Insect Biochemistry and Molecular Biology 113 (2019) 103210

Contents lists available at ScienceDirect

Insect Biochemistry and Molecular Biology

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

CYP303A1 has a conserved function in adult eclosion in *Locusta migratoria* and *Drosophila melanogaster*

Lixian Wu[a,b,c], Qiangqiang Jia[c], Xubo Zhang[a], Xueyao Zhang[a], Suning Liu[c], Yoonseong Park[d], René Feyereisen[e,f], Kun Yan Zhu[d], Enbo Ma[a], Jianzhen Zhang[a,*], Sheng Li[c,**]

[a] Research Institute of Applied Biology, Shanxi University, Taiyuan, Shanxi 030006, China
[b] College of Life Science, Shanxi University, Taiyuan, Shanxi 030006, China
[c] Guangdong Provincial Key Laboratory of Insect Development Biology and Applied Technology, Institute of Insect Science and Technology, School of Life Sciences, South China Normal University, Guangzhou 510631, China
[d] Department of Entomology, Kansas State University, Manhattan, KS 66506, USA
[e] Department of Plant and Environmental Sciences, University of Copenhagen, Copenhagen 1017, Denmark
[f] Department of Plant and Crops, Ghent University, B-9000 Ghent, Belgium

ARTICLE INFO

Keywords:
CYP303A1
Adult eclosion
Locusta migratoria
Drosophila melanogaster
Embryonic development

ABSTRACT

Insect cytochrome P450 monooxygenases (CYPs) play essential roles in both xenobiotic metabolism and developmental processes. However, the exact physiological function of many *CYP* genes remains largely unknown. Screening the expression of the *CYP* genes from the CYP2 and mitochondrial CYP clans of *Drosophila melanogaster* revealed that *Cyp303a1* is highly expressed in the pupal stage. Knockdown of *CYP303A1* transcripts by RNAi using the Gal4/UAS system with a ubiquitous driver (*tubulin-Gal4*) in *Drosophila* or by dsRNA injection in the last nymph stage of *Locusta migratoria* resulted in severe defects in eclosion and lethality during and after adult emergence. In *Drosophila*, tissue-specific RNAi of *Cyp303a1* with a wing-specific driver (*MS1096-Gal4*) revealed that *Cyp303a1* was essential for wing extension. Stage-specific RNAi of *Cyp303a1* using Gal80^{ts} for thermal-dependent-suppression found that the expression of *Cyp303a1* at the middle pupal stage was absolutely required. Meanwhile, *Cyp303a1* mutants exhibited more than 80% lethality at the late embryonic development stages. Embryonic lethality of the *Cyp303a1* mutants was fully rescued by the ubiquitous overexpression of exogenous *Cyp303a1*. Taken together, we conclude that *Cyp303a1* is indispensable for embryonic development and adult eclosion in *D. melanogaster*, the latter role being conserved over 400 million years of insect evolution.

1. Introduction

Cytochrome P450 monooxygenases (CYPs or P450s) are an ancient and conserved superfamily of heme-containing enzymes found in virtually all living organisms from bacteria to human (Werck-Reichhart and Feyereisen, 2000). They play a critical role in oxidation reactions of many physiological and developmental processes. In addition to their roles in endogenous metabolism of hormones, steroids, pheromones and fatty acids, they also participate in metabolism of exogenous molecules, such as drugs, insecticides and plant secondary metabolites (Li et al., 2007; Nelson, 2009; Feyereisen, 2012).

Sequences of numerous insect genomes have revealed that the number of *CYP* genes in different insect species varies greatly, and they are distributed in four phylogenetically distinct clades. According to the P450 nomenclature, these are called the CYP3, CYP4, CYP2 and mitochondrial CYP clans (Feyereisen, 2006). In insects, the gene numbers in the CYP2 and mitochondrial CYP clans are relatively stable, whereas those in the CYP3 and CYP4 clans largely vary (Feyereisen, 2012). While many insect CYP enzymes have been well studied for their roles in xenobiotic metabolism, phytochemical detoxification and insecticide resistance, their potential functions in developmental processes are relatively understudied yet.

The fruit fly, *Drosophila melanogaster*, is a genetic model organism which has more than 80 distinct *CYP* genes (Tijet et al., 2001). A number of *Drosophila CYP* genes are regulated in a developmentally specific manner, suggesting the roles related to developmental processes. It has been reported that several CYP enzymes mediate the biosynthesis of the insect molting hormone (Iga and Kataoka, 2012). *CYP* genes including *Cyp302a1* (*Disembodied, Dib*), *Cyp306a1* (*Phantom, Phm*), *Cyp315a1* (*Shadow, Sad*) and *Cyp307a2* expressed in the

* Corresponding author.
** Corresponding author.
E-mail addresses: zjz@sxu.edu.cn (J. Zhang), lisheng@scnu.edu.cn (S. Li).

https://doi.org/10.1016/j.ibmb.2019.103210
Received 1 May 2019; Received in revised form 15 July 2019; Accepted 15 July 2019
Available online 15 August 2019
0965-1748/

prothoracic glands are involved in the ecdysone biosynthetic pathway along with *Cyp307a1* (*Spook/Spo*) expressed in embryos and ovaries, while *Cyp314a1* (*Shade, Shd*) has a broader tissue expression and is involved in the conversion from ecdysone (E) to 20-hydroxyecdysone (20 E) (Ono et al., 2006; Niwa et al., 2004; Warren et al., 2002; Rewitz et al., 2006; Petryk et al., 2003). These *CYP* genes are generally referred to as "Halloween genes", as knockout of these genes causes embryonic lethality as a result of ecdysteroid biosynthesis deficiency. These genes belong to the CYP2 clan (*Cyp307*, *Cyp306a1*) and the mitochondrial CYP clan (*Cyp302a1*, *Cyp315a1*, *Cyp314a1*). In addition to the previously defined Halloween genes, *Cyp18a1*, which belongs to the CYP2 clan, is involved in 20 E inactivation (Guittard et al., 2011; Rewitz et al., 2010). *Cyp301a1*, which belongs to the mitochondrial CYP clan, was described as an important gene involved in the formation of the adult cuticle, but its biochemical function is unknown (Sztal et al., 2012). Another CYP2 clan member, *Cyp303a1* (*nompH*) is expressed in the socket cells of sensory bristles in *D. melanogaster*, and is essential for the development and structure of external sensory organs (Willingham and Keil, 2004). This P450 is highly conserved in insects with 48% sequence identity between the fly enzyme and the *Locusta migratoria* enzyme. In the locust, the *CYP303A1* gene is highly expressed in embryos with a peak on day 5 of 13 (Zhang et al., 2018).

Here we show that in *Drosophila Cyp303a1* has a similar expression pattern in embryos and is also highly expressed in the wings at the pupal stage. RNAi in both *L. migratoria* and *D. melanogaster* cause a similar lethal phenotype at adult eclosion, with failure to expand wings and complete ecdysis. In addition, the lethal phenotype of *Drosophila Cyp303a1* mutants implies that the gene is also essential for embryonic development. This conserved P450 therefore plays a heretofore unrecognized role in insect development.

2. Materials and methods

2.1. Fly strains and genetic experiments

All fly strains were kept on standard cornmeal/molasses/agar medium at 25 °C unless otherwise stated. The transgenic line *UAS-Cyp303a1-RNAi* (v107902) was obtained from the Vienna *Drosophila* Resource Center (VDRC). w^{1118}, *tubulin-Gal4*, *actin-Gal4*, *Aug21-Gal4* and *Phm-Gal4* were reported previously (Zhang et al., 2013; Wen et al., 2015; Liu et al., 2018). *UAS-Dicer2*, the mutant *Cyp303a1* $^{<ck1>}$ and other *Gal4* lines were obtained from the Bloomington *Drosophila* Stock Center (BDSC). The mutant *Cyp303a1* $^{<AS96>}$ was obtained from the Kyoto Stock Center. The list of fly strains used in this study is in Table S1.

The *UAS-Cyp303a1* construct was generated using pUAST vector by the homologous recombination of an EcoRI/XbaI site fragment containing the full open reading frame (ORF) of *Cyp303a1* cDNA. The transgenic fly *UAS-Cyp303a1* was produced by P-element-mediated germline transformation. The embryos were injected by Core Facility of *Drosophila* Resource and Technology at Shanghai Institute of Biochemistry and Cell Biology (Shanghai, China). Other flies used in this study were generated by recombination.

2.2. Growing and collection of fruit fly

Drosophila wild-type (w^{1118}) embryos were laid at 25 °C for 2 h on standard food in bottles. Groups of animals were collected in triplicate at 24 h intervals immediately after the beginning of the embryonic stage and up to adult eclosion. For pupal stage, groups of pupae were collected in triplicate at 24 h intervals after puparium formation. For egg collections, eggs were laid at 25 °C for 2 h on grape juice agar plate supplemented with yeast. Groups of eggs were collected at every 2 h. Therefore, the sampling time indicated in the data is in the range between the time indicated and the time + 2 h. The collected eggs, larvae, pupae and adults were washed twice with PBS and frozen in liquid nitrogen and stored at − 80 °C until the samples were used for analyses.

2.3. Total RNA extraction and reverse transcription quantitative PCR

Total RNA was extracted from whole body sampled at each developmental stage or from different tissues dissected from white pepupal stage in wild-type (w^{1118}) using TRIzol (Invitrogen, USA). Potential genomic DNA contamination was removed by DNaseI (Invitrogen, USA). Reverse transcription reaction was made from total RNA (2 μg) with polyT primer by using M-MLV reverse transcriptase kit (TaKaRa, Japan). Reverse transcription quantitative PCR (RT-qPCR) was performed using the IQ SYBR Green Supermix (Bio-Rad, USA) and ABI 7300 Real-Time PCR System (Applied Biosystems, USA). RT-qPCR was performed as described previously (Zhao et al., 2018). *Rp49* was used as an internal reference gene (Jia et al., 2017). The primers used for RT-qPCR are listed in Table S2.

2.4. Embryo immunostaining

Embryos were collected at 25 °C on grape juice agar plate 18 h after egg laying (AEL). The eggs were dechorionated in 50% bleach for 2 min, and fixed in formaldehyde -heptane (1:1) for 40 min (Yuan and O'Farrell, 2016). The fixative was then replaced by methanol, the embryos shaken vigorously for 75s and washed three times by methanol. The treated embryos were stored at − 20 °C for immunostaining. Before immunostaining, the embryos were rehydrated gradually (5 min each in 1:3, 1:1, and 3:1 PBT: methanol and then five times for 10 min each in PBT) (PBT consisted of PBS supplemented with 0.5% Triton X-100). They were incubated with anti-Fasciclin III [7G10; the Developmental Studies Hybridoma Bank, (DSHB)] as primary antibody (1:50 in PBT) overnight 4 °C (Enya et al., 2014). The embryos were washed four times for 5 min each in PBT and incubated with Alexa Fluor 594 goat anti-mouse IgG (1:200, A11032; Invitrogen) as secondary antibody for 2 h in the dark at room temperature. They were then washed four times for 5 min each in PBT, incubated with 4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI, 0.5μg/mL, C1002; Beyotime) for 20 min, and washed three times for 5 min each in PBT.

2.5. Imaging

Drosophila phenotypes were photographed using a Nikon DS-Ri2 camera. The embryo immunostaining signals were captured with an Olympus Fluoview FV1000 confocal microscope (Japan) (Liu et al., 2018).

2.6. RNAi of CYP303A1 in L. migratoria

The *LmCYP303A1* and green fluorescent protein (*GFP*) genes were amplified by PCR using dsRNA primers containing the T7 RNA polymerase promoter. The primers used for RNAi analysis are shown in Table S2. Double-stranded RNA (dsRNA) was synthesized using T7 RiboMAX™ Express RNAi System (Promega, USA) as previously described (Yu et al., 2016). The synthesized dsRNA was dissolved in ddH_2O, and the final concentration of dsRNA was adjusted to 2 μg/μl. Approximately 10 μg (5 μl) of ds*LmCYP303A1* or ds*GFP* was injected into the hemocoel between the second and third abdominal segments of 5th instar nymph on the second day using a manual microsyringe (Ningbo, China). dsGFP-injected nymphs were used as control. To determine the suppression level of *LmCYP303A1* transcripts, cDNAs were synthesized from total RNA isolated from whole nymphs 72 h after dsRNA injections. The total RNA was independently isolated for each of the five replications. All data were analyzed by Student's t-test.

2.7. Statistical analysis

All data were analyzed using Student's *t*-test and one-way ANOVA. The values are presented as the mean ± standard deviation of three to six independent experiments. Statistical significance of differences

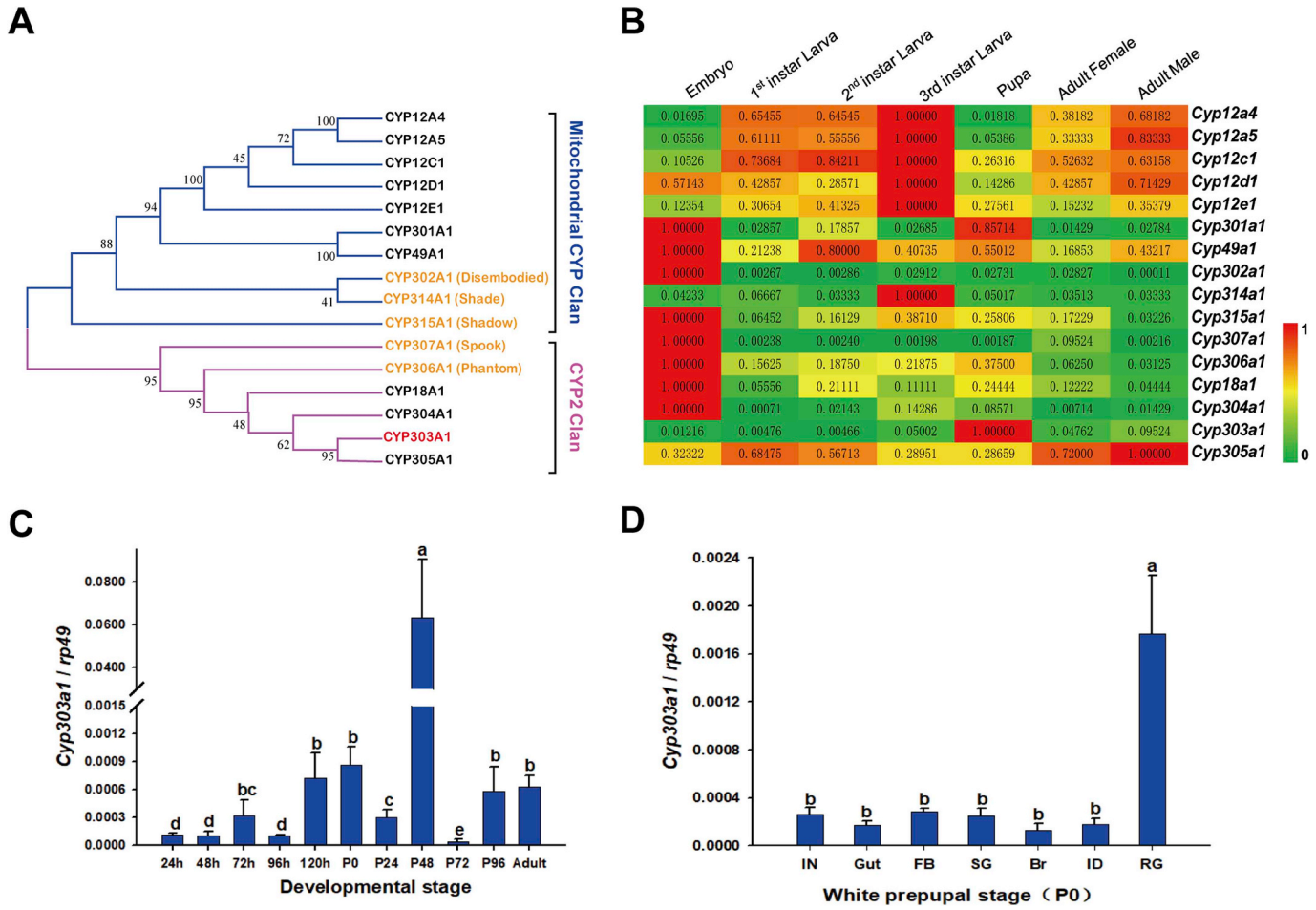

Fig. 1. Phylogenetic tree and expression profiles of members of the *CYP* gene families (CYP2 and Mitochondrial CYP Clans) in *D. melanogaster*. A. Phylogenetic analysis of *D. melanogaster* CYPs from the CYP2 and mitochondrial CYP clans. The phylogenetic tree was constructed with the neighbor joining method of MEGA 5 using the pairwise deletion of indels. Bootstrap support is based on 1000 resampled data sets. **B.** Expression of CYPs at different developmental stages as detected by RT-qPCR. **C.** Expression of *Cyp303a1* at different days as detected by RT-qPCR. Larval stage: 24–120 h after egg laying (AEL); Pupal stage: P0–P96h, 0–96 h after puparium formation. **D.** Expression of *Cyp303a1* in different tissues at white prepupal stage (P0) as detected by RT-qPCR. Different tissues are listed as below. IN: Integument; Gut; FB: Fatbody; SG: Salivary gland; Br: Brain; ID: Imaginal disc; RG: Ring gland. The data are presented as the mean ± standard deviations of three independent biological replications, and bars labeled with different lowercase letters indicate significant differences.

between two groups was determined with the 2-tailed unpaired Student's *t*-test, and $p < 0.05$ was considered statistically significant ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$). The *Cyp303a1* relative expression levels were analyzed using one-way ANOVA followed by Tukey's honest significant difference test ($p < 0.05$). Different lowercase letters on the bars indicate significant difference.

3. Results

3.1. Tissue and development-specific expression of Drosophila Cyp303a1

The CYP2 and mitochondrial CYP clans are relatively well-conserved in insects. A phylogenetic tree for these CYP clans in *D. melanogaster* combined with their expression profiles revealed an interesting pattern (Fig. 1A and 1B). *Cyp18a1*, *Cyp304a1* and four Halloween genes *Cyp302a1* (*disembodied*), *Cyp315a1* (*shadow*), *Cyp307a1* (*spook*) and *Cyp306a1* (*phantom*) were highly expressed in the embryonic stage, whereas *Cyp314a1* (*shade*) was expressed highly in third-instar larvae. CYP12 subfamily genes, containing *Cyp12a4*, *Cyp12a5*, *Cyp12c1*, *Cyp12d1* and *Cyp12e1*, were expressed in both larval and adult stages. In contrast, *Cyp301a1* and *Cyp49a1* were expressed in the embryonic and pupal stages. *Cyp305a1* was constitutively expressed in all developmental stages. Interestingly, we found that *Cyp303a1* was markedly expressed in the pupal stage, suggesting the involvement of this gene in metamorphic development.

The expression pattern of *Cyp303a1* in *Drosophila* was further investigated at a finer scale in the pupal stage and in different tissues. The highest expression was observed at 48 h after puparium formation (48 h APF or P48), followed by white prepupal stage (P0) and 120 h AEL (120 h) (Fig. 1C). Its expression level at P48 is hundreds of times more than in larval or adult stage, confirming its pupa-specific expression pattern (Fig. 1B). The tissue-specific expression pattern at P0 revealed that *Cyp303a1* was expressed in all tested tissues and had its highest relative expression level in the ring gland (RG), which contains the prothoracic gland (PG), corpora allata (CA) and corpora cardiaca (CC) (Fig. 1D).

3.2. Defects in eclosion after silencing of Cyp303a1 in drosophila and L. migratoria

To study *Cyp303a1* function during *Drosophila* development, we examined the phenotypes of flies after loss- or gain of function for *Cyp303a1* by the Gal4/UAS system. *Tubulin-Gal4* is a ubiquitous driver that is expressed throughout the development in all tissues. Interestingly, insects with globally overexpressed *Cyp303a1* (*tub-Gal4 > UAS-Cyp303a1*) molted to adults during the pupal-adult

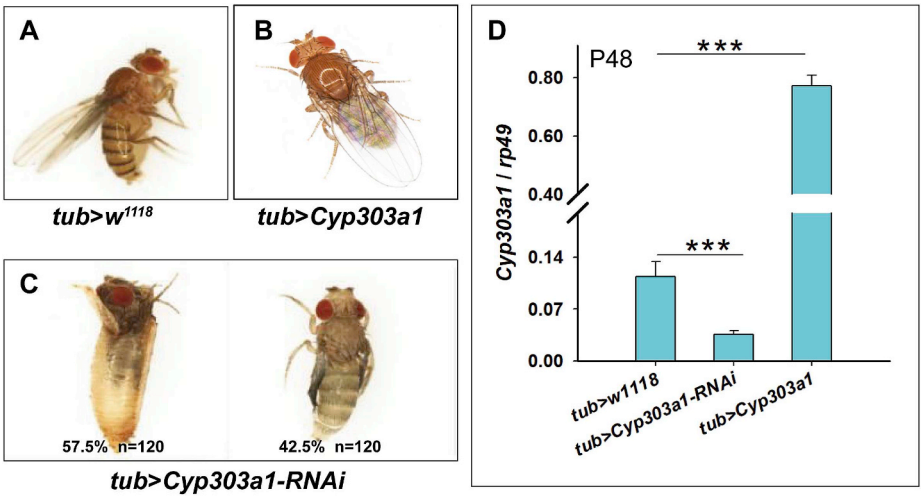

Fig. 2. Gal4/UAS-mediated overexpression or knockdown of *Cyp303a1* in *Drosophila*. A. The adult of *tub* > w^{1118} as control developed normally. **B.** The phenotype of flies with overexpressed *Cyp303a1* by a ubiquitous *Gal4* driver (*tub* > *Cyp303a1*). **C.** The two types of phenotype after knockdown *Cyp303a1* in whole body (*tub* > *Cyp303a1*-RNAi). These flies eventually died. Some flies failed to emerge as adults. Other flies eclosed with unexpanded wings. **D.** Relative expression level of *Cyp303a1* at 48 h APF overexpression or knockdown as detected by RT-qPCR. The data are presented as the mean ± standard deviation of four independent biological replications, asterisks indicate significant differences, (*, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$).

transition without observable phenotype (Fig. 2B). However, silencing the expression of *Cyp303a1* gene globally (*tub-Gal4* > *UAS-Cyp303a1-RNAi*) resulted in some embryonic lethality (see below, Fig. 5B) while the remainder showed phenotypic deficiencies in adult eclosion that can be divided into two subtypes (Fig. 2C). Some flies (~57%) failed to escape from the puparium and died, whereas other flies (~43%) were able to escape from the puparium, but unable to spread the wings and died within 3 h after adult emergence. The expression levels of *Cyp303a1* were compared at P48 by RT-qPCR (Fig. 2D). It was significantly increased in *tub-Gal4* driven overexpression (7 fold) and decreased in RNAi knockdown (by 73%) compared to the control flies (*tub-Gal4* > w^{1118}).

Because CYP303A1 is conserved in insects, we took advantage of feasibility of RNAi by injection in the locust (Yu et al., 2016; Zhao et al., 2018) to determine if the eclosion phenotype was specific to *Drosophila* or more general. Injection of ds*LmCYP303A1* in fifth (last)-instar nymphs was highly effective in suppressing *CYP303A1* mRNA levels (Fig. 3A). It caused a lethal phenotype in 26 of 28 insects. These were trapped in the old cuticle leading to death. Ecdysis was initiated by dorsal rupture of the old pronotum cuticle, but the wings failed to expand and to escape from the pterotheca (Fig. 3B). In contrast, all ds*GFP* injected nymphs molted and ecdysed normally into adults. This phenotype at adult eclosion was similar to that seen in the fly.

Next, we focused on *Drosophila* because it is genetically more tractable than the locust. We performed *Cyp303a1* RNAi experiments by using tissue specific *Gal4* drivers that have been previously characterized. These *Gal4* lines were chosen for expression in wing disc, muscles, gut, fat body, Malpighian tubules, hemolymph, trachea, central nervous system, prothoracic gland, corpora allata and corpora cardiaca (Table 1). In addition to another ubiquitous driver *actin-Gal4* showing the similar phenotype to *tubulin-Gal4*, only wing-specific knockdown of *Cyp303a1* resulted in flies emerging with unextended wings (Fig. 4C–D). Moreover, *Cyp303a1* expression levels in the wings were compared with whole body (without wings) at 48 h APF by RT-qPCR (Fig. 4E). *Cyp303a1* was significantly expressed in the wings. Taken together, these results imply that *Cyp303a1* plays a key role during the process of wing extension and adult eclosion.

3.3. *Cyp303a1* suppression in mid-pupal stage causes eclosion defects

To further determine the periods of *Cyp303a1* requirement during metamorphic development, we exploited a temperature-sensitive protein Gal80^{ts} to suppress expression of the *Cyp303a1* gene at different developmental stages (Li et al., 2017). The Gal80^{ts} is functional as negative regulator of Gal4 at low temperature (18 °C), while it loses function at the shifted temperature (29 °C) (McGuire and Davis, 2003). When thermal shifts to 29 °C were made to suppress *Cyp303a1* expression only in the embryonic or larval stages, insects normally emerged into adults (Fig. 5a-b). However, when thermal shifts were made to inhibit *Cyp303a1* expression only in the pupal stage, insects

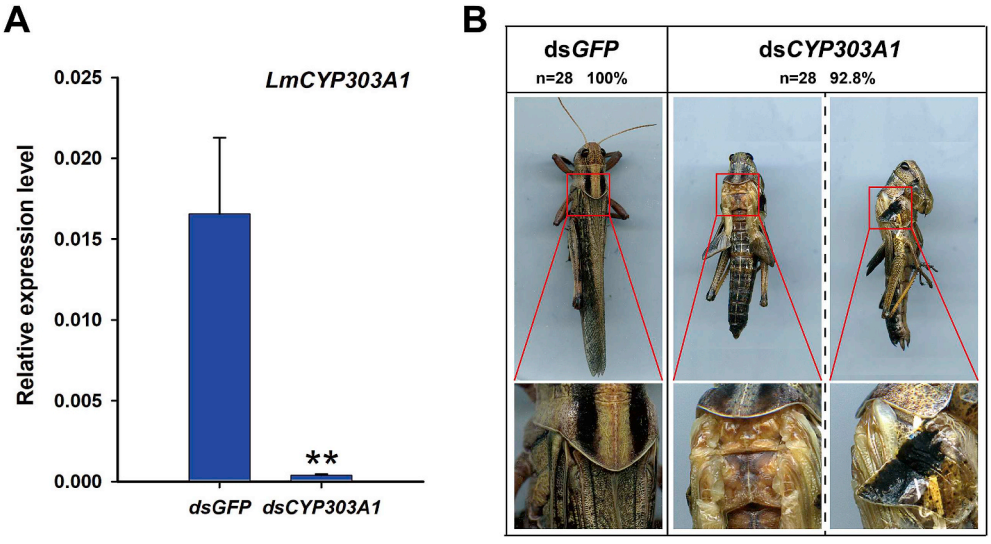

Fig. 3. Effects of ds*LmCYP303A1* injection in 5th instar nymphs on the *LmCYP303A1* transcript level and development of *L. migratoria*. A. Relative expression level of *LmCYP303A1* after the ds*LmCYP303A1* injection as detected by RT-qPCR. The data are presented as the mean ± standard deviation of four independent biological replications, asterisks indicate significant differences, (*, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$). B. The phenotypes of the nymphs after ds*LmCYP303A1* injection. All 5th instar nymphs after the ds*GFP* injection developed into adults successfully. 92.8% 5th instar nymphs after the ds*LmCYP303A1* injection could not develop into adults successfully, and were trapped in the old cuticle leading to death.

Table 1
The effect of targeted expression of *UAS-Cyp303a1-RNAi* using several *Gal4* drivers on flies.

Parental genotype	Expression specificity	Lethality
UAS-Cyp303a1-RNAi × *Tubulin-Gal4*	Ubiquitous	Eclosion lethality
UAS-Cyp303a1-RNAi × *Actin-Gal4*	Ubiquitous	Eclosion lethality
UAS-Cyp303a1-RNAi × *MS1096-Gal4*	Wing disc	Defected in the wing extension
UAS-Cyp303a1-RNAi × *c601-Gal4*	Gut, Malpighian tubules	Fully viable
UAS-Cyp303a1-RNAi × *Cg-Gal4*	Hemolymph, fat body,	Fully viable
UAS-Cyp303a1-RNAi × *Hml-Gal4*	Hemolymph	Fully viable
UAS-Cyp303a1-RNAi × *ppl-Gal4*	Fat body	Fully viable
UAS-Cyp303a1-RNAi × *btl-Gal4*	Tracheal cells	Fully viable
UAS-Cyp303a1-RNAi × *Mef2-Gal4*	Muscles	Fully viable
UAS-Cyp303a1-RNAi × *CNS-Gal4*	Nervous system	Fully viable
UAS-Cyp303a1-RNAi × *phm-Gal4*	Prothoracic gland	Fully viable
UAS-Cyp303a1-RNAi × *Aug21-Gal4*	Corpora allata	Fully viable
UAS-Cyp303a1-RNAi × *AKH-Gal4*	Corpora cardiaca	Fully viable

Transgenic *UAS-Cyp303a1-RNAi* virgin females were crossed to males of several *Gal4* drivers. The expression pattern and lethal stage are noted.

were deficient in eclosion (Fig. 5c). Because the *Drosophila* pupal stage is about 9 days at 18 °C, we further silenced *Cyp303a1* at different time points in the pupal stage, including 48 h APF (Figs. 4d), 96 h APF (Figs. 5e), 144 h APF (Figs. 5f) and 192 h APF (Fig. 5g). Interestingly, insects with *Cyp303a1* suppression from 192 h APF could emerge into adults successfully, whereas other groups were abnormal. These results suggest that *Cyp303a1* plays a role at mid-pupal stage.

3.4. Deficiency in embryonic development in Cyp303a1 mutants

Previous studies have reported that *Cyp303a1* is mainly expressed at fourth- and fifth-day of egg stage in *Locusta migratoria* (Zhang et al., 2018). We investigated the expression pattern of *Cyp303a1* in *D. melanogaster* embryonic development using RT-qPCR, which revealed two major peaks. The first peak occurred at 6–12 h AEL, and the second smaller peak appeared at 20 h AEL (Fig. 6A). To define whether *Cyp303a1* gene expression is required at the embryonic stage, the phenotypes of two mutants from one spontaneous and one EMS-induced allele, renamed for convenience as *Cyp303a1* $^{<ck1>}$ and *Cyp303a1* $^{<AS96>}$, respectively, were examined (Ashburner et al., 1999). We identified the point mutations in the open reading frame of *Cyp303a1* in the mutants, while previous studies have not described molecular lesions of these mutants. The *Cyp303a1* $^{<AS96>}$ mutant line carried a P28L mutation, while *Cyp303a1* $^{<ck1>}$ contained a P350L mutation (Fig. S2A). The P28L mutation would affect the "hinge" or "PPGP" region between the transmembrane N-terminal and the globular CYP enzyme, thus destabilizing the membrane association of the CYP. The P350L mutation is predicted in helix J. It is possible that the mutations seen in the open reading frame of *Cyp303a1* are not themselves responsible for the phenotypes observed, but that they are markers for *Cyp303a1* haplotypes in which mutations in the 5' UTR would negatively affect expression. The homozygous mutants *Cyp303a1* $^{<ck1>}$ and *Cyp303a1* $^{<AS96>}$ exhibited a compelling 87.3% and 90.3% lethality at the embryonic stage, respectively (Fig. 6B). To rule out possible effects of mutations outside the *Cyp303a1* gene, we used transheterozygous mutants (*Cyp303a1* $^{<ck1>}$ / *Cyp303a1* $^{<AS96>}$). These also showed high lethality of 82.5%, and the rest died during or immediately after adult eclosion (Fig. 6B). Meanwhile, besides the majority of lethality during or immediately after adult eclosion, silencing the expression of *Cyp303a1* gene globally (*tub-Gal4* > *UAS-Cyp303a1-RNAi*) resulted in 21.3% lethality at the embryonic stage. Moreover, increasing interference efficiency (~97%) by driving *Dicer2* (Fig. S1), it caused 53.9% lethality at embryonic stage (Fig. 5B).

We further investigated the arrested embryonic stage by using immunohistochemistry of Fasciclin III (Fas III) in the homozygous mutant embryos of *Cyp303a1* $^{<ck1>}$ and *Cyp303a1* $^{<AS96>}$. Anti-Fasciclin III staining revealed that the developmental arrest occurred in the late embryonic development at stage 16–17 (18–20 h AEL) in the *Cyp303a1* mutants showing an abnormal dorsal vessel (Fig. 6C). Compared to the known ecdysteroidogenic genes exhibiting a typical Halloween-class embryonic phenotype (Niwa et al., 2004; Warren et al., 2002; Rewitz et al., 2006; Petryk et al., 2003), the *Cyp303a1* mutants have a later stage of arrest in the embryonic development after near completion of dorsal closure. The phenotypic characteristics of *Cyp303a1* $^{<ck1>}$ and *Cyp303a1* $^{<AS96>}$ were indistinguishable, dying shortly before dorsal closure completion (Fig. 6C).

3.5. Cyp303a1 mutants are rescued by overexpression of exogenous Cyp303a1

To verify the function of *Cyp303a1* in embryonic development, we conducted rescue experiment. When *Cyp303a1* was overexpressed using *tubulin-Gal4* driver, all homozygous mutants (*Cyp303a1* $^{<ck1>}$ and *Cyp303a1* $^{<AS96>}$) and transheterozygous mutants (*Cyp303a1* $^{<ck1>}$ / *Cyp303a1* $^{<AS96>}$) embryos developed normally and emerged into adults (Table 2). For *Cyp303a1* $^{<ck1>}$ homozygous mutant, in the off-

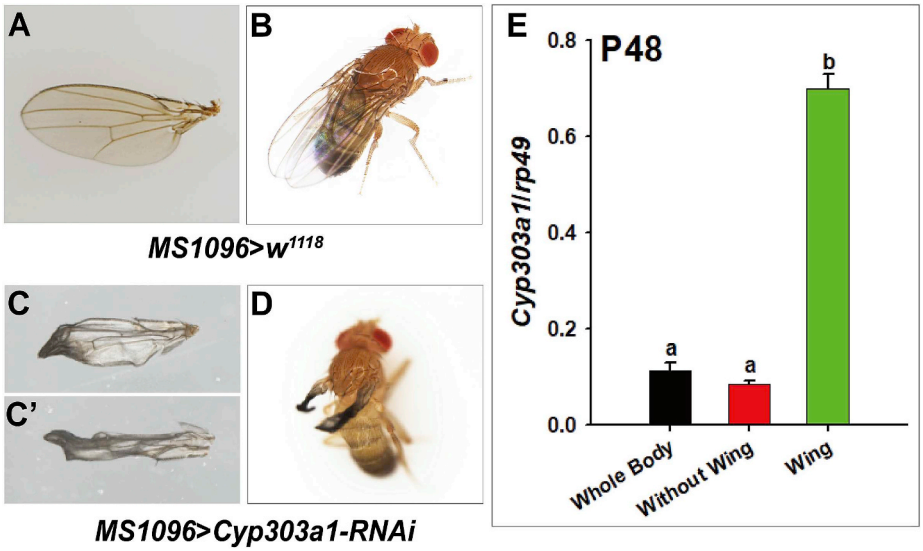

Fig. 4. The effects of wing-specific knockdown of *Cyp303a1* in *D. melanogaster*. **A-B.** The wing and adult of *MS1096* > w^{1118} as control developed normally. **C-D.** The wing phenotypes after wing-specific knock-down *Cyp303a1* (*MS1096* > *Cyp303a1*-RNAi). **E.** Relative expression level of *Cyp303a1* at 48 h APF in whole body, wing and without wing of w^{1118} flies by RT-qPCR. Lowercase letters indicate significant differences ($p < 0.05$) according to ANOVA followed by Tukey's HSD test. The data are presented as the mean ± standard deviation of four independent biological replications.

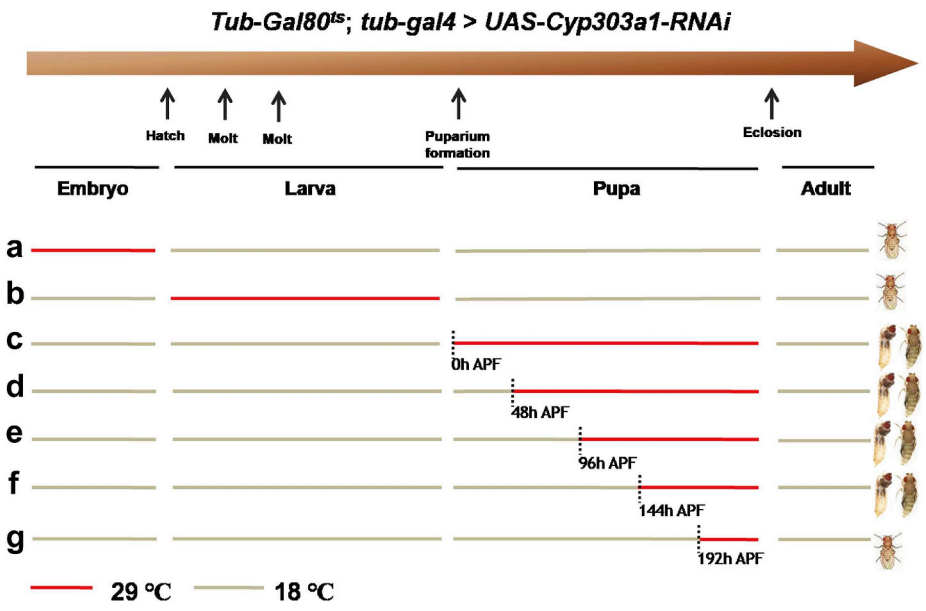

Fig. 5. *Cyp303a1* is required at pupal stage for development in *D. melanogaster*. a-b. No visible phenotype was observed when silencing *Cyp303a1* at embryonic stage or larval stage. **c.** The *Cyp303a1-RNAi* phenotype occurred when silencing *Cyp303a1* at whole pupal stage. *Drosophila* pupal stage is about 9 days at 18 °C. **d-g.** *Cyp303a1* gene was silenced at different time points during the pupal stage, including 48 h APF (**d**), 96 h APF (**e**), 144 h APF (**f**) and 192 h APF (showing no visible phenotype) (**g**). Gal80ts binds *tub-Gal4* and inhibits its transcription activity at 18 °C, but relieves the inhibition of *tub-Gal4* and allows its binding to the UAS sites to drive the expression of *UAS-Cyp303a1-RNAi* at 29 °C. The red line indicates that fruit flies are raised at 29 °C, and the brown line represents that fruit flies are kept at 18 °C. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

springs of *Cyp303a1* $^{<ck1>}$ */cyo-GFP;tub-Gal4/TM6B* × *Cyp303a1* $^{<ck1>}$ */cyo-GFP;UAS-Cyp303a1*, a total of 38 overexpressed (*tub-Gal4/UAS-Cyp303a1*) progenies were recovered, of which 13 were rescued homozygous adults, *Cyp303a1* $^{<ck1>}$ */Cyp303a1* $^{<ck1>}$ *;tub-Gal4/UAS-Cyp303a1* was found showing neither *Cyo* (wings curling upward and outward) and *TM6B* (larvae, pupae short and stout) phenotypes (Fig. S2B).

For the *Cyp303a1* $^{<AS96>}$ homozygous mutant, a total of 51 overexpressed progenies were recovered, of which 15 *Cyp303a1* $^{<AS96>}$ */Cyp303a1* $^{<AS96>}$ *;tub-Gal4/UAS-Cyp303a1* homozygous adults was observed (Fig. S2C). Meanwhile, transheterozygous mutant (*Cyp303a1* $^{<ck1>}$ */Cyp303a1* $^{<AS96>}$), a total of 68 overexpressed progenies were recovered, of which 21 *Cyp303a1* $^{<ck1>}$ */*

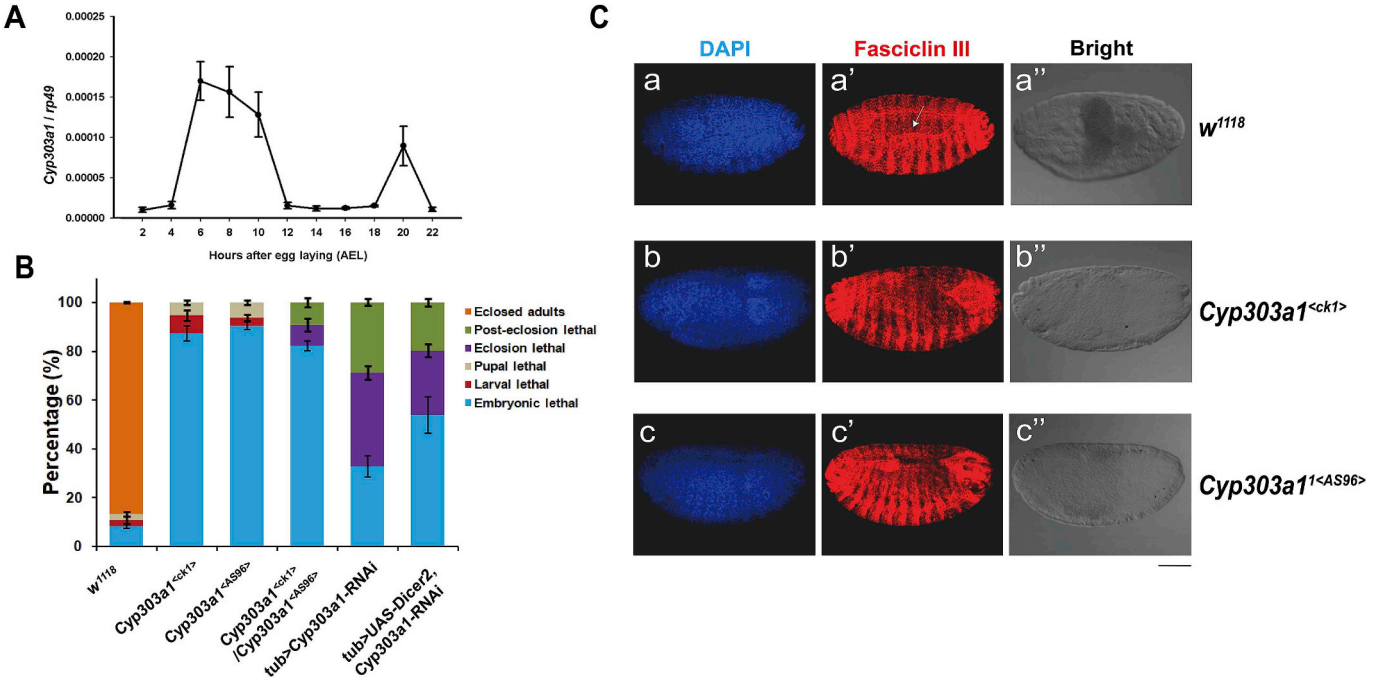

Fig. 6. *Cyp303a1* is also essential for embryonic development in *Drosophila*. A. Relative expression levels of *Cyp303a1* as determined by RT-qPCR at embryonic stage in *Drosophila*. The data are presented as the mean ± standard deviation of five independent biological replications. **B.** Lethality of homozygous mutants (*Cyp303a1* $^{<ck1>}$ and *Cyp303a1* $^{<AS96>}$), transheterozygous mutants (*Cyp303a1* $^{<ck1>}$ */Cyp303a1* $^{<AS96>}$) and knockdown of *Cyp303a1* globally. Lethality of *Cyp303a1* $^{<ck1>}$ homozygous mutant during the embryonic, larval, and pupal stages is 87.3, 7.5 and 5.2%, respectively. Lethality of *Cyp303a1* $^{<AS96>}$ homozygous mutant during the embryonic, larval, and pupal stages is 90.3, 3.6 and 6.1%, respectively. Lethality of transheterozygous mutants (*Cyp303a1* $^{<ck1>}$ */Cyp303a1* $^{<AS96>}$) during the embryonic, eclosion and post-eclosion is 82.25, 8.5 and 9.25%, respectively. Lethality of *tub* > *Cyp303a1-RNAi* during the embryonic, eclosion and post-eclosion stages is 21.4, 44.9 and 33.7%, respectively. Lethality of *tub* > *UAS-Dicer2,Cyp303a1-RNAi* during the embryonic, eclosion and post-eclosion stages is 53.9, 26.3 and 19.8%, respectively. *w*^{1118} was used as control. **C.** The phenotype of homozygous *Cyp303a1* mutant at embryonic stages 16–17. (a-a") The wild-type (*w*^{1118}) embryos. (b-b") Homozygous *Cyp303a1* $^{<ck1>}$ embryos. (c-c") Homozygous *Cyp303a1* $^{<AS96>}$ embryos. DAPI labeling Nuclei (blue), Fasciclin III antibody staining to highlight overall embryo morphology (red). The white arrow indicated the dorsal vessels. Scale bars: 100 μm for all images. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 2
UAS-Cyp303a1 rescue of Cyp303a1 mutants.

Parental genotype (female × male)	F1 Genotype	
	Homozygote/Transheterozygote (without *cyo-GFP*)	Heterozygote (with *cyo-GFP*)
Cyp303a1 $^{<ck1>}$*/cyo-GFP; UAS-Cyp303a1* × *Cyp303a1* $^{<ck1>}$*/cyo-GFP; tub-Gal4/TM6B*	13	25
Cyp303a1 $^{<AS96>}$*/cyo-GFP; UAS-Cyp303a1* × *Cyp303a1* $^{<AS96>}$*/cyo-GFP; tub-Gal4/TM6B*	15	36
Cyp303a1 $^{<ck1>}$*/cyo-GFP; UAS-Cyp303a1* × *Cyp303a1* $^{<AS96>}$*/cyo-GFP; tub-Gal4/TM6B*	21	47

Data are analyzed by Chi-square test, and $\chi^2 < \chi^2_{0.05,}$ $P > 0.05$, these results fit into 1:2 expected ratio.

Cyp303a1 $^{<AS96>}$*;tub-Gal4/UAS-Cyp303a1* adults was observed. Exogenous *Cyp303a1* overexpression rescued the embryonic lethality of *Cyp303a1* mutants, indicating that *Cyp303a1* is indispensable for normal embryonic development. Furthermore, *Phm-Gal4 > UAS-Cyp303a1* was used to attempt to rescue the mutants, but these insects still showed the similar lethal phenotypes and were not rescued.

4. Discussion

CYPs play important roles in physiological and developmental processes. Some conserved *CYP* genes are involved in biosynthesis and inactivation of ecdysteroids (Niwa et al., 2004; Warren et al., 2002; Rewitz et al., 2006, 2010; Petryk et al., 2003; Guittard et al., 2011). In addition, CYP4G subfamily genes have been shown to be essential for the synthesis of cuticular hydrocarbons (Qiu et al., 2012; Yu et al., 2016; Chen et al., 2016; Balabanidou et al., 2016; Kefi et al., 2019), and the CYP15 enzymes catalyze the epoxidation of sesquiterpenoid precursors to juvenile hormones in corpora allata (Helvig et al., 2004; Daimon et al., 2012; Daimon and Shinoda, 2013). However, although most CYPs remain functional "orphans", many probably have precise physiological functions (Feyereisen, 2015). In this study, we report that the *Drosophila Cyp303a1* gene, a member of the CYP2 clan, is highly expressed at the pupal stage and in the wings at 48 h APF. We also show that *Cyp303a1* is essential for embryonic development and adult eclosion in *Drosophila*, with an RNAi phenotype that is very similar to that elicited at adult eclosion in the locust. The conservation of the CYP303A1 sequence is therefore linked to the conservation of its physiological function over 400 MY of insect evolution.

CYP303 is a highly conserved CYP450 with a single ortholog in most insect species studied to date (Feyereisen, 2012). In *D. melanogaster*, by comparing RG with whole body transcripts, it has been shown that *Cyp303a1* has high RG-specific expression (Ou et al., 2016), which is consistent with our results (Fig. 1D). It is important to note that *Cyp303a1* expression in the wings at 48 h APF is hundreds of times higher than that in RG at P0 (Figs. 1C and 4E). Moreover, when *UAS-Dicer2* was combined with *phantom-Gal4* to enhance RNAi efficiency specifically in the PG, no phenotypic changes were observed either. The data indicate that CYP303A1 might play little or non-essential role in the PG or RG. In that respect, *Cyp303a1* is not alone or remarkable, as seven other non-Halloween genes are overexpressed in the PG (Christesen et al., 2017), yet fail to show a phenotype by phantom-directed RNAi (Danielsen et al., 2016), while four other P450 genes do have such a phenotype without being overexpressed in the ring gland. It has been reported that *Cyp303a1* (*nompH*) is expressed in the apical region of the socket cell in sensory bristles and is essential for the development and structure of these sensory organs in adult *Drosophila* (Willingham and Keil, 2004). Our phenotypes show that this is not the only, or perhaps the most important role of CYP303A1. These authors did not report other phenotypes, although mentioning obscurely some adult mortality in hemizygous mutant flies. Chung et al. (2009) reported expression in trachea and head of late stage embryos but did not report an RNAi phenotype. Other expression surveys (FlyBase FBgn0001992) indicate overall low or undetectable expression, except in the late pupal stage. The embryonic and eclosion phenotypes we report are more far reaching than these earlier observations.

In our study, *Cyp303a1* is highly expressed in the wings at 48 h APF, and wing-specific silencing of *Cyp303a1* expression (*MS1096 > UAS-Cyp303a1-RNAi*) results in flies emerging with unextended wings. It is well known that bursicon, through its receptor rickets (rk), controls post-eclosion development, and *Bursicon* and *rk* mutations failed to spread their wings following eclosion (Dewey et al., 2004; Diao and White, 2012). As a similar phenotype was observed in the flies when *Cyp303a1* was silenced, our results imply that *Cyp303a1* might be involved in this developmental process.

RNAi of the orthologous *CYP303A1* in *L. migratoria* by injection of ds*LmCYP303A1* had profound effects. The locust nymphs initiated ecdysis but were trapped in the old cuticle leading to death. Critically, lack of wing expansion did not allow escape from the pterotheca. There are mechanical differences in ecdysis from a pupal cuticle (flies) and from a last nymphal cuticle (locusts), and the control of the ecdysis process has undergone changes during insect evolution (Arakane et al., 2008). Nonetheless, the phenotypes are highly similar and will need to be more closely analyzed in the locust. This similarity may explain why *CYP303A1* is one of the most conserved *CYP* genes in insect.

During the embryonic development in *L. migratoria*, *CYP303A1* is highly expressed at early egg stage (fourth and fifth-day of total 13 embryonic developmental days) (Zhang et al., 2018). It was thus reasonable to hypothesize that *CYP303A1* plays a role in embryonic development of *Drosophila* as well. Indeed we demonstrated that *Cyp303a1* is required for embryonic development in *D. melanogaster* with three crucial evidences. Firstly, zygotic expression *Cyp303a1* occurred at the early and late embryonic stages. Secondly, two mutant strains and transheterozygous mutant of *Cyp303a1* exhibit more than 80% lethality at the late embryonic stage. Thirdly, the embryonic phenotype of mutants was rescued by overexpression of *Cyp303a1* in whole body. A majority of *tub > Cyp303a1-RNAi* animals were able to survive through the embryonic stage, but when interference efficiency was increased by the simultaneous overexpression of *Dicer2*, over half the embryos died. It is likely that RNAi cannot completely eliminate the transcripts or that the enzyme itself has a long half-life at that stage.

The substrate of CYP303A1 is unknown so far. The phylogeny shows that CYP303A1 is closely related in the CYP2 clan to the enzymes in ecdysteroid biosynthesis (CYP307, CYP306A1) and inactivation (CYP18A1). It is also closely related to the CYP15 enzymes of JH synthesis, which, however, have no representative in *Drosophila*. Similarly, several CYP2 clan CYP450s in vertebrates such as CYP2J1, CYP2R1, CYP17A1, CYP21A1 are also involved in sterol, steroid and secosteroid (vitamin D) metabolism (Nebert and Dalton, 2006). The recent finding of a unique expansion of the CYP303 family in the firefly *Photinus pyralis*, including 11 *CYP303* genes and two pseudogenes (Fallon et al., 2018), provides additional arguments for a role in sterol/steroid metabolism. Indeed the CYP303 in the firefly may be associated with synthesis of lucibufagins, which are polyhydroxylated compounds derived from cholesterol. In addition, the embryonic phenotype of *Cyp303a1* mutants, arrested at the late dorsal closure phase after the 20 E peak (Fig. 6C), also suggests a possible role of *Cyp303a1* in ecdysteroid metabolism during embryonic development. High expression of *Cyp303a1* with a number of other *CYP* genes including Halloween genes implies that *Cyp303a1* may play a role in the complex ecdysteroid metabolic process. We note however that the RG-specific RNAi of

Cyp303a1 did not show any phenotype, confirming the observation of Danielsen et al. (2016). This is possibly due to redundant metabolic pathways or to incomplete suppression by RNAi. The fact that *Cyp303a1* is also expressed outside the RG further suggests that this peripheral expression can compensate for the deficit in RG expression, or that RG expression is not essential to *Cyp303a1* function and insect viability. Low level expression in many tissues may be sufficient for CYP303A1 to fulfill its function locally, and may explain why the most prominent RNAi effects are seen with strong, global drivers. Taken together, more specifically than the hypothesized role in metabolizing a small signal molecule (Feyereisen, 2012), we propose that *Cyp303a1* might play a role in metabolism of an ecdysteroid-like molecule. Identifying the substrate of CYP303A1 and the precise pathway affected by CYP303A1 are the object of our current research. In addition, it is also worthwhile to characterize the underlying molecular mechanism in which *Cyp303a1* regulate eclosion and embryonic development in *Drosophila*. Our work adds *Cyp303a1* to the growing list of *CYP* genes involved in essential physiological functions and points to a gap in our knowledge of identity of small signal molecules that are typical CYP substrates.

Acknowledgements

This research was supported by a grant from the National Natural Science Foundation of China (International Cooperation and Exchange Program Grant No. 31320103921 and 31620103917) and the Graduate Outstanding Innovation Projected of Shanxi Province (Grant No. 2017BY017).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ibmb.2019.103210.

References

Arakane, Y., Li, B., Muthukrishnan, S., Beeman, R.W., Kramer, K.J., Park, Y., 2008. Functional analysis of four neuropeptides, EH, ETH, CCAP and bursicon, and their receptors in adult ecdysis behavior of the red flour beetle, *Tribolium castaneum*. Mech. Dev. 125 (11–12), 984–995.

Ashburner, M., Misra, S., Roote, J., Lewis, S.E., Blazej, R., Davis, T., Doyle, C., Galle, R., George, R., Harris, N., Hartzell, G., Harvey, D., Hong, L., Houston, K., Hoskins, R., Johnson, G., Martin, C., Moshrefi, A., Palazzolo, M., Reese, M.G., Spradling, A., Tsang, G., Wan, K., Whitelaw, K., Kimmel, B., Celniker, S., Rubin, G.M., 1999. An exploration of the sequence of a 2.9-Mb region of the genome of *Drosophila melanogaster*: the *Adh* region. Genetics 153 (1) 179.

Balabanidou, V., Kampouraki, A., MacLean, M., Blomquist, G.J., Tittiger, C., Juárez, M.P., Mijailovsky, S.J., Chalepakis, G., Anthousi, A., Lynd, A., Antoine, S., Hemingway, J., Ranson, H., Lycett, G.J., Vontas, J., 2016. Cytochrome P450 associated with insecticide resistance catalyzes cuticular hydrocarbon production in *Anopheles gambiae*. Proc. Natl. Acad. Sci. U.S.A. 113, 9268–9273.

Chen, N., Fan, Y., Bai, Y., Li, X., Zhang, Z., Liu, T., 2016. Cytochrome P450 gene, *CYP4G51*, modulates hydrocarbon production in the pea aphid, *Acyrthosiphon pisum*. Insect Biochem. Mol. Biol. 76, 84–94.

Christesen, D., Yang, Y.T., Somers, J., Robin, C., Sztal, T., Batterham, P., Perry, T., 2017. Transcriptome analysis of *Drosophila melanogaster* third instar larval ring glands points to novel functions and uncovers a cytochrome P450 required for development. G3(Bethesda) 7 (2), 467–479.

Chung, H., Sztal, T., Pasricha, S., Sridhar, M., Batterham, P., Daborn, P.J., 2009. Characterization of *Drosophila melanogaster* cytochrome P450 genes. Proc. Natl. Acad. Sci. U.S.A. 106, 5731–5736.

Daimon, T., Shinoda, T., 2013. Function, diversity, and application of insect juvenile hormone epoxidases (CYP15). Biotechnol. Appl. Biochem. 60 (1), 82–91.

Daimon, T., Kozaki, T., Niwa, R., Kobayashi, I., Furuta, K., Namiki, T., Uchino, K., Banno, Y., Katsuma, S., Tamura, T., Mita, K., Sezutsu, H., Nakayama, M., Itoyama, K., Shimada, T., Shinoda, T., 2012. Precocious metamorphosis in the juvenile hormone–deficient mutant of the silkworm, *Bombyx mori*. PLoS Genet. 8 (3), e1002486.

Danielsen, E.T., Moeller, M.E., Yamanaka, N., Ou, Q., Laursen, J., Soenderholm, C., Zhuo, R., Phelps, B., Tang, K., Zeng, J., Kondo, S., Nielsen, C.H., Harvald, E.B., Faergeman, N.J., Haley, M.J., O'Connor, K.A., King-Jone, K., O'Connor, M.B., Rewitz, K.F., 2016. A *Drosophila* genome-wide screen identifies regulators of steroid hormone production and developmental timing. Dev. Cell 37 (6), 558–570.

Dewey, E.M., Mcnabb, S.L., Ewer, J., Kuo, G.R., Takanishi, C.L., Truman, J.W., Honegger, H.W., 2004. Identification of the gene encoding bursicon, an insect neuropeptide responsible for cuticle sclerotization and wing spreading. Curr. Biol. 14 (13), 1208–1213.

Diao, F., White, B.H., 2012. A novel approach for directing transgene expression in *Drosophila*: T2A-Gal4 in-frame fusion. Genetics 190, 1139–1144.

Enya, S., Ameku, T., Igarashi, F., Iga, M., Kataoka, H., Shinoda, T., Niwa, R., 2014. A Halloween gene noppera-bo encodes a glutathione S-transferase essential for ecdysteroid biosynthesis via regulating the behaviour of cholesterol in *Drosophila*. Sci. Rep. 4, 6586.

Fallon, T.R., Lower, S.E., Chang, C., Bessho-Uehara, M., Martin, G.J., Bewick, A.J., Behringer, M., Debat, H.J., Wong, I., Day, J.C., Suvorov, A., Silva, C.J., Stanger-Hall, K.F., Hall, D.W., Schmitz, R.J., Nelson, D.R., Lewis, S.M., Shigenobu, S., Bybee, S.M., Larracuente, A.M., Oba, Y., Weng, J., 2018. Firefly genomes illuminate parallel origins of bioluminescence in beetles. eLife 7, e36495.

Feyereisen, R., 2006. Evolution of insect P450. Biochem. Soc. Trans. 34 (6), 1252–1255.

Feyereisen, R., 2012. Insect CYP Genes and P450 Enzymes. Insect Molecular Biology and Biochemistry. Academic Press, pp. 236–316 (Chapter 8).

Feyereisen, R., 2015. Insect P450 inhibitors and insecticides: challenges and opportunities. Pest Manag. Sci. 71 (6), 793–800.

Guittard, E., Blais, C., Maria, A., Parvy, J.P., Pasricha, S., Lumb, C., Lafont, R., Daborn, P.J., Dauphin-Villemant, C., 2011. CYP18A1, a key enzyme of *Drosophila* steroid hormone inactivation, is essential for metamorphosis. Dev. Biol. 349 (1), 35–45.

Helvig, C., Koener, J.F., Unnithan, G.C., Feyereisen, R., 2004. CYP15A1, the cytochrome P450 that catalyzes epoxidation of methyl farnesoate to juvenile hormone III in cockroach corpora allata. Proc. Natl. Acad. Sci. U.S.A. 101 (12), 4024–4029.

Iga, M., Kataoka, H., 2012. Recent studies on insect hormone metabolic pathways mediated by cytochrome P450 enzymes. Biol. Pharm. Bull. 35 (6), 838–843.

Jia, Q., Liu, S., Wen, D., Cheng, Y., Bendena, W.G., Wang, J., Li, S., 2017. Juvenile hormone and 20-hydroxyecdysone coordinately control the developmental timing of matrix metalloproteinase–induced fat body cell dissociation. J. Biol. Chem. 292 (52), 21504–21516.

Kefi, M., Balabanidou, V., Douris, V., Lycett, G., Feyereisen, R., Vontas, J., 2019. Two functionally distinct CYP4G genes of *Anopheles gambiae* contribute to cuticular hydrocarbon biosynthesis. Insect Biochem. Mol. Biol. 110, 52–59.

Li, K., Zhang, X., Zuo, Y., Liu, W., Zhang, J., Moussian, B., 2017. Timed Knickkopf function is essential for wing cuticle formation in *Drosophila melanogaster*. Insect Biochem. Mol. Biol. 89, 1–10.

Li, X., Schuler, M.A., Berenbaum, M.R., 2007. Molecular mechanisms of metabolic resistance to synthetic and natural xenobiotics. Annu. Rev. Entomol. 52 (1), 231–253.

Liu, S., Li, K., Gao, Y., Liu, X., Chen, W., Ge, W., Feng, Q., Palli, S.R., Li, S., 2018. Antagonistic actions of juvenile hormone and 20-hydroxyecdysone within the ring gland determine developmental transitions in *Drosophila*. Proc. Natl. Acad. Sci. U.S.A. 115 (1), 139–144.

McGuire, S.E., Davis, R.L., 2003. Spatiotemporal rescue of memory dysfunction in Drosophila. Science 302 (5651), 1765–1768.

Nebert, D.W., Dalton, T.P., 2006. The role of cytochrome P450 enzymes in endogenous signalling pathways and environmental carcinogenesis. Nat. Rev. Cancer 6 (12), 947–960.

Nelson, D.R., 2009. The cytochrome P450 homepage. Hum. Genom. 4 (1), 59–65.

Niwa, R., Matsuda, T., Yoshiyama, T., Namiki, T., Mita, K., Fujimoto, Y., Kataoka, H., 2004. CYP306A1, a cytochrome P450 enzyme, is essential for ecdysteroid biosynthesis in the prothoracic glands of *Bombyx* and *Drosophila*. J. Biol. Chem. 279 (34), 35942–35949.

Ono, H., Rewitz, K.F., Shinoda, T., Itoyama, K., Petryk, A., Rybczynski, R., Jarcho, M., Warren, J.T., Marqués, G., Shimell, M.J., Gilbert, L.I., O'Connor, M.B., 2006. Spook and spookier code for stage-specific components of the ecdysone biosynthetic pathway in diptera. Dev. Biol. 298 (2) 0-570.

Ou, Q., Zeng, J., Yamanaka, N., Brakken-Thal, C., O'Connor, M.B., King-Jones, K., 2016. The insect prothoracic gland as a model for steroid hormone biosynthesis and regulation. Cell Rep. 16 (1), 247–262.

Petryk, A., Warren, J.T., Marqués, G., Jarcho, M.P., Gilbert, L.I., Kahler, J., Parvy, J.P., Li, Y., Dauphin-Villemant, C., O'Connor, M.B., 2003. Shade is the *Drosophila* P450 enzyme that mediates the hydroxylation of ecdysone to the steroid insect molting hormone 20-hydroxyecdysone. Proc. Natl. Acad. Sci. U.S.A. 100 (24), 13773–13778.

Qiu, Y., Tittiger, C., Wicker-Thomas, C., Le Goff, G., Young, S., Wajnberg, E., Fricaux, T., Taquet, N., Blomquist, G.J., Feyereisen, R., 2012. An insect-specific P450 oxidative decarbonylase for cuticular hydrocarbon biosynthesis. Proc. Natl. Acad. Sci. U.S.A. 109 (37), 14858–14863.

Rewitz, K.F., Rybczynski, R., Warren, J.T., Gilbert, L.I., 2006. The Halloween genes code for cytochrome P450 enzymes mediating synthesis of the insect moulting hormone. Biochem. Soc. Trans. 34 (6), 1256–1260.

Rewitz, K.F., Yamanaka, N., O'Connor, M.B., 2010. Steroid hormone inactivation is required during the juvenile-adult transition in *Drosophila*. Dev. Cell 19 (6), 895–902.

Sztal, T., Chung, H., Berger, S., Currie, P.D., Batterham, P., Daborn, P.J., 2012. A cytochrome P450 conserved in insects is involved in cuticle formation. PLoS One 7 (5), e36544.

Tijet, N., Helvig, C., Feyereisen, R., 2001. The cytochrome P450 gene superfamily in *Drosophila melanogaster*: annotation, intron–exon organization and phylogeny. Gene 262, 189–198.

Warren, J.T., Petryk, A., Marques, G., Jarcho, M., Parvy, J.P., Dauphinvillemant, C., O'Connor, M.B., Gilbert, L.I., 2002. Molecular and biochemical characterization of two P450 enzymes in the ecdysteroidogenic pathway of *Drosophila melanogaster*. Proc. Natl. Acad. Sci. U.S.A. 99 (17), 11043–11048.

Wen, D., Riveraperez, C., Abdou, M., Jia, Q., He, Q., Liu, X., Zyaan, O., Xu, J., Bendena, W.G., Tobe, S.S., Noriega, F.G., Palli, S.R., Wang, J., Li, S., 2015. Methyl farnesoate plays a dual role in regulating *Drosophila metamorphosis*. PLoS Genet. 11 (3), e1005038.

Werck-Reichhart, D., Feyereisen, R., 2000. Cytochromes P450: a success story. Genome

Biol. 1 (6), 1–9.
Willingham, A.T., Keil, T., 2004. A tissue specific cytochrome P450 required for the structure and function of *Drosophila* sensory organs. Mech. Dev. 121, 1289–1297.
Yu, Z., Zhang, X., Wang, Y., Moussian, B., Zhu, K.Y., Li, S., Ma, E., Zhang, J., 2016. *LmCYP4G102*: an oenocyte-specific cytochrome P450 gene required for cuticular waterproofing in the migratory locust, *Locusta migratoria*. Sci. Rep. 6 29980.
Yuan, K., O'Farrell, P.H., 2016. TALE-light imaging reveals maternally guided, H3K9me2/3-independent emergence of functional heterochromatin in *Drosophila* embryos. Genes Dev. 30 (5), 579–593.
Zhang, X., Kang, X., Wu, H., Silver, K., Zhang, J., Ma, E., Zhu, K.Y., 2018. Transcriptome-wide survey, gene expression profiling and exogenous chemical-induced transcriptional responses of cytochrome P450 superfamily genes in migratory locust (*Locusta migratoria*). Insect Biochem. Mol. Biol. 100, 66–77.
Zhang, X., Luo, D., Pflugfelder, G.O., Shen, J., 2013. Dpp signaling inhibits proliferation in the *Drosophila* wing by Omb-dependent regional control of *bantam*. Development 140 (14), 2917–2922.
Zhao, X., Qin, Z., Liu, W., Liu, X., Moussian, B., Ma, E., Li, S., Zhang, J., 2018. Nuclear receptor HR3 controls locust molt by regulating chitin synthesis and degradation genes of *Locusta migratoria*. Insect Biochem. Mol. Biol. 92, 1–11.