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Ecdysone receptor strongly influences larval–pupal–adult transition and melanization in *Tuta absoluta*

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Abstract

Background Tomato leaf miner (*Tuta absoluta*) is a quarantined pest that damages Solanaceae crops worldwide. The overuse of traditional pesticides negatively affects both human health and the environment. RNA interference (RNAi), based on double-stranded RNA (dsRNA) induction, can be beneficial in the control of *Tuta absoluta*; one of the key points of using this technique is the selection of target genes. Exploring the ecdysone receptors (EcR) associated with the growth and development of tomato leaf miners is an important research topic and the primary aim of this study.

Methods In this study, RNA extraction, cDNA synthesis, gene cloning, bioinformatics analysis, and quantitative real-time polymerase chain reaction were used to obtain the full length, conserved domain, and relative expression levels of the *EcR*. RNAi was used to explore the effects of *EcR* on larval growth and development, pupal weight, and emergence rate.

Results The full-length cDNA of *T. absoluta TaEcR* was 1859 bp, and the coding region including the ZnF_C4 and HOLI domains was relatively conserved. The relative expression of *EcR* in the early pupal stage was substantially higher than that in the other instars. Approximately 70% of *TaEcR* RNAi larvae died or pupated abnormally. In the few successful pupations, the pupa weights were substantially lower (36.44%) than those of the control group. The color of the pupae was abnormal, and they did not enter their normal black state; the emergence rate of pupae was reduced by 43.45% compared to that of the control group.

Conclusion These results indicate that *TaEcR* inhibition can affect larval metamorphosis, pupation, melanism, eclosion abnormalities, and, ultimately, lead to death. The results of this study suggest that *TaEcR* may be a candidate factor for developing environmentally-friendly RNAi pesticides that have practical value in field control.

Keywords *Tuta absoluta*, Ecdysone receptor, RNA interference, Metamorphosis, Melanization

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Background

Tomato leaf miner (*Tuta absoluta*) (*Meyrick*) (*Lepidoptera: Gelechiidae*) is a quarantined pest located widely across Asia, Europe, Africa, North America, and South America (Araújo Soares and Ribeiro Campos 2022). They have a wide range of hosts, high reproductive rates, and strong adaptability (Zhang et al. 2021). Overuse of chemical pesticides increases resistance in pests and makes their control increasingly difficult (Campos et al. 2014; Silva et al. 2016; Pereira et al. 2023). Therefore, it is necessary to use new drug action modes to control the spread and harm caused by tomato leaf miners, such as RNA interference (RNAi)-based biopesticides that target key genes necessary for insect growth and development (Wang et al. 2023a). RNAi and double-stranded RNA (dsRNA) have been used to perform the functional analysis of arthropods. Using dsRNA as a medium, it is possible to design sequences that match target genes, are specific to target insects, and show low or no toxicity to non-target organisms. In recent years, RNA-based technologies have shown considerable potential in protecting crops from prominent agricultural pests (Wang et al. 2021, in Chinese).

Insect development is regulated by ecdysone and juvenile hormones (Wang et al. 2023b), and ecdysone receptors (EcR) are key signaling molecules in insect growth and developmental regulation. EcR is a nuclear receptor (NR) that acts as a ligand-controlled transcription factor (Koelle et al. 1991). EcR binds the ultraspiracle protein (USP) and 20-hydroxyecdysone (20E) to form an EcR-USP complex (Oro et al. 1990; Yao et al. 1993; Wang et al. 2000). The EcR-USP complex mediates 20E signaling and regulates several life activities, such as molting and metamorphosis, reproduction, diapause, and innate immunity (Brown and Truman 2009; Gautam and Tapadia 2010; König et al. 2011; Mirth et al. 2009; Schwedes et al. 2011; Tian et al. 2010; Wang et al. 2011). The EcR contains a highly conserved DNA-binding domain (DBD) and a structurally conserved ligand-binding domain (LBD) (Bain et al. 2007), the latter being a unique structure that covalently binds transcription factors to NRsand effectively regulates transcriptional cascades (Cruz et al. 2006). Therefore, elucidating the structure and function of EcR in insects will assist in developing gene switches for controlling notable agricultural pests and can be successfully applied as an environmentally friendly or "green" pest control method.

In previous study, researchers administered *dsEcR* to the third (penultimate) and fourth (final) instar larvae of *Leptinotarsa decemlineata*, which resulted in the failure of larval–pupal and pupal–adult ecdysis; this confirmed that EcR is necessary for regulating Colorado potato beetle steroidogenesis and mediating 20E signaling in an isometric-dependent pattern (Xu et al. 2020). Following RNAi, the *HvEcR* gene was applied to the third and fourth instar larvae of *Henosepilachna vigintioctopunctata*; the growth and development of the larvae were impeded, and the larvae remained in prepupae or abnormal pupae form and failed to develop into adults (Wu et al. 2021). After silencing *TcEcR* in the larvae of *Tetranychus cinnabarinus*, 73.1% of the mites did not survive in the larval stage, and the probability of mites successfully developing into adults was 11.7%, which was significantly lower than that of the control group (Shen et al. 2019). Using dsRNA-induced expression in *Drosophila melanogaster*, EcR was demonstrated to be essential for larval molting and metamorphosis (Lam and Thummel 2000). In addition, RNAi experiments have demonstrated that *EcR* is necessary for specific developmental processes in adult *Blattella germanica*, including wing development, prothoracic gland degeneration, and normal choriogenesis (Cruz et al. 2006). These findings suggest that *EcR* is essential for molting and both complete and incomplete insect metamorphosis. However, the role of *EcR* in the growth and development of tomato leaf miners has not been clearly defined.

In the present study, we used the completely metamorphosed insect *Tuta absoluta* as a model to explore the role of the *EcR* gene in growth, development, and metamorphosis.

Materials and methods

Insect rearing and host plants

The tomato leaf miner (*Tuta absoluta*) colony used in this experiment was originally collected from Yuxi, Yunnan Province in August 2018. The tomato variety planted was Maofen. In a laboratory, tomato leaf miners were reared in an insectary at 25 ± 2 °C under 50–60% relative humidity under a 14 h:10 h light:dark cycle. The host plants were individually grown in 9 cm diameter pots under the same conditions as the tomato leaf miner.

RNA extraction, cDNA synthesis and *TaEcR* cloning

Total RNA was isolated and extracted using the Trizol method. A NanoPhotometer™ P330 (Implen, Munich, Germany) and 1% agarose gel electrophoresis were used to detect the quality and concentration of the RNA, and the band size was determined. The first-strand cDNA was generated from 2.0 μg RNA using the Super Script First-Strand Synthesis System (TransGen, Beijing, China).

Full-length cDNA was obtained using a Taq DNA Polymerase Amplification Kit (TransGen, Beijing, China) according to the manufacturer's instructions. The *EcR* homologous genes of *Bombyx mori* (NM_001043866.2) and *Helicoverpa armigera* (KY328717.1) were used to query the transcriptome dataset of *T. absoluta* using

tblastn. According to the corresponding sequence of *T. absoluta*, Primer 5.0 was used to design primers for full-length amplification, quantitative real-time PCR (RT-qPCR), and double-stranded RNA synthesis (Table 1). The amplified fragments were purified using a DNA Gel Extraction Kit (Genstone Biotech, Beijing, China). Distinct single-band amplification products were cloned into the pEASY-T3 vector (TransGen) and sequenced.

Sequence analysis of *TaEcR*

The full-length *EcR* coding region was obtained using DNAMAN (version 5.0; Lynnon BioSoft, QC, Canada) for sequence translation and splicing. SMART software was used to identify the conserved functional domains of *EcR*. SWISS-MODEL (https://swissmodel.expasy.org/) was used to predict the tertiary structure of EcR proteins through homology modeling, and GeneDoc software (version 2.7.0.0) was used for multiple protein sequence alignment and gene-conserved domain analysis. ExPASy (http://web.expasy.org/protparam/) was used to calculate molecular weights (Mw) and theoretical isoelectric points (pI). A phylogenetic tree was constructed using the maximum likelihood method in the MAGE software (version 7.0) to evaluate the molecular evolutionary relationships among various insects. Bootstrap majority consensus values for 1000 replicates are shown at each branch point (%). The EcR amino acid sequences of different insects were downloaded from the NCBI database (https://www.ncbi.nlm.nih.gov/).

Quantitative real-time PCR analysis of relative expression levels

The relative expression levels of dsRNA were analyzed, and the expression profiles were determined at different developmental stages, including eggs, first to fourth instar larvae, early pupae, late pupae, newly emerging females and males, and mature females and males. In addition, the relative expression of genes after application of dsRNA was measured. Relative mRNA expression levels were evaluated using reverse transcription polymerase chain reaction (RT-PCR) analysis. The primer sequences are listed in Table 1. The reactions were performed using an ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). All amplifications were confirmed by sequencing, and the specificity of RT-qPCR was estimated using melting curve analysis. PCR assays were prepared to a final volume of 20.0 µL with 1.0 µL of the cDNA template, 10.0 µL of Hieff® qPCR SYBR Green Master Mix, 0.4 µL of the forward primer (10 µM), 0.4 µL of the Reverse primer (10 µM), and 8.2 µL ddH_2O. A thermocycler was programmed with the following cycling conditions: (1) 94 °C for 5 min, followed by (2) 40 cycles at 95 °C for 10 s and 60 °C for 34 s. There were three replicates for each treatment or control, with four larvae in each replicate, and each replicate was assessed in triplicate (technical replicates). *RpL5* (large subunit 5 ribosomal protein) was used as the reference gene. The amplification efficiency was validated by constructing a standard curve using seven serial dilutions of cDNA. The relative quantification of mRNA expression was calculated using the mathematical model described by Livak and Schmittgen (2001) and Pfaffl (2001), which simplifies to $2^{-\Delta\Delta CT}$ and is as follows: ($\Delta\Delta CT$ = (Ct target − Ct reference) treatment − (Ct target − Ct reference) control).

Table 1 Primers used for cDNA cloning, quantitative real-time PCR (RT-qPCR), and double-stranded RNA (dsRNA) synthesis

Primer name	Primer sequence (5′→3′)	Amplicon length/bp
EcR- F	TCAACAAAGTTTAAGTTTTC	1860
EcR- R	ACGATCTGAATCTCCTTG	
dsEcR-F	taatacgactcactatagggGCGACCAACCAAGCCTACAC	406
dsEcR-R	taatacgactcactatagggAAGACACATCGGCGACATCC	
qEcR-F	AAGTGACTTCGTCTAGTGCGTTGG	119
qEcR-R	GCTGTTGATGCCGTCGTCGTAG	

Production of dsRNA transcription templates and synthesis of dsRNA

To generate dsRNA, three fragment templates of *EcR* were amplified by PCR using previously cloned cDNA templates with forward and reverse primers containing the T7 primer sequence at the 5′ ends. Amplification reactions were conducted in 50 µL mixes containing 37.5 µL of ddH_2O, 5 µL of 10 × buffer, 4 µL of dNTPs (10 mM for each nucleotide), 1.0 µL of forward primer (10 mM/µL), 1.0 µL of reverse primer (10 mM/µL), 1 µL of cDNA template, and 0.5 µL of Taq DNA Polymerase. The PCR cycling conditions were as follows: 94 °C for 5 min, followed by 35 cycles at 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s, and a final extension step of 72 °C for 10 min. Amplification of PCR products was confirmed via electrophoresis on 1.5% agarose gels and visualized by staining with ethidium bromide under UV light. The sequences were verified through Sangon Biotech sequencing. dsRNA was synthesized using the MEGAscript RNAi Kit (Ambion, Austin, TX, USA), and 1 µg of PCR product was used as the transcription template. The dsRNAs were resuspended in RNase-free water, analyzed using agarose gel electrophoresis and quantified spectrophotometrically. The dsRNA was stored at − 80 °C prior to further use.

RNAi experiments

The petioles of isolated leaves of Maofen tomatoes were immersed in the dsRNA solution at a concentration of 5 μg/200 μL, and dsRNA was administered to tomato leaf miner larvae via feeding. The treatment group was treated with dsRNA of the *EcR* gene and the control group was treated with dsRNA of the *EGFP* gene, and each group had three replicates. At least 3–4 h elapsed before the tomato leaves absorbed the dsRNA solution. Immediately after uptake, the second-instar larvae (n = 25) were gently placed on the leaves for feeding, and individuals in the treatment and control groups were sampled 48 h later. The effects of RNAi on larvae were evaluated using real-time fluorescence quantitative PCR.

Phenotypic observation and detection of growth and development

Following the RNAi experiments, the larvae and pupae were observed every 24 h under a research-level microscope (Stemi 508, ZEISS, Germany), and the number of dead larvae, pupal weight, and emergences were calculated. The phenotypes were observed and photographed using a three-dimensional ultra-depth field microscope (vhx-2000).

Statistical analysis

The relative expression of genes was calculated using $2^{-\Delta\Delta Ct}$ (Pfaffl 2001). One-way analysis of variance (ANOVA) was used to analyze gene expression profiles in SPSS 20.0, and differences were considered significant at $P < 0.05$. RNAi efficiency, larval mortality, pupal weight, and emergence rate were analyzed through t-test detection. *Represents $P < 0.05$, **represents $P < 0.01$, and ***represents $P < 0.001$, indicating significant ($0.01 < P < 0.05$) and extremely significant ($P < 0.01$) differences, respectively.

Results

Cloning of *TaEcR*

The full-length cDNA of *T. absoluta TaEcR* was 1859-bp and contained a 173-bp 5′-untranslated region (5′-UTR) (positions 1–173), a 21-bp 3′-UTR (positions 1839–1859), and a 1665-bp open reading frame (ORF) (positions 174–1838). The ORF encoded a polypeptide of 555 amino acids with a calculated molecular weight of 135.16 kDa and an isoelectric point (pI) of 4.92 (Fig. 1).

Sequence analysis of *TaEcR*

The EcR protein sequence, including the ZnF_C4 and HOLI domains, was analyzed using SMART online software (Fig. 2). The ZnF_C4 domain is a nuclear hormone receptor and a relatively small protein motif consisting of multiple finger-like protrusions in tandem with the target molecule, located between amino acids 139 and 210 of the EcR protein sequence. The HOLI domain is a hormone receptor LBD located at amino acids 338 to 498 in the EcR protein. The prototypical NR has a common structural organization with a variable N-terminal domain containing a constitutive activity activation function (AF)-1 domain, a conserved DBD consisting of two zinc fingers, a linker region, and a C-terminal LBD, also known as the HOLI domain (Robinson-Rechavi et al. 2003; Brelivet et al. 2004; Xie et al. 2014). We also used Swiss-Model online software to predict the three-dimensional structures of EcR and confirmed that it contained the conserved domains ZnF_C4 and HOLI (Fig. 3). The specific circumferential spatial conformation formed by ECR protein folding can intuitively reveal the location of the conserved domain, which is conducive to our understanding of the structural characteristics and functions of proteins.

We selected five representative species (*B. mori* NP_001166846.1, *Grapholitha molesta* ALG36653.1, *H. armigera* XP_021181316.1, *Plutella xylostella* NP_001296080.1, and *Manduca sexta* XP_030038409.1) for multiple sequence alignment and found that the homology of the EcR protein was as high as 80.86% (Fig. 4), indicating that this protein is highly conserved in insects.

To explore the evolutionary history of the evaluated genes, a phylogenetic tree was constructed using the maximum likelihood method with 1000 bootstrap replications in MEGA 7.0. Insects of the same order were gathered in the same branch, indicating that these genes were relatively conserved throughout the evolutionary process. The EcR of *T. absoluta* and *Lepidoptera* such as *Papilio machaon*, *P. xuthus*, *Melitaea cinxia*, *P. xylostella*, *H. armigera*, *Heliothis virescens*, *Spodoptera litura*, *B. mori*, *B. mandarina*, were clustered in the same branch (Fig. 5). The results showed that *EcR* was conserved throughout evolution, which is consistent with traditional taxonomy.

Expression profiles of *TaEcR* during different developmental stages

To detect the expression patterns of *TaEcR* during different developmental stages, a pair of primers for *EcR* was designed (Table 1), and qRT-PCR was performed. The relative expression levels of *EcR* in eggs, first-to-fourth-instar larvae, early pupae, late pupae, emerging males and females, and mature males and females were determined using real-time fluorescence quantitative PCR. The results revealed that *TaEcR* transcripts were detectable from the embryo (egg) to the adult stage. The relative expression of *EcR* in the early pupal stage was significantly higher than that in the other instars; however, there was no significant change in *EcR* expression in the

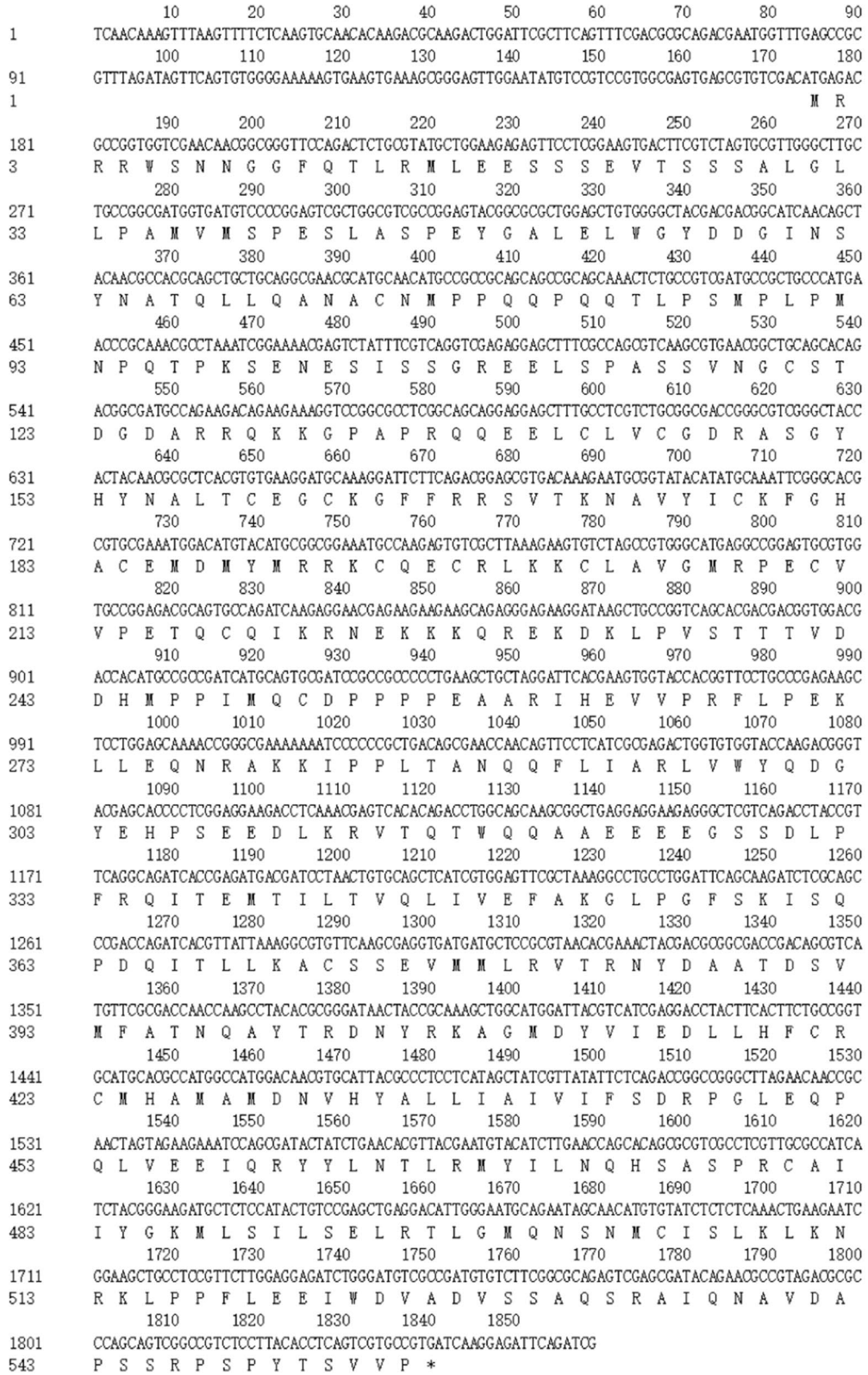

Fig. 1 Full-length cDNA sequence of *Tuta absoluta TaEcR* and its deduced amino acid sequence. The full-length cDNA of *T. absoluta TaEcR* was 1859-bp, and the open reading frame (174–1838 bp) encoded a polypeptide of 555 amino acids

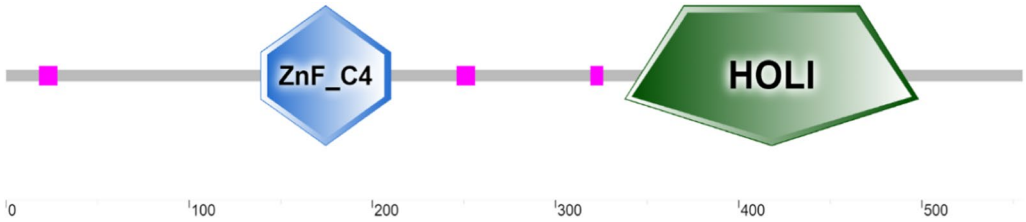

Fig. 2 Conserved domains of *EcR* in *Tuta absoluta*

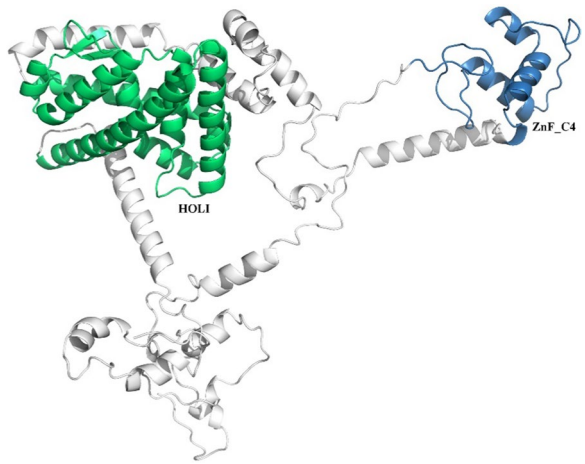

Fig. 3 Predicted tertiary structure of the EcR protein

late pupal stage until emergence. The expression level at the egg stage was significantly higher than that at the larval stage; however, there was no significant difference in expression levels between the first and fourth instars (Fig. 6).

The regulatory effect of *EcR* on growth and development

The silencing efficiency of *EcR* was tested 48 h after feeding, and there was a significant downregulation in *EcR* expression in the *dsEcR* group; the relative expression of *TaECR* transcripts was significantly reduced by 44.53% ($t=2.752$, $df=6$, $P<0.05$) compared to that in the control group (Fig. 7). The *TaEcR* reduction significantly affected the growth and development of tomato leaf miners. First,there was a significant difference in the larval phenotype. Larvae with normal growth were fully and

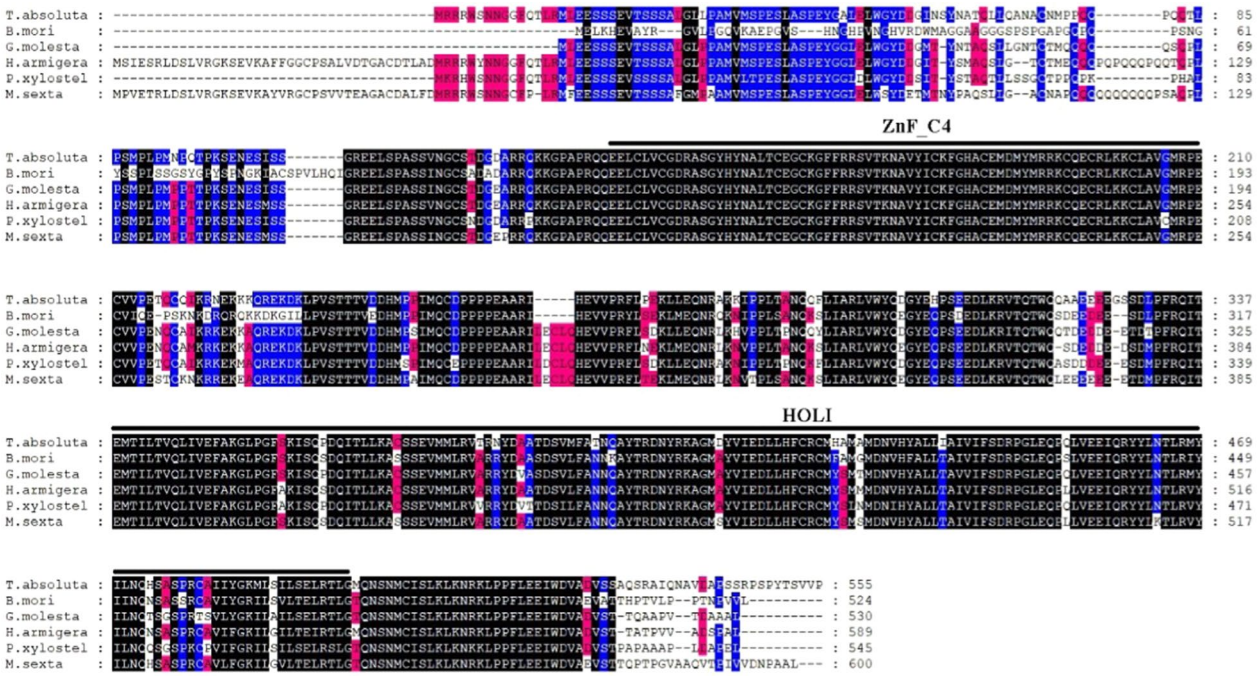

Fig. 4 Multiple alignments of the EcR protein sequences from *Tuta absoluta* and other insects

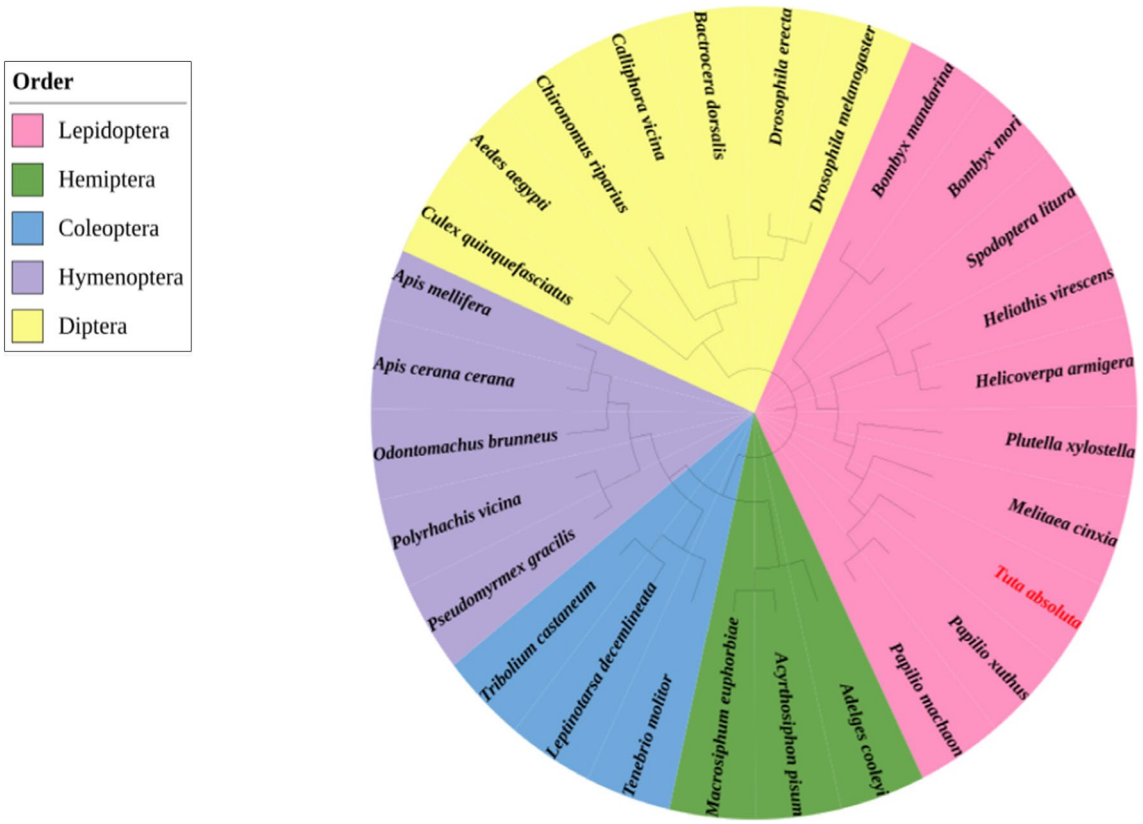

Fig. 5 Phylogenetic tree of the homologous amino acid sequences of *EcR* gene of *Tuta absoluta* and other insects. The phylogenetic tree was generated via the maximum likelihood method based on the Poisson correction mode and was used to determine the relationships between different insects, including *Bombyx mandarina* (XP_028037880.1), *Bombyx mori* (BAA07890.1), *Spodoptera litura* (ABX79143.1), *Heliothis virescens* (CAA70212.1), *Helicoverpa armigera* (ASK12085.1), *Plutella xylostella* (NP_001296080.1), *Melitaea cinxia* (XP_045445429.1), *Papilio xuthus* (KPI91595.1), *Papilio machaon* (KPJ06186.1), *Adelges cooleyi* (XP_050421434.1), *Acyrthosiphon pisum* (NP_001152832.1), *Macrosiphum euphorbiae* (QUS47837.1), *Tenebrio molitor* (CAA72296.1), *Leptinotarsa decemlineata* (BAD99296.1), *Tribolium castaneum* (NP_001107650.1), *Pseudomyrmex gracilis* (XP_020286030.1), *Polyrhachis vicina* (AFN06393.1), *Odontomachus brunneus* (XP_032676768.1), *Apis cerana* (PBC26988.1), *Apis mellifera* (NP_001152827.1), *Culex quinquefasciatus* (EDS37702.1), *Aedes aegypti* (XP_021710217.1), *Chironomus riparius* (AHM10271.1), *Calliphora vicina* (AAG46050.1), *Bactrocera dorsalis* (AFO64336.1), *Drosophila erecta* (XP_026834957.1), and *Drosophila melanogaster* (NP_001163061.1)

actively fed (Fig. 8A). The epidermis of the RNAi larvae exhibited appeared as black spots before insects entered the pupal stage (Fig. 8B), and the color of the body surface deepened to black at the time of death (Fig. 8C). After the deaths of some larvae, the epidermis was broken upon contact, indicating a state of swelling and decay (Fig. 8D); some insects did not complete the pupation process and died before pupation; in these cases, the body was deformed (Fig. 8E, F), or the tail was shrunken and wrinkled (Fig. 8G). Larval mortality increased significantly and was 39.48% ($t=5.652$, $df=4$, $P<0.01$) higher than that of the control group (Fig. 8H). Second, there was a significant difference in the pupal weights. After successful pupation of larvae that did not die during the larval stage, the average pupal weight of the treatment group was 1.56 mg ($t=4.817$, $df=27$, $P<0.001$), which was significantly reduced by 36.44% compared to that of the control group. In addition, the pupae in the control group were significantly darker than those in the treatment group (Fig. 9). Third, there was a significant difference in emergence rates. The eclosion rate of the control group was 95.83%, while that of the treatment group was 52.38% ($t=9.055$, $df=4$, $P<0.001$), which was 43.45% lower than that of the control group (Fig. 10).

Discussion

The ecdysone pathway is a complex regulatory process that includes ecdysone biosynthesis, EcR signaling, chitin/cuticle synthesis and degradation, ecdysis behavior, and genes associated with cuticle tanning (Song et al. 2017). Ecdysone triggers downstream responses by activating EcR, a subfamily of NRs that crucially influences the ecdysone pathway.

In the present study, we cloned the full-length *EcR* gene of *Tuta absoluta*. A high degree of sequence similarity (>80%) was found by comparing the amino acid

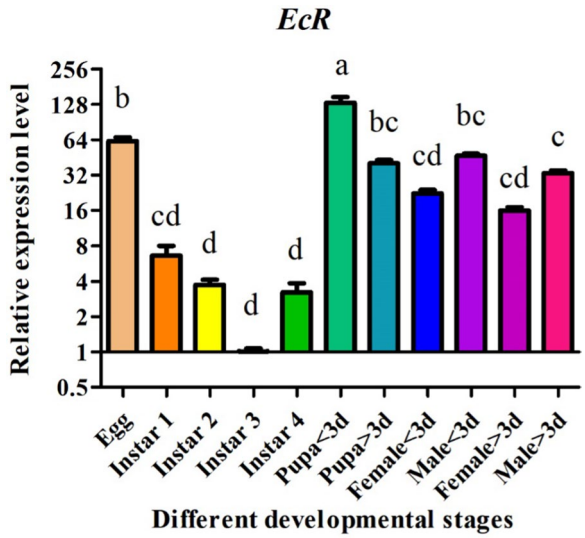

Fig. 6 Relative expression levels of *TaEcR* in eggs, first to fourth instars, early to late pupae, and newly emerged to mature females and males. The different letters (a, b, c, d) represent groups with significant differences according to the ANOVA results (Tukey's test, F = 52.15, df = 32, $P < 0.05$)

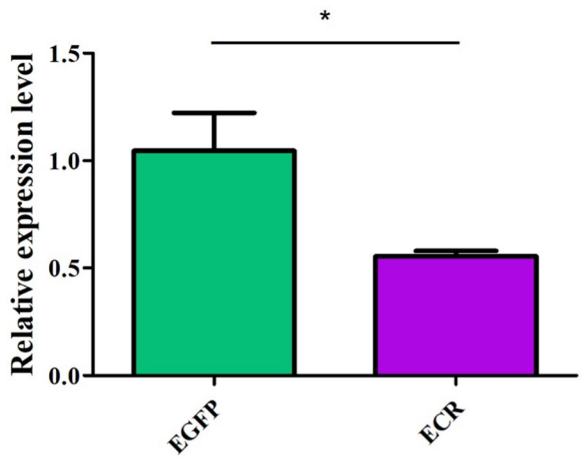

Fig. 7 Effects of dsRNA treatments on mRNA expression in *Tuta absoluta*. *On the horizontal line represents the difference in relative gene expression between insects under different treatment conditions (*$P < 0.05$)

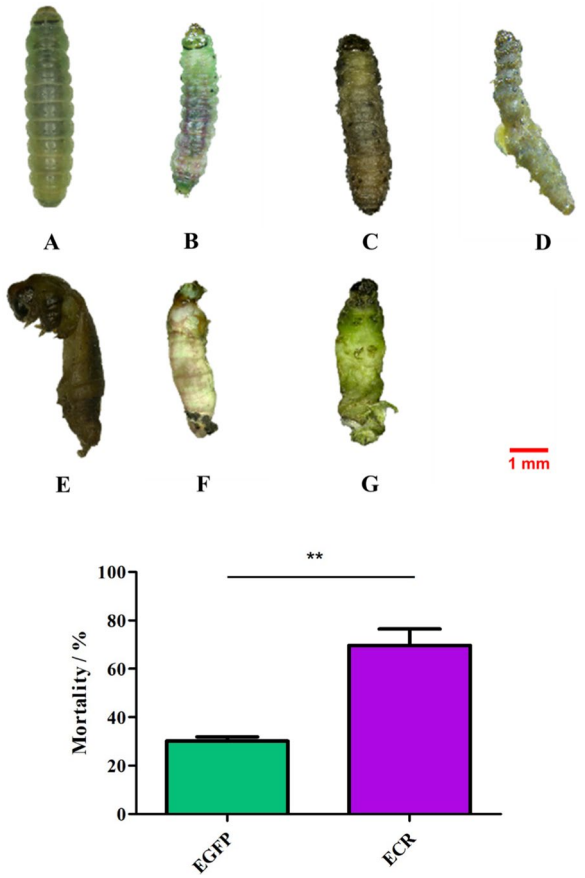

Fig. 8 Effects of *dsEcR* on *Tuta absoluta* larvae. **A** Phenotype of*Tuta absoluta* fed with *dsEGFP*. **B**–**G** Phenotype of *Tuta absoluta* after feeding with *dsEcR*. **H** Mortality of *Tuta absoluta* after feeding *dsEcR*. *On the horizontal line represents the difference in relative gene expression between insects under different treatment conditions (**$P < 0.01$)

sequences derived from *TaEcR* with the EcRs of five species: *B. mori*, *G. molesta*, *H. armigera*, *M. sexta*, and *Plutella xylostell*. The highly conserved ZnF_C4 and HOLI domains were preserved in the *TaEcR* sequence. The C4 zinc finger of nuclear hormone receptors, HOLI, is the LBD of hormone receptor proteins. NRs are an important transcriptional regulatory superfamily involved in various physiological functions, including embryonic development, cell differentiation, and in vivo homeostasis (Bain et al. 2007). NR proteins consist of two structural subunits. One of the structural subunits is the HOLI domain, which contains an internal binding pocket specifically targeting homologous hormones or ligands and is also responsible for the ligand-regulated transcriptional activation function (AF-2), which is necessary for recruiting various activator proteins. Subsequently, co-activators can interact with chromatin-remodeling proteins and general transcriptional activation mechanisms (Xu and Li 2003). The other structural subunit is a highly conserved DBD located at the center, which is connected to the C-terminal LBD through a linker, triggering activation or inhibition, as well as a zinc-finger structure (Moehren et al. 2004; Claessens and Gewirth 2004). Zinc-binding motifs are stable structures that rarely undergo conformational changes after binding to target proteins. They have specialized functions, such as facilitating gene transcription, translation, mRNA transport, cytoskeleton

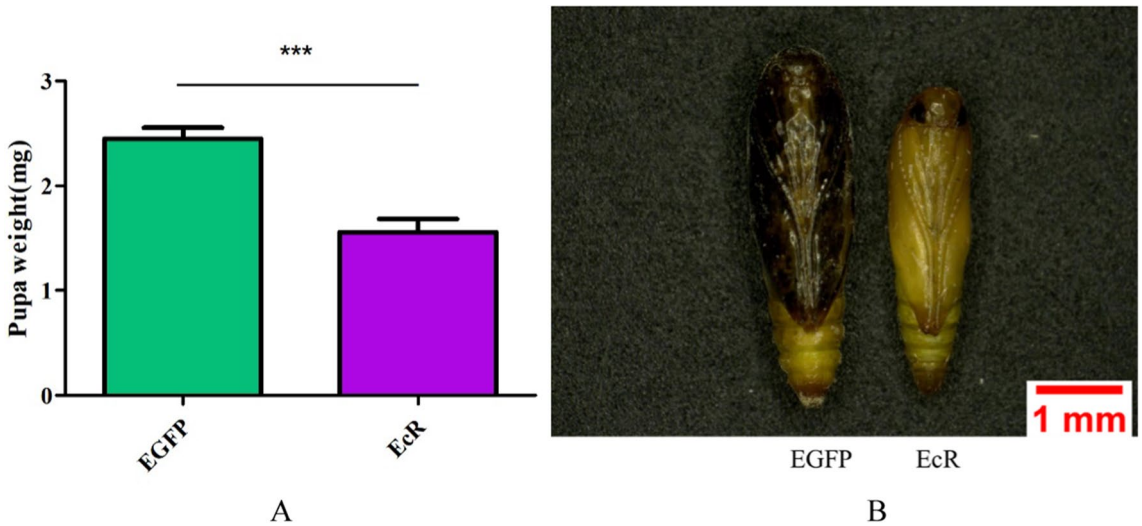

Fig. 9 Effects of feeding *dsEcR* on *Tuta absoluta* pupae. **A** Pupa weights were significantly different between different groups. ***On the horizontal line indicates the difference in the emergence rate of tomato leaf miners under different treatment conditions (***$P < 0.001$). **B** Phenotype of tomato leaf miners after feeding with dsRNA. *dsEGFP* was fed to the control group on the left, and *dsEcR* was fed to the treatment group on the right

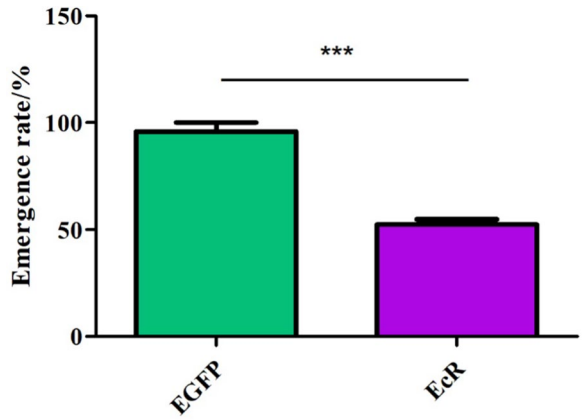

Fig. 10 Effect of feeding *dsEcR* to *Tuta absoluta* on emergence rate. ***On the horizontal line indicates the differences in the emergence rates of tomato leaf miners under different treatment conditions (***$P < 0.001$)

organization, epithelial development, cell adhesion, protein folding, chromatin remodeling, and zinc sensing (Laity et al. 2001).

The *TaEcR* gene is expressed throughout the bodies of *T. absoluta* eggs, larvae, pupae, and adults. In the present study, we determined that the expression of *TaEcR* was significantly higher in the early pupal stage than that in the other instars and was relatively lower in the larval stage. Similarly, a study on *H. vigintioctopunctata* found that *HvEcR* expression levels were high in the pupal and larval stages and relatively low in the eggs, prepupae, and adults (Wu et al. 2021). In a study on the carmine spider mite *T. cinnabarinus*, *TcEcR* mRNA levels were higher in larvae but relatively lower in pupae and adults (Shen et al. 2019). The temporal expression patterns of *EcR* indicate that it strongly influences the completion of larval and pupal develpoment. The inhibition of its expression has a significant effect on the physiological processes of insects.

We analyzed the role of *EcR* in the larval–pupal–adult transition using RNAi in *Tuta absoluta*. Compared with the control group *dsEGFP*, larvae treated with *dsEcR* showed developmental arrest, higher mortality, abnormal pupation, and failure to emerge. In a previous study, after silencing *LmEcR*, the developmental time of *Locusta migratoria* was significantly prolonged, and insects died during the nymph stage, with a mortality rate as high as 100% (Liu et al. 2018). After *dsEcR* injection into the final instar of *Tribolium castaneum* larvae, all the larvae died during the quiescent stage (Tan and Palli 2008). After RNAi of *S. exigua* fifth instar larvae, the mortality rate before pupation was as high as 81.11% (Yao et al. 2010). These findings also demonstrate that *EcR* is essential for the larval-to-pupa transition process. In addition, following RNAi of *TaEcR*, except for those with decreased pupa weights, the color of the pupae did not turn black with the extension of pupation time, and instead, the pupae remained in the brown-yellow stage, and these pupae often showed difficulty in emerging. In many insects, changes in morphological color are regulated by growth and development hormones, such as ecdysone and juvenile hormones (Hiruma and Riddiford 2009). In a study of *M. sexta* in *Lepidoptera*, the cuticular melanization

of larvae during molting was controlled by a cascade of molting hormones (Riddiford et al. 2003). In a study on *Laodelphgax striatellus*, the nymphs of the control group developed normally, and the epidermis developed wing melanin; however, *EcR* silencing blocked melanin production in the wings of the larvae (Wu et al. 2012). These cases have elucidated the molecular mechanism regulating wing melanogenesis, indicating that *EcR* is a key receptor in the ecdysone signaling-mediated melanogenesis cascade. It also directly affects the synthesis of melanin during the development of tomato leaf miners and directly mediates successful emergence in the later developmental stages; this finding also provides evidence for a new function of *EcR* in regulating melanin in completely metamorphosed insects. The key role of *EcR* in the growth and development of *Tuta absoluta* has been verified in this experiment; however, elucidating the cascade regulation relationship between EcR and USP complex regulating 20-hydroxyecdysone requires further experiments. Ecdysone and juvenile hormone jointly regulate moulting and metamorphosis of insects. Their combination to reveal their complex regulatory network is of great significance for pest control. Our study has merely initiated the elucidation of this complex regulatory mechanism, and further mechanistic investigations through future experiments are warranted.

Insect growth regulators are insecticides that mimic hormones, including chitin synthesis inhibitors, juvenile hormone agonists, and molting hormone agonists, which have selective inhibition, low toxicity to mammals, and low resistance in insects (Masih and Ahmad 2019). Several synthetic ecdysone agonists have been developed and commercialized. In 1998, Rohm and Haas first reported a nonsteroidal ecdysone agonist of EcR, a new dibenzoylhydrazine compound, RH-5849 (Wing et al. 1988). The insecticidal properties of commercial dibenzoylhydrazines (e.g., halofenozide, tebufenozide, methoxyfenozide, and chromafenozide) are structurally similar to that of the natural substrate 20E (Hu et al. 2017); dibenzoylhydrazines have good physical and chemical properties and interact with the EcR LBD by competing with 20E, eventually disrupting the molting signaling pathway of the insect (Retnakaran et al. 2003; Song et al. 2017). EcR can be used as the action site of commercially useful chemical insecticides, and a variety of modeling methods, such as homology modeling, molecular docking, MD simulation, binding free energy calculation, and per-residue binding free energy decomposition, are used to design new steroid receptor agonists (Hu et al. 2017). With the emergence of environmental and virulence issues, new requirements have been proposed for the development of insecticides that are environmentally friendly and have novel modes of action (Casida and Bryant 2017; Sparks et al. 2019), among which RNAi is considered to be a favorable technology for eco-friendly and species-specific pest control (Liu et al. 2020). *EcR*, as a key target gene of tomato leaf miners, has great potential for pest control against lepidoptera insects. The application of dsRNA that carries EcR genes is a promising method to achieve effective pest control, and this green control method has been initially explored in tomato leaf miners (Wang et al. 2023b). It is expected to realize the promotion and application of dsRNA/nanoparticle complexes in the future.

Conclusions

The results of this study provide new insights into the biological functions of *EcR* in *Tuta absoluta*. Ecdysone is a hormone unique to arthropods, and EcR plays a key role in insect molting, metamorphosis, reproduction, diapause, and innate immunity. In the present study, we used gene cloning, bioinformatics, and RNAi technologies to elucidate the role of *TaEcR* in the larval–pupal–adult transition and melanization of tomato leaf miners. This gene affects the metamorphosis of insects, and a lack of this gene leads to larval death or pupation failure. However, *TaEcR* also has a marked effect on the synthesis and accumulation of melanin in the pupal stage, and the lack of this gene leads to emergence failure. How the heterodimer complex of two NRs-EcR and USP—mediates 20E signaling and regulates molting, reproduction, diapause, and immunity in insects must be studied further. However, these genes are potential targets for RNAi-based pest control to provide green, safe, and effective pest prevention and control methods.

Abbreviations

RNAi	RNA interference
EcR	Ecdysone receptors
USP	Ultraspiracle
DBD	DNA-binding domain
LBD	Ligand-binding domain
RT-qPCR	Real-time quantitative polymerase chain reaction
ANOVA	Analysis of variance
ORF	Open reading frame
NR	Nuclear receptor

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Author contributions

XW analyzed the results and wrote the original draft preparation, JW did the RNAi experiment and made phenotypic observations, JG helped to revise the introduction and discussion, NY helped to analyze the results, FW helped to revise the discussion, ZL was responsible for the experiment design and the manuscript's revision, WL helped to revise the introduction and results.

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Availability of data and materials
The data presented in this study are available upon request from the corresponding author.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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