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The molecular basis for the neofunctionalization of the juvenile hormone esterase duplication in Drosophila

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ABSTRACT

The *Drosophila melanogaster* enzymes juvenile hormone esterase (DmJHE) and its duplicate, DmJHEdup, present ideal examples for studying the structural changes involved in the neofunctionalization of enzyme duplicates. DmJHE is a hormone esterase with precise regulation and highly specific activity for its substrate, juvenile hormone. DmJHEdup is an odorant degrading esterase (ODE) responsible for processing various kairomones in antennae. Our phylogenetic analysis shows that the JHE lineage predates the hemi/holometabolan split and that several duplications of JHEs have been templates for the evolution of secreted β-esterases such as ODEs through the course of insect evolution. Our biochemical comparisons further show that DmJHE has sufficient substrate promiscuity and activity against odorant esters for a duplicate to evolve a general ODE function against a range of mid-long chain food esters, as is shown in DmJHEdup. This substrate range complements that of the only other general ODE known in this species, Esterase 6. Homology models of DmJHE and DmJHEdup enabled comparisons between each enzyme and the known structures of a lepidopteran JHE and Esterase 6. Both JHEs showed very similar active sites despite low sequence identity (30%). Both ODEs differed drastically from the JHEs and each other, explaining their complementary substrate ranges. A small number of amino acid changes are identified that may have been involved in the early stages of the neofunctionalization of DmJHEdup. Our results provide key insights into the process of neofunctionalization and the structural changes that can be involved.

1. Introduction

Adaptation and the development of new physiological functions are driven by a combination of structural changes to proteins and changes in gene regulatory sequences (Carroll, 2005). There has been much discussion about how these two factors interact to effect the neofunctionalization of enzyme duplicates, particularly in higher eukaryotes, where tissue and temporal expression can be so varied (Carroll, 2005; Hoekstra and Coyne, 2007; Juneja et al., 2016; Tangwancharoen et al., 2018). The evolution of insect hormone and semiochemical processing enzymes provides an ideal system to study this interaction due to their reliance on both precise regulation and specific *in vivo* biochemistry (Oakeshott et al., 2005). Earlier work identified a group of four related clades of insect carboxylesterases (CBEs) that generally have roles in the processing of the sesquiterpenoid ester, juvenile hormone (JH), which plays a vital role in preventing precocious metamorphosis, or various pheromone or food esters received by sensory tissues, which trigger various behavioral changes (Oakeshott et al., 2010; Sadd et al., 2015). The temporal and tissue specificity required of the JH esterases (JHEs) is very different from that required of the odorant degrading esterases (ODEs) and their respective physiological roles impose very distinct requirements on their substrate specificities and kinetics (Oakeshott et al., 2005).

JHEs have been identified in more than twenty species of insect from at least six insect orders (El-Sheikh, 2015; Elayidam and Muraleedharen, 2008; Kamita et al., 2011; Kamita and Hammock, 2010; Kontogiannatos et al., 2013, 2011; Valaitis, 1991; Zhu et al., 2017), with the best characterized enzymes, from Lepidoptera and Diptera, falling into two different clades; clade G for the lepidopteran JHEs and F for the dipteran JHEs. These JHEs have been defined biochemically, in terms of their activity, by demonstrating a high k_{cat}/K_M against JH, predominantly due to a low apparent K_M, in the presence

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Abbreviations: CBE, Carboxylesterase; Dm, *Drosophila melanogaster*; Ms, *Manduca sexta*; JHE, Juvenile hormone esterase; ODE, Odorant degrading esterase; EST6, Esterase 6

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and absence of a carrier protein (Hammock, 1985). Notwithstanding their distribution across different phylogenetic clades, a range of active site residues are highly conserved amongst the JHEs, including $R_{72}F$, $D_{190}Q$, $GxxH_{476}xxD/E$, E_{351}, T_{309} and in particular $GQS_{218}AG$ which defines the residues around the catalytic serine (Note: Residues are labelled by their location in the amino acid sequence of *Drosophila melanogaster* JHE) (Kamita and Hammock, 2010; Tsubota et al., 2010b). Intriguingly, the *D. melanogaster* genome project discovered a closely related duplicate of its JHE gene (*DmJHE*) immediately upstream of it (Campbell et al., 2001). Subsequent physiological and behavioral work showed the product of the duplicate gene (DmJHEdup) functioned not as another JHE but as an antennal ODE active against certain food acetates, including isoamyl acetate, ethyl butyrate and ethyl propionate (Steiner et al., 2017). DmJHEdup does not possess the GQSAG motif and has been shown *in vitro* to have negligible activity with JHIII (Crone et al., 2007), however, the wrong start codon for DmJHEdup was used in the latter study, producing an unstable enzyme and compromising this result (Crone et al., 2007). Phylogenetic analysis of dipteran JHEs and JHEdups suggests the JHEdup duplication occurred early in the Brachycera (true fly) suborder, ie after the higher Diptera (flies) diverged from the lower Diptera (mosquitoes) (Steiner et al., 2017).

The only other well characterized *D. melanogaster* CBE in the four clades in question is Esterase 6 (DmEST6), which also acts as an antennal ODE (Younus et al., 2017, 2014), albeit it may also have other functions (Chertemps et al., 2012). DmEST6 lies in clade E, secreted β-esterases, which contains secreted CBEs from a range of insect orders that are involved in degrading hormones, pheromones and odorants (Oakeshott et al., 2010, 2005). Comprehensive biochemical analysis of DmEST6 shows it has relatively high activity against short-mid chain food esters and a crystal structure of the enzyme has provided key insights into the biophysical basis of its ODE function (Younus et al., 2017).

Here we present a revamped phylogenetic analysis of the JHE- and ODE-containing clades of insect CBEs, a detailed biochemical comparison of the activities of DmJHE and DmJHEdup with a range of esters, and a comparison of homology modelled structures for DmJHE and DmJHEdup and the known structures of a lepidopteran JHE and DmEST6. These analyses allow us to infer the key biochemical and structural differences among the enzymes and the sequence differences underpinning their functional divergence. Combining these results with recently published transcriptomic data for the enzymes allows us to infer key structural and regulatory changes associated with the neofunctionalization of DmJHEdup (Steiner et al., 2017; Younus et al., 2014).

2. Materials and methods

2.1. Phylogenetic analysis of DmJHE and DmJHEdup

A phylogenetic tree was constructed using a total of 47 sequences obtained from official gene sets of the respective species on NCBI, two of which (AmelCCEFYa, AmelCCEFXa) were manually curated from GB11403 and GB18660 (BeeBase), respectively. *D. melanogaster* acetylcholinesterase was used as an outgroup. Sequences include: all identified hormone/semiochemical CBEs from *Helicoverpa armigera, Bombyx mori* and *D. melanogaster*; sequences homologous to DmJHE and DmJHEdup in other insect orders; identified insect JHEs; and a selection of sequences that defined the clades D, E, F and G in previous phylogenies (Oakeshott et al., 2010, 2005). The protein alignments were carried out using the MAFFT program (Katoh et al., 2005) ("–localpair") and trimmed with trimAL (Capella-Gutierrez et al., 2009) to remove gaps. The phylogeny was constructed with IQ-TREE (Nguyen et al., 2015) using the "LG + R5" model identified to be the best-fit model by ModelFinder (Kalyaanamoorthy et al., 2017). The phylogenies were then plotted using the iTOL portal (Letunic and Bork, 2016).

2.2. Activity assays

The expression of DmJHE and DmJHEdup (FlyBase Release 6 version) using the baculovirus system has been previously described (Younus et al., 2014). Enzyme concentration was determined through titration using varying concentrations of the inhibitor, Dibrom (Sigma-Aldrich) and by incubating at room temperature for 20 min before using α-napthyl acetate (Sigma-Aldrich) to determine the remaining activity. All assays were performed at room temperature.

2.2.1. Artificial esters

Michaelis-Menten kinetics were determined for both DmJHE and DmJHEdup with 4-nitrophenyl esters with saturated acyl chain lengths varying from 2 to 12 carbon atoms long (Sigma-Aldrich) as well as with α-naphthyl acetate. Assays were performed in triplicate with varying substrate concentrations (0–2 mM) in 25 mM Tris-HCl buffer (pH 7.5) with ethanol (5% v/v). Enzyme concentrations varied from 10 to 25 nM. The formation of 4-nitrophenol was measured at 405 nm and the concentration was determined using a molar extinction coefficient (ε) of 18400 $M^{-1}\,cm^{-1}$. The formation of α-naphthol was measured at 325 nm and the concentration was determined using a ε of 1114 $M^{-1}\,cm^{-1}$. Michaelis-Menten kinetics were calculated from the initial velocity data using nonlinear regression.

2.2.2. Odorant and hormone esters

The activities of both DmJHE and DmJHEdup were determined against a range of odorant and hormone esters (Sigma-Aldrich) using gas chromatography-mass spectrometry (GC-MS) assays and GC with flame ionization detection (GC-FID) assays modified from Younus et al. (2014). Briefly, substrate concentration was kept at 200 μM in 25 mM Tris-HCl buffer (pH 7.5) with ethanol (5% v/v). Enzyme concentrations were varied from 0.1 to 90 nM. Reactions were stopped at specific time points by the addition of ice-cold hexane (containing 200 μM heptanone as an external standard) and vortexed for 10 min. The hexane layer was extracted and substrate loss was determined using GC-MS and GC-FID.

2.2.3. Comparative chiral activity assay

Qualitative JHE preference for Juvenile hormone III stereoisomer was determined through a high-performance liquid chromatography (HPLC) chiral separation technique modified from Ichikawa et al. (2007). Briefly, activity assays were performed as described above. Extracted samples were separated using a chiralpak IA column (DAICEL Co., Ltd) under normal-phase conditions; mobile phase: hexane/EtOH = 99.5/0.5; flow rate 0.5 ml/min; detection: UV 230 nm. Stereoisomers were identified through HPLC profile comparison with Ichikawa et al. (2007).

2.2.4. Inhibition assays

The inhibition constant, K_i, of DmJHE and DmJHEdup towards the odorant and hormone esters was determined using a competitive assay modified from Younus et al. (2014) and based on the concept that competitive substrates can be treated as inhibitors of each other (Cornish-Bowden, 1995; Younus et al., 2014). Briefly, the full kinetics towards the substrate α-naphthyl acetate were determined for both DmJHE and DmJHEdup in 25 mM Tris-HCl buffer (pH 7.5) with ethanol (5% v/v). Then, competitive assays were performed between each of the odorant and hormone esters at a range of concentrations (0–32 mM) against a single concentration of α-napthyl acetate (500 μM). K_i was determined using the following formula:

$$K_i = \frac{\frac{[I]}{(1-\alpha)} - [I]}{1 + \frac{[S]}{K_M}}$$

Where $\alpha = v_i/v_0$ = relative activity; v_i = the initial velocity at a given substrate concentration, [S], and in the presence of inhibitor at a certain concentration, [I]; and v_0 = the initial velocity at the same [S] in the absence of inhibitor (Cornish-Bowden, 1995; Segel, 1993).

2.3. Homology modelling and docking

The structural modelling of both DmJHE and DmJHEdup was performed using the ROBETTA server (Kim et al., 2004). These were compared with other homology models using the QMEAN server and found to be of the highest quality (Benkert et al., 2008). These structures were compared with structurally determined enzymes with similar functions using PyMol, which was also used to make all protein structure images (Schrodinger LLC, 2010). To determine the potential acyl-enzyme complexes of DmJHEdup and DmJHE formed with JHIII, automated covalent docking using flexible residues was performed using AutoDock4 as described by Bianco et al. (2016) and Morris et al. (2009). To determine the potential tetrahedral intermediates formed between a selection of odorant esters and DmJHE and DmJHEdup, covalent docking was conducted with CovDock (Zhu et al., 2014).

3. Results

3.1. Proposed revision of the phylogeny of insect JHEs

A phylogeny was constructed using 47 functionally annotated sequences of insect CBEs that preliminary analyses indicated would sit in one of the four clades originally identified as containing hormone and semiochemical processing enzymes (Fig. 1). Those four original clades were: clade D, integument esterases; clade E, secreted β-esterases; clade F, dipteran-type JHEs; and clade G, lepidopteran-type JHEs (Oakeshott et al., 2010, 2005). The 47 sequences were drawn from seven insect orders, namely the Diptera, Coleoptera, Lepidoptera, Hemiptera, Hymenoptera, Orthoptera and Blattodea. Each major branch in our new phylogeny is supported by a bootstrap value of at least 60, with the majority of branches supported by bootstrap values above 98. Our phylogeny breaks the CBEs analyzed into seven distinct groups. The integrity of clades G and D are retained and clade G is confirmed as the ancestral clade. However, clades E and F containing DmJHE, DmJHEdup and DmEST6 are now re-arranged and partitioned into five subclades: E − 1; E − 2; F-1; F-2; and F-3.

There are now four robust groups of sequences that include JHEs: the first, clade G, contains lepidopteran JHEs and β-esterases; the second, clade F-1, contains solely coleopteran JHEs; the third, clade F-2, contains DmJHE and DmJHEdup, other dipteran and orthopteran JHEs and other dipteran β-esterases; and the fourth, clade F-3, contains hemipteran, hymenopteran and blattodean JHEs along with other dipteran, lepidopteran and hymenopteran β-esterases. The branching of these groups and their divergence order is not related to the splits of the organismal orders, with two groups (F-2 and F-3) containing JHEs from both hemi- and holometabolous insects (Misof et al., 2014). This establishes that the JHE lineage predates the hemi/holometabolan split and has given rise to many different subclades with JHE functions (Misof et al., 2014).

Although the integrity of clade D, integument esterases, is retained, its evolutionary relationship with other clades is altered in this phylogeny (Oakeshott et al., 2010, 2005). Before clade D was thought to exist as a monophyletic clade distinct from clades E and F, whereas this phylogeny suggests that it evolved within the JHE lineage.

While the previous phylogenetic studies suggested clade E is the only secreted β-esterase clade, our phylogenetic analysis suggests a more complex situation. There are two groups, E − 1 and E − 2, that contain solely secreted β-esterases and three groups, G, F-2 and F-3, with both β-esterases and JHEs. While DmJHEdup and DmEST6 share a common function as ODEs in *D. melanogaster,* they occur in distinct groups (F-2 and F-3, respectively), both of which contain insect JHEs. This demonstrates that JHEs and JHE-like CBEs can act as templates for the development of enzymes with secreted β-esterase functions over evolutionary time.

3.2. Kinetic comparison between DmJHEdup and DmJHE

We assayed DmJHEdup and DmJHE against a range of artificial 4-nitrophenyl esters with varying acyl chain lengths at a range of substrate concentrations, allowing the determination of full Michaelis-Menten kinetics (Table 1). DmJHEdup has an optimal k_{cat} at an acyl chain length of six, where it is $>$ 15-fold higher than that of DmJHE. DmJHE has an optimal k_{cat} value with an acyl chain length of only two, albeit DmJHEdup still has similar activity with that substrate. Both enzymes have their lowest K_M values, in the low micromolar to high nanomolar range, somewhat lower for DmJHEdup than DmJHE, with longer (8 or 10) acyl moiety substrates. Specificity constants are also highest for both enzymes on these substrates, those for DmJHEdup nearly ten-fold higher than those for DmJHE.

Assays were also carried out against a racemic mixture of JHIII (the major form of JH for most insects, including *Drosophila* (Kamita et al., 2003; Kamita and Hammock, 2010; Noriega, 2014)) and some other natural bioactive esters, mainly food odorants to which *D. melanogaster* is known to respond (Younus et al., 2017). These assays were restricted by substrate solubility, so activities were determined at a set substrate concentration (200 μM) (Table 2). To determine the Michaelis-Menten constant, K_M, we utilized the fact that in a reaction including two CBE substrates, each can be treated as a competitive inhibitor of the other and that the determined K_i for a substrate is equivalent to its K_M (Cornish-Bowden, 1995; Younus et al., 2014). Thus, while we were unable to determine Michaelis-Menten kinetics directly, we were able to determine the K_i with JHIII and the other bioactive esters by utilizing a competitive inhibition assay between each substrate and the fluorometric, artificial substrate, α-naphthyl acetate (Table 2).

Consistent with its known physiological function, DmJHE has significant activity and a low micromolar K_i with JHIII (Table 2, Fig. 2). By contrast, DmJHEdup has no measurable activity or K_i with this substrate, confirming that it has no role as a JHE physiologically. Chiral separation of the reaction between DmJHE and racemic JHIII confirmed that DmJHE preferentially reacts with the biologically active (10R)-2E JHIII enantiomer (Fig. S1.) (Campbell et al., 1998; Crone et al., 2007). Notably, DmJHE shows less or no activity with the JH precursor, methyl farnesoate, and the JH analog, methoprene, although it still has K_i values in the micromolar range with both these substrates (Table 2, Fig. 2). The K_i result with methoprene is noteworthy in light of its known role as an insect growth regulator insecticide with an antagonist mode of action (Barry et al., 2008; Wilson and Fabian, 1986; Yin et al., 1987). DmJHEdup has no measurable activity with either methyl farnesoate or methoprene and K_i values in the high micromolar range with both, again consistent with it having a completely different physiological function from DmJHE (Table 2, Fig. 2).

The activities of both enzymes with the other bioactive esters confirm their respective structural substrate preferences. Of these esters, DmJHE shows the highest activity with methyl decanoate, an ester of a similar length and configuration as JHIII, and measurable activity with some other esters with shorter acyl chains (Table 2, Fig. 2). By contrast, DmJHEdup shows minimal activity with methyl decanoate, relatively low activity towards esters with shorter acyl and alcohol groups such as ethyl butyrate and propyl propionate, but high activity towards esters with mid-long length acyl and alcohol groups such as pentyl hexanoate and octyl butyrate (Table 2). Again, consistent with the 4-nitrophenol ester results, both enzymes give high micromolar/low millimolar K_M values with most of the esters with either short or mid-length acyl and alcohol groups. Neither enzyme displayed any activity against the important sex pheromone, *cis*-vaccenyl acetate (Table 2, Fig. 2) (Chertemps et al., 2012; Younus et al., 2017).

3.3. Structural comparison between DmJHEdup, DmJHE and other JHEs

Homology models were generated for both DmJHEdup and DmJHE using the ROBETTA server, which automatically selected the structure

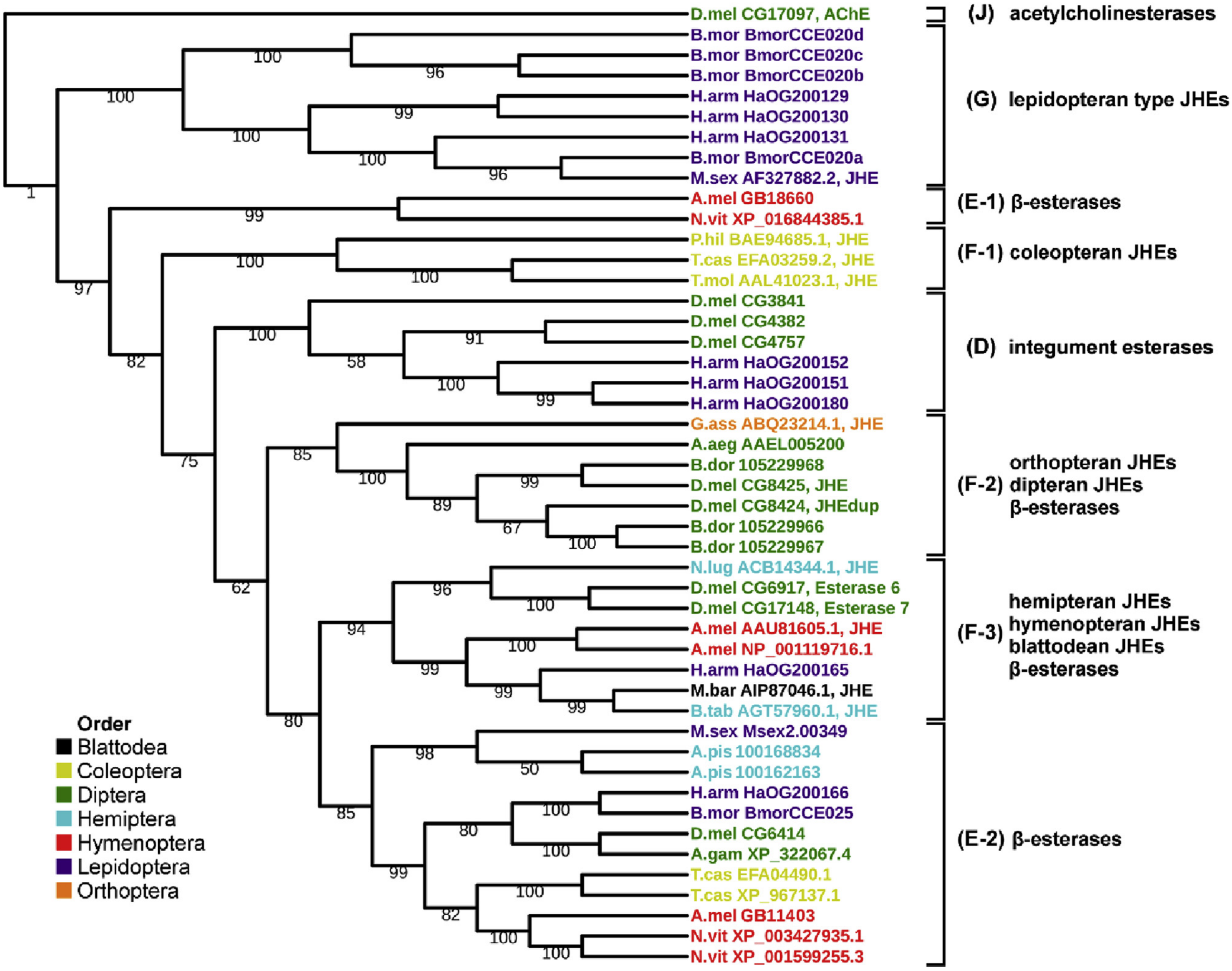

Fig. 1. A phylogenetic tree of the evolutionary relationship between insect JHEs and other hormone/semiochemical processing CBEs. *D. melanogaster* acetylcholinesterase was used as an outgroup. Sequences include all identified hormone/semiochemical CBEs from *Helicoverpa armigera, Bombyx mori* and *D. melanogaster*, homologous sequences to DmJHE and DmJHEdup in other insect orders, identified insect JHEs and CBEs and a selection of sequences that defined the clades D, E, F and G in previous phylogenies (Oakeshott et al., 2010, 2005). Sequences are first labelled by a shorthand for species name: D.mel, *D. melanogaster*; B.mor, *B. mori*; H.arm, *H. armigera*; M.sex, *Manduca sexta*; A.mel, *Apis mellifera*; N.vit, *Nasonia vitripennis*; P.hil, *Psacothea hilaris*; T.cas, *Tribolium castaneum*; T.mol, *Tenebrio molitor*; G.ass, *Gryllus assimilis*; A.aeg, *Aedes aegypti*; B.dor, *Bactrocera dorsalis*; N.lug, *Nilaparvata lugens*; M.bar, *Macrotermes barneyi*; B.tab, *Bemisia tabaci*; A.pis, *Acyrthosiphon pisum*; and A.gam, *Anopheles gambiae*. Sequences are then labelled either by the appropriate genome annotation number, by genbank/NCBI gene ID, or NCBI reference number, followed by generic name, if appropriate. Sequences are colored based on insect order: Diptera, green; Lepidoptera, purple; Hymenoptera, red; Coleoptera, yellow; Orthoptera, orange; Hemiptera, blue; and Blattodea, black. Sequences are grouped based on suggested phylogenetic clades shown in brackets. Percentage bootstrap values are shown prior to each node. Phylogenetic groups are labelled.

Table 1

Kinetic parameters of DmJHEdup and DmJHE with 4-nitrophenyl esters with varying acyl chain length.

4-nitrophenyl acyl chain length	k_{cat} (min^{-1})		KM (μM)		k_{cat}/K_M (M^{-1}.s^{-1})	
	DmJHEdup	DmJHE	DmJHEdup	DmJHE	DmJHEdup	DmJHE
2	834 ± 38	816 ± 59	$2.14 \times 10^2 \pm 0.14 \times 10^2$	$2.22 \times 10^3 \pm 0.20 \times 10^3$	$6.50 \times 10^4 \pm 0.14 \times 10^4$	$6.13 \times 10^3 \pm 0.09 \times 10^3$
4	1190 ± 20	123 ± 0.4	$1.36 \times 10^1 \pm 0.03 \times 10^1$	$1.84 \times 10^2 \pm 0.01 \times 10^2$	$1.46 \times 10^6 \pm 0.02 \times 10^6$	$1.12 \times 10^4 \pm 0.01 \times 10^4$
6	1540 ± 16	90.4 ± 3.0	$8.10 \times 10^0 \pm 0.02 \times 10^0$	$3.87 \times 10^1 \pm 0.36 \times 10^1$	$3.16 \times 10^6 \pm 0.03 \times 10^6$	$3.91 \times 10^4 \pm 0.25 \times 10^4$
8	554 ± 11	182 ± 2	$1.38 \times 10^0 \pm 0.08 \times 10^0$	$3.03 \times 10^0 \pm 0.11 \times 10^0$	$6.73 \times 10^6 \pm 0.50 \times 10^6$	$9.98 \times 10^5 \pm 0.27 \times 10^5$
10	268 ± 2	345 ± 7	$5.40 \times 10^{-1} \pm 0.92 \times 10^{-1}$	$6.55 \times 10^0 \pm 0.40 \times 10^0$	$8.41 \times 10^6 \pm 1.36 \times 10^6$	$8.79 \times 10^5 \pm 0.42 \times 10^5$
12	79.1 ± 0.5	101 ± 2	$1.44 \times 10^0 \pm 0.13 \times 10^0$	$7.43 \times 10^0 \pm 1.12 \times 10^0$	$9.20 \times 10^5 \pm 0.89 \times 10^5$	$2.29 \times 10^5 \pm 0.30 \times 10^5$

of α-Esterase 7 from *Lucilia cuprina* as the template (PDB: 5CH3 and PDB: 5TYM, respectively) (Correy et al., 2016; Kim et al., 2004). The quality of each model was assessed using the QMEAN server (Benkert et al., 2008). The QMEAN6 Z-scores of DmJHEdup and DmJHE were −1.80 and −1.29, respectively, indicating models of sufficient quality to interpret the kinetic and phylogenetic results in a structural sense and draw comparisons with known insect CBE structures such as MsJHE and DmEST6 (Wogulis et al., 2006; Younus et al., 2017).

Both the modelled structure of DmJHE and the crystal structure of MsJHE (Wogulis et al., 2006) have binding pockets in the form of long, narrow tunnels in the same orientation (Fig. 3A). Covalent docking simulations (Fig. 3B, D) show both also utilize similar residues to

Table 2
The specific activities and inhibition constants determined for DmJHE and DmJHEdup against a range of food odorant, green plant odorant, pheromonal and hormonal esters. Substrates have been grouped based on both source and alcohol and acyl chain length (C_x-C_y, C_a-C_b, respectively).

Source	Substrates	Specific activity (min^{-1})		K_i (μM)	
		JHEdup	DmJHE	JHEdup	DmJHE
Food odorant (C_1-C_3, C_1-C_4)	Ethyl butyrate	8.27 ± 4.29	4.92 ± 0.30	62600 ± 5600	11300 ± 200
	Propyl propionate	162 ± 44	310 ± 34	395000 ± 8000	18300 ± 800
Food odorant (C_5-C_8, C_4-C_6)	Pentyl hexanoate	2050 ± 690	0	588 ± 78	36.6 ± 3.5
	Octyl butyrate	2540 ± 490	186 ± 18	257 ± 15	330 ± 27
Food odorant (C_5-C_{10}, C_1-C_2)	Pentyl formate	240 ± 55	13.6 ± 3.6	20600 ± 2400	9470 ± 360
	Decyl acetate	316 ± 66	0	272 ± 52	55.1 ± 10.0
Plant odorant (C_1-C_4, C_{10}-C_{12})	Methyl decanoate	42.0 ± 12.9	492 ± 16	1990 ± 360	46.1 ± 9.1
	Methyl myristate	0	12.2 ± 1.2	0	13.9 ± 0.6
Food odorant (aromatic ester)	Phenethyl acetate	107 ± 35	38.9 ± 24.4	3370 ± 320	10400 ± 1200
Insecticide	Methoprene	0	0	226 ± 78	203 ± 69
Hormone	Methyl farnesoate	0	6.64 ± 2.70	93.2 ± 12.9	5.88 ± 0.20
	JHIII (racemate)	0	87.7 ± 4.5	0	3.35 ± 0.09
Pheromone	*cis*-Vaccenyl acetate	0	0	0	0

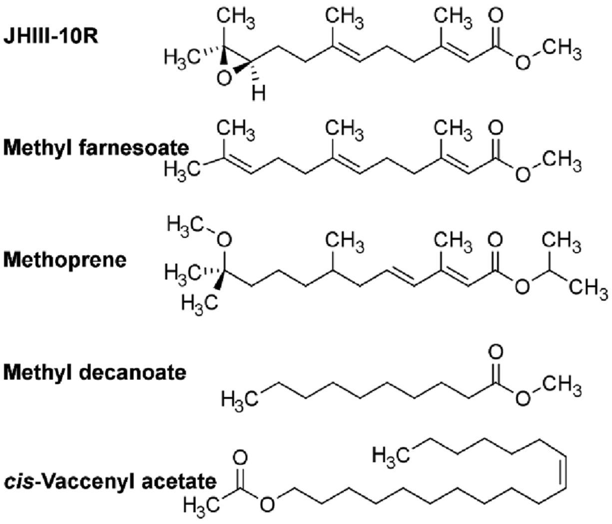

Fig. 2. The chemical structures of a number of key substrates reacted with DmJHE and DmJHEdup.

interact with JHIII. These include two phenylalanine residues (F251 and F427 in DmJHE and F259 and F425 in MsJHE) that form π-π interactions with the conjugated portions of JHIII and a threonine residue (T309 in DmJHE and T314 in MsJHE) that forms hydrogen bonding interactions with the epoxide moiety of JHIII. These residues are conserved among other insect JHEs and are known to be key in JHIII binding (Kamita et al., 2010, 2003; Kamita and Hammock, 2010). The glutamine residue (Q217 in DmJHE and Q225 in MsJHE) that precedes the catalytic serine in the primary sequence is also highly conserved between insect JHEs (Kamita et al., 2010, 2003; Kamita and Hammock, 2010). In MsJHE, Q225 is shown to be in hydrogen bonding distance to the bound JHIII's acyl group (Fig. 3D); this interaction may be important in orienting and stabilizing JHIII. In DmJHE, the docking simulation did not orient the conserved glutamine to interact with JHIII, however, none of the surrounding residues prevent interaction, suggesting this poor orientation may be an artifact from the simulation (Fig. 3B). Overall, both the position and nature of the key residues in the active site are consistent between the two enzymes even though they possess very low sequence identity (30%) (Altschul et al., 1990).

While the modelled structures of DmJHEdup and DmJHE use the same template, their structures show significant differences. In contrast to the long, narrow binding pocket of DmJHE, the binding pocket of DmJHEdup is wider and more open, consistent with the broader substrate specificity observed in the kinetic data (Table 1, Table 2, Fig. 3A). The CASTp server determined that the volumes of the binding pockets of DmJHEdup and DmJHE are 1374.1 Å^3 and 460.0 Å^3, respectively (Dundas et al., 2006). Superimposition of JHIII bound DmJHE over the active site of DmJHEdup also shows why the latter does not interact with JHIII. (Fig. 3C). All the key residues for JHIII binding in DmJHE are either mutated or shifted in DmJHEdup: F251 and Q217 in DmJHE are replaced by A240 and H206 in DmJHEdup, respectively; L299 in DmJHEdup replaces T309 in DmJHE and directly clashes with JHIII binding; and while F427 in DmJHE aids JHIII binding, the equivalent residue in DmJHEdup, F414, is shifted away. Both DmJHE and DmJHEdup possess predominantly hydrophobic binding pockets, however all of the residues of DmJHEdup are shifted further from the catalytic serine, which would allow easier diffusion of substrates into the active site and accommodate the broader substrate range observed for DmJHEdup.

Both the mutations from T309 in DmJHE to L299 in DmJHEdup and from Q217 in DmJHE to H206 in DmJHEdup are of particular significance due to the high conservation of these residues across insect JHEs (Kamita and Hammock, 2010; Tsubota et al., 2010b). While the mutation from T309 to L299 would require two nucleotide changes, a multiple sequence alignment of JHE and JHEdup pairs in representatives of the major families of Schizophora (Fig. S2) shows that these changes are largely conserved: Each JHEdup has a mutation from T to a hydrophobic residue (L, G, C and V), all of which would require at least two nucleotide changes; and each JHEdup, except for *Lucilia cuprina* JHEdup, has the mutation from Q to H. This suggests that these mutations are involved in the neofunctionalization of DmJHEdup from DmJHE.

3.4. Biochemical and structural comparison between DmJHEdup and DmEST6

We also compared the biochemistry of DmJHEdup with that of the only other well studied *Drosophila* ODE, DmEST6 (Younus et al., 2017). Both enzymes show poor activity with odorant esters that possess small alcohol groups, irrespective of the length of the acyl group (Younus et al., 2017). However, DmEST6 has its greatest activity with esters with propionate acyl groups and alcohol groups from 3 to 8 carbon atoms long (Younus et al., 2017), whereas DmJHEdup has greatest activity against substrates with longer acyl groups, butyrate and hexanoate, albeit with a similar preference for alcohol groups (Table 2). The differences in acyl group preferences suggest the two enzymes may have complementary roles in processing the array of food esters to which *D. melanogaster* responds.

The modelled binding pocket of DmJHEdup was also compared to

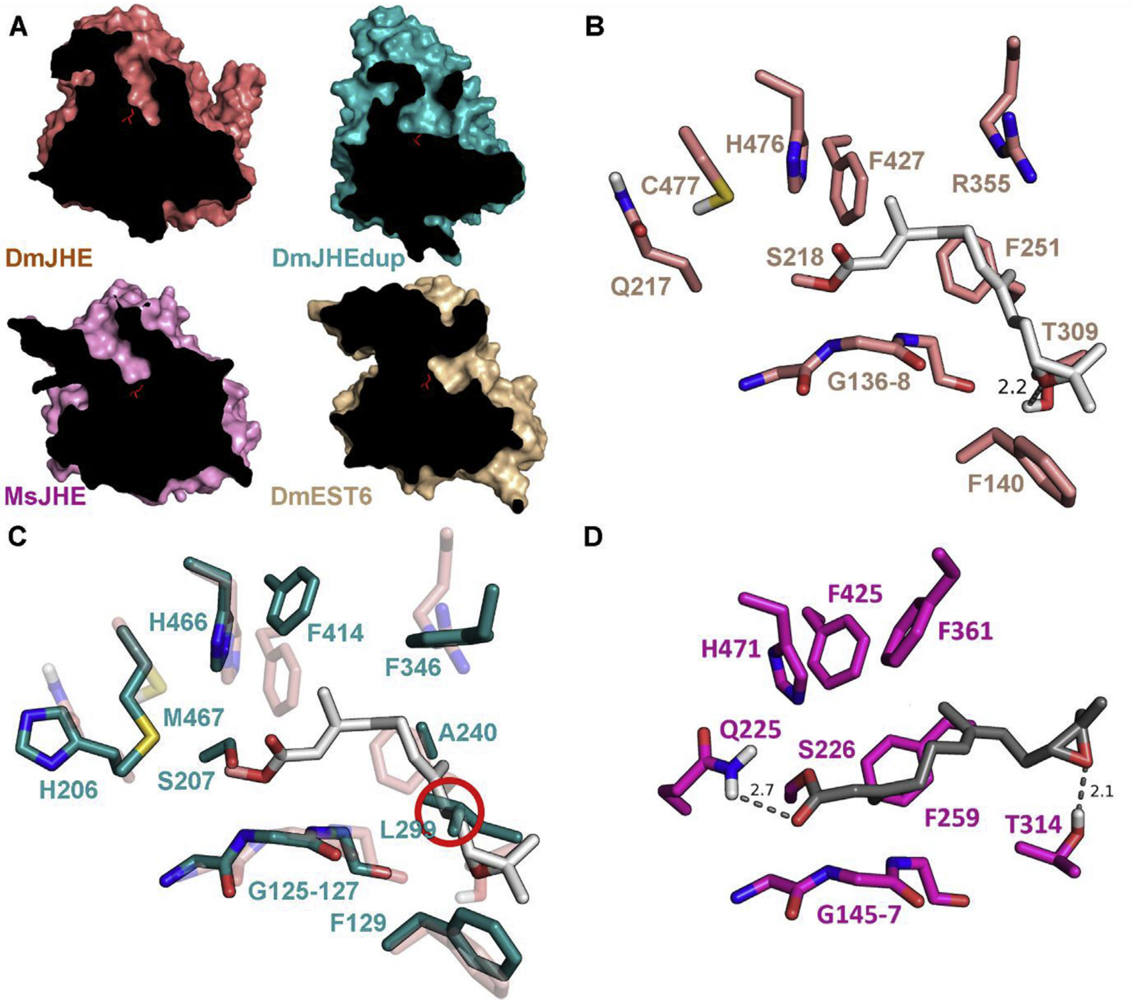

Fig. 3. **(A)** A comparison between the predicted binding pockets of DmJHE (red) and DmJHEdup (cyan) against the pockets of the solved structures of MsJHE (pink) and DmEST6 (sand). **(B)** The docked interaction between JHIII and the modelled active site of DmJHE indicating key residues involved in binding. **(C)** The active site of the modelled DmJHEdup indicating key residues. A transparent representation of the DmJHE active site with JH bound is overlaid and the location of a key clash in the DmJHEdup structure is indicated. **(D)** The covalently docked interaction between the crystal structure of MsJHE and JHIII with key residues involved in binding labelled.

the crystal structure of DmEST6. Both enzymes share a different active site entrance from the JHEs (Fig. 3A). However, the two enzymes also differ significantly from each other in their binding pockets. The DmEST6 binding pocket is much smaller (227.8 $Å^3$) than that of DmJHEdup (1374.1 $Å^3$) and it also differs significantly in shape (Fig. 3A) (Dundas et al., 2006). The acyl binding pocket of DmEST6 consists of a range of bulky, hydrophobic residues such as F276 and F397 and is primarily restricted by W221 (Fig. 4C and D), whereas the buried acyl binding pocket of DmJHEdup is larger, being defined by smaller and more flexible hydrophobic residues such as I211, L238, A240, M241, and L299 (Fig. 4A and B) and with no residue equivalent to W221 to restrict it. These differences could explain why DmJHEdup is better suited to substrates with longer acyl groups. The alcohol binding pockets of both enzymes include their active site entrances and are quite broad with both consisting of predominantly bulky, hydrophobic residues (Fig. 4). These similarities can explain their similar preferences for ester alcohol groups.

4. Discussion

JH plays an essential role in the regulation of development in every insect which has been studied, covering a range of hemi- and holometabolous orders (Bai et al., 2007; Cornette et al., 2008; Hinton and Hammock, 2003; Kamita and Hammock, 2010; Li, 2007; Robinson et al., 1991; Teal et al., 2014). It also carries out similar functions in various other arthropods (Cusson et al., 1991; Jindra et al., 2013; Smykal et al., 2014). It is therefore assumed that JHE has also provided an essential function since early arthropod evolution (Cusson et al., 1991; Jindra et al., 2013; Smykal et al., 2014). While earlier phylogenetic studies, using fewer identified JHEs, suggested that they could be classified into at least two distinct CBE clades, our phylogeny now shows them to be spread across four CBE lineages, most of which include enzymes with non-JHE functions (Oakeshott et al., 2010, 2005). Moreover, the topology of these lineages does not correspond well with the topology of a phylogeny for the respective insect orders (Misof et al., 2014). We suggest that several different daughter lineages of the ancestral JHE have retained the JHE function, whilst also independently duplicating to evolve various other functions. We favor this interpretation over an alternative hypothesis that the ancestral JHE was replaced by other neofunctionalized esterases in some lineages; it seems unlikely that either a) an organismal lineage would survive the loss of a fully functional JHE while it evolved an alternative in a different esterase lineage, or b) that it would evolve a ready-made alternative while it still had a fully functional version of the original JHE.

Significantly there is no evidence from any study for more than one functional JHE in a given insect, so we further suggest that there has

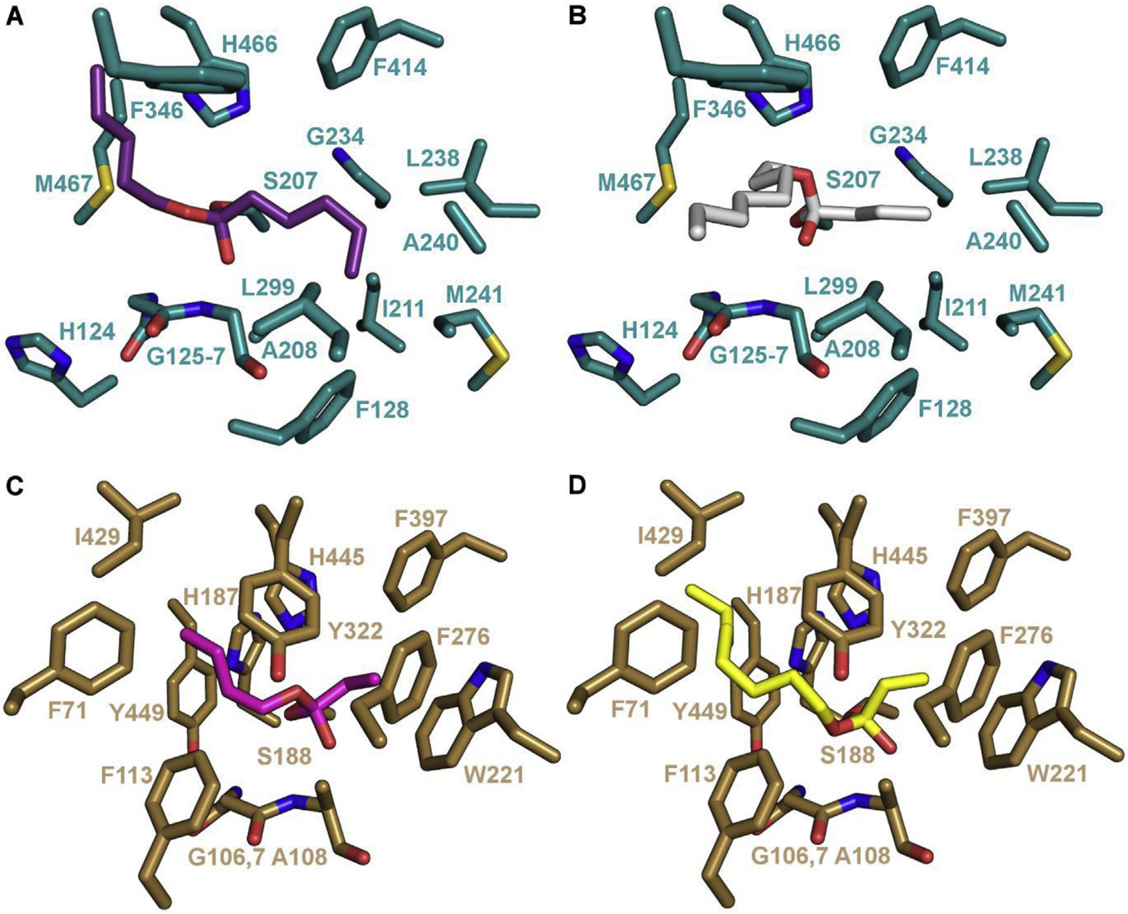

Fig. 4. Covalent docking simulations showing the tetrahedral intermediates formed between DmJHEdup and its most efficiently hydrolyzed substrates; pentyl hexanoate (purple) (**A**) and octyl butyrate (grey) (**B**), and between DmEST6 and its most efficiently hydrolyzed substrates; butyl propionate (pink) (**C**) and octyl propionate (yellow) (**D**). All simulations were aligned and have been shown from the same direction to highlight the interactions between the substrate and each enzyme's acyl binding pocket (on the right) and alcohol binding pocket/active site entrance (on the left).

been selection against the retention of JHE function in any duplicate of a functional JHE through the course of insect evolution (Crone et al., 2007; Gilbert et al., 2000; Kamita and Hammock, 2010; Oakeshott et al., 2005; Tsubota et al., 2010a; Tsubota and Shiotsuki, 2010). Further evidence for this is that transgenic manipulation of JHE activity or expression profiles has been shown to disrupt insect development (Hajós et al., 1999; Hammock et al., 1990).

DmJHEdup is a relatively recent example of the divergent evolution of a duplicate JHE, having arisen in the Brachycera lineage of the Diptera, before the emergence of the Schizophora (Steiner et al., 2017; Yeates and Wiegmann, 1999). In particular, we extend earlier work showing its function does not overlap with DmJHE. The previous work had shown that it has evolved a very different tissue and temporal expression profile from DmJHE: DmJHEdup is the most highly expressed CBE in the antenna where DmJHE has low expression; and DmJHE has high expression in the adipose tissue during the pupal stage of development where DmJHEdup has no detectable expression (Younus et al., 2014). Our study shows that despite a relatively wide substrate range, at least *in vitro*, DmJHEdup also lacks detectable amounts of JHIII hydrolytic activity or even binding. Thus, DmJHEdup is incapable of interfering with the physiological function of DmJHE on both regulatory and structural grounds. Equally, we now know, from the work of Younus et al. (2014), that DmJHEdup has the expression profile and, from the current study, that it has the kinetic capability to function as an ODE in a complementary fashion to the only other known Drosophila ODE, DmEST6 (Younus et al., 2017, 2014). DmJHEdup thus exemplifies the neofunctionalization that is central in the theory of the evolution of new biochemical functions by gene duplication (Hahn et al., 2007; Kondrashov, 2012; Oakeshott et al., 1993).

The question then arises as to what sort of mutation could have occurred early in the life of the DmJHEdup gene that would have obliterated any physiologically relevant JHIII hydrolytic function whilst at the same time retaining some expression and activity as a template from which selection could then act to evolve its eventual role as an effective ODE? Without some such utility the cognate gene would accumulate disabling mutations through neutral evolution, becoming irreversibly ‘pseudogenized’ and eventually lost (Copley, 2010; DePristo et al., 2005; Li et al., 1981; Podlaha and Zhang, 2010).

We suspect that a crucial early step in the evolution of DmJHE would have been a cis-inherited regulatory change that eliminated the expression profile associated with *in vivo* JHIII degradation whilst bestowing the adult antennal expression from which the new ODE function might evolve. For example, an antennal-specific enhancer might have been inserted into the promoter of the gene (perhaps via a transposable element) in such a way as to disrupt an element required for the ancestral JHE expression profile (Kidwell and Lisch, 1997; Levin and Moran, 2011; Wittkopp and Kalay, 2012). The promiscuous activities of the duplicated JHE for a range of mid-long chain esters might then have had some immediate ODE utility and at the same time served as a template for further evolution to optimize its substrate range for an ODE function. We note that the kinetics of DmJHE for some such substrates (estimated K_M values in the range 10–600 μM) are not qualitatively different from the range of values found for some known ODEs from other insects (K_M values in the range 1 μM–10 mM), suggesting a duplicate expressed in the antennae could then have had immediate utility (Durand et al., 2010a,b; Durand et al., 2011; He et al., 2014; Ishida and Leal, 2005, 2008).

An alternative model for the crucial early step in the neofunctionalization of DmJHEdup might propose a structural mutation which obliterated activity against JHIII but retained potentially useful promiscuous activities as a template for further evolution. We cannot discount this possibility and indeed our data suggest changes such as L299/T309 could affect such a transition. A possible problem with this scenario may be that without a concomitant change in expression profile to produce the enzyme in antennae, it is not clear what immediate utility the new mutation would confer that would prevent the gene from decaying to pseudogene status and retain it as a template for further evolution.

Two broad functional classes of ODEs, specific and general ODEs, have been recognized in insect antennae and other sensory organs (Leal, 2013). Specific ODEs only act on specific substrates and play a dynamic role in refreshing the sensory system to continually respond to new incoming signals (Leal, 2013). The best characterized examples act on sex pheromones, where males in flight must be able to react to changes in the concentration of a female's pheromone plume on a millisecond scale (Durand et al., 2010a,b; Durand et al., 2010a,b; Vogt et al., 1985). The ApolPDE enzyme of *Antheraea polyphemus*, for example, has a specificity constant for its substrate, E6Z11-16:acetate, of $1.00 \times 10^8\,M^{-1}\,s^{-1}$, with a K_M of 1.27 µM and k_{cat} of 127 s^{-1} (Ishida and Leal, 2005). On the other hand, general ODEs act on a broader range of substrates and do not have kinetics optimized for a specific substrate. As an example, DmEST6 is proposed to be a general ODE that enables the fly to locate foods emitting various volatile short-mid chain esters and it has specificity constants for these esters estimated to be in the range of 10^5–$10^6\,M^{-1}\,s^{-1}$, with K_M values in the range of 121–880 µM (Younus et al., 2017). While our data show DmJHEdup prefers mid-long chain esters, we can now also see it has evolved kinetics for those esters that are in a very comparable range. In combination with the electrophysiological and behavioral data of Steiner et al. (2017), our results show DmJHEdup has effectively neofunctionalized to an ODE role with a complementary set of food esters (Steiner et al., 2017).

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ibmb.2019.01.001.

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References

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. J. Mol. Biol. 215, 403–410.

Bai, H., Ramaseshadri, P., Palli, S.R., 2007. Identification and characterization of juvenile hormone esterase gene from the yellow fever mosquito, *Aedes aegypti*. Insect Biochem. Mol. Biol. 37, 829–837.

Barry, J., Wang, S., Wilson, T.G., 2008. Overexpression of Methoprene-tolerant, a *Drosophila melanogaster* gene that is critical for juvenile hormone action and insecticide resistance. Insect Biochem. Mol. Biol. 38, 346–353.

Benkert, P., Tosatto, S.C.E., Schomburg, D., 2008. QMEAN: a comprehensive scoring function for model quality assessment. Proteins Struct. Funct. Bioinforma. 71, 261–277.

Bianco, G., Forli, S., Goodsell, D.S., Olson, A.J., 2016. Covalent docking using autodock: Two-point attractor and flexible side chain methods. Protein Sci. 25, 295–301.

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.

Campbell, P.M., Oakeshott, J.G., Healy, M.J., 1998. Purification and kinetic characterisation of juvenile hormone esterase from *Drosophila melanogaster*. Insect Biochem. Mol. Biol. 28, 501–515.

Capella-Gutierrez, S., Silla-Martinez, J.M., Gabaldon, T., 2009. trimAl: a tool for automated alignment trimming in large-scale phylogenetic analyses. Bioinformatics 25, 1972–1973.

Carroll, S.B., 2005. Evolution at two levels: on genes and form. PLoS Biol. 3, e245.

Chertemps, T., François, A., Durand, N., Rosell, G., Dekker, T., Lucas, P., Maïbèche-Coisne, M., 2012. A carboxylesterase, Esterase-6, modulates sensory physiological and behavioral response dynamics to pheromone in Drosophila. BMC Biol. 10, 56.

Copley, S.D., 2010. Evolution and the enzyme. In: Barton, D., Meth-Cohn, D. (Eds.), Comprehensive Natural Products Chemistry II. Elsevier, pp. 9–46.

Cornette, R., Gotoh, H., Koshikawa, S., Miura, T., 2008. Juvenile hormone titers and caste differentiation in the damp-wood termite *Hodotermopsis sjostedti* (Isoptera, Termopsidae). J. Insect Physiol. 54, 922–930.

Cornish-Bowden, A., 1995. Fundamentals of Enzyme Kinetics. Portland Press, London.

Correy, G.J., Carr, P.D., Meirelles, T., Mabbitt, P.D., Fraser, N.J., Weik, M., Jackson, C.J., 2016. Mapping the accessible conformational landscape of an insect carboxylesterase using conformational ensemble analysis and kinetic crystallography. Structure 24, 977–987.

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.

Cusson, M., Yagi, K.J., Ding, Q., Duve, H., Thorpe, A., McNeil, J.N., Tobe, S.S., 1991. Biosynthesis and release of juvenile hormone and its precursors in insects and crustaceans: the search for a unifying arthropod endocrinology. Insect Biochem. 21, 1–6.

DePristo, M.A., Weinreich, D.M., Hartl, D.L., 2005. Missense meanderings in sequence space: a biophysical view of protein evolution. Nat. Rev. Genet. 6, 678–687.

Dundas, J., Ouyang, Z., Tseng, J., Binkowski, A., Turpaz, Y., Liang, J., 2006. CASTp: computed atlas of surface topography of proteins with structural and topographical mapping of functionally annotated residues. Nucleic Acids Res. 34, 116–118.

Durand, N., Carot-Sans, G., Bozzolan, F., Rosell, G., Siaussat, D., Debernard, S., Chertemps, T., Maïbèche-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., Bozzolan, F., Party, V., Renou, M., Debernard, S., Rosell, G., Maïbèche-Coisne, M., 2010a. Characterization of an antennal carboxylesterase from the pest moth *Spodoptera littoralis* degrading a host plant odorant. PLoS One 5, e15026.

Durand, N., Carot-Sans, G., Chertemps, T., Montagné, N., Jacquin-Joly, E., Debernard, S., Maïbèche-Coisne, M., 2010b. A diversity of putative carboxylesterases are expressed in the antennae of the noctuid moth *Spodoptera littoralis*. Insect Mol. Biol. 19, 87–97.

El-Sheikh, E.A., 2015. Characterization and kinetics of juvenile hormone esterase from *Spodoptera littoralis* (Boisd.) and *S. frugiperda* (J. E. Smith) (Lepidoptera: noctuidae). Egypt. J. Biol. Pest Control 25, 625–630.

Elayidam, U.G., Muraleedharen, D., 2008. Identification and partial characterization of juvenile hormone esterase from cotton pest *Dysdercus cingulatus*. Indian J. Biochem. Biophys. 45, 121–125.

Gilbert, L.I., Granger, N.A., Roe, R.M., 2000. The juvenile hormones: historical facts and speculations on future research directions. Insect Biochem. Mol. Biol. 30, 617–644.

Hahn, M.W., Han, M.V., Han, S.G., 2007. Gene family evolution across 12 Drosophila genomes. PLoS Genet. 3, e197.

Hajós, J.P., Vermunt, A.M., Zuidema, D., Kulcsár, P., Varjas, L., de Kort, C.A., Závodszky, P., Vlak, J.M., 1999. Dissecting insect development: baculovirus-mediated gene silencing in insects. Insect Mol. Biol. 8, 539–544.

Hammock, B.D., 1985. Regulation of juvenile hormone titer: Degradation. In: Kerkut, G.A., Gilbert, L.I. (Eds.), Comprehensive Insect Physiology, Biochemistry, and Pharmacology. Pergamon Press, New York, NY, USA, pp. 431–472.

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., He, P., 2014. Functional characterization of an antennal esterase from the noctuid moth, *Spodoptera exigua*. Arch. Insect Biochem. Physiol. 86, 85–99.

Hinton, A.C., Hammock, B.D., 2003. Juvenile hormone esterase (JHE) from *Tenebrio molitor*: full-length cDNA sequence, in vitro expression, and characterization of the recombinant protein. Insect Biochem. Mol. Biol. 33, 477–487.

Hoekstra, H.E., Coyne, J.A., 2007. The locus of evolution: evo devo and the genetics of adaptation. Evolution 61, 995–1016.

Ichikawa, A., Ono, H., Furuta, K., Shiotsuki, T., Shinoda, T., 2007. Enantioselective separation of racemic juvenile hormone III by normal-phase high-performance liquid chromatography and preparation of [$^2H_3$]juvenile hormone III as an internal standard for liquid chromatography–mass spectrometry quantification. J. Chromatogr. A 1161, 252–260.

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. U. S. A 105, 9076–9080.

Ishida, Y., Leal, W.S., 2005. Rapid inactivation of a moth pheromone. Proc. Natl. Acad. Sci. U. S. A 102, 14075–14079.

Jindra, M., Palli, S.R., Riddiford, L.M., 2013. The juvenile hormone signaling pathway in insect development. Annu. Rev. Entomol. 58, 181–204.

Juneja, P., Quinn, A., Jiggins, F.M., 2016. Latitudinal clines in gene expression and cis-regulatory element variation in *Drosophila melanogaster*. BMC Genomics 17, 981.

Kalyaanamoorthy, S., Minh, B.Q., Wong, T.K.F., von Haeseler, A., Jermiin, L.S., 2017. ModelFinder: fast model selection for accurate phylogenetic estimates. Nat. Methods 14, 587–589.

Kamita, S.G., Hammock, B.D., 2010. Juvenile hormone esterase: biochemistry and structure. J. Pestic. Sci. 35, 265–274.

Kamita, S.G., Hinton, A.C., Wheelock, C.E., Wogulis, M.D., Wilson, D.K., Wolf, N.M., Stok, J.E., Hock, B., Hammock, B.D., 2003. Juvenile hormone (JH) esterase: why are you so JH specific? Insect Biochem. Mol. Biol. 33, 1261–1273.

Kamita, S.G., Samra, A.I., Liu, J.-Y., Cornel, A.J., Hammock, B.D., 2011. Juvenile hormone (JH) esterase of the mosquito *Culex quinquefasciatus* is not a target of the JH analog insecticide methoprene. PLoS One 6, e28392.

Kamita, S.G., Wogulis, M.D., Law, C.S., Morisseau, C., Tanaka, H., Huang, H., Wilson, D.K., Hammock, B.D., 2010. Function of phenylalanine 259 and threonine 314 within the substrate binding pocket of the juvenile hormone esterase of *Manduca sexta*. Biochemistry 49, 3733–3742.

Katoh, K., Kuma, K., Toh, H., Miyata, T., 2005. MAFFT version 5: improvement in accuracy of multiple sequence alignment. Nucleic Acids Res. 33, 511–518.

Kidwell, M.G., Lisch, D., 1997. Transposable elements as sources of variation in animals and plants. Proc. Natl. Acad. Sci. U. S. A 94, 7704–7711.

Kim, D.E., Chivian, D., Baker, D., 2004. Protein structure prediction and analysis using the Robetta server. Nucleic Acids Res. 32, W526–W531.

Kondrashov, F.A., 2012. Gene duplication as a mechanism of genomic adaptation to a changing environment. Proc. R. Soc. B 279, 5048–5057.

Kontogiannatos, D., Michail, X., Kourti, A., 2011. Molecular characterization of an ecdysteroid inducible carboxylesterase with GQSCG motif in the corn borer, *Sesamia nonagrioides*. J. Insect Physiol. 57, 1000–1009.

Kontogiannatos, D., Swevers, L., Maenaka, K., Park, E.Y., Iatrou, K., Kourti, A., 2013. Functional characterization of a juvenile hormone esterase related gene in the moth *Sesamia nonagrioides* through RNA interference. PLoS One 8, e73834.

Leal, W.S., 2013. Odorant reception in insects: roles of receptors, binding proteins, and degrading enzymes. Annu. Rev. Entomol. 58, 373–391.

Letunic, I., Bork, P., 2016. Interactive tree of life (iTOL) v3: an online tool for the display and annotation of phylogenetic and other trees. Nucleic Acids Res. 44, W242–W245.

Levin, H.L., Moran, J.V., 2011. Dynamic interactions between transposable elements and their hosts. Nat. Rev. Genet. 12, 615–627.

Li, W.H., Gojobori, T., Nei, M., 1981. Pseudogenes as a paradigm of neutral evolution. Nature 292, 237–239.

Li, X., 2007. Juvenile hormone and methyl farnesoate production in cockroach embryos in relation to dorsal closure and the reproductive modes of different species of cockroaches. Arch. Insect Biochem. Physiol. 66, 159–168.

Misof, B., Liu, S., Meusemann, K., Peters, R.S., Donath, A., Mayer, C., Frandsen, P.B., Ware, J., Flouri, T., Beutel, R.G., Niehuis, O., Petersen, M., Izquierdo-Carrasco, F., Wappler, T., Rust, J., Aberer, A.J., Aspöck, U., Aspöck, H., Bartel, D., Blanke, A., Berger, S., Böhm, A., Buckley, T.R., Calcott, B., Chen, J., Friedrich, F., Fukui, M., Fujita, M., Greve, C., Grobe, P., Gu, S., Huang, Y., Jermiin, L.S., Kawahara, A.Y., Krogmann, L., Kubiak, M., Lanfear, R., Letsch, H., Li, Y., Li, Z., Li, J., Lu, H., Machida, R., Mashimo, Y., Kapli, P., McKenna, D.D., Meng, G., Nakagaki, Y., Navarrete-Heredia, J.L., Ott, M., Ou, Y., Pass, G., Podsiadlowski, L., Pohl, H., von Reumont, B.M., Schütte, K., Sekiya, K., Shimizu, S., Slipinski, A., Stamatakis, A., Song, W., Su, X., Szucsich, N.U., Tan, M., Tan, X., Tang, M., Tang, J., Timelthaler, G., Tomizuka, S., Trautwein, M., Tong, X., Uchifune, T., Walzl, M.G., Wiegmann, B.M., Wilbrandt, J., Wipfler, B., Wong, T.K.F., Wu, Q., Wu, G., Xie, Y., Yang, S., Yang, Q., Yeates, D.K., Yoshizawa, K., Zhang, Q., Zhang, R., Zhang, W., Zhang, Y., Zhao, J., Zhou, C., Zhou, L., Ziesmann, T., Zou, S., Li, Y., Xu, X., Zhang, Y., Yang, H., Wang, J., Wang, J., Kjer, K.M., Zhou, X., 2014. Phylogenomics resolves the timing and pattern of insect evolution. Science 346, 763–767.

Morris, G.M., Huey, R., Lindstrom, W., Sanner, M.F., Belew, R.K., Goodsell, D.S., Olson, A.J., 2009. AutoDock4 and AutoDockTools4: Automated docking with selective receptor flexibility. J. Comput. Chem. 30, 2785–2791.

Nguyen, L.T., Schmidt, H.A., von Haeseler, A., Minh, B.Q., 2015. IQ-TREE: a fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies. Mol. Biol. Evol. 32, 268–274.

Noriega, F.G., 2014. Juvenile hormone biosynthesis in insects: what is new, what do we know, and what questions remain? Int. Sch. Res. Not. 2014, 1–16.

Oakeshott, J.G., Claudianos, C., Campbell, P.M., Newcomb, R.D., Russell, R.J., 2005. Biochemical genetics and genomics of insect esterases. In: Gilbert, L., Iatrou, K., Gill, S. (Eds.), Comprehensive Molecular Insect Science. Elsevier, pp. 309–381.

Oakeshott, J.G., Johnson, R.M., Berenbaum, M.R., Ranson, H., Cristino, A.S., Claudianos, C., 2010. Metabolic enzymes associated with xenobiotic and chemosensory responses in *Nasonia vitripennis*. Insect Mol. Biol. 19, 147–163.

Oakeshott, J.G., van Papenrecht, E.A., Boyce, T.M., Healy, M.J., Russell, R.J., 1993. Evolutionary genetics of Drosophila esterases. Genetica 90, 239–268.

Podlaha, O., Zhang, J., 2010. Pseudogenes and their evolution. In: Encyclopedia of Life Sciences. John Wiley & Sons, Ltd, Chichester, UK, pp. 1–8.

Robinson, G.E., Strambi, C., Strambi, A., Feldlaufer, M.F., 1991. Comparison of juvenile hormone and ecdysteroid haemolymph titres in adult worker and queen honey bees (*Apis mellifera*). J. Insect Physiol. 37, 929–935.

Sadd, B.M., Barribeau, S.M., Bloch, G., de Graaf, D.C., Dearden, P., Elsik, C.G., Gadau, J., Grimmelikhuijzen, C.J.P., Hasselmann, M., Lozier, J.D., Robertson, H.M., Smagghe, G., Stolle, E., Van Vaerenbergh, M., Waterhouse, R.M., Bornberg-Bauer, E., Klasberg, S., Bennett, A.K., Câmara, F., Guigó, R., Hoff, K., Mariotti, M., Munoz-Torres, M., Murphy, T., Santesmasses, D., Amdam, G.V., Beckers, M., Beye, M., Biewer, M., Bitondi, M.M.G., Blaxter, M.L., Bourke, A.F.G., Brown, M.J.F., Buechel, S.D., Cameron, R., Cappelle, K., Carolan, J.C., Christiaens, O., Ciborowski, K.L., Clarke, D.F., Colgan, T.J., Collins, D.H., Cridge, A.G., Dalmay, T., Dreier, S., du Plessis, L., Duncan, E., Erler, S., Evans, J., Falcon, T., Flores, K., Freitas, F.C.P., Fuchikawa, T., Gempe, T., Hartfelder, K., Hauser, F., Helbing, S., Humann, F.C., Irvine, F., Jermiin, L.S., Johnson, C.E., Johnson, R.M., Jones, A.K., Kadowaki, T., Kidner, J.H., Koch, V., Köhler, A., Kraus, F.B., Lattorff, H.M.G., Leask, M., Lockett, G.A., Mallon, E.B., Antonio, D.S.M., Marxer, M., Meeus, I., Moritz, R.F.A., Nair, A., Näpflin, K., Nissen, I., Niu, J., Nunes, F.M.F., Oakeshott, J.G., Osborne, A., Otte, M., Pinheiro, D.G., Rossié, N., Rueppell, O., Santos, C.G., Schmid-Hempel, R., Schmitt, B.D., Schulte, C., Simões, Z.L.P., Soares, M.P.M., Swevers, L., Winnebeck, E.C., Wolschin, F., Yu, N., Zdobnov, E.M., Aqrawi, P.K., Blankenburg, K.P., Coyle, M., Francisco, L., Hernandez, A.G., Holder, M., Hudson, M.E., Jackson, L., Jayaseelan, J., Joshi, V., Kovar, C., Lee, S.L., Mata, R., Mathew, T., Newsham, I.F., Ngo, R., Okwuonu, G., Pham, C., Pu, L.-L., Saada, N., Santibanez, J., Simmons, D., Thornton, R., Venkat, A., Walden, K.K.O., Wu, Y.-Q., Debyser, G., Devreese, B., Asher, C., Blommaert, J., Chipman, A.D., Chittka, L., Fouks, B., Liu, J., O'Neill, M.P., Sumner, S., Puiu, D., Qu, J., Salzberg, S.L., Scherer, S.E., Muzny, D.M., Richards, S., Robinson, G.E., Gibbs, R.A., Schmid-Hempel, P., Worley, K.C., 2015. The genomes of two key bumblebee species with primitive eusocial organization. Genome Biol. 16, 76.

Schrodinger, L.L.C., 2010. The PyMOL Molecular Graphics System, Version 1.3r1.

Segel, I.H., 1993. Enzyme Kinetics: Behavior and Analysis of Rapid Equilibrium and Steady State Enzyme Systems. John Wiley & Sons.

Smykal, V., Bajgar, A., Provaznik, J., Fexova, S., Buricova, M., Takaki, K., Hodkova, M., Jindra, M., Dolezel, D., 2014. Juvenile hormone signaling during reproduction and development of the linden bug, *Pyrrhocoris apterus*. Insect Biochem. Mol. Biol. 45, 69–76.

Steiner, C., Bozzolan, F., Montagné, N., Maïbèche, M., Chertemps, T., 2017. Neofunctionalization of “juvenile hormone esterase duplication” in Drosophila as an odorant-degrading enzyme towards food odorants. Sci. Rep. 7, 12629.

Tangwancharoen, S., Moy, G.W., Burton, R.S., 2018. Multiple modes of adaptation: regulatory and structural evolution in a small heat shock protein gene. Mol. Biol. Evol. 35, 2110–2119.

Teal, P.E.A., Jones, D., Jones, G., Torto, B., Nyasembe, V., Borgemeister, C., Alborn, H.T., Kaplan, F., Boucias, D., Lietze, V.U., 2014. Identification of methyl farnesoate from the hemolymph of insects. J. Nat. Prod. 77, 402–405.

Tsubota, T., Minakuchi, C., Nakakura, T., Shinoda, T., Shiotsuki, T., 2010a. Molecular characterization of a gene encoding juvenile hormone esterase in the red flour beetle, *Tribolium castaneum*. Insect Mol. Biol. 19, 527–535.

Tsubota, T., Shimomura, M., Ogura, T., Seino, A., Nakakura, T., Mita, K., Shinoda, T., Shiotsuki, T., 2010b. Molecular characterization and functional analysis of novel carboxyl/cholinesterases with GQSAG motif in the silkworm *Bombyx mori*. Insect Biochem. Mol. Biol. 40, 100–112.

Tsubota, T., Shiotsuki, T., 2010. Genomic analysis of carboxyl/cholinesterase genes in the silkworm *Bombyx mori*. BMC Genomics 11, 377.

Valaitis, A.P., 1991. Characterization of hemolymph juvenile hormone esterase from *Lymantria dispar*. Insect Biochem. 21, 583–595.

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. U. S. A 82, 8827–8831.

Wilson, T.G., Fabian, J., 1986. A *Drosophila melanogaster* mutant resistant to a chemical analog of juvenile hormone. Dev. Biol. 118, 190–201.

Wittkopp, P.J., Kalay, G., 2012. Cis-regulatory elements: molecular mechanisms and evolutionary processes underlying divergence. Nat. Rev. Genet. 13, 59–69.

Wogulis, M., Wheelock, C.E., Kamita, S.G., Hinton, A.C., Whetstone, P.A., Hammock, B.D., Wilson, D.K., 2006. Structural studies of a potent insect maturation inhibitor bound to the juvenile hormone esterase of *Manduca sexta*. Biochemistry 45, 4045–4057.

Yeates, D.K., Wiegmann, B.M., 1999. Congruence and controversy: toward a higher-Level phylogeny of Diptera. Annu. Rev. Entomol. 44, 397–428.

Yin, C.M., Takeda, M., Wang, Z.S., 1987. A juvenile hormone analogue, methoprene as a circadian and developmental modulator in *Diatraea grandiosella* (Pyralidae). J. Insect Physiol. 33, 95–102.

Younus, F., Chertemps, T., Pearce, S.L., Pandey, G., Bozzolan, F., Coppin, C.W., Russell, R.J., Maïbèche-Coisne, M., Oakeshott, J.G., 2014. Identification of candidate odorant degrading gene/enzyme systems in the antennal transcriptome of *Drosophila melanogaster*. Insect Biochem. Mol. Biol. 53, 30–43.

Younus, F., Fraser, N.J., Coppin, C.W., Liu, J.-W., Correy, G.J., Chertemps, T., Pandey, G., Maïbèche, M., Jackson, C.J., Oakeshott, J.G., 2017. Molecular basis for the behavioral effects of the odorant degrading enzyme Esterase 6 in Drosophila. Sci. Rep. 7, 46188.

Zhu, K., Borrelli, K.W., Greenwood, J.R., Day, T., Abel, R., Farid, R.S., Harder, E., 2014. Docking covalent inhibitors: a parameter free approach to pose prediction and scoring. J. Chem. Inf. Model. 54, 1932–1940.

Zhu, L., Yin, T.Y., Sun, D., Liu, W., Zhu, F., Lei, C.L., Wang, X.P., 2017. Juvenile hormone regulates the differential expression of putative juvenile hormone esterases via methoprene-tolerant in non-diapause-destined and diapause-destined adult female beetle. Gene 627, 373–378.