Ecdysone receptor isoforms play distinct roles in larval–pupal–adult transition in *Leptinotarsa decemlineata*

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**Abstract** A heterodimer of two nuclear receptors, ecdysone receptor (EcR) and ultraspiracle, mediates 20-hydroxyecdysone (20E) signaling to modulate many aspects in insect life, such as molting and metamorphosis, reproduction, diapause and innate immunity. In the present paper, we intended to determine the isoform-specific roles of EcR during larval–pupal–adult transition in the Colorado potato beetle. Double-stranded RNAs (ds-RNAs) were prepared using the common (dsEcR) or isoform-specific (dsEcRA, dsEcRB1) regions of EcR as templates. Ingestion of either dsEcR or dsEcRA, rather than dsEcRB1, by the penultimate (3rd) and final (4th) instar larvae caused failure of larval–pupal and pupal–adult ecdysis. The RNA interference (RNAi) larvae remained as prepupae, or became deformed pupae and adults. Determination of messenger RNA (mRNA) levels of EcR isoforms found that LdEcRA regulates the expression of LdEcRB1. Moreover, silencing the two EcR transcripts, LdEcRA or LdEcRB1 reduced the mRNA levels of Ldspo and Ldsad, and lowered 20E titer. In contrast, the expression levels of HR3, HR4, E74 and E75 were significantly decreased in the LdEcR or LdEcRA RNAi larvae, but not in LdEcRB1 depleted specimens. Dietary supplement with 20E did not restore the expression of five 20E signaling genes (USP, HR3, HR4, E74 and E75), and only partially alleviated the pupation defects in dsEcR- or dsEcRA-fed beetles. These data suggest that EcR plays isoform-specific roles in the regulation of ecdysteroidogenesis and the transduction of 20E signal in *L. decemlineata*.

**Key words** ecdysone receptor; *Leptinotarsa decemlineata*; metamorphosis; RNA interference

**Introduction**

In insects, steroid hormone 20-hydroxyecdysone (20E) modulates many aspects in life, such as reproduction, embryogenesis, molting and metamorphosis, diapause and innate immunity (Yamanaka *et al.*, 2013; Mazina *et al.*, 2017). In larval stages, ecdysone is synthesized in prothoracic glands (PGs) from dietary sterols such as cholesterol and phytosterols, under the catalysis of a series of cytochrome P450 monooxygenases (CYPs) encoded by Halloween genes including *spook* (*spo*), *phantom* (*phm*), *disembodied* (*dib*) and *shadow* (*sad*). Ecdysone is then released from PGs into the hemolymph. It is transported to peripheral tissues, and is converted to 20E by another CYP, the product of the Halloween gene *shade* (*shd*) (Iga & Kataoka, 2012; Niwa & Niwa, 2014). 20E exerts its effects through a heterodimer of ecdysone receptor (EcR) and ultraspiracle (USP) (Mazina *et al.*, 2017).

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2017). The 20E/EcR/USP complex binds to ecdysone response elements (EcREs) to activate early 20E-response genes, for instance, Broad-Complex (BrC), Ecdysone-induced protein 75 (E75) and E74. The complex and the products of early genes then trigger the expression of early-late genes such as HR3 and HR4. HR3 in turn inhibits E74A, E75A and BrC, permitting a developmental switch (Beckstead et al., 2005; King-Jones & Thummel, 2005; Ruaud et al., 2010), and induces late gene βFTZ-F1 (Reinking et al., 2005; Palanker et al., 2006) to trigger pupation.

The physiological importance of EcR during larval metamorphosis has been well explored in the common fruit fly Drosophila melanogaster (Koelle et al., 1991; Talbot et al., 1993; Bender et al., 1997; Schubiger et al., 1998; Davies et al., 2005). Moreover, a very small fraction of EcRs from non-drosophilid insects have been functionally characterized by in vivo RNA interference (RNAi) (Cruz et al., 2006; Tan & Palli, 2008; Yao et al., 2010; Wu et al., 2012; Weng et al., 2013; Tan et al., 2015a, b) and in vitro analysis (Chen et al., 2017). Despite these findings, the molecular aspects for EcR to control juvenile development in non-drosophilid species have not been well studied. At least two questions remain to be addressed. First, knockdown of SgEcR reduces the transcript levels of Halloween genes (spo, sad and shd) and 20E titer in the desert locust Schistocerca gregaria (Lenaerts et al., 2016). In D. melanogaster, depletion of EcRA in the PGs allows normal development, with lengthened developing period (48 h). In contrast, depletion of EcRB1 and all three isoforms in the PGs halts development of 40% and 100% resultant larvae. They remain at the last instar stage over 25 days without entering metamorphosis (Mansilla et al., 2016). Do the phenotypic defects in the EcR RNAi non-drosophilid larvae principally result from deficiency of 20E, or inhibition of 20E signaling, or both?

Second, three functional isoforms, EcRA, EcRB1 and EcRB2, are produced from EcR gene in D. melanogaster (Koelle et al., 1991; Talbot et al., 1993). During the last larval instar, each of the EcR isoforms is expressed in a spatiotemporal-specific manner that coordinates the onset of major metamorphic changes during pupal development. EcRA is predominantly expressed in imaginal discs that develop into adult-specific structures (Bender et al., 1997; Davies et al., 2005), EcRB1 is mainly transcribed in those larval structures fated to apoptosis (Schubiger et al., 1998; Cherbas et al., 2003; Davies et al., 2005), whereas EcRB2 is found in Malpighian tubules (Gautam et al., 2015). Each EcR isoform exerts specific functions during development (Bender et al., 1997; Davies et al., 2005; Gautam et al., 2015). Similar results have been documented in the red flour beetle Tribolium castaneum (Tan & Palli, 2008), the Japanese pine sawyer Monochamus alternates (Weng et al., 2013) and the cotton mirid bug Apolygus lucorum (Tan et al., 2015a, b). Which EcR isoform is mainly responsible for the transduction of 20E signal in non-drosophilid insect species? Are there any differences of EcR isoforms that mediate 20E signaling among insects?

We have characterized the main ecdysteroidogenesis (Wan et al., 2013; Kong et al., 2014) and 20E signaling (Guo et al., 2015, 2016; Xu et al., 2018a, b) genes in the Colorado potato beetle Leptinotarsa decemlineata. In the present paper, we intended to address the two questions. We first measured the transcription patterns of LdEcR isoforms at whole development excursion and in different tissues. For functional analyses, we examined the defects after silencing either LdEcRA or LdEcRB1 using RNAi, or both of them. We found that silencing either LdEcRA or LdEcRB1 reduced 20E titer. In contrast, silencing two isoforms, or LdEcRA, but not LdEcRB1, repressed the 20E signal and caused lethality due to failure of ecdysis in the L. decemlineata penultimate and final instar larvae. Our results reveal that EcR is undoubtedly necessary for regulation of ecdysteroidogenesis and for mediation of 20E signaling, in an isoform-dependent pattern, in L. decemlineata.

Materials and methods

Insects

The L. decemlineata beetles were kept in an insectary according to a previously described method (Shi et al., 2013), with potato foliage at the vegetative growth or young tuber stages in order to assure sufficient nutrition. At this feeding protocol, the larvae progressed through four distinct instars, with approximate periods of the 1st-, 2nd-, penultimate-, and final-instar stages of 2, 2, 2 and 4 days, respectively. Upon reaching full size, the final larval instars stopped feeding, dropped to the ground, burrowed to the soil and entered the prepupal stage. The prepupae took approximately 3 days to pupate. The pupae lasted about 5 days and the adults emerged.

Preparation of dsRNAs

Specific primers used to clone the fragments of double-stranded RNAs (dsRNAs) are listed in Table S1. These dsRNAs were individually expressed using Escherichia coli HT115 (DE3) competent cells lacking RNase III following the established method (Kong et al., 2014). Individual colonies were inoculated, and grown until cultures
reached an OD600 value of 1.0. The colonies were then induced to express dsRNA by addition of isopropyl β-D-1-thiogalactopyranoside to a final concentration of 0.1 mmol/L. The expressed dsRNA was extracted and confirmed by electrophoresis on 1% agarose gel. Bacteria cells were centrifuged at 5000 × g for 10 min, and resuspended in an equal original culture volume of 0.05 mol/L phosphate buffered saline (PBS, pH 7.4). The bacterial solutions (at a dsRNA concentration of about 0.5 μg/mL) were used for the experiment.

**Dietary introduction of dsRNA**

The same method as previously reported (Kong et al., 2014) was used to introduce dsRNA into larvae. Potato leaves were immersed with a bacterial suspension containing a dsRNA for 5 s, removed, and dried for 2 h under airflow on filter paper. The PBS- and dsgefp (enhanced green fluorescent protein)-dipped leaves were used as controls. Five treated leaves were then placed in Petri dishes (9 cm diameter and 1.5 cm height). The newly ecysed penultimate- and final-instar larvae were starved for at least 4 h prior to the experiment. Then, 10 larvae were transferred to each dish as a repeat. For each treatment, nine repeats were set. Three replicates were used to observe the pupation and adult emergences by allowing the larvae to feed on dsRNA-dipped foliage for 3 days (replaced with freshly treated ones each day), and on untreated foliage until reaching the wandering stage. For extraction of total RNA and 20E, six replicates were respectively collected after being continuously fed on treated foliage for 3 days.

**Rescuing experiment by 20E**

20E (Sigma-Aldrich, St Louis, MS, USA) was dissolved in distilled water with added surfactant (Tween 20, 1 g/L) to give stock solutions of 100 ng/mL. It was further serially diluted 10-fold with distilled water before the bioassy.

A bioassay was carried out as previously described (Kong et al., 2014) using newly ecysed final-instar larvae, to test the rescuing effect of 20E at the concentration of 10 ng/mL. The larvae were first allowed to ingest potato foliage immersed with dsgefp and dsEcR for 2 days, and then to consume leaves dipped with dsgefp, dsgefp+20E, dsEcR, dsEcR+20E, for an additional day. The larvae were then transferred to untreated foliage if necessary. For each treatment, six repeats were set. Three replicates were used to observe the pupation and adult emergences and three repeats were used for quantitative real-time polymerase chain reaction (qPCR).

**Real-time quantitative PCR (qRT-PCR)**

For temporal expression analysis, RNA templates were derived from eggs, the larvae from the 1st through final instars, wandering larvae, pupae (5 days after burrowing into soil) and adults (5 days after emerging). For analysis of the tissue expression patterns, RNA templates were derived from the brain-corpora cardiaca-corpora allata complex, prothoracic glands, foregut, midgut, hindgut, Malpighian tubules, fat body, and epidermis of day 4 final-instar larvae, and adult (5 days after emerging) ovary and testis. For analysis of the effects of treatments, total RNA was extracted from treated larvae. Each sample contained 5–10 individuals and repeated three times. The RNA was extracted using SV Total RNA Isolation System Kit (Promega, Madison, WI, USA). Purified RNA was subjected to DNase I to remove any residual DNA according to the manufacturer’s instructions. Quantitative messenger RNA (mRNA) measurements were performed by qPCR in technical triplicate, using four internal control genes (LdRP4, LdRP18, LdARF1 and LdARF4, the primers listed in Table S1) according to our published results (Shi et al., 2013). An reverse transcriptase negative control (without reverse transcriptase) and a non-template negative control were included for each primer set to confirm the absence of genomic DNA and to check for primer-dimer or contamination in the reactions, respectively.

According to a previously describes method (Bustin et al., 2009), the generation of specific PCR products was confirmed by gel electrophoresis. The primer pair for each gene was tested with a 10-fold logarithmic dilution of a complementary DNA (cDNA) mixture to generate a linear standard curve (crossing point [CP] plotted vs. log of template concentration), which was used to calculate the primer pair efficiency. All primer pairs amplified a single PCR product with the expected sizes, showed a slope less than –3.0, and exhibited efficiency values ranging from 2.3 to 2.5. Data were analyzed by the 2−ΔΔCT or 2−ΔΔCT method, using the geometric mean of the four internal control genes for normalization.

**Quantitative determination of 20E**

20E was extracted according to a ultrasonic-assisted extraction method (Liu et al., 2014), and its titer (ng per g body weight) was analyzed by liquid chromatography tandem mass spectrometry-mass spectrometry (LC-MS/MS).
Fig. 1 The exon/intron and mRNA structures of the *Leptinotarsa decemlineata* ECR gene (A) and the two isoforms (B). Boxes mark exons and lines represent introns. The red and green lines below isoform A and B indicate the sequences for exon-specific double-stranded DNA (dsRNAs), whereas the black lines from exon 6 to 8 show the sequence for dsRNA against both isoforms.

using a protocol the same as described in Zhou et al. (2011).

Data analysis

We used SPSS for Windows (Chicago, IL, USA) for statistical analyses. The averages (±SE) were submitted to analysis of variance with the Tukey–Kramer test.

Results

Identification of the EcR isoforms

EcR gene in *L. decemlineata* contains eight exons (Fig. 1A). Comparison of the EcR gene and its corresponding cDNA sequences revealed that alternative exon usage forms two variants: EcRA consists of exons 1, 3, 5, 6, 7 and 8, whereas EcRB1 is composed of exons 2, 3, 5, 6, 7 and 8 (Fig. 1B).

The expression profiles of LdEcR isoforms

*LdEcRA* and *LdEcRB1* transcripts were detectable from the egg to adult. *LdEcRA* was abundantly expressed in the final larval instars, wandering prepupae, pupae and adults. It peaked at day 4 final instar stage. In contrast, *LdEcRB1* was mainly transcribed from the embryo (eggs) to day 1 final instar larvae and in the pupae. Its peak appeared just after the molt of the final instar larvae (Fig. 2A).

The tissue-specific expression patterns of *LdEcR* isoforms were also tested by qPCR. The results clearly showed that both isoforms were expressed in the brain-corpora cardiaca-corpora allata complex, prothoracic glands, foregut, midgut, hindgut, Malpighian tubules, fat body and epidermis of the day 4 final instar larvae. It was also expressed in adult ovary and testis. *LdEcRA* was highly expressed in the brain-corpora cardiaca-corpora allata complex, midgut and epidermis, moderately transcribed in the prothoracic glands and hindgut. Similarly, the *LdEcRB1* transcripts were high in the brain-corpora cardiaca-corpora allata complex and epidermis, were moderate in the prothoracic glands, midgut and hindgut (Fig. 2B). The spatiotemporal data are compatible with the common idea that EcR functions in 20E signaling.

Knockdown of LdEcR in the final instar larvae impairs pupation

In order to investigate the biological function of EcR isoforms in metamorphosis, dsRNAs from isoform-specific (dsEcRA, dsEcRB) (marked with red and green lines) and the common (dsEcR) (marked with black lines) sequences were dietarily introduced to the final instar larvae (Fig. 1B). In the larvae having fed for 3 days on foliage immersed with dsEcR or dsEcRA, the mRNA levels of *LdEcRA+LdEcRB1* (hereafter *LdEcR*), *LdEcRA* and *LdEcRB1* were significantly decreased. In beetles having ingested dsEcRB1, the transcript levels of *LdEcR* and *LdEcRB1* were significantly reduced, whereas the expression level of *LdEcRA* was greatly increased (Fig. 3A–C).

Ingestion of dsEcR, dsEcRA or dsEcRB1 for 3 days had little effect on larval survival (Fig. 3D). In contrast, consumption of dsEcR or dsEcRA caused derailment of development (Fig. 3E, 3F). While the control (CK) and the larvae having ingested dsEcRB1, pupated (Fig. 3G, 3H) and emerged as adults 8 and 13 days after initiation of bioassay, almost all dsEcR- or dsEcRA-fed larvae were arrested in development (Fig. 3E, 3F). Old larval cuticles within the prothorax of these treated larvae were first broken transversely (Fig. 3J, 3O) or along the dorsal middle line (Fig. 3K, 3P). And then, pieces of the broken larval cuticle were dropped off, exposing prothorax, mesothorax and metathorax (Fig. 3M) and shortened fore, middle, and hind legs (Fig. 3N vs. 3G). Whereas the larvae having ingested dsegfp or dsEcRB1 pupae had elongated wings (Fig. 3G, 3H), the forewings and hindwings were only small protrusions on the mesothorax and metathorax of the dsEcR- and dsEcRA-fed pupae (Fig. 3L, 3Q).

Silencing LdEcR in the penultimate-instar larvae causes similar defects

We repeated the bioassay using the newly ecdysed penultimate instar larvae. When compared to their levels in control and dsegfp-fed insects, the *LdEcR* and
Fig. 2 Temporal (A, B) and tissue (C, D) expression of the isoforms from ecdysone receptor gene (LdEcRA and LdEcRB1) in Leptinotarsa decemlineata. For temporal expression analysis, complementary DNA templates were derived from egg (day 3), the 1st, 2nd, 3rd and final (D0 indicates newly ecdysed larvae) larval instars, wandering larvae (W), pupae (P, day 4) and adults (A, day 7). For tissue expression analysis, the relative transcripts were measured in the brain-corpora cardiaca-corpora allata complex (BCC), prothoracic glands (PG), foregut (FG), midgut (MG), hindgut (HG), Malpighian tubules (MT), fat body (FB), and epidermis (EP) of the day 4 final instar larvae, and adult ovary (OV) and testis (TE). For each sample, three independent pools of 5–10 individuals were measured in technical triplicate using quantitative real-time polymerase chain reaction. The values were calculated using the 2−ΔΔC_T method. The columns represent averages with vertical lines indicating SE.

LdEcRB mRNA levels in the larvae having ingested dsEcR, dsEcRA or dsEcRB were lower. Similarly, the LdEcRA mRNA levels were reduced in the larvae having ingested dsEcR or dsEcRA. In contrast, dsEcRB ingestion significantly increased the expression level of LdEcRA (Fig. 4A–4C).

Ingestion of dsEcR, dsEcRA or dsEcRB1 for 3 days did not cause larval lethality (Fig. 4D). However, feeding of dsEcR or dsEcRA, rather than dsEcRB1, caused the failure of pupation and adult emergence (Fig. 4E, 4F). Only approximately 30% and 45% resultant larvae having fed on dsEcR and dsEcRA pupated and around 30% and 45% of resultant pupae emerged as adults, respectively. The remaining treated beetles remained as prepupae (Fig. 4H, 4I), or became deformed pupae (Fig. 4J–4L).
Fig. 3  Ingestion of double-stranded RNA (dsRNA) from LdEcR by the final instar larvae isoform specifically affects larval performance in Leptinotarsa decemlineata. The newly ecdysed final instar larvae had ingested phosphate-buffered saline (PBS) (CK)-, ds egfp-, dsEcR-, dsEcRA- and dsEcRB1-dipped leaves for 3 days. The expression levels of LdEcR (A), LdEcRA (B) and LdEcRB1 (C) were tested. Relative transcripts were calculated using the $2^{-\Delta\Delta C_T}$ method. They are the ratios of relative copy numbers in treated individuals to PBS-fed controls (CK), which is set as 1. The larval survival, pupation and emergence rates were recorded during a 4-week trial period (D–F). The bars represent values ($\pm$SE). Different letters indicate significant differences at $P$-value $< 0.05$. While the CK and dsEcRB1-fed larvae pupate and emerge as adults 8 (E, G, H) and 13 days (F) after initiation of bioassay, nearly all the LdEcR and LdEcRA RNA interference (RNAi) larvae failed to ecdyse. Seven days after initiation of experiment, the beetles remained as prepupae (I); old larval cuticles within the prothorax were broken transversely (J, O) or along the dorsal middle line (K, P). Ten days after initiation of experiment, partial larval cuticle was dropped off (M, N), exposing prothorax (PRT), mesothorax (MST) and metathorax (MTT) (M) and fore (FL), middle (ML), and hindlegs (HL) (N). Moreover, the control and dsEcRB1-fed pupae had elongated wings (G, H), whereas the forewings (FW) and hindwings (HW) were only small protrusions on the mesothorax and metathorax of the LdEcR and LdEcRA RNAi pupae (L, Q).

RNAi of LdEcR isoforms reduces ecdysteroidogenesis and represses 20E signaling in the final instar larvae The defects in LdEcR or LdEcRA RNAi larvae were similar to the phenotypes in 20E-deficient beetles (Kong et al., 2014). Therefore, we tested the expression levels of several Halloween genes Ldspo, Ldphm, Lddib, Ldsad and Ldshd (Wan et al., 2013; Kong et al., 2014), and measured the 20E titer in LdEcR, LdEcRA or LdEcRB depleted animals. Our results showed that knockdown of LdEcR, LdEcRA or LdEcRB reduced the mRNA levels of Ldspo and Ldsad, and lowered 20E titer (Fig. 5A–5E, 5K).
Fig. 4 Ingestion of double-stranded RNA (dsRNA) from LdEcR by the 3rd-instar larvae isoform specifically affects larval performance in Leptinotarsa decemlineata. The newly ecdysed 3rd-instar larvae had ingested phosphate-buffered saline (PBS) (CK)-, dsegfp-, dsEcR-, dsEcRA- and dsEcRB1-dipped leaves for 3 days. The expression levels of LdEcR (A), LdEcRA (B) and LdEcRB1 (C) were calculated using the $2^{-\Delta\Delta C_T}$ method. Relative transcripts are the ratios of relative copy numbers in treated individuals to PBS-fed controls (CK), which is set as 1. The larval survival, pupation and emergence rates were recorded during a 4-week trial period (D–F). The bars represent values (±SE). Different letters indicate significant differences at $P$-value $< 0.05$. While the CK larvae pupate 11 days (G) after initiation of bioassay, most of the LdEcR and LdEcRA RNA interference larvae remain as prepupae (H, I), or became deformed pupae (J, K). Sixteen days after initiation of experiments, the beetles were dried and darkened (L).

The mRNA levels of five 20E signaling genes, USP, HR3, HR4, E74 and E75 (Ogura et al., 2005; Guo et al., 2015, 2016; Xu et al., 2018a, b), were quantified in treated larvae. Compared with the control (CK) and the larvae having ingested dsegfp, the expression levels of HR3, HR4, E74 and E75 were significantly decreased in the LdEcR or LdEcRA RNAi larvae, in contrast to those in the LdEcRB depleted specimens (Fig. 5F–5J).

Rescuing effect by 20E in the final instar LdEcR RNAi larvae 20E ingestion by the larvae having fed on dsEcR or dsEcRA rescued neither the decreased LdEcR, LdEcRA and LdEcRB1 expression levels (Fig. 6A–6C), nor the expression levels of four other genes mediating 20E signal, that is, LdUSP, LdE75, LdHR3 and LdE74 (Fig. 6D–6G). Moreover, the decreased pupation rates and the defects in dsEcR- and dsEcRA-fed larvae were only partially alleviated. A few pupae the larvae of which had ingested dsEcRA+20E finally became adults (Fig. 6H, 6I). A portion of LdEcR+20E and LdEcRA1+20E beetles remained as prepupae (Fig. 6J), or became deformed pupae (Fig. 6K). After removal of the apolysed larval cuticle, the prepupae subjected to LdEcR+20E and LdEcRA1+20E treatment had developing adult structures such as mouthparts, fore, middle and hind legs (Fig. 6L), and shortened fore and hind wings (Fig. 6M).

Discussion

LdEcR is critical for larval metamorphosis

In the present paper, we found that ingestion of dsEcR caused failure of larval–pupal and pupal–adult ecdysis in L. decemlineata. Consistent with our results, mutations that block all three EcR isoforms in D. melanogaster lead to embryonic lethality, and result in an early-to-mid-stage
Fig. 5 Ingestion of double-stranded RNA (dsRNA) from LdEcR by the final instar larvae inhibits ecdysteroidogenesis and represses 20E signal in Leptinotarsa decemlineata. The newly ecdysed final instar larvae had ingested phosphate-buffered saline (PBS) (CK)-, dsegfp-, dsEcR-, dsEcRA- and dsEcRB1-dipped leaves for 3 days. The expression levels of five Halloween genes Ldspo (A), Ldphm (B), Lddib (C), Ldsad (D) and Ldsad (E), and five 20E signaling genes LdUSP (F), LdHR3 (G), LdHR4 (H), LdE74 (I), and LdE75 (J) were calculated using the $2^{-\Delta\Delta C_T}$ method. Relative transcripts are the ratios of relative copy numbers in treated individuals to PBS-fed controls (CK), which are set as 1. The 20E titer was measured (K). The bars represent values (±SE). Different letters indicate significant differences at $P$-value < 0.05.

pupal arrest (Bender et al., 1997). Correspondingly, knockdown of all EcR isoforms in S. exigua (Yao et al., 2010), the brown planthopper Nilaparvata lugens (Wu et al., 2012), A. lucorum (Tan et al., 2015a), the German cockroach Blattella germanica (Cruz et al., 2006), T. castaneum (Tan & Palli, 2008) and M. alternates (Weng et al., 2013) cause molting defects and juvenile lethality.

In this study, two layers of cuticle were found in dsEcR-fed larvae, with the old one partially or completely covering the new. In agreement with our results, the new cuticle is present underneath the old one in surviving SgEcR RNAi 5th nymphs in S. gregaria (Lenaerts et al., 2016).

Our results revealed that the LdEcR RNAi prepupae possessed short forewings and hindwings after removal of the apolysed larval cuticle. Similarly, in the adults developing from T. castaneum larvae injected with dsEcR or dsEcRA during the final instar larval stage, both the forewings and hindwings are short (Tan & Palli, 2008). Moreover, knockdown of both EcR isoforms results in wing defects in morphogenesis and melanization in the nymphs of the small brown planthopper Laodelphax striatellus (Wu et al., 2012).

All these findings demonstrate that EcR is critical for insect molting and metamorphosis in both holometabolon and hemimetabolon insect species (Yamanaka et al., 2013; Mazina et al., 2017).

LdEcRA is more important in larval metamorphosis in Coleopterans

Our results revealed that EcR plays isoform-specific roles in L. decemlineata. Silencing dsEcRA, but not dsEcRB1, mimicked the negative effects on larval–pupal and pupal–adult ecdysis in the larvae where two EcR
isoforms were knocked down. Consistent with our results, although all the larvae injected with dsEcR or dsEcRA arrested their development during the quiescent stage and die, around 80% and 60% of the larvae injected with dsEcRB progressed in larval development and survived to the adult stage (Tan & Palli, 2008). Similarly, most larvae depleted of both EcR isoforms or EcRA die prior to adulthood in M. alternates, whereas about half of the EcRB RNAi larvae become adults (Weng et al., 2013). Therefore, EcRA plays critical roles in insect molting and metamorphosis in three Coleopteran insect species L. decemlineata (this study), T. castaneum (Tan & Palli, 2008) and M. alternates (Weng et al., 2013).

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In contrast, EcRB1 isoform appears important in juvenile development in some insect species. In H. armigera, for example, 20E rapidly enhances PKCδ expression via EcRB1 and USP1. PKCδ then phosphorylates EcRB1 at Thr468 to form the EcR/B1/USP1 transcriptional complex to promote apoptosis (Chen et al., 2017).

In holometabolon and hemimetabolon insect representatives, both EcRA and EcRB are essential for juvenile development. In D. melanogaster, for instance, loss-of-function mutations in EcR4 cause an early-to-mid-pupal arrest (Bender et al., 1997; Davies et al., 2005). EcRB1 mutants fail to pupariate (Bender et al., 1997; Davies et al., 2005). EcRB mutants having removed both EcRB1 and EcRB2 show defects in larval molting, arresting at the boundaries between the three larval stages (Schubiger et al., 1998). Similarly, silencing either EcRA or EcRB1 inhibits growth and increases juvenile mortalities in A. lucorum (Tan et al., 2015a, b).

EcRA is required for complete expression of EcRB1 in Coleopterans

In the present paper, we provide three lines of evidence to support that LdEcRA is required for complete expression of LdEcRB1 in L. decemlineata. First, both LdEcR isoforms showed similar spatiotemporal expression patterns. Similar temporal expression patterns of EcRA and EcRB1 are found in T. castaneum (Tan & Palli, 2008).

Second, knockdown of EcR and EcRA causes failure of larval–pupal and pupal–adult ecdysis, whereas knockdown of EcRB1 does not affect larval–pupal–adult transition in L. decemlineata (this study), T. castaneum (Tan & Palli, 2008) and M. alternates (Weng et al., 2013).

Lastly, ingestion of dsEcRA significantly repressed the expression of LdEcRB1. In contrast, feeding of dsEcRB1 significantly enhanced the expression of LdEcRA in L. decemlineata. Since we used exon-specific dsRNAs in the present paper, it may not be from the off-target effect. Consistent with our results, dsEcRB1 injection does not affect EcR4 mRNA levels, whereas EcRB levels are lower in larvae injected with dsEcRA in T. castaneum (Tan & Palli, 2008) and M. alternates (Weng et al., 2013).

Therefore, EcRA regulates the expression of EcRB in at least three Coleopteran insect species: L. decemlineata (this study), T. castaneum (Tan & Palli, 2008) and M. alternates (Weng et al., 2013).

In contrast, high levels of EcR4 have a moderate effect on suppressing EcRB1 expression. By contrast, EcRA expression is strongly down-regulated by EcRB1 or EcRB2 over-expression in Drosophila (Schubiger et al., 2003). It appears that different isoforms may interact to regulate the ratio of receptors in non-Coleopteran insects.

EcRA acts as an activator in 20E signaling in Coleopterans

Our results revealed that silencing either LdEcRA or LdEcRB1 inhibited ecdysteroidogenesis, whereas RNAi of LdEcRA but not LdEcRB1 decreased transcript levels of LdHR3, LdHR4, LdE74 and LdE75. Indeed, expression levels of LdHR3, LdE74 and LdE75 were rather enhanced in EcRB1 RNAi larvae, consistent with the fact that EcR4 expression was increased in these animals. It is suggested that LdEcRA acts as an activator to stimulate 20E signaling. Similar results have been documented in T. castaneum (Tan & Palli, 2008) and M. alternates (Weng et al., 2013).

In contrast, Drosophila EcRA is a weak activator (Hu et al., 2003) and has some repressive activity (Mouillet et al., 2001). For example, the expression levels of DmHR3 and DmE75B are lowered by over-expression of EcRA (Schubiger et al., 2003).

Both LdEcRA and LdEcRB1 are involved in the regulation of ecdysteroidogenesis

Two lines of experimental evidence support that both LdEcRA and LdEcRB1 are associated with ecdysteroidogenesis in L. decemlineata larvae. First, we found in L. decemlineata that the two LdEcR isoforms were widely expressed in PGs where ecdysone is biosynthesized, and peripheral tissues including brain-corpora cardica-corpora allata complex, prothoracic glands, foregut, midgut, hindgut, Malpighian tubules, fat body and epidermis where 20E is produced. In agreement with our results, SgEcR in S. gregaria is expressed in the prothoracic glands and Malpighian tubules (Lenaerts et al., 2016). The tissue expression patterns suggest that EcR is critical for the regulation of ecdysteroidogenesis in insects.

Second, knockdown of two EcR transcripts, LdEcRA or LdEcRB reduced the mRNA levels of Ldspo and Ldsad, and lowered 20E titer in L. decemlineata larvae. Similarly, knockdown of SgEcR isoforms results in lower transcript levels of Sgspo, Sgsad and Sgshd, and lower titer of 20E (Lenaerts et al., 2016). In D. melanogaster, a dominant-negative mutation of EcR in the PG of larvae leads to reduced transcript levels of phm, dib and sad, and lowered ecdysteroid biosynthesis (Moeller et al., 2013). Moreover, EcR is required for the survival of prothoracic gland cells during metamorphosis via an ecdysone-independent pathway (Mansilla et al., 2016). Furthermore, knockdown of EcR lowers circulating ecdysteroid levels in a hemimetabolon B. germanica (Cruz et al., 2006).
Therefore, both EcRA and EcRB1 are involved in the stimulation of ecdysteroidogenesis in *L. decemlineata* larvae.

In the present paper, we perform rescuing experiments by dietary supplement with 20E. We found that the mRNA levels of *LdHR3*, *LdHR4*, *LdE74*, and *LdE75* were not restored by 20E ingestion in the *LdEcRA* RNAi larvae. Moreover, the pupation defect was only partially alleviated in dsEcR- or dsEcRA-fed beetles. Our rescuing experiment further reveals that *LdEcRA* functions as an activator to mediate 20E signal. However, our results come from knockdown of EcR or specific isoforms, whereas most consequences in *Drosophila* are from null mutants (Mouillet *et al.*, 2001; Hu *et al.*, 2003; Schubiger *et al.*, 2003). Any comparative conclusions should be further verified.

It is known that some EcR-mediated activities do not require USP, suggesting that EcR may dimerize with other partners, form homodimers, and/or form protein complexes that have yet to be described (Costantino *et al.*, 2008; Cheng *et al.*, 2018). In *D. melanogaster* male adults, for example, EcR (but not USP) depleted accessory glands fail to make seminal proteins and have dying cells. The active receptor may be a homodimer, probably involving all the isoforms of EcR (Sharma *et al.*, 2017). A similar paradigm is observed in the expression of genes encoding glue proteins in the salivary glands (Costantino *et al.*, 2008). Interestingly, EcR apparently does not require USP as a heterodimeric partner in scorpions (Nakagawa *et al.*, 2007). Our results in the present paper do not exclude the possibility that *LdEcR* plays a role in larval metamorphosis independent of USP-E75-HR3 cascade in *L. decemlineata*.

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**Discloser**

The authors have declared that no competing interest exists.

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**Supporting Information**

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Table S1.** Primers used in dsRNA synthesis and qRT-PCR.