea specifically tuned for detection of odorants and pheromones (Fig. 4).

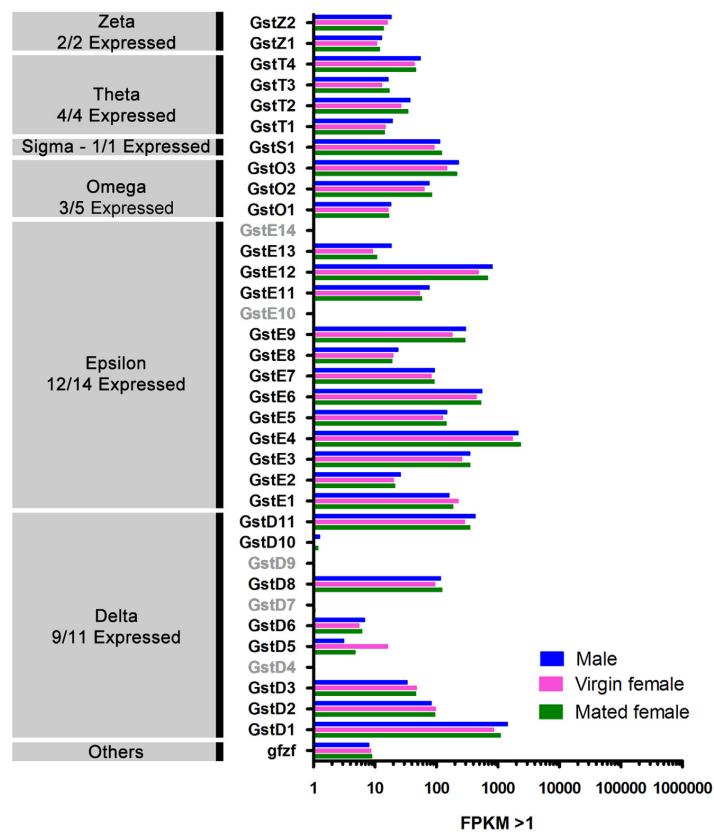


Fig. 6. Expression level of glutathione S-transferases (GSTs) in the antennae of *D. melanogaster*. The genes were divided into seven groups, based on (Low et al., 2007). As in Fig. 2, genes not expressed in any of the three treatments are highlighted in grey. Expression values are the mean of the Cufflinks-determined FPKM values for the two samples in each treatment.

All the antennally expressed CYPs are also expressed in other tissues (Fig. 5) and, as in *S. littoralis* (Pottier et al., 2012), the CYP3s and CYP4s are commonly found in gut or fat body. Their occurrence in other tissues does not preclude these enzymes from specific antennal functions; recent studies have found that CYP6a20, which is highly expressed across all the three types of antennae in our study, but has also been found by others in other tissues, can regulate pheromone sensitivity and influence aggressiveness (Wang et al., 2008). Nevertheless, we found one P450, CYP308a1, which shows a high degree of selectivity for antennal tissue, as confirmed by qPCR analysis of various tissues (Fig. 2). This enzyme is now a prime candidate for further investigation to determine its precise role in the antenna. Interestingly, five CYPs (CYP6a8, CYP6t3, CYP9f2, CYP4d2 and CYP4e2) that are expressed well in the antennae (FPKM>10 in all cases) have been shown previously to be up-regulated in the presence of ethanol or methanol in *D. melanogaster* larvae (Giraud et al., 2010; Morozova et al., 2006; Wang et al., 2012). In fact only recently it has been reported that CYPs are also responsible in adult *D. melanogaster* for the elimination of methanol, the most abundant short chain alcohol present in the oviposition sites for the flies (Wang et al., 2013). It is possible that such CYPs are also induced in the antennae to metabolise the alcohols produced by the action of esterases on incoming ester volatiles.

3.4. Glutathione S-transferases (GSTs)

GSTs are classified phylogenetically into seven groups (Low et al., 2007) and a majority of each group is expressed in the

antennae at FPKM>1, and generally at FPKM>10 (Fig. 6). Delta (GSTD) and Epsilon (GSTE) GSTs are insect-specific and to date are the only GST groups to have been implicated in insecticide resistance (Low et al., 2007). Interestingly three of them, GSTD8, GSTD11 and GSTE11, are preferentially expressed in the antennae and another one, GSTE4, appears to be antennal specific, at least by comparison to other tissues (Fig. 7). GSTE4 also shows a higher level of antennal expression than any of the other GSTs. When tested against a model GST substrate CDNB (1-chloro-2-4-dinitrobenzene), GSTE4, GSTE11 and GSTD11 all showed high catalytic efficiency (K_{cat} 83.7 s⁻¹, 60.2 s⁻¹ and 360 s⁻¹ respectively) whereas GSTD8 had a lower activity (K_{cat} 4.49 s⁻¹) (Saisawang et al., 2012). More importantly, all of these four GSTs with preferential or specific expression in the antennae show catalytic activity against naturally occurring substrates such as 4-HNE (4-hydroxynonenal) and phenethyl isothiocyanate (PEITC) (Saisawang et al., 2012). 4-HNE is a secondary cytotoxic product of lipid peroxidation which is also known to function as a signalling molecule (Awasthi et al., 2005) while PEITC is an anti-carcinogen found in edible plants (van Lieshout et al., 1998).

Among the other GSTs exhibiting high expression levels in the antennae, GSTD1 plays a critical role in metabolism of the insecticide DDT (Tang and Tu, 1994) and GSTS1 (Fig. 6) is generally associated with indirect flight muscle (Clayton et al., 1998). GSTS1 is reported to have high catalytic activity (K_{cat} 7.2 s⁻¹) in the conjugation of lipid peroxidation end products, such as 4-HNE, produced during oxidative stress (Singh et al., 2001).

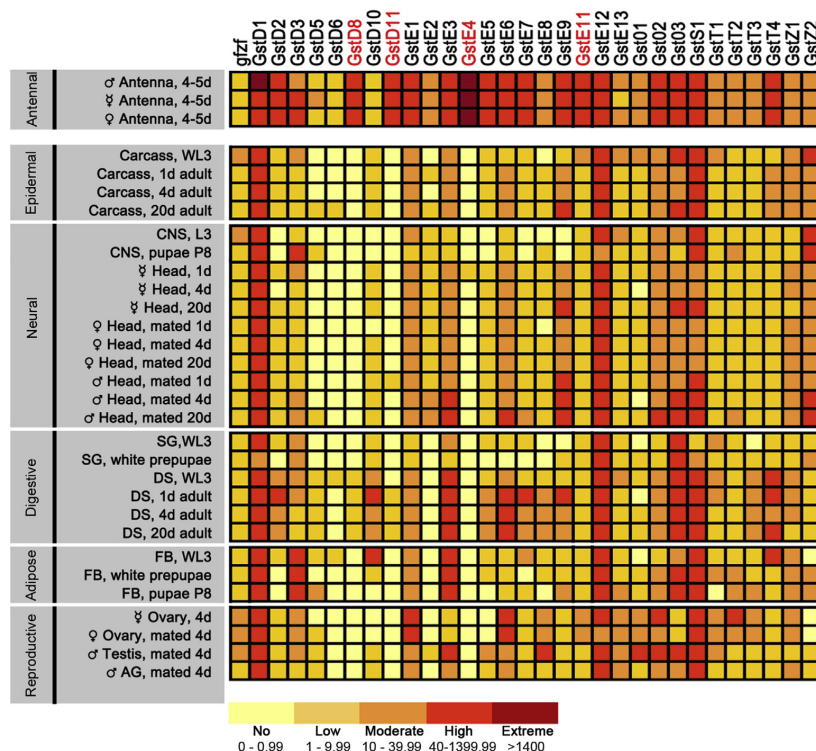


Fig. 7. Heat map comparing expression levels of 31 GSTs expressed in the antennae across different tissues. The classifications and description of the tissues and sources of data are as described in Fig. 3. GSTD8, GSTD11 and GSTE11 highlighted in red appear to be preferentially expressed compared to GSTE4 which shows antennal specific expression.

3.5. UDP – glycosyltransferases (UGTs)

Nine UGTs were found to be expressed in the antenna at FPKM>1, four of them at FPKM>10 (Fig. 8). Two of them, UGT35a and UGT35b had also previously been shown to be preferentially expressed in the third antennal segment of *D. melanogaster*, the latter suggesting possible involvement in odorant turnover (Wang et al., 1999). Fig. 9 show that four of the more highly expressed UGTs have some level of preferential expression in the antenna. UGT35b is also expressed in mated male reproductive tract. UGT35a has been suggested to participate primarily in the detoxification (Wang et al., 1999) and is also expressed highly in the digestive systems (Fig. 9).

3.6. Differences between male and female antennae and effects of mating

Our analyses identified 391 genes that are differentially expressed in the antennae of virgin males and females (Supplementary Fig. 4). Fourteen of these belong to five of the families described above (OBPs, ORs, IRs, CYPs and GSTs) (Supplementary Fig. 2). Those showing ≥ 2.5 -fold differences included one OR, one IR, two OBPs, four P450s and one GST.

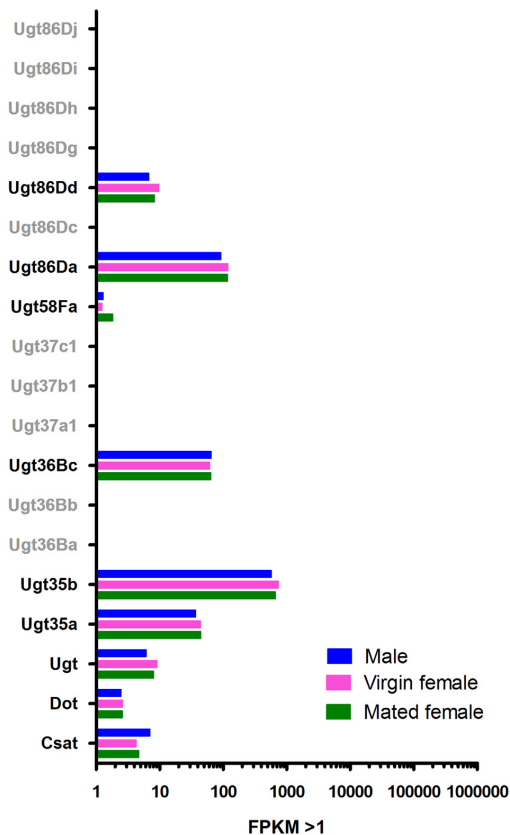


Fig. 8. Expression level of UDP-glycosyltransferase (UGTs) in the antennae of *D. melanogaster*. As in Fig. 2, genes not expressed in any of the three treatments are highlighted in grey. Expression values are the mean of the Cufflinks-determined FPKM values for the two samples in each treatment.

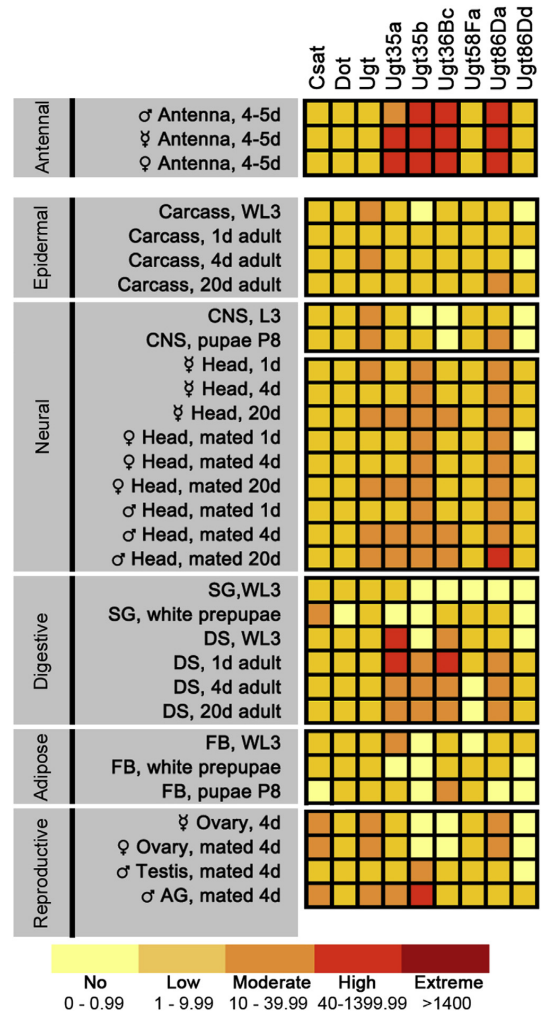


Fig. 9. Heat map comparing expression levels of 9 UGTs expressed in the antennae across various tissues. The classifications and description of the tissues and sources of data are as described in Fig. 3.

OBP99a, CYP4e3, IR75c, OR13a and GSTD5 are expressed significantly more in virgin female antennae while CYP311a1, CYP313a1, CYP4d21 and OBP8a are expressed more in male antennae. The expression of OBP99a and OBP8a has also previously been reported to be sexually dimorphic (Anholt et al., 2003; Arya et al., 2010), but predominantly located in reproductive tissue (Chintapalli et al., 2007; Takemori and Yamamoto, 2009). The expression in the latter sites suggests pleiotropic functions of these OBPs (Flint et al., 2009). Among the P450s preferentially expressed in male antennae, CYP4d21 was already known to be expressed with a strong male bias in heads (Fujii and Amrein, 2002). This bias is now also found in antennae (Fig. 6). CYP4d21 plays a function in male courtship and mating (Fujii et al., 2008) but its substrates are still unknown. Interestingly, the CYP4d21 expression pattern includes antennal chemosensory sensilla (Fujii et al., 2008), where its function also remains to be determined. GSTD5, which shows five-

fold higher expression in virgin females compared to males, is up-regulated in the presence of heavy metals and is thought to play a role in protection against heavy metal stress and maintaining metal homeostasis (Yepiskoposyan et al., 2006).

The genes showing the greatest differences in expression between the antennae of the two sexes are the yolk proteins, YP1, YP2 and YP3 (showing 117- and 136- fold greater expression in females, respectively) (Supplementary Fig. 4). These proteins were also shown to be essentially female-specific in Fujii and Amrein (2002), where they were shown to be expressed in the head. Yolk proteins have previously been found to be largely confined to female fat body tissue and ovarian follicle cells (Kraus et al., 1988) where they are associated with neurogenesis and vitellogenesis (Bownes, 1986; Burtis et al., 1991). Intriguingly, Le Goff et al. (2006) have reported that phenobarbital strongly induces all three yolk proteins in *D. melanogaster* males, whereas the herbicide, atrazine, down-regulates the same genes in the female. These authors have suggested that such sex differences in gene expression may be related to sex differences in xenobiotic metabolism.

Only 35 genes were identified as differentially expressed in the antennae of mated and unmated females (Supplementary Fig. 4B). This relatively low number may have been because of a larger difference between the transcriptional profiles of the two replicates for the mated females compared to the other two sources of antennae. Only one of the 35 cognate proteins, CYP313a1, had previously been implicated in olfactory signalling processes (Giraud et al., 2010; Willingham and Keil, 2004). Three of them were related to defence response (CG2736 and the dipterics, Dpt and DptB). CG2736 belongs to the insect CD36 protein family, members of which have diverse functions, such as carotenoid transport (ninaD, Santa Maria), removal of apoptotic cells and bacteria (Crq, Pes) but also chemoreception (SNMP) (Nichols and Vogt, 2008). Dipterics are antimicrobial peptides active against Gram-negative bacteria (Leclerc and Reichhart, 2004). Courtship is known to regulate various antennal innate immunity and olfactory signalling genes, involved either in signal perception or in the molecular interactions that occur between the sexes after mating (Immonen and Ritchie, 2012). In particular, DptB has been shown to be down-regulated in female heads after mating, as observed here in mated female antennae.

4. Conclusion

Our study presents the first comprehensive transcriptome of *D. melanogaster* antennae. We find that 55–61% of the total genome is expressed in our transcriptome at FPKM>1, and 27–30% at FPKM>10. Similar proportions of the four candidate ODE families were also expressed in the antennal transcriptome (71% of the esterases, 64% of the P450s, 81% of the GSTs and 47% of the UGTs with FPKM>1, whereas 46%, 41%, 62% and 21%, respectively, with FPKM>10 as a threshold), so the four detox families as a whole are only marginally more commonly expressed in the antennae than the average for the genome.

Consistent with this conclusion, almost all the esterases, P450s, GSTs and UGTs found in the *D. melanogaster* antennal transcriptomes have also been reported in transcriptomes from other tissues. This might support the view that many have general detoxification functions in the antennae rather than acting on specific odorant substrates. Nonetheless, this does not preclude these enzymes from having specific olfactory functions. For example, CYP6a20 and Est6, which we have found to be highly expressed in antennae and were also expressed at high levels in a range of other tissues (including those without direct exposure to the environment), have been previously linked to specific pheromone processing and behavioural phenotypes.

Strikingly however, our study did identify one esterase (JHEdup), a cytochrome P450 (CYP308a1) and a GST (GSTe4) as antennae-selective, so these enzymes become good candidates to play specific antennal functions. Our biochemical characterization of JHEdup *in vitro* supports this. Interestingly, JHEdup differs from previously characterized ODEs in that it degrades food odorants. Further work is now needed to characterize the biochemical properties and physiological functions of these three enzymes and determine the precise roles they play in chemoreception.

Competing interests

The authors have declared that no competing interests exist.

Author contributions

FY, TC, CWC, FB performed experiments; FY, TC, MMC, CWC, GP, RJR, JGO designed research, FY, SP, CWC, GP, JGO analysed data; FY, MMC and JGO wrote the manuscript.

Acknowledgements

We thank Olivia Leitch and Madeleine Gane for helping us with the fly experiments and Kaiman Peng from the John Curtin School of Medical Research at the Australian National University for her support with the transcriptome sequencing. The research was funded in part by a CSIRO OCE Postgraduate Scholarship and the French-Australian Science and Technology (FAST) Program.

Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.ibmb.2014.07.003>.

References

- Ahn, S.J., Vogel, H., Heckel, D.G., 2012. Comparative analysis of the UDP-glycosyltransferase multigene family in insects. *Insect Biochem. Mol.* 42, 133–147.
- Anders, S., Huber, W., 2010. Differential expression analysis for sequence count data. *Genome Biol.* 11, R106.
- Anholt, R.R.H., Dilda, C.L., Chang, S., Fanara, J.J., Kulkarni, N.H., Ganguly, I., Rollmann, S.M., Kamdar, K.P., Mackay, T.F.C., 2003. The genetic architecture of odor-guided behavior in *Drosophila*: epistasis and the transcriptome. *Nat. Genet.* 35, 180–184.
- Anholt, R.R.H., Williams, T.L., 2010. The soluble proteome of the *Drosophila* antenna. *Chem. Senses* 35, 21–30.
- Arya, G.H., Weber, A.L., Wang, P., Magwire, M.M., Negron, Y.L., Mackay, T.F., Anholt, R.R., 2010. Natural variation, functional pleiotropy and transcriptional contexts of odorant binding protein genes in *Drosophila melanogaster*. *Genetics* 186, 1475–1485.
- Awasthi, Y.C., Ansari, G.A.S., Awasthi, S., 2005. Regulation of 4-hydroxynonenal mediated signaling by glutathione S-transferases. *Method Enzymol.* 401, 379–407.
- Benton, R., Sachse, S., Michnick, S.W., Vosshall, L.B., 2006. Atypical membrane topology and heteromeric function of *Drosophila* odorant receptors *in vivo*. *PLoS Biol.* 4, e20.
- Bownes, M., 1986. Expression of the genes coding for vitellogenin (yolk protein). *Annu. Rev. Entomol.* 31, 507–531.
- Burtis, K.C., Coschigano, K.T., Baker, B.S., Wensink, P.C., 1991. The doublesex proteins of *Drosophila melanogaster* bind directly to a sex-specific yolk protein gene enhancer. *EMBO J.* 10, 2577–2582.
- Campbell, P.M., Harcourt, R.L., Crone, E.J., Claudianos, C., Hammock, B.D., Russell, R.J., Oakeshott, J.G., 2001. Identification of a juvenile hormone esterase gene by matching its peptide mass fingerprint with a sequence from the *Drosophila* genome project. *Insect Biochem. Mol. Biol.* 31, 513–520.
- Catalan, A., Hutter, S., Parsch, J., 2012. Population and sex differences in *Drosophila melanogaster* brain gene expression. *BMC Genomics* 13, 1–12.
- Chang, P.L., Dunham, J.P., Nuzhdin, S.V., Arbeitman, M.N., 2011. Somatic sex-specific transcriptome differences in *Drosophila* revealed by whole transcriptome sequencing. *BMC Genomics* 12, 364.
- Chertermpis, T., Francois, A., Durand, N., Rosell, G., Dekker, T., Lucas, P., Maibèche-Coisne, M., 2012. A carboxylesterase, Esterase-6, modulates sensory

- physiological and behavioral response dynamics to pheromone in *Drosophila*. *BMC Biol.* 10, 56.
- Chintapalli, V.R., Wang, J., Dow, J.A.T., 2007. Using FlyAtlas to identify better *Drosophila melanogaster* models of human disease. *Nat. Genet.* 39, 715–720.
- Claudianos, C., Ranson, H., Johnson, R.M., Biswas, S., Schuler, M.A., Berenbaum, M.R., Feyereisen, R., Oakeshott, J.G., 2006. A deficit of detoxification enzymes: pesticide sensitivity and environmental response in the Honeybee. *Insect Mol. Biol.* 15, 615–636.
- Clayton, J.D., Cripps, R.M., Sparrow, J.C., Bullard, B., 1998. Interaction of troponin-H and glutathione S-transferase-2 in the indirect flight muscles of *Drosophila melanogaster*. *J. Muscle Res. Cell. M.* 19, 117–127.
- Coppin, C.W., Jackson, C.J., Sutherland, T., Hart, P.J., Devonshire, A.L., Russell, R.J., Oakeshott, J.G., 2012. Testing the evolvability of an insect carboxylesterase for the detoxification of synthetic pyrethroid insecticides. *Insect Biochem. Mol.* 42, 343–352.
- Cornish-Bowden, A., 1995. *Fundamentals of Enzyme Kinetics*. Portland Press, London, pp. 105–108.
- Couto, A., Alenius, M., Dickson, B.J., 2005. Molecular, anatomical, and functional organization of the *Drosophila* olfactory system. *Curr. Biol.* 15, 1535–1547.
- Cox, M.P., Peterson, D.A., Biggs, P.J., 2010. SolexaQA: at-a-glance quality assessment of Illumina second-generation sequencing data. *BMC Bioinformatics* 11, 458.
- Crone, E.J., Sutherland, T.D., Campbell, P.M., Coppin, C.W., Russell, R.J., Oakeshott, J.G., 2007. Only one esterase of *Drosophila melanogaster* is likely to degrade juvenile hormone *in vivo*. *Insect Biochem. Mol. Biol.* 37, 540–549.
- Daborn, P., Boundy, S., Yen, J., Pittendrigh, B., Ffrench-Constant, R., 2001. DDT resistance in *Drosophila* correlates with CYP6g1 over-expression and confers cross-resistance to the neonicotinoid imidacloprid. *Mol. Genet. Genomics* 266, 556–563.
- Daborn, P.J., Yen, J.L., Bogwitz, M.R., Le Goff, G., Feil, E., Jeffers, S., Tijet, N., Perry, T., Heckel, D., Batterham, P., Feyereisen, R., Wilson, T.G., Ffrench-Constant, R.H., 2002. A single p450 allele associated with insecticide resistance in *Drosophila*. *Science* 297, 2253–2256.
- David, J.R., Van Herreweghe, J., De Scheemaeker-Louis, M., Pla, E., 1981. *Drosophila* alcohol dehydrogenase: detoxification of isopropanol and acetone, substances not used in energy metabolism. *Hered. (Edinb)* 47, 263–268.
- de Bruyne, M., Warr, C.G., 2006. Molecular and cellular organization of insect chemosensory neurons. *Bioessays* 28, 23–34.
- Despres, L., David, J.P., Gallet, C., 2007. The evolutionary ecology of insect resistance to plant chemicals. *Trends Ecol. Evol.* 22, 298–307.
- Ding, X.X., Kaminsky, L.S., 2003. Human extrahepatic cytochromes P450: function in xenobiotic metabolism and tissue-selective chemical toxicity in the respiratory and gastrointestinal tracts. *Annu. Rev. Pharmacol.* 43, 149–173.
- Durand, N., Carot-Sans, G., Bozzolan, F., Rosell, G., Siauxsat, D., Debernard, S., Chertemps, T., Maibèche-Coisne, M., 2011. Degradation of pheromone and plant volatile components by a same odorant-degrading enzyme in the cotton leafworm, *Spodoptera littoralis*. *Plos One* 6, e29147.
- Durand, N., Carot-Sans, G., Chertemps, T., Montagne, N., Jacquin-Joly, E., Debernard, S., Maibèche-Coisne, M., 2010. A diversity of putative carboxylesterases are expressed in the antennae of the noctuid moth *Spodoptera littoralis*. *Insect Mol. Biol.* 19, 87–97.
- Eisenthal, R., Danson, M.J., Hough, D.W., 2007. Catalytic efficiency and k(cat)/K_m: a useful comparator? *Trends Biotechnol.* 25, 247–249.
- Enayati, A.A., Ranson, H., Hemingway, J., 2005. Insect glutathione transferases and insecticide resistance. *Insect Mol. Biol.* 14, 3–8.
- Feyereisen, R., 2006. Evolution of insect P450. *Biochem. Soc. Trans.* 34, 1252–1255.
- Feyereisen, R., 2012. 8-Insect CYP genes and P450 enzymes. In: Gilbert, L.L. (Ed.), *Insect Molecular Biology and Biochemistry*. Academic Press, San Diego, pp. 236–316.
- Flint, J., Zhou, S., Stone, E.A., Mackay, T.F.C., Anholt, R.R.H., 2009. Plasticity of the chemoreceptor repertoire in *Drosophila melanogaster*. *PLoS Genet.* 5, e1000681.
- Fujii, S., Amrein, H., 2002. Genes expressed in the *Drosophila* head reveal a role for fat cells in sex-specific physiology. *EMBO J.* 21, 5353–5363.
- Fujii, S., Toyama, A., Amrein, H., 2008. A male-specific fatty acid omega-hydroxylase, *SXE1*, is necessary for efficient male mating in *Drosophila melanogaster*. *Genetics* 180, 179–190.
- Gilbert, D.G., 1981. Studies of Esterase-6 in *Drosophila melanogaster*. 8. ejaculate esterase-6 and initial sperm use by female *Drosophila melanogaster*. *J. Insect Physiol.* 27, 641–650.
- Gilbert, L.L., 2004. Halloween genes encode P450 enzymes that mediate steroid hormone biosynthesis in *Drosophila melanogaster*. *Mol. Cell. Endocrinol.* 215, 1–10.
- Giraud, M., Unnithan, G.C., Le Goff, G., Feyereisen, R., 2010. Regulation of cytochrome P450 expression in *Drosophila*: genomic insights. *Pestic. Biochem. Physiol.* 97, 115–122.
- Graveley, B.R., Brooks, A.N., Carlson, J., Duff, M.O., Landolin, J.M., Yang, L., Artieri, C.G., van Baren, M.J., Boley, N., Booth, B.W., Brown, J.B., Cherbas, L., Davis, C.A., Dobin, A., Li, R.H., Lin, W., Malone, J.H., Mattiuzzo, N.R., Miller, D., Sturgill, D., Tuch, B.B., Zaleski, C., Zhang, D.Y., Blanchette, M., Dudoit, S., Eads, B., Green, R.E., Hammonds, A., Jiang, L.C., Kapranov, P., Langton, L., Perrimon, N., Sandler, J.E., Wan, K.H., Willingham, A., Zhang, Y., Zou, Y., Andrews, J., Bickel, P.J., Brenner, S.E., Brent, M.R., Cherkas, P., Gingeras, T.R., Hoskins, R.A., Kaufman, T.C., Oliver, B., Celisner, S.E., 2011. The developmental transcriptome of *Drosophila melanogaster*. *Nature* 471, 473–479.
- Hammock, B.D., Bonning, B.C., Possee, R.D., Hanzlik, T.N., Maeda, S., 1990. Expression and effects of the juvenile hormone esterase in a baculovirus vector. *Nature* 344, 458–461.
- He, P., Zhang, J., Li, Z.-Q., Zhang, Y.-N., Yang, K., Dong, S.-L., 2014. Functional characterization of an antennal esterase from the noctuid moth, *Spodoptera exigua*. *Arch. Insect Biochem. Physiol.* 86, 85–99.
- Heydel, J.M., Holsztynska, E.J., Legendre, A., Thiebaut, N., Artur, Y., Le Bon, A.M., 2010. UDP-glucuronosyltransferases (UGTs) in neuro-olfactory tissues: expression, regulation, and function. *Drug. Metab. Rev.* 42, 74–97.
- Huang, F., C. C., Zhang, Z., Liu, Z., Dai, F., Lu, C., 2008. The UDP-glucosyltransferase multigene family in *Bombyx mori*. *BMC Genomics* 9, 563.
- Immonen, E., Ritchie, M.G., 2012. The genomic response to courtship song stimulation in female *Drosophila melanogaster*. *P. Roy. Soc. B-Biol. Sci.* 279, 1359–1365.
- Ishida, Y., Leal, W.S., 2005. Rapid inactivation of a moth pheromone. *Proc. Natl. Acad. Sci. USA* 102, 14075–14079.
- Ishida, Y., Leal, W.S., 2008. Chiral discrimination of the Japanese beetle sex pheromone and a behavioral antagonist by a pheromone-degrading enzyme. *Proc. Natl. Acad. Sci. USA* 105, 9076–9080.
- Jackson, C.J., Liu, J.W., Carr, P.D., Younus, F., Coppin, C., Meirles, T., Lethier, M., Pandey, G., Ollis, D.L., Russell, R.J., Weik, M., Oakeshott, J.G., 2013. Structure and function of an insect alpha-carboxylesterase (alphaEsterase7) associated with insecticide resistance. *Proc. Natl. Acad. Sci. USA* 110, 10177–10182.
- Jordan, M.D., Stanley, D., Marshall, S.D., De Silva, D., Crowhurst, R.N., Gleave, A.P., Greenwood, D.R., Newcomb, R.D., 2008. Expressed sequence tags and proteomics of antennae from the tortricid moth, *Epiphyas postvittana*. *Insect Mol. Biol.* 17, 361–373.
- Kamikouchi, A., Morioka, M., Kubo, T., 2004. Identification of honeybee antennal proteins/genes expressed in a sex and/or caste selective manner. *Zool. Sci.* 21, 53–62.
- Kraus, K.W., Lee, Y.H., Lis, J.T., Wolfner, M.F., 1988. Sex specific control of *Drosophila melanogaster* yolk protein 1 gene expression is limited to transcription. *Mol. Cell. Biol.* 8, 4756–4764.
- Laughlin, J.D., Ha, T.S., Jones, D.N.M., Smith, D.P., 2008. Activation of pheromone sensitive neurons is mediated by conformational activation of pheromone binding protein. *Cell* 133, 1255–1265.
- Lazard, D., Zupko, K., Poria, Y., Nef, P., Lazarovits, J., Horn, S., Khen, M., Lancet, D., 1991. Odorant signal termination by olfactory Udp glucuronosyl transferase. *Nature* 349, 790–793.
- Le Goff, G., Boundy, S., Daborn, P.J., Yen, J.L., Sofer, L., Lind, R., Sabourault, C., Madi-Ravazzi, L., Ffrench-Constant, R.H., 2003. Microarray analysis of cytochrome P450 mediated insecticide resistance in *Drosophila*. *Insect Biochem. Mol.* 33, 701–708.
- Le Goff, G., Hilliou, F., Siegfried, B.D., Boundy, S., Wajnberg, E., Sofer, L., Audant, P., Ffrench-Constant, R.H., Feyereisen, R., 2006. Xenobiotic response in *Drosophila melanogaster*: sex dependence of P450 and GST gene induction. *Insect Biochem. Mol.* 36, 674–682.
- Leclerc, V., Reichhart, J.M., 2004. The immune response of *Drosophila melanogaster*. *Immunol. Rev.* 198, 59–71.
- Li, X.C., Schuler, M.A., Berenbaum, M.R., 2007. Molecular mechanisms of metabolic resistance to synthetic and natural xenobiotics. *Annu. Rev. Entomol.* 52, 231–253.
- Low, W.Y., Ng, H.L., Morton, C.J., Parker, M.W., Batterham, P., Robin, C., 2007. Molecular evolution of glutathione S-transferases in the genus *Drosophila*. *Genetics* 177, 1363–1375.
- Luque, T., Okano, K., O'Reilly, D.R., 2002. Characterization of a novel silkworm (*Bombyx mori*) phenol UDP-glucosyltransferase. *Eur. J. Biochem.* 269, 819–825.
- Maibèche-Coisne, M., Jacquin-Joly, E., Francois, M.C., Nagnan-Le Meillour, P., 2002. cDNA cloning of biotransformation enzymes belonging to the cytochrome P450 family in the antennae of the noctuid moth *Mamestra brassicae*. *Insect Mol. Biol.* 11, 273–281.
- Maibèche-Coisne, M., Nikonov, A.A., Ishida, Y., Jacquin-Joly, E., Leal, W.S., 2004. Pheromone anosmia in a scarab beetle induced by *in vivo* inhibition of a pheromone-degrading enzyme. *Proc. Natl. Acad. Sci. USA* 101, 11459–11464.
- Mane, S.D., Tompkins, L., Richmond, R.C., 1983. Male Esterase 6 catalyzes the synthesis of a sex pheromone in *Drosophila melanogaster* females. *Science* 222, 419–421.
- Marygold, S.J., Leyland, P.C., Seal, R.L., Goodman, J.L., Thurmond, J., Strelets, V.B., Wilson, R.J., the FlyBase, c. 2013. FlyBase: Improvements to the bibliography. *Nucleic Acids Res.* 41, D751–DD757.
- Merlin, C., Rosell, G., Carot-Sans, G., Francois, M.C., Bozzolan, F., Pelletier, J., Jacquin-Joly, E., Guerrero, A., Maibèche-Coisne, M., 2007. Antennal esterase cDNAs from two pest moths, *Spodoptera littoralis* and *Sesamia nonagrioides*, potentially involved in odourant degradation. *Insect Mol. Biol.* 16, 73–81.
- Mi, H.Y., Muruganujan, A., Thomas, P.D., 2013. PANTHER in 2013: modeling the evolution of gene function, and other gene attributes, in the context of phylogenetic trees. *Nucleic Acids Res.* 41, D377–D386.
- Morozova, T.V., Anholt, R.R.H., Mackay, T.F.C., 2006. Transcriptional response to alcohol exposure in *Drosophila melanogaster*. *Genome Biol.* 7, R95.
- Nichols, Z., Vogt, R.G., 2008. The SNMP/CD36 gene family in Diptera, Hymenoptera and Coleoptera: *Drosophila melanogaster*, *D. pseudoobscura*, *Anopheles gambiae*, *Aedes aegypti*, *Apis mellifera*, and *Tribolium castaneum*. *Insect Biochem. Mol. Biol.* 38, 398–415.
- Oakeshott, J.G., Claudianos, C., Campbell, P.M., Newcomb, R.D., Russell, R.J., 2005. 5.10-Biochemical genetics and genomics of insect esterases. In: Lawrence, I.G.,

- Kostas, I., Sarjeet, S.G. (Eds.), *Comprehensive Molecular Insect Science*. Elsevier, Amsterdam, pp. 309–381.
- Pedra, J.H.F., McIntyre, L.M., Scharf, M.E., Pittendrigh, B.R., 2004. Genome-wide transcription profile of field- and laboratory-selected dichlorodiphenyltrichloroethane (DDT)-resistant *Drosophila*. *Proc. Natl. Acad. Sci. U. S. A.* 101, 7034–7039.
- Pitts, R.J., Rinker, D.C., Jones, P.L., Rokas, A., Zwiebel, L.J., 2011. Transcriptome profiling of chemosensory appendages in the malaria vector *Anopheles gambiae* reveals tissue- and sex-specific signatures of odor coding. *BMC Genomics* 12.
- Pottier, M.A., Bozzolan, F., Chertemps, T., Jacquin-Joly, E., Lalouette, L., Siauxat, D., Maibèche-Coisne, M., 2012. Cytochrome P450s and cytochrome P450 reductase in the olfactory organ of the cotton leafworm *Spodoptera littoralis*. *Insect Mol. Biol.* 21, 568–580.
- Richmond, R.C., Gilbert, D.G., Sheehan, K.B., Gromko, M.H., Butterworth, F.M., 1980. Esterase-6 and reproduction in *Drosophila melanogaster*. *Science* 207, 1483–1485.
- Robertson, H.M., Martos, R., Sears, C.R., Todres, E.Z., Walden, K.K., Nardi, J.B., 1999. Diversity of odourant binding proteins revealed by an expressed sequence tag project on male *Manduca sexta* moth antennae. *Insect Mol. Biol.* 8, 501–518.
- Rogers, M.E., Jani, M.K., Vogt, R.G., 1999. An olfactory-specific glutathione-S-transferase in the sphinx moth *Manduca sexta*. *J. Exp. Biol.* 202, 1625–1637.
- Rytz, R., Croset, V., Benton, R., 2013. Ionotropic receptors (IRs): chemosensory ionotropic glutamate receptors in *Drosophila* and beyond. *Insect Biochem Mol. Biol.* 43, 888–897.
- Saisawang, C., Wongsantichon, J., Ketterman, A.J., 2012. A preliminary characterization of the cytosolic glutathione transferase proteome from *Drosophila melanogaster*. *Biochem J.* 442, 181–190.
- Scott, D., 1986. Inhibition of female *Drosophila melanogaster* remating by a seminal fluid protein (Esterase-6). *Evolution* 40, 1084–1091.
- Segel, I.H., 1993. *Enzyme Kinetics. Behavior and Analysis of Rapid Equilibrium and Steady-State Enzyme Systems*. John Wiley & Sons, New York, p. 105.
- Shanbhag, S.R., Park, S.K., Pikielny, C.W., Steinbrecht, R.A., 2001. Gustatory organs of *Drosophila melanogaster*: Fine structure and expression of the putative odorant-binding protein PBPRP2. *Cell. Tissue Res.* 304, 423–437.
- Simon, P., 2003. Q-Gen: processing quantitative real-time RT-PCR data. *Bioinformatics* 19, 1439–1440.
- Singh, S.P., Coronella, J.A., Benes, H., Cochrane, B.J., Zimniak, P., 2001. Catalytic function of *Drosophila melanogaster* glutathione S-transferase DmGST1-1 (GST-2) in conjugation of lipid peroxidation end products. *Eur. J. Biochem* 268, 2912–2923.
- Starostina, E., Xu, A.G., Lin, H.P., Pikielny, C.W., 2009. A *Drosophila* protein family implicated in pheromone perception is related to Tay-Sachs GM2-Activator protein. *J. Biol. Chem.* 284, 585–594.
- Stensmyr, M.C., Giordano, E., Balloi, A., Angioy, A.M., Hansson, B.S., 2003. Novel natural ligands for *Drosophila* olfactory receptor neurones. *J. Exp. Biol.* 206, 715–724.
- Takemori, N., Yamamoto, M.T., 2009. Proteome mapping of the *Drosophila melanogaster* male reproductive system. *Proteomics* 9, 2484–2493.
- Tang, A.H., Tu, C.P.D., 1994. Biochemical-characterization of *Drosophila* glutathione S-transferases D1 and D21. *J. Biol. Chem.* 269, 27876–27884.
- Tijet, N., Helvig, C., Feyereisen, R., 2001. The cytochrome P450 gene superfamily in *Drosophila melanogaster*: annotation, intron-exon organization and phylogeny. *Gene* 262, 189–198.
- Trappnell, C., Pachter, L., Salzberg, S.L., 2009. TopHat: discovering splice junctions with RNA-Seq. *Bioinformatics* 25, 1105–1111.
- Trappnell, C., Williams, B.A., Pertea, G., Mortazavi, A., Kwan, G., van Baren, M.J., Salzberg, S.L., Wold, B.J., Pachter, L., 2010. Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation. *Nat. Biotech.* 28, 511–515.
- van Lieshout, E.M.M., Bedaf, M.M.G., Pieter, M., Ekkel, C., Nijhoff, W.A., Peters, W.H.M., 1998. Effects of dietary anticarcinogens on rat gastrointestinal glutathione S-transferase theta 1-1 levels. *Carcinogenesis* 19, 2055–2057.
- Vandermeer, R.K., Obin, M.S., Zawistowski, S., Sheehan, K.B., Richmond, R.C., 1986. A re-evaluation of the role of *cis*-vaccenyl acetate, *cis*-vaccenol and Esterase-6 in the regulation of mated female sexual attractiveness in *Drosophila melanogaster*. *J. Insect Physiol.* 32, 681–686.
- Vogt, R.G., 2005. Molecular basis of pheromone detection in insects. In: *Comprehensive Insect Physiology, Biochemistry, Pharmacology and Molecular Biology*. The Genetics Society, pp. 753–804.
- Vogt, R.G., Riddiford, L.M., 1981. Pheromone binding and inactivation by moth antennae. *Nature* 293, 161–163.
- Vogt, R.G., Riddiford, L.M., Prestwich, G.D., 1985. Kinetic properties of a sex pheromone-degrading enzyme: the sensillar esterase of *Antheraea polyphemus*. *Proc. Natl. Acad. Sci. USA* 82, 8827–8831.
- Vosshall, L.B., Wong, A.M., Axel, R., 2000. An olfactory sensory map in the fly brain. *Cell* 102, 147–159.
- Wang, C.W., Sun, Y.H., 2012. Segregation of eye and antenna fates maintained by mutual antagonism in *Drosophila*. *Development* 139, 3413–3421.
- Wang, L., Dankert, H., Perona, P., Anderson, D.J., 2008. A common genetic target for environmental and heritable influences on aggressiveness in *Drosophila*. *Proc. Natl. Acad. Sci. USA* 105, 5657–5663.
- Wang, Q., Hasan, G., Pikielny, C., 1999. Preferential expression of biotransformation enzymes in the olfactory organs of *Drosophila melanogaster*, the antennae. *J. Biol. Chem.* 274, 10309–10315.
- Wang, S.P., He, G.L., Chen, R.R., Li, F., Li, G.Q., 2012. The involvement of cytochrome P450 monooxygenases in methanol elimination in *Drosophila melanogaster* larvae. *Arch. Insect Biochem. Physiol.* 79, 264–275.
- Wang, S.P., Hu, X.X., Meng, Q.W., Muhammad, S.A., Chen, R.R., Li, F., Li, G.Q., 2013. The involvement of several enzymes in methanol detoxification in *Drosophila melanogaster* adults. *Comp. Biochem. Phys. B* 166, 7–14.
- Willingham, A.T., Keil, T., 2004. A tissue specific cytochrome P450 required for the structure and function of *Drosophila* sensory organs. *Mech. Dev.* 121, 1289–1297.
- Wojtasek, H., Leal, W.S., 1999. Degradation of an alkaloid pheromone from the pale-brown chafer, *Phyllopertha diversa* (Coleoptera: Scarabaeidae), by an insect olfactory cytochrome P450. *FEBS Lett.* 458, 333–336.
- Yepiskoposyan, H., Egli, D., Fergestad, T., Selvaraj, A., Treiber, C., Multhaup, G., Georgiev, O., Schaffner, W., 2006. Transcriptome response to heavy metal stress in *Drosophila* reveals a new zinc transporter that confers resistance to zinc. *Nucleic Acids Res.* 34, 4866–4877.
- Zhou, S.S., Stone, E.A., Mackay, T.F.C., Anholt, R.R.H., 2009. Plasticity of the chemoreceptor repertoire in *Drosophila melanogaster*. *PLoS Genet.* 5 e1000681.

Chapter 3: An antennal carboxylesterase from *Drosophila melanogaster*, Esterase 6, is a candidate odorant-degrading enzyme toward food odorants



An antennal carboxylesterase from *Drosophila melanogaster*, esterase 6, is a candidate odorant-degrading enzyme toward food odorants

Thomas Chertemps¹, Faisal Younus^{2,3}, Claudia Steiner¹, Nicolas Durand¹, Chris W. Coppin², Gunjan Pandey², John G. Oakeshott² and Martine Maibèche^{1*}

¹ Sorbonne Universités UPMC - Univ Paris 06, Institut d'Ecologie et des Sciences de l'Environnement de Paris, INRA, CNRS, IRD, UPEC, Paris, France, ² Commonwealth Scientific and Industrial Research Organisation (CSIRO) Land and Water Flagship, Canberra, ACT, Australia, ³ Research School of Chemistry, ANU College of Physical and Mathematical Sciences, Australian National University, Canberra, ACT, Australia

OPEN ACCESS

Edited by:

Monique Gauthier,
University Paul Sabatier Toulouse 3,
France

Reviewed by:

Yael Grosjean,
Centre National de la Recherche
Scientifique, France
Marcus Carl Stensmyr,
Lund University, Sweden

*Correspondence:

Martine Maibèche
martine.maibèche@upmc.fr

Specialty section:

This article was submitted to
Invertebrate Physiology,
a section of the journal
Frontiers in Physiology

Received: 27 May 2015

Accepted: 19 October 2015

Published: 05 November 2015

Citation:

Chertemps T, Younus F, Steiner C,
Durand N, Coppin CW, Pandey G,
Oakeshott JG and Maibèche M (2015)
An antennal carboxylesterase from
Drosophila melanogaster, esterase 6,
is a candidate odorant-degrading
enzyme toward food odorants.
Front. Physiol. 6:315.
doi: 10.3389/fphys.2015.00315

Reception of odorant molecules within insect olfactory organs involves several sequential steps, including their transport through the sensillar lymph, interaction with the respective sensory receptors, and subsequent inactivation. Odorant-degrading enzymes (ODEs) putatively play a role in signal dynamics by rapid degradation of odorants in the vicinity of the receptors, but this hypothesis is mainly supported by *in vitro* results. We have recently shown that an extracellular carboxylesterase, esterase-6 (EST-6), is involved in the physiological and behavioral dynamics of the response of *Drosophila melanogaster* to its volatile pheromone ester, *cis*-vaccenyl acetate. However, as the expression pattern of the *Est-6* gene in the antennae is not restricted to the pheromone responding sensilla, we tested here if EST-6 could play a broader function in the antennae. We found that recombinant EST-6 is able to efficiently hydrolyse several volatile esters that would be emitted by its natural food *in vitro*. Electrophysiological comparisons of mutant *Est-6* null flies and a control strain (on the same genetic background) showed that the dynamics of the antennal response to these compounds is influenced by EST-6, with the antennae of the null mutants showing prolonged activity in response to them. Antennal responses to the strongest odorant, pentyl acetate, were then studied in more detail, showing that the repolarization dynamics were modified even at low doses but without modification of the detection threshold. Behavioral choice experiments with pentyl acetate also showed differences between genotypes; attraction to this compound was observed at a lower dose among the null than control flies. As EST-6 is able to degrade various bioactive odorants emitted by food and plays a role in the response to these compounds, we hypothesize a role as an ODE for this enzyme toward food volatiles.

Keywords: carboxylesterase, olfaction, odorant-degrading enzyme, *Drosophila melanogaster*, enzyme activity assays, electroantennogram, behavior

INTRODUCTION

Our understanding of the molecular basis of insect olfaction has improved greatly over the last few years, in large part through the application of modern genomic technologies and advances in associated physiology. Much of the focus has been on the molecular events occurring during early olfactory processing (i.e., within the olfactory organs, also called perireceptor events Getchell et al., 1984). The steps by which odorants are bound by Odorant-Binding Proteins (OBPs) and transported to the olfactory receptors (ORs), and the ORs then activated, are now well documented (reviewed in Leal, 2013). However, the subsequent step of odorant inactivation that sustains the kinetics of the olfactory system response is still not well understood. Two sets of hypotheses are still under debate: one proposes that odorant-degrading enzymes (ODEs) are principally responsible for the rapid degradation of the odorant molecules, on a millisecond timescale (Vogt and Riddiford, 1981; Ishida and Leal, 2005; Chertemps et al., 2012), while the other invokes more complex processes also involving OBPs, ORs, or as yet unknown scavenger molecules (reviewed in Rützler and Zwiebel, 2005; Kaissling, 2009, 2014).

In vitro experiments have demonstrated that several important insect pheromones can be rapidly degraded by candidate ODEs belonging to various detoxification enzyme families, including esterases, cytochromes P450s, aldehyde oxidases, and glutathione S-transferases (reviewed in Vogt, 2005; Leal, 2013). Given the diversity of detoxification enzymes expressed in insect antennae (reviewed in Siauxsat et al., 2014) and the variety of their potential physiological roles, functional characterizations of particular candidate ODEs are still relatively scarce. However, several antennal esterases from a range of species have now been shown *in vitro* to efficiently degrade particular sex pheromones (Durand et al., 2011; Leal, 2013; He et al., 2014a,b) and plant volatiles (Durand et al., 2010; He et al., 2014a,b). In *Drosophila melanogaster*, esterase 6 (EST-6) has been reported to degrade the pheromone cis-vaccenyl acetate (CVA; Mane et al., 1983) and more recently, a protein encoded by a duplication of the *Juvenile hormone esterase* gene (*Jhe-dup*) has been shown to hydrolyse various ester odorants for this species (Younus et al., 2014).

We have previously used comparisons between *D. melanogaster* strains carrying *Est-6* wild-type vs. null alleles (on the same genetic background) to show that EST-6 plays a role in the physiological and behavioral responses of *D. melanogaster* males to CVA (Chertemps et al., 2012). This supports the *in vitro* evidence above that this enzyme is an ODE in male antennae. Also consistent with this evidence, transcriptomic analysis of the olfactory organ shows *Est-6* and *Jhe-dup* are the most highly expressed of all the esterase genes in the antennae of this species (Younus et al., 2014).

Intriguingly however, we also find that EST-6 is widely distributed within the third antennal segment, including in sensilla tuned to other odorants, in addition to those sensitive to CVA (Chertemps et al., 2012). This suggests that EST-6 could play a broader role in the antennae than CVA processing, perhaps functioning as an ODE for other bioactive ester

volatiles. *D. melanogaster* is indeed known to detect a large number of volatile esters, although their possible functions and ecological relevance are still under investigation (Mansourian and Stensmyr, 2015).

To explore this issue further, we have produced recombinant EST-6 protein using the baculovirus system and characterized its activity against eight volatile esters produced by decomposing fruits and other plant tissues (Stensmyr et al., 2003). We find that it can efficiently process most of them *in vitro*. The physiological responses of the antennae to these compounds were therefore then measured by electroantennography (EAG) on *Est-6* wild-type vs. null flies (on the same genetic background, as above). Consistent differences between the two strains were found for the six compounds for which the recombinant EST-6 had the greatest activity. For one of these, pentyl acetate, which was the strongest odorant, dose-response studies revealed that the repolarization dynamics were also modified at low doses. Behavioral studies using pentyl acetate as the bioactive molecule confirmed that EST-6 is indeed involved in the perception of this compound. These data suggest that EST-6 may function as an ODE for a variety of bioactive volatile esters in this species.

MATERIALS AND METHODS

Fly Strains

The three strains used in this study were described in full in Chertemps et al. (2012). One is an *Est-6* null mutant strain (*Est-6*[°]; Bloomington stock 4211), where EST-6 expression is abolished, and the other is a rescue strain, *Est-6*⁺, which has the same genetic background as the *Est-6*[°] strain but with a fully functional *Est-6* copy inserted independently. *Canton-S* (CS) flies were also used as a second wild-type strain in the study of pentyl acetate responses.

All flies were raised at 25°C on standard yeast/cornmeal/agar medium in a 12-h light/12-h dark cycle, with 50–60% relative humidity.

Assays of EST-6 Activity

Three acetate esters, two propionate esters, one butyrate ester, and two methyl esters of mid-long chain fatty acids (Table 1) were tested. Octyl acetate, methyl decanoate, and methyl myristate are odorants produced by green plant tissue and the others are volatile products of rotting fruit. All eight esters were purchased in the highest available purity from Sigma Aldrich or, in the case of heptyl propionate and octyl propionate, Vigon International (USA).

A wild-type form of EST-6 (EST-6^F; from strain Sengwa 24; accession KR014246) was expressed commercially (Genscript, USA) behind its own signal peptide using the BacuVance™ baculovirus expression system. An inactive EST-6 was expressed in the same way as a negative control; the gene for this was identical to that above except that the catalytic Ser²⁰⁹TCC codon was changed to Gly²⁰⁹GGG. Both proteins were concentrated ~10-fold, by passing the media through a 30 K Amicon filter. The titer of the active variant was determined using the fluorometric methods of Coppin et al. (2012).

TABLE 1 | *In vitro* activities of recombinant EST-6 toward the eight esters tested.

	Compound	Specificity activity (S ⁻¹)	Kinetics			OR (sensillar type)
			Kcat (S ⁻¹ ± SE)	Km (Mm ± SE)	Specific constant (M ⁻¹ s ⁻¹)	
Good substrates	Octyl propionate	268.6	4519 ± 1683	3.16 ± 1.05	1.43 × 10 ⁶	Unknown
	Hexyl propionate	210.9	≥21,304	≥20	1.07 × 10 ⁶	Unknown
	Heptyl acetate	115.9	≥11,704	≥20	5.85 × 10 ⁵	67b, 13a, 92a (basiconic)
	Octyl acetate	83.3	≥8412	≥20	4.21 × 10 ⁵	45a, 35a (coeloconic)
	Pentyl acetate	61.9	969 ± 215	2.93 ± 0.49	3.30 × 10 ⁵	47a, 35a, 85c, 85b, 98a, 22a, 67a (ab5B ab3B)
Poor Substrates	Propyl butyrate	18	1671 ± 748	18.33 ± 8.10	9.12 × 10 ⁴	19a (at3)
	Methyl decanoate	4.4	≥447	≥20	2.24 × 10 ⁴	Unknown
	Methyl myristate	3.1	≥309	≥20	1.54 × 10 ⁴	88a (at4c)

The OR and sensillar types were taken from the OR response data bases (<http://neuro.uni-konstanz.de/DoOR/default.html> and <http://neuro.uni-konstanz.de/DoOR/2.0/>) and from Dweck et al. (2015).

The activity of EST-6 toward the test odorants was monitored in triplicate by gas chromatography/mass spectrometry (GC-MS) assays of substrate loss using methods modified from those described in Jackson et al. (2013). Each reaction mixture (200 µl) consisted of odorant substrate (200 µM), enzyme (0.2–3.6 nM), BSA (5 µg, for enzyme stability), and ethanol (5% v/v) in 25 mM Tris-HCl buffer (pH 8.0). Reactions were stopped by the addition of 0.5 volumes of hexane at intervals from 3 min to 20 min. The upper hexane layer was removed from the vial and analyzed by GC-MS (7890 Series, Agilent Technologies, USA) on a J&W DB-WAX column (30 m × 0.25 mm × 0.25 µm, Agilent Technologies, USA) with He (2 ml/min) as the carrier gas. The oven temperature was initially set at 50°C for 2 min and then subsequently increased over a gradient of 10°–275°C and held for 10 min. The injector and detector temperature was set at 250°C with a 10:1 split ratio.

K_m -values were determined using the competitive inhibition method with 4-nitrophenyl acetate as substrate as described in Younus et al. (2014). This assay circumvents technical challenges that make the direct determination of the Michaelis–Menten kinetics impossible in the GC-MS assay above. It does this by treating the odorant ester as a competitive inhibitor of a chromogenic ester substrate for which a more facile continuous microplate assay exists. The inhibition constant, K_i , of the “inhibitor” is equivalent to its Michaelis constant, K_m (Cornish-Bowden, 1995; Eisenthal et al., 2007). With the data on K_m and the activity at a single known substrate concentration (from the GC-MS method), the Michaelis–Menten equation can then be used to derive K_{cat} .

EAG of Antennal Responses to Various Esters

EAG was carried out to compare the responses of *Est-6*^o and *Est-6*⁺ males to the eight esters above. Recordings were performed on a pClamp 10 (Molecular Devices) at 22°C on 5-day old males previously kept in individual tubes, as described in Chertemps et al. (2012). Antennae were first stimulated for 3 s with each ester (all with >95% purity, diluted 1:1000 in paraffin oil, excepting

methyl myristate which was diluted in hexane). Pure hexane (>98% purity, Carlo-Erba) and paraffin oil (Sigma Aldrich) were used as negative controls. Propionic acid (Sigma Aldrich), which is not hydrolysed by esterases and is detected by another type of sensilla (Rytz et al., 2013), was used as positive control (ddH₂O as solvent). To further analyse the antennal responses to pentyl acetate, shorter stimulations (0.5 s) at the same dose of odorant were also performed following the same protocol. In addition, the responses to various doses (10⁻⁴ up to 10⁻²) of this compound were also recorded. Stimulus cartridges were changed between the tests on different insects.

Several parameters were measured. The peak amplitudes of EAG responses were measured at the maximum negative voltage deflection from the baseline (max amplitude, in millivolts) and reflect the intensity of the responses. It is generally accepted that such EAG amplitudes represent the sum of the generator potentials created by individual receptors' neurons within all the responsive sensilla carried by the antennae (Haase et al., 2011; Kaissling, 2014). The dynamics of the repolarization during and after the stimulation was estimated by three parameters: (i) a repolarization rate during the stimulation, which was calculated as [(maximum amplitude of depolarization – amplitude of depolarization at the end of stimulation)/maximum amplitude] × 100; (ii) the time at which 3/4 of maximum amplitude was recorded in seconds (3/4 repolarization time), and (iii) the value of the EAG decay slope (mV/s).

Behavioral Responses to Pentyl Acetate

Flies were maintained on standard yeast/cornmeal/agar medium at 25°C in a 12-h light/12-h dark cycle, 50–60% relative humidity. Newly enclosed male flies were collected and aged for 6–8 d, then wet-starved for 5 h before testing. Choice tests were then performed to assess the responses of the flies to pentyl acetate in a two-choice T-maze apparatus adapted from Stensmyr et al. (2003). All tests were performed at 25°C with 50–60% humidity and under dim red light to exclude visual effects. Responses to the control odorant propionic acid, known to trigger attraction (Knaden et al., 2012), were measured in the same conditions.

The T-maze was made of three 1 mL, 6 cm long pipettes connected with a Three-way splitter (E765.1, Roth) and tightly sealed with parafilm. Solutions of pentyl acetate ranging from 10^{-1} (i.e., 920 ng of pentyl acetate) up to 10^{-9} were prepared. For propionic acid, solutions ranging from 10^{-2} (i.e., 99 ng of propionic acid) up to 10^{-8} were used. Ten microliters of the corresponding solution was applied to a piece of filter paper (5×5 mm), which was then placed in a 1.5 mL tube (no. EA83.1; Roth). This tube was then joined to one arm of the T-maze, and a control tube with the solvent (i.e., paraffin oil) on an equivalent piece of filter paper was then embedded on another arm. A single male was introduced into the third arm of the T-maze by gentle aspiration and allowed to move through the maze for 2 min, after which the arm in which it was located was recorded. At least 70 replications with different males for each strain and pentyl acetate or propionic acid concentration were performed and the position of the stimulation and control arm was alternated between trials. The response index (RI) was calculated as (number of flies in odorant arm/total number of tested flies). An RI of 1 represents full attraction, a value of 0 represents full avoidance, and 0.5 indifference to the odor.

Statistical Analysis

All statistical analyses were performed using the Graphpad® Prism 5 software. For the EAG study with the eight esters, Mann-Whitney or Student's *t*-tests were used for pairwise comparisons depending on the distribution of the EAG variables. For the EAG study with pentyl acetate, ANOVA analyses (both parametric and non-parametric according to the data distribution) followed by *post-hoc* tests (Tukey or Dunns), were performed. For behavioral analysis, the Wilcoxon signed rank test was used to test data from the behavioral choice experiment against the null hypothesis of indifference to the odorant and non-parametric ANOVA followed by Dunns *post-hoc* tests were used for comparisons between genotypes.

RESULTS

In Vitro EST-6 Activity toward Various Esters

Recombinantly expressed EST-6 showed detectable specific activity against all eight naturally occurring esters tested. However a strong preference for acyl groups containing no more than three carbons was evident; propyl butyrate yielded activities at least three fold lower than the five acetate and propionate substrates, while the values for methyl decanoate and methyl myristate were several fold lower again (Table 1). Consistent with expectations for an enzyme with a relatively broad substrate range, the kinetic data showed high K_m -values (in the mM range) for all substrates, indicating relatively loose enzyme-substrate affinities. However, k_{cat} -values were also quite high, particularly for the substrates with the shorter acyl groups, although even methyl decanoate and methyl myristate yielded non-negligible estimates. Despite the high K_m -values, the high k_{cat} -values, and the high specificity constants (k_{cat}/K_m) that follow, would suggest that the enzyme could effectively turn over the locally high

concentrations of several of the substrates that might be expected in the vicinity of the ORs in the sensilla.

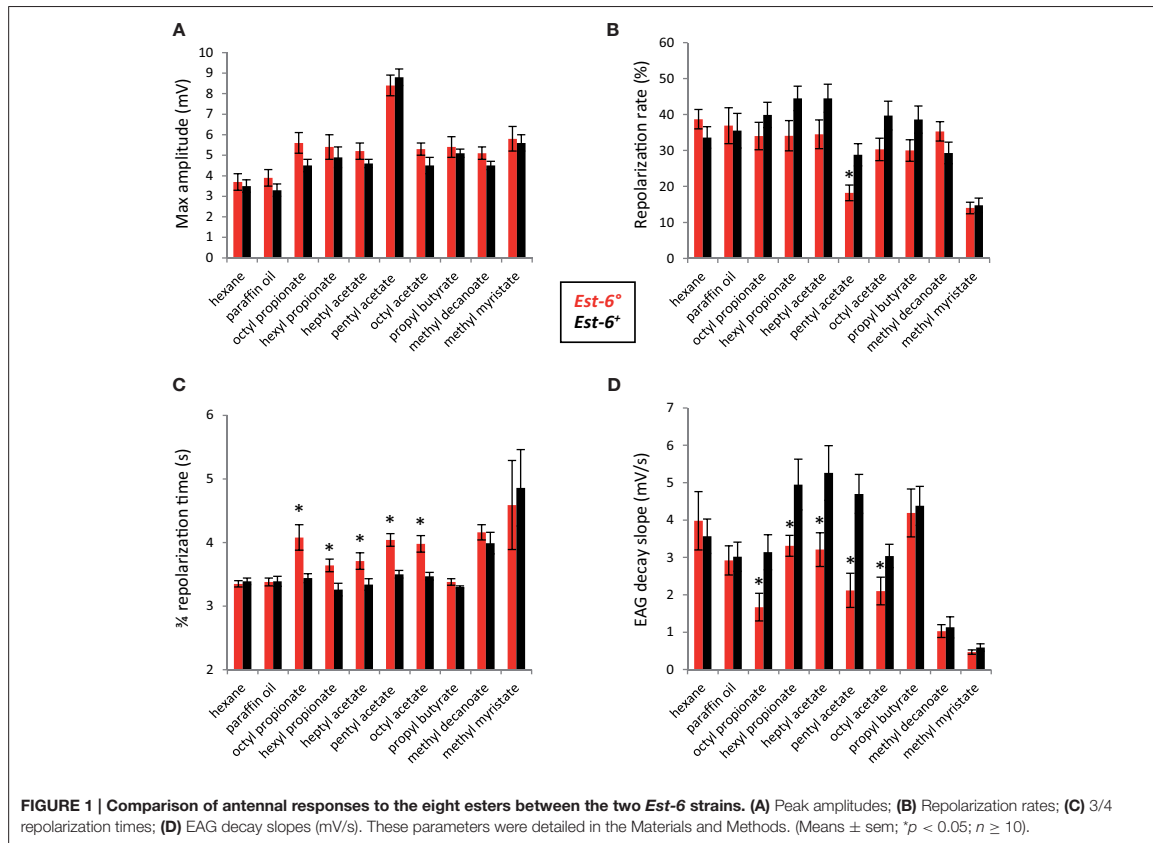
Global Comparison of EAG Responses to the Eight Esters

As a first step in testing whether the *in vitro* activities above were indeed relevant *in vivo*, we compared the olfactory responses of *Est-6^o* and *Est-6⁺* males to the eight esters by EAG. We used a dose of each odorant that should induce a high response and gave a relatively long stimulation (3 s) in order to represent an overstimulation of the antennae. Five of the chosen esters (pentyl, octyl, and heptyl acetate, propyl butyrate and methyl myristate) were previously known to be detected by the fly (Cobb and Dannel, 1994; Stensmyr et al., 2003; Schlieff and Wilson, 2007; Dweck et al., 2015; <http://neuro.uni-konstanz.de/DoOR/2.0/>), with no data on the question available for the other three (Table 1). Moreover, the responses to octyl and heptyl acetate have only been reported for larvae and at the behavioral level (Cobb and Dannel, 1994). Methyl myristate has been shown recently to be involved in short-range attraction in both sexes (Dweck et al., 2015). Here, we first showed that all eight of the compounds tested were detected by the antennae of the different strains (Figure 1A) and could thus be possible odorants. The maximum EAG amplitude values for both strains were indeed statistically different from the responses induced by the paraffin oil and hexane controls, the latter two being identical between the two strains ($p < 0.05$, Student's *t*-test). Pentyl acetate induced the strongest EAG responses (max amplitude >8 mV) in both strains.

No variation in the peak amplitude of depolarization was observed between the two strains for any of the compounds tested (Figure 1A), suggesting that EST-6 does not affect the intensity of the response. However, the repolarization dynamics during the stimulation are significantly different in the case of pentyl acetate (repolarization rate of 18.2 cf 28.8% in *Est-6^o* and *Est-6⁺* males, respectively; Figure 1B). This parameter was also lower in *Est-6^o* males for the other esters for which EST6 had good activity (referred as "good substrates" in Table 1) but with no statistically significant differences. After stimulation, the 3/4 repolarization time and the decay slope values also differed significantly between the two strains for all these five compounds (Figures 1C,D), whereas the latter measures of the dynamics of the response were not affected for the three other compounds. Responses of the different strains to the control odorant propionic acid were similar (Figure S1).

Further Analysis of EAG Responses to Pentyl Acetate

As pentyl acetate elicited the strongest antennal responses (Figure 1A), recordings with this compound were also performed using short stimulation duration and different doses. A second wild-type strain, CS, was also used in these experiments. The differences in the responses of the null and wild-type strains were again seen when the antennae were stimulated with pentyl acetate at 10^{-3} for a shorter period (0.5 cf. 3.0 s, Figure 2). Compared to the *Est-6⁺* and CS males, the *Est-6^o*



males showed a slower repolarization rate during the stimulation (2.6 vs. 8%, and 8.9%, respectively), and a slower repolarization, further supporting a role for EST-6 in the temporal dynamics of antennal responses to this compound. Responses to several doses of pentyl acetate were also compared following 3 s stimulations (Figure 3). The peak amplitude was again not modified but the repolarization dynamics were altered even at the lowest dose (10^{-4} dilution).

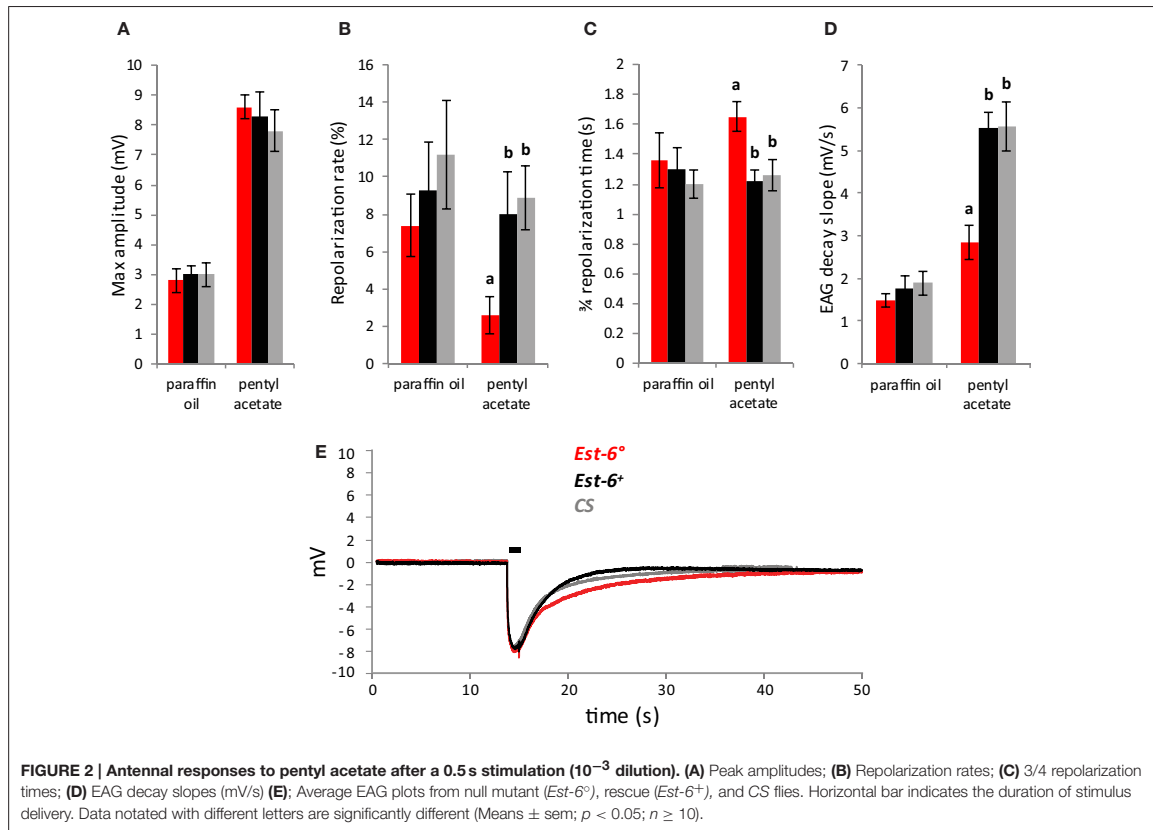
Behavioral Responses to Pentyl Acetate

As pentyl acetate was the only compound tested here already known to induce a clear behavioral response in adults and as its antennal detection was clearly impaired in the *Est-6°* mutants, we performed a choice assay in order to test if the lack of EST-6 could also alter their behavioral responses. As shown in Figure 4, the overall responses of all strains to the odorant followed the pattern expected for a bioactive volatile (Stensmyr et al., 2003), with indifference to low concentrations, attraction to intermediate concentrations and repulsion to high concentrations. The attractive response was clearly modified in the *Est-6°* mutant flies, with a threshold attractive dose of 10^{-7} compared to 10^{-5} in the two control strains. No significant

difference was seen between *Est-6°* and *Est-6+* strains in the threshold for the repulsion response, which was at 10^{-4} dilution in both cases, even if at 10^{-5} the *Est-6°* mutant flies were already in the repulsive part of the response. The CS flies were repulsed at higher dose (10^{-3}), a difference that could be explained by their different genetic background compared to *Est-6°* and *Est-6+* flies (because of which the *Est-6+* strain is a more reliable control strain.) Thus the *Est-6°* males had a 100-fold lower threshold for their attractive response to this odorant than did the *Est-6+* and CS males and a tenfold lower threshold for their avoidance response than did the CS flies. Responses of the different strains to the control odorant propionic acid were similar (Figure S2).

DISCUSSION

High temporal resolution of the chemical signal within the olfactory system is required to allow an accurate spatial location of odorant sources by the insect. Inactivation of odorant molecules which are a few milliseconds old in the vicinity of the ORs is a necessity for the dynamics of the response. The data accumulating in the literature suggest that ODEs could be involved at least in part in odorant inactivation (reviewed in

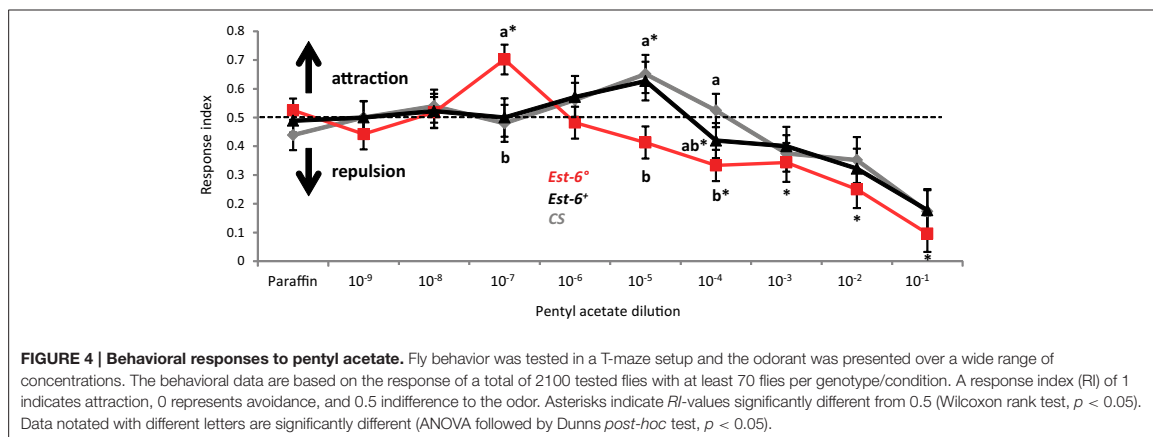
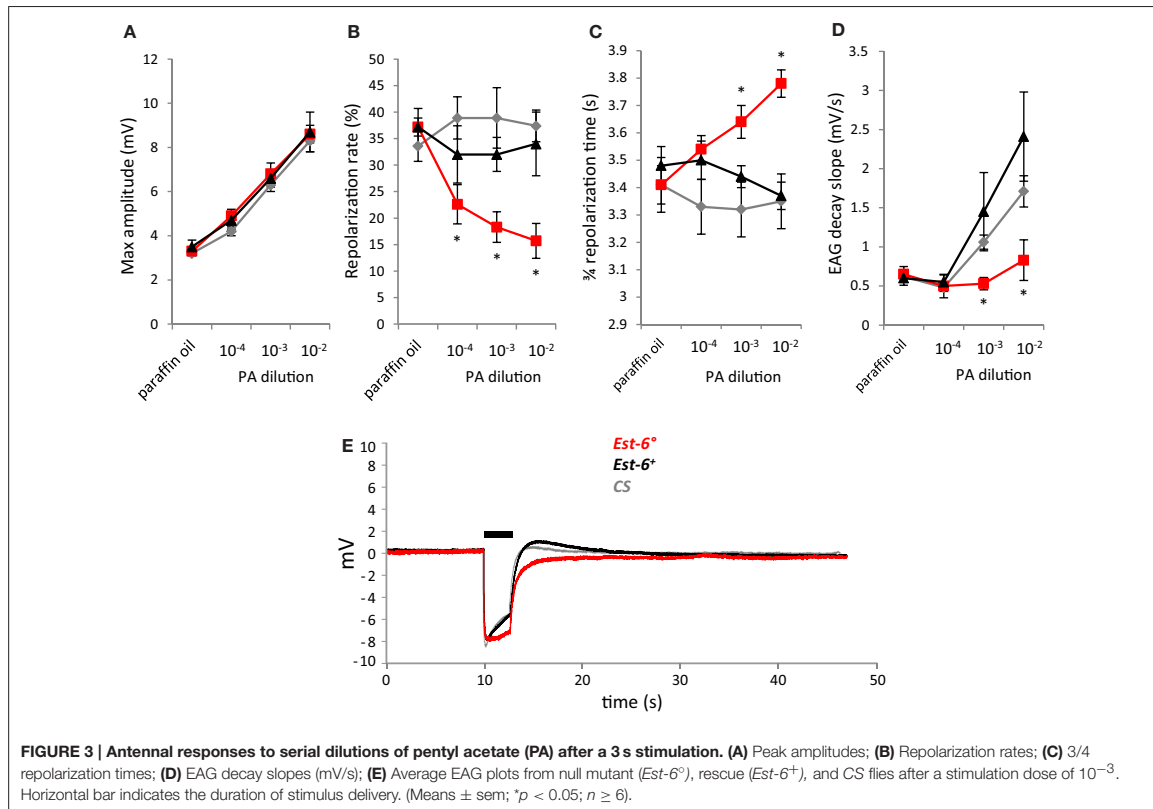


Leal, 2013). Pheromone or plant odorant degradation *in vitro* by antennal extracts or recombinant enzymes (reviewed in Jacquin-Joly and Maibèche-Coisne, 2009; Leal, 2013), as well as *in vivo* inhibition (Chertemps et al., 2012) support this hypothesis.

Most of the data that support the role of ODEs have been obtained on extracellular antennal esterases from moths (reviewed Leal, 2013; He et al., 2014a,b) and *Drosophila melanogaster* (Chertemps et al., 2012; Younus et al., 2014). Several sets of physiological and behavioral data for this latter species have previously suggested that an antennal extracellular carboxylesterase, EST-6, acts as an ODE in the detection and perception of the volatile pheromone CVA. However, its widespread expression through the third antennal segment (Chertemps et al., 2012) suggests a more general role in odorant processing. We show here that this carboxylesterase is indeed able to degrade a range of volatile esters emitted from natural *D. melanogaster* food sources *in vitro* and that it plays a role in antennal responses to those esters. Notably, those esters which were good substrates for the enzyme *in vitro* were also those which affected antennal responses, while those which were poor substrates, such as the pheromonal compound methyl myristate (Dweck et al., 2015), did not affect antennal responses. In the

case of pentyl acetate, we further show that the effects of EST-6 translate to behavioral changes in the presence of the odorant. A previous study of purified EST-6 had also shown *in vitro* activity against several other volatile esters which could be emitted by rotting fruit (e.g., ethyl acetate and butyl acetate; Danford and Beardmore, 1979). It appears that EST-6 may act as a general rather than specific ODE.

The kinetics of EST-6 toward the five preferred substrates among the eight esters studied here are in the range reported for other insect esterases that have been proposed as ODEs against their respective odorants (reviewed in Younus et al., 2014). Given the abundance of EST-6 within the antennae (Anholt and Williams, 2010), this suggests that EST-6 should be an efficient ODE for processing these odorants. Interestingly, JHedup, the other *D. melanogaster* esterase proposed to have an ODE function, was also found to have kinetics in this range for the five acetate esters of various primary, secondary and unsaturated esters that were tested (Younus et al., 2014). And, in the case of the one substrate also tested here, pentyl acetate, its kinetics were comparable to those of EST-6. While more work is needed to establish the extent of the overlap in substrate range, the pentyl acetate results suggest that more than one



esterase ODE could act on the same volatile, depending on their expression patterns within the antennae. Such an overlap could reduce the size of the *in vivo* effects evident from mutation or inhibition of any one of the ODEs in question.

Few data have been available to date on the effect of mutation or inhibition of candidate ODEs on the electrophysiological

responses of insect antennae. Several pharmacological carboxylesterase inhibitors (trifluoroketones) have been used in experiments with different lepidopteran species to test whether they affected responses to pheromones, but the effects reported remain controversial (reviewed in Vogt, 2005); the targets of these inhibitors, i.e., esterases but also putatively OBPs or ORs,

are still debated. In vertebrates, a potential role of esterases in the nasal mucus has also been revealed by pharmacological inhibition approaches which show that enzymatic conversion of odorants could be fast enough to affect the intensity and dynamics of the olfactory responses (Nagashima and Touhara, 2010; Thiebaud et al., 2013). More recently, we have shown that a null mutation of EST-6 in *D. melanogaster* modifies the neuronal responses of males to the pheromone CVA, prolonging the period of response, as might be expected in the absence of the relevant ODE (Chertemps et al., 2012). Similarly, our EAG data indicate that the temporal dynamics of antennal responses to various food odors that are good substrates for EST-6 are altered in EST-6 null males. A delay in signal termination was found for each of these compounds and, for pentyl acetate, a slower rate of recovery was evident even during the stimulation period (for both the stimulation periods tested).

Direct comparison between a behavioral and an electrophysiological response to an odorant should always be interpreted carefully, as the contexts of response recordings are different. These EAG results on pentyl acetate detection are nevertheless consistent with the differences we found between the two *Est-6* genotypes in the behavior induced by this odorant. We found that *Est-6*^o males had lower thresholds of attractive response to this volatile than did wild-type males in our behavioral choice experiments. This mirrors our previous finding that *Est-6*^o males also have lower thresholds for behavioral responses (including enhanced anti-aphrodisiac effects) to the pheromone CVA (Chertemps et al., 2012). Taken together, the data suggest that *Est-6*^o flies are more sensitive to both these ester compounds. We expect that the lack of EST-6 in the mutant antennae may delay the degradation of these esters in the sensillar lymph, which in turn could delay signal termination and modulate, at least partially, the corresponding behaviors.

REFERENCES

- Anholt, R., and Williams, T. (2010). The soluble proteome of the *Drosophila* antennae. *Chem. Senses* 35, 21–30. doi: 10.1093/chemse/bjp073
- Chertemps, T., François, A., Durand, N., Rosell, G., Dekker, T., Lucas, P., et al. (2012). A carboxylesterase, Esterase-6, modulates sensory physiological and behavioral response dynamics to pheromone in *Drosophila*. *BMC Biol.* 10:56. doi: 10.1186/1741-7007-10-56
- Cobb, M., and Dannel, F. (1994). Multiple genetic control of acetate-induced olfactory responses in *Drosophila melanogaster* larvae. *Heredity* 73, 444–455.
- Coppin, C., Jackson, C., Sutherland, T., Hart, P., Devonshire, A., Russell, R., et al. (2012). Testing the evolvability of an insect carboxylesterase for the detoxification of synthetic pyrethroid insecticides. *Insect Biochem. Mol. Biol.* 42, 343–352. doi: 10.1016/j.ibmb.2012.01.004
- Cornish-Bowden, A. (1995). *Fundamentals of Enzyme Kinetics*. London; Brookfield, VT; Portland: Ashgate Pub. Co.
- Danford, N., and Beardmore, J. (1979). Biochemical properties of esterase 6 in *Drosophila melanogaster*. *Biochem. Genet.* 17, 1–22. doi: 10.1007/BF00484470
- Durand, N., Carot-Sans, G., Bozzolan, F., Rosell, G., Siauxsat, D., Debernard, S., et al. (2011). Degradation of pheromone and plant volatile components by a same odorant-degrading enzyme in the cotton leafworm, *Spodoptera littoralis*. *PLoS ONE* 6:e29147. doi: 10.1371/journal.pone.0029147
- Durand, N., Carot-Sans, G., Chertemps, T., Bozzolan, F., Party, V., Renou, M., et al. (2010). Characterization of an antennal carboxylesterase from the pest moth *Spodoptera littoralis* degrading a host plant odorant. *PLoS ONE* 5:e15026. doi: 10.1371/journal.pone.0015026
- Dweck, H., Ebrahim, S., Thoma, M., Mohamed, A., Keesey, I., Trona, F., et al. (2015). Pheromones mediating copulation and attraction in *Drosophila*. *Proc. Natl. Acad. Sci. U.S.A.* 112, E2829–E2835. doi: 10.1073/pnas.1504527112
- Eisenthal, R., Danson, M. J., and Hough, D. W. (2007). Catalytic efficiency and k(cat)/K-M: a useful comparator? *Trends Biotechnol.* 25, 247–249. doi: 10.1016/j.tibtech.2007.03.010
- Getchell, T., Margolis, F., and Getchell, M. (1984). Perireceptor and receptor events in vertebrate olfaction. *Prog. Neurobiol.* 23, 317–345. doi: 10.1016/0301-0082(84)90008-X
- Haase, A., Rigosi, E., Frasnelli, E., Trona, F., Tassarolo, F., Vinegoni, C., et al. (2011). A multimodal approach for tracing lateralisation along the olfactory pathway in the honeybee through electrophysiological recordings, morpho-functional imaging, and behavioural studies. *Eur. Biophys. J.* 40, 1247–1258. doi: 10.1007/s00249-011-0748-6
- He, P., Zhang, Y., Li, Z., Yang, K., Zhu, J., Liu, S., et al. (2014b). An antennae-enriched carboxylesterase from *Spodoptera exigua* displays degradation activity in both plant volatiles and female sex pheromones. *Insect Mol. Biol.* 23, 475–486. doi: 10.1111/imb.12095

ACKNOWLEDGMENTS

This work was supported by ANR-12-BSV7-0024-01 ChemSenz, a doctoral grant from the Doctoral School ABIES for CS. We thank Coline Sentenac for her participation in the behavioral analysis. FY was supported by the French-Australian Science and Technology Program (FAST) and a CSIRO OCE Postgraduate scholarship.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fphys.2015.00315>

Figure S1 | Antennal responses to propionic acid (10⁻¹ dose) after a 3 s-stimulation. (A) Peak amplitudes; (B) Repolarization rates; (C) ^{3/4} repolarization times; (D) EAG decay slopes (mV/s). Means ± sem; *p* < 0.05; *n* ≥ 7.

Figure S2 | Behavioral responses to propionic acid. A response index (RI) of 1 indicates attraction, 0 represents avoidance, and 0.5 indifference to the odor. Asterisks indicate *F*-values significantly different from 0.5. At least 70 flies per genotype/condition were tested (Wilcoxon rank test, *p* < 0.05; ANOVA followed by Dunns *post-hoc* test, *p* < 0.05).

- He, P., Zhang, J., Li, Z., Zhang, Y., Yang, K., Dong, S., et al. (2014a). Functional characterization of an antennal esterase from the noctuid moth, *Spodoptera exigua*. *Arch. Insect Biochem. Physiol.* 86, 85–99. doi: 10.1002/arch.21164
- Ishida, Y., and Leal, W. S. (2005). Rapid inactivation of a moth pheromone. *Proc. Natl. Acad. Sci. U.S.A.* 102, 14075–14079. doi: 10.1073/pnas.0505340102
- Jackson, C., Liu, J., Carr, P., Younus, F., Coppin, C., Meirrelles, T., et al. (2013). Structure and function of an insect α -carboxylesterase (α Esterase7) associated with insecticide resistance. *Proc. Natl. Acad. Sci. U.S.A.* 10, 10177–10182. doi: 10.1073/pnas.1304097110
- Jacquin-Joly, E., and Maïbèche-Coisne, M. (2009). “Chapter 8: Molecular mechanisms of sex pheromone reception in Lepidoptera,” in *Short Views on Insect Molecular Biology*, ed R. Chandrasekar (Tamil Nadu: Bharathidasan University), 147–158.
- Kaissling, K. (2009). Olfactory perireceptor and receptor events in moths: a kinetic model revised. *J. Comp. Physiol. A Neuroethol. Sens. Neural Behav. Physiol.* 195, 895–922. doi: 10.1007/s00359-009-0461-4
- Kaissling, K.-E. (2014). “Pheromone Reception in Insects,” in *Neurobiology of Chemical Communication*, ed C. Mucignat-Caretta (Boca-Raton, FL: CRC Press), 99–138. doi: 10.1201/b16511-5
- Knaden, M., Strutz, A., Ahsan, J., Sachse, S., and Hansson, B. (2012). Spatial representation of odorant valence in an insect brain. *Cell Rep.* 19, 392–399. doi: 10.1016/j.celrep.2012.03.002
- Leal, W. (2013). Odorant reception in insects: roles of receptors, binding proteins, and degrading enzymes. *Annu. Rev. Entomol.* 58, 373–391. doi: 10.1146/annurev-ento-120811-153635
- Mane, S., Tompkins, M., and Richmond, R. (1983). Male Esterase 6 catalyzes the synthesis of a sex pheromone in *Drosophila melanogaster* females. *Science* 28, 419–421. doi: 10.1126/science.222.4622.419
- Mansourian, S., and Stensmyr, M. (2015). The chemical ecology of the fly. *Curr. Opin. Neurobiol.* 34, 95–102. doi: 10.1016/j.conb.2015.02.006
- Nagashima, A., and Touhara, K. (2010). Enzymatic conversion of odorants in nasal mucus affects olfactory glomerular activation patterns and odor perception. *J. Neurosci.* 30, 16392–16398. doi: 10.1523/JNEUROSCI.2527-10.2010
- Rützler, M., and Zwiebel, L. (2005). Molecular biology of insect olfaction: recent progress and conceptual models. *J. Comp. Physiol. A Neuroethol. Sens. Neural Behav. Physiol.* 191, 777–790. doi: 10.1007/s00359-005-0044-y
- Rytz, R., Croset, V., and Benton, R. (2013). Ionotropic receptors (IRs): chemosensory ionotropic glutamate receptors in *Drosophila* and beyond. *Insect Biochem. Mol. Biol.* 43, 888–897. doi: 10.1016/j.ibmb.2013.02.007
- Schlieff, M. L., and Wilson, R. I. (2007). Olfactory processing and behavior downstream from highly selective receptor neurons. *Nat. Neurosci.* 10, 623–630. doi: 10.1038/nn1881
- Siaussat, D. C., hertemps, T., and Maïbèche, M. (2014). “Detoxication, stress and immune responses in insect antenna: new insights from transcriptomics,” in *Short Views on Insect Biochemistry and Molecular Biology*, ed C. Raman (Tamil Nadu: Bharathidasan University), 75–98.
- Stensmyr, M., Giordano, E., Balloi, A., Angioy, A., and Hansson, B. (2003). Novel natural ligands for *Drosophila* olfactory receptor neurons. *J. Exp. Biol.* 206, 715–724. doi: 10.1242/jeb.00143
- Thiebaud, N., Veloso Da Silva, S., Jakob, I., Sicard, G., Chevalier, J., Ménétrier, F., et al. (2013). Odorant metabolism catalyzed by olfactory mucosal enzymes influences peripheral olfactory responses in rats. *PLoS ONE* 8:e59547. doi: 10.1371/journal.pone.0059547
- Vogt, R. G. (2005). “Molecular basis of pheromone detection in insects,” in *Comprehensive Insect Physiology, Biochemistry, Pharmacology and Molecular Biology*, eds L. Gilbert, K. Iatrou and S. Gill (London: Elsevier), 753–804.
- Vogt, R. G., and Riddiford, L. M. (1981). Pheromone binding and inactivation by moth antennae. *Nature* 293, 161–163. doi: 10.1038/293161a0
- Younus, F., Chertemps, T., Pearce, S., Pandey, G., Bozzolan, F., Coppin, C., et al. (2014). Identification of candidate odorant degrading gene/enzyme systems in the antennal transcriptome of *Drosophila melanogaster*. *Insect Biochem. Mol. Biol.* 53, 30–43. doi: 10.1016/j.ibmb.2014.07.003

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2015 Chertemps, Younus, Steiner, Durand, Coppin, Pandey, Oakeshott and Maïbèche. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

Chapter 4: Molecular basis for the behavioral effects of the odorant degrading enzyme Esterase 6 in *Drosophila*

SCIENTIFIC REPORTS

OPEN

Molecular basis for the behavioral effects of the odorant degrading enzyme Esterase 6 in *Drosophila*

Received: 15 December 2016
Accepted: 10 March 2017
Published: 10 April 2017

Faisal Younus^{1,2}, Nicholas J. Fraser², Chris W. Coppin¹, Jian-Wei Liu¹, Galen J. Correy², Thomas Chertemps³, Gunjan Pandey¹, Martine Maibèche³, Colin J. Jackson² & John G. Oakeshott¹

Previous electrophysiological and behavioural studies implicate esterase 6 in the processing of the pheromone *cis*-vaccenyl acetate and various food odorants that affect aggregation and reproductive behaviours. Here we show esterase 6 has relatively high activity against many of the short-mid chain food esters, but negligible activity against *cis*-vaccenyl acetate. The crystal structure of esterase 6 confirms its substrate-binding site can accommodate many short-mid chain food esters but not *cis*-vaccenyl acetate. Immunohistochemical assays show esterase 6 is expressed in non-neuronal cells in the third antennal segment that could be accessory or epidermal cells surrounding numerous olfactory sensilla, including basiconics involved in food odorant detection. Esterase 6 is also produced in trichoid sensilla, but not in the same cell types as the *cis*-vaccenyl acetate binding protein LUSH. Our data support a model in which esterase 6 acts as a direct odorant degrading enzyme for many bioactive food esters, but not *cis*-vaccenyl acetate.

Insects' olfactory systems are both primary drivers of their interactions with the environment and an emerging model for studying the molecular basis of eukaryote signaling processes. They are also of enormous interest in applied entomology because they are the targets for various pest control strategies based on mating disruption¹. Many aspects of insects' olfactory system have recently been elucidated but others, such as their odorant degrading enzymes (ODEs), are still poorly understood^{2,3}. It is proposed that ODEs are vital in the maintenance of the ongoing sensitivity of the olfactory system to incoming signals through the rapid inactivation of the relevant pheromones and kairomones once they have activated their receptors^{2,4}. However few of these have yet been characterized in any detail and fundamental questions remain about their modes of action. In particular there is ongoing debate, both about whether individual ODEs are specific for particular odorants or act generally against many², and about whether they act alone or in combination with odorant binding proteins (OBPs)^{2,5}. OBPs have been strongly implicated in the transport of incoming odorants through the sensillar lymph to their corresponding receptors, but any subsequent role for them in the deactivation process remains controversial².

Most of the work to date on ODEs has been done on certain Lepidoptera that have antennae large enough for classical biochemical and physiological studies⁴. One of the best characterized is the antennal specific esterase Apo1SE from the giant silk moth *Antheraea polyphemus*, which is estimated to have a k_{cat} of 127 s^{-1} for its natural E6Z11-16:acetate pheromone substrate⁶, but little activity for other isomers of this compound or for several other volatile esters tested. Relatively high k_{cat} values for their putative pheromone ester substrates have also been reported for a few other lepidopteran antennal esterases, although in at least two of these cases their substrate ranges seem to be less specific^{3,7,8}, perhaps suggesting broad rather than specific ODE functions.

By far the best characterized ODE for the model insect *Drosophila melanogaster* is esterase 6 (EST6). This enzyme was originally reported to degrade the major volatile sex and aggregation pheromone *cis*-vaccenyl acetate (cVA)⁹. Subsequent electrophysiological comparisons of EST6 wildtype and null flies on comparable genetic backgrounds have confirmed a role for the enzyme in the dynamics of cVA processing¹⁰. A specific OBP, LUSH, has been identified for cVA in *D. melanogaster* but the latest genetic evidence suggests that the interaction of cVA with its receptor OR67d is independent of LUSH¹¹. Notably, the distribution of EST6 in the third antennal

¹CSIRO Land and Water, Black Mountain, Canberra, ACT, 2601, Australia. ²Research School of Chemistry, Australian National University, Canberra, ACT, 2601, Australia. ³Université Pierre et Marie Curie, Institut d'Ecologie et des Sciences de l'Environnement de Paris, 75252, Paris, France. Correspondence and requests for materials should be addressed to J.G.O. (email: john.oakeshott@csiro.au)

segment also includes sensilla that are responsive to other odorants but not to cVA¹⁰, and further biochemical, electrophysiological and behavioral comparisons of the EST6 active and null strains indicate that the enzyme also acts on various short chain fatty acid food esters^{12,13}. There is indeed some relationship between the level of EST6 activity for the different esters and the size of the electrophysiological effect¹³, suggesting that EST6 does act as a general ODE with activities for several ester odorants.

As further evidence for pleiotropic effects of the enzyme, EST6 is also known to be expressed at high levels in the male ejaculatory duct, from where it is transferred to the female reproductive tract during mating¹⁴. It is then rapidly (within minutes) translocated to her hemolymph, where it remains for several days. Comparisons of females mated with null and wildtype EST6 males indicate it acts in the female to stimulate her egg-laying and delay her receptivity to re-mating^{15,16}. Early claims that this effect was mediated by EST6 action on endogenous cVA⁹ have since been refuted¹⁷, but the substrate responsible for the effect nevertheless remains unknown.

EST6 is a member of the carboxyl/cholinesterase (CCE) family of proteins¹⁸, which is represented by 30–110 different gene/enzyme systems encoding diverse functions in the insect genomes so far sequenced¹⁹. However, the juvenile hormone esterase from the moth *Manduca sexta* (MsJHE)²⁰, an insecticide metabolizing carboxylesterase from the blowfly *Lucilia cuprina* (LcαE7)²¹ and acetylcholinesterase from *D. melanogaster* (DmAChE)²² are the only insect CCEs for which crystal structures have been determined, so relatively little is known of the structure-function relationships underlying their diverse functions. The structural features of EST6 have so far been inferred from the structure of the *D. melanogaster* AChE or its orthologue from the electric ray *Torpedo californica*, but the low sequence similarity between EST6 and AChE (27%) means that the fine structural features of the enzyme responsible for its substrate specificity have not yet been understood²³.

In this paper, we present a comprehensive analysis of the substrate range of semi-purified EST6, showing it has significant activity for a range of short chain fatty acid esters but negligible activity for long chain fatty acid esters. In particular, we find that EST6 is not active against cVA, either in the presence or absence of LUSH, but does degrade various volatiles emitted by rotting fruits and the yeasts therein on which the flies naturally live; these volatiles have recently been shown to be key regulators of *Drosophila* mating behavior²⁴. We also present a crystal structure for the enzyme which, together with *in silico* docking studies, supports the kinetic data and shows that its active site can readily accommodate short chain fatty acid esters, including the yeast and fruit volatiles above, but not long chain fatty acid esters like cVA. A unique active site location and entry is identified, which appears to explain the enzyme's substrate preferences. Finally, we present data from immunohistochemical and behavioral assays with RNAi knock-down constructs that localize the expression of EST6 to a large proportion of non-neuronal cells surrounding the olfactory neurons of almost all the olfactory sensilla, but in different cells than those producing LUSH in the trichoid sensilla.

Results

Enzyme kinetics. Wildtype EST6 was tested for activity against 85 bioactive ester odorants and two model substrates; 4-nitrophenyl acetate (4NPA) and 2-naphthyl acetate (2NA). It showed detectable activity (generally, a specificity constant $k_{cat}/K_M^{Est} > 1.5 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$) for 47 of the bioactive esters as well as the two model substrates (Fig. 1 and Supplementary Table S1). Specificity constants for most (42) of these 49 were above $1 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$, although none exceeded $1.3 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$, consistent with typical k_{cat}/K_M values for enzymatic reactions in secondary metabolism²⁵. The highest activities were seen with esters containing longer ($C > 6$) or more complex (branched, unsaturated or cyclic) leaving groups and acetate or propionate acid moieties, although a combination of mid-length leaving groups and acid groups (butyl decanoate) was also a relatively good substrate in these assays. The 38 compounds for which little or no activity could be detected were mainly methyl or ethyl esters or those with more complex acidic groups. cVA, which has a very long leaving group, was not hydrolysed at significant rates.

Precise K_M values for most substrates could not be calculated because of low substrate solubility. However, estimates of K_M values could be obtained for some of the more soluble esters (4NPA, 2NA, benzyl acetate, phenyl acetate, phenethyl acetate) and were found to be in the range 121–880 μM under these assay conditions, which included 5% ethanol (Supplementary Table S1). Previous kinetic analyses of EST6 with 2NA²⁶ and 4NPA (Supplementary Fig. S1) indicated the K_M values were ~5–20 fold lower in the absence of 5% ethanol. The K_M values that were obtained generally exceed the concentration of substrate in the reaction mixtures (200 μM), which means that the k_{cat}/K_M^{Est} calculated will be a reasonable approximation of the true k_{cat}/K_M value (in those cases where K_M is lower than 200 μM , the estimated value will underestimate the true k_{cat}/K_M for the assay conditions used – see Methods). Given the measured K_M values are typically $>100 \mu\text{M}$, the measured k_{cat}/K_M values therefore imply relatively high k_{cat} values (in some cases $>1,000 \text{ s}^{-1}$). These results indicate that EST6 is a relatively “fast” enzyme (high k_{cat} values) that displays broad specificity, working moderately efficiently with a very wide range of natural esters. In comparison, the related enzyme acetylcholinesterase catalyses acetylcholine hydrolysis with very high efficiency but has an extraordinarily narrow substrate range, essentially catalysing a single substrate¹⁸.

The assays with cVA were repeated in the presence of the cVA binding protein LUSH, which again indicated negligible activity, even in the presence of a great excess of EST6 (57 nM compared with the 3 nM used previously). The only other known pheromone among the compounds tested was the fatty acid ester methyl myristate, which is also a plant volatile and functions as an attractant to *D. melanogaster*²⁷. EST6 also had relatively little activity with this compound ($\sim 1.5 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$).

Apart from the two pheromones and two model substrates, all the esters tested for which EST6 was found to have significant activity are food odorants that are known to be bioactive against *D. melanogaster* in *in vivo* (behavioral) and/or *in vitro* (receptor binding) assays (Supplementary Table S2)^{27–29}. Five of the major odorant receptors in this species that are known to have affinity for ester ligands (Or10a, Or22a, Or35a, Or67a and Or98a) all bind a variety of such esters, with substantially overlapping ranges^{30,31}. Notably, many of the alcohol and aldehyde metabolites of these esters are also known ligands for various *D. melanogaster* odorant receptors³².

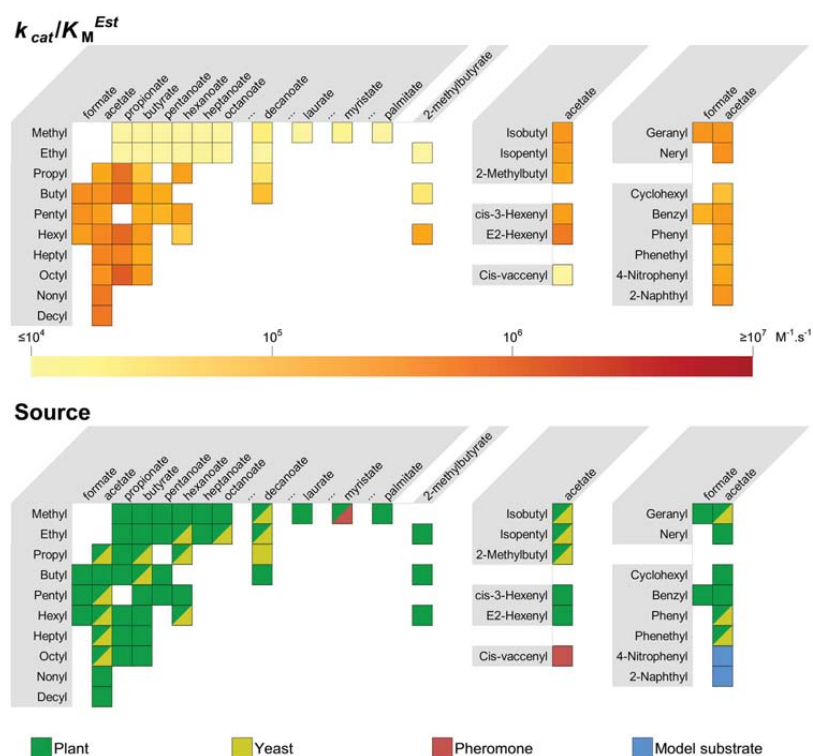


Figure 1. EST6 k_{cat}/K_M^{Est} and biological source of the most active substrates tested and other substrates of particular structural or physiological significance. Alcohol moieties are listed on the vertical and are grouped according to structural similarity. Acid moieties are listed on the horizontal. An ellipsis (...) demarcates a break in an otherwise incremental series. Data on the biological source of the substrates are taken from Supplementary Table S2. Activity results for all 87 compounds tested are given in Supplementary Table S1.

Structure determination of EST6. One of the main barriers to crystallizing EST6 was its very low soluble expression in *Escherichia coli*. To address this, we used the same approach as we did to solve the structures of the α -Esterase 7 carboxylesterase from *Lucilia cuprina*²¹. Briefly, we utilized directed evolution to screen libraries of EST6 variants lacking the N-terminal signal peptide³³ for enhanced activity (as a result of enhanced soluble expression) in *E. coli* (Supplementary Fig. S2). After six rounds of directed evolution, the EST6 variant with greatest soluble expression (EST6-1) contained 16 mutations; K15V, V145L, R208K, G229E, N237S, T247A, D290G, I292F, I335V, E383G, S400G, A416V, F450S, F456S, N485D, I511T (note that amino acids are numbered from the first residue of the mature EST6 protein as it would be processed in its native form within the fly³³ and omits the start methionine included to permit heterologous expression in *E. coli*). Four of these mutations have been found in EST6 from several *Drosophila* species (V145A, R208K, T247A and I292F)^{23,34}. Importantly, the catalytic activity of EST6-1 was very similar to that of EST6-WT (Supplementary Fig. S3), suggesting that the 16 mutations principally affected folding, rather than function, consistent with their being located remote from the active site.

Using the Origami B strain of *E. coli*, a cell line that has been designed to enhance disulfide bond formation in the cytoplasm in prokaryotic systems³⁵, high levels of soluble EST6-1 were expressed (~ 20 mg.l⁻¹) (Supplementary Fig. S4). Expression of EST6-WT in *E. coli* Origami B cells resulted in substantially lower soluble expression (~ 0.5 mg.l⁻¹). Size exclusion chromatography showed EST6-1 eluted primarily as a monomer, although there was secondary peak present that indicated a small amount of dimer (Supplementary Fig. S4). Crystallization trials of the EST6-1 monomer fraction at two different concentrations did not yield crystals. We then performed surface lysine methylation, which has been shown to increase the propensity of proteins to crystallize³⁶, which yielded crystals in conditions of 0.2 M ammonium acetate, 0.1 M Tris pH 8.5 and 25% w/v PEG 3,350 that diffracted to 2.1 Å resolution.

The structure of EST6-1 contains 520 amino acids, 353 water molecules, 32 surface carboxylated lysines and one monomer per asymmetric unit. All but the first four N-terminal amino acids are present in reasonable electron density. EST6-1 adopts an α/β -hydrolase fold, including the conserved catalytic triad and oxyanion hole (Fig. 2a,b). The eight-stranded β -sheet (β 1–8) surrounded by six α -helices (A–F), that comprises the canonical fold is present, along with the two antiparallel β -strands at the start and two antiparallel β -strands at the end of the structure that are found in the other three insect carboxylesterases whose structures have been solved^{20–22}. The

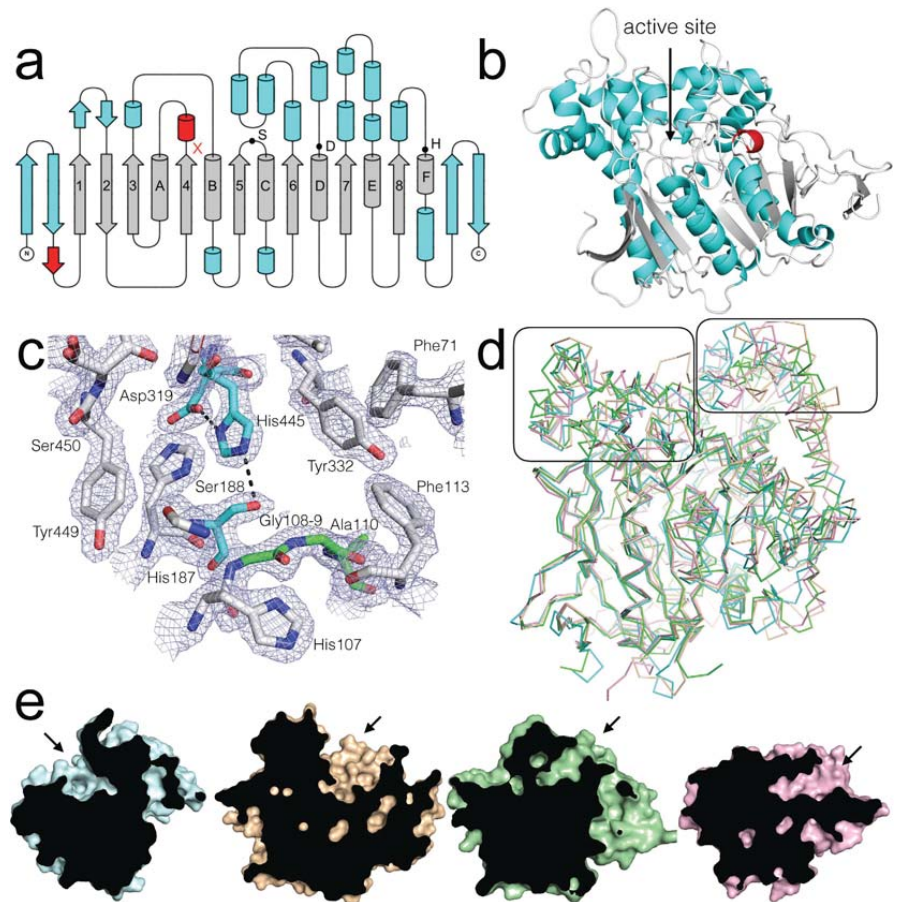


Figure 2. The structure of EST6 from *D. melanogaster*. (a) Topology representation of EST6 displaying the conserved α/β -hydrolase fold (grey), secondary structure found in the structurally similar proteins (blue) and unique secondary structure (red). S, D, H represent the Ser188, Asp319 and His445 residues that make up the catalytic triad. The oxyanion hole is located in the loop following sheet 4 (marked by a red x). (b) Cartoon diagram of EST6 with features shown in the topology model similarly coloured. The location of the active site is indicated. (c) The active site of EST6 with $2mF_o - dF_c$ electron density contoured at 1.5σ . The active site serine and histidine from the catalytic triad are coloured cyan, the oxyanion hole (Gly108, Gly109, Ala110) is coloured green. (d) An overlay of EST6 (cyan), *LcoE7* (tan; 4FNM), *DmAChE* (green; 1QO9) and *MsJHE* (pink; 2FJ0). Conservation of the core β -sheet and conserved α -helices is apparent, but the structures diverge in the region that forms the active site entrance. These regions, either side of the active site, are boxed for clarity. (e) A superposition of EST6, *LcoE7*, *DmAChE* and *MsJHE*, with cut-aways through the middle of the active site. The location of the active site entrance difference between EST6 (cyan) and the other related insect carboxylesterases *LcoE7* (tan; 4FNM), *DmAChE* (green; 1QO9) and *MsJHE* (pink; 2FJ0).

entrance of the active site is formed by loops following $\beta 1$, loops and two helices following $\beta 4$, and loops following $\beta 8$, including helix F that makes up part of the canonical α/β hydrolase fold. The active site itself is formed from the catalytic triad (Ser188, His445 and Asp319), oxyanion hole (Gly108, Gly109 and Ala189) and additional residues (Tyr322 Tyr449, Phe450, Asn455, Phe456 and Val457) from a helix after $\beta 7$ and a loop following $\beta 5$ (Fig. 2c). There are three intramolecular disulfide bonds present (65–84, 240–252, and 493–514) on surface loop regions. The first two disulfide bonds are also seen in the other two insect carboxylesterase structures containing disulfides (AChE, JHE), but the third disulfide is unique to EST6, which also has a shortened C-terminus relative to the other three carboxylesterases structures.

Comparison to known structures. Analysis of the ESTHER database³⁷, which comprehensively describes the α/β hydrolase fold across a wide range of organisms, reveals that EST6-1 falls into Block C, which also includes the other three known insect carboxylesterase structures. Amongst the insect carboxylesterases,

Protein	Active Site Volume (Å ³)	Distance from surface to active site Serine (Å)
EST6 WT FoldX Model	408	15.1
EST6-1 Crystal Structure	935	15.1
<i>LcoE7</i> (4FNG)	2727	20.2
<i>DmAChE</i> (1QO9)	782	17.2
<i>MsjHE</i> (2FJ0)	1308	18.1
Lipase (1AQL)	3074	17.4

Table 1. Active site volume calculated using the CASTp server.

which Oakeshott *et al.*¹⁸ have divided into 14 Clades, EST6-1 falls into Clade E, with *LcoE7* (PDB - 4FNM) in Clade B, *MsjHE* (PDB - 2FJ0) in Clade G and *DmAChE* (PDB - 1QO9) in Clade J. Application of the SALAMI server³⁸ confirmed there were no structural homologues in PDB closer to EST6-1 than these three enzymes (Supplementary Table S3). The four clades are well separated from one another phylogenetically (26–29% amino acid identity) but all four structures superimpose well over the canonical fold (2.27, 2.09 and 2.41 Å C- α r.m.s.d. for the other three compared with EST6-1, respectively). In contrast, in the loop regions above the canonical β -sheet and α -helices, there is significant variance between the structures (Fig. 2d).

Closer inspection of the structures and alignment revealed that EST6-1 is missing the C-terminal helix present in *DmAChE*, *LcoE7* and *MsjHE*. Another feature of interest is the length and composition of the surface-exposed loop regions after strands β 1, β 6 and β 8, which contribute to the active site entrance in the other three proteins. In the latter three, the opening of the active site is formed from helices after β 6 and β 7 and loops and helices after β 1 and β 8, but this region has closed over in EST6-1. Its active site entry is instead formed by loops and helices after β 1, β 4, and β 8 on the opposite face of the protein (Fig. 2e). The result is a narrower and shorter active site entrance in EST6-1 in comparison to the open and accessible active site in *LcoE7* and the deep gorges leading to the catalytic triads in AChE and JHE.

A comparison of the four structures using the CASTp server³⁹ also revealed that the active site volume of EST6-1 was significantly less than in *LcoE7* (Table 1). The relative sizes of the active sites of *DmAChE*, *LcoE7* and *MsjHE* reflect their native substrate preferences: *LcoE7* natively hydrolyses a wide range of medium chain fatty acid methyl esters and has a large active volume (2727 Å³)²¹, while AChE and JHE both have narrower substrate specificities, for the smaller acetylcholine and juvenile hormone molecules respectively, and have much smaller active site volumes, of 782 and 1308 Å³, respectively. The active site volume of EST6-1 is estimated to be 935 Å³, which is consistent with the observed preference of EST6 for smaller substrates than *LcoE7* (Fig. 1).

The substrate binding pocket. Given that EST6-1 is ~97% identical to EST6-WT, and the mutations distinguishing them are all remote from the active site, it is highly likely that the structures will be essentially identical in this region. Nevertheless, for analysis of the substrate binding site, a model of EST6-WT was produced using the empirical structure of EST6-1 and the FoldX force field, which has been developed to allow accurate modeling of point mutations, among other things⁴⁰. As noted above, the conserved catalytic triad of EST6 consists of Ser188, His445 and Asp319, while the backbone NH groups of Gly108, Gly109 and Ala189 create the oxyanion hole (Fig. 2c). His187 is adjacent to the catalytic serine and as with the other three structures its side chain extends into the active site; in the others it has been suggested to affect substrate specificity²⁶. EST6 has an asymmetrical binding pocket with a very small, hydrophobic and buried sub-site consisting of Ala110, Trp221, Phe276, Tyr322, Phe397 and His445 that could accommodate the carboxyl group. Opposite this, there is a larger cavity (the putative alcohol leaving group site) that extends into the active site exit/entrance and is slightly less hydrophobic, consisting of Gln70, Phe71, Phe113, Gly114, Gln118, Asn119, Ile429, Tyr449, Phe450, Asn455, Phe456 and Val457 (Fig. 3a).

A representative range of potential substrates that EST6 was tested with were docked into the active site of EST6 using flexible docking with DOCKOvalent⁴¹, which is able to screen binding modes for substrates or inhibitors that form covalent bonds with the target enzyme (Fig. 3b, Supplementary Fig. S5 and Supplementary Table S4). The docking results are entirely consistent with the kinetic data, in so much as acylated enzyme intermediates for substrates that were hydrolyzed at significant rates were well accommodated by the substrate binding pocket, whereas no suitable binding poses (without steric clashes) could be obtained for the acylated enzyme intermediates that would result from reaction with compounds that were shown not to be substrates of EST6 (such as cVA). A clear trend is evident: the small sub-site can easily accommodate chains of 1–6 carbons, while the leaving group site has a preference for longer saturated chains, such as hexyl and octyl, over smaller chains, such as methyl and ethyl, but not as large as cVA (C18). This is also consistent with the high activity and complementary binding of geranyl and neryl acetate, with the short carboxyl side chains being accommodated in the small sub-site and the unsaturated leaving group being accommodated in the leaving group site. Likewise, those substrates with aromatic leaving groups and short carboxyl groups are also well accommodated (Supplementary Fig. S5). This analysis provides a molecular explanation for the observed substrate preference for typical food odorants with carboxyl groups of 0–6 carbons and leaving alcohol groups up to ~10 carbons, including branched and aromatic moieties. This structural analysis also strongly supports the kinetic analysis and the initially surprising observation that cVA does not appear to be a physiological substrate for EST6, in that it is clearly far too large for the EST6 substrate binding pocket.

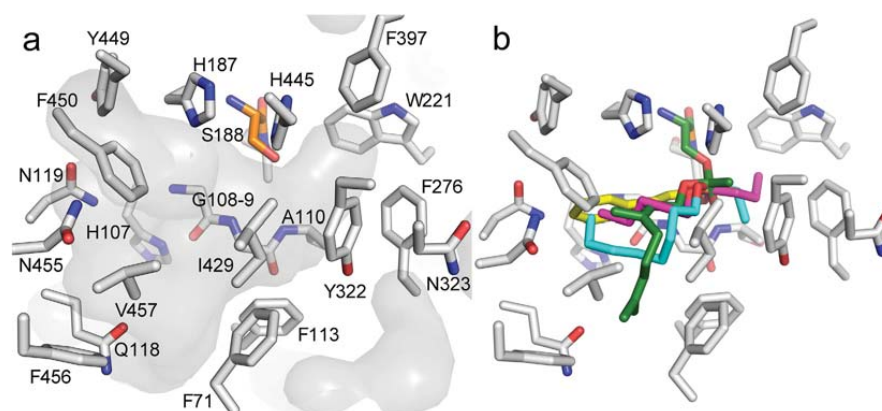


Figure 3. The substrate binding site of EST6. (a) The surface of the substrate binding site is shaded grey and the residues that comprise the small and large pockets are shown (grey) as is the catalytic serine (orange). The small site consists of Ala110, Trp221, Phe276, Tyr322, Phe397 and His445, and the large site consists of Gln70, Phe71, Phe113, Gly114, Gln118, Asn119, Ile429, Tyr449, Phe450, Asn455, Phe456, and Val457. (b) An overlay of representative acylated enzyme intermediates covalently docked into EST6: the efficiently hydrolyzed substrates pentyl butyrate (magenta), octyl propionate (cyan), geranyl acetate (green) and phenethyl acetate (yellow) all produce acylated intermediates that are accommodated by the substrate binding site.

Localization of EST6 in the antennae. *Est6* is known to be highly expressed in the antenna⁸, in particular in the third antennal segment¹⁰, but its expression in this tissue at the cellular level was unknown. Labelling of EST6 with anti-EST6 antibody and of Red Fluorescent Protein (RFP) under the control of the *Orco* promoter (*Orco* encodes the universal odorant co-receptor *Orco*) in transgenic adults showed EST6 immunoreactivity in numerous cells at the base of olfactory sensilla throughout the third antennal segment whereas, as expected⁴², the *Orco* promoter directed expression in numerous olfactory receptor neurons (ORNs) and in cilia entering the sensillar lumen (Fig. 4). As was earlier suggested by Chertemps *et al.*¹⁰, there was thus no co-localization of the two signals, showing that EST6 is not expressed in ORNs. Similarly, a complementary experiment showed no co-localization of EST6 and the neuron-specific expression of Green Fluorescent Protein (GFP) under the control of the *elav* promoter⁴³ (Supplementary Fig. S7). Given that EST6 is a secreted enzyme, this confirms that the enzyme surrounds the *Orco*⁺ dendrites within the sensillar lymph of various sensilla.

Co-labelling of EST6 and *lush* was then performed to investigate whether the location of EST6 in the sensillar lymph includes the T1 trichoid sensilla involved in cVA detection. LUSH is known to be expressed in all trichoid types⁴⁴. Labelling of EST6 with anti-EST6 antibody and of RFP under the control of the *lush* promoter in transgenic adults found that both signals were closely associated but with no co-localization of the two. RFP was found at the base of trichoid sensilla in accessory cells (Supplementary Fig. S8) that could correspond to trichogen and tormogen cells⁴⁴, whereas EST6 was apparently produced by different support cells for the trichoid sensilla than the LUSH-producing cells⁴⁵, and possibly also by the epidermal cells surrounding the sensilla. To corroborate this result we also performed RNAi knock-down experiments. These results are also consistent with *Est6* is not being co-expressed with *lush* (Supplementary text).

Altogether, these data show that EST6 is produced by non-neuronal cells in the olfactory sensilla, most probably in a large population of accessory cells surrounding ORNs. Its localization in the sensillar lymph is compatible with a function of a general ODE in the basiconic sensilla involved in the detection of almost all the substrates tested here⁴⁶. Its function in the T1 trichoid sensilla is not yet clear but its effect on cVA processing in the absence of any direct hydrolytic activity for the compound may reflect a general scavenging role for other ester odorants which might otherwise impede the processing of cVA by its own, as yet unknown, ODE. It is possible that it also plays an equivalent broad scavenging role in some of the other sensilla where it is abundant, although its strong hydrolytic activity for many ester kairomones suggests it has a direct ODE function for several of them.

Discussion

Notwithstanding the genetic evidence that EST6 contributes to cVA processing *in vivo*¹⁰, we find that the enzyme has negligible activity ($<1.5 \text{ M}^{-1} \cdot \text{s}^{-1}$) for this substrate *in vitro*, with or without LUSH in the assay mix. Our results in fact confirm the only other direct measure of its *in vitro* activity, by Mane *et al.*⁹; their estimation of 55 picomoles of cVA per min per g of purified EST6, or $3.4 \text{ M} \cdot \text{min}^{-1} \cdot \text{M}^{-1}$, (in the absence of LUSH) is in the range that was too low to measure accurately in our assays. We concur with Vandermeer *et al.*¹⁷ that activity in this range is most unlikely to be physiologically relevant. This indicates that the *in vivo* effects of EST6 on cVA processing seen by Chertemps *et al.*¹⁰ must be indirect.

While we found that EST6 had low activity against cVA, it clearly has physiologically significant ($k_{\text{cat}}/K_M > 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$)²⁵ activity with a wide range of esters with acyl chains up to six carbons in length and alcohol groups from mid length (3–10 carbon atoms), aliphatic moieties to branched, secondary, unsaturated,

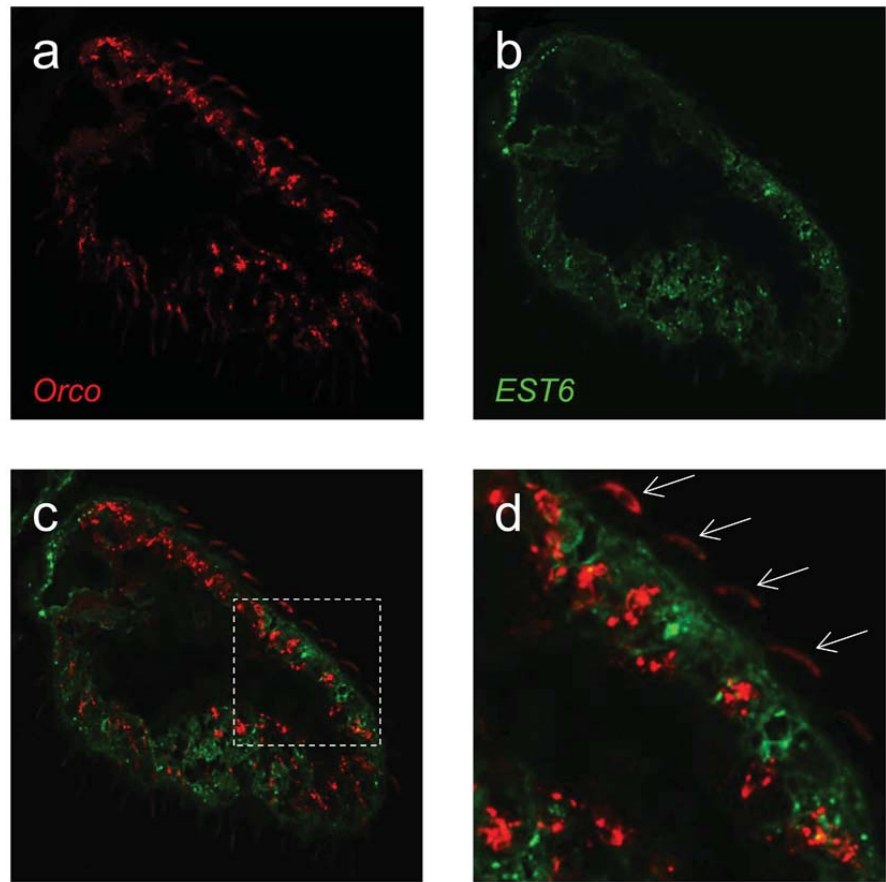


Figure 4. EST6 and *Orco* expression in the third antennal segment, longitudinal sections. (a) Membrane-tethered RFP expressed with the *Orco* promoter (*Orco^{Gal4}/UAS-mCD8::RFP* transgenic flies). (b) EST6 protein localization in the same section. (c) Merge image of (a,b): Est-6 and *Orco* are not expressed in the same cells. (d) Higher magnifications of (c): EST6 protein surrounds the *Orco*⁺ dendrites. Arrows indicate the dendrites of *Orco* expressing ORNs. Western blots and immunohistochemistry showing the specificity of the anti-EST6 antibody are shown in Supplementary Fig. S6.

cyclic and aromatic groups. These substrates include many fruit and yeast volatiles that are known to be bioactive against *Drosophila*, consistent with the results of electrophysiological and behavioural comparisons of wildtype and EST null flies by Chertemps *et al.*¹⁰, which show that the enzyme contributes to the processing of many such molecules *in vivo*. As such, our biochemical data support the proposition that EST6 is a general, rather than specific, odorant degrading enzyme (ODE), but with a substrate range tuned to various volatile esters with relatively short chain acyl groups that are commonly emitted by the food sources for the flies.

Significantly, the bioactivity of many of these better substrates for EST6 involves attraction behaviours⁴⁷. For example, fruity smelling acetate esters such as isopentyl and pentyl acetate, which are produced by both plants and yeasts, are highly attractive to *Drosophila*⁴⁸, wherein they activate several fairly broadly tuned odorant receptors, such as Or43b, Or47a and Or85b^{46,49}. Likewise, the phenolic yeast volatile phenethyl acetate elicits an attraction response from the fly⁵⁰ and activates its Or85d receptor⁴⁹. Notably, some of these attraction behaviors also manifest as effects on reproductive traits; for example, citrus fruits emit many short-mid chain volatile acetates (e.g. propyl, hexyl, heptyl, nonyl, decyl, neryl and geranyl acetates⁵¹), which attract females to lay eggs²⁸.

It has been shown that several food odors, including ester substrates for EST6, can act synergistically with cVA in both aggregation and courtship bioassays^{52–55}. Indeed, some evidence suggests that cVA only acts as an aggregation pheromone in the presence of attractive food odors⁵⁵. It is suggested that the co-processing of pheromonal and kairomonal stimuli would help coordinate feeding and oviposition site selection with reproductive behaviors⁵³. However, we cannot see how this synergism would explain the indirect effects of EST6 activity on cVA processing observed by Chertemps *et al.*¹⁰. One reason is that the experimental design of that previous study meant

that food odors would not have been present in the cVA atmospheres they tested. Furthermore, the co-processing of the signals from cVA and the food odors must occur downstream of their receptors, since they have different receptors and the signals from their receptors are transmitted to different glomeruli in the brain, but the effects of EST6 on EAG responses to cVA seen by Cherteremps *et al.*¹⁰ must occur prior to or at the time when the cVA interacts with its receptors. Other indirect effects of EST6 on cVA processing must therefore explain the data of Cherteremps *et al.*¹⁰. For example, as noted above, EST6 may facilitate cVA processing simply by removing other potential substrates (or inhibitors) of the ODE that does degrade cVA. As noted, the latter ODE may be a lipase, and indeed, with an 18-carbon leaving group, cVA is more like a typical lipase substrate than an esterase substrate. Our localization studies would certainly allow for that, given the broad distribution of the enzyme through the sensillar lymph. Further work is needed to elucidate the molecular basis for the effects seen by Cherteremps *et al.*¹³.

Our biochemical studies also bear on the question of the molecular basis for the effects on female oviposition and remating behaviors due to the ejaculatory duct EST6 transferred from their mates^{15,16}. This enzyme is known to be transferred from the female's reproductive tract to her haemolymph within minutes of mating¹⁴, but its fate from there and its substrate in the female are unknown. Our results indicate that a wide variety of esters of terpene or aromatic alcohol groups and short-mid chain acids could be candidate substrates. Notably, some such compounds are precursors for various hormones and other key molecules in the fly^{56,57}. Modern metabolomic technologies may be useful in identifying the *in vivo* substrate for the transferred EST6, particularly given the availability of the *Est6*^o flies and wildtype revertants on the same genetic background¹⁰.

EST6, in Clade E of the carboxylcholinesterase gene family, is not closely related in sequence (26–29% amino acid identity) to any of the three insect esterases for which structures have been solved previously (in Clades B, G and J). While its overall structure is similar to the other three, we noted several significant differences in relation to its active site. Of particular note was the appearance of an active site entrance on the opposite face of the protein to that containing the active site entrance in the other three structures. Interestingly, the entrance in EST6 corresponds to the alternative 'back door' entrance that has been proposed for AChE⁵⁸. Moreover, the corresponding surface of the catalytically inactive ligand-binding 'esterase' neuroigin is the site to which its ligand binds⁵⁹.

Transcriptomic analyses of sensory tissues in various insects have shown as many as half of the catalytically competent carboxyl/cholinesterases in some insects may be expressed at readily detectable levels in their sensory tissues^{8,60}. The few for which there is any empirical support for ODE functions have spanned four major Clades (A, D, E and G)^{18,61}, suggesting that esterase ODEs may have evolved independently on several occasions. However, there is a concentration of putative esterase ODEs in the particular lineage within Clade E that contains EST6 (31% amino acid identity)¹⁸. This lineage contains esterases from at least four insect orders, including one of the best-understood ODE's at a physiological level, the Apo1PDE from the silkworm *Antheraea polyphemus*. Apo1PDE is highly specific ODE for a particular sex pheromone substrate⁶², whereas we find EST6 has both broad activity for many kairomones and an indirect effect on cVA processing whose mechanism we currently do not understand. Further work on this lineage could elucidate a range of biochemical, physiological and evolutionary phenomena concerning the function of esterases in insect antennae.

Methods

EST6 activity assays. The expression of wildtype EST6 and an inactive EST6 variant in the baculovirus system has been described previously¹³. These two enzymes were assayed here for activity against 85 ester odorants of potential ecological relevance^{49,63,64} and two other model substrates (listed in Supplementary Table S1). All these esters were purchased in the highest available purity.

Eighty two of the esters were first subjected individually to gas chromatography-mass spectrometry (GC-MS, 7890 series, Agilent Technologies, USA) to determine their respective retention times. A J&W DB-WAX column (30 m × 0.25 mm × 0.25 μm, Agilent Technologies, USA) was used with He (2 ml. min⁻¹) as the carrier gas. The oven temperature was initially set at 50 °C for 2 mins and then subsequently increased over a gradient of 10 °C to 275 °C and held for 10 mins. The injector and detector temperature was set at 250 °C with a 10:1 split ratio.

Mixtures of up to 17 compounds with non-overlapping GC-MS retention times were then made in Tris HCl buffer pH 8.0 for a set of preliminary 'group assays'. Each group included pentyl acetate as a common ester substrate standard. All compounds had been dissolved in ethanol to give a 5% v/v final solvent concentration; preliminary assays on some of the more water-soluble esters showed that this ethanol concentration increased K_M by 5–20 fold (see below) but lower concentrations of ethanol were insufficient to solubilize some compounds and equivalent concentrations of other organic solvents tested were more disruptive to EST6 activity. Several reactions were set up at 25 °C in Tris-HCl buffer pH 8.0 with each ester in the mixture at a final concentration of 200 μM and the enzyme (added last) at 8.2 nM. Individual reactions were then stopped by the addition of 0.5 volumes of ice-cold hexane containing 200 μM heptanone as an internal non-ester standard at intervals from 5 to 65 mins. The concentrations of the various esters remaining were then determined by GC-MS as above. EST6 activity was calculated from the difference in substrate usage between the wildtype and null enzymes, but all values for the latter were essentially negligible.

Subsequently, 43 substrates from the group assay, including all the better substrates, were assayed individually in order to obtain estimates of k_{cat}/K_M using equation (1):

$$k_{cat}/K_M^{Est} = V_0/([E] \cdot [S]) \quad (1)$$

where [E] and [S] are the starting enzyme and substrate concentrations respectively, and V_0 is the initial velocity of the reaction⁶⁵.

Aside from the single substrate, these assays were the same as those for the group assays, except that a lower enzyme concentration was used (0.1 to 3.6 nM). The appropriate enzyme concentration was inferred from the enzyme's activity towards each substrate in the group assay. Three other esters that were not included in the

group assays but with closely similar chain lengths and structures to some of the best substrates were also assayed individually in this way. Individual substrate assays with two model esters, 4 NPA and 2 NA, were also carried out using previously described 420 and 390 nm UV/vis protocols for monitoring substrate loss^{8,21}. K_M estimates could be obtained from these data for a few substrates and a few were also obtained using the competitive inhibition method with 4 NPA as substrate as described in Younus *et al.*⁸. All the above assays were conducted in triplicate.

Assays with LUSH. Some assays were also conducted in the presence of the odorant binding protein LUSH. In preparation for this the *lush* coding region was synthesized by Invitrogen and cloned into the expression vector pETMCSI⁶⁶. The LUSH protein was overexpressed in inclusion bodies of *E. coli* BL21 (DE3) star (Invitrogen) cells after overnight growth in Lysogeny Broth (LB) broth containing 100 mg.l⁻¹ of ampicillin at 37 °C. The cells were harvested by centrifugation at 5,000 g for 5 min at 4 °C, the cells lysed by three passages through a French Press, and the inclusion bodies collected by centrifugation at 10,000 g for 20 min at 4 °C. The inclusion bodies were then solubilized and refolded following the method of Kruse *et al.*⁶⁷ using a cysteine-cysteine redox reaction in the presence of 1% v/v ethanol. The only modifications to this method were that 8 M urea was used to solubilize the inclusion bodies and the soluble protein was dialyzed in 20 mM Tris pH 7.4, 50 mM NaCl. The soluble LUSH was further purified by using a Superdex 200 preparation size exclusion column (GE Healthcare, UK) and assayed for binding activity with the model ligand N-phenyl-1-naphthylamine (NPN) according to the method described by Katti *et al.*⁶⁸. This involved titrating LUSH (1 μM) with increasing amounts of NPN to final concentrations ranging from 0.5 μM to 20 μM. A saturable NPN fluorescence change was recorded by a fluorometer and the dissociation constant was found to be 2.39 μM. Katti *et al.*⁶⁸ showed that LUSH does not display a saturable NPN fluorescence change if it is not fully refolded.

Assays to investigate the activity of EST6 towards cVA in the presence of LUSH were set up the same as those for the group assays except for changes to the substrate (150 μM) and enzyme (3 and 57 nM) concentrations, and the addition of LUSH (300 μM). Duplicate reaction mixtures were set up without LUSH as controls. Equivalent reactions using a better, mid-chain ester substrate, decyl acetate, were also set up as further controls.

Protein engineering and expression. Six generations of directed evolution were undertaken to improve the soluble expression of *E. coli*-expressed wildtype EST6. The method followed Jackson *et al.*²¹, but in this case the coding region of *Est6* from the iso-1 *y'cn'bw'sp'* reference strain (<http://flybase.org/reports/FBsn0000272.html>), omitting the 63 bp encoding the N-terminal signal peptide³³, was cloned into the expression vector pETMCSIII⁶⁶ between the *Nde*I and *Eco*RI sites in frame with the ATG start codon of the *Nde*I site. Adequate expression of *Est6* could be achieved by 'leaky expression' because of the presence of trace amounts of lactose in the LB media used. The error-prone PCR protocol used to construct the initial mutant library involved a reaction mixture comprising 100–200 ng of pETMCSIII-*Est6*, 1 μM primers pET3 and pET4 (5'CGACTCACTATAGGGAGACCACCAC3' and 5'CCTTTCGGGCTTGT'TAGCAG3'), 1 × Taq DNA polymerase buffer, 5 mM MgCl₂, 0.1–0.4 mM MnCl₂, 0.5 mM dNTPs, 5U Taq DNA polymerase, and milliQ H₂O to a final volume of 50 μl. Thermocycling involved 30 cycles of 94 °C for 10 s, 45 °C for 10 s and 30 s at 72 °C. The *Nde*I- and *Eco*RI-digested PCR product was gel extracted, ligated back into pETMCSIII, and then used to transform competent BL21 (DE3) star cells. Transformed cells were plated onto LB plates containing 100 mg.l⁻¹ ampicillin. After incubation at 30 °C overnight, the colonies were blotted onto 3 M filter papers and esterase activity was assayed by staining the filter paper with a solution consisting of 10 ml of 0.1% w/v Fast Red and 0.2 ml of 1% w/v 2 NA in 0.1 M Tris pH 7.0. Between 200–300 (approximately 1%) of the colonies generating the most intense red colour were then picked by hand and grown overnight in 500 μl of LB, 100 mg.l⁻¹ ampicillin, in 96-well culture plates. 50 μl of each of these cultures was then added to the corresponding well of a 96-well assay plate that contained 250 μl of a reaction mixture consisting of 0.5 mM 2 NA, 0.5 mM Fast Red, and 0.1 M Tris pH 7.0. The reaction was monitored with a spectrophotometer at 490 nm, and the 10–20 colonies generating the highest activities were sequenced and used as parents for the next generation of mutation and selection. The protocols for generations 2 to 6 followed those above. The sixth generation mutant generating the highest activity in the spectrophotometric assay, denoted EST6-1, was used for crystallization.

EST6-1 Crystallization and Computational Analysis. EST6-1 was expressed in *E. coli* Origami B (DE3) pLys Cells (Merck) grown in LB media with 100 μg.ml⁻¹ ampicillin to an optical density of 0.6. The cells were induced with 700 μM IPTG and harvested after 18 hours at 25 °C. The cells were then lysed by sonication in 50 mM Hepes (pH 7.5), 300 mM NaCl, 10 mM imidazole (buffer A). The soluble lysate was separated by centrifugation at 23,000 g and filtered with a 0.45 μM filter before being loaded onto a 5 ml Ni-NTA column. The protein was eluted from the column with buffer A supplemented with 300 mM imidazole. Fractions were pooled after confirmation by SDS-PAGE and further purified by size exclusion chromatography in 20 mM Hepes (pH 7.5) and 50 mM NaCl (buffer B) using a Hiload 26/600 Superdex 200 pg column (GE Healthcare). The concentration of EST6-1 was determined at 280 nm with the Nanodrop 1000 (Thermo Scientific) using an extinction coefficient of 74,635 M⁻¹ cm⁻¹ estimated by the ProtParam server⁶⁹.

Surface lysine residues of purified EST6-1 (1 mg.ml⁻¹) were methylated following the protocol of Walter *et al.*³⁶, and the reaction was quenched with 1 M glycine, followed by concentration of methylated EST6-1 to 18.2 mg.ml⁻¹ and dialysis into buffer B. Crystals of methylated EST6 were grown by the sitting drop diffusion technique with a reservoir solution containing 0.2 M ammonium acetate, 0.1 M Tris pH 8.5 and 25% w/v PEG 3,350. 35% w/v PEG 3,350 was used as a cryoprotectant during flash cooling of the crystals in nitrogen at 100 K. Diffraction data were collected at the MX2 beamline at the Australian Synchrotron, Victoria, Australia with a wavelength of 0.9655 Å. Data collection methods and statistics as well as details of the informatics methods used to solve the enzymes structure are given in Supplementary Table S5.

A model EST6-WT was built from the 97% identical structure of EST6-1 using FoldX⁴⁰. Covalent docking was performed with DOCKovalent, a covalent version of DOCK3.7⁴¹. The program pre-generates a set of conformations for each ligand, covalently attaches the ligand to a receptor, and exhaustively samples ligand orientations around the covalent bond. Ligands are then ranked via a physics-based scoring function. Esters investigated in this work were represented as SMILES strings with the covalent attachment to the catalytic serine O γ marked with a dummy atom. The esters were docked in the form of a tetrahedral intermediate, after nucleophilic addition of the serine O γ to the carbonyl carbon and prior to departure of the alcohol, with the carbonyl oxygen bearing a negative charge. The generation of ligand conformations and preparation of the receptor (EST6-WT model) was carried out as described previously⁴¹. The catalytic histidine was represented in its doubly protonated form. The selected esters were covalently docked onto the Ser188 O γ with a O γ -ligand bond length of 1.6 ± 0.1 Å sampled at 0.05 Å increments and with the C β -O γ -ligand and O γ -ligand-ligand bond angles set to $109.5 \pm 5^\circ$ and sampled at 1° increments. The lowest energy pose for each ligand was selected for analysis. Protein structure images were produced with PyMol V 1.3 and a topology diagram was generated using TOPDRAW⁷⁰.

Immunohistochemistry. *Flies.* *Orco*^{Gal4} flies were generously provided by G. Galizia (University of Konstanz, Germany), *lush*^{Gal4} flies (originally from R Benton, Université de Lausanne, Switzerland) from J-F. Ferveur (CSGA, Dijon, France) and *elav*^{LexA}, *LexAOP-mCD8::GFP*, *UAS-mCD8::RFP* and *Est6* null mutant flies from the Bloomington Stock Center (stocks 52676, 32203, 27392 and 4211 respectively). All flies were raised at 25°C on standard yeast/cornmeal/agar medium in a 12-hr light/12-hr dark cycle, 50–60% relative humidity.

Generation of anti-EST6 antiserum. Preparation of denatured EST6 antigen and production of polyclonal antibody followed the methods of Han *et al.*⁷¹. Briefly, wildtype EST6 was overexpressed in inclusion bodies in *E. coli* using the expression vector pETMCS III as above. Cells were harvested and lysed and inclusion bodies collected as above. The latter were then dissolved in 6 M guanidine HCl in a buffer containing 20 mM phosphate pH 7.4 and the solubilized denatured proteins loaded onto a 5 ml Ni-NTA column. The EST6 was eluted from the column with a gradient of buffer containing 6 M guanidine HCl, 20 mM phosphate, 0.5 M imidazole, pH 7.4. Fractions containing EST6 were identified from the presence of a 59.7 kDa band on denaturing PAGE and then pooled and loaded onto a Superdex 200 preparative scale exclusion column (GE Healthcare) equilibrated with 6 M of guanidine HCl, 20 mM phosphate buffer, pH 7.4. EST6 fractions from this column were concentrated to 1 mg ml⁻¹ using an Amicon Ultra-15 centrifugal device (Millipore, US) and the guanidine HCl removed by dialysis in 20 mM phosphate buffer, pH 7.4. The purified denatured EST6 was used as antigen for polyclonal antibody production by IMVS Veterinary Services, South Australia. Four doses of 0.5 mg antigen were administered to a rabbit at 3 weekly intervals. The polyclonal antibodies were purified from antiserum using an IgG affinity column and the protein concentration was estimated at 3 mg ml⁻¹.

The specificity of the antiserum was then tested by western blotting against extracts from heads of wild-type (*Canton S*) and *Est6*^o null mutant flies. Mass homogenates of heads from each strain in 20 mM Tris-HCl buffer (pH 7.4) were briefly sonicated, centrifuged at 8,000 g for 10 min and the supernatants isolated. Twenty µg of protein from each homogenate were then separated by SDS-PAGE and blotted onto a polyvinylidene fluoride (PVDF) membrane. After blocking in Tris Buffered Saline-Tween 10% (TBST-10%) blocking reagent (Invitrogen), membranes were incubated overnight at 4°C with the anti EST6 antibody (1:3,000), then incubated with rabbit-peroxidase-labelled antibody (1:10,000). Blots were then washed and incubated with chemiluminescent substrate (ECL Plus Western Detection Kit, GE Healthcare).

Localization of EST6 within antennae. To localize EST6 in the antenna, we performed immunohistochemistry with the anti-EST6 antibody above on transgenic flies expressing RFP under the control of either the *Orco* or *lush* promoter or GFP under the control of the *elav* promoter. *Est6* null mutant flies were used as a control for the specific labelling of the antibody. Specifically, heads with antennae still attached from 5-day-old *Orco*^{Gal4}/*UAS-mCD8::RFP*, *elav*^{LexA}/*LexAOP-mCD8::GFP*, *lush*^{Gal4}/*UAS-mCD8::RFP* or *Est6* null mutant males were fixed for 3 h in 4% paraformaldehyde with 0.2% Triton X-100, then washed for 1 h with PBS containing 0.2% Triton X-100 (PBST). The heads were then embedded in Tissue-Tek™ (CellPath) and cryosections (15 µm) were set in cell culture insert (Greiner Bio-one). After blocking with 3% normal goat serum and 1% BSA in PBST (1 h at room temperature), the anti-EST6 antibody was diluted from 1:3,000 to 1:750 (v:v) in the blocking solution (3% normal goat serum in PBST) and incubated overnight at room temperature. After a brief rinse in PBST, an anti-mouse conjugated Alexa-488 or Alexa-596 (Invitrogen) was applied at a concentration of 1:800 (v:v) in the blocking solution for 4 h at room temperature. Tissues were mounted in Slowfade reagent with DAPI (Invitrogen). Images were captured on a Leica SP5 confocal microscope and analysed using ImageJ 1.47 v (<http://imagej.nih.gov/ij>).

References

- Carey, A. F. & Carlson, J. R. Insect olfaction from model systems to disease control. *Proc. Natl. Acad. Sci. USA* **108**, 12987–12995, doi: 10.1073/pnas.1103472108 (2011).
- Leal, W. Odorant reception in insects: Roles of receptors, binding proteins, and degrading enzymes. *Annu. Rev. Entomol.* **58**, doi: 10.1146/annurev-ento-120811-153635 (2013).
- Durand, N. *et al.* Degradation of pheromone and plant volatile components by a same odorant-degrading enzyme in the cotton leafworm, *Spodoptera littoralis*. *PLoS One* **6**, doi: 10.1371/journal.pone.0029147 (2011).
- Vogt, R. G. Molecular Basis of Pheromone Detection in Insects. In *Comprehensive Molecular Insect Science* Vol. 3 (ed. Gilbert, L. I.) 753–803 (Elsevier, 2005).
- Vogt, R. G., Callahan, F. E., Rogers, M. E. & Dickens, J. C. Odorant binding protein diversity and distribution among the insect orders, as indicated by LAP, an OBP-related protein of the true bug *Lygus lineolaris* (Hemiptera, Heteroptera). *Chem. Senses* **24**, 481–495, doi: 10.1093/chemse/24.5.481 (1999).

6. Vogt, R., Riddiford, L. & Prestwich, G. Kinetic properties of a sex pheromone-degrading enzyme: the sensillar esterase of *Antheraea polyphemus*. *Proc. Natl. Acad. Sci. USA* **82**, doi: 10.1073/pnas.82.24.8827 (1985).
7. He, P. *et al.* Functional characterization of an antennal esterase from the noctuid moth, *Spodoptera exigua*. *Arch. Insect Biochem. Physiol.* **86**, doi: 10.1002/arch.21164 (2014).
8. Younus, F. *et al.* Identification of candidate odorant degrading gene/enzyme systems in the antennal transcriptome of *Drosophila melanogaster*. *Insect Biochem. Mol. Biol.* **53**, doi: 10.1016/j.ibmb.2014.07.003 (2014).
9. Mane, S. D., Tompkins, L. & Richmond, R. C. Male esterase 6 catalyzes the synthesis of a sex pheromone in *Drosophila melanogaster* females. *Science* **222**, doi: 10.1126/science.222.4622.419 (1983).
10. Chertemps, T. *et al.* A carboxylesterase, Esterase-6, modulates sensory physiological and behavioral response dynamics to pheromone in *Drosophila*. *BMC Biol.* **10**, doi: 10.1186/1741-7007-10-56 (2012).
11. Gomez-Diaz, C., Reina, J., Cambillau, C. & Benton, R. Ligands for pheromone-sensing neurons are not conformationally activated odorant binding proteins. *PLoS Biol.* **11**, doi: 10.1371/journal.pbio.1001546 (2013).
12. Benton, R. Sensitivity and specificity in *Drosophila* pheromone perception. *Trends Neurosci.* **30**, 512–519, doi: 10.1016/j.tins.2007.07.004 (2007).
13. Chertemps, T. *et al.* An antennal carboxylesterase from *Drosophila melanogaster*, esterase 6, is a candidate odorant-degrading enzyme toward food odorants. *Front. Physiol.* **6**, doi: 10.3389/fphys.2015.00315 (2015).
14. Richmond, R., Gilbert, D., Sheehan, K., Gromko, M. & Butterworth, F. Esterase 6 and reproduction in *Drosophila melanogaster*. *Science* **207**, doi: 10.1126/science.6767273 (1980).
15. Gilbert, D. Ejaculate esterase 6 and initial sperm use by female *Drosophila melanogaster*. *J. Insect Physiol.* **27**, doi: 10.1016/0022-1910(81)90112-8 (1981).
16. Scott, D. Inhibition of female *Drosophila melanogaster* remating by a seminal fluid protein (Esterase 6). *Evolution* **40**, doi: 10.2307/2408766 (1986).
17. Vander Meer, R., Obin, M., Zawistowski, S., Sheehan, K. & Richmond, R. A reevaluation of the role of cis-vaccenyl acetate, cis-vaccenol and esterase 6 in the regulation of mated female sexual attractiveness in *Drosophila melanogaster*. *J. Insect Physiol.* **32**, doi: 10.1016/0022-1910(86)90109-5 (1986).
18. Oakeshott, J. G., Claudianos, C., Campbell, P. M., Newcomb, R. D. & Russell, R. J. Biochemical Genetics and Genomics of Insect Esterases. In *Comprehensive Molecular Insect Science* Vol. 5 (ed. Gilbert, L. I.) 309–381 (Elsevier, 2005).
19. Rane, R. V. *et al.* Are feeding preferences and insecticide resistance associated with the size of detoxifying enzyme families in insect herbivores? *Curr. Opin. Insect Sci.* **13**, 70–76, doi: 10.1016/j.cois.2015.12.001 (2016).
20. Wogulis, M. *et al.* Structural studies of a potent insect maturation inhibitor bound to the juvenile hormone esterase of *Manduca sexta*. *Biochemistry* **45**, 4045–4057, doi: 10.1021/bi0521644 (2006).
21. Jackson, C. J. *et al.* Structure and function of an insect α -carboxylesterase (α Esterase7) associated with insecticide resistance. *Proc. Natl. Acad. Sci. USA* **110**, 10177–10182, doi: 10.1073/pnas.1304097110 (2013).
22. Harel, M. *et al.* Three-dimensional structures of *Drosophila melanogaster* acetylcholinesterase and of its complexes with two potent inhibitors. *Protein Sci.* **9**, doi: 10.1110/ps.9.6.1063 (2000).
23. Oakeshott, J. G. *et al.* An episode of accelerated amino acid change in *Drosophila* esterase-6 associated with a change in physiological function. *Genetica* **110**, 231–244, doi: 10.1023/A:1012727814167 (2000).
24. Becher, P. *et al.* Yeast, not fruit volatiles mediate *Drosophila melanogaster* attraction, oviposition and development. *Funct. Ecol.* **26**, doi: 10.1111/j.1365-2435.2012.02006.x (2012).
25. Bar-Even, A. *et al.* The moderately efficient enzyme: evolutionary and physicochemical trends shaping enzyme parameters. *Biochemistry* **50**, 4402–4410, doi: 10.1021/bi2002289 (2011).
26. Myers, M. A., Healy, M. J. & Oakeshott, J. G. Effects of the residue adjacent to the reactive serine on the substrate interactions of *Drosophila* esterase 6. *Biochem. Genet.* **31**, 259–278, doi: 10.1007/bf00553170 (1993).
27. Dweck, H. *et al.* Pheromones mediating copulation and attraction in *Drosophila*. *Proc. Natl. Acad. Sci. USA* **112**, doi: 10.1073/pnas.1504527112 (2015).
28. Dweck, H. *et al.* Olfactory preference for egg laying on citrus substrates in *Drosophila*. *Curr. Biol.* **23**, doi: 10.1016/j.cub.2013.10.047 (2013).
29. Galizia, C., Münch, D., Strauch, M., Nissler, A. & Ma, S. Integrating heterogeneous odor response data into a common response model: A DoOR to the complete olfactome. *Chem. Senses* **35**, doi: 10.1093/chemse/bjq042 (2010).
30. Mansourian, S. & Stensmyr, M. The chemical ecology of the fly. *Curr. Opin. Neurobiol.* **34**, doi: 10.1016/j.conb.2015.02.006 (2015).
31. Münch, D. & Galizia, C. DoOR 2.0 - Comprehensive mapping of *Drosophila melanogaster* odorant responses. *Sci. Rep.* **6**, doi: 10.1038/srep21841 (2016).
32. Mathew, D. *et al.* Functional diversity among sensory receptors in a *Drosophila* olfactory circuit. *Proc. Natl. Acad. Sci. USA* **110**, 43, doi: 10.1073/pnas.1306976110 (2013).
33. Cooke, P. H. & Oakeshott, J. G. Amino acid polymorphisms for esterase-6 in *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. USA* **86**, 1426–1430 (1989).
34. Karotam, J., Delves, A. C. & Oakeshott, J. G. Conservation and change in structural and 5' flanking sequences of esterase 6 in sibling *Drosophila* species. *Genetica* **88**, 11–28 (1993).
35. de Marco, A. Strategies for successful recombinant expression of disulfide bond-dependent proteins in *Escherichia coli*. *Microb. Cell Fact.* **8**, doi: 10.1186/1475-2859-8-26 (2009).
36. Walter, T. *et al.* Lysine methylation as a routine rescue strategy for protein crystallization. *Structure* **14**, doi: 10.1016/j.str.2006.09.005 (2006).
37. Lenfant, N. *et al.* ESTHER, the database of the α/β -hydrolase fold superfamily of proteins: tools to explore diversity of functions. *Nucleic Acids Res.* **41**, doi: 10.1093/nar/gks1154 (2013).
38. Margraf, T., Schenk, G. & Torda, A. The SALAMI protein structure search server. *Nucleic Acids Res.* **37**, doi: 10.1093/nar/gkp431 (2009).
39. Binkowski, A. T., Naghibzadeh, S. & Liang, J. CASTp: Computed Atlas of Surface Topography of proteins. *Nucleic Acids Res.* **31**, 3352–3355, doi: 10.1093/nar/gkg512 (2003).
40. Schymkowitz, J. *et al.* The FoldX web server: an online force field. *Nucleic Acids Res.* **33**, doi: 10.1093/nar/gki387 (2005).
41. London, N. *et al.* Covalent docking of large libraries for the discovery of chemical probes. *Nat. Chem. Biol.* **10**, 1066–1072, doi: 10.1038/nchembio.1666 (2014).
42. Larsson, M. C. *et al.* Or83b encodes a broadly expressed odorant receptor essential for *Drosophila* olfaction. *Neuron* **43**, 703–714, doi: 10.1016/j.neuron.2004.08.019 (2004).
43. Yao, K. M. & White, K. Neural specificity of elav expression: defining a *Drosophila* promoter for directing expression to the nervous system. *J. Neurochem.* **63**, 41–51, doi: 10.1046/j.1471-4159.1994.63010041.x (1994).
44. Shanbhag, S. R. *et al.* Expression mosaic of odorant-binding proteins in *Drosophila* olfactory organs. *Microsc. Res. Tech.* **55**, 297–306, doi: 10.1002/jemt.1179 (2001).
45. Kim, M. S., Repp, A. & Smith, D. P. LUSH odorant-binding protein mediates chemosensory responses to alcohols in *Drosophila melanogaster*. *Genetics* **150**, 711–721 (1998).
46. Hallem, E., Ho, M. & Carlson, J. The molecular basis of odor coding in the *Drosophila* antenna. *Cell* **117**, doi: 10.1016/j.cell.2004.05.012 (2004).

47. Knaden, M., Strutz, A., Ahsan, J., Sachse, S. & Hansson, B. S. Spatial representation of odorant valence in an insect brain. *Cell Rep.* **1**, 392–399, doi: 10.1016/j.celrep.2012.03.002 (2012).
48. Christiaens, J. *et al.* The fungal aroma gene ATF1 promotes dispersal of yeast cells through insect vectors. *Cell Rep.* **9**, doi: 10.1016/j.celrep.2014.09.009 (2014).
49. Hallem, E. & Carlson, J. Coding of odors by a receptor repertoire. *Cell* **125**, doi: 10.1016/j.cell.2006.01.050 (2006).
50. Stöckl, J. *et al.* A deceptive pollination system targeting drosophilids through olfactory mimicry of yeast. *Curr. Biol.* **20**, doi: 10.1016/j.cub.2010.09.033 (2010).
51. González-Mas, M., Rambla, J., Alamar, M., Gutiérrez, A. & Granell, A. Comparative analysis of the volatile fraction of fruit juice from different citrus species. *PLoS One* **6**, doi: 10.1371/journal.pone.0022016 (2011).
52. Bartelt, R. J., Schaner, A. M. & Jackson, L. L. Cis-Vaccenyl acetate as an aggregation pheromone in *Drosophila melanogaster*. *J. Chem. Ecol.* **11**, doi: 10.1007/BF01012124 (1985).
53. Ejima, A. Pleiotropic actions of the male pheromone cis-vaccenyl acetate in *Drosophila melanogaster*. *J. Comp. Physiol. A Neuroethol. Sens. Neural Behav. Physiol.* **201**, doi: 10.1007/s00359-015-1020-9 (2015).
54. Griffith, L. & Ejima, A. Courtship learning in *Drosophila melanogaster*: Diverse plasticity of a reproductive behavior. *Learn. Mem.* **16**, doi: 10.1101/lm.956309 (2009).
55. Grosjean, Y. *et al.* An olfactory receptor for food-derived odours promotes male courtship in *Drosophila*. *Nature* **478**, 236–240, doi: 10.1038/nature10428 (2011).
56. Schlieff, M. & Wilson, R. Olfactory processing and behavior downstream from highly selective receptor neurons. *Nat. Neurosci.* **10**, doi: 10.1038/nn1881 (2007).
57. Strutz, A. *et al.* Decoding odor quality and intensity in the *Drosophila* brain. *Elife* **3**, doi: 10.7554/eLife.04147 (2014).
58. Gilson, M. *et al.* Open “back door” in a molecular dynamics simulation of acetylcholinesterase. *Science* **263**, 1276–1278, doi: 10.1126/science.8122110 (1994).
59. Biswas, S. *et al.* Bridging the synaptic gap: Neuroligins and neuexin I in *Apis mellifera*. *PLoS One* **3**, doi: 10.1371/journal.pone.0003542 (2008).
60. Jordan, M. *et al.* Expressed sequence tags and proteomics of antennae from the tortricid moth, *Epiphyas postvittana*. *Insect Mol. Biol.* **17**, doi: 10.1111/j.1365-2583.2008.00812.x (2008).
61. Durand, N. *et al.* A diversity of putative carboxylesterases are expressed in the antennae of the noctuid moth *Spodoptera littoralis*. *Insect Mol. Biol.* **19**, doi: 10.1111/j.1365-2583.2009.00939.x (2010).
62. Ishida, Y. & Leal, W. Rapid inactivation of a moth pheromone. *Proc. Natl. Acad. Sci. USA* **102**, doi: 10.1073/pnas.0505340102 (2005).
63. Silbering, A. F. *et al.* Complementary function and integrated wiring of the evolutionarily distinct *Drosophila* olfactory subsystems. *J. Neurosci.* **31**, doi: 10.1523/JNEUROSCI.2360-11.2011 (2011).
64. Stensmyr, M., Giordano, E., Balloi, A., Angioy, A.-M. & Hansson, B. Novel natural ligands for *Drosophila* olfactory receptor neurons. *J. Exp. Biol.* **206**, doi: 10.1242/jeb.00143 (2003).
65. Eysenath, R., Danson, M. J. & Hough, D. W. Catalytic efficiency and kcat/KM: a useful comparator? *Trends Biotechnol.* **25**, 247–249, doi: 10.1016/j.tibtech.2007.03.010 (2007).
66. Love, C. A., Lilley, P. E. & Dixon, N. E. Stable high-copy-number bacteriophage lambda promoter vectors for overproduction of proteins in *Escherichia coli*. *Gene* **176**, 49–53 (1996).
67. Kruse, S. W., Zhao, R., Smith, D. P. & Jones, D. N. M. Structure of a specific alcohol-binding site defined by the odorant binding protein LUSH from *Drosophila melanogaster*. *Nat. Struct. Biol.* **10**, 694–700, doi: 10.1038/nsb960 (2003).
68. Katti, S., Lokhande, N., González, D., Cassill, A. & Renthal, R. Quantitative analysis of pheromone-binding protein specificity. *Insect Mol. Biol.* **22**, doi: 10.1111/j.1365-2583.2012.01167.x (2013).
69. Gasteiger, E. *et al.* Protein Identification and Analysis Tools on the ExPASy Server. In *The Proteomics Protocols Handbook* (ed. J. M. Walker) 571–607 (Humana Press, 2005).
70. Bond, C. S. TopDraw: a sketchpad for protein structure topology cartoons. *Bioinformatics* **19**, 311–312 (2003).
71. Han, Y. *et al.* Proteomic and molecular analyses of esterases associated with monocrotophos resistance in *Helicoverpa armigera*. *Pestic. Biochem. Physiol.* **104**, doi: 10.1016/j.pestbp.2012.09.005 (2012).

Acknowledgements

Our thanks to G. Galizia (University of Konstanz, Germany), R. Benton (Université de Lausanne, Switzerland), J.-F. Ferveur (CSGA, Dijon, France) and the Bloomington Stock Center and NIG Stock Center (Japan) for the provision of fly strains. FY was supported by the French-Australian Science and Technology Program (FAST) and a CSIRO OCE Postgraduate scholarship. MM and TC were supported by ANR-12-BSV7-0024-01. CJJ acknowledges beamtime from the Australian Synchrotron (MX2) and a Future Fellowship from the Australian Research Council.

Author Contributions

F.Y., N.J.F., C.W.C., J.W.L., G.P., T.C., C.J.J. performed experiments; F.Y., N.J.F., C.J.J., C.W.C., J.W.L., G.J.C., M.M.C., G.P., J.G.O., T.H. designed research, F.Y., N.J.F., C.W.C., G.P., C.C., C.J.J., J.G.O. analyzed data; F.Y., N.J.F., C.J.J. and J.G.O. wrote the manuscript.

Additional Information

Supplementary information accompanies this paper at <http://www.nature.com/srep>

Competing Interests: The authors declare no competing financial interests.

How to cite this article: Younus, F. *et al.* Molecular basis for the behavioral effects of the odorant degrading enzyme Esterase 6 in *Drosophila*. *Sci. Rep.* **7**, 46188; doi: 10.1038/srep46188 (2017).

Publisher's note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.



This work is licensed under a Creative Commons Attribution 4.0 International License. The images or other third party material in this article are included in the article's Creative Commons license, unless indicated otherwise in the credit line; if the material is not included under the Creative Commons license, users will need to obtain permission from the license holder to reproduce the material. To view a copy of this license, visit <http://creativecommons.org/licenses/by/4.0/>

© The Author(s) 2017

Chapter 5: General discussion

5.1. EST6 and JHEdup as ODEs

The chemosensory system of *D. melanogaster* has become a prominent model in neuroscience to answer fundamental questions as to how sensory stimuli are processed in the brain to generate appropriate responses. Stimuli such as pheromones released by an insect together with kairomones present in the environment alter the physiology and behaviour of an insect and contribute to its Darwinian fitness. Substantial progress has been made in understanding insect pheromone processing in recent years, particularly in *D. melanogaster*, but one remaining major hurdle has been to identify and elucidate the specific roles played by ODEs in processing these volatile molecules. Technological issues, such as the electrophysiology equipment required to monitor responses of neurons to incoming odorants and difficulties in obtaining sufficient antennal tissues for biochemical analysis, have meant that progress in understanding the roles of ODEs has remained painfully slow. The studies presented here have overcome some of these issues and helped to elucidate the roles of two candidate ODEs in the sensillar lymph of *D. melanogaster*.

The first paper (Chapter 2) presents comprehensive transcriptomes of *D. melanogaster* antennae for the first time. It finds that 55–61% of the total gene content of the fly is expressed in the antennae with values of FPKM>1, and 27–30% at values of FPKM>10. Even higher proportions of the four candidate ODE families were expressed in the antennae (71% of the esterases, 64% of the P450s, 81% of the GSTs and 47% of the UGTs at FPKM>1, and 46%, 41%, 62% and 21%, respectively, at FPKM>10). However, almost all the esterases, P450s, GSTs and UGTs have also been reported in transcriptomes from other tissues. This strengthens the view that many of these enzymes may have general detoxification/scavenging functions broadly in the antenna, rather than acting specifically on one substrate⁶. To what extent they function as ODEs thus remains moot.

However, the paper identified one esterase (*JHEdup*), a cytochrome P450 (*CYP308a1*) and a GST (*GSTE4*) as antennae-selective, making them good candidates for specific antennal functions. *In vitro* biochemical characterization of *JHEdup* supports this notion.

Notably, JHEdup was shown to differ from previously characterized ODEs as it was able to degrade a chemically diverse array of food odorants *in vitro*.

Two issues now become priorities for future research on JHEdup. The first concerns the paradox that JHEdup might have a broad ODE function in antennae whereas its close relative JHE is known to have a very different and a very precise physiological function and substrate. The second is that there is actually no direct evidence yet that JHEdup is a physiologically relevant ODE.

To address the first issue our CSIRO Canberra group is currently conducting a detailed *in vitro* comparison of the kinetics of JHE and JHEdup against a wide range of bioactive esters, including many of those studied in the third paper (Chapter 4), plus the physiologically relevant isomers of JH. Crone et al.¹¹⁸ has previously undertaken a small scale version of such a study but their results were compromised by the use of the wrong N-terminus in their heterologously expressed version of JHEdup. The resultant protein was particularly unstable, losing all activity within 30 minutes at 30°C¹¹⁸.

To address the second issue our colleagues in France have also initiated a range of physiological and behavioural studies comparing wild type and JHEdup null mutant flies. Preliminary results support the contention that JHEdup is indeed a physiological ODE. Thus they have shown that the enzyme is located in the basiconic sensilla where the processing of food odour occurs. Moreover, they found behavioural differences between the wild type and mutant flies towards food esters such as ethyl butyrate and ethyl propionate.

The first paper herein had also shown that EST6 is expressed widely through the antennae. Notwithstanding all the evidence reviewed in Section 1.5 regarding other roles for EST6 expressed in other tissues, the second paper (Chapter 3) therefore undertook to test whether EST6 is able to degrade a range of volatile esters emitted from various food sources *in vitro* and *in vivo*. Remarkably, esters that were found to be good substrates for the enzyme *in vitro* were also found to differentially affect antennal responses of EST6 wild type and null flies. Conversely, those that were poor substrates *in vitro*, such as the recently identified pheromonal compound methyl myristate¹²³, did not register notable antennal response differences between the wild type and mutant flies. Interestingly, using a major fruit volatile, pentyl acetate, we also managed to show

that the effects of EST6 are translated into behavioural changes in the presence of that odorant. One important experiment that now needs to be done involves single sensillum recordings¹²⁴ of the wild type and mutant flies to elucidate more precisely how EST6 is affecting the pentyl acetate response.

The kinetics of EST6 toward the five preferred substrates among the eight chemically diverse esters studied in the second paper were comparable with those of other insect esterases reported so far to act as ODEs against their respective odorants. Interestingly, JHEdup had shown similar kinetics for the five esters against which it had been characterized in the first paper above. Notably for pentyl acetate, its kinetics were very similar to those of EST6, raising the possibility that more than one esterase could act as an ODE on the same volatile.

Given the evidence from the second paper that EST6 has activity against several volatile esters, a key question addressed by the third paper was then just how broad a range of plant and yeast volatiles it could act on. This was addressed in a comprehensive survey of 85 bioactive esters *in vitro*, together with a complementary analysis of the active site of the enzyme which was enabled by the solving of its crystal structure. At the same time the capabilities developed in this work enabled us to categorically address the question about the enzyme's activity against cVA.

Considering first the latter, the third paper showed unequivocally that EST6 has negligible hydrolytic activity against cVA, whether or not the OBP LUSH is present. This finding corroborates Van der Meer *et al.*¹⁰⁰ who had previously reported values for EST6 activity against cVA that are highly unlikely to be physiologically relevant. It follows that the observed effect of EST6 on cVA perception gathered from electrophysiological studies¹¹² must be an indirect one. Possibly relevant to the latter is the other major finding of the third paper, that EST6 has activity against a broad range of volatile food esters, many of which are known to affect aggregation behaviours around breeding sites. Thus, substrates against which EST6 has better activities, such as isopentyl acetate, pentyl acetate and phenethyl acetate, are known to elicit strong attraction behaviours⁸. It also has good activity against propyl, hexyl, heptyl, nonyl, decyl and geranyl acetate which are also known to be attractants for *D. melanogaster* in the wild¹²⁵. This suggests that EST6 may mediate the effects of various food volatiles on the reproductive

behaviour of flies at natural breeding sites. However, it also suggests there could be synergistic interactions between cVA and food odours mediated by EST6 as a result of its role in repriming the ORNs for the food odours. It is not clear how much such an effect could explain the finding that EST6 affects cVA responses because the studies involved have appeared to use methodologies that would eliminate food odour effects ¹¹². Nevertheless, it would seem highly worthwhile now to test for interactive effects between the food odours and cVA in comparisons between the wild type and EST6 null strains.

5.2. Some broader perspectives

The findings of the three papers presented previously also invite further consideration of some other aspects of EST6 biology. One of these concerns the substrate and function of the male donated EST6 that is transferred to the female during mating. The finding of the third paper, together with advances in modern metabolomics ¹²⁶, now make it worthwhile and feasible to investigate the *in vivo* substrate of this enzyme in the female. Assisting here is the fact that haemolymph is a relatively amenable material for metabolomic analysis ¹²⁷ and the availability of EST6 null flies provides important negative controls. Additionally the ability to purify EST6 to homogeneity from either *in vivo* sources or heterologous expression systems means that ‘spiking’ of null haemolymph with wild type EST6 becomes possible. In fact, my CSIRO colleagues and I, together with Metabolomics Australia have begun such experiments, but the results were not available in time to be included in this thesis.

Some evolutionary questions also now arise in relation to EST6 function. Knowing that the ejaculate function of EST6 has arisen relatively recently within the melanogaster subgroup of species, the question arises as to whether the ancestral function of the enzyme included antennal ODE roles, or are these also recently derived. Antennal transcriptomes of species such as *D. yakuba* would be informative in this respect, as would a survey of the *in vitro* substrate range of *D. yakuba* EST6 as per the third paper above.

Also notable here is that EST7 expression is highly localized to late larval cuticle in *D. melanogaster* and may also be in several other *Drosophila* species ⁸⁹. This raises the

question of whether EST7 has a relatively 'old' function as an ODE in larval olfaction. Larvae also respond to acetate esters ¹²⁸, so performing behavioural and electrophysiological studies with EST7 wild type and null variant larvae might lead to some interesting insights in the largely neglected field of larval olfaction. Also worthwhile here may be some comparative substrate range and structural analyses of EST6 and EST7 as per the third chapter.

Another, obvious, question arising from my work concerns the identity of the ODE for cVA if it is not, as my work shows it cannot be, EST6. Interestingly, our transcriptomic study indicates the presence of several lipases in *D. melanogaster* antennae that may play a role in breaking down such long chain fatty acid esters. Preliminary biochemical studies in our laboratory with a lipase from the apple moth *Epiphyas postvittana* indicate that it may have good activity against cVA. Further work now remains to purify one or more *D. melanogaster* antennal lipases, or heterologously express these enzymes, and test their effectiveness against cVA.

Finally here I consider what relevance my findings may have for the development of effective control strategies for pests such as *Drosophila suzukii* based on olfactory disruption. Closely related to *D. melanogaster*, this highly invasive Asian pest has now established in Europe and North America ¹²⁹. Unlike their *D. melanogaster* counterparts, *D. suzukii* female flies drill through undamaged ripening fruit with their serrated ovipositor and lay eggs inside the fruit ¹³⁰. Reported losses of crops such as grapes, strawberries, raspberries, blueberries and cherries are as high as 80% and, with several biocontrol measures yet to be proven successful, *D. suzukii* is causing the horticultural industry in many countries to lose millions of dollars every year ¹³¹. Recent data suggest *D. suzukii* also facilitates *D. melanogaster* infestation and favours the outbreak of deadly sour rot diseases in French vineyards ^{132,133}. These diseases have been reported to decimate almost 80% of some soft fruits harvested in France since the introduction of *D. suzukii* ¹³⁴. Several chemical insecticides have been banned from use against *D. suzukii* around the world and there is considerable interest in the development of biological alternatives such as olfactory disruption ⁴.

Transcriptomic studies suggest that *D. suzukii* also contains numerous esterases ¹³⁵. It will now be important to investigate how many of these are expressed in the antennae,

whether they act as ODEs, and what their relationships are to EST6 and JHEdup in *D. melanogaster*. The development of specific blocking agents or antagonists against these other essential components of the system could disrupt the recognition of plant hosts or sexual partners, interfering with fundamental activities such as feeding and reproduction. There are clearly many issues in respect of efficacy, selectivity, etc. that would need to be addressed prior to implementation. However, even without detailed mechanistic understanding of their modes of action, an increasing number of interventions based on olfactory disruption are now proving successful against various insect pests around the world¹³⁶.

References

- 1 Buck, L. B. Information coding in the vertebrate olfactory system. *Annu. Rev. Neurosci.* **19**, 517-544, (1996).
- 2 Hallem, E. A. & Carlson, J. R. The odor coding system of *Drosophila*. *Trends. Genet.* **20**, 453-459, (2004).
- 3 Kehat, M. & Dunkelblum, E. Sex pheromones: Achievements in monitoring and mating disruption of cotton pests in Israel. *Arch. Insect Biochem. Physiol.* **22**, 425-431, (1993).
- 4 Carey, A. F. & Carlson, J. R. Insect olfaction from model systems to disease control. *Proc. Natl. Acad. Sci. USA* **108**, 12987-12995, (2011).
- 5 Robertson, H. M. & Wanner, K. W. The chemoreceptor superfamily in the honey bee, *Apis mellifera*: Expansion of the odorant, but not gustatory, receptor family. *Gen. Res.* **16**, 1395-1403, (2006).
- 6 Leal, W. Odorant reception in insects: Roles of receptors, binding proteins, and degrading enzymes. *Annu. Rev. Entomol.* **58**, (2013).
- 7 Becher, P., Flick, G., Rozpędowska, E., Schmidt, A., Hagman, A., Lebreton, S. *et al.* Yeast, not fruit volatiles mediate *Drosophila melanogaster* attraction, oviposition and development. *Funct. Ecol.* **26**, (2012).
- 8 Christiaens, J., Franco, L., Cools, T., Meester, L., Michiels, J., Wenseleers, T. *et al.* The fungal aroma gene *ATF1* promotes dispersal of yeast cells through insect vectors. *Cell Reports* **9**, (2014).
- 9 Hallem, E. & Carlson, J. Coding of odors by a receptor repertoire. *Cell* **125**, (2006).
- 10 Dweck, H. K., Ebrahim, S. A., Farhan, A., Hansson, B. S. & Stensmyr, M. C. Olfactory proxy detection of dietary antioxidants in *Drosophila*. *Curr. Biol.* **25**, 455-466, (2015).
- 11 Silbering, A. F., Rytz, R., Grosjean, Y., Abuin, L., Ramdya, P., Jefferis, G. *et al.* Complementary function and integrated wiring of the evolutionarily distinct *Drosophila* olfactory subsystems. *J. Neurosci.* **31**, (2011).
- 12 Min, S., Ai, M., Shin, S. A. & Suh, G. S. Dedicated olfactory neurons mediating attraction behavior to ammonia and amines in *Drosophila*. *Proc. Natl. Acad. Sci. USA* **110**, E1321-1329, (2013).
- 13 de Bruyne, M., Foster, K. & Carlson, J. R. Odor coding in the *Drosophila* antenna. *Neuron* **30**, 537-552, (2001).
- 14 Stensmyr, M., Giordano, E., Balloi, A., Angioy, A.-M. & Hansson, B. Novel natural ligands for *Drosophila* olfactory receptor neurones. *J. Exp. Biol.* **206**, (2003).
- 15 Bartelt, R. J., Schaner, A. M. & Jackson, L. L. cis-Vaccenyl acetate as an aggregation pheromone in *Drosophila melanogaster*. *J. Chem. Ecol.* **11**, (1985).
- 16 Grosjean, Y., Rytz, R., Farine, J.-P., Abuin, L., Cortot, J., Jefferis, G. S. X. E. *et al.* An olfactory receptor for food-derived odours promotes male courtship in *Drosophila*. *Nature* **478**, 236-240, (2011).
- 17 Jackson, L. L., Arnold, M. T. & Blomquist, G. J. Surface lipids of *Drosophila melanogaster*: comparison of the lipids from female and male wild type and sex-linked yellow mutant. *Insect Biochem.* **11**, 87-91, (1981).
- 18 Brieger, G. & Butterworth, F. M. *Drosophila melanogaster*: Identity of male lipid in reproductive system. *Science* **167**, 1262-1262, (1970).
- 19 Liu, W., Liang, X., Gong, J., Yang, Z., Zhang, Y. H., Zhang, J. X. *et al.* Social regulation of aggression by pheromonal activation of Or65a olfactory neurons in *Drosophila*. *Nat. Neurosci.* **14**, 896-902, (2011).
- 20 Wang, L. & Anderson, D. J. Identification of an aggression promoting pheromone and its receptor neurons in *Drosophila*. *Nature* **463**, 227-231, (2010).
- 21 Symonds, M. R. & Wertheim, B. The mode of evolution of aggregation pheromones in *Drosophila* species. *J. Evol. Biol.* **18**, 1253-1263, (2005).
- 22 Kurtovic, A., Widmer, A. & Dickson, B. J. A single class of olfactory neurons mediates behavioural responses to a *Drosophila* sex pheromone. *Nature* **446**, 542-546, (2007).

- 23 Jallon, J., Antony, C. & Benamar, O. Un anti-aphrodisiaque produit par les mâles de *Drosophila melanogaster* et transféré aux femelles lors de la copulation. *CR Acad. Sci. Paris* **292**, 1147-1149, (1981).
- 24 Ejima, A. Pleiotropic actions of the male pheromone cis-vaccenyl acetate in *Drosophila melanogaster*. *J. Comp. Physiol. A: Neuroethology, Sensory, Neural, and Behavioral Physiology* **201**, (2015).
- 25 Shanbhag, S. R., Müller, B. & Steinbrecht, R. A. Atlas of olfactory organs of *Drosophila melanogaster*. *Int. J. Insect Morph. Embryol.* **28**, 377-397, (1999).
- 26 Laissue, P. P. & Vosshall, L. B. The olfactory sensory map in *Drosophila*. *Adv. Exp. Med. Biol.* **628**, 102-114, (2008).
- 27 Yao, C. A., Ignell, R. & Carlson, J. R. Chemosensory coding by neurons in the coeloconic sensilla of the *Drosophila* antenna. *J. Neurosci.* **25**, 8359-8367, (2005).
- 28 van der Goes van Naters, W. & Carlson, J. R. Receptors and neurons for fly odors in *Drosophila*. *Curr. Biol.* **17**, 606-612, (2007).
- 29 Larsson, M. C., Domingos, A. I., Jones, W. D., Chiappe, M. E., Amrein, H. & Vosshall, L. B. Or83b encodes a broadly expressed odorant receptor essential for *Drosophila* olfaction. *Neuron* **43**, 703-714, (2004).
- 30 Clyne, P. J., Warr, C. G., Freeman, M. R., Lessing, D., Kim, J. & Carlson, J. R. A novel family of divergent seven-transmembrane proteins. *Neuron* **22**, 327-338, (1999).
- 31 Gao, Q. & Chess, A. Identification of candidate *Drosophila* olfactory receptors from genomic DNA sequence. *Genomics* **60**, 31-39, (1999).
- 32 Vosshall, L. B., Amrein, H., Morozov, P. S., Rzhetsky, A. & Axel, R. A spatial map of olfactory receptor expression in the *Drosophila* antenna. *Cell* **96**, 725-736, (1999).
- 33 Smart, R., Kiely, A., Beale, M., Vargas, E., Carraher, C., Kralicek, A. V. *et al.* *Drosophila* odorant receptors are novel seven transmembrane domain proteins that can signal independently of heterotrimeric G proteins. *Insect Biochem. Mol. Biol.* **38**, 770-780, (2008).
- 34 Benton, R., Sachse, S., Michnick, S. W. & Vosshall, L. B. Atypical membrane topology and heteromeric function of *Drosophila* odorant receptors *in vivo*. *PLoS Biol.* **4**, e20, (2006).
- 35 Robertson, H. M., Warr, C. G. & Carlson, J. R. Molecular evolution of the insect chemoreceptor gene superfamily in *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. USA* **100**, 14537-14542, (2003).
- 36 Touhara, K. & Vosshall, L. B. Sensing odorants and pheromones with chemosensory receptors. *Annu. Rev. Physiol.* **71**, 307-332, (2009).
- 37 Mombaerts, P. Molecular Biology of odorant receptors in vertebrates *Annu. Rev. Neurosci.* **22**, 487-509, (1999).
- 38 Vosshall, L. B., Wong, A. M. & Axel, R. An olfactory sensory map in the fly brain. *Cell* **102**, 147-159, (2000).
- 39 Krieger, J., Raming, K., Dewer, Y. M. E., Bette, S., Conzelmann, S. & Breer, H. A divergent gene family encoding candidate olfactory receptors of the moth *Heliothis virescens*. *Eur. J. Neurosci.* **16**, 619-628, (2002).
- 40 Hill, C. A., Fox, A. N., Pitts, R. J., Kent, L. B., Tan, P. L., Chrystal, M. A. *et al.* G protein-coupled receptors in *Anopheles gambiae*. *Science* **298**, 176-178, (2002).
- 41 Pitts, R. J., Fox, A. N. & Zwiebel, L. J. A highly conserved candidate chemoreceptor expressed in both olfactory and gustatory tissues in the malaria vector *Anopheles gambiae*. *Proc. Natl. Acad. Sci. USA* **101**, 5058-5063, (2004).
- 42 Jones, W. D., Nguyen, T.-A. T., Kloss, B., Lee, K. J. & Vosshall, L. B. Functional conservation of an insect odorant receptor gene across 250 million years of evolution. *Curr. Biol.* **15**, R119-R121, (2005).
- 43 Neuhaus, E. M., Gisselmann, G., Zhang, W., Dooley, R., Stortkuhl, K. & Hatt, H. Odorant receptor heterodimerization in the olfactory system of *Drosophila melanogaster*. *Nat. Neurosci.* **8**, 15-17, (2005).

- 44 Pelz, D., Roeske, T., Syed, Z., Bruyne, M. d. & Galizia, C. G. The molecular receptive range of an olfactory receptor *in vivo* *Drosophila melanogaster* Or22a. *J. Neurobiol.* **66**, 1544-1563, (2006).
- 45 Stensmyr, M. C., Dweck, H. K. M., Farhan, A., Ibba, I., Strutz, A., Mukunda, L. *et al.* A conserved dedicated olfactory circuit for detecting harmful microbes in *Drosophila*. *Cell* **151**, 1345-1357.
- 46 Mathew, D., Martelli, C., Kelley-Swift, E., Brusalis, C., Gershow, M., Samuel, A. D. T. *et al.* Functional diversity among sensory receptors in a *Drosophila* olfactory circuit. *Proc. Natl. Acad. Sci. USA* **110**, 43, (2013).
- 47 Vogt, R. G. & Riddiford, L. M. Pheromone binding and inactivation by moth antennae. *Nature* **293**, 161-163, (1981).
- 48 Klein, U. Sensillum-lymph proteins from antennal olfactory hairs of the moth *Antheraea polyphemus* (Saturniidae). *Insect Biochem.* **17**, 1193-1204, (1987).
- 49 Pelosi, P., Mastrogiacomo, R., Iovinella, I., Tuccori, E. & Persaud, K. C. Structure and biotechnological applications of odorant binding proteins. *App. Microbiol. Biotech.* **98**, 61-70, (2014).
- 50 Steinbrecht, R. A. Odorant binding proteins: Expression and function. *Annal. New York Acad. Sci.* **855**, 323-332, (1998).
- 51 Hekmat-Scafe, D. S., Scafe, C. R., McKinney, A. J. & Tanouye, M. A. Genome wide analysis of the odorant binding protein gene family in *Drosophila melanogaster*. *Gen. Res.* **12**, 1357-1369, (2002).
- 52 Xu, P., Atkinson, R., Jones, D. N. M. & Smith, D. P. *Drosophila* OBP LUSH is required for activity of pheromone sensitive neurons. *Neuron* **45**, 193-200, (2005).
- 53 Kim, M. S., Repp, A. & Smith, D. P. LUSH odorant-binding protein mediates chemosensory responses to alcohols in *Drosophila melanogaster*. *Genetics* **150**, 711-721, (1998).
- 54 Laughlin, J. D., Ha, T. S., Jones, D. N. M. & Smith, D. P. Activation of pheromone sensitive neurons is mediated by conformational activation of pheromone binding protein. *Cell* **133**, 1255-1265, (2008).
- 55 Benton, R. Sensitivity and specificity in *Drosophila* pheromone perception. *Trends Neurosci.* **30**, 512-519, (2007).
- 56 Benton, R., Vannice, K. S., Gomez-Diaz, C. & Vosshall, L. B. Variant ionotropic glutamate receptors as chemosensory receptors in *Drosophila*. *Cell* **136**, 149-162, (2009).
- 57 Rytz, R., Croset, V. & Benton, R. Ionotropic receptors (IRs): Chemosensory ionotropic glutamate receptors in *Drosophila* and beyond. *Insect Biochem. Mol. Biol.* **43**, 888-897, (2013).
- 58 Ai, M., Min, S., Grosjean, Y., Leblanc, C., Bell, R., Benton, R. *et al.* Acid sensing by the *Drosophila* olfactory system. *Nature* **468**, 691-695, (2010).
- 59 Senthilan, P. R., Piepenbrock, D., Ovezmyradov, G., Nadrowski, B., Bechstedt, S., Pauls, S. *et al.* *Drosophila* auditory organ genes and genetic hearing defects. *Cell* **150**, 1042-1054, (2012).
- 60 Ueno, K., Kohatsu, S., Clay, C., Forte, M., Isono, K. & Kidokoro, Y. Gs α is involved in sugar perception in *Drosophila melanogaster*. *J. Neurosci.* **26**, 6143-6152, (2006).
- 61 Ishimoto, H., Takahashi, K., Ueda, R. & Tanimura, T. G-protein gamma subunit 1 is required for sugar reception in *Drosophila*. *EMBO J.* **24**, 3259-3265, (2005).
- 62 Kwon, J. Y., Dahanukar, A., Weiss, L. A. & Carlson, J. R. Molecular and cellular organization of the taste system in the *Drosophila* larva. *J. Neurosci.* **31**, 15300-15309, (2011).
- 63 Benton, R. Chemical sensing in *Drosophila*. *Curr. Op. Neurobiol.* **18**, 357-363, (2008).
- 64 Thorne, N. & Amrein, H. Atypical expression of *Drosophila* gustatory receptor genes in sensory and central neurons. *J. Comp. Neurol.* **506**, 548-568, (2008).
- 65 Montell, C. A taste of the *Drosophila* gustatory receptors. *Curr. Opi. Neurobiol.* **19**, 345-353, (2009).

- 66 Depetris-Chauvin, A., Galagovsky, D. & Grosjean, Y. Chemicals and chemoreceptors: Ecologically relevant signals driving behavior in *Drosophila*. *Front. Ecol. Evol.* **3**, (2015).
- 67 Weiss, L. A., Dahanukar, A., Kwon, J. Y., Banerjee, D. & Carlson, J. R. The molecular and cellular basis of bitter taste in *Drosophila*. *Neuron* **69**, 258-272, (2011).
- 68 Ling, F., Dahanukar, A., Weiss, L. A., Kwon, J. Y. & Carlson, J. R. The molecular and cellular basis of taste coding in the legs of *Drosophila*. *J. Neurosci.* **34**, 7148-7164, (2014).
- 69 Dahanukar, A., Lei, Y.-T., Kwon, J. Y. & Carlson, J. R. Two Gr genes underlie sugar reception in *Drosophila*. *Neuron* **56**, 503-516, (2007).
- 70 Shim, J., Lee, Y., Jeong, Y. T., Kim, Y., Lee, M. G., Montell, C. *et al.* The full repertoire of *Drosophila* gustatory receptors for detecting an aversive compound. *Nat. Comm.* **6**, 8867, (2015).
- 71 Benton, R., Vannice, K. S. & Vosshall, L. B. An essential role for a CD36-related receptor in pheromone detection in *Drosophila*. *Nature* **450**, 289-293, (2007).
- 72 Rać, M. E., Safranow, K. & Poncyljusz, W. Molecular basis of human CD36 gene mutations. *Mol. Med.* **13**, 288-296, (2007).
- 73 Jin, X., Ha, T. S. & Smith, D. P. SNMP is a signaling component required for pheromone sensitivity in *Drosophila*. *Proc. Natl. Acad. Sci. USA* **105**, 10996-11001, (2008).
- 74 Nichols, Z. & Vogt, R. G. The SNMP/CD36 gene family in Diptera, Hymenoptera and Coleoptera: *Drosophila melanogaster*, *D. pseudoobscura*, *Anopheles gambiae*, *Aedes aegypti*, *Apis mellifera*, and *Tribolium castaneum*. *Insect Biochem. Mol. Biol.* **38**, 398-415, (2008).
- 75 Vogt, R. G., Riddiford, L. M. & Prestwich, G. D. Kinetic properties of a sex pheromone-degrading enzyme: the sensillar esterase of *Antheraea polyphemus*. *Proc. Natl. Acad. Sci. USA* **82**, 8827-8831, (1985).
- 76 Ishida, Y. & Leal, W. S. Rapid inactivation of a moth pheromone. *Proc. Natl. Acad. Sci. USA* **102**, 14075-14079, (2005).
- 77 Merlin, C., Rosell, G., Carot-Sans, G., Francois, M. C., Bozzolan, F., Pelletier, J. *et al.* Antennal esterase cDNAs from two pest moths, *Spodoptera littoralis* and *Sesamia nonagrioides*, potentially involved in odorant degradation. *Insect. Mol. Biol.* **16**, 73-81, (2007).
- 78 Durand, N., Carot-Sans, G., Bozzolan, F., Rosell, G., Siauxat, D., Debernard, S. *et al.* Degradation of pheromone and plant volatile components by a same odorant-degrading enzyme in the cotton leafworm, *Spodoptera littoralis*. *PLoS One* **6**, (2011).
- 79 Vogt, R. G. Molecular basis of pheromone detection in insects. In *Comprehensive Insect Physiology, Biochemistry, Pharmacology and Molecular Biology*, 753-804, The Genetics Society, (2005).
- 80 Vogt, R. G. Biochemical diversity of odor detection: OBPs, ODEs and SNMPs. *Insect pheromone biochemistry and molecular biology*, 391-445, (2003).
- 81 Ishida, Y. & Leal, W. S. Chiral discrimination of the Japanese beetle sex pheromone and a behavioral antagonist by a pheromone-degrading enzyme. *Proc. Natl. Acad. Sci. USA* **105**, 9076-9080, (2008).
- 82 Maibeche-Coisne, M., Nikonov, A. A., Ishida, Y., Jacquin-Joly, E. & Leal, W. S. Pheromone anosmia in a scarab beetle induced by *in vivo* inhibition of a pheromone-degrading enzyme. *Proc. Natl. Acad. Sci. USA* **101**, 11459-11464, (2004).
- 83 He, P., Zhang, J., Li, Z. Q., Zhang, Y. N., Yang, K., Dong, S. L. *et al.* Functional characterization of an antennal esterase from the noctuid moth, *Spodoptera exigua*. *Arch. Insect Biochem. Physiol.* **86**, (2014).
- 84 Jordan, M. D., Stanley, D., Marshall, S. D., De Silva, D., Crowhurst, R. N., Gleave, A. P. *et al.* Expressed sequence tags and proteomics of antennae from the tortricid moth, *Epiphyas postvittana*. *Insect Mol. Biol.* **17**, 361-373, (2008).

- 85 Kamikouchi, A., Morioka, M. & Kubo, T. Identification of honeybee antennal proteins/genes expressed in a sex- and/or caste selective manner. *Zoolog. Sci.* **21**, 53-62, (2004).
- 86 Oakeshott, J. G., Claudianos, C., Campbell, P. M., Newcomb, R. D. & Russell, R. J. Biochemical genetics and genomics of insect esterases. In *Comprehensive Molecular Insect Science* Vol. 5, 309-381, Elsevier, (2005).
- 87 Coates, P. M., Mestrjner, M. A. & Hopkinson, D. A. A preliminary genetic interpretation of the esterase isozymes of human tissues. *Annals Human Genet.* **39**, 1-20, (1975).
- 88 Holmes, R. S. & Masters, C. J. The developmental multiplicity and isoenzyme status ofavian esterases. *Biochem. Biophys. Acta.* **132**, 379-399, (1967).
- 89 Healy, M. J., Dumancic, M. M. & Oakeshott, J. G. Biochemical and physiological studies of soluble esterases from *Drosophila melanogaster*. *Biochem. Genet.* **29**, 365-388, (1991).
- 90 Wright, T. R. F. The genetics of an esterase in *Drosophila melanogaster*. *Genetics* **48**, 787-801, (1963).
- 91 Claudianos, C., Crone, E., Coppin, C., Russell, R. & Oakeshott, J. A genomics perspective on mutant aliesterases and metabolic resistance to organophosphates. In *Agrochemical Resistance* Vol. 808, 90-101, American Chemical Society, (2001).
- 92 Montella, I. R., Schama, R. & Valle, D. The classification of esterases: an important gene family involved in insecticide resistance - A review. *Memórias do Instituto Oswaldo Cruz* **107**, 437-449, (2012).
- 93 Oakeshott, J. G., Healy, M. J. & Game, A. Y. Regulatory evolution of β -carboxyl esterases in *Drosophila*. In *Ecological and Evolutionary Genetics of Drosophila*, 359-387, Springer US, (1990).
- 94 Oakeshott, J. G., van Papenrecht, E. A., Claudianos, C., Morrish, B. C., Coppin, C. & Odgers, W. A. An episode of accelerated amino acid change in *Drosophila* esterase-6 associated with a change in physiological function. *Genetica* **110**, 231-244, (2000).
- 95 Balakirev, E. S., Chechetkin, V. R., Lobzin, V. V. & Ayala, F. J. DNA polymorphism in the β -esterase gene cluster of *Drosophila melanogaster*. *Genetics* **164**, 533-544, (2003).
- 96 Dumancic, M. M., Oakeshott, J. G., Russell, R. J. & Healy, M. J. Characterization of the EstP protein in *Drosophila melanogaster* and its conservation in Drosophilids. *Biochem. Genet.* **35**, 251-271, (1997).
- 97 Arbeitman, M. N., Furlong, E. E. M., Imam, F., Johnson, E., Null, B. H., Baker, B. S. *et al.* Gene expression during the life cycle of *Drosophila melanogaster*. *Science* **297**, 2270-2275, (2002).
- 98 Meikle, D. B., Sheehan, K. B., Phillis, D. M. & Richmond, R. C. Localization and longevity of seminal-fluid esterase 6 in mated female *Drosophila melanogaster*. *J. Insect Physiol.* **36**, 93-101, (1990).
- 99 Richmond, R. C., Nielsen, K. M., Brady, J. P. & Snella, E. M. Physiology, biochemistry and molecular biology of the Est-6 locus in *Drosophila melanogaster*. In *Ecological and Evolutionary Genetics of Drosophila*, 273-292, Springer US, (1990).
- 100 Vander Meer, R., Obin, M., Zawistowski, S., Sheehan, K. & Richmond, R. A re-evaluation of the role of cis-vaccenyl acetate, cis-vaccenol and Esterase 6 in the regulation of mated female sexual attractiveness in *Drosophila melanogaster*. *J. Insect Physiol.* **32**, (1986).
- 101 Richmond, R., Gilbert, D., Sheehan, K., Gromko, M. & Butterworth, F. Esterase 6 and reproduction in *Drosophila melanogaster*. *Science* **207**, (1980).
- 102 Gilbert, D. Ejaculate Esterase 6 and initial sperm use by female *Drosophila melanogaster*. *J. Insect Physiol.* **27**, (1981).
- 103 Oakeshott, J. H., Boyce, T. M., Russell, R. J. & Healy, M. J. Molecular insights into the evolution of an enzyme; Esterase 6 in *Drosophila*. *Trend. Ecol. Evol.* **10**, 103-110, (1995).

- 104 Morton, R. & Singh, R. Biochemical properties, homology, and genetic variation of
Drosophila "nonspecific" esterases. *Biochem. Genet.* **23**, 959-973, (1985).
- 105 Anholt, R. R. H. & Williams, T. I. The soluble proteome of the *Drosophila* antenna.
Chem. Senses **35**, 21-30, (2009).
- 106 Tamarina, N. A., Ludwig, M. Z. & Richmond, R. C. Divergent and conserved features in
the spatial expression of the *Drosophila pseudoobscura* Esterase 5B gene and the
Esterase 6 gene of *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. USA* **94**, 7735-7741,
(1997).
- 107 Chapman, T., Bangham, J., Vinti, G., Seifried, B., Lung, O., Wolfner, M. F. *et al.* The sex
peptide of *Drosophila melanogaster*: Female post-mating responses analyzed by using
RNA interference. *Proc. Natl. Acad. Sci. USA* **100**, 9923-9928, (2003).
- 108 Liu, H. & Kubli, E. Sex-peptide is the molecular basis of the sperm effect in *Drosophila*
melanogaster. *Proc. Natl. Acad. Sci. USA* **100**, 9929-9933, (2003).
- 109 Mane, S. D., Tompkins, L. & Richmond, R. C. Male Esterase 6 catalyzes the synthesis of
a sex pheromone in *Drosophila melanogaster* females. *Science* **222**, (1983).
- 110 Sheehan, K., Richmond, R. C. & Cochrane, B. J. Studies of Esterase 6 in *Drosophila*
melanogaster. III. The developmental pattern and tissue distribution. *Insect Biochem.*
9, 443-450, (1979).
- 111 Langley, C. H., Voelker, R. A., Brown, A. J. L., Ohnishi, S., Dickson, B. & Montgomery, E.
Null allele frequencies at allozyme loci in natural populations of *Drosophila*
melanogaster. *Genetics* **99**, 151-156, (1981).
- 112 Chertemps, T., François, A., Durand, N., Rosell, G., Dekker, T., Lucas, P. *et al.* A
carboxylesterase, Esterase-6, modulates sensory physiological and behavioral
response dynamics to pheromone in *Drosophila*. *BMC Biol.* **10**, (2012).
- 113 Campbell, P. M., Harcourt, R. L., Crone, E. J., Claudianos, C., Hammock, B. D., Russell, R.
J. *et al.* Identification of a juvenile hormone esterase gene by matching its peptide
mass fingerprint with a sequence from the *Drosophila* genome project. *Insect Biochem.*
Mol. Biol. **31**, 513-520, (2001).
- 114 Hammock, B. D., Bonning, B. C., Possee, R. D., Hanzlik, T. N. & Maeda, S. Expression
and effects of the juvenile-hormone esterase in a *Baculovirus* vector. *Nature* **344**, 458-
461, (1990).
- 115 Yamamoto, K., Chadarevian, A. & Pellegrini, M. Juvenile hormone action mediated in
male accessory glands of *Drosophila* by calcium and kinase C. *Science* **239**, 916-919,
(1988).
- 116 Rachinsky, A. & Hartfelder, K. Corpora allata activity, a prime regulating element for
caste specific juvenile hormone titre in honey bee larvae (*Apis mellifera carnica*). *J.*
Insect Physiol. **36**, 189-194, (1990).
- 117 Denlinger, D. L. & Tanaka, S. Cycles of juvenile hormone esterase activity during the
juvenile hormone-driven cycles of oxygen consumption in pupal diapause of Flesh flies.
Experientia **45**, 474-476, (1989).
- 118 Crone, E. J., Sutherland, T. D., Campbell, P. M., Coppin, C. W., Russell, R. J. &
Oakeshott, J. G. Only one esterase of *Drosophila melanogaster* is likely to degrade
juvenile hormone *in vivo*. *Insect Biochem. Mol. Biol.* **37**, 540-549, (2007).
- 119 Kong, E. C., Allouche, L., Chapot, P. A., Vranizan, K., Moore, M. S., Heberlein, U. *et al.*
Ethanol regulated genes that contribute to ethanol sensitivity and rapid tolerance in
Drosophila. *Alc. Clinical Exp. Res.* **34**, 302-316, (2010).
- 120 Morozova, T. V., Anholt, R. R. H. & Mackay, T. F. C. Transcriptional response to alcohol
exposure in *Drosophila melanogaster*. *Genome Biol.* **7**, (2006).
- 121 Su, C.-Y., Martelli, C., Emonet, T. & Carlson, J. R. Temporal coding of odor mixtures in
an olfactory receptor neuron. *Proc. Natl. Acad. Sci. USA* **108**, 5075-5080, (2011).
- 122 Kundu, S., Ganguly, A., Chakraborty, T. S., Kumar, A. & Siddiqi, O. Synergism and
combinatorial coding for binary odor mixture perception in *Drosophila*. *eNeuro* **3**,
ENEURO.0056-0014.2016, (2016).

- 123 Dweck, H. K. M., Ebrahim, S. A. M., Thoma, M., Mohamed, A. A. M., Keeseey, I. W.,
Trona, F. *et al.* Pheromones mediating copulation and attraction in *Drosophila*. *Proc.*
Natl. Acad. Sci. USA **112**, E2829-E2835, (2015).
- 124 Pellegrino, M., Nakagawa, T. & Vosshall, L. B. Single sensillum recordings in the insects
Drosophila melanogaster and *Anopheles gambiae*. *J. Vis. Exp. : JoVE*, 1725, (2010).
- 125 González-Mas, M. C., Rambla, J. L., Alamar, M. C., Gutiérrez, A. & Granell, A.
Comparative analysis of the volatile fraction of fruit juice from different citrus species.
PLoS One **6**, e22016, (2011).
- 126 Johnson, C. H., Ivanisevic, J. & Siuzdak, G. Metabolomics: Beyond biomarkers and
towards mechanisms. *Nat. Rev. Mol. Cell. Biol.* **17**, 451-459, (2016).
- 127 Piyankarage, S. C., Featherstone, D. E. & Shippy, S. A. Nanoliter haemolymph sampling
and analysis of individual adult *Drosophila melanogaster*. *Anal. Chem.* **84**, 4460-4466,
(2012).
- 128 Kreher, S. A., Kwon, J. Y. & Carlson, J. R. The molecular basis of odor coding in the
Drosophila larva. *Neuron* **46**, 445-456, (2005).
- 129 Walsh, D. B., Bolda, M. P., Goodhue, R. E., Dreves, A. J., Lee, J., Bruck, D. J. *et al.*
Drosophila suzukii (Diptera: Drosophilidae): Invasive pest of ripening soft fruit
expanding its geographic range and damage potential. *J. Integr. Pest Manag.* **2**, G1-G7,
(2011).
- 130 Atallah, J., Teixeira, L., Salazar, R., Zaragoza, G. & Kopp, A. The making of a pest: the
evolution of a fruit-penetrating ovipositor in *Drosophila suzukii* and related species.
Proc. Roy. Soc. B-Biol Sci. **281**, 20132840, (2014).
- 131 Asplen, M. K., Anfora, G., Biondi, A., Choi, D.-S., Chu, D., Daane, K. M. *et al.* Invasion
biology of spotted wing *Drosophila* (*Drosophila suzukii*): A global perspective and
future priorities. *J. Pest Sci.* **88**, 469-494, (2015).
- 132 Ioriatti, C., Walton, V., Dalton, D., Anfora, G., Grassi, A., Maistri, S. *et al.* *Drosophila*
suzukii (Diptera: Drosophilidae) and its potential impact to wine grapes during harvest
in two cool climate wine grape production regions. *J. Econ. Entomol.* **108**, 1148-1155,
(2015).
- 133 Chabert, S., Allemand, R., Poyet, M., Eslin, P. & Gibert, P. Ability of European
parasitoids (*Hymenoptera*) to control a new invasive Asiatic pest, *Drosophila suzukii*.
Biol. Control **63**, 40-47, (2012).
- 134 Maderspacher, F. & Stensmyr, M. C. Sensory evolution: Trouble in the cherry orchard.
Curr. Biol. **27**, R218-R220, (2017).
- 135 Chiu, J. C., Jiang, X., Zhao, L., Hamm, C. A., Cridland, J. M., Saelao, P. *et al.* Genome of
Drosophila suzukii, the Spotted Wing *Drosophila*. *G3: Genes|Genomes|Genetics* **3**,
2257-2271, (2013).
- 136 Muema, J. M., Bargul, J. L., Njeru, S. N., Onyango, J. O. & Imbahale, S. S. Prospects for
malaria control through manipulation of mosquito larval habitats and olfactory-
mediated behavioural responses using plant-derived compounds. *Parasites & Vectors*
10, 184, (2017).

Appendix: Structure and function of an insect α -carboxylesterase (α Esterase7) associated with insecticide resistance

Structure and function of an insect α -carboxylesterase (α Esterase7) associated with insecticide resistance

Colin J. Jackson^{a,b,1}, Jian-Wei Liu^c, Paul D. Carr^a, Faisal Younus^c, Chris Coppin^c, Tamara Meirelles^a, Mathilde Lethier^b, Gunjan Pandey^c, David L. Ollis^a, Robyn J. Russell^c, Martin Weik^b, and John G. Oakeshott^c

^aResearch School of Chemistry, Australian National University, Canberra, ACT 0200, Australia; ^bInstitut de Biologie Structurale, Commissariat à l'Énergie Atomique, F-38027 Grenoble, France; and ^cCommonwealth Scientific and Industrial Research Organization (Australia) Ecosystems Science, Canberra, ACT 0200, Australia

Edited by Bruce D. Hammock, University of California, Davis, CA, and approved May 2, 2013 (received for review March 6, 2013)

Insect carboxylesterases from the α Esterase gene cluster, such as α E7 (also known as E3) from the Australian sheep blowfly *Lucilia cuprina* (*Lc* α E7), play an important physiological role in lipid metabolism and are implicated in the detoxification of organophosphate (OP) insecticides. Despite the importance of OPs to agriculture and the spread of insect-borne diseases, the molecular basis for the ability of α -carboxylesterases to confer OP resistance to insects is poorly understood. In this work, we used laboratory evolution to increase the thermal stability of *Lc* α E7, allowing its overexpression in *Escherichia coli* and structure determination. The crystal structure reveals a canonical α/β -hydrolase fold that is very similar to the primary target of OPs (acetylcholinesterase) and a unique N-terminal α -helix that serves as a membrane anchor. Soaking of *Lc* α E7 crystals in OPs led to the capture of a crystallographic snapshot of *Lc* α E7 in its phosphorylated state, which allowed comparison with acetylcholinesterase and rationalization of its ability to protect insects against the effects of OPs. Finally, inspection of the active site of *Lc* α E7 reveals an asymmetric and hydrophobic substrate binding cavity that is well-suited to fatty acid methyl esters, which are hydrolyzed by the enzyme with specificity constants ($\sim 10^6 \text{ M}^{-1} \text{ s}^{-1}$) indicative of a natural substrate.

protein engineering | directed evolution | ali-esterase

The demand for greater productivity from agriculture and the avoidance of insect-borne diseases has made efficient control of insect pests increasingly important; in 2007, the world market for insecticides was estimated to be in the order of \$11 billion US (1). However, the effectiveness of insecticides is decreasing, with over 500 documented instances of insecticide resistance (2). The development of resistance to organophosphate (OP) (Fig. 1A) pesticides through the action of α -carboxylesterases (α -CBEs) has been documented in many species (3), including the Australian sheep blowfly *Lucilia cuprina* (4). OPs inhibit acetylcholinesterase (AChE) at cholinergic synapses in the central and peripheral nervous systems, which leads to interminable nerve signal transduction and death (5). However, α -CBEs, such as α E7 in *L. cuprina* (*Lc* α E7), confer a significant protective effect on insects owing to their ability to bind and slowly hydrolyze OPs (6). It has also been shown that this CBE-mediated OP resistance can be increased through G137D and W251L point mutations (4, 7).

Insect α -CBEs, including *Lc* α E7, are thought to play important physiological roles in lipid and xenobiotic metabolism (6, 8). They are one of the most abundant protein families in insects, with between 20 and 40 active CBEs predicted to be expressed by the fruit fly *Drosophila melanogaster* (9, 10). Although the physiological substrates of insect α -CBEs are unknown, recent MS results have shown that *D. melanogaster* α E7 is expressed in the fat body lipid droplet proteome (11), implying a role in lipid or cholesterol metabolism. This work has been extended to show that *D. melanogaster* α E7-null mutants exhibit reduced lifespan, lowered insecticide tolerance, and reduced lipid storage capacity (6).

Intense efforts over the last 50 y have shed light on the critical role that CBEs have played in the evolution of insecticide resistance (12). However, in the absence of a molecular structure of

an insect CBE, the interactions of these enzymes with pesticides or natural substrates and the effects of mutations and sequence polymorphisms could not be understood at a molecular level. In this work, we describe the directed evolution of *Lc* α E7 for increased stability and heterologous expression in *Escherichia coli*, which allowed us to grow crystals and solve the structure. By soaking crystals of *Lc* α E7 in OPs, we have been able to capture it in its phosphorylated state after OP hydrolysis, allowing us to understand the structural features that confer insecticide resistance to the fly. Finally, this structure allowed us to identify the probable natural substrate for these enzymes.

Results and Discussion

Evolving a Stable Variant of *Lc* α E7 for Structure Determination.

Previous attempts to purify and crystallize WT *Lc* α E7 have been hampered by the instability of the protein, and therefore, we performed a laboratory (directed) evolution experiment to increase its stability and allow its overexpression in *E. coli*. This process involved successive rounds of mutagenesis of the gene using error-prone PCR before the variants were screened for increased thermal stability. Approximately 100,000 random variants of *Lc* α E7 were plated onto agar plates and then replica-plated onto filter paper, which was incubated at various temperatures for 1 h. After incubation, the filter paper was immersed in a solution of the model ester substrate 2-naphthyl acetate and fast-red dye, which forms a red complex with the naphthol product of hydrolysis. Colonies that displayed the greatest activity were selected as positive clones and pooled for the next round of random mutagenesis. After four rounds, an enhanced variant was obtained that contained six mutations (M364L, I419F, A472T, I505T, K530E, and D554G). Bacterial colonies in which this variant was expressed displayed significant esterase activity after incubation at 54 °C for 1 h, whereas colonies expressing the WT enzyme displayed no activity after this heat treatment.

The best *Lc* α E7 variant, hereafter termed *Lc* α E7-4, was purified from *E. coli*. Measurement of its thermal stability and comparison with WT *Lc* α E7 confirmed that the mutations provided stabilizing effects. Specifically, we observed a 5.6 °C increase in the apparent temperature at which the protein lost one-half of its activity through thermal denaturation (T_{m50}^{app} ; from 41.9 °C in the WT to 47.5 °C in *Lc* α E7-4) (Fig. S1). The kinetic parameters of *Lc* α E7-4 were very similar to the parameters of the WT enzyme ($k_{\text{cat}}/K_M = 1.1 \times 10^6 \text{ s}^{-1} \text{ M}^{-1}$ vs. $1.8 \times 10^6 \text{ s}^{-1} \text{ M}^{-1}$),

Author contributions: C.J.J., J.-W.L., D.L.O., R.J.R., M.W., and J.G.O. designed research; C.J.J., J.-W.L., P.D.C., F.Y., C.C., T.M., M.L., and G.P. performed research; C.J.J., M.W., and J.G.O. analyzed data; and C.J.J. and J.G.O. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Freely available online through the PNAS open access option.

Data deposition: The crystallography, atomic coordinates, and structure factors have been deposited in the Protein Data Bank, www.pdb.org (PDB ID codes 4FG5, 4FNG, and 4FNM).

¹To whom correspondence should be addressed. E-mail: cjackson@rsc.anu.edu.au.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1304097110/-DCSupplemental.

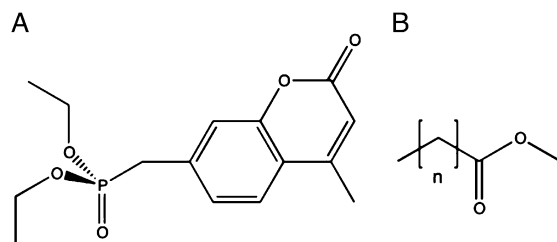


Fig. 1. Chemical structures of the substrates used in this work. (A) The OP diethyl-4-methylumbelliferyl phosphate (DEUP). (B) The generic structure of FAMES. Hexanoate, $n = 4$; octanoate, $n = 6$; decanoate, $n = 8$; laurate, $n = 10$; myristate, $n = 12$.

suggesting that the mutations have not significantly affected the structure.

Size exclusion chromatography revealed that *LcaE7-4* existed in equilibrium between monomeric and dimeric forms (Fig. S2). Each of these peaks was concentrated to 8 mg/mL and screened for crystallization. Crystals of both the monomeric and the dimeric forms of the enzyme were obtained and diffracted to 2.2 and 1.95 Å, respectively (Table 1). The structure was solved by molecular replacement using the structure of mouse AChE (13), which shares 29% amino acid identity. Several AChE structures were used in the molecular replacement search, with this structure being the only one to give a correct solution. There were no significant differences between the monomeric and dimeric structures.

Overall Structure of *LcaE7*. *LcaE7* adopts an α/β -hydrolase superfamily fold (14), which is shown in Fig. 2A. The canonical core of the α/β -hydrolase fold is present as well as the conserved catalytic Ser-His-Glu triad. In addition to eight canonical β -strands, the central β -sheet in *LcaE7* also contains two antiparallel β -strands at the start and two antiparallel β -strands at the end. The six canonical α -helices are also present, although in some instances (α -b, α -d, and α -f), additional short helices are also present. The structure diverges significantly from the canonical α/β -hydrolase fold with the presence of a large α -helix at the N terminus, which

will be discussed in detail later, and two large subdomains on either side of the active site cleft on the upper face of the protein. Subdomain 1 is formed by a short antiparallel β -sheet insertion after β 1, two α -helices inserted after β 3, and four α -helices inserted after β 6. Subdomain 2 is comprised of four α -helices inserted after β 7 and two α -helices at the C terminus of the protein (Fig. 2A).

The monomeric and dimeric *LcaE7* protein structures were analyzed by the PISA server (http://www.ebi.ac.uk/msd-srv/prot_int/cgi-bin/piserver) (15). The total surface area buried at the dimer interface is 2,040 Å², which is predicted to result in free energy of dimer formation of -7.4 kcal/mol and free energy of dissociation ΔG^{diss} of 0.9 kcal/mol. The relatively small energy barrier to dissociation is consistent with its equilibrium with monomeric protein (Fig. S2). The dimer interface, which is symmetrical, is formed by subdomains 1 and 2 and dominated by symmetrical interactions between subdomain 1 from each monomer (Fig. S3).

Of six mutations that were found in the stabilized variant, three mutations (Ile419Phe, Ala472Thr, and Ile505Thr) are in the interior of the protein, and three mutations (Met364Leu, Lys530Glu, and Asp554Gly) are on its surface (Fig. S4). It is clear that the Ile419Phe mutation results in a hydrophobic cavity in the interior of the protein being filled, which is a common and established mode of protein stabilization (16). However, the other mutations do not obviously conform to established modes of protein stabilization.

N-Terminal Membrane Anchor. Analysis of the structure of *LcaE7* shows the presence of an amphipathic α -helix at the N terminus (Fig. 2A and B). α -CBEs have long been known to be associated with the microsomal fraction (17). However, it was unknown how this association was maintained at a structural level. The crystal structure presented here provides an explanation: the N-terminal helix of *LcaE7* is rich in positively charged and hydrophobic amino acids, with an amphipathic distribution that is ideal for membrane association. This conclusion is supported by sequence analysis from the MEMPype server (18), which predicted the protein to be associated with the intracellular membrane through the N-terminal helix. The helix is packed only loosely against the body of the protein in the crystal structure through hydrophobic interactions, which will allow it to easily dissociate.

Table 1. Data collection and refinement statistics for structures reported in this work

	Apo- <i>LcaE7</i> P2 ₁	Apo- <i>LcaE7</i> C22 ₁	DEUP+ <i>LcaE7</i> C22 ₁
Space group	P1 21 1	C 2 2 21	C 2 2 21
Unit cell parameters			
a (Å)	61.77	48.62	50.65
b (Å)	108.96	100.51	102.74
c (Å)	92.17	221.74	226.40
β (°)	90.36		
Data collection			
Wavelength (Å)	0.8266	0.9393	0.8266
Resolution range (Å)*	46.9–2.19 (2.31–2.19)	19.7–1.95 (2.00–1.95)	42.5–1.80 (1.85–1.80)
No. of unique reflections	62,125	40,150	54,447
Redundancy	3.3 (3.2)	6.6 (5.7)	7.3 (6.7)
Completeness (%)	97.9 (100)	99.8 (100.0)	99.1 (97.5)
$R_{\text{merge}}(\%)$	0.093 (0.439)	0.076 (0.631)	0.109 (0.60)
Mean $\langle I/\sigma(I) \rangle$	12.7 (4.0)	17.6 (2.7)	15.6 (3.0)
Refinement			
No. reflections (total)	58,544	40,152	54,447
Resolution range	46.9–2.19 (2.24–2.19)	19.7–1.95 (2.02–1.95)	42.5–1.80 (1.83–1.80)
$R_{\text{work}}/R_{\text{free}}$	17.06/20.91 (23.7/28.3)	17.75/22.50 (24.83/32.60)	16.96/20.45 (21.54/21.86)
rmsd			
Bond lengths (Å)	0.020	0.008	0.006
Bond angles (°)	2.24	1.09	1.04
Protein Data Bank ID code	4FG5	4FNG	4FNM

*Values in parenthesis are for the highest-resolution shell.

binding site. Overall, this comparison reveals that the active site of *LcαE7* is much more open than the active sites of its relatives and shows how small insertions in highly homologous protein structures can have significant impact on the overall topology of substrate binding sites.

Active Site and Natural Substrate Preference. The canonical catalytic triad of the α/β -hydrolase fold is conserved in *LcαE7*, with Ser218 at the end of $\beta 4$ hydrogen bonded to His471 at the end of $\beta 7$, which is, in turn, hydrogen bonded to Glu351 at the end of $\beta 6$. The structure also reveals the presence of an oxyanion hole consisting of the backbone amide groups of Ala219, Gly136, and Gly137 (Fig. 4). Thus, the catalytic groups in *LcαE7* are essentially identical to those groups in most other structural homologs, such as AChE.

The differences and similarities between the active sites of *LcαE7*, AChE, and JHE were used to identify plausible natural substrates. First, like JHE and AChE, the substrate binding cavity of *LcαE7* is also extremely asymmetrical, with a small binding pocket, which is comprised of Phe354, Tyr457, Met460, and Thr472, and a large pocket lined by the side chains of Trp251, Met308, Phe309, Phe355, and Phe421 (Fig. 4). This asymmetry strongly suggests that, like juvenile hormone and acetylcholine, the carboxylesters that *LcαE7* hydrolyzes most likely have an acyl group. Second, unlike AChE but like JHE, the binding cavity is almost entirely hydrophobic, suggesting that the leaving acid group will be strongly hydrophobic. Additionally, its association with the fat droplet (6, 17) limits the possible esters that it may encounter to triglycerides, cholesterol esters, retinyl esters, and fatty acid methyl esters (FAMES) (Fig. 1B) (24). Of these esters, retinyl esters and triglycerides are far too large for the binding pocket. Thus, cholesterol acetate and FAMES were tested as substrates. No activity was observed with cholesterol acetate, consistent with the curved shape of the pocket making it unsuited for substrates with long rigid leaving groups. Docking analysis suggested that the active site is ideally shaped for FAMES of medium length (Fig. 4). *LcαE7* efficiently catalyzed the hydrolysis of these compounds with high-specificity constants (up to $1.4 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ for methyl decanoate) (Table 2). A specificity constant in the order of $10^6 \text{ M}^{-1} \text{ s}^{-1}$ is highly indicative of a natural substrate, especially in metabolic scenarios (25). This finding is also in keeping with a role in lipid metabolism and the observation that *MdαE7*-null *Musca domestica* KOs are deficient in fatty acid accumulation (6).

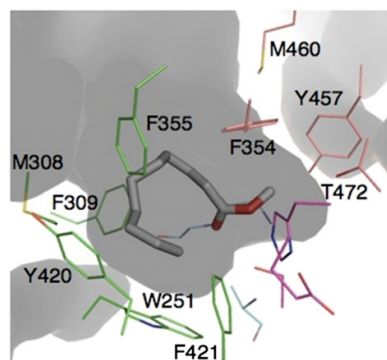


Fig. 4. The substrate binding pocket of *LcαE7*. The pocket can be divided into sections: a small pocket (pink) comprising Y457, M460, F354, and T472 and a larger pocket (green) comprising F355, Y420, W251, M308, F309, and F421. The FAME methyl decanoate has been docked in the active site. The catalytic triad is colored magenta, and the oxyanion hole is colored sky blue.

Structural Basis for OP Resistance. CBEs, such as *LcαE7*, that belong to the *αEsterase* gene cluster are known to provide protection against OPs, even as WT enzymes (6). Both AChE and *LcαE7* are inhibited by OPs as a result of phosphorylation of the catalytic serine. However, although this process is often irreversible in AChE as a result of a secondary aging reaction, in which the phosphorylated serine is dealkylated (26), *LcαE7* is capable of hydrolytic turnover of OPs at significant, albeit low, rates (Table 2) (27). To investigate why this process does not occur in *LcαE7*, we captured *LcαE7* in its intermediate, phosphorylated state by flash-cooling crystals of *LcαE7* that had been soaked in 1 mM diethyl 4-methylumbelliferyl phosphate. These crystals diffracted to 1.8 Å (Table 1), and as shown in Fig. 5 and Fig. S5, no evidence of aging was observed (27). This finding is consistent with previous work that has shown mammalian CBEs to be resistant to the aging reaction (28, 29).

Aging of AChE, involving dealkylation of the phosphorylated serine, has been proposed to occur through either hydrolysis or carbocation bond scission (26, 30). To investigate why *LcαE7* is resistant to aging, we compared the structural features that are known to make AChE prone to aging (Fig. 5) (26, 30–32). First, activation and stabilization of the alkyl side chain is thought to involve the catalytic histidine, which is positioned close to the alkyl side chain and stabilized in a position to allow this interaction by Glu199 and Phe331 (*Torpedo californica* AChE numbering). In comparison, the catalytic histidine (His471) in *LcαE7* is farther away from the alkyl side chain (3.2 vs. 2.5 Å) and not well-oriented to interact with this group during dealkylation; the catalytic histidine in human CBE is also poorly positioned to interact with the alkyl side chain of a phosphorylated serine (28). Although Glu217 is present in a similar position to Glu199 in AChE, there is no residue present in *LcαE7* in the same position as Phe331 in AChE, meaning that the catalytic histidine cannot be stabilized in the required position to catalyze dealkylation. Second, the Ω -loop of AChE, containing Trp84, is known to play an essential role in catalysis of acetylcholine hydrolysis and aging through cation- π interactions between the choline or carbocation leaving group and the indole moiety of Trp84 (23, 30). Like Phe331, this residue is not present in *LcαE7*; the Ω -loop is, in fact, replaced by the short antiparallel sheet after strand $\beta 1$ (Fig. 2B and C). In *LcαE7*, a tyrosine residue (Tyr427) is present in a similar position, but the electronegative hydroxyl substitution weakens the cation- π interaction in contrast to the substituted indole, which is known to be particularly activated (33).

Therefore, the ability of *LcαE7* to confer OP resistance derives from two structural properties. First, the immediate active site of *LcαE7* is strikingly similar to AChE (Fig. 5) and highly complementary to OPs. Indeed, studies have shown that the binding affinity of *LcαE7* for the OP paraoxon is ~ 10 -fold higher than the binding affinity of *LcAChE* (22 vs. 244 nM) (4, 34). Second, specific residues required for the aging reaction to occur in AChE (Trp84 and Phe331) are absent in *LcαE7*, allowing for slow turnover of the OP rather than irreversible inhibition. The differential cost to the organism of *LcαE7* vs. AChE inhibition is also important; although AChE is essential, loss of $\alpha E7$ CBE activity in *D. melanogaster* and *L. cuprina* is tolerated (6, 35). Additionally, $\alpha E7$ CBEs are expressed in relatively high abundance across the larvae and adult life stages during which

Table 2. Kinetic parameters for substrate turnover by *LcαE7*

Substrate	k_{cat}/K_M ($10^6 \text{ M}^{-1} \text{ s}^{-1}$)
Methyl hexanoate	0.28 ± 0.02
Methyl octanoate	0.83 ± 0.07
Methyl decanoate	1.38 ± 0.02
Methyl laurate	0.2 ± 0.03
Methyl myristate	0.061 ± 0.001
Diethyl 4-methylumbelliferyl phosphate	0.05 ± 0.005

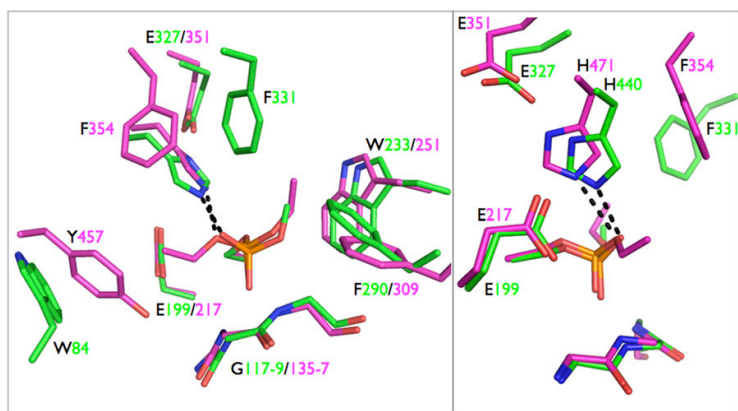


Fig. 5. Comparison between *LcaE7*-diethylphosphate (magenta) and AChE-methylphosphate (green) after aging of AChE-dimethylphosphate (3GEL; *T. californica*). Right is rotated 90° from Left. The active sites and substrate binding sites are generally highly conserved. The key differences between the structures include the closer distance (2.7 vs. 3.2 Å) between H440 and the side chain oxygen of the adduct in AChE, the absence of a residue at an analogous position to AChE F331 in *LcaE7*, and the replacement of W84 in AChE by Y457 in *LcaE7*.

the insects will be exposed to insecticides (36, 37). Their abundant expression allows α E7 CBEs to act as scavengers for OPs, sequestering the poison and slowly detoxifying it. Although the turnover rates of OPs by *LcaE7* are currently much lower than other enzymes that have been engineered for OP hydrolysis (38), its high affinity for OPs makes *LcaE7* a good starting point for the development of a high-specificity enzyme for OP poisoning. Indeed, in its native host, the G137D mutation is seen to increase the rate of OP turnover by two orders of magnitude, resulting in a large protective effect (4).

Conclusion

Hundreds of studies have been published on CBE-mediated OP resistance since the discovery of this phenomenon by Openoorth and van Apseren in 1960 (39). This first structure of an insect CBE has allowed us to identify a probable natural substrate for the enzyme, and it revealed the molecular basis for the ability of insect CBEs to hydrolyze and detoxify OP insecticides. This work shows the power of laboratory evolution for generating slightly modified versions of important proteins that can be heterologously expressed in *E. coli* at high levels and more easily studied and crystallized, allowing their molecular structures to be solved.

Materials and Methods

All chemicals were purchased from Sigma-Aldrich unless otherwise specified.

Cloning and Laboratory Evolution. The internal *Nde*I restriction site in the WT *LcaE7* gene was removed using primers E31 and E32 (Table S1) before the gene was cloned into the pETMCSIII vector (40) between the *Nde*I (New England Biolabs) and *Eco*RI (New England Biolabs) sites using the E33 and E34 primers (Table S1). Random mutations were introduced to the coding region of *LcaE7* by error-prone PCR. Briefly, the PCR was performed using primers pET1 and pET2, Taq DNA polymerase buffer (New England Biolabs), 5 mM MgCl₂, 0.1–0.4 mM MnCl₂, 0.5 mM dNTPs (New England Biolabs), 5 U Taq DNA polymerase (New England Biolabs), and milliQ H₂O to a final volume of 50 μ L. Thermocycling was performed with 30 cycles of 94 °C (10 s), 45 °C (10 s), and 72 °C (30 s). The PCR product was digested with *Nde*I, and *Eco*RI-digested PCR product was gel extracted, ligated into pETMCS III, and then used to transform BL21 (DE3)-competent cells (Invitrogen).

For library screening, ~100,000 colonies were plated onto LB-agar plates supplemented with 100 μ g/mL ampicillin. Colonies were blotted onto Whatman grade 3 filter paper (GE Healthcare) and incubated for 1 h at 50 °C (increased to 55 °C, 60 °C, and 70 °C for the subsequent generations). The residual esterase activity was assayed by spraying heat-treated colonies with substrate solution, which consisted of 0.8% (wt/vol) Fast Red, 10 mM β -naphthyl acetate, and 100 mM Tris (pH 7.0). The most stable mutants were identified as those colonies that produced the most intense red color. A secondary screen of the best variants was carried out, in which the best variants from the plate screen were picked and grown in 96-deep well plate formats. The overnight cultures were heat stressed for 1 h at the same temperatures used in the primary screen, and 25 mL overnight cultures were

assayed using a Molecular Devices plate reader at 490 nm in the presence of 0.5 mM β -naphthyl acetate, 0.5 mM Fast Red dye, and 100 mM Tris (pH 7.0). The best 5–10 variants of each generation were carried forward to the next generation, and the final product *LcaE7-4* was sequenced at the Micromon Sequencing Facility, Melbourne, Australia.

Protein Expression and Purification. WT and mutant *LcaE7* were expressed in *E. coli* BL21-DE3 (Invitrogen) cells grown at 30 °C overnight in Overnight Express TB Media (Merck) supplemented with 100 μ g/mL ampicillin. Cells were pelleted at 5,000 \times g and lysed using 1 \times Bugbuster protein extraction reagent (Merck) in 100 mM Tris-Cl (pH 8) and 200 mM NaCl. Lysate was filtered and passed over a 5-mL Ni-NTA column (Qiagen), and protein was eluted using lysis buffer supplemented with 500 mM imidazole. SDS/PAGE analysis of pooled active fractions indicated that purified *LcaE7* was essentially homogeneous. The active fraction was then subjected to size exclusion chromatography in 20 mM Hepes (pH 7.5) and 50 mM NaCl using a Sephacryl S300 column (GE Healthcare). Protein concentration was determined by measuring absorbance at 280 nm using an extinction coefficient of 91,510 M⁻¹ cm⁻¹ calculated using the Protparam server (41) (none of the mutations in *LcaE7-4* affect absorbance at 280 nm).

Enzyme Assays. FAME assays. FAMES were purchased in the highest available purity. The reaction mixture (200 μ L) consisted of substrate (200 μ M) and enzyme in 25 mM Tris-HCl (pH 8.0) supplemented with 0.2% BSA and 0.01% gum arabic as an emulsifying agent. The reaction mix was emulsified by sonication for 5 min in a water bath. The reaction was carried out in a silanized screw-top vial (Agilent) at 25 °C and quenched at specific time points with equivalent amounts of ice-cold hexane (containing 250 μ m heptanone as an external standard). The tubes were then placed in a vortex shaker for 15 min at maximum speed (MS1 minishaker; IKA). The upper (hexane) layer was then carefully transferred with a glass pipette to a deactivated glass insert (Agilent) for analysis with an Agilent 7890 series GC/MS and GC with flame ionization detection. The system also consisted of an Agilent 597 series MSD standalone capillary detector together with a CTC PAL autosampler (G6500 Combi PAL; CTC Analytics AG). The compounds were separated on a J&W DB-WAX column (30 m \times 0.25 mm \times 0.25 μ m; Agilent Technologies) with He (2 mL/min) as the carrier gas. The oven temperature was initially set at 50 °C for 2 min, then subsequently increased over a gradient of 10 °C to 250 °C, and held for 5 min. The injector and detector temperatures were set at 250 °C with a 15:1 split ratio. Quantities of FAMES were calculated using response factors as per standard protocol. All assays were conducted in duplicate or triplicate. Apparent k_{cat}/K_M values were estimated using Eq. 1:

$$k_{cat}/K_M^{app} = v_o / ([E]/[S]). \quad [1]$$

OP and carboxylester assays. Purified enzymes were incubated with substrates (diethyl 4-methylumbelliferyl phosphate and 4-nitrophenyl acetate) in 100 mM Hepes (pH 8.0) and 100 mM NaCl. The rate at which the substrates were hydrolyzed was determined by monitoring product formation using a molecular devices 96-well plate reader. For 4-nitrophenyl acetate, the assay was followed at 405 nm, and product concentration was determined using a molar extinction coefficient (ϵ = 14,800 M⁻¹ cm⁻¹). For diethyl 4-methylumbelliferyl

phosphate, the product fluorescence was followed at $\lambda_{\text{exc}} = 360$ nm and $\lambda_{\text{em}} = 455$ nm, and product concentration was determined through calculation of a standard curve of 4-methylumbelliferone in the assay buffer. The k_{cat} and K_M values were determined by fitting the initial velocity data to the Michaelis–Menten equation. Assays were conducted in duplicate.

Crystallization, Structure Determination, Substrate Soaking, and Docking. Crystals of L α E74 were grown using the hanging-drop vapor diffusion method, with reservoir solutions of 100 mM Mes (pH 6.5) and 20% PEG 2K MME or 100 mM sodium-acetate (pH 4.6) and 20% PEG 2K MME. The concentration of PEG 2K MME was increased to 35% for use as a cryoprotectant during flash cooling to 100 K of the crystals under a stream of nitrogen gas. Diffraction data were collected at beamlines ID 14–4 and 23–2 of the European Synchrotron Radiation Facility with wavelengths of 0.9393 and 0.8266 Å. Diffraction data were indexed, integrated, and scaled using the XDS package (42). Data collection statistics are shown in Table 1. Phases were obtained using molecular replacement with the program *MOLREP* and structure of mouse AChE (13). Iterative model building was performed using *COOT* (43) and

BUCCANEER (44). Refinement (including twin refinement) was undertaken using *REFMAC* (45), which was implemented in the *CCP4* suite of programs (46).

Crystals L α E7-4 from pH 4.6 conditions were soaked with 1 mM diethyl 4-methylumbelliferyl phosphate for time periods between 1 h and 3 d before data collection. The presence of the ligand was confirmed through investigation of omit electron density maps. Input structures for docking were generated with the AutoDock Tools package (47), and rigid docking was performed using Autodock Vina (48). The searching space was centered on the catalytic serine, and a search box of $36 \times 52 \times 48$ Å was used to encompass the entire substrate binding cavity. All protein structure images were produced using PyMol (25). Protein structure diagrams were produced using *TOPDRAW* (49).

ACKNOWLEDGMENTS. This work was supported by Defense Threat Reduction Agency Contract HDTRA1-11-C-0047. C.J.J. was supported by a Marie Curie International Incoming Fellowship.

- Grube A, Donaldson D, Kiely T, Wu L (2011) *Pesticide Industry Sales and Usage* (United States Environmental Protection Agency, Washington, DC).
- Whalon ME, Mota-Sanchez D, Hollingworth RM, eds (2008) *Global Pesticide Resistance in Arthropods* (CAB International, Oxfordshire, United Kingdom).
- Jackson CJ, Oakeshott JG, Sanchez-Hernandez J, Wheelock C (2010) Carboxylesterases in the metabolism and toxicity of pesticides. *Acetylcholinesterase Pesticides: Metabolism, Neurotoxicity and Epidemiology*, eds Satoh T, Gupta RC (Wiley, New York), pp 57–77.
- Newcomb RD, et al. (1997) A single amino acid substitution converts a carboxylesterase to an organophosphorus hydrolase and confers insecticide resistance on a blowfly. *Proc Natl Acad Sci USA* 94(14):7464–7468.
- Marrs TC (1993) Organophosphate poisoning. *Pharmacol Ther* 58(1):51–66.
- Birner-Gruenberger R, et al. (2012) Functional fat body proteomics and gene targeting reveal in vivo functions of *Drosophila melanogaster* α -Esterase-7. *Insect Biochem Mol Biol* 42(3):220–229.
- Campbell PM, Newcomb RD, Russell RJ, Oakeshott JG (1998) Two different amino acid substitutions in the α -esterase, E3, confer alternative types of organophosphorus insecticide resistance in the sheep blowfly, *Lucilia cuprina*. *Insect Biochem Mol Biol* 28(3):139–150.
- Campbell PM, et al. (2003) Developmental expression and gene/enzyme identifications in the alpha esterase gene cluster of *Drosophila melanogaster*. *Insect Mol Biol* 12(5):459–471.
- Healy MJ, Dumancic MM, Oakeshott JG (1991) Biochemical and physiological studies of soluble esterases from *Drosophila melanogaster*. *Biochem Genet* 29(7–8):365–388.
- Tweedie S, et al. (2009) FlyBase: Enhancing *Drosophila* Gene Ontology annotations. *Nucleic Acids Res* 37(Database issue):D555–D559.
- Beller M, et al. (2006) Characterization of the *Drosophila* lipid droplet subproteome. *Mol Cell Proteomics* 5(6):1082–1094.
- Ranson H, et al. (2002) Evolution of supergene families associated with insecticide resistance. *Science* 298(5591):179–181.
- Bourne Y, Taylor P, Marchot P (1995) Acetylcholinesterase inhibition by fasciculin: Crystal structure of the complex. *Cell* 83(3):503–512.
- Carr PD, Ollis DL (2009) ω 1 hydrolase fold: An update. *Protein Pept Lett* 16(10):1137–1148.
- Krisinell E, Henrick K (2007) Inference of macromolecular assemblies from crystalline state. *J Mol Biol* 372(3):774–797.
- Ishikawa K, Nakamura H, Morikawa K, Kanaya S (1993) Stabilization of *Escherichia coli* ribonuclease HI by cavity-filling mutations within a hydrophobic core. *Biochemistry* 32(24):6171–6178.
- Spackman ME, Oakeshott JG, Smyth KA, Medveczky KM, Russell RJ (1994) A cluster of esterase genes on chromosome 3R of *Drosophila melanogaster* includes homologues of esterase genes conferring insecticide resistance in *Lucilia cuprina*. *Biochem Genet* 32(1–2):39–62.
- Pierleoni A, et al. (2011) MemPype: A pipeline for the annotation of eukaryotic membrane proteins. *Nucleic Acids Res* 39(Web Server issue):W375–W380.
- Margraf T, Schenk G, Torda AE (2009) The SALAMI protein structure search server. *Nucleic Acids Res* 37(Web Server issue):W480–W484.
- Wogulis M, et al. (2006) Structural studies of a potent insect maturation inhibitor bound to the juvenile hormone esterase of *Manduca sexta*. *Biochemistry* 45(13):4045–4057.
- Sussman JL, et al. (1991) Atomic structure of acetylcholinesterase from *Torpedo californica*: A prototypic acetylcholine-binding protein. *Science* 253(5022):872–879.
- Lenfant N, et al. (2013) ESTHER, the database of the α/β -hydrolase fold superfamily of proteins: Tools to explore diversity of functions. *Nucleic Acids Res* 41(Database issue):D423–D429.
- Velan B, et al. (1996) Structural modifications of the omega loop in human acetylcholinesterase. *FEBS Lett* 395(1):22–28.
- Gilbert LI, Chino H (1974) Transport of lipids in insects. *J Lipid Res* 15(5):439–456.
- Bar-Even A, et al. (2011) The moderately efficient enzyme: Evolutionary and physicochemical trends shaping enzyme parameters. *Biochemistry* 50(21):4402–4410.
- Nachon F, Asojo OA, Borgstahl GE, Masson P, Lockridge O (2005) Role of water in aging of human butyrylcholinesterase inhibited by echothiophate: The crystal structure suggests two alternative mechanisms of aging. *Biochemistry* 44(4):1154–1162.
- Heidari R, et al. (2004) Hydrolysis of organophosphorus insecticides by in vitro modified carboxylesterase E3 from *Lucilia cuprina*. *Insect Biochem Mol Biol* 34(4):353–363.
- Fleming CD, et al. (2007) Crystal structures of human carboxylesterase 1 in covalent complexes with the chemical warfare agents soman and tabun. *Biochemistry* 46(17):5063–5071.
- Maxwell DM, Brecht KM (2001) Carboxylesterase: Specificity and spontaneous re-activation of an endogenous scavenger for organophosphorus compounds. *J Appl Toxicol* 21(1 Suppl 1):S103–S107.
- Shafferman A, et al. (1996) Aging of phosphorylated human acetylcholinesterase: Catalytic processes mediated by aromatic and polar residues of the active centre. *Biochem J* 318(Pt 3):833–840.
- Carletti E, et al. (2008) Aging of cholinesterases phosphorylated by tabun proceeds through O-dealkylation. *J Am Chem Soc* 130(47):16011–16020.
- Millard CB, et al. (1999) Crystal structures of aged phosphorylated acetylcholinesterase: Nerve agent reaction products at the atomic level. *Biochemistry* 38(22):7032–7039.
- Dougherty DA (1996) Cation- π interactions in chemistry and biology: A new view of benzene, Phe, Tyr, and Trp. *Science* 271(5246):163–168.
- Chen 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 Mol Biol* 31(8):805–816.
- McKenzie J, O'Farrell K (1993) Modification of developmental instability and fitness: Malathion-resistance in the Australian sheep blowfly *Lucilia cuprina*. *Genetica* 89(3):67–76.
- Parker AG, Campbell PM, Spackman ME, Russell RJ, Oakeshott JG (1996) Comparison of an esterase associated with organophosphate resistance in *Lucilia cuprina* with an orthologue not associated with resistance in *Drosophila melanogaster*. *Pestic Biochem Physiol* 55(2):85–99.
- Parker AG, Russell RJ, Delves AC, Oakeshott JG (1991) Biochemistry and physiology of esterases in organophosphate-susceptible and -resistant strains of the Australian sheep blowfly, *Lucilia cuprina*. *Pestic Biochem Physiol* 41(3):305–318.
- Goldsmith M, et al. (2012) Evolved stereoselective hydrolases for broad-spectrum G-type nerve agent detoxification. *Chem Biol* 19(4):456–466.
- Oppenoorth FJ, van Asperen (1960) Allelic genes in the housefly producing modified enzymes that cause organophosphate resistance. *Science* 132(3422):298–299.
- Neylon C, et al. (2000) Interaction of the *Escherichia coli* replication terminator protein (Tus) with DNA: A model derived from DNA-binding studies of mutant proteins by surface plasmon resonance. *Biochemistry* 39(39):11989–11999.
- Gasteiger E, et al. (2005) Protein identification and analysis tools on the ExPASy Server. *The Proteomics Protocols Handbook*, ed Walker JM (Humana Press, Clifton, NJ), pp 571–607.
- Kabsch W (2010) XDS. *Acta Crystallogr D Biol Crystallogr* 66(Pt 2):125–132.
- Emsley P, Lohkamp B, Scott WG, Cowtan K (2010) Features and development of Coot. *Acta Crystallogr D Biol Crystallogr* 66(Pt 4):486–501.
- Cowtan K (2006) The Buccaneer software for automated model building. 1. Tracing protein chains. *Acta Crystallogr D Biol Crystallogr* 62(Pt 9):1002–1011.
- Murshudov GN, et al. (2011) REFMACS for the refinement of macromolecular crystal structures. *Acta Crystallogr D Biol Crystallogr* 67(Pt 4):355–367.
- Collaborative Computational Project, Number 4 (1994) The CCP4 suite: Programs for protein crystallography. *Acta Crystallogr D Biol Crystallogr* 50(Pt 5):760–763.
- Morris GM, et al. (2009) AutoDock4 and AutoDockTools4: Automated docking with selective receptor flexibility. *J Comput Chem* 30(16):2785–2791.
- Trott O, Olson AJ (2010) AutoDock Vina: Improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. *J Comput Chem* 31(2):455–461.
- Bond CS (2003) TopDraw: A sketchpad for protein structure topology cartoons. *Bioinformatics* 19(2):311–312.
- Colletier JP, et al. (2006) Structural insights into substrate traffic and inhibition in acetylcholinesterase. *EMBO J* 25(12):2746–2756.
- Gautier R, Douguet D, Antony B, Drin G (2008) HELIQUEST: A web server to screen sequences with specific alpha-helical properties. *Bioinformatics* 24(18):2101–2102.