Received: 23 February 2024 | Accepted: 16 October 2024

DOI: 10.1111/imb.12968

RESEARCH ARTICLE

Insect Molecular Biology

Pleiotropic effects of *Ebony* on pigmentation and development in the Asian multi-coloured ladybird beetle, *Harmonia axyridis* (Coleoptera: Coccinellidae)

Jing Lin[1,2] | Da Xiao[2,3] | Mengmeng Wu[1] | Xu Chen[1] | Qingxuan Xu[2,3] | Su Wang[2,3] | Liansheng Zang[1]

[1]State Key Laboratory of Green Pesticide; Key Laboratory of Green Pesticide and Agricultural Bioengineering, Ministry of Education, Center for R&D of Fine Chemicals of Guizhou University, Guiyang, China

[2]Institute of Plant Protection, Beijing Academy of Agriculture and Forestry Sciences, Beijing, China

[3]Key Laboratory of Natural Enemies Insects, Ministry of Agriculture and Rural Affairs, Beijing, China

Correspondence
Su Wang, Institute of Plant Protection, Beijing Academy of Agriculture and Forestry Science, Beijing 100097, China.
Email: wangsu@ippbaafs.cn

Liansheng Zang, State Key Laboratory of Green Pesticide; Key Laboratory of Green Pesticide and Agricultural Bioengineering, Ministry of Education, Center for R&D of Fine Chemicals of Guizhou University, Guiyang 550025, China.
Email: lsz0415@163.com

Funding information
Technical Innovation Program of Beijing Academy of Agriculture and Forestry Sciences, Grant/Award Number: KJCX20230115; National Key Research and Development Program of China, Grant/Award Number: 2023YFD1400600

Associate Editor: Haobo Jiang

Abstract

Melanin plays a pivotal role in insect body pigmentation, significantly contributing to their adaptation to diverse biotic and abiotic environmental challenges. Several genes involved in insect melanin synthesis showed pleiotropic effects on insect development and reproduction. Among these, the N-β-alanyl dopamine synthetase gene (*Ebony*) is integral to the pigmentation process. However, the full spectrum of its pleiotropic impacts is not yet thoroughly understood. In this study, we identified and characterised the *HaEbony* gene in the Asian multi-coloured ladybird beetle (*Harmonia axyridis*) and found that *HaEbony* gene is a conserved gene within the Coleoptera order. We aimed to further explore the multiple roles of *HaEbony* in the physiology and behaviour in *H. axyridis*. The CRISPR/Cas9 system was applied to generate multiple *HaEbony* knockout allele (*HaEbony*$^{+/-}$), showing nucleotide deletion in the G_0 and G_1 generations. Remarkably, the resultant *HaEbony*$^{+/-}$ mutants consistently displayed darker pigmentation than their wild-type counterparts across larval, pupal and adult stages. Furthermore, these *HaEbony*$^{+/-}$ individuals (G_0) demonstrated an enhanced predatory efficiency, evidenced by a higher number of aphids consumed compared to the wild type. A significant finding was the reduced egg hatchability in both G_0 and G_1 generations of the *HaEbony*$^{+/-}$ group, highlighting a potential reproductive fitness cost associated with *HaEbony* deficiency. In conclusion, our study not only sheds light on the multifaceted roles of *HaEbony* in *H. axyridis* but also highlights the potential of employing CRISPR/Cas9-targeted modifications of the *Ebony* gene. Such genetic interventions could enhance the environmental adaptability and predatory efficacy of ladybirds, presenting a novel strategy in biological control application.

KEYWORDS
CRISPR/Cas9, *Ebony*, *Harmonia axyridis*, pigmentation

INTRODUCTION

Phenotypic diversity is pivotal in enhancing breeding efficiency, ensuring population reproduction, adapting to seasonal fluctuations, coping with environmental stresses and mitigating predation risks (Badejo et al., 2020; Protas & Patel, 2008). Among the various phenotypic variations in insects, melanism is a prominent variant influenced by both genetic and environmental factors. A critical element of this diversity is the cuticle tanning process, which encompass pigmentation and sclerotization (Andersen, 2012). This transformative process

Jing Lin and Da Xiao made equal contribution to this article.

involves the darkening and hardening of the insect cuticle. It involves melanin deposition and protein crosslinking, culminating in a hardened, mature cuticle that provides essential structural support and protection (Sterkel et al., 2019).

Melanin, a predominant class of pigment, plays a central role in the coloration and sclerotization of the insect cuticle (Andersen, 2012; True, 2003). Furthermore, melanin plays an important role in insect wound healing and immune process, providing physical or immune protection to insects (Sugumaran & Barek, 2016). The biosynthetic pathway of melanin and sclerotin originates from tyrosine (Arakane et al., 2016). Tyrosine is enzymatically converted into L-3, 4-dihydroxyphenylalanine (DOPA) by tyrosine hydroxylase (TH) (Gorman & Arakane, 2010). Subsequently, DOPA decarboxylase (DDC) catalyses the conversion of DOPA into dopamine (Tsukioka et al., 2017). Both DOPA and dopamine, essential precursors in melanin biosynthesis pathway, undergo oxidation by phenoloxidase laccases, resulting in melanin formation (Barek et al., 2018). Concurrently, the enzyme N-β-alanyl dopamine synthetase, encoded by the *Ebony* gene, catalyses the synthesis N-β-alanyl dopamine (NBAD) from β-alanine and dopamine, leading to the production of NBAD sclerotin. This biochemical cascade underscores the complex of the cuticle tanning process in insects.

Tyrosine metabolism is crucial for insect cuticle formation and plays a significant role in broader aspects of insect life, including development, feeding and fecundity. This importance is further underscored by the derivation of key neurotransmitters from this pathway, including L-DOPA, dopamine and octopamine (Blenau & Baumann, 2001; Daubner et al., 2011; Eriksson et al., 2017; Li et al., 2020). Consequently, genes involved in the tyrosine metabolism pathway exhibit pleiotropic effects, impacting multiple traits and behaviours in insects. Notably, the pale (TH) gene encodes tyrosine hydroxylase, the rate-limiting enzyme in this pathway, and is indispensable for insect development. The absence of TH leads to severe lethality, highlighting its critical role (Bai et al., 2023; Gorman & Arakane, 2010; Xiao et al., 2020). Additionally, dopa decarboxylase (DDC) is another vital gene within this pathway, displaying pleiotropic effects by regulating ecdysis, survival and reproduction in insects (Shen et al., 2020; Sterkel et al., 2019; Xiao et al., 2020). Similarly, the gene *Ebony* plays a predominant role in development and fecundity due to its involvement in neurotransmitter metabolism. Research has demonstrated that *Ebony* influences various physiological processes in insects, including embryogenesis and behaviours such as courtship, locomotion and circadian rhythm (Sun et al., 2023; Xu et al., 2021). Furthermore, *Ebony* serves as a visible marker gene in gene-editing system in insect research, as evidenced by the distinctively darker phenotype of *Ebony* mutant individuals across different species (Cheng et al., 2023; Osanai-Futahashi et al., 2012; Xu et al., 2021). This multifunctional importance underscores the critical role of tyrosine metabolism in the complex web of insect life, from molecular mechanisms to observable behaviours and phenotypes.

The Asian multi-coloured ladybird beetle, *Harmonia axyridis* (Pallas) (Coleoptera: Coccinellidae), is renowned for its significant predatory efficacy against various pests, as highlighted in previous research (Chen et al., 2019; Koch, 2003). Additionally, *H. axyridis* is notable for its high variation in elytra patterns, a characteristic thoroughly investigated in recent studies (Ando et al., 2018; Ando & Niimi, 2019; Niimi & Ando, 2021). Among these, four common colour patterns of elytra are *succinea*, *axyridis*, *spectabilis* and *conspicua* (Gautier et al., 2018; Xiao et al., 2020). The variation in *H. axyridis* elytra phenotype, particularly in shades of melanin and orange, makes it an ideal subject for analysing the molecular mechanism underlying body colour transitions. In our previous studies, we established that dopamine melanin is the predominant form of melanin in *H. axyridis*. Furthermore, we identified TH and DDC as critical genes in the developmental process of *H. axyridis* (Chen et al., 2019; Wu et al., 2022; Xiao et al., 2020). Other research has shown that the *HaEbony* specifically controls the size and number of spots solely during the pupal stage through RNA interference experiments in *H. axyridis* (Zhang, Wang, Feng, Cong, et al., 2020; Zhang, Wang, Feng, Tian, et al., 2020). Despite these advancements, one aspect that remains unclear is how the *Ebony* gene influences behaviour in *H. axyridis*. This knowledge gap presents an intriguing avenue for further research, potentially shedding light on the complex interplay between genetics and behaviour in this species.

Studies across various model organisms consistently highlight the crucial role of the *Ebony* gene in the cuticle tanning process, demonstrating its remarkable conservation across species. To deepen our understanding of the biological functions of *Ebony* in diverse insect species, our research focused on three key objectives: Firstly, we sequenced and characterised cDNA putatively encoding *Ebony* from the Asian multi-coloured ladybird (*Harmonia axyridis*), an emerging model organism for studying phenotype variation mechanism. Secondly, we examined the developmental stage expression profiles of *HaEbony*. Lastly, we explored the roles of *HaEbony* in cuticle tanning, development and fecundity by using the CRISPR/Cas9 gene-editing technique. Our findings showed that disruptions in *HaEbony* lead to noticeable alternations not only in cuticle coloration but also in predatory ability. For the first time, our results provide critical evidence suggesting that *Ebony* could be considered a target gene in improving the germplasm resources of predatory ladybirds. Employing CRISPR/Cas9 to enhance their environmental adaptability and predatory ability could represent a novel strategy. This research contributes significantly to our understanding of the underlying molecular mechanisms of phenotypic diversity, particularly in relation to cuticle tanning and pigmentation processes in insects.

RESULTS

Analysis of *HaEbony* cDNA, deduced amino acid and genomic sequences

We utilised the *Ebony* gene sequence available in the InsectBase database (Haxy005633) as a reference to clone and sequence this gene. Subsequently, the obtained sequence was compared with those in NCBI (LOC123680340) and InsectBase using the blastn tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi), showing homology of 96.5% and

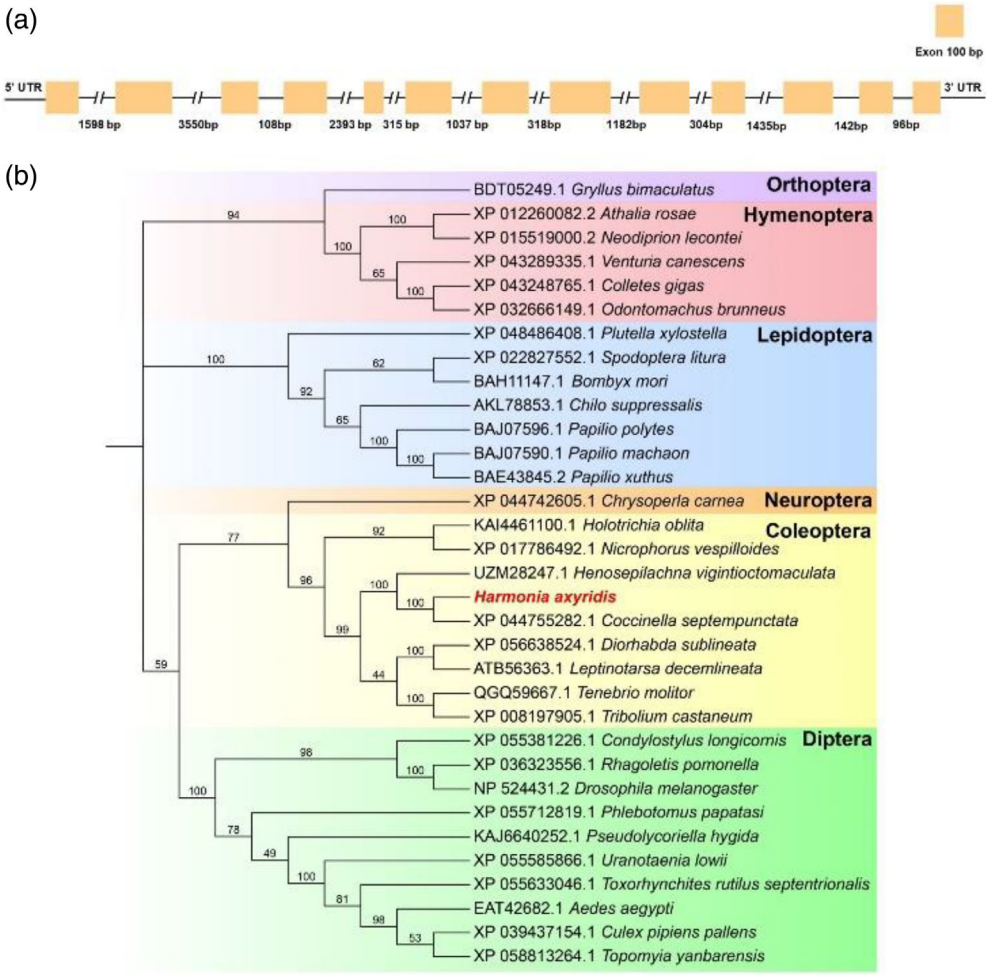

FIGURE 1 Molecular analyses of the β–alanyl-dopamine synthase (*HaEbony*) in *H. axyridis*. (a) Schematic diagram showing the organisation of *HaEbony* gene (38,901 bp). (b) The rooted phylogenetic tree of deduced Ebony protein sequences from 33 insect species as constructed by the neighbour-jointing method.

98.2%, respectively. Based on these findings, we concluded that the obtained sequence is the *Ebony* gene sequence. The sequencing results revealed that the full length of the *HaEbony* cDNA comprises 38, 901 base pairs (bp), including an open reading frame (ORF) of 2538 bp, which encodes a protein comprising 845 amino acid residues. Additionally, we identified non-coding regions at both ends of the cDNA: a 23, 676-nucleotide segment at the 5′-end and a 209-nucleotide segment at the 3′-end (Figure 1a). We deduced that the molecular mass of the predicted *HaEbony* protein is approximately 9.54 kDa, with an isoelectric point (PI) estimated to be around 5.68.

Phylogenetic relationship of *H. axyridis Ebony* to other insect *Ebonys*

The *HaEbony* along with other 32 insect *Ebonys* which were retrieved from GenBank formed six distinctive groups representing different orders (Figure 1b): Coleoptera including *Coccinella septempunctata* (CsEbony, amino acid sequence identity to HaEbony 90.8%), *Henosepilachna vigintioctomaculata* (HvEbony, 79.4%), *Holotrichia oblita* (HoEbony,56.8%), *Tribolium castaneum* (TcEbony, 62.9%), *Diorhabda sublineata* (DsEbony, 61.9%), *Tenebrio molitor* (TmEbony, 63.6%), *Nicrophorus vespilloides* (NvEbony, 55.6%) and *Leptinotarsa decemlineata* (LdEbony, 62.4%); Diptera including *Pseudolycoriella hygida* (PhEbony, 52.0%), *Phlebotomus papatasi* (PpEbony, 53.1%), *Condylostylus longicornis* (ClEbony, 52.9%), *Uranotaenia lowii* (UlEbony, 52.6%), *Topomyia yanbarensis* (TyEbony, 52.8%), *Culex pipiens* pallens (CpEbony, 52.1%), *Toxorhynchites rutilus* septentrionalis (TrEbony, 52.7%), *Rhagoletis pomonella* (RpEBony, 52.8%), *Drosophila melanogaster* (DmEbony, 51.9%) and *Aedes aegypti* (AaEbony,52.4%); Hymenoptera including *Neodiprion lecontei* (NlEbony, 53.6%), *Athalia rosae* (ArEbony, 52.1%), *Colletes gigas* (CgEbony, 51.7%), *Odontomachus brunneus* (ObEbony,49.5%) and *Venturia canescens* (VcEbony, 50.0%), Lepidoptera including *Plutella xylostella* (PxEbony, 48.8%), *Bombyx mori* (BmEbony, 46.3%), *Spodoptera litura* (SlEbony, 38.1%), *Papilio xuthus* (PxEbony, 47.4%), *Papilio machaon* (PmEbony, 47.8%), *Papilio polytes* (PpEbony, 47.5%) and *Chilo suppressalis* (CsEbony, 49.3%) (Figure 1b). The remaining two Ebonys were from two

different orders including Neuroptera for *Chrysoperla carnea* (CcEbony, 54.1%) and Orthoptera for *Gryllus bimaculatus* (GbEbony, 52.4%). *HaEbony* clusters closely with *C. septempunctata* and *H. vigintioctomaculata*, suggesting a highly conserved evolutionary pathway for the *Ebony* gene within the Coccinellidae family. This close relationship indicates that these species share similar mechanism for melanin synthesis.

Developmental stage and tissue expression patterns of *HaEbony*

We investigated the temporal expression patterns of *HaEbony* in different developmental stages of *H. axyridis* by using RT-qPCR from the embryo through adult stages (Figure 2a). Our findings indicate a consistent expression of *HaEbony* throughout all the developmental stages. Notably, the highest expression level was observed in 4-day-old pupae. This peak suggests a significant role for *HaEbony* during this critical phase of metamorphosis. In contrast, the initial stages of embryogenesis, specifically in 1- and 2-day-old eggs, exhibited the lowest expression levels of *HaEbony*. However, there was a marked increase in expression during the later stages of embryogenesis, particularly in 3-day-old eggs. This heightened expression level was sustained throughout the larval stage. After the pupal stage, a significant decrease in *HaEbony* expression was observed in 1-day-old adults. Moreover, the second highest expression level was observed in the adult stage. It was observed that the expression levels fluctuate significantly across different stages, with a marked peak during the late pupal stage. This indicates a crucial role for the *Ebony* gene in the processes associated with cuticle sclerotization and development. To gain a more detailed understanding of its temporal regulation, we further examined the expression level at specific time points. At 0 h post-eclosion, *Ebony* gene expression remains at a high level, comparable to that observed on day 4 of the pupal stage. By 4 h, the expression level decreases that approaching that observed at 24-h post-eclosion. By 6 h, expression stabilises, aligning with the 24-h post-eclosion level, indicating that the cuticle sclerotization process is nearing completion, and *Ebony* gene expression progressively transitions to a lower state.

Given that the *Ebony* gene exhibited its highest expression on day 4 of the pupal stage in *H. axyridis*, we selected 4-day pupae to perform the tissue-specific expression profile analysis. The tissues- specific expression profile of the *Ebony* gene in 4-day pupae of *H. axyridis* revealed distinct patterns across various tissues. The highest expression was observed in the head tissues, where the *Ebony* gene was significantly upregulated compared to other tissues. Moderate expression levels were detected in the epidermis, indicating that *Ebony* may also contribute to general cuticle pigmentation and hardening in other parts of the body. In contrast, expression in the midgut, fat body and Malpighian tubules was minimal, suggesting that the *Ebony* gene does not play a significant role in these internal tissues during the late pupal stage (Figure 2b).

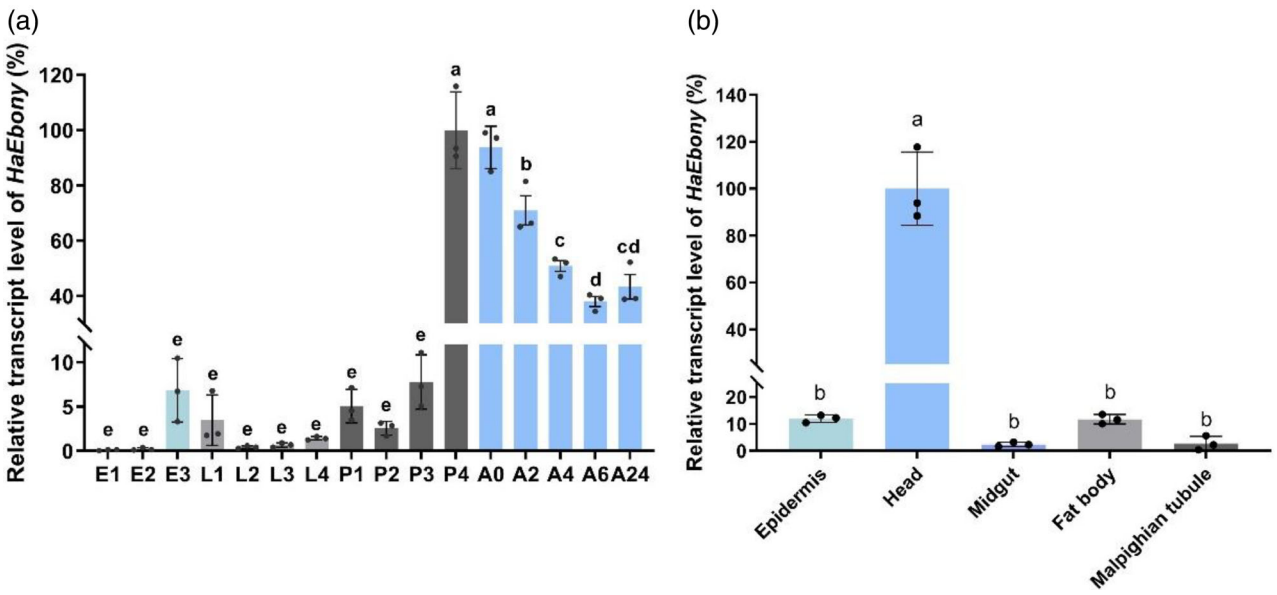

FIGURE 2 The expression patterns of *HaEbony* at different developmental stages and in various tissues of 4-day pupae. (a) Developmental stage-dependent expression pattern of *HaEbony* determined by RT-qPCR. E1, E2, E3 represent 1-, 2- and 3-day eggs; L1, L2, L3 and L4 represent the first, second, third and fourth instar larvae; P1, P2, P3 and P4 represent 1-, 2-, 3- and 4-day pupae; and A0, A2, A4, A6 and A24 represent 0-, 2-, 4-, 6- and 24-h adults, respectively. (b) Tissue-specific expression pattern of *HaEbony* determined by RT-qPCR. The resulted are presented as mean and standard errors of three replicates. Different letters above the standard error bars indicate significant differences based on ANOVA followed by Tukey's HSD multiple comparison test ($p < 0.05$). *H. axyridis* ribosomal protein 49 (*Harp49*) was used as an internal reference gene to normalise the differences among the samples. Relative expression levels for *HaEbony* at different development stages and tissues were calculated based on the highest expressions of *HaEbony* in 4-day pupae (P4) and head as 100%, respectively.

CRISPR/Cas9-mediated knockout of *HaEbony* in *H. axyridis*

In our study, we focused on disrupting the function of the *Ebony* gene in *H. axyridis*. To achieve this, we employed the CHOPCHOP V3.00 (Labun et al., 2019) to design sgRNA targeting exon 7 of the *HaEbony* (Figure 2a). In total, 1866 eggs were microinjected with a complex of Cas9 protein and the designed sgRNA. Additionally, 250 eggs were injected with water to serve as a control group. In the *HaEbony-sgRNA* injected group, the hatching success rate was observed to be 28.6% (534/1866), a significant decrease compared to the to 49.6% (124/250) hatching rate in the control group. The pupation rate in the *HaEbony-sgRNA* injected group was also affected, recorded at 57.3% (306/534), which was lower than the 66.9% (83/124) observed in the control group. However, the eclosion rate in the *HaEbony-sgRNA* injected group was 98.4% (301/306), showing no significant difference when compared to the control group of 98.8% (82/83). Ultimately, this phase of the experiment resulted in the successful generation of 30 mosaic adults (comprising 14 males and 16 females) in the *HaEbony-sgRNA* injected group (Table 1). These individuals were then selected for mating to produce the first generation (G_1) to analysis fitness cost of *HaEbony* mutation on their offspring.

Mutant phenotypes of the HaEbony knockout strain (G_0)

In our comparative study, the *HaEbony* knockout individuals were designated as *HaEbony*$^{+/-}$ (G_0), exhibited notably darker pigmentation at various developmental stages compared to their wild-type counterparts. This difference was apparent in the late larval, pupal and adult stages (Figure 3b). The mosaic phenotypes of the *HaEbony*$^{+/-}$ (G_0)

TABLE 1 Number (and proportion) of specimens reaching different developmental stages after microinjection.

Injection	gRNA/Cas9 concentration (ng/μL)	Injected embryos	Hatched larvae	Pupae	Adults	Mosaic adults
Control (H_2O)	-	250	124 (49.6%)	83 (66.9%)	82 (98.8%)	-
HaEbony gRNA/Cas9	300/300	1866	534 (28.6%)	306 (57.3%)	301 (98.4%)	30 (14♂/16♀)

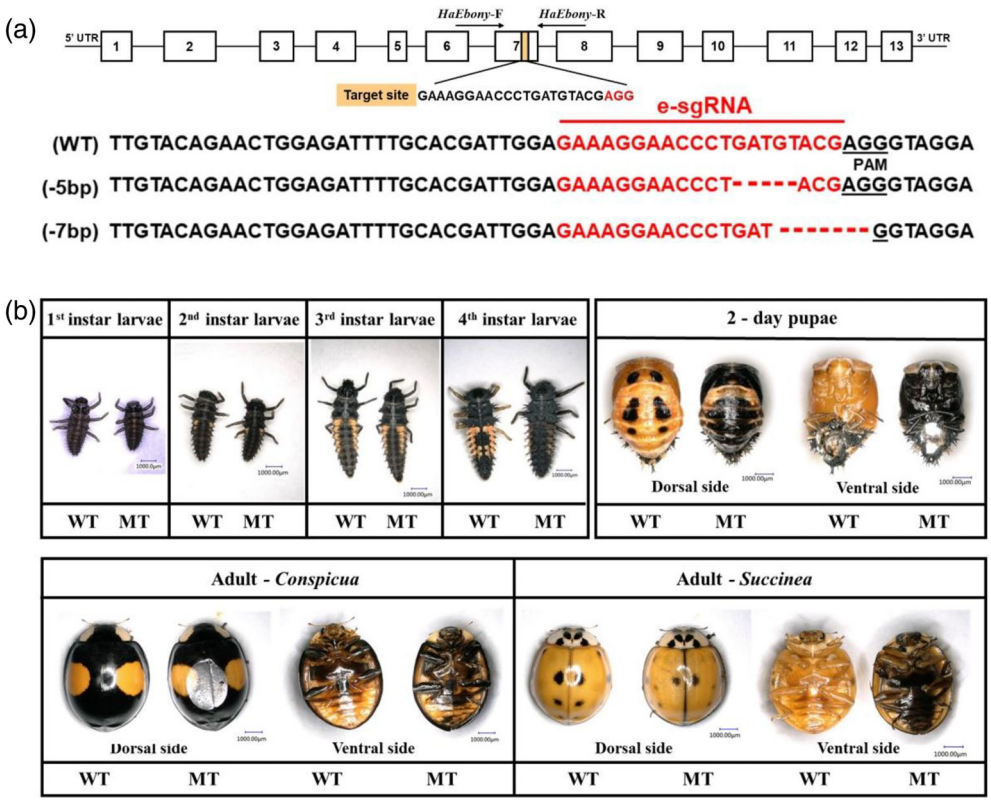

FIGURE 3 Genotypes and phenotype of the *Ebony* mutation induced by sgRNA and Cas9 injection in *H. axyridis* (G_0). (a) Identification of the mutant genotypes of *Ebony* gene in *H. axyridis*. Utilising flat end cloning techniques, two distinct types of mutations were identified: deletions of 5 and 7 bases, respectively. Additionally, the PAM sites that Cas9/sgRNA recognises are underlined for clarity. (b) Anterior view comparison between the wild type (WT) and mutant type (MT) across various developmental stages: the second day of the first, second, third and fourth instar larvae. Furthermore, it includes a top view of both the dorsal and ventral sides of the WT and MT on the second day of the pupae and adult stages. The scale bar is set at 1000 μm for reference.

mutants were distinctly different from those of the wild type, making them easily identifiable. During the 4th instar larval stage, the *HaEbony*$^{+/-}$ (G_0) mutants showed increased melanin deposition in their cuticle. This was particularly evident in the orange furcella on the abdomen, which turned black in the mutant individuals, making it challenging to distinguish the instar of *H. axyridis* larvae based on morphological characteristics alone. Furthermore, in the pupal stage, there was an increase in melanin levels, resulting in noticeably darker pigmentation on both the dorsal and ventral sides of the cuticle compared to the wild type. Additionally, adult *HaEbony*$^{+/-}$ (G_0) individuals also demonstrated a deeper dark phenotype. Direct visual screening revealed that the legs, abdomen and elytra margins of *HaEbony*$^{+/-}$ individuals in the *succinea* subgroup were darker than those of the wild type. However, it was observed that the *HaEbony*$^{+/-}$ mutant ladybirds exhibited normal elytral development processes and maintained typical elytra morphology, similar to the wild type. To validate that the observed mutant phenotype was a direct result of genomic mutagenesis of the *Ebony* gene, we extracted genomic DNA from the exuviae of several larvae of *HaEbony*$^{+/-}$ (G_0). This DNA served as a template for PCR amplification of the genomic DNA fragment. Sequencing results confirmed the presence of deleted nucleotide (5 and 7) at the target site in the mutant individuals (Figure 3a).

Fitness analysis of the *HaEbony* mutant strain (G_0)

The phenotype of *HaEbony*$^{+/-}$ (G_0) ladybirds exhibited a notably darker cuticle compared to the wild type. This change is attributed to the functional disruption of the *Ebony* gene in the sclerotization pathway, which lead to an accumulation of dopamine. Dopamine plays dual role: it is involved not only in the cuticle tanning process but also serves as a crucial neurotransmitter in the central nervous system of organism. Consequently, an increase in dopamine levels may impact insect physiology. Our study delves into the fitness costs associated with the *HaEbony*$^{+/-}$ (G_0) phenotype. In terms of development, the durations for *HaEbony*$^{+/-}$ (G_0) were generally consistent with those of the wild type, encompassing most larval and pupal stages. The average development time of the first instar larva was 2.33 ± 0.48 days in mutant *H. axyridis* which was significantly longer than that of wild-type *H. axyridis*, with 2.10 ± 0.31 days ($t = 2.249$, $p = 0.028$) (Figure 4a). In order to assess the effects of *ebony* on behaviour, we have undertaken a more detailed observation and comprehensive statistical analysis, focusing specifically on their predatory and mating behaviours in *HaEbony*$^{+/-}$ (G_0) mutants. Predatory ability, a key factor in assessing the pest-control capability of predatory natural enemies, was also evaluated between *HaEbony*$^{+/-}$ (G_0) and wild-

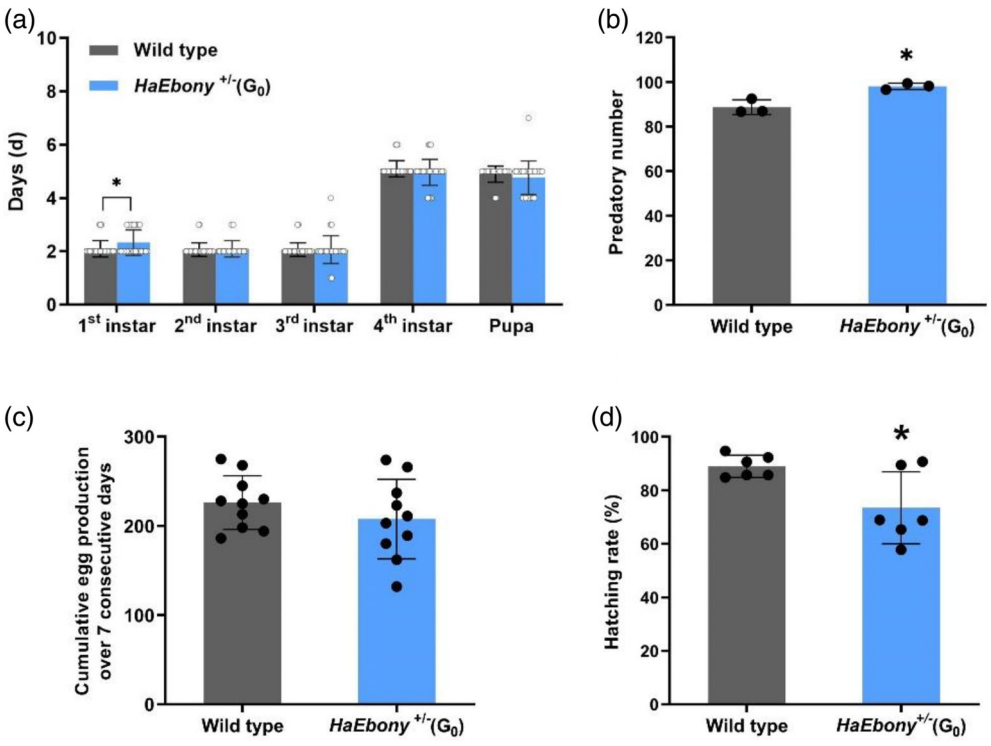

FIGURE 4 (a) Effects of *HaEbony* knockout on developmental duration of G_0 generation in *H. axyridis*. The results are presented as mean and standard errors of 30 replicates, with 1 ladybird beetle in each replicate. Asterisk above the standard error bars indicate significantly differences between wild and mutant type based on Student's *t* test ($p < 0.05$). (b) Effects of *HaEbony* knockout on predatory number of G_0 generation in *H. axyridis*. The results are presented as mean and standard errors of three replicates, with 10 ladybird beetles in each replicate. Asterisk above the standard error bars indicate significantly differences between wild and mutant type based on Student's *t* test ($p < 0.05$). (c) Effects of *HaEbony* knockout on fecundity of G_0 generation in *H. axyridis*. The results of fecundity are presented as mean and standard error of 10 replicates, with one pair of ladybird beetles in each replicate. (d) Effects of *HaEbony* knockout on hatching rate of G_0 generation in *H. axyridis*. The results of hatching rate are presented as mean and standard error of six replicates, with five egg masses in each replicate. Asterisk above the standard error bars indicate significantly differences between wild and mutant type based on Student's *t* test ($p < 0.05$).

type individuals. Our results revealed that the adult of *HaEbony*$^{+/-}$ (G_0) consumed more *M. crassicauda* prey compared to the wild type ($t = 4.532$, $p = 0.011$) (Figure 4b). Fecundity, another critical parameter for evaluating the fitness cost of mutant strains in insects, was assessed by mating female and male from the *HaEbony*$^{+/-}$ (G_0) and recording egg production. The fecundity results indicated no significant difference between *HaEbony*$^{+/-}$ (G_0), with an average of 207.7 ± 14.09 eggs of a pair ladybird within 7 days, and an average of 226.2 ± 9.50 eggs in the wild type ($t = 1.089$, $p = 0.291$) (Figure 4c). However, a significant decrease was observed in the egg hatching rate of offspring in *HaEbony*$^{+/-}$ (G_0), which was 73.49 ± 5.51%, compared to 89.40 ± 1.58% in the wild type ($t = 2.776$, $p = 0.0196$) (Figure 4d).

Mutant phenotypes and fitness costs of the *HaEbony* knockout strain (G_1)

The fecundity results of *HaEbony*$^{+/-}$ (G_0) demonstrated that *Ebony* disruption does not impact mating and egg laying behaviours in *H. axyridis*. Consequently, we select 12 egg masses, comprising 490 eggs, offspring from the *HaEbony*$^{+/-}$ (G_0) intercrossed to investigate any potential fitness effects caused by *HaEbony* knockout. In the G_1 generation, among 490 eggs, 332 eggs were hatched successfully, the mutant ratio of *HaEbony* was 63.6% (211/332), as confirmed by both sequencing and phenotype observations. Sequencing of *HaEbony*$^{+/-}$ (G_1) confirmed the presence of same nucleotide deletions (5 and 7 bp) at the target site with *HaEbony*$^{+/-}$ (G_0) (Figure 5a). The phenotype of *HaEbony*$^{+/-}$ (G_1) is same with mutant individuals of their parent generation that more melanin deposited in the cuticle as compared with wild type. Notably, these darker phenotypes were evident in the larval, pupal and adult stages (Figure 5b). This allows for the establishment of a stable genetic lineage with the *Ebony* mutation in *H. axyridis*. Developmental duration analysis revealed that the fourth instar larvae stage was significantly longer in *HaEbony*$^{+/-}$ (G_1) (6.23 ± 1.08 days) compared to wild-type ladybirds (5.03 ± 0.18 days) ($t = 8.493$, $p < 0.001$), and the second instar larvae stage was longer in mutants (1.77 ± 0.50 days) compared to wild type (2.07 ± 0.25 days) ($t = 4.154$, $p < 0.001$) (Figure 6a). We assessed the predatory ability on aphid *M. crassicauda* using fourth instar larvae of both *HaEbony*$^{+/-}$ (G_1) and wild type. Our findings indicated no significant difference in predation rates between *HaEbony*$^{+/-}$ (G_1) and wild type ($t = 0.2894$, $p = 0.7867$) (Figure 6b). In addition, a significantly decrease in pupation rate was observed in *HaEbony*$^{+/-}$ (G_1) of 71.67 ± 2.20% compared to the wild type of 97.5 ± 1.44% ($t = 9.803$, $p < 0.001$) (Figure 6c). Meanwhile, there was no significant difference in the eclosion rate between the wild and mutant type (G_1) *H. axyridis* ($t = 0.707$, $p = 0.5185$) (Figure 6d).

The *Ebony* gene plays a crucial role in dopamine metabolism by catalysing the conversion of dopamine to NBAD. When *Ebony* is knocked out may disrupt this pathway that leading to the potential

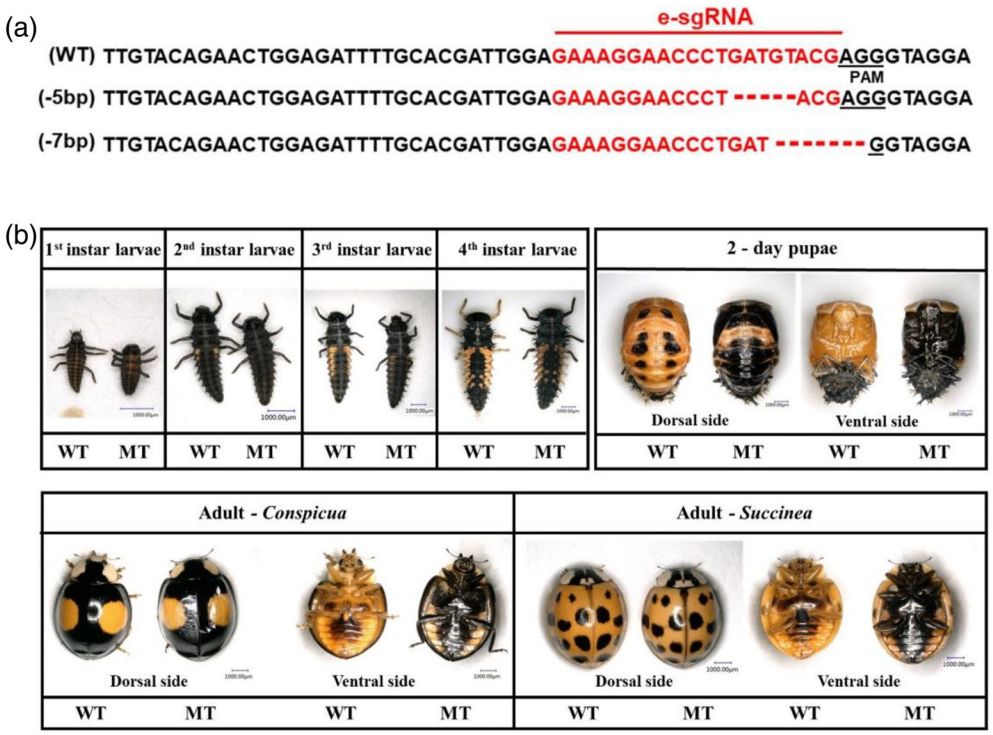

FIGURE 5 Phenotype and genotypes of the Ebony mutation induced by sgRNA and Cas9 injection in *H. axyridis* (G_1). (a) Identification of the mutant genotypes of Ebony gene in *H. axyridis*. Utilising flat end cloning techniques, two distinct types of mutations were identified: deletions of 5 and 7 bases, respectively. Additionally, the PAM sites that Cas9/sgRNA recognises are underlined for clarity. (b) Anterior view comparison between the wild type (WT) and mutant type (MT) across various developmental stages: the second day of the first, second, third and fourth instar larvae. Furthermore, it includes a top view of both the dorsal and ventral sides of the WT and MT on the second day of the pupae and adult stages. The scale bar is set at 1000 μm for reference.

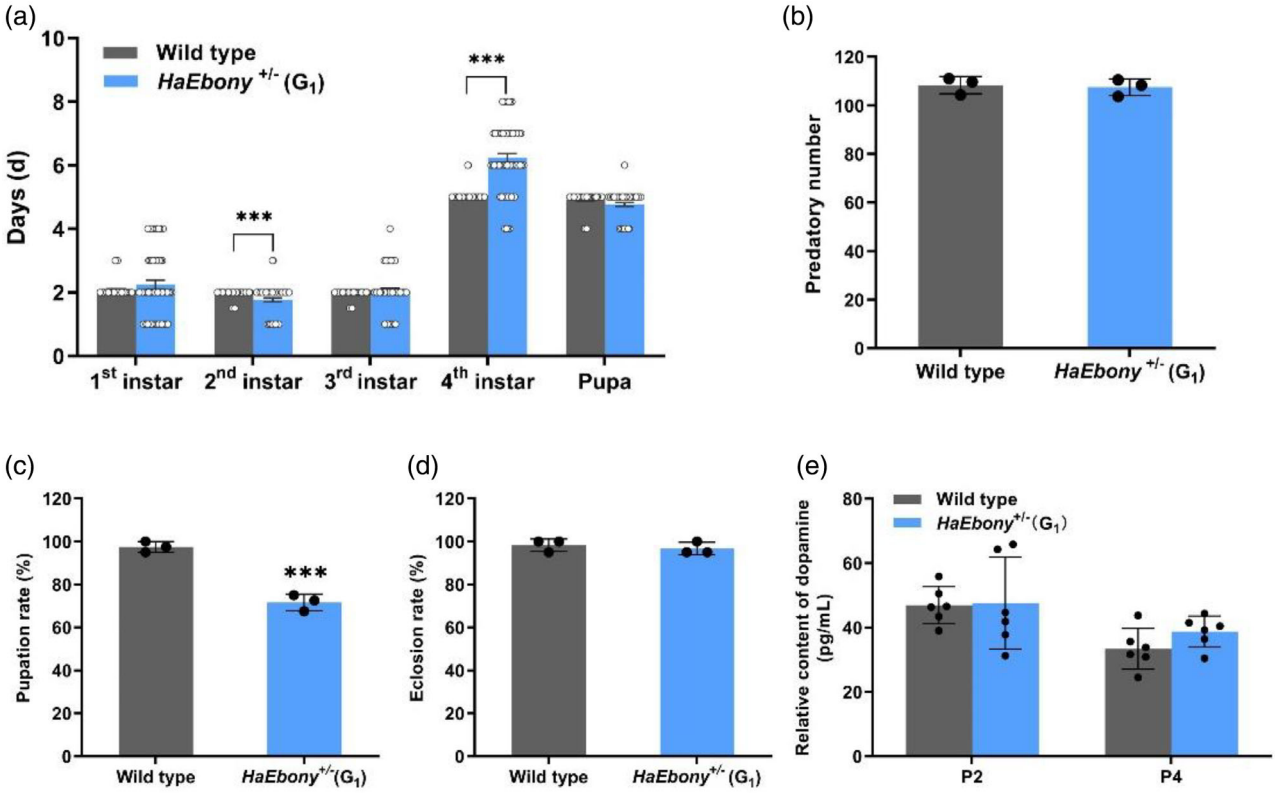

FIGURE 6 Effects of *HaEbony* knockout on developmental time of each instar larvae and pupa in G_1 generation in *H. axyridis*. (a) The results are presented as the mean and standard errors of 60 replicates, with 1 beetle in each replicate. Asterisk above the standard error bars indicate significantly differences based on Student's *t* test ($p < 0.05$). (b) Effects of *HaEbony* knockout on predation ability of the 2-day of fourth instar larvae in *H. axyridis*. The results are presented as mean and standard errors of three replicates, with a minimum of 20 beetles per replicate. (c) Effects of *HaEbony* knockout on pupation rate in G_1 generation in *H. axyridis*. The results are presented as mean and standard errors of three replicates, with a minimum of 20 beetles per replicate. Asterisk above the standard error bars indicate significantly differences based on Student's *t* test ($p < 0.05$). (d) Effects of *HaEbony* knockout on eclosion rate in G_1 generation in *H. axyridis*. The results are presented as mean and standard errors of three replicates, with a minimum of 20 beetles per replicate. (e) Relative content of dopamine of *HaEbony* knockout in G_1 generation in *H. axyridis*. P2 and P4 represent 2- and 4-day pupae. The results are presented as mean and standard errors of six replicates, with 3 beetles per replicate.

accumulation of dopamine. Notably, both G_0 and G_1 of *HaEbony*$^{+/-}$ exihibit darker pigmentation phenotype, which is indicative of increased melanin synthesis and suggest a possible rise in dopamine levels. To investigate this, we measured dopamine content in *HaEbony*$^{+/-}$(G_1) and wild-type *H. axyridis* at two pupal stages (P2 and P4). At both stages, the *HaEbony* mutants exhibited elevated dopamine content, with greater variability observed in the P4 stage (Figure 6e). However, statistical analysis indicated that these differences were not significant ($p > 0.05$), suggesting that while partial loss of *Ebony* gene function may affect dopamine metabolism, the observed increase in dopamine levels does not reach statistical significance.

DISCUSSION

Tyrosine metabolism plays a crucial role in insect development, particularly as key precursor in melanin synthesis. This process imparts coloration to various body parts, including the cuticle, eyes and wings, significantly affecting the insect's appearance and adaptability (Arakane et al., 2016). Tyrosine metabolism also contributes to cuticle formation and sclerotization, the latter enhancing cuticle strength and protection, which is vital for the insect's physical integrity and defence against environmental stressors (Andersen, 2010). Furthermore, tyrosine is a precursor for the synthesis of important neurotransmitters, such as dopamine and octopamine, essential for insect nervous system functioning (Costa & Schoenbaum, 2022). Consequently, tyrosine metabolism regulation is intricately tuned to the diverse developmental stages and environmental conditions insects encounter throughout their life cycle. In this intricate metabolic pathway, the *Ebony* gene emerges as a key player, with a broad impact on various aspects of insect development. However, the specific functions and mechanisms of the *Ebony* gene, particularly in the context of the Asian multicoloured ladybird beetle, remain largely unexplored. Understanding the role of *Ebony* in this species is crucial, as it may reveal novel insights into the complex interplay of genetics, development and environmental adaptation in insects.

Phylogenetic analysis has revealed that *HaEbony* is a conserved gene, potentially playing a pivotal role in the insect cuticle tanning

process (Figure 1b). Upon investigating the expression patterns of *HaEbony* throughout developmental stages, we found consistent expression from embryogenesis through to the adult stage. Notably, a significant increase in *HaEbony* expression levels was observed during the later stages of the pupal phase (Figure 2a). The pupal stage on day 4 marks the completion of metamorphosis and the transition to adulthood. The high expression of the *Ebony* gene at this stage is positively correlated with the peak in melanin deposition and cuticle hardening, suggesting that this gene plays a key role in the sclerotization and pigmentation of the exoskeleton in *H. axyridis*. Given the high expression of the *Ebony* gene in day 4 pupae, we further analysed its tissue-specific expression during this period. We dissected and isolated tissues from the 4-day pupae of *H. axyridis*, and found that *Ebony* was significantly expressed in head tissues compared to other tissues parts (Figure 2b). Our findings highlight the tissue specific roles of *Ebony* in the developmental processes of *H. axyridis*, with its expression being most prominent in regions related to cuticle pigmentation and hardening, particularly in the head and epidermis. In *Spodoptera litura*, the researchers investigated the mRNA levels of *ebony* at the 3rd day of the 5th instar in different tissues and the highest levels of *Slebony* were observed in the head (Bi et al., 2019). These studies suggest a critical role for the *Ebony* gene in the head during this development stage, likely related to the regulation of cuticle melanization and sclerotization processes that are essential for the formation and hardening of the head exoskeleton.

Ebony plays a crucial role in the cuticle tanning process, primarily by facilitating the formation of N-β-alanyl dopamine (NBAD), derived from dopamine and β-alanine (Wittkopp et al., 2002, 2003). Consequently, dysfunction in *Ebony* can lead to the accumulation of either dopamine or β-alanine. Our research reveals that a null mutation of *Ebony* in *H. axyridis* does not impede elytral development or sclerotization, as evidenced by the normal elytra morphology in *HaEbony*$^{+/-}$ individuals of G_0 and G_1 generation (Figures 3b and 5b). Previous studies utilising the CRISPR/Cas9 technique to disrupt *Ebony* expression have consistently shown deeper pigmentation in mutant individuals across various species. For instance, *Drosophila melanogaster* exhibited dark body pigmentation (Massey et al., 2019). Similarly, *Bombyx mori* showed smoky larvae and black pupae and *Papilio xuthus* demonstrated enhanced melanic pigmentation in fifth instar larvae (Futahashi et al., 2008; Li et al., 2015). Likewise, deep coloration of the pupae in *Spodoptera litura* and darker pigmentation in larvae, pupae and adults in *Plutella xylostella* (Bi et al., 2019; Xu et al., 2021). Furthermore, deep black coloured of pupae in *Spodoptera frugiperda* (Cheng et al., 2023), and darkened body colour at all stages in *Gryllus bimaculatus* (Inoue et al., 2023). In accordance with previous study, knocking out of *Ebony* in *H. axyridis* using CRISPR/Cas9 also showed obvious melanin accumulated in cuticle tissue at the larval, pupal and adult stages in both G_0 and G_1 mosaic generation (Figures 3b and 5b). The loss of *Ebony* gene function not only disrupted the melanin metabolic balance in the G_0 generation but also transmitted this metabolic abnormality to the G_1 generation through genetic mechanisms. This reflects the genetic stability of an organism in regulating key metabolic pathways. Similar study in *H. axyridis* also showed notably darker pigmentation during pupal stage when *HaEbony* was knocked out in the 4th instar larvae using RNAi technique (Zhang, Wang, Feng, Tian, et al., 2020). The above studies demonstrate that with *Ebony* dysfunction, dopamine fails to combine with alanine to form NBAD. Instead, the accumulated dopamine leads to excess melanin production, resulting in a darker phenotype. Moreover, our findings along with previous studies, confirm that disrupting the sclerotization process in insects results in surplus dopamine being diverted into the pigmentation pathway, thereby enhancing dopamine-melanin synthesis. Overall, these results underscore the conserved systemic function of *Ebony* in cuticle pigmentation across a wide range of insect species.

Disruption of *Ebony* allele function has been observed to produce diverse effects on embryonic and larval development stages across different insect species. For instance, in *S. frugiperda*, *Sfebony* homozygous mutants did not show significant differences in hatching, pupation and eclosion rates compared to the wild type (Cheng et al., 2023). In contrast, *ebony* mutants of *P. xylostella* and *B. mori* exhibit reduced embryo hatchability and larval survival rates (Sun et al., 2023; Xu et al., 2021). Our results were consistent with these reports, as we observed a significant decrease in the hatching rate of *HaEbony*$^{+/-}$ offspring compared to that of their wild-type counterparts (Figure 4d). In addition, the developmental duration of *HaEbony*$^{+/-}$ (G_1) suggested that disruption of *Ebony* could lead to an extended larval developmental period that was also reported in *P. xylostella* (Xu et al., 2021). These collective studies highlight the profound impact that disruption of tyrosine metabolism enzyme function can have on critical embryogenesis and larval development processes. Moreover, they emphasise the pleiotropic role of *Ebony* in insect development, highlighting its influence across various species and developmental stages. Such findings are crucial for understanding the genetic factors underpinning insect development and survival.

Disruption of genes within the tyrosine metabolism pathway often resulted in altered levels of melanin precursors, such as dopa, dopamine, NADA and NBAD. A partial loss of *Ebony* function can limit NBAD synthesis, leading to dopamine accumulation. As a vital neurotransmitter, dopamine significantly influences various physiological pathways and behaviours, including locomotion, feeding, mating, learning, and more (Costa & Schoenbaum, 2022; Liu et al., 2008; Sasaki, 2016; Sugumaran & Barek, 2016; Watanabe & Sasaki, 2021). A previous study in *D. melanogaster* revealed that *Ebony* mutants exhibited not only increased aggressive behaviour, such as boxing, but also a decrease in courtship behaviours and an increase in sleep duration (Pantalia et al., 2023). Notably, *HaEbony*$^{+/-}$ ladybirds did not exhibit the aggressive behaviours observed in *D. melanogaster* mutants. Instead, these ladybirds displayed normal courtship behaviour and fecundity (Figure 4c). Intriguingly, these mutants showed enhanced predatory ability, consuming more aphids than their wild type (Figure 4b). Previous studies have documented that dopamine is involved in regulating appetite. It can influence the brain circuits that control hunger and satiety (Verlinden, 2018). The disruption of *HaEbony*, resulted in dopamine accumulation, may lead to reduce feeling of fullness and enhance desire to eat, thus contributing to higher food intake. Based on the increased melanin phenotype observed in *HaEbony*$^{+/-}$ individuals, it is inferred that their dopamine levels may be higher than those of the wild type, which could subsequently

influence their prey consumption. To test this hypothesis, we collected samples from day 2 and day 4 of the pupal stage for dopamine content analysis. The results showed a trend of increased dopamine content in the *HaEbony*$^{+/-}$ compared to the wild type. However, statistical analysis demonstrated that the differences were not significant (Figure 6e). In study of *D. melanogaster*, it has been shown that dopamine levels tend to decrease following the knockout of the *E*bony gene (Pantalia et al., 2023). Our study aligns with above study found that dopamine levels did not show an expected increase following *Ebony* gene knockout. Two speculated reasons may explain these unexpected results. Firstly, the time points selected for measurement may have coincided with a phase where dopamine levels were stable. Initially, dopamine levels may have increased after gene knockout, but due to the dynamic regulation of metabolic pathways, a new homeostasis might have been restored. Secondly, the inhibition of dopamine degradation pathways may have triggered alternative metabolic pathways, thereby reducing overall fluctuations in dopamine levels. These alternative pathways could divert dopamine into other metabolic processes, ensuring its stability within the organism.

Insects, as ectotherms, adjust their cuticle composition to environmental temperature fluctuations (Trullas et al., 2007). A previous study has indicated that melanic phenotypes in *H. axyridis* are more prevalent in colder climates (Wang et al., 2009). Furthermore, studies have documented that *H. axyridis* can increase the melanin content in its cuticle when exposed to cold temperatures (Michie et al., 2011, 2020). Melanin can absorb and retain heat, significantly influencing the insect's body temperature and playing a crucial role in thermoregulation in colder environments (Pinkert & Zeuss, 2018). In the current study, we have successfully produced darker cuticle mutant of *H. axyridis* by knocking out the *Ebony* gene (Figure 3b). Remarkably, not only did these G_1 mutants retain the darkened phenotype, but they also showed stable inheritance of this trait (Figure 5b). Consequently, disrupting the *Ebony* allele function resulted in darker phenotypes in *H. axyridis*, it provides a viable method for breeding darker cuticle strains through a gene-editing approach, potentially enhancing biological efficiency in colder environments.

In our previous study, we investigated the effects of knocking out the dopa decarboxylase (DDC) gene, a key component in the melanin synthesis pathway in *H. axyridis* (Wu et al., 2022). The knockout of this gene led to a striking reduction in melanin across all developmental stages, except during embryogenesis. However, this absence of DDC gene expression led to severe fitness costs, as all mutant ladybird beetles died in the adult stage. This finding underscores the unsuitability of the DDC gene for developing a genetic manipulation system in *H. axyridis*, given the severe impact on survival. In contrast, our current study presents the *Ebony* gene as a more promising candidate for establishing a CRISPR/Cas9-based gene editing system. Notably, the knockout of the *Ebony* gene resulted in a clearly identifiable phenotype, while inflicting minimal fitness costs on the insects. This observation aligns with previous research, which has underscored the potential of the *Ebony* gene as a visual marker in genome editing-based functional studies and genetics-based pest management strategies (Bi et al., 2019; Cheng et al., 2023; Osanai-Futahashi et al., 2012; Xu et al., 2021). Our findings suggest that targeting the *Ebony* gene could be a more viable approach for genetic studies and applications in *H. axyridis*, balancing phenotypic visibility with organismal viability.

In summary, our extensive research on the role of the *Ebony* gene in the tyrosine metabolism pathway has illuminated its multifaceted impact on insect development and adaptation. Our finding demonstrated that the *Ebony* gene's influence extends beyond physical attributes, affecting crucial development stages and behaviours, such as embryonic viability, predatory efficiency and neurotransmitter regulation. Our research has also highlighted the potential of gene editing, specifically using CRISPR/Cas9, as a promising tool for enhancing biological control strategies and adapting insects to environmental changes. For instance, creating darker cuticle phenotypes in ladybirds could optimise their thermal regulation in colder climates, a critical advantage for ecological adaptability. Furthermore, our comparison with the dopa decarboxylase gene knockout underscores the *Ebony* gene's suitability for genetic manipulation due to its minimal impact on overall fitness. Collectively, these insights not only contribute to our understanding of insect molecular biology but also open new avenues for applied research in pest management and ecological conservation.

EXPERIMENTAL PROCEDURES

Insect culture

H. axyridis specimens were collected from wheat fields located at 39°950 N, 116°280 E, within the experimental grounds of the Beijing Academy of Agriculture and Forestry Sciences (BAAFS), Beijing, China. Upon collection, these ladybird beetles were transported to the laboratory and reared on the bean aphid, *Megoura crassicauda* Mordvilko (Hemiptera: Aphididae) to establish the experimental population. The rearing environment was meticulously controlled: the temperature was maintained at a consistent 25 ± 2°C; relative humidity was set at 65%; and the light cycle consisted of 16 h of light followed by 8 h of darkness. This photoperiod was regulated by an automated device, the L-100 model from Suntech, Beijing, China. The ladybird beetles were maintained in custom-built culturing cages (50 cm × 50 cm × 50 cm; 100 mesh plastic fabric on aluminium frames) at a density of 40 pairs to a cage. The beetles were fed daily with bean aphids, which were cultivated on freshly germinated seedlings of the broad bean variety *Vicia faba* L. 'LinCan-5'.

Total RNA isolation and reverse transcription

Total RNA was isolated from *H. axyridis* samples using TRIzol reagent (Invitrogen, Carlsbad, CA), and RNA concentration was measured using NanoDrop ONE spectrophotometer (Thermo Fisher Scientific, Waltham, MA) at 260 nm. After the total RNA (1.0 μg) was used for the first-strand cDNA synthesis using PrimeScript™ RT reagent Kit with gDNA Eraser (Takara, Dalian, China) in a 20 μL reaction system. The first-strand cDNA was used in following analyses.

Subcloning and sequencing of HaEbony coding sequence

Two pairs of gene-specific primers were designed based on the *HaEbony* gene prediction in InsectBase (accession number: Haxy005633) (Chen et al., 2021) and NCBI (Accession number: LOC123680340) to amplify overlapping fragments by PCR for assembling the full-length cDNA corresponding to the entire protein coding regions (Table 2). The PCR products were subjected to electrophoresis on 1% agarose gel. The PCR bands were excised and purified using QIAEX II Agarose Gel Extraction Kit (Qiagen, Valencia, CA). The purified PCR fragment was ligated into a pMD™18-T Vector (Takara, Dalian, China). The ligation mixtures were then transformed into DH5α bacterial cells. Plasmids were isolated from the bacterial cells and sequenced by Sangon Biotech Co. (Shanghai, China).

Analyses of *HaEbony* cDNA, deduced amino acid and genomic sequences

The amino acid sequence of a putative *HaEbony* protein was deduced from its cDNA, and molecular mass and isoelectric point of the deduced protein were calculated by using online tools of ExPASy website (http://www.expasy.org/tools/). Multiple amino acid sequence alignment of all known insect Ebonys found in GenBank was carried out using ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2/). The phylogenetic tree of the insect species available in GenBank was generated using the neighbour-joining algorithm by using Mega 11 software. To evaluate the branch strength of the tree, a bootstrap analysis of 1000 replications was performed. The exon/intron organisation of *HaEbony* was revealed by comparing the full-length cDNA sequence with its corresponding genomic sequence (http://v2.insect-genome.com/).

Analysis of developmental and tissue expression profiles by reverse transcription quantitative PCR

The developmental expression profile of *HaEbony* gene was analysed with the samples prepared from different developmental stages including embryos (1-, 2- and 3-day eggs), larvae (1st, 2nd, 3rd and 4th instar larvae), pupae (1-, 2-, 3- and 4-day pupae) and A0, A2, A4, A6 and A24 represent 0-, 2-, 4-, 6- and 24-h adults (post-ecdysis) (24-h adults contain female and male). The tissue expression profile of *HaEbony* gene was analysed with the samples prepared from multiple tissues of the 4-day pupae (epidermis, head, midgut, fat body and Malpighian tubules). Total RNA was extracted from each stage or different tissues by using TRIzol reagent (Invitrogen), and 1.0 μg of total RNA was used for cDNA synthesis by using PrimeScript™ RT reagent Kit with gDNA Eraser (Takara, Dalian, China). The gene-specific primer was designed by using the Primer3Plus and ribosomal protein 49 (*Harp49*) in *H. axyridis* was used as an internal reference gene (Table 2).

The transcript levels of *HaEbony* were determined by reverse transcription quantitative PCR (RT-qPCR) using SYBR Green by using

TABLE 2 Sequences and relevant parameters of PCR primers used for gene cloning, expression level analysis, sgRNA synthesis and genotype analysis.

Primer name	Sequence (5′–3′)	Tm (°C)	Product size (bp)
PCR for cDNA sequencing			
HaEbony-1-F	TTTGAAAGGACCTACCAG	47.7	1309
HaEbony-1-R	AGAGTCCGTCCTACCCTC	50.3	
HaEbony-2-F	GATCCTACCTACGGTAAATT	48.3	1226
HaEbony-2-R	GCAGAACTTCGTACTTGA	45.2	
Reverse transcription quantitative PCR (RT-qPCR)			
HaEbony(Q)-F	GAAGTGGACATCAGTTCAGG	60.0	162
HaEbony(Q)-R	GACCAATATGTTTTCCCTCG	60.0	
Harp49-F	GCCGTTTCAAGGGACAGTAT	56.7	84
Harp49-R	TGAATCCAGTAGGAAGCATGTG	57.8	
PCR for sgRNA synthesis			
SgRNA-F	GAAATTAATACGACTCACTATAGAAAGGAACCCTGATGTACGGTTTTAGAGCTAGAAATAGC	55.0	115
SgRNA-ComR	AAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTT ATTTTAACTTGCTATTTCTAGCTCTAAAAC	55.0	
PCR for genotype analysis			
HaEbony-F	CTCTGAGTTTCGACCAG	44.2	214
HaEbony-R	CTGAGAGTCCGTCCTAC	42.9	

the QuantStudio™ 7 Flex Real-time PCR system (Thermo Fisher Scientific, Waltham, MA, USA) based on the method of Giulietti (Giulietti et al., 2001). The optimised quantitative PCR program consisted of an initial denaturation at 95°C for 30 s, followed by 40 cycles of 95°C for 10 s, 60°C for 30 s. At the end of the PCR, amplification specificity was verified by obtaining the dissociation curve, in which the samples were followed by one repeat of 95°C for 15 s, 60°C for 1 min and 95°C for 15 s. The specificity of each reaction was evaluated based on the melting temperatures of the PCR products. RT-qPCR was performed with three biological replicates, each with three technical replicates. The transcript levels of *HaEbony* were expressed as normalised transcript abundance using *Harp49* as an internal reference gene. The relative *Harp49* transcript levels were calculated according to the $2^{-\triangle\triangle Ct}$ method.

CRISPR guide design and guide RNA synthesis

sgRNA target sequences was designed in exon 7 of the *HaEbony* genes via the CHOPCHOP V3.00. To prepare the template DNA for in vitro sgRNA synthesis, fusion PCR was then performed with two oligonucleotides using the High-Fidelity DNA Polymerase PCR Mix (NEB, Ipswich, MA, USA). Two specific oligonucleotides were regarded as the forward primer that encoded the T7 polymerase binding site and the sgRNA target sequence, and the other common oligonucleotide served as the reverse primer and encoded the remaining sgRNA sequences (Table 1). The hybridization PCR (in a total volume of 50 μL) contained 0.5 μL of Phusion DNA Polymerase, 2.5 μL of SgRNA-F (10 μM), 2.5 μL of SgRNA-R (10 μM), 1 μL of dNTPs (10 μM) and 33.5 μL of Nuclease-free H_2O. The PCR program was conducted with the following parameters: one cycle of 98°C for 30s; 35 cycles of 98°C for 10 s, 60°C for 30s and 72°C for 15 s; and a final cycle of 72°C for 10 min. The PCR products were purified with the MinElute PCR Purification Kit (Qiagen, Hilden, Germany). The purity and concentration of the generated DNA templates were detected with a NanoDrop ONE spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Whereafter, in vitro transcription was performed to produce the sgRNAs with the T7 MEGAscript Kit (Ambion, Foster City, CA, USA) following the manufacturer's protocol, and the generated sgRNAs were further purified by the MEGAclear™ Transcription Clean-Up Kit (Ambion, Foster City, CA, USA). The synthesised sgRNA samples were used immediately or stored in aliquots at −80°C until use. The recombinant Cas9 protein from *Streptococcus pyogenes* used in this study was obtained commercially (Thermo Fisher Scientific, Waltham, MA, USA).

H. axyridis egg collection and microinjection

There are 40 pairs of *H. axyridis* adults in every cage were fed daily on cowpea aphids, *M. crassicauda* Mordvilko (Hemiptera: Aphididae) on leaves of seedlings of broad bean, *V. faba* L. Fresh pre-blastoderm stage eggs (15 min) were collected on double-sided adhesive on microscope slides (24 × 50 mm). Mixtures of sgRNA (300 ng/μL) and Cas9 (300 ng/μL) were injected into the germ cells located at the posterior pole of each egg within 30 min after oviposition using a Femto-Jet 4i and an InjectMan 4 microinjection system (Eppendorf, Hamburg, Germany) attached to glass needles (Sutter Instrument, Novato, CA, USA) pulled by a P—2000 micropipette puller (Sutter Instrument) and polished by an EG-401 micro-grinder (Narishige, Tokyo, Japan). After microinjection, the eggs were placed in a petri dish, then transfer into normal condition at 25°C, RH 65%, L/D = 16:8 h. After 24 h, the eggs were sprinkled with flour to prevent the hatching larvae from being stuck by the double-sided adhesive. After the eggs hatching, larvae fed with *M. crassicauda* Mordvilko for normal rearing in small square plastic containers (side length 9.4 cm × high 6.5 cm) individually.

gDNA isolation and mutagenesis detection

In order to detect the indel (insertion or deletion) mutations of the *HaEbony* gene induced by the CRISPR/Cas9 genome editing system, genomic DNA (gDNA) samples were extracted from the exuvium of the third instar larvae transform to the fourth instar larvae in *H. axyridis*. Trace amounts of gDNA were isolated individually using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. Then, the gDNA fragments of *HaEbony* (214 bp) were amplified by PCR using the corresponding gene-specific primer pairs flanking the sgRNA target sites (Table 1). The PCRs (in a total volume of 50 μL) contained 25 μL of Premix Taq™ (Takara, Dalian, China), 1 μL of *HaEbony*-F (20 μM), 1 μL of *HaEbony*-R (20 μM), 300 ng of gDNA template and ddH_2O up to 23 μL. The PCR program was as follows: one cycle of 94°C for 5 min; 35 cycles of 94°C for 30 s, 49°C for 30 s and 72°C for 30 s; and a final cycle of 72°C for 10 min. The PCR products were excised and purified using QIAEX II Agarose Gel Extraction Kit (Qiagen, Hilden, Germany). The purified PCR fragment was ligated into the pMD™18-T cloning vector (Takara, Dalian, China). The ligation mixtures were then used to transform *DH5α* bacterial cells (Cwbio, Jiangsu, China). Plasmids were isolated from the bacterial cells and sequenced for exact indel mutation type detection (Sangon Biotech, Beijing, China).

Phenotype images were taken using Keyence Digital Microscope VHX-6000 (Keyence, Osaka, Japan), all using the same magnification exposure time, and light intensity. Images were then selected for depiction of the most representative phenotypes.

Fitness analysis of *HaEbony* mutants (G_0)

To evaluate the effects of the *HaEbony* mutation on the fitness of *H. axyridis* population, eggs subjected to *HaEbony*-sgRNA and Cas9 injections were meticulously collected. After the eggs hatching, larvae were then individually reared in small square plastic containers (side length 9.4 cm × high 6.5 cm), under the previously detailed ambient

conditions. For daily sustenance, each *H. axyridis* larva was provided with a broad bean leaf infested with 50–120 *M. crassicauda* aphids. We conducted rigorous monitoring, recording survival rates and the developmental duration of each instar on a daily basis. This experiment included 30 biological replicates for both the wild-type and mutant strains of *H. axyridis*, with a minimum of 1 ladybird beetle per replicate. *HaEbony*$^{+/-}$ mutant individuals were selected to mating and recorded eggs laying daily. The experiment of fecundity included 10 biological replicates for both the wild-type and mutant strains of *H. axyridis*, with one pair of ladybird beetles per replicate. Egg hatchability was assessed 3 days post-collection. This aspect of the study included six biological replicates for each group, with five egg masses in each replicate.

Furthermore, to compare predation abilities between the mutant and wild-type adults, an assay was performed on 3-day-old adults. Each ladybird beetle, both from mutant and wild-type groups, was subjected to a 24-h starvation period. Then, each beetle was provided with 120 second instar nymphs of *M. crassicauda* in a petri dish. After 24 h, we counted the remaining *M. crassicauda* nymphs to assess the predatory efficiency. This aspect of the study also included three biological replicates for each group, with a minimum of 10 beetles per replicate.

Fitness analysis of HaEbony mutants (G_1)

To evaluate the effects of the *HaEbony* knockout on the fitness of *H. axyridis* mutant strain (G_1), the developmental time, pupation rate, eclosion rate and predatory number of *HaEbony* mutants (G_1) were recorded. Twelve egg masses, comprising 490 eggs derived from the mutant G_0 generation were selected to explore the associated fitness cost. Upon hatching, each larva was kept in a small square plastic container (side length 9.4 cm × high 6.5 cm), provisioned daily with a broad bean leaf infested with 50–120 *M. crassicauda* aphids. Concurrently, rigorous monitoring of all hatched larvae (332 larvae) was undertaken, with daily records of each instar's developmental duration, and tracking of individual pupation and eclosion events to analyse their respective rates. *HaEbony* mutants are confirmed by both sequencing and phenotype observations, and according to this, the data of mutants were separated and counted. The above experiments encompassed three biological replicates, each comprising a minimum of 20 ladybirds. The experiment of developmental duration included 60 biological replicates for both the wild-type and mutant strains of *H. axyridis*, with 1 ladybird beetle per replicate.

To compare predation abilities between mutant and wild-type ladybirds, we conducted an assay on 2-day-old fourth instar larvae. Each ladybird beetle, from both mutant and wild-type groups, was subjected to a 24-h starvation period. Subsequently, each beetle was provided with 120 second instar nymphs of *M. crassicauda* in a petri dish. After 24 h, the remaining *M. crassicauda* nymphs were counted to evaluate the predatory efficiency. This section of the study also included three biological replicates for each group, with a minimum of 20 beetles per replicate.

The relative content of dopamine profile was analysed with the samples prepared from *HaEbony* mutants (G_1) and wild types including 2- and 4-day pupae. This experiment included six biological replicates for both the wild-type and mutant strains of *H. axyridis*, with 3 beetles per replicate. These insects were homogenised thoroughly in PBS buffer (1x) and centrifuged at 8000 rpm for 5 min. Subsequently, the supernatant was extracted for dopamine level detection. The quantification of dopamine content was measured using Insect DA ELISA Kit (Gene lab, Beijing, China) according to the manufacturer's recommendations. For the determination of absorbance, both the diluted test sample and standards were measured at the wavelength of 450 nm, utilising an enzyme-linked immunosorbent assay reader (PerkinElmer, Massachusetts, USA).

Statistical analysis

The raw data encompassing various metrics such as hatching rate, mutation rate, developmental time, predation ability, pupation rate and eclosion rate were analysed using GraphPad Prism software (version 8, GraphPad Software, CA, USA). Means and standard error of development time and predation ability were estimated by the GraphPad Prism (v.8, GraphPad Software, CA, USA). Differences in development time, fecundity, hatching rates, predation ability, pupation rates and eclosion rates between groups were subjected to Student's *t* test using SPSS software (v. 22, IBM Corp. Armonk, NY, USA).

AUTHOR CONTRIBUTIONS

Jing Lin: Software; writing – original draft; data curation; methodology. **Da Xiao:** Software; formal analysis; writing – review and editing; writing – original draft. **Mengmeng Wu:** Data curation; formal analysis; investigation. **Xu Chen:** Software; data curation; investigation. **Qingxuan Xu:** Methodology; investigation. **Su Wang:** Funding acquisition; writing – review and editing; project administration; resources. **Liansheng Zang:** Formal analysis; resources; writing – review and editing.

ACKNOWLEDGEMENTS

This research was supported by the National Key Research and Development Program (2023YFD1400600) and Technical Innovation Program of Beijing Academy of Agriculture and Forestry Sciences (KJCX20230115).

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the research of this study are available from the corresponding authors upon reasonable request.

ORCID

Jing Lin https://orcid.org/0009-0002-9778-3146
Su Wang https://orcid.org/0000-0001-5166-0250
Liansheng Zang https://orcid.org/0000-0002-9964-0512

REFERENCES

Andersen, S.O. (2010) Insect cuticular sclerotization: a review. *Insect Biochemistry and Molecular Biology*, 40(3), 166–178. Available from: https://doi.org/10.1016/j.ibmb.2009.10.007

Andersen, S.O. (2012) Cuticular sclerotization and tanning. In: *Insect molecular biology and biochemistry*. Amsterdam, Netherlands: Elsevier, pp. 167–192. Available from: https://doi.org/10.1016/B978-0-12-384747-8.10006-6

Ando, T., Matsuda, T., Goto, K., Hara, K., Ito, A., Hirata, J. et al. (2018) Repeated inversions within a *pannier* intron drive diversification of intraspecific colour patterns of ladybird beetles. *Nature Communications*, 9(1), 3843. Available from: https://doi.org/10.1038/s41467-018-06116-1

Ando, T. & Niimi, T. (2019) Development and evolution of color patterns in ladybird beetles: a case study in *Harmonia axyridis*. *Development, Growth & Differentiation*, 61(1), 73–84. Available from: https://doi.org/10.1111/dgd.12592

Arakane, Y., Noh, M.Y., Asano, T. & Kramer, K.J. (2016) Tyrosine metabolism for insect cuticle pigmentation and sclerotization. In: Cohen, E. & Moussian, B. (Eds.) *Extracellular composite matrices in arthropods*. Cham: Springer International Publishing, pp. 165–220. Available from: https://doi.org/10.1007/978-3-319-40740-16

Badejo, O., Skaldina, O., Gilev, A. & Sorvari, J. (2020) Benefits of insect colours: a review from social insect studies. *Oecologia*, 194(1–2), 27–40. Available from: https://doi.org/10.1007/s00442-020-04738-1

Bai, Y., He, Y., Shen, C.Z., Li, K., Li, D.L. & He, Z.Q. (2023) CRISPR/Cas9-mediated genomic knock out of tyrosine hydroxylase and *yellow* genes in cricket *Gryllus bimaculatus*. *PLoS One*, 18(4), e0284124. Available from: https://doi.org/10.1371/journal.pone.0284124

Barek, H., Sugumaran, M., Ito, S. & Wakamatsu, K. (2018) Insect cuticular melanins are distinctly different from those of mammalian epidermal melanins. *Pigment Cell & Melanoma Research*, 31(3), 384–392. Available from: https://doi.org/10.1111/pcmr.12672

Bi, H., Xu, J., He, L., Zhang, Y., Li, K. & Huang, Y. (2019) CRISPR/Cas9-mediated *ebony* knockout results in puparium melanism in *Spodoptera litura*. *Insect Science*, 26(6), 1011–1019. Available from: https://doi.org/10.1111/1744-7917.12663

Blenau, W. & Baumann, A. (2001) Molecular and pharmacological properties of insect biogenic amine receptors: lessons from *Drosophila melanogaster* and *Apis mellifera*. *Archives of Insect Biochemistry and Physiology*, 48(1), 13–38. Available from: https://doi.org/10.1002/arch.1055

Chen, M., Mei, Y., Chen, X., Chen, X., Xiao, D., He, K. et al. (2021) A chromosome-level assembly of the harlequin ladybird *Harmonia axyridis* as a genomic resource to study beetle and invasion biology. *Molecular Ecology Resources*, 21(4), 1318–1332. Available from: https://doi.org/10.1111/1755-0998.13342

Chen, X., Xiao, D., Du, X., Guo, X. & Wang, S. (2019) The role of the dopamine melanin pathway in the ontogeny of elytral melanization in *Harmonia axyridis*. *Frontiers in Physiology*, 10, 1066. Available from: https://doi.org/10.3389/fphys.2019.01066

Cheng, Y., Wang, P., Zeng, Y., An, W., Wang, T. & Xiao, Y. (2023) Characterization of five pigmentation genes as transgenic markers in *Spodoptera frugiperda* (lepidoptera: Noctuidae). *International Journal of Biological Macromolecules*, 242, 124981. Available from: https://doi.org/10.1016/j.ijbiomac.2023.124981

Costa, K.M. & Schoenbaum, G. (2022) Dopamine. *Current Biology*, 32(15), R817–R824. Available from: https://doi.org/10.1016/j.cub.2022.06.060

Daubner, S.C., Le, T. & Wang, S. (2011) Tyrosine hydroxylase and regulation of dopamine synthesis. *Archives of Biochemistry and Biophysics*, 508(1), 1–12. Available from: https://doi.org/10.1016/j.abb.2010.12.017

Eriksson, A., Raczkowska, M., Navawongse, R., Choudhury, D., Stewart, J. C., Tang, Y.L. et al. (2017) Neuromodulatory circuit effects on *drosophila* feeding behaviour and metabolism. *Scientific Reports*, 7(1), 8839. Available from: https://doi.org/10.1038/s41598-017-08466-0

Futahashi, R., Sato, J., Meng, Y., Okamoto, S., Daimon, T., Yamamoto, K. et al. (2008) *Yellow* and *ebony* are the responsible genes for the larval color mutants of the silkworm *Bombyx mori*. *Genetics*, 180(4), 1995–2005. Available from: https://doi.org/10.1534/genetics.108.096388

Gautier, M., Yamaguchi, J., Foucaud, J., Loiseau, A., Ausset, A., Facon, B. et al. (2018) The genomic basis of color pattern polymorphism in the harlequin ladybird. *Current Biology*, 28(20), 3296–3302.e7. Available from: https://doi.org/10.1016/j.cub.2018.08.023

Gorman, M.J. & Arakane, Y. (2010) Tyrosine hydroxylase is required for cuticle sclerotization and pigmentation in *Tribolium castaneum*. *Insect Biochemistry and Molecular Biology*, 40(3), 267–273. Available from: https://doi.org/10.1016/j.ibmb.2010.01.004

Giulietti, A., Overbergh, L., Valckx, D., Decallonne, B., Bouillon, R. & Mathieu, C. (2001) An overview of real-time quantitative PCR: applications to quantify cytokine gene expression. *Methods*, 25(4), 386–401. Available from: https://doi.org/10.1006/meth.2001.1261

Inoue, S., Watanabe, T., Hamaguchi, T., Ishimaru, Y., Miyawaki, K., Nikawa, T. et al. (2023) Combinatorial expression of *ebony* and *tan* generates body color variation from nymph through adult stages in the cricket, *Gryllus bimaculatus*. *PLoS One*, 18(5), e0285934. Available from: https://doi.org/10.1371/journal.pone.0285934

Koch, R.L. (2003) The multicolored Asian lady beetle, *Harmonia axyridis*: a review of its biology, uses in biological control, and non-target impacts. *Journal of Insect Science*, 3(1), 32. Available from: https://doi.org/10.1093/jis/3.1.32

Labun, K., Montague, T.G., Krause, M., Torres Cleuren, Y.N., Tjeldnes, H. & Valen, E. (2019) CHOPCHOP v3: expanding the CRISPR web toolbox beyond genome editing. *Nucleic Acids Research*, 47(W1), W171–W174. Available from: https://doi.org/10.1093/nar/gkz365

Li, F., Li, K., Wu, L.J., Fan, Y.L. & Liu, T.X. (2020) Role of biogenic amines in oviposition by the diamondback moth, *Plutella xylostella* L. *Frontiers in Physiology*, 11, 475. Available from: https://doi.org/10.3389/fphys.2020.00475

Li, X., Fan, D., Zhang, W., Liu, G., Zhang, L., Zhao, L. et al. (2015) Outbred genome sequencing and CRISPR/Cas9 gene editing in butterflies. *Nature Communications*, 6(1), 8212. Available from: https://doi.org/10.1038/ncomms9212

Liu, T., Dartevelle, L., Yuan, C.Y., Wei, H.P., Wang, Y., Ferveur, J. et al. (2008) Increased dopamine level enhances male-male courtship in *drosophila*. *The Journal of Neuroscience*, 28(21), 5539–5546. Available from: https://doi.org/10.1523/jneurosci.5290-07.2008

Massey, J.H., Akiyama, N., Bien, T., Dreisewerd, K., Wittkopp, P.J., Yew, J. Y. et al. (2019) Pleiotropic effects of *ebony* and *tan* on pigmentation and cuticular hydrocarbon composition in *Drosophila melanogaster*. *Frontiers in Physiology*, 10, 518. Available from: https://doi.org/10.3389/fphys.2019.00518

Michie, L.J., Mallard, F., Majerus, M.E.N. & Jiggins, F.M. (2020) Melanic through nature or nurture: genetic polymorphism and phenotypic plasticity in *Harmonia axyridis*. *Journal of Evolutionary Biology*, 23(8), 1699–1707. Available from: https://doi.org/10.1111/j.1420-9101.2010.02043.x

Michie, L.J., Masson, A., Ware, R.L. & Jiggins, F.M. (2011) Seasonal phenotypic plasticity: wild ladybirds are darker at cold temperatures. *Evolutionary Ecology*, 25(6), 1259–1268. Available from: https://doi.org/10.1007/s10682-011-9476-8

Niimi, T. & Ando, T. (2021) Evo-devo of wing colour patterns in beetles. *Current Opinion in Genetics and Development*, 69, 97–102. Available from: https://doi.org/10.1016/j.gde.2021.02.007

Osanai-Futahashi, M., Ohde, T., Hirata, J., Uchino, K., Futahashi, R., Tamura, T. et al. (2012) A visible dominant marker for insect transgenesis. *Nature Communications*, 3(1), 1295. Available from: https://doi.org/10.1038/ncomms2312

Pantalia, M., Lin, Z., Tener, S.J., Qiao, B., Tang, G., Ulgherait, M. et al. (2023) *Drosophila* mutants lacking the glial neurotransmitter-modifying enzyme *ebony* exhibit low neurotransmitter levels and altered behavior. *Scientific Reports*, 13(1), 10411. Available from: https://doi.org/10.1038/s41598-023-36558-7

Pinkert, S. & Zeuss, D. (2018) Thermal biology: melanin-based energy harvesting across the tree of life. *Cell Press*, 28(16), R887–R889. Available from: https://doi.org/10.1016/j.cub.2018.07.026

Protas, M.E. & Patel, N.H. (2008) Evolution of coloration patterns. *Annual Review of Cell and Developmental Biology*, 24(1), 425–446. Available from: https://doi.org/10.1146/annurev.cellbio.24.110707.175302

Sasaki, K. (2016) Nutrition and dopamine: an intake of tyrosine in royal jelly can affect the brain levels of dopamine in male honeybees (Apis mellifera L.). *Journal of Insect Physiology*, 87, 45–52. Available from: https://doi.org/10.1016/j.jinsphys.2016.02.003

Shen, Z.J., Liu, Y.J., Zhu, F., Cai, L.M., Liu, X.M., Tian, Z.Q. et al. (2020) MicroRNA-277 regulates dopa decarboxylase to control larval-pupal and pupal-adult metamorphosis of *Helicoverpa armigera*. *Insect Biochemistry and Molecular Biology*, 122, 103391. Available from: https://doi.org/10.1016/j.ibmb.2020.103391

Sterkel, M., Ons, S. & Oliveira, P.L. (2019) DOPA decarboxylase is essential for cuticle tanning in *Rhodnius prolixus* (Hemiptera: Reduviidae), affecting ecdysis, survival and reproduction. *Insect Biochemistry and Molecular Biology*, 108, 24–31. Available from: https://doi.org/10.1016/j.ibmb.2019.03.006

Sugumaran, M. & Barek, H. (2016) Critical analysis of the melanogenic pathway in insects and higher animals. *International Journal of Molecular Science*, 17(10), 1753. Available from: https://doi.org/10.3390/ijms17101753

Sun, J., Zheng, X., Yang, G., Qian, H. & Chen, A. (2023) *Ebony* plays an important role in egg hatching and 30k protein expression of silkworm (*Bombyx mori*). *Archives of Insect Biochemistry and Physiology*, 113(3), e22014. Available from: https://doi.org/10.1002/arch.22014

True, J.R. (2003) Insect melanism: the molecules matter. *Trends in Ecology & Evolution*, 18(12), 640–647. Available from: https://doi.org/10.1016/j.tree.2003.09.006

Trullas, S.C., Wyk, J.H.V. & Spotila, J.R. (2007) Thermal melanism in ectotherms. *Journal of Thermal Biology*, 32(5), 235–245. Available from: https://doi.org/10.1016/j.jtherbio.2007.01.013

Tsukioka, D., Izumi, S. & Adachi, T. (2017) Cloning and expression analysis of a novel tissue-specific dopa decarboxylase mRNA splicing variant in *Bombyx mori*. *Bioscience, Biotechnology, and Biochemistry*, 81(3), 555–557. Available from: https://doi.org/10.1080/09168451.2016.1258987

Verlinden, H. (2018) Dopamine signalling in locusts and other insects. *Insect Biochemistry and Molecular Biology*, 97, 40–52. Available from: https://doi.org/10.1016/j.ibmb.2018.04.005

Wang, S.U., Michaud, J.P., Runzhi, Z., Fan, Z. & Shuang, L. (2009) Seasonal cycles of assortative mating and reproductive behaviour in polymorphic populations of *Harmonia axyridis* in China. *Ecological Entomology*, 34(4), 483–494. Available from: https://doi.org/10.1111/j.1365-2311.2008.01075.x

Watanabe, T. & Sasaki, K. (2021) Regulation of dopamine production in the brains during sexual maturation in male honey bees. *Journal of Insect Physiology*, 132, 104270. Available from: https://doi.org/10.1016/j.jinsphys.2021.104270

Wittkopp, P.J., Carroll, S.B. & Kopp, A. (2003) Evolution in black and white: genetic control of pigment patterns in *drosophila*. *Trends in Genetics*, 19(9), 495–504. Available from: https://doi.org/10.1016/S0168-9525(03)00194-X

Wittkopp, P.J., True, J.R. & Carroll, S.B. (2002) Reciprocal functions of the *drosophila yellow* and *ebony* proteins in the development and evolution of pigment patterns. *Development*, 129(8), 1849–1858. Available from: https://doi.org/10.1242/dev.129.8.1849

Wu, M., Chen, X., Xu, Q., Zang, L., Wang, S., Li, M. et al. (2022) Melanin synthesis pathway interruption: CRISPR/Cas9-mediated knockout of dopa decarboxylase (DDC) in *Harmonia axyridis* (coleoptera: Coccinellidae). *Journal of Insect Science*, 22(5), 1–9. Available from: https://doi.org/10.1093/jisesa/ieac048

Xiao, D., Chen, X., Tian, R., Wu, M., Zhang, F., Zang, L. et al. (2020) Molecular and potential regulatory mechanisms of melanin synthesis in *Harmonia axyridis*. *International Journal of Molecular Sciences*, 21(6), 2088. Available from: https://doi.org/10.3390/ijms21062088

Xu, X., Harvey-Samuel, T., Yang, J., You, M. & Alphey, L. (2021) CRISPR/-Cas9-based functional characterization of the pigmentation gene *ebony* in *Plutella xylostella*. *Insect Molecular Biology*, 30(6), 615–623. Available from: https://doi.org/10.1111/imb.12730

Zhang, Y., Wang, X., Feng, Z.J., Cong, H., Chen, Z.S., Li, Y. et al. (2020) Superficially similar adaptation within one species exhibits similar morphological specialization but different physiological regulations and origins. *Frontiers in Cell and Developmental Biology*, 8, 300. Available from: https://doi.org/10.3389/fcell.2020.00300

Zhang, Y., Wang, X.X., Feng, Z.J., Tian, H.G., Feng, Y. & Liu, T.X. (2020) Aspartate-β-alanine-NBAD pathway regulates pupal melanin pigmentation plasticity of ladybird *Harmonia axyridis*. *Insect Science*, 28(6), 1651–1663. Available from: https://doi.org/10.1111/1744-7917.12877

How to cite this article: Lin, J., Xiao, D., Wu, M., Chen, X., Xu, Q., Wang, S. et al. (2024) Pleiotropic effects of *Ebony* on pigmentation and development in the Asian multi-coloured ladybird beetle, *Harmonia axyridis* (Coleoptera: Coccinellidae). *Insect Molecular Biology*, 1–15. Available from: https://doi.org/10.1111/imb.12968