CrossMark

Bulletin of Entomological Research (2019) **109**, 534–543
doi:10.1017/S0007485318000895
© Cambridge University Press 2019

Latrophilin mediates insecticides susceptibility and fecundity through two *carboxylesterases, esterase4* and *esterase6*, in *Tribolium castaneum*

L. Wei†, S. Gao†, W. Xiong, J. Liu, J. Mao, Y. Lu, X. Song and B. Li*

Jiangsu Key Laboratory for Biodiversity and Biotechnology, College of Life Sciences, Nanjing Normal University, Nanjing 210023, China‡

Abstract

Latrophilin (LPH) is known as an adhesion G-protein-coupled receptor which involved in multiple physiological processes in organisms. Previous studies showed that *lph* not only involved the susceptibility to anticholinesterase insecticides but also affected fecundity in *Tribolium castaneum*. However, its regulatory mechanisms in these biological processes are still not clear. Here, we identified two potential downstream carboxylesterase (*cce*) genes of *Tclph*, *esterase4* and *esterase6*, and further characterized their interactions with *Tclph*. After treatment of *T. castaneum* larvae with carbofuran or dichlorvos insecticides, the transcript levels of *Tcest4* and *Tcest6* were significantly induced from 12 to 72 h. RNAi against *Tcest4* or *Tcest6* led to the higher mortality compared with the controls after the insecticides treatment, suggesting that these two genes play a vital role in detoxification of insecticides in *T. castaneum*. Furthermore, with insecticides exposure to *Tclph* knockdown beetles, the expression of *Tcest4* was upregulated but *Tcest6* was downregulated, indicating that beetles existed a compensatory response against the insecticides. Additionally, RNAi of *Tcest6* resulted in 43% reductions in female egg laying and completely inhibited egg hatching, which showed the similar phenotype as that of *Tclph* knockdown. These results indicated that *Tclph* affected fecundity by positively regulating *Tcest6* expression. Our findings will provide a new insight into the molecular mechanisms of *Tclph* involved in physiological functions in *T. castaneum*.

Keywords: *Latrophilin*, *Carboxylesterase*, insecticide susceptibility, fecundity, RNA interference, *Tribolium castaneum*

(Accepted 25 October 2018; First published online 21 February 2019)

Introduction

Latrophilin (LPH) belongs to a subfamily of neuronal adhesion G-protein-coupled receptors (aGPCRs). It was originally isolated from bovine brain and known as receptors of α-latrotoxin, a neurotoxic component of black widow spider venom (Krasnoperov *et al.*, 1997; Lelianova *et al.*, 1997; Mee *et al.*, 2004). Typically, the LPHs family comprise three isoforms LPH-1, LPH-2, and LPH-3 in most vertebrates, whereas only one in insects (Sugita *et al.*, 1998; Boucard *et al.*, 2014). As an important component of GPCRs, LPHs can transmit multiple signals to regulate the growth, development, and reproduction in eukaryotes (Meza-Aguilar & Boucard, 2014). Studies in vertebrates showed LPHs play an important role in nervous system. For instance, LPH-1 was reported to be closely associated with the mental disorders such as schizophrenia and bipolar disorder in human and mice (Chen & Chen, 2005; Kellendonk *et al.*, 2009; Bonaglia *et al.*, 2010). Null mutant of *lph-3* mice led to a hyperactive phenotype in

*Author for correspondence
E-mail: libin@njnu.edu.cn, libinlb@hotmail.com
†These authors contributed equally to this work
‡ Postal address: Nanjing Normal University, #1 Wenyuan Road, Nanjing, China.

https://doi.org/10.1017/S0007485318000895 Published online by Cambridge University Press

behavioral tests which accompanied by increasing the levels of dopamine and serotonin in the dorsal striatum (Wallis *et al*., 2012). It was also reported that *lph-3* mutation in exon 20 was the reason of equine degenerative myeloencephalopathy, which caused degeneration of the motor neurons in the spinal cord and progressive development of symmetric ataxia predominantly in the hind limbs (Posbergh, 2015). Furthermore, in *Danio rerio*, the loss of *lph-3.1* function caused a reduction and misplacement of dopamine-positive neurons in the ventral diencephalon and further caused hyperactive/impulsive motor phenotype (Lange *et al*., 2012; van der Voet *et al*., 2016). All these results suggested that *lphs* are essential for normal development of nervous system. In addition to the role in mental disorders and nervous system, it has been reported that *lphs* are also essential for the embryogenesis in vertebrates. Null mutant of *lph-1* mice causes embryonic lethal, and *lph-2* is essential for the normal development of embryonic chicken heart which has been regarded as a novel regulator of the epithelial–mesenchymal transition (Doyle *et al*., 2006; Silva & Ushkaryov, 2010). These results suggested that *lphs* play an important role in nervous system and embryogenesis in vertebrates.

Although there is only one *lph* existed in insects, it still plays essential roles in regulating nervous system and embryogenesis. In *Drosophila melanogaster*, RNA interference (RNAi) against CG8639/*lph* induced hyperactivity and lost sleep (Van Der Voet *et al*., 2016). RNAi of *lat-1* (a homolog of *lph-1*) in *Caenorhabditis elegans* resulted in embryo lethality by affecting establishment of anterior–posterior tissue polarity (Langenhan & Russ, 2010; Muller *et al*., 2015). These results revealed the conserved functions of *lph* between the vertebrates and invertebrates. Interestingly, recent studies in invertebrates showed novel functions of *lph*. Further, in *C. elegans*, RNAi against *lat-1* led to a higher paralysis rate than control after exposure to aldicarb (Mee *et al*., 2004), which was an acetylcholinesterase inhibitor and killed insects by overstimulation of the nervous system (Oh & Kim, 2017). This result suggested that *lph* may play an important role in the susceptibility to anticholinesterase insecticides. Simultaneously, our recent studies in *T. castaneum* showed that RNAi of *Tclph* significantly increased susceptibility to organophosphates (OPs) and carbamates, further caused the higher mortalities compared with control groups (Gao *et al*., 2018). Knockdown of *Tclph* caused the decline on egg laying in females (Gao *et al*., 2017), which suggested *Tclph* plays important roles in anticholinesterase susceptibility and fecundity in *T. castaneum*. However, it is still not clear how does *lph* play such important roles in *T. castaneum*. To elucidate these issues, we further performed transcriptome profiling analysis between the control and ds-*lph* insects. It showed that RNAi of *Tclph* changed a large amount of multiple metabolism detoxification enzymes which were participated in cellular detoxification processes. Among these detoxification enzyme, two carboxylesterase (*cce*, also called *est*/*ces*/*carE*) genes, *Tces4* and *Tcest6*, as typical metabolism detoxification enzyme gene were changed markedly in ds-*lph* insects (Gao *et al*., 2018).

Notably, in insects, *cces* were involved in resistance for a larger number of insecticides including OPs, carbamates, and pyrethroids (Kim *et al*., 2015; Zhang *et al*., 2015). In *Locusta migratoria*, RNAi against *Lmcesa1* or *Lmcesa2* caused the significantly increased susceptibility of the nymphs to chlorpyrifos (Langenhan & Russ, 2010; Muller *et al*., 2015). In *Anopheles gambiae*, the activities of CCEs in the carbofuran-, bendiocarb-, and pyrethroid-resistant strains were higher than those of the susceptible strains, suggesting that the elevated activities of CCEs in the resistant strains may be responsible for resistance to these insecticides. In *Nilaparvata lugens*, OPs, and carbamate resistance is based on the amplification of a *cce* gene, *Nlest1*. Besides, *cces* also affect reproduction process of insects (Robin *et al*., 2009; Durand *et al*., 2014). In *Sogatella furcifera*, suppression of *cce* precursor *Sfest1* expression led to dramatically reduced egg laying, oviposition period, and longevity of female (Ge *et al*., 2017). In *Drosophila ananassae*, null mutant fly of *est4* caused the reduction of fertile (Krishnamoorti & Singh, 2017). These results indicated that *lph* and *cces* may have some intrinsic link on insecticides tolerance and reproduction of organisms.

Here, we focused attention on two *cce* genes, *Tcest4* and *Tcest6*, and characterized the interactions between these two genes and *Tclph*, to clarify the potential regulatory mechanisms of *Tclph* in *T. castaneum*. We first investigated the possible roles of *Tcest4* and *Tcest6* in insecticide resistance. Then, we tested the response of these two genes in ds-*lph* insects after exposure to carbofuran and dichlorvos insecticides. Finally, the possible mechanism of *Tcest4* and *Tcest6* in *Tclph*-regulated reproduction was also investigated. Our results will provide a novel clue on how that *Tclph* affected the insecticides susceptibility and fecundity through regulating *Tcest4* and *Tcest6* in *T. castaneum*.

Materials and methods

Insect strains

A laboratory colony of *T. castaneum* Georgia-1 (GA-1) line insects were used for all these experiments and reared 40% relative humidity in whole wheat flour with 5% brewer yeast powder at 30°C under standard conditions (Song *et al*., 2017; Xiong *et al*., 2018).

RNA extraction and cDNA preparation

Samples for early eggs (1 day old), late eggs (3 days old), early larvae (1 day old), late larvae (20 days old), early pupae (1 day old), late pupae (5 days old), early adults (1 day old), late adults (7 days old) were collected during eight developmental stages. Central nervous system, gut, fat body, epidermis and hemolymph were dissected from late larvae of *T. castaneum*. While, epidermis, central nervous system, tentacle, elytra, fat body, malpighian tubule, gut, ovary, and testis were dissected from late adults of *T. castaneum*. Total RNAs were extracted from these samples and tissues by using RNAiso™Plus (TaKaRa, Dalian, China) following the standard protocol. The yields of the isolated RNAs were determined by NanoDrop 2000 Spectrophotometer (Thermo Scientific, Waltham, MA, USA). The RNAs were only used when the Abs260 nm/Abs280 nm ratio was >1.8 and the RNAs integrity was further evaluated by 1% agarose gel electrophoresis. Then, 500 ng of total RNAs was converted to cDNAs by HiScript II Reverse Transcriptase (Vazyme, Nanjing, China).

Quantitative real-time PCR

Quantitative real-time PCR (qRT-PCR) was performed with ChamQ SYBR qPCR Master Mix (High ROX Premixed) (Vazyme) by StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The qRT-PCR was

https://doi.org/10.1017/S0007485318000895 Published online by Cambridge University Press

programmed at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min, finally at 95°C for 15 s, 60°C for 1 min, and 95°C for 15 s. The data are expressed here as the relative mRNA levels normalized to a control gene, *T. castaneum* ribosomal protein S3 (*rps3*) (Begum *et al.*, 2009), using the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001; Wang *et al.*, 2018). The primers were listed in table 1.

Double-strand RNA synthesis and injection

For preparing double-strand RNAs (dsRNAs), gene-specific primers containing a T7 polymerase recognition promoter were designed with the Primer Premier 5.0 (table 1). The primers were used for PCR amplification and PCR products were further used as templates for dsRNAs synthesis with TranscriptAid™ T7 High Yield Transcription Kit (Fermentas, Vilnius, Lithuania). A total of 200 ng of dsRNAs in 200 nl was injected into the body cavity of each larva. Insects injected with an equal volume of physiological buffer only (IB) and non-injected wild type (WT) insects were denoted as negative controls. At least three biological replications were performed for independent injection.

Induction analysis in response to insecticides

Two selected insecticides including carbofuran (purity 98%) and dichlorvos (purity 98%) (Sigma-Aldrich, Munich, Germany) were used to evaluate contact toxicity to late larvae (20 days old). Median lethal concentration (LC50) diluted with acetone were used for each insecticide as follows: 1 mg ml^{-1} for carbofuran and 10 mg ml^{-1} for dichlorvos. At the time point showing the highest RNAi efficiency, approximately 170 µl of each insecticide solution or acetone (control) was used to treat the 50 late larvae from ds-*lph* or control (IB and WT) group for 1 min. Then the treated larvae were placed on a Whatman filter paper for drying in the air (about 2 min), they were transferred into an 8 ml glass vial and kept under the standard conditions as previously described (Lu *et al.*, 2012). After the insecticide treatment, surviving beetles were randomly selected to determine the expression of *Tcest4* and *Tcest6* at each of six follow-up time points (12, 24, 36, 48, 60 and 72 h) by using qRT-PCR. Three biological replicates were performed for each treatment.

Bioassay of insecticide susceptibility

At the time point showing the highest RNAi efficiency, approximately 50 µl of each of two insecticide solution including carbofuran (1 mg ml^{-1}) or dichlorvos (10 mg ml^{-1}) was applied into 15 beetles from ds-*est4*, ds-*est6* or control (IB and WT) group following the procedure described. The mortalities of the treated and control larvae were assessed every 12 h after the insecticide treatment in 3 days. Here, the insects were considered dead if they were unable to move and no response when disturbed with a tweezer or brush, and each bioassay was replicated three times.

Behavior analysis

The larval injections were followed by the observation of the noticeable morphological defects, mortality, and female egg-laying and egg-hatching rate. Eight days adults from six replications of larval RNAi injections were utilized for single pair mating (average 8–10 pairs per replication). Three of these replications were used to examine reproductive recovery by backcrossing with the WT beetles. Eggs laid over 3 days were collected, counted, and held for hatchability measurement and observation of development. The hatching rate of the offspring was investigated at ∼15 days after the eggs were collected.

Imaging of gonads and measurement of eggs size

The gonads were dissected from the injected beetles on eighth day post-adult eclosion. Eggs laid over 3 days were collected following the procedure described in 'Behavior analysis' section for length and width measurement of eggs. Images of gonads and eggs were taken using an Olympus SZX16 (Olympus Corporation, Tokyo, Japan) stereomicroscope. Image-Pro-Express software (Media Cybernetics, Silver Spring, MD, USA) was used to control the microscope, image acquisition, and exportation of TIFF files. The images were processed using Photoshop CS4 software (Adobe Systems Inc., San Jose, CA, USA) for converting RAW format to EPS format, which then were compatible with the Adobe Illustrator version 4.0 software (Adobe Systems Inc.) vector graphics image editor program. The length and width of eggs was measured by using ImageJ software (Schneider *et al.*, 2012).

Determination of enzyme activity

For enzymatic activity determinations, ∼15 larvae were homogenized in 10 volumes of 0.15 M NaCl. The equal amount of homogenate was used to measure the activities of CCEs by spectrophotometrical method following the commercial kits instructions (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Statistical analysis

All data were expressed as the mean ± SE. Fold changes in gene expression between control and treated beetles, differences among the tissues and development stages, and insecticides bioassay were subjected to Student's *t*-test and one-way analysis of variance (ANOVA) in combination with a Fisher's least significant difference (LSD) multiple comparison tests, respectively, by using the SPSS statistics program (Chicago, USA). ***/### indicates $P < 0.001$, **/## indicates $P < 0.01$, and */# indicates $P < 0.05$.

Results

Tclph *negatively regulated* Tcest4 *and positively regulated* Tcest6 *transcripts*

In order to identify the interaction of *Tcest4* and *Tcest6* with *Tclph*, RNAi was performed. QRT-PCR analyses showed *Tclph*, *Tcest4*, and *Tcest6* were effectively knockdown by the corresponding dsRNA injection (fig. S1). RNAi against *Tclph* further upregulated *Tcest4* expression (2.9-fold, $P < 0.05$) and downregulated *Tcest6* (1.79-fold, $P < 0.05$), respectively. While knockdown *Tcest4* and *Tcest6* did not affect the expression of *Tclph* (fig. 1). However, depression of *Tcest4* remarkably elevated the expression level of *Tcest6*, and *Tcest6* RNAi also increased the expression of *Tcest4*. These results implied that *Tcest4* and *Tcest6* are two downstream genes for *Tclph*,

https://doi.org/10.1017/S0007485318000895 Published online by Cambridge University Press

Table 1. Primers used for qRT-PCR analysis and dsRNA synthesis in this study.

Genes	Sequence (5′–3′)	Product size (bp)	Remarks
RT-PCR			
Tclph	F:ATGTCGCCAAAATCTTTGC R:CCGATACTTTGGGAGGAGGA	262	Q-RT-PCR
Tcest4	F:CGAGACAAGTCAGTAAAGGCATTC R:TGTACCGTCCTTGGTACGAAAAC	192	Q-RT-PCR
Tcest6	F:CGGGATTTGCAAGATGAGGT R:AGTTTGGCGGTTTTGACGATA	143	Q-RT-PCR
TcRp3	F:TCAAATTGATCGGAGGTTTG R:GTCCCACGGCAACATAATCT	260	Q-RT-PCR
dsRNA synthesis			
Tclph	F:taatacgactcactatagggGCTTATGGACTTCCGTTGGT R:taatacgactcactatagggCTTCGAACAACGCAAACACT	483	RNAi
Tcest4	F:taatacgactcactatagggCTACACTTTTTCCCTTTTTCA R:taatacgactcactatagggGCAGTTGTTCCTTTATTCTCA	124	RNAi
Tcest6	F:taatacgactcactatagggGAGTTGGTGGCGATGTTTTCA R:taatacgactcactatagggGTCTCGGTGGTTTCTGCTTGT	237	RNAi

F means forward primers; R means reverse primers. Letters in lowercase are the T7 promoters for dsRNA synthesis.

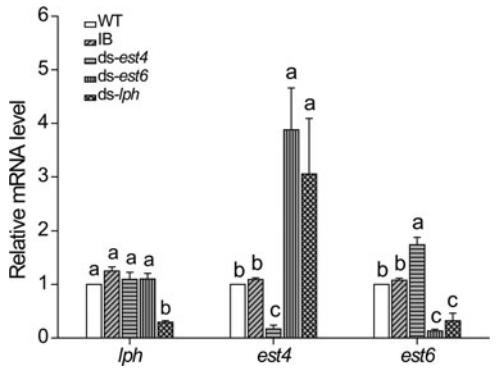

Fig. 1. Interaction of *Tclph*, *Tcest4*, and *Tcest6*. Late larvae were injected with ds-*lph*, ds-*est4*, and ds-*est6*. Physiological buffer-injected beetles (IB) and wild type beetles (WT) provided controls. The induction of *Tclph*, *Tcest4*, and *Tcest6* was separately examined on the five groups (WT, IB, ds-*est4*, ds-*est6*, and ds-*lph*) using qRT-PCR. Data were shown as mean ± SE from three independent experiments. Different letters above the standard error bars indicated significant differences based on the one-way ANOVA followed by Fisher's LSD multiple comparison test ($P < 0.05$).

and *Tclph* negatively regulates *Tcest4* and positively regulates *Tcest6*.

Temporal and spatial expression pattern of Tcest4 *and* Tcest6 *in* T. castaneum

These two *cce* genes were expressed throughout all the stages of development. Typically, *Tcest4* was highly expressed at the late larvae stage, followed by early pupae stage. Whereas *Tcest6* was abundantly accumulated at the late adults stage, followed by early and late pupae stage (fig. 2a). The spatial expression patterns showed *Tcest4* was mainly expressed in central nervous system, as well as slightly expression in other tissues. However, *Tcest6* was predominantly expressed in fat body and hemolymph (fig. 2b).

The induction of Tcest4 *and* Tcest6 *by insecticides treatment*

To investigate how *Tcest4* and *Tcest6* were involved in the detoxification, the expression of *Tcest4* and *Tcest6* were examined at 12, 24, 36, 48, 60, and 72 h after exposure to carbofuran and dichlorvos insecticides. *Tcest4* was increased 2.4-, 2.3-, 1.8-fold at 48, 60, 72 h after exposure to carbofuran treatments, respectively, compared with acetone treatments; it was increased 2.2-, 5.2-, 2.9-fold at 48, 60, 72 h after treated with dichlorvos, respectively (figs 3a, b). While, the expression of *Tcest6* was also elevated 3.5-, 4-, 11.5-, 2.7-fold at 24, 36, 48, 72 h after treated with carbofuran, respectively; and increased 4.9-, 2.3-fold at 48, 72 h after carbofuran treatment, respectively (figs 3c, d).

RNAi against Tcest4 *and* Tcest6 *increased the susceptibility to insecticides*

Knockdown efficiencies of *Tcest4* and *Tcest6* were reached at ∼87 and ∼84% reduction in gene expression, respectively, on the fourth day after RNAi injection (fig. S1). Then, we detected the *T. castaneum* susceptibility to insecticides by treating with LC50 carbofuran and dichlorvos. The mortality statistical data show that the cumulative mortalities were 74.6 and 63.4% in the group injected with ds-*est4* and ds-*est6*, respectively, after treated with carbofuran 72 h (fig. 4a). After treated with dichlorvos 72 h, the cumulative mortalities were 58.7 and 58.6% in ds-*est4* and ds-*est6* injected group, respectively (fig. 4b). All these results showed either *Tcest4* or *Tcest6* knockdown beetles exhibited a significantly faster and higher mortality response compared with controls, which suggested *Tcest4* and *Tcest6* were important in metabolic carbofuran and dichlorvos detoxification in *T. castaneum*.

Tcest4 *was promoted and* Tcest6 *was repressed after insecticides treatment in ds-*lph*-injected beetles*

To further explore that whether *Tclph* participated in insecticides resistance through regulation of *Tcest4* and *Tcest6* in *T. castaneum*, the *Tcest4* and *Tcest6* transcript levels were examined at 12, 24, 36, 48, 60, and 72 h after insecticides treatment in ds-*lph*-injected beetles. As shown in figs 3a, b, when compared with insecticides treatment only, the expressions of *Tcest4*

https://doi.org/10.1017/S0007485318000895 Published online by Cambridge University Press

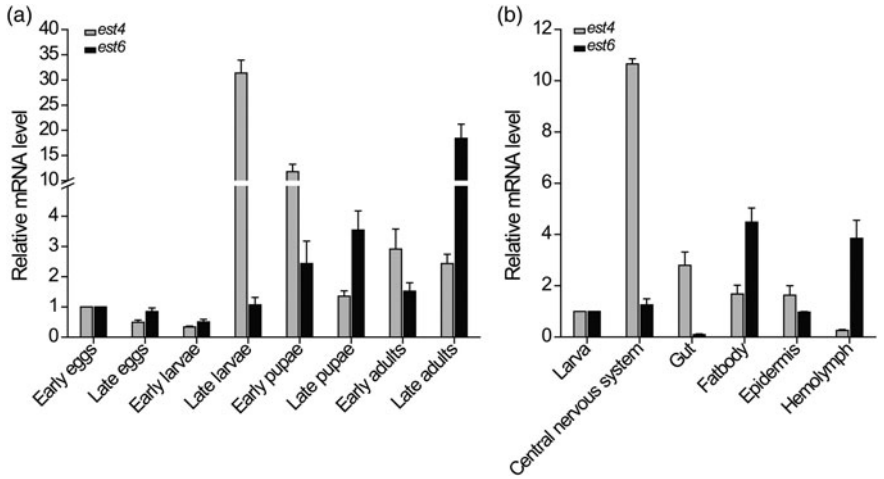

Fig. 2. Temporal (a) and larvae spatial expression patterns (b) of *Tcest4* and *Tcest6* in *T. castaneum* by qRT-PCR. *Tribolium* ribosomal protein 3 (*rps3*) transcript with the same cDNA template served as an internal control. Tissues were isolated from late larvae. Data were shown as mean ± SE from three independent experiments.

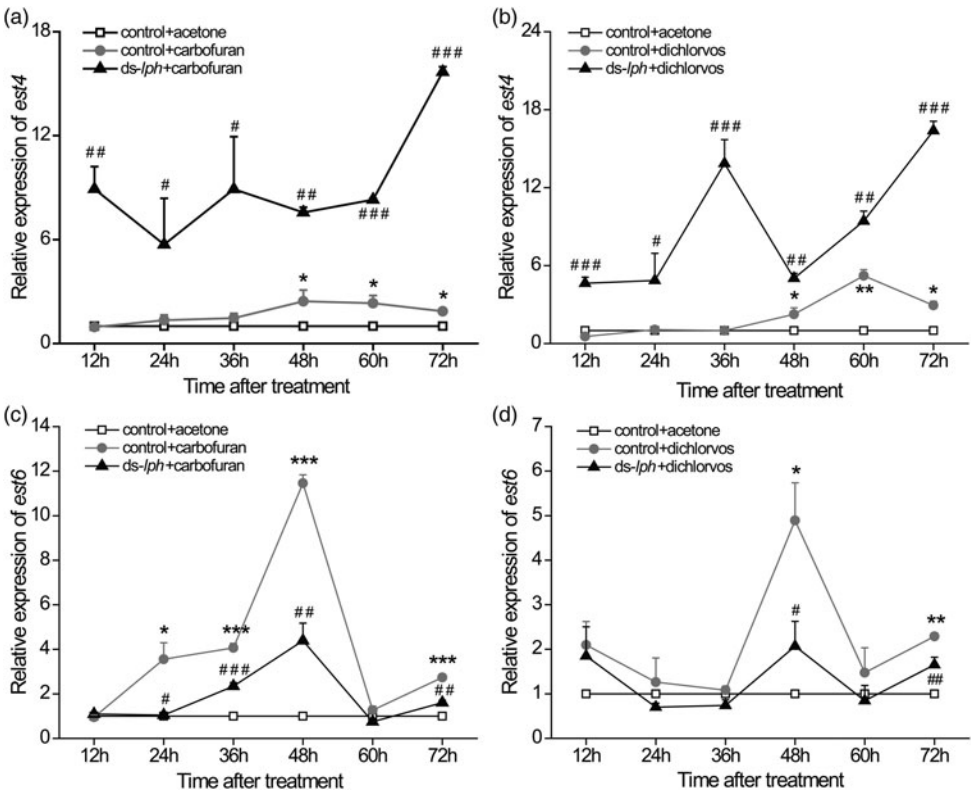

Fig. 3. The response of *Tcest4* and *Tcest6* to insecticides exposure with/without RNAi of *Tclph*. Late larvae of ds-*lph* and control group treated with both carbofuran (10 mg ml^{-1}) and dichlorvos (1 mg ml^{-1}) solution. Acetone-treated IB beetles provided negative controls, which had been ascribed an arbitrary value of 1. The mRNA amounts *Tcest4* and *Tcest6* were determined by qRT-PCR after 12, 24, 36, 48, 60, 72 h later insecticides treated. Data were shown as mean ± SE from three independent experiments. Significant differences between the acetone treated and insecticides treated were identified with '*'; significant differences between insecticides treatment and insecticides treated after ds-*lph* injected were identified with '#'. Three replicates were employed per time point. Statistical significance was assessed by one-way ANOVA followed by Fisher's LSD multiple comparison test (***/###$P < 0.001$, **/##$P < 0.01$, */#$P < 0.05$).

https://doi.org/10.1017/S0007485318000895 Published online by Cambridge University Press

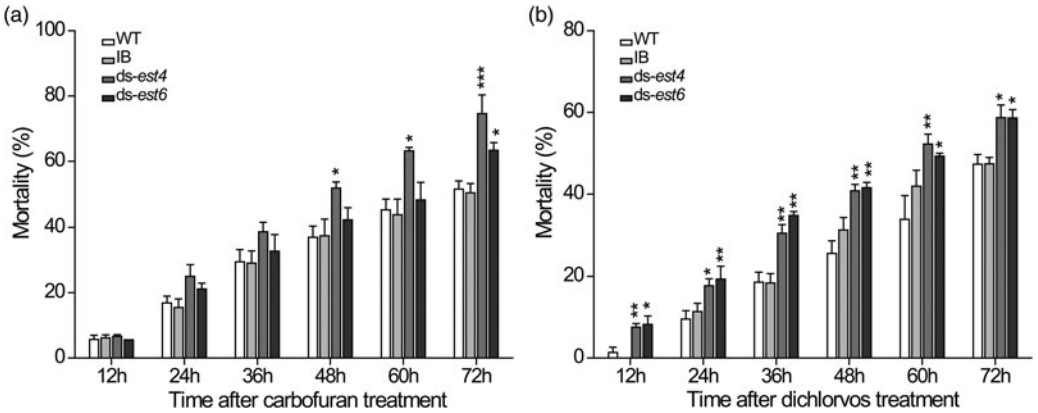

Fig. 4. Susceptibility of *T. castaneum* to carbofuran (a) and dichlorvos (b) after *Tcest4* and *Tcest6* was knockdown. IB and WT served as negative control. Ds-*est4* and ds-*est6* beetles were injected with dsRNA against *Tcest4* and *Tcest6*, respectively. Data were shown as mean ± SE from three independent experiments. '*' indicated significant differences in mortality rates between dsRNA-treated beetles and IB or WT. Statistical significance was assessed by one-way ANOVA followed by Fisher's LSD multiple comparison test (*** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$).

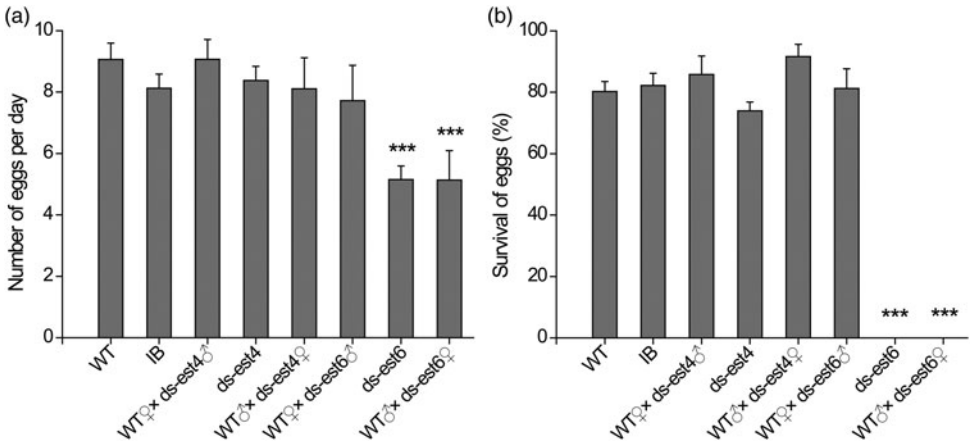

Fig. 5. The effect of RNAi for *Tcest4 and Tcest6* on female egg-laying (a) and egg-hatching rate (b). ♂ and ♀ represented males and females, respectively. WT♀ × ds-*est4*♂, ds-*est4* male crossed with WT female adult; WT♂ × ds-*est4*♀, ds-*est4* female crossed with WT male adult; WT♀ × ds-*est6*♂, ds-*est6* male crossed with WT female adult; WT♂ × ds-*est6*♀, ds-*est6* female crossed with WT male adult. Female egg laying was presented as egg number per female per day. Egg-hatching rate was calculated as the percentage of the number of hatched larvae vs the total number of the eggs laid. Each bar represented mean ± SE of more than 20 pairs of beetles from three independent experiments. Statistical significance between dsRNA-treated beetles and control was assessed by one-way ANOVA followed by Fisher's LSD multiple comparison test (*** $P < 0.001$).

were increased 4.6- to 16.38-fold in ds-*lph* group after carbofuran and dichlorvos treated 12–72 h. However, the expressions of *Tcest6* were reduced 70.5, 42.4, 61.7, and 41.4% in ds-*lph* group after carbofuran treated 24, 36, 48, 72 h, respectively (fig. 3c); and reduced 57.9 and 27.6% after exposure to dichlorvos 48 and 72 h, respectively.

RNAi of Tcest6 *gene reduced female egg-laying and egg-hatching rate*

To identify whether *Tcest4* and *Tcest6* are involved in the fecundity deficiency of *Tclph* in *T. castaenum*, RNAi was performed by knocking down the expression of *Tcest4* and *Tcest6* in 15 days old larvae. Ds-*est6* beetles laid an average of 5.15 ± 0.44 eggs/day/female, while 9.06 ± 0.54 eggs were produced by the WT females per day (fig. 5a). Furthermore, all of the ds-*est6* eggs could not hatch into larva, whereas WT hatching rate is 80.2% (fig. 5b). Meanwhile, the inhibition of number of eggs and survival rate were recovered by backcrossing with WT females, but not WT males (figs 5a, b). However, RNAi of *Tcest4* had no influence on female egg-laying and egg-hatching rate. These results showed that the effect of *Tcest6* RNAi on *T. castaneum* adult fecundity was female specific as well as ds-*lph*. It indicated that *Tclph* may affect the fecundity through the *Tcest6* in *T. castaneum*.

RNAi of Tcest6 *gene affected ovary development and eggs size*

The dissections results showed, in ds-*est6* beetles, the fully developed eggs stuck into the lateral oviducts (75%, $n = 28$).

https://doi.org/10.1017/S0007485318000895 Published online by Cambridge University Press

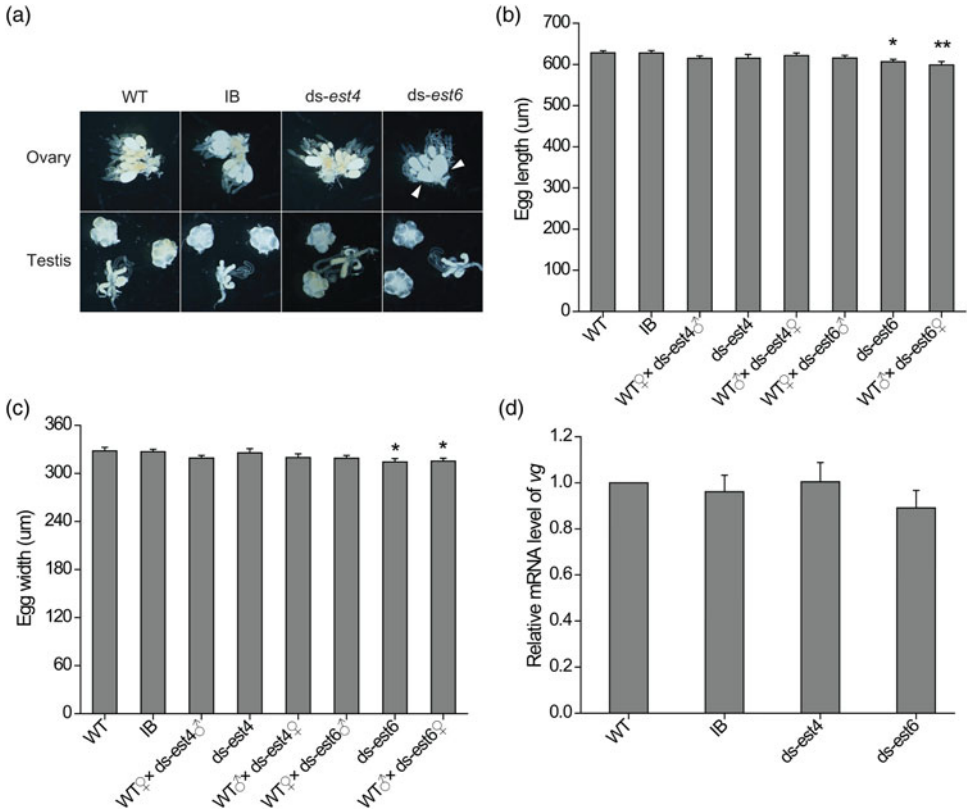

Fig. 6. The effect of *Tcest4* and *Tcest6* on ovary phenotype (a), egg length (b), egg width (c), *vg* expression (d) by larval RNAi. IB and WT served as negative control. Ds-*est4* and ds-*est6* beetles were injected with dsRNA against *Tcest4* and *Tcest6*, respectively. Arrowheads in A indicated the lateral oviducts clogged by mature eggs. ♂ and ♀ represented males and females, respectively. WT♀ × ds-*est4*♂, ds-*est4* male crossed with WT female adult; WT♂ × ds-*est4*♀, ds-*est4* female crossed with WT male adult; WT♀ × ds-*est6*♂, ds-*est6* male crossed with WT female adult; WT♂ × ds-*est6*♀, ds-*est6* female crossed with WT male adult. The mRNA amounts *vg* were determined by qRT-PCR. Each bar represented mean ± SE of more than 15 pairs of beetles. Statistical significance between dsRNA-treated beetles and control was assessed by one-way ANOVA followed by Fisher's LSD multiple comparison test (**$P < 0.01$, *$P < 0.05$).

In ds-*est6* beetles, the mean size of eggs was 606.50 ± 6.05 μm length and 314.43 ± 4.20 μm width, which were significantly shorter than 628.03 ± 5.36 μm length and 328.01 ± 4.50 μm width in WT (WT, $n = 35$) (figs 6a, b, c). Whereas ds-*est4* have no effect on ovarian and egg development (figs 6a, b, c). To identify whether fecundity deficiency of ds-*est6* was due to affecting the *vitellogenin* (*vg*) production, the same as *Tclph*, the expression of *vg* was measured using qRT-PCR. However, the expression of *vg* in ds-*est6* beetles has no significant difference with the control groups (fig. 6d).

Discussion

Our previous study has shown that *Tclph* was involved in the anticholinesterase insecticides susceptibility and fecundity in *T. castaneum* (Gao *et al.*, 2017; Gao *et al.*, 2018). To further identify the regulatory mechanisms of *Tclph* in these important biological processes, we performed the transcriptome analysis between the control and ds-*lph* beetles. The results showed that multiple detoxification enzyme genes including cytochrome P450s (*cyps*), *cces*, odorant-binding proteins (*obps*), and chemosensory proteins (*csps*) were affected in ds-*lph* beetles. Among these, the expression of two genes of *cce* family, *Tcest4* and *Tcest6*, were significantly changed. In this study, we identified the interaction between *Tcest4*, *Tcest6*, and *Tclph* to elucidate the regulation mechanism of these two genes in the *Tclph*-mediated signal pathway.

After carbofuran or dichlorvos treatment, the transcripts of *Tcest4* and *Tcest6* were significantly induced. Knockdown *Tcest4* or *Tcest6*, the mortality of beetles was significantly increased compared with the controls (figs 3 and 4), indicating that *Tcest4* and *Tcest6* were involved in insecticides susceptibility in *T. castaneum*. It is well known that one of the common mechanisms of increased insecticides susceptibility in insects was decreased detoxifications. As the essential members of three detoxification metabolism enzyme systems, *cces* have shown mediated insecticides detoxification in several species. For instance, *cce1* and *cce2* RNAi led to the increased mortality in *L. migratoria* (20.9 and 14.5%, respectively) when chlorpyrifos was applied to this insect (Zhang *et al.*, 2013). Furthermore, *L. migratoria* nymphs injected with *cce9* and *cce25* dsRNAs followed by malathion exposures increased the mortality from 34 to 65% and 54%, respectively (Zhang *et al.*, 2013). In *Bactrocera dorsalis*, *BdcarE4* or *BdcarE6* knockdown flies exhibited a significantly faster and higher mortality response to malathion when compared with controls (Wang *et al.*, 2015). Additionally, it has been shown that insect *cces* could also be involved in resistance to many insecticides through gene amplification, upregulation of mRNA and point mutation. In *Culex quinquefasciatus*, duplication of *est3*

https://doi.org/10.1017/S0007485318000895 Published online by Cambridge University Press

and *est2* in resistant strains contribute to insecticides resistance (De Silva & Hemingway, 2002; Hawkes & Hemingway, 2002). In *Aedes mosquito*, *cceae3a* has been implicated in conferring temephos resistance with upregulation more than 60-fold in resistance population compared with the susceptible population (Poupardin *et al*., 2014). In *L. cuprina* and *M. domestica*, Gly137Asp mutation *of LcαE7* or *MdαE7* in diazinon-type resistant strains led to OPs resistance (Newcomb *et al*., 1997; Claudianos *et al*., 1999). Considering the induced expression of *Tcest4* and *Tcest6* after beetles exposure to insecticides, it suggested that both *Tcest4* and *Tcest6* were involved in insecticides susceptibility in *T. castaneum*.

The spatial expression pattern of *Tcest4* showed that it was abundantly expressed in central nervous system. The similar phenomenon has also been found in *L. migratoria*, *A. gambiae Oxya chinensis*. The *cces* in these species were tissue-specific highly expressed in central nervous system, which have been known to protect the brain and other neural tissues from damages of the peroral xenogenous toxicity (Strode *et al*., 2006; Zhang *et al*., 2014*a*; Zhang *et al*., 2014*b*). While *Tcest6* was highly expressed in fat body and hemolymph, which are known as the primary detoxification organs in insects with many enzymes to digest, neutralize ingested toxins and defend against toxic substances (Yu *et al*., 2009; Zhang *et al*., 2014*a*; Yang, 2016). These results suggest that *Tcest4* and *Tcest6* may differentiate their function but they still share the similar function in detoxification of insecticides.

Further, in ds-*lph* beetles, although *Tcest4* was promoted while *Tcest6* was repressed after insecticides treatment, the CCEs enzyme activity was still reduced compared with the IB, which supports *Tclph* participates in insecticides susceptibility by positively regulating *Tcest6* and negatively regulating *Tcest4* (Fig. S3). But the enhanced expression of *Tcest4* after *Tclph* knockdown (on both cases, either with or without insecticide treatment) is the compensatory effect to the down-expression of *Tcest6*, while it did not completely compensate the effects of the down-expression of *Tcest6*. Moreover, the reduced CCE enzyme activity caused the decreased detoxifications in *T. castaneum* which did not change the trend of beetle's resistance to insecticides after *Tclph* RNAi. Similarly, *Tcest6* also just partially compensate to the effects of RNAi of *Tcest4*, but those beetles still decreased the resistance to insecticides. Interestingly, the compensatory effect between *Tcest4* and *Tcest6* is also been found in other members of *cce* genes. In human, the expression of *cce3* increased after *cce1* silencing in macrophages (Zhao *et al*., 2012). In house flies, loss of *αe7* gene function confers overproduction of the CYP6A1 protein (Sabourault *et al*., 2001). These results indicated that *Tclph* was involved in insecticide susceptibility through positively regulating *Tcest6*, as well as it could partially compensate for insecticide susceptibility by negatively regulating *Tcest4* when the *T. castaneum* larvae losses *lph*.

Moreover, larval RNAi of *Tcest6* in *T. casataneum* reduced female egg-laying and egg-hatching rate with inflate ovarioles, which further confirmed that it had an effect on reproduction (fig. 5 and 6). The similar function has also been found with other members of *cces*. In *S. furcifera*, knockdown *cce* precursor gene *est1* dramatically reduced egg laying and oviposition period, accompanying reduced soluble protein content in ovary and soluble sugar in adult females (Ge *et al*., 2017). In *D. ananassae*, deletion of *est4* caused the female fecundity deficiency with reduced triglycerides level in larval hemolymph (Krishnamoorti & Singh, 2017). These results indicate the role of *cce* genes in reproduction has been interconnected with protein synthesis and lipid metabolism. While some secreted CCEs including JHE and β-esterase regulate reproduction through regulating the hormone and pheromones signal (Oakeshott *et al*., 2005). JHE stimulates reproduction maturation at the adult stage by degrading the content of JH in hemolymph (Feng *et al*., 1999; Hirai *et al*., 2002). β-esterase affected reproductive function though disturbed the pheromone signaling (Ishida and Leal, 2005; Oakeshott *et al*., 2005). However, in *D. melanogaster*, *est6* regulate the fecundity with a novel mechanism. The *est6* in the sperm ejaculatory duct of the adult male fly is transferred to the female fly during mating and modifies its subsequent egg-laying and remating behaviors (Gilbert & Richmond, 1982). Thus, these results demonstrated that *Tclph* RNAi affect *T. castaneum* female reproduction probably mediated by *Tcest6* and further indicated that negatively regulated *Tcest4* most likely is just compensated to downexpression of *Tcest6*.

In this study, we demonstrate two members of *cce* family: *Tcest4* and *Tcest6* are positively involved in the insecticide tolerance of *T. castaneum*. There is a compensatory mechanism existed once one of these two genes was knockdown. *Tclph* participates in the insecticides susceptibility most likely by positively regulating *Tcest6* and negatively regulating *Tcest4* to reduce detoxifications of *T. castaneum*. Moreover, *Tcest6* play important roles in the *Tclph*-mediated reproduction process. Our study provides a new insight to the regulatory mechanism of *Tclph* in insecticides susceptibility and fecundity of *T. castaneum*, we also provide a new potential target for the insecticides. Future study may need to focus on other *Tclph*-downstream genes to fully demonstrate the functional mechanism of *Tclph* in *T. castaneum*.

Supplementary material

The supplementary material for this article can be found at https://doi.org/10.1017/S0007485318000895

Acknowledgements

This work was supported by the National Natural Science Foundation of China (grant number 31572326 & 31872970).

Conflict of interest

None.

References

Begum, K., Li, B., Beeman, R.W. & Park, Y. (2009) Functions of ion transport peptide and ion transport peptide-like in the red flour beetle *Tribolium castaneum*. *Insect Biochemistry and Molecular Biology* **39**, 717–725.

Bonaglia, M.C., Marelli, S., Novara, F., Commodaro, S., Borgatti, R., Minardo, G., Memo, L., Mangold, E., Beri, S. & Zucca, C. (2010) Genotype–phenotype relationship in three cases with overlapping 19p13. 12 microdeletions. *European Journal of Human Genetics* **18**, 1302–1309.

Boucard, A.A., Maxeiner, S. & Südhof, T.C. (2014) Latrophilins function as heterophilic cell-adhesion molecules by binding to teneurins: regulation by alternative splicing. *Journal of Biological Chemistry* **289**, 387–402.

Chen, M.-L. & Chen, C.-H. (2005) Microarray analysis of differentially expressed genes in rat frontal cortex under chronic risperidone treatment. *Neuropsychopharmacology* **30**, 268–277.

https://doi.org/10.1017/S0007485318000895 Published online by Cambridge University Press

Claudianos, C., Russell, R.J. & Oakeshott, J.G. (1999) The same amino acid substitution in orthologous esterases confers organophosphate resistance on the house fly and a blowfly. *Insect Biochem Mol Biol* **29**, 675–686.

De Silva, D. & Hemingway, J. (2002) Structural organization of the estalpha3(1) gene in a Colombian strain of *Culex quinquefasciatus* differs from that in Cuba. *Medical and Veterinary Entomology* **16**, 99–105.

Doyle, S.E., Scholz, M.J., Greer, K.A., Hubbard, A.D., Darnell, D.K., Antin, P.B., Klewer, S.E. & Runyan, R.B. (2006) Latrophilin-2 is a novel component of the epithelial-mesenchymal transition within the atrioventricular canal of the embryonic chicken heart. *Dev Dyn* **235**, 3213–3221.

Durand, N., Chertemps, T., Francois, A., Rosell, G., Dekker, T., Lucas, P. & Maibeche-Coisne, M. (2014) In vivo characterization of an antennal carboxylesterase involved in pheromone response in Drosophila. In Proceedings of the Congress of the European-Chemoreception-Research-Organization, pp. 95.

Feng, Q.L., Ladd, T.R., Tomkins, B.L., Sundaram, M., Sohi, S.S., Retnakaran, A., Davey, K.G. & Palli, S.R. (1999) Spruce budworm (*Choristoneura fumiferana*) juvenile hormone esterase: hormonal regulation, developmental expression and cDNA cloning. *Molecular and Cellular Endocrinology* **148**, 95–108.

Gao, S., Liu, X., Liu, J., Xiong, W., Song, X., Wei, W., Wei, L. & Li, B. (2017) Identification and evolution of latrophilin receptor gene involved in *Tribolium castaneum* development and female fecundity. *Genesis* **55**(12).

Gao, S., Xiong, W., Wei, L., Liu, J., Liu, X., Xie, J., Song, X., Bi, J. & Li, B. (2018) Transcriptome profiling analysis reveals the role of latrophilin in controlling development, reproduction and insecticide susceptibility in *Tribolium castaneum*. *Genetica* **146**, 287–302.

Ge, L.Q., Huang, B., Jiang, Y.P., Gu, H.T., Xia, T., Yang, G.Q., Liu, F. & Wu, J.C. (2017) Carboxylesterase precursor (EST-1) mediated the fungicide jinggangmycin-suppressed reproduction of *Sogatella furcifera* (Hemiptera: Delphacidae). *Journal of Economic Entomology* **110**, 2199–2206.

Gilbert, D.G. & Richmond, R.C. (1982) Esterase 6 in Drosophila melanogaster: reproductive function of active and null males at low temperature. *Proc Natl Acad Sci U S A* **79**, 2962–2966.

Hawkes, N.J. & Hemingway, J. (2002) Analysis of the promoters for the beta-esterase genes associated with insecticide resistance in the mosquito *Culex quinquefasciatus*. *Biochimica et Biophysica Acta* **1574**, 51–62.

Hirai, M., Kamimura, M., Kikuchi, K., Yasukochi, Y., Kiuchi, M., Shinoda, T. & Shiotsuki, T. (2002) cDNA cloning and characterization of *Bombyx mori* juvenile hormone esterase: an inducible gene by the imidazole insect growth regulator KK-42. *Insect Biochemistry and Molecular Biology* **32**, 627–635.

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

Kellendonk, C., Simpson, E.H. & Kandel, E.R. (2009) Modeling cognitive endophenotypes of schizophrenia in mice. *Trends in Neurosciences* **32**, 347–358.

Kim, Y.H., Issa, M.S., Cooper, A.M.W. & Zhu, K.Y. (2015) RNA interference: Applications and advances in insect toxicology and insect pest management. *Pesticide Biochemistry & Physiology* **120**, 109–117.

Krasnoperov, V.G., Bittner, M.A., Beavis, R., Kuang, Y., Salnikow, K.V., Chepurny, O.G., Little, A.R., Plotnikov, A. N., Wu, D. & Holz, R.W. (1997) α-Latrotoxin stimulates exocytosis by the interaction with a neuronal G-protein-coupled receptor. *Neuron* **18**, 925.

Krishnamoorti, K. & Singh, A.K. (2017) Fitness differences due to allelic variation at Esterase-4 locus in *Drosophila ananassae*. *Journal of Genetics* **96**, 625–631.

Lange, M., Norton, W., Coolen, M., Chaminade, M., Merker, S., Proft, F., Schmitt, A., Vernier, P., Lesch, K. & Bally-Cuif, L. (2012) The ADHD-susceptibility gene lphn3. 1 modulates dopaminergic neuron formation and locomotor activity during zebrafish development. *Molecular psychiatry* **17**, 946–954.

Langenhan, T. & Russ, A.P. (2010) Latrophilin signalling in tissue polarity and morphogenesis. *Advances in Experimental Medicine and Biology* **706**, 37–48.

Lelianova, V.G., Davletov, B.A., Sterling, A., Rahman, M.A., Grishin, E.V., Totty, N.F. & Ushkaryov, Y.A. (1997) Alpha-latrotoxin receptor, latrophilin, is a novel member of the secretin family of G protein-coupled receptors. *Journal of Biological Chemistry* **272**, 21504–21508.

Livak, K.J. & Schmittgen, T.D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2 (-Delta Delta C(T)) Method. *Methods* **25**, 402–408.

Lu, Y., Park, Y., Gao, X., Zhang, X., Yao, J., Pang, Y.P., Jiang, H. & Zhu, K.Y. (2012) Cholinergic and non-cholinergic functions of two acetylcholinesterase genes revealed by gene-silencing in *Tribolium castaneum*. *Scientific Reports* **2**, 288.

Mee, C.J., Tomlinson, S.R., Perestenko, P.V., De, P.D., Duce, I.R., Usherwood, P.N. & Bell, D.R. (2004) Latrophilin is required for toxicity of black widow spider venom in *Caenorhabditis elegans*. *Biochemical Journal* **378**, 185–191.

Meza-Aguilar, D.G. & Boucard, A.A. (2014) Latrophilins updated. *Biomolecular Concepts* **5**, 457–478.

Muller, A., Winkler, J., Fiedler, F., Sastradihardja, T., Binder, C., Schnabel, R., Kungel, J., Rothemund, S., Hennig, C., Schoneberg, T. & Promel, S. (2015) Oriented cell division in the *C. elegans* embryo is coordinated by G-protein signaling dependent on the adhesion GPCR LAT-1. *PLoS Genetics* **11**, e1005624.

Newcomb, R.D., Campbell, P.M., Ollis, D.L., Cheah, E., Russell, R.J. & Oakeshott, J.G. (1997) A single amino acid substitution converts a carboxylesterase to an organophosphorus hydrolase and confers insecticide resistance on a blowfly. *Proc Natl Acad Sci U S A* **94**, 7464–7468.

Oakeshott, J.G., Claudianos, C., Campbell, P.M., Newcomb, R.D. & Russell, R.J. (2005) 5.10 – biochemical genetics and genomics of insect esterases. *Comprehensive Molecular Insect Science* **5**, 309–381.

Oh, K.H. & Kim, H. (2017) Aldicarb-induced paralysis assay to determine defects in synaptic transmission in *Caenorhabditis elegans*. *Bio-protocol* **7**(14).

Posbergh, C.J. (2015) A non-synonymous change in latrophilin-3 associated with equine degenerative myeloencephalopathy. In *Proceedings of Plant and Animal Genome Conference (PAG XXIV), San Diego, CA, 10 - 14 January.*, p. 0338

Poupardin, R., Srisukontarat, W., Yunta, C. & Ranson, H. (2014) Identification of carboxylesterase genes implicated in temephos resistance in the dengue vector *Aedes aegypti*. *PLoS Neglected Tropical Diseases* **8**, e2743.

Robin, C., Bardsley, L.M.J., Coppin, C. & Oakeshott, J.G. (2009) Birth and Death of Genes and Functions in the β-Esterase Cluster of Drosophila. *Journal of Molecular Evolution* **69**, 10–21.

Sabourault, C., Guzov, V.M., Koener, J.F., Claudianos, C., Plapp, F.W. Jr. & Feyereisen, R. (2001) Overproduction of a P450 that metabolizes diazinon is linked to a loss-of-function in the

https://doi.org/10.1017/S0007485318000895 Published online by Cambridge University Press

chromosome 2 ali-esterase (MdalphaE7) gene in resistant house flies. *Insect Molecular Biology* **10**, 609–618.

Schneider, C.A., Rasband, W.S. & Eliceiri, K.W. (2012) NIH image to ImageJ: 25 years of Image Analysis. *Nature Methods* **9**, 671–675.

Silva, J.P. & Ushkaryov, Y.A. (2010) The latrophilins, "split-personality" receptors. *Adv Exp Med Biol* **706**, 59–75.

Song, X., Huang, F., Liu, J., Li, C., Gao, S., Wu, W., Zhai, M., Yu, X., Xiong, W., Xie, J. & Li, B. (2017) Genome-wide DNA methylomes from discrete developmental stages reveal the predominance of non-CpG methylation in *Tribolium castaneum*. *DNA Research* **24**, 445–457.

Strode, C., Steen, K., Ortelli, F. & Ranson, H. (2006) Differential expression of the detoxification genes in the different life stages of the malaria vector Anopheles gambiae. *Insect Mol Biol* **15**, 523–530.

Sugita, S., Ichtchenko, K., Khvotchev, M. & Südhof, T.C. (1998) alpha-Latrotoxin receptor CIRL/latrophilin 1 (CL1) defines an unusual family of ubiquitous G-protein-linked receptors. G-protein coupling not required for triggering exocytosis. *Journal of Biological Chemistry* **273**, 32715.

Van Der Voet, M., Harich, B., Franke, B. & Schenck, A. (2016) ADHD-associated dopamine transporter, latrophilin and neurofibromin share a dopamine-related locomotor signature in Drosophila. *Molecular Psychiatry* **21**, 565–573.

Wallis, D., Hill, D.S., Mendez, I.A., Abbott, L.C., Finnell, R.H., Wellman, P.J. & Setlow, B. (2012) Initial characterization of mice null for Lphn3, a gene implicated in ADHD and addiction. *Brain Research* **1463**, 85–92.

Wang, L.L., Huang, Y., Lu, X.P., Jiang, X.Z., Smagghe, G., Feng, Z.J., Yuan, G.R., Wei, D. & Wang, J.J. (2015) Overexpression of two alpha-esterase genes mediates metabolic resistance to malathion in the oriental fruit fly, *Bactrocera dorsalis* (Hendel). *Insect Molecular Biology* **24**, 467–479.

Wang, S., Liu, Y., Zhou, J.J., Yi, J.K., Pan, Y., Wang, J., Zhang, X.X., Wang, J.X., Yang, S. & Xi, J.H. (2018) Identification and tissue expression profiling of candidate UDP-glycosyltransferase genes expressed in *Holotrichia parallela motschulsky* antennae. *Bulletin of Entomological Research* **108**, 807–816.

Xiong, W., Zhai, M., Yu, X., Wei, L., Mao, J., Liu, J., Xie, J. & Li, B. (2018) Comparative RNA-sequencing analysis of ER-based HSP90 functions and signal pathways in *Tribolium castaneum*. *Cell Stress & Chaperones* **23**, 29–43.

Yang, X.Q. (2016) Gene expression analysis and enzyme assay reveal a potential role of the carboxylesterase gene CpCE-1 from Cydia pomonella in detoxification of insecticides. *Pesticide Biochemistry & Physiology* **129**, 56–62.

Yu, Q.Y., Lu, C., Li, W.L., Xiang, Z.H. & Zhang, Z. (2009) Annotation and expression of carboxylesterases in the silkworm, Bombyx mori. *BMC Genomics* **10**, 553.

Zhang, J., Li, D., Ge, P., Guo, Y., Zhu, K.Y., Ma, E. & Zhang, J. (2014a) Molecular and functional characterization of cDNAs putatively encoding carboxylesterases from the migratory locust, Locusta migratoria. *PLoS One* **9**, e94809.

Zhang, J., Ge, P., Li, D., Guo, Y., Zhu, K.Y., Ma, E. & Zhang, J. (2015) Two homologous carboxylesterase genes from Locusta migratoria with different tissue expression patterns and roles in insecticide detoxification. *Journal of Insect Physiology* **77**, 1–8.

Zhang, J., Li, D., Ge, P., Yang, M., Guo, Y., Zhu, K.Y., Ma, E. & Zhang, J. (2013) RNA interference revealed the roles of two carboxylesterase genes in insecticide detoxification in *Locusta migratoria*. *Chemosphere* **93**, 1207–1215.

Zhang, Y.P., Song, D.N., Wu, H.H., Yang, H.M., Zhang, J.Z., Li, L. J., Ma, E.B. & Guo, Y.P. (2014b) Effect of dietary cadmium on the activity of glutathione S-transferase and carboxylesterase in different developmental stages of the Oxya chinensis (Orthoptera: Acridoidea). *Environ Entomol* **43**, 171–177.

Zhao, B., Bie, J., Wang, J., Marqueen, S.A. & Ghosh, S. (2012) Identification of a novel intracellular cholesteryl ester hydrolase (carboxylesterase 3) in human macrophages: compensatory increase in its expression after carboxylesterase 1 silencing. *Am J Physiol Cell Physiol* **303**, C427–435.

https://doi.org/10.1017/S0007485318000895 Published online by Cambridge University Press