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The role of tyrosine hydroxylase and dopa decarboxylase in melanin biosynthesis and metamorphosis in *Coccinella septempunctata*

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With 4 figures

Abstract: Melanin has been recognized as a crucial factor in the evolution, polymorphisms, and environmental adaption of insects. Within the Coleoptera family, ladybird beetles exhibit remarkable phenotypic variation characterized by the number and size of black spots on their elytra. This distinctive characteristic makes ladybird as ideal models for studying melanin synthesis and its associated physiological effects. In this study, we focused on tyrosine hydroxylase (*CsTH*) and dopa decarboxylase (*CsDDC*), which participate in melanin biosynthesis in the monomorphic seven-spotted ladybird beetle, *Coccinella septempunctata*. Spatial expression profiles in adults revealed that *CsTH* was predominantly expressed in the elytra, while *CsDDC* was primarily expressed in elytra and ovaries. RNA interference (RNAi)-mediated silencing of these genes in fourth instar larvae led to reduced melanin on the cuticle, complete mortality shortly after eclosion, and disrupted eclosion processes. Furthermore, dopamine levels significantly decreased following the silencing of *CsTH*. Silencing *CsTH* during early pupal stages prevented eclosion, while later silencing permitted eclosion but resulted in melanin deficiency. In contrast, silencing *CsDDC* did not affect adult cuticle melanin synthesis. Our findings highlighted the vital roles of *CsTH* and *CsDDC* in melanin synthesis and metamorphosis in *C. septempunctata*, demonstrating that dopamine melanin is the primary component of cuticle melanin and underscoring the multifunctional effects of these genes on metamorphosis. The results highlight some mechanisms underneath insect melanization during cuticle formation and provide evidence of the multifunctional effects of essential genes in metamorphosis process in insects.

Keywords: Melanin synthesis; seven-spotted ladybird beetle; dopamine; holometabolous; cuticle

1 Introduction

The phenomenon of insect cuticle melanization is a significant aspect of insect biology and has garnered considerable attention from researchers over several decades (Kramer et al. 1984; Noh et al. 2016; Ando et al. 2019). Melanization refers to the accumulation of melanin, a pigment responsible for dark coloration, within the insect cuticle (Noh et al. 2016; Wittkopp et al. 2009), and this process plays a crucial role in insect adaptation, survival, and ecological interactions (Noh et al. 2016; Bezzerides et al. 2007). The ability of insects to regulate cuticle melanization is a highly dynamic and context-dependent process (Blount et al. 2012), influenced by a complex interplay of intrinsic and extrinsic factors. Hormonal signaling pathways, notably involving ecdysteroids and juvenile hormones, are known to play a critical role in the regulation of melanin synthesis (Bitondi et al. 1998; Tang et al. 2022). Moreover, environmental stimuli such as light exposure, temperature fluctuations, and humidity levels have all been suggested as potential contributors to the melanization process (MK et al. 2022).

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In insects, melanin is a nitrogen-containing polyphenolic pigment, and its synthesis typically starts from the amino acid tyrosine (Kramer et al. 1987). Through the catalysis of tyrosine hydroxylase (TH), tyrosine is hydroxylated to form L-3,4-dihydroxyphenylalanine (Dopa) (Wang et al. 2022). Dopa can synthesize dopa-melanin directly or enzymatically be converted to dopamine by dopa decarboxylase (DDC) (Arakane et al. 2009). Under the catalysis of phenol oxidases (PO), dopamine is oxidized to form dopamine quinone. Dopamine quinone acts as the final crucial intermediate and undergoes a series of reactions to eventually produce melanin (True 2003). As pivotal enzymes in the process of insect melanin synthesis, TH and DDC have been firmly established to exert critical functions in numerous species of insects (Wang et al. 2022; Arakane et al. 2009). For example, they significantly influence larval and adult cuticle tanning and development in *Blattella germanica*, which consequentially impact their cuticular permeability (Bai et al. 2022). Similarly, the silencing expression of *DDC* results in the absence of melanin in *Rhodnius prolixus*, further affecting ecdysis, survival and reproduction (Sterkel et al. 2019). The investigation of elytral patterns in Coleoptera represents a widely explored and intriguing domain within entomological research (Kramer et al. 1987). Previous studies demonstrated that the primary reason led to *Harmonia axyridis* with various phenotypes is melanin distribution diversity on the body wall and confirmed that dopamine melanin is the primary melanin in their cuticle (Wu et al. 2022; Chen et al. 2019; Xiao et al. 2020). Similar to *H. axyridis*, the melanin in exoskeleton of *Henosepilachna vigintioctopunctata* is synthesized via the tyrosine metabolic pathway (Ze et al. 2022). This research illuminated distinct adaptive and evolutionary mechanisms inherent to each species. Nevertheless, it remains crucial to further investigate the functionality of identical genes within diverse biological contexts.

In this study, the seven-spot ladybird beetle, *Coccinella septempunctata* (Coleoptera: Coccinellidae) plays a significant role in biological pest control within agricultural ecosystems (Angalet et al. 1979; Hodek et al. 2008). Its distinctive appearance includes reddish-orange elytra with seven black spots. Nevertheless, despite its ecological importance and visual distinctiveness, the precise mechanistic intricacies and underlying regulatory factors governing melanin synthesis have not been fully elucidated. This investigation aims to delineate the specific functions and regulatory mechanisms of the *TH* and *DDC* genes in the melanogenesis pathway of *C. septempunctata*. This study explored the molecular pathways and mechanisms by which these genes influence melanin biosynthesis, while also examining their physiological and developmental roles to enhance understanding of genetic regulation in insect melanization.

2 Materials & methods

2.1 Insect collection and rearing

The insect colony was collected from corn fields (39°95′N, 116°28′E) in Guiyang, Guizhou province, China and a breeding population was established in a controlled laboratory environment. Insects were reared in aluminum frame cages (50 cm × 50 cm × 50 cm) covered with 100-mesh plastic gauze and maintained at 25 ± 1 ℃, 60% relative humidity and a 16 h:8 h (light: dark) photoperiod. Ladybirds were provided with cowpea aphids, *Aphis craccivora* Koch (Hemiptera: Aphidoidea) on leaves of seedlings of broad bean, *Vicia faba* L., cv. "LinCan-5".

2.2 RNA extraction and cDNA synthesis

The total RNA from each sample was isolated by TRIzol reagent (Invitrogen, Carlsbad, CA, United States), following standard protocols and assessed using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, United States) at 260 nm. To ensure the removal of potential genomic DNA contamination, 1.0 μg of total RNA was treated with gDNA Eraser (Takara, Dalian, China) in a specific enzymatic reaction. Subsequently, the first-strand cDNA synthesis was carried out using the First Strand cDNA Synthesis Kit (Takara, Dalian, China) with oligo $(dT)_{18}$ as the primer in a 20 μL reaction system. This cDNA synthesis method allowed for the conversion of mRNA into cDNA, facilitating downstream gene expression analysis. The resulting first-strand cDNA served as the template for all subsequent molecular analyses.

2.3 Molecular cloning and sequence analysis

The amino acid sequences of the annotated TH and DDC proteins from *H. vigintioctopunctata* and *H. axyridis* were downloaded from the National Center for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov/) reference sequence database. These two protein sequences were subjected to a BLAST search (E-value = $1e^{-5}$) against *C. septempunctata* genome data retrieved from InsectBase v2.0 (http://v2.insect-genome.com/). The identification of open reading frames (ORFs) was conducted using the ORFfinder tool from NCBI (https://www.ncbi.nlm.nih.gov/orffinder/). Gene-specific primers for obtaining the full-length cDNA of *CsTH* and *CsDDC* were designed based on Primer-BLAST from NCBI (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). Incorporating TA cloning, we successfully cloned the cDNA encoding both the *CsTH* and *CsDDC* genes and uploaded to NCBI database with accession number PP982312 and PP982319. Concurrently, qPCR-specific primers were designed using the Primer-BLAST, while primers for the synthesis of double-stranded RNAs were generated with the E-RNAi website (https://www.dkfz.de/signaling/e-rnai3/) (Table S1).

The tertiary protein structures of CsTH and CsDDC were predicted with AlphaFold2 Protein Structure Database (Jumper et al. 2021) and visualized using PyMOL v2.5.8 (https://pymol.org/2/). Protein sequences were aligned by MUSCLE v3.8.1551. The phylogenetic trees of CsTH and CsDDC in 18 insect species from Coleoptera, Diptera, Lepidoptera, Hemiptera and Hymenoptera were constructed with the neighbor-jointing method by IQ-TREE v2.1.2 and 1000 rapid bootstrap replicates to assess the robustness of the trees. Sequences used in the trees were downloaded from Uniport and iTOL (https://itol.embl.de/) was utilized to visualize and enhance the appearance of the phylogenetic trees.

2.4 Real-time quantitative PCR (RT-qPCR)

To investigate spatial and temporal expression patterns, complementary DNAs (cDNAs) were synthesized from total RNA extracted from various developmental stages, ranging from 1-day-old eggs to emergence-12h adults. Adults were used for tissue expression analysis (leg, head, midgut, elytra and ovary) because their tissues and organs are fully differentiated, providing more accurate gene expression profiles, particularly for neurotransmitter synthesis and melanin synthesis processes. The primer sets utilized for qPCR were meticulously designed using the Primer-BLAST tool (Table S1). The optimized quantitative PCR (qPCR) program was performed on Bio-Rad CFX96 Real-Time PCR Detection System (Bio-Rad, USA) and conducted as follows: an initial denaturation step at 95 °C for 10 minutes, followed by 40 amplification cycles consisting of denaturation at 95 °C for 15 seconds and annealing/extension at 60 °C for 1 minute. To verify the specificity of the amplification, dissociation curves were generated by cooling the samples to 55 °C after denaturation, and melting curves were obtained by increasing the temperature by 0.5 °C every 10 seconds for each cycle, totaling 80 cycles until reaching 95 °C to denature the double-stranded DNA. For each experimental condition, three biological replicates were analyzed, and each biological replicate was measured in triplicate. *Actin* was used as a reference gene and relative transcript levels were calculated using the $2^{-\Delta\Delta Ct}$ method.

2.5 RNA interference of *CsTH* and *CsDDC*

RNAi was performed to investigate the functional significance of *CsTH* and *CsDDC* in the developmental process and elytral melanization of *C. septempunctata*. To initiate the RNAi process, dsRNAs were synthesized using the MEGAscript RNAi Kit (Invitrogen, Carlsbad, CA, United States) following the manufacturer's protocols. Detailed information regarding the primers used for dsRNA synthesis can be found in Table S1. For the RNAi experiments, fourth-instar larvae, 1-, 2- and 3-day pupae were carefully chosen for the injections of *CsTH* and *CsDDC* double-stranded RNA (ds*CsTH* and ds*CsDDC*) at a precise dose of 300 ng per individual and similar numbers of the same life stages were injected with dsRNA of the green fluorescent protein gene (GFP) at the same dose to serve as controls. The injected insects were raised under standardized conditions, and their phenotypes were diligently observed and recorded daily following the injection.

2.6 Enzyme-linked immunosorbent assay (ELISA) of dopamine content

Samples of *C. septempunctata* were collected at 3, 4, 5 and 6 days after ds*CsTH* were injected in the fourth instar larvae. These insects were subsequently homogenized in PBS buffer (1×) and then centrifuged for 20 min at 2, 000 rpm. Following this, the supernatant was extracted for dopamine level detection. The quantification of dopamine content was measured using an insect dopamine enzyme-linked immunosorbent assay (ELISA) kit (Jin En Lai, Beijing, China) in accordance with the manufacturer's protocol. For the determination of absorbance, both the diluted test sample and standards were measured at the wavelength of 450 nm using an enzyme-linked immunosorbent assay reader (PerkinElmer, Massachusetts, USA).

2.7 Image acquisition and photography

Microscopy images were acquired using the Zeiss Microscope SteREO Discovery V20 (Carl Zeiss, Germany). All images were captured under consistent conditions, maintaining the same magnification, exposure time, and light intensity settings throughout the imaging process. Subsequently, images were selected to represent the most characteristic and representative phenotypes observed during the study.

2.8 Data analysis

In the context of RT-qPCR analysis, the quantification of *CsTH* and *CsDDC* transcript levels was expressed as a percentage relative to the control group. This normalization process involved calculating the relative expression value (REV) in the ds*CsTH*- and ds*CsDDC*-injected insects and then dividing it by the REV in the ds*GFP*-injected insects, followed by multiplication with 100 to obtain the final percentage value. To ensure statistical rigor, the percentage data from both developmental stages analysis and RNAi experiments were subjected to an arcsine square-root transformation. One-way ANOVA followed by Tukey's honestly significant difference (HSD) test was performed by SPSS software to analyze the expression pattern ($P < 0.05$). Student's *t*-test ($P < 0.05$) was utilized to compare the difference between control and RNAi treatments. The figures in this experiment were generated using GraphPad v9.0.

3 Results

3.1 Sequence and phylogenetic analysis of *CsTH* and *CsDDC*

The ORF of the *CsTH* gene were 1608 base pairs (bp), encoding a protein of 535 amino acids with a molecular weight of 61.08 kDa and an isoelectric point of 5.75. Analysis of the *CsTH* genome disclosed the presence of seven exons interspersed with six introns (Fig. S1a). The secondary and tertiary structure analysis showed that the CsTH comprised four different subunits (Fig. S1b). The phylogenetic tree of CsTH was constructed using amino acid sequences from eight Coleoptera species, two Diptera species, two Lepidoptera species, three Hemiptera species and two Hymenoptera species. The results demonstrated that tyrosine hydroxylase (TH) genes exhibit a high degree of conservation within orders, as evidenced by strong bootstrap support. Notably, sequence from *C. septempunctata, H. axyridis* and *H. vigintioctopunctata* converged into a distinct clade (Fig. S1d).

The ORF of the *CsDDC* gene are 1458 bp and is predicted to translate into a protein comprising 485 amino acids, with a molecular weight of 53.93 kDa and an isoelectric point of 5.71. This gene's structure includes eight exons separated by seven intervening introns (Fig. S1a). The secondary and tertiary structure analysis showed that the CsDDC comprised 25 α-helices and four β-sheets (Fig. S1c). The phylogenetic analysis of CsDDC involved amino acid sequences from six Coleoptera species, four Diptera species, two Lepidoptera species, four Hemiptera species and two Hymenoptera species. *C. septempunctata, H. axyridis* and *H. vigintioctopunctata* were grouped into one cluster. (Fig. S1e).

3.2 Expression patterns of *CsTH* and *CsDDC*

To reveal the temporal and spatial expression profiles of *CsTH* and *CsDDC*, comprehensive developmental stage-specific and tissue-specific expression analyses were conducted for two genes. Based on RT-qPCR results, we observed that *CsTH* was expressed at all developmental stages. *CsTH* is expressed during the embryonic stages, with no significant difference among E1, E2 and E3 (Fig. 1a). Then, the expression level of *CsTH* was significantly decreased in the 1st instar larvae. As the second instar larvae emerge, the expression level of *CsTH* gene increase, showing no significant difference compared to its expression during embryonic development. In the later stages of larval development, the expression level of *CsTH* was decreased. *CsTH* expression showed variability during the pupal stages. P1 had a high expression level with no significant difference as compared with early embryonic stages. In adult females and males, the expression level of *CsTH* were significantly lower compared to most other developmental stages, including embryonic, larval, and early pupal stages (Fig. 1a). The transcript level of *CsDDC* was also present throughout different developmental stages. However, expression level reached the highest in 4-day pupae and the lowest at E2 (2-day-old eggs) (Fig. 1b). To cover a diverse range of physiological systems and organs within adult ladybird, we investigated the expression in leg, head, midgut, elytra and ovary. The result of tissue-specific expression of *CsTH* showed that the highest expression level is in the elytra ($F_{4,\ 10} = 14.57$, $P = 0.0004$, Fig. 1c). Nevertheless, *CsDDC* exhibited the highest expression in the ovary, with notably lower expression levels in the leg, head, elytra and midgut ($F_{4,\ 10} = 395.3$, $P < 0.0001$, Fig. 1d).

3.3 RNAi of *CsTH* and *CsDDC* in the fourth instar larvae and its effect on pupation and eclosion

We observed considerable suppression of *CsTH* transcript levels in the fourth instar larvae following injection with ds*CsTH* as compared with control on day 2 and 4. The *CsTH* transcript levels were suppressed by 95.9% on day 4 after ds*CsTH* injection ($t_4 = 3.336$, $P = 0.0281$, Fig. 2a). Injection of ds*CsDDC* also demonstrated a significant silencing effect on its target gene silencing. The transcript level of *CsDDC* was suppressed by 87.0 % on day 2 post- injection ($t_4 = 4.337$, $P = 0.0023$, Fig. 2b).

The silencing of *CsTH* in the fourth instar larvae did not affect the final pupation rate of *C. septempunctata* (day 6: $t_4 = 1.949$, $P = 0.123$, Fig. 2c), however, it seriously impacted the eclosion rate that all injected individuals failed to eclosion (day 10: $t_4 = 14.46$, $P = 0.0001$, Fig. 2d). Unexpectedly, these pupae that failed to undergo normal metamorphosis exhibited signs of activity beyond the typical time point of metamorphosis. Specifically, this was evidenced by the test insects were only able to lie flat with observable movements in their legs. Furthermore, these malformed individuals eventually died within 5 days after emergence (day 15: $t_4 = 38.18$, $P < 0.0001$, Fig. 2e). These results indicated that *CsTH* is involved in regulating eclosion process in *C. septempunctata*. Conversely, the silencing of *CsDDC* did not impact either the pupation (day 6: $t_4 = 2.283$, $P = 0.0845$, Fig. 2f) or eclosion rates (day 10: $t_4 = 1.357$, $P = 0.2463$, Fig. 2g) in *C. septempunctata*. Despite successful eclosion, these ladybirds displayed symptoms of sluggish movement and an inability to prey, leading to their eventual death (day 15: $t_4 = 35.36$, $P < 0.0001$, Fig. 2h).

3.4 RNAi of *CsTH* and *CsDDC* in the fourth instar larvae and its effect on melanin synthesis

Silencing of *CsTH* in fourth instar larvae resulted in complete pale pupae with normal morphology, in contrast to the ds*GFP* injected group (Fig. 3a). Additionally, silencing of *CsDDC* in fourth instar larvae resulted in melanin deficiency during the initial pupal stage (1–5 hours), unlike the ds*GFP* injected group that melanin deposition on the cuticle was observed 1 h after pupation. In ds*CsDDC* injected group, melanin deposition on the pupae cuticle commenced approximately 24 h after pupation. Furthermore, silencing *CsDDC* also affected the timing and extent of melanin deposited in the adult cuticles, with minimal melanin observed on

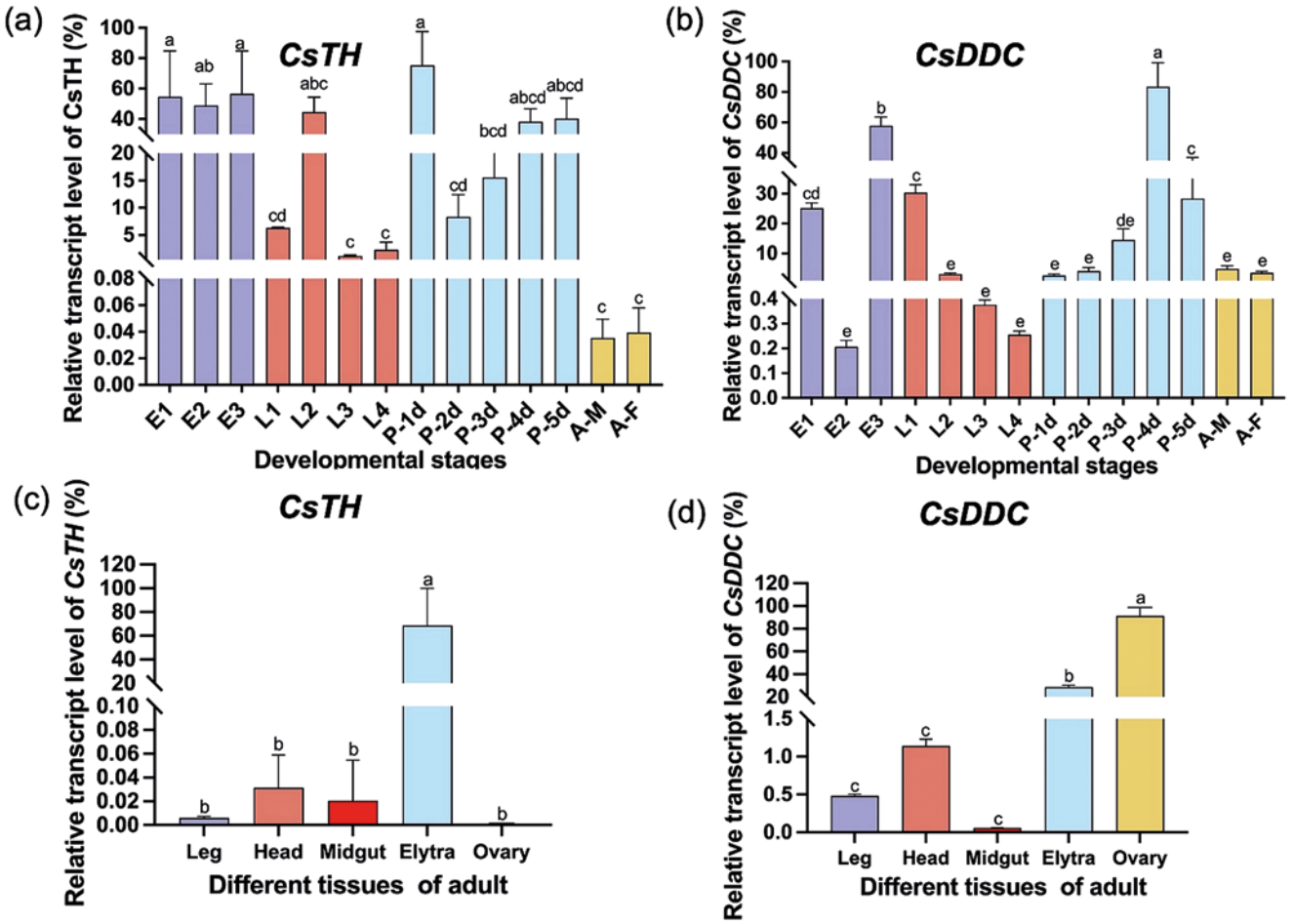

Fig. 1. Developmental-stage and tissue-specific expression pattern of *CsTH* and *CsDDC* in *Coccinella septempunctata*. (a) The relative transcript levels of *CsTH* and (b) *CsDDC* at different developmental stages performed by RT-qPCR. E1, E2, E3 represent 1-, 2-, 3-day-old eggs; L1, L2, L3 and L4 represent 1st, 2nd, 3rd and 4th instar larvae; P-1d, P-2d, P-3d and P-4d represent 1-, 2-, 3- and 4-day-old pupae; A-M represents 1-day-old male adult and A-F represents 1-day-old female adult. (c) The relative transcript levels of *CsTH* and (d) *CsDDC* in different tissues (leg, head, midgut, elytra and ovary) of female adult performed by RT-qPCR. Each tissue sample was independently replicated three times. Different letters indicate significant difference (ANOVA followed by Tukey-test, $P < 0.05$).

the head and pronotum approximately 24h after eclosion (Fig. 3b). Comprehensive analysis of the phenotype between *CsTH* and *CsDDC* silenced, it is inferred that dopamine melanin is the primary component of the cuticle melanin in the *C. septempunctata*.

3.5 RNAi of *CsTH* and *CsDDC* in the pupal stage and its effect on eclosion and melanin synthesis

To comprehensively elucidate the melanin synthesis mechanism in *C. septempunctata*, gene silencing of *CsTH* and *CsDDC* was performed in 1-, 2- and 3-day old pupae. A robust RNAi–mediated suppression of gene expression was observed during the pupal stage (Fig. S2a–f). Specifically, the transcript levels of *CsTH* were suppressed by 95.88% on day 2 following the injection of ds*CsTH* in 3-day old pupae ($t_4 = 4.519$, $P = 0.0107$, Fig. S2c). Additionally, the transcript levels of *CsDDC* were suppressed by 97.30% on day 4 after ds*CsDDC* injected in 2-day old pupae ($t_4 = 28.39$, $P < 0.0001$, Fig. S2e).

Silencing *CsTH* at various pupal stages induced a range of phenotypes in *C. septempunctata* (Fig. 4a). When *CsTH* was silenced in 1-day-old pupae, it led to complete eclosion failure. In addition, silencing *CsTH* in 2-day-old pupae resulted in two distinct phenotypes: approximately 34% of the ladybirds exhibited unsuccessful eclosion with abnormalities similar to those silenced at 1-day-old. Conversely, around 66% of the ladybirds managed successful eclosion but demonstrated total melanin deficiency and presented with deformed elytra, which were unable to close properly at the ends. Furthermore, silencing *CsTH* in 3-day-old pupae resulted in adults that successfully enclosed yet displayed complete melanin deficiency in their cuticle. Overall, all ladybirds derived from *CsTH*-silenced pupae died within

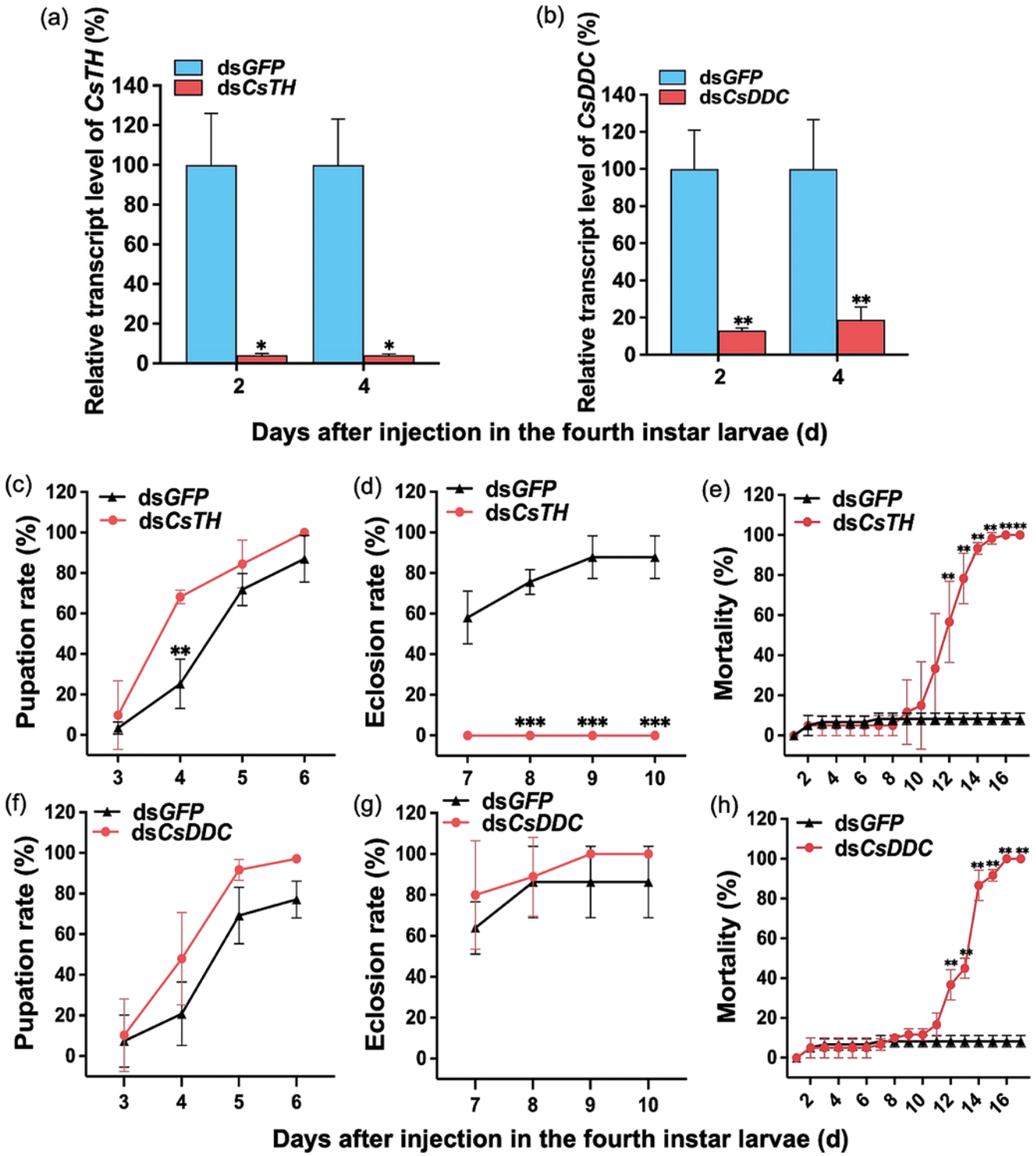

Fig. 2. The relative transcript level of (a) *CsTH* and (b) *CsDDC* following the injection of ds*CsTH* and ds*CsDDC* in the fourth instar larvae of *Coccinella septempunctata*. ds*GFP* was injected as a control. The results are presented as mean and standard errors with three biological replicates, each with three technical replicates of three ladybirds, respectively. Asterisks above the standard error bars indicate significant differences as determined by Student's *t*-test ($P < 0.05$). Time-dependent pupation rates, eclosion rates and mortality observed in the fourth instar larvae injected with (c, d and e) ds*CsTH* and (f, g and h) ds*CsDDC*. The percentages for pupation and eclosion were calculated from three independent replicates, each comprising a minimum of 40 ladybirds. ***: $P < 0.001$, **: $P < 0.01$, *: $P < 0.05$ (Student's *t*-test).

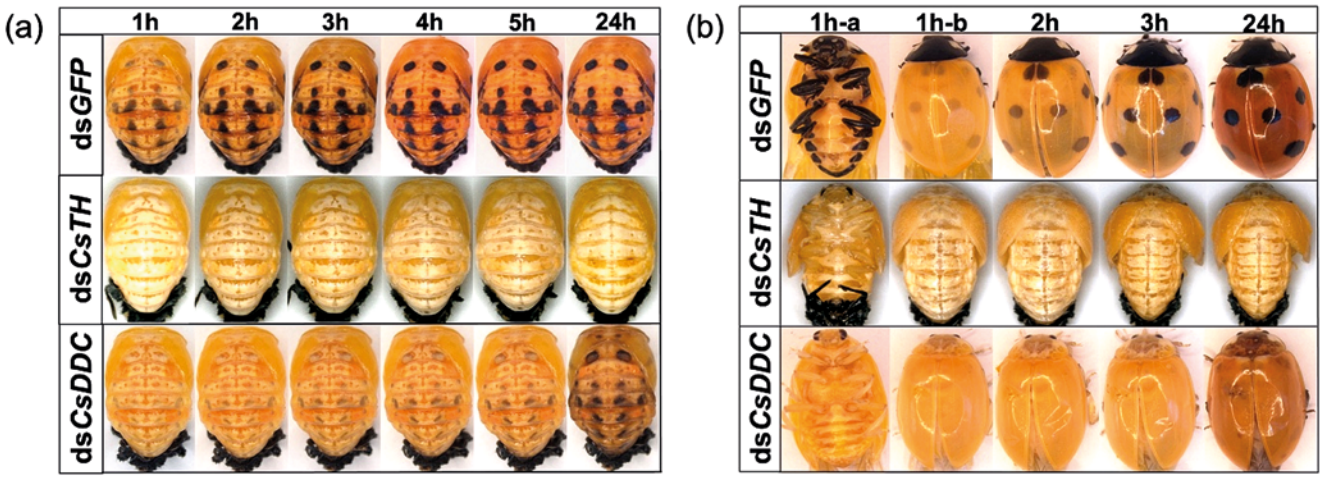

Fig. 3. Dorsal phenotypes of pupae following the injection of ds*CsTH* and ds*CsDDC* in the fourth instar larvae of *Coccinella septempunctata*. (a) Ranging from 1 to 24 h after pupation. (b) The phenotypes of adults from 1 to 24 h after emergence, with 1h-a representing ventral views and 1h-b representing dorsal views, respectively. The phenotype was recorded for three independent replicates, each comprising a minimum of 30 ladybirds.

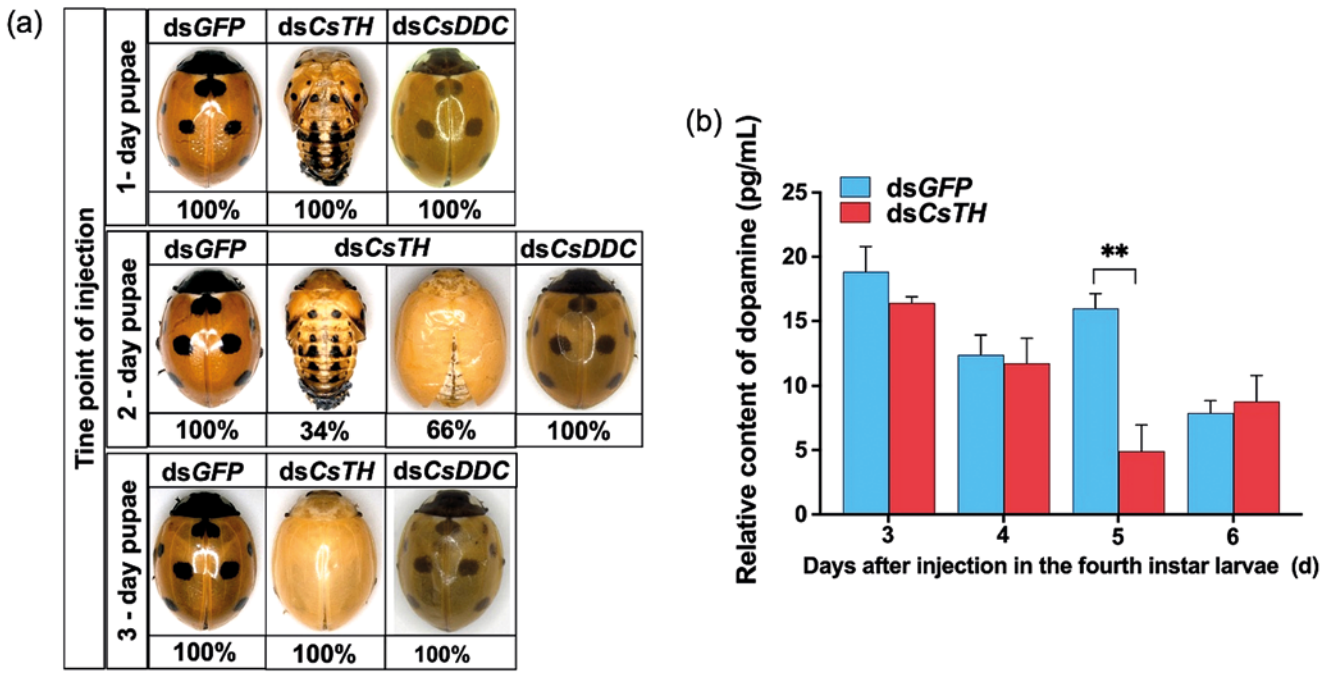

Fig. 4. (a) The percentage below each image represents the proportion of phenotype following the injection of ds*CsTH* and ds*CsDDC* after 2- and 4-days in the 1-, 2- and 3-day pupae of *Coccinella septempunctata*. The phenotype was recorded for three independent replicates, each comprising a minimum of 30 ladybirds. (b) The relative content of dopamine after injection in the fourth instar larvae of *Coccinella septempunctata*. The result is presented as mean and standard errors with three biological replicates, each with three technical replicates of three ladybirds, respectively. **: $P < 0.01$ (Student's *t*-test).

5 days post -eclosion. On the other hand, silencing *CsDDC* during the pupal stage led to all adults successfully undergoing metamorphosis with no deformities, albeit with a slight reduction in melanin content.

3.6 RNAi of *CsTH* in the fourth instar larvae and its effect on dopamine content

The silencing of *CsTH* in the fourth instar larvae significantly impacts cuticle melanin deposition in both pupae and adults. Concurrently, it adversely affects eclosion processes, ultimately leading to death. To elucidate the regulatory mechanism of *CsTH* on melanin synthesis and eclosion processes, we measured dopamine levels between ds*CsTH* and ds*GFP* injected group. Notably, dopamine levels were significantly reduced in the *CsTH*-silenced group as compared with ds*GFP*-injected group on fifth day post injection (t_4 = 4.727, P = 0.0091, Fig. 4b). This study suggests that TH may participate in the eclosion process via a dopamine-mediated signaling regulation network in *C. septempunctata*.

4 Discussion

The melanin present in the exoskeleton of ladybirds not only imparts a distinctive appearance but also reflects their complex ecological adaptability, defense mechanism, and evolutionary history. Therefore, they serve as representative insects for studying the synthesis and function of melanin, as well as the contribution to fitness (Noh et al. 2016). This study clarified the critical roles of *CsTH* and *CsDDC* in melanin production in *C. septempunctata*, and emphasized the importance of *CsTH* in adult development and elytra formation. Insects are known to produce two distinct types of melanin: dopamine melanin utilizing dopamine as a substrate, and dopa melanin derived from dopa (Kramer et al. 1987). Research focusing on closely related species of the *C. septempunctata*, specifically polymorphic *H. axyridis* and phytophagous *H. vigintioctopunctata*, has revealed that the predominant melanin type in these species is dopamine melanin (Chen et al. 2019; Xiao et al. 2020; Zhang et al. 2020). In *C. septempunctata*, when *CsTH* expression was inhibited in fourth instar larvae resulted in a complete absence of melanin in the exoskeletons of pupae and adults (Fig. 3). Considering that *CsTH* is an upstream enzyme responsible for converting tyrosine to dopa, it was hypothesized that the exoskeleton's melanin primarily comprises either dopa melanin or dopamine melanin. Conversely, inhibiting *CsDDC* synthesis resulted in reduced melanin production in pupae and adults, thereby confirming dopamine melanin as the primary melanin component in the exoskeleton in *C. septempunctata*. Consequently, it is evident that the melanin synthesis pathway in the exoskeleton of Coleoptera Coccinellidae insects is conserved.

Tyrosine hydroxylase (TH), the rate-limiting enzyme in the tyrosine metabolic pathway, is crucial for cuticle tanning and metamorphosis (Xiao et al. 2020; Zhang et al. 2019), particularly in elytral formation in adult beetles (Arakane et al. 2011). In this study, *CsTH* suppression in fourth-instar larvae allowed normal pupation (Fig. 3a), but eclosion failed (Fig. 2d), leading to the death of malformed adults (Fig. 2e), highlighting *CsTH*'s essential role in eclosion. Similarly, *TH* suppression in *H. axyridis* larvae did not affect eclosion but resulted in adult mortality post-eclosion (Xiao et al. 2020), suggesting *TH*'s conserved function in ladybird development. When *CsTH* was suppressed during early pupation, eclosion was unsuccessful, emphasizing its critical role in eclosion initiation. However, suppression during later pupal stages allowed normal eclosion (Fig. 4a), though all subjects died post-emergence, underscoring *CsTH*'s importance for adult survival in *C. septempunctata*.

Dopamine is a key downstream product of tyrosine hydroxylase (TH) in the tyrosine metabolism pathway, plays a crucial role in metamorphosis in holometabolous insects (Malhotra et al. 2023). In this study, dopamine levels significantly decreased three days after ds*CsTH* injection in fourth instar larvae, coinciding with the onset of the pupal stage (Fig. 4b). During pupation, insects undergo metabolic shifts for tissue reconstruction and growth, requiring energy and complex signal transduction for gene expression (Martín et al. 2021). Dopamine interacts with GPCRs, activating second messenger systems like cAMP, which triggers gene expression (Vallone et al. 2000). We hypothesize that reduced *CsTH* expression lowers dopamine levels, impairing signal transduction during tissue reconstruction, affecting genes crucial for ecdysone synthesis and wing development, and leading to eclosion failure in *C. septempunctata*.

In the polymorphic *H. axyridis*, dopa decarboxylase (DDC) also worked as a crucial enzyme in dopamine melanin synthesis and playing an integral role in the cuticle tanning process (Chen et al. 2019; Xiao et al. 2020). Our study investigates the role of the *CsDDC* gene in developmental processes, revealing that its suppression in fourth-instar larvae markedly impedes melanin synthesis (Fig. 3), although pupation and eclosion proceed normally in the monomorphic *C. septempunctata* (Fig. 2f & g). Furthermore, suppression of *CsDDC* gene expression during the pupal stage resulted in normal eclosion and survival rate of the insects, albeit with a marginal decrease in melanin content. This observation further underscores the critical role of the *DDC* gene in melanin synthesis. Totally, these findings indicate that the conserved *DDC* gene not only contributes to melanization of the ladybird beetle's cuticle but also plays a role in the cuticle hardening process.

Peaks in *TH* and *DDC* gene expression during the pupal stage in both polymorphic *H. axyridis* and monomorphic *C. septempunctata* suggested their crucial role in adult development (Chen et al. 2019; Xiao et al. 2020). Our study and previous reports indicated that the impact of *TH* gene silencing on adult phenotype diminishes as pupation progresses. In *H. axyridis*, silencing *HaTH* on day five of the pupal stage does not affect adult melanization, whereas earlier silencing

leads to a complete lack of melanin. In *C. septempunctata*, early silencing of *CsTH* results in failed eclosion, while later silencing increases eclosion rates but still prevents melanin synthesis (Fig. 4a). *CsDDC* silencing has minimal impact on adult melanin in *C. septempunctata* (Fig. 4a). Suppression of *DDC* led to increased dopa content, which might be directly converted to dopa melanin in the absence of *DDC*, resulting in a dark exoskeleton. These findings suggest that while *TH* is critical for adult development, its activation occurs later than *DDC*.In summary, this research substantially advances our comprehension of the roles and mechanisms of *CsTH* and *CsDDC* in the melanin synthesis and developmental processes of the monomorphic seven-spot ladybird beetle. *CsTH* is vital for successful eclosion and adult development, its influence diminishes as the pupal stage progresses, indicating a nuanced involvement in developmental timing. Conversely, the role of *CsDDC*, primarily in melanin synthesis and cuticle hardening, is consistent throughout development, underscoring its importance in the physiological resilience. These findings highlight the essentiality of both *TH* and *DDC* in the melanin synthesis pathway, notably with their expression reaching a peak during the pupal stage, which is pivotal for adult insect development. The results delineate the distinct roles of these enzymes in the epidermal melanization process, an essential factor for insect survival and adaptability.

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Table S1; Fig. S1, S