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SHORT COMMUNICATION

The proteins involved in larval colouration in the cuticle and haemolymph of two strains of *Antheraea pernyi* (Lepidoptera: Saturniidae)

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Abstract. The proteome profiles of the cuticle and haemolymph of two strains of the Chinese Oak silk moth *A. pernyi*, with cyan and yellow coloured bodies, were compared in order to identify the differentially expressed proteins (DEPs) that determine the differences in the colouration of the two strains. A total of 324 DEPs, including 124 up-regulated and 200 down-regulated proteins, were identified in the cuticles of the cyan and yellow coloured individuals, and 286 DEPs including 79 up-regulated and 207 down-regulated proteins in the haemolymph of the cyan and yellow coloured individuals. Several DEPs associated with the colour of larvae of *A. pernyi* were screened, including an ommochrome-binding protein, juvenile hormone esterase, protein yellow, L-dopachrome tautomerase yellow-f2 and fumarylacetoacetase, the expression levels in the cuticle and haemolymph of the yellow larvae were higher than those in the cyan coloured larvae, indicating their possible roles underlying the colouration of the yellow larvae of *A. pernyi*.

INTRODUCTION

In insects, the diversity of colour patterns enables them to adapt behaviourally to different environments in terms of camouflage, warning, mimicry or thermo-regulation (Badejo et al., 2020; Jin et al., 2020). The pigments in the cuticle or underlying epidermis are important factors for insect colouration (Wittkopp & Beldade, 2009; Nishikawa et al., 2013; Shamim et al., 2014). Flavonoids are related to the wing colour of the butterfly *Polyommatus icarus* (Burghardt et al., 2001) and the bristle colour of *Parasemia plantaginis* (Lindstedt et al., 2010). The different amounts of carotenoid, biliverdin and β-carotene are associated with body colour polyphenism of green solitary and black gregarious individuals of the locust, *Locusta migratoria* (Yang et al., 2019). Biochemical and genetic studies indicate that more than ten genes are involved in the larval pigmentation of *Bombyx mori*, including *tyrosine hydroxylase* (Liu et al., 2010), *ebony* and *yellow* (Futahashi et al., 2008), *tan* (Futahashi et al., 2010), *yellow-e* (Ito et al., 2010), *BmMFS* (Ito et al., 2012), *guanylyl cyclase* (Yuasa et al., 2016), *sepiapterin reductase* (Meng et al., 2009) and *6-pyruvoyl-tetrahydropterin synthase* (Fujii et al., 2013). In *Papilio xuthus*, the genes encoding tyrosine hydroxylase, dopa decarboxylase, yellow, ebony and guanosine triphosphate cyclohydrolase I are associated with melanin pigmentation at specific development stages (Futahashi & Fujiwara, 2005, 2006, 2007, 2008a). The co-expression of bilin-binding protein and yellow-related gene contribute to the green colour of swallowtail butterflies (Shirataki et al., 2010), and the switches in the larval pattern of *P. xuthus* are modulated by juvenile hormone (Futahashi & Fujiwara, 2008b), which also regulates the larval colour of the tobacco hornworm *Manduca sexta* (Suzuki & Nijhout, 2006).

A. pernyi is important economically and has various colour forms. The colour of cuticle is consistent with that of the haemolymph in different strains of *A. pernyi*. Recent research indicates that the lower production of lutein, β-carotene and zeaxanthin in white *A. pernyi* may result in its body colour differing from that of blue individuals (Su et al., 2021). In this study, the mechanisms underlying the colouration of the larvae of *A. pernyi*, especially differences in protein levels in the larvae of different colours, was investigated by 4D label-free quantitative proteomics to compare the proteome profiles of the cuticle and haemolymph in order to determine the differentially expressed proteins (DEPs) associated with cyan and yellow coloured larvae of *A. pernyi*. The DEPs involved in the colouration of *A. pernyi* were screened via bioinformatic analysis. This data provides novel insights into the molecular mechanisms determining the colour of insects.

MATERIAL AND METHODS

Sample collection

Two strains of *A. pernyi*, Xuanda, and Shenhuang, the larvae of which are coloured cyan (C) and yellow (Y), respectively (Fig. 1A and B), were used in this study. The larvae were reared on fresh leaves of *Quercus liaotungensis* under a photoperiod of 12L : 12D, at 23 ± 2°C and a relative humidity of 70 ± 5%.

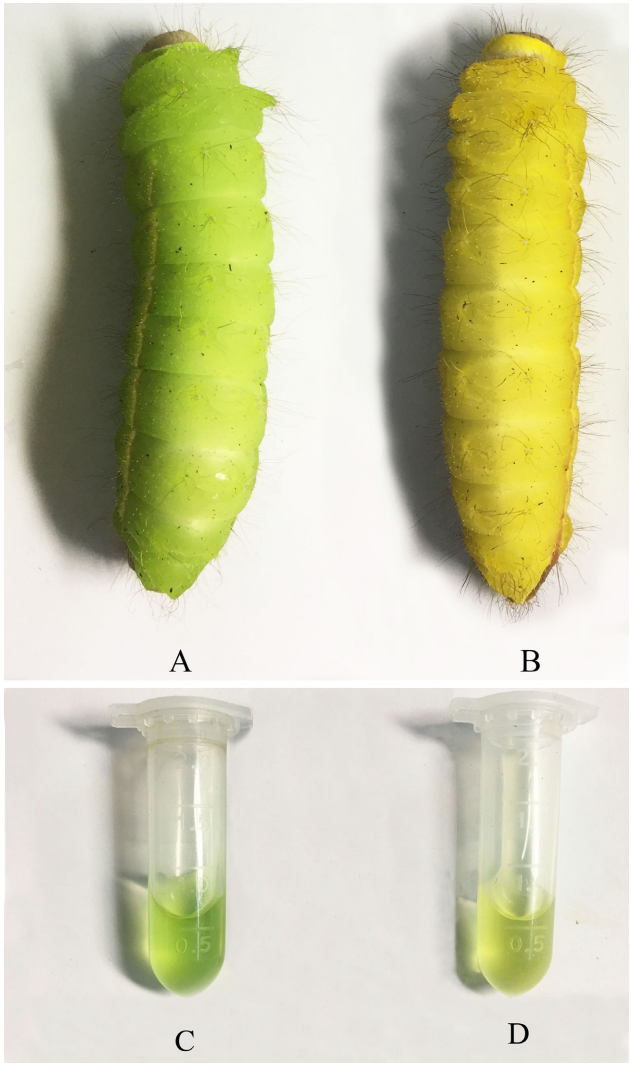

Fig. 1. The larvae of *A. pernyi* strains with cyan (A) and yellow (B) body colours, and that of the colour of their haemolymph, cyan (C) and yellow (D).

Newly moulted fifth instar larvae were randomly separated into two groups (C v. Y) based on their colour, with 20 larvae per group. Samples of cuticle and haemolymph (Fig. 1C and D) were obtained from the larvae in each group and stored at –80°C for further use.

Protein extraction and digestion

The samples were powdered using liquid nitrogen and homogenized with lysis buffer (Beyotime, Shanghai). The homogenate was sonicated on ice and then centrifuged at 12,000 *g* for 10 min. The supernatant was collected after filtering and quantified using a BCA kit (Thermo, USA). A 10 µg sample of proteins was separated by SDS-PAGE and then stained with Coomassie Brilliant Blue.

For protein digestion, 5 mM dithiothreitol was incubated with the above protein solution for 30 min at 55°C and placed on ice to cool to room temperature. Then the corresponding volume of iodoacetamide was added and the mixture was kept in the dark for 15 min. Six times the volume of acetone was added to precipitate the protein and the mixture stored at –20°C overnight. The final mixture was subjected to 10 min centrifugation under 8000 *g* at 4°C for collecting the precipitate, which was dissolved in 100 µL NH_4HCO_3 (50 mM) and then incubated with the corresponding volume of enzymolysis diluent (protein: enzyme = 50 : 1) for 12 h at 37°C. The samples were lyophilized following enzymolysis.

Liquid chromatography-tandem mass spectrometry (LC-MS/MS)

The LC-MS/MS analysis was carried out by Shanghai Luming biological technology Co., Ltd (Shanghai, China). A Tims TOF Pro mass spectrometer (Bruker, GER) was used to do the analyses. Samples were loaded onto a Nano Elute system (Bruker, GER) using a C18 column (25 cm, RP-C18, ionopticks) at a flow rate of 300 nL/min. The mobile phase consisted of solvent A (water : formic acid = 99.9 : 0.1) and solvent B (acetonitrile : formic acid = 99.9 : 0.1) with a linear gradient of 2–22% solvent B (0–75 min), 22–37% solvent B (75–80 min), 37–80% solvent B (80–85 min) and 80% solvent B (85–90 min). The MS parameters were set as follows: capillary voltage, 1.4 kV; drying gas temperature, 180°C; flow rate of drying gas, 3.0 L/min; MS scanning range, $100 \leq m/z \leq 1700$; ion mobility, 0.6–1.6 Vs/cm^2; collision energy, 20–59 eV.

Database search

MS/MS spectra were searched against the proteome on-line database of *A. pernyi* (https://bigd.big.ac.cn/gwh/, accession number: GWHABGR00000000) (Duan et al., 2020) using MaxQuant (1.6.17.0). The parameters of database search were set as follows: FDR, 0.01; missed cleavage, 2; fixed modifications,

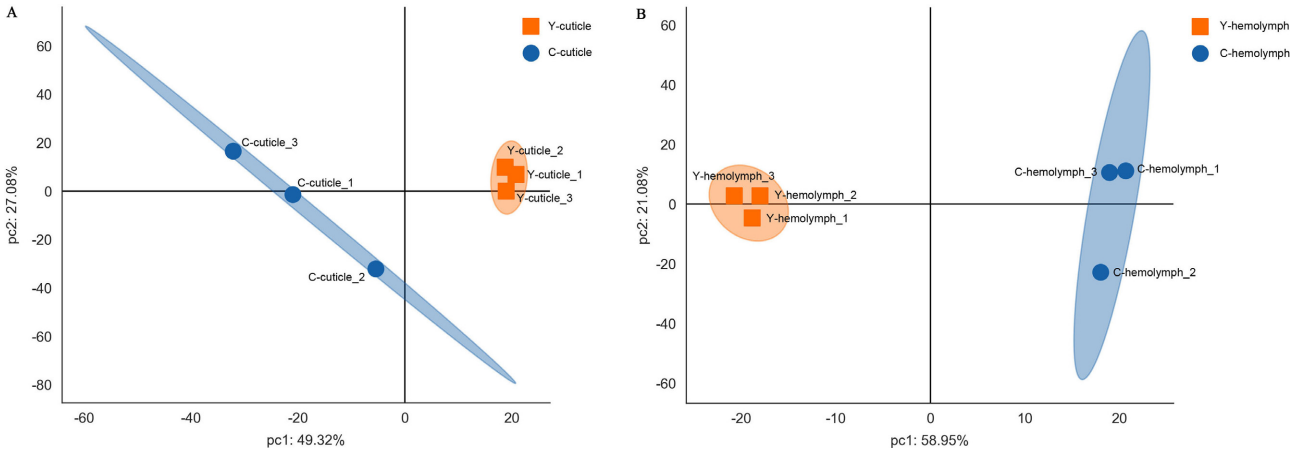

Fig. 2. Principal component analysis of the cuticle (A) and haemolymph (B) samples from the cyan and yellow strains of *A. pernyi*.

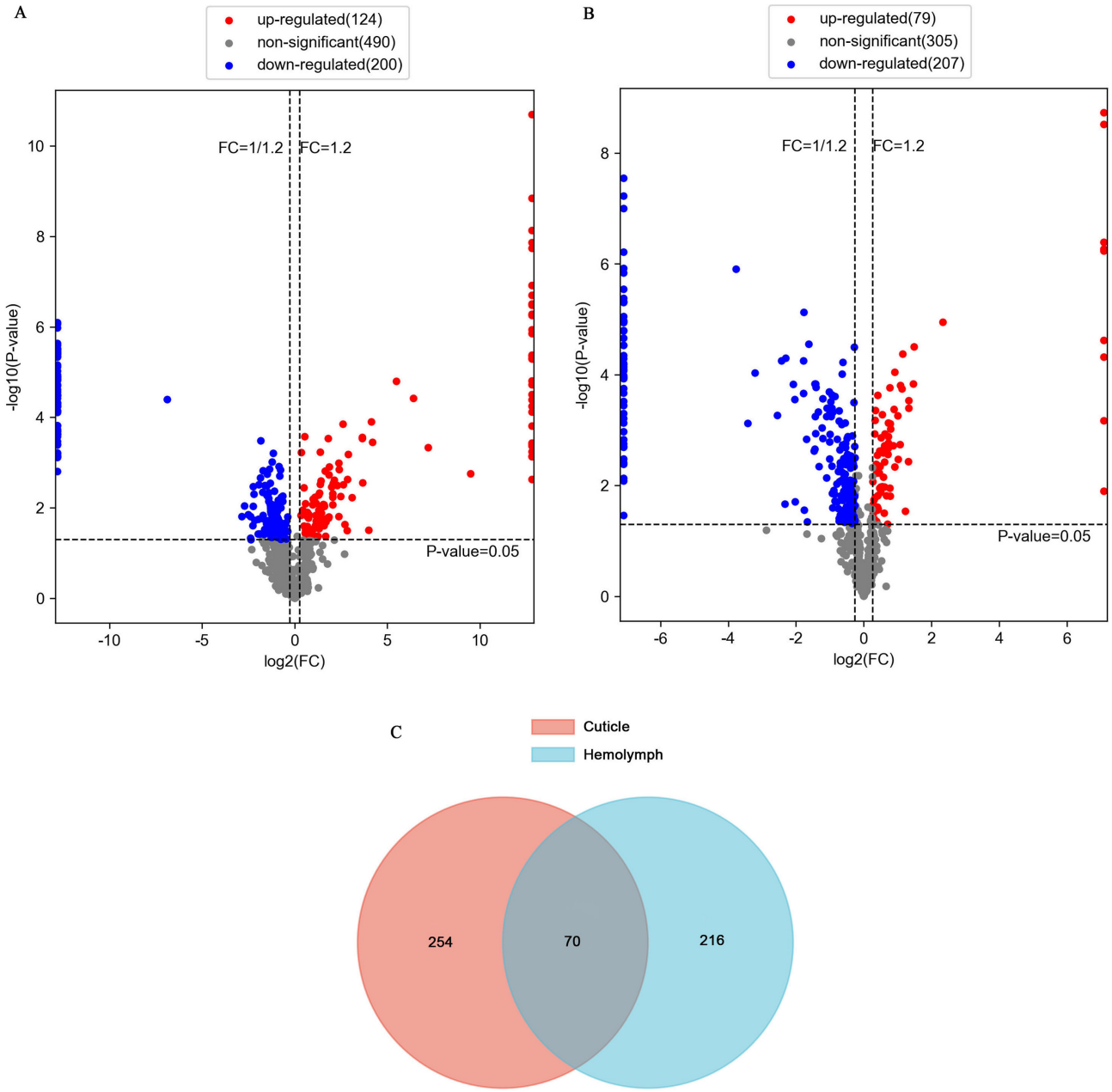

Fig. 3. Identification of the differentially expressed proteins (DEPs). A and B respectively represent the volcano plots of the DEPs in the cuticle and haemolymph of the cyan and yellow strains. C – Venn diagram of the DEPs in the cuticle and haemolymph of *A. pernyi*.

carbamidomethyl (C); variable modification, oxidation (M) and acetyl (protein N-term); decoy database pattern, reverse; enzyme, trypsin; first search peptide tolerance, 20 ppm; main search peptide tolerance, 10 ppm. Principal component analysis (PCA) was performed to detect outliers (if any) and evaluate the batch effect with the pre-processed datasets.

Statistical analyses

Fold change criteria (the ratio of the level of protein expression in the Y and C groups) > 1.2 or < 0.83 and *P*-value < 0.05 were adopted for identifying DEPs. GO (http://www.blast2go.com/b2ghome; http://geneontology.org/) and KEGG pathway (http://www.genome.jp/kegg/) enrichment analyses were performed for the DEPs.

RESULTS AND DISCUSSION

PCA results indicated that the samples from each group exhibited high intra-repeatability and were inter-separated between the groups (Fig. 2A and B). Based on the proteome data sets, a total of 894 proteins were obtained from the cuticle sample. In addition, 324 DEPs including 124 up-regulated and 200 down-regulated proteins were recorded in the Y cuticle compared to C group (control) (Fig. 3A). There were 629 proteins in the total haemolymph samples from *A. pernyi*, with 286 DEPs including 79 up-regulated and 207 down-regulated proteins (Fig. 3B). The DEPs from the cuticle (Fig. 4A) and haemolymph (Fig. 4B) from the Y and C groups were hierarchically clustered in order to determine their expression patterns.

Based on the bioinformatics analysis, 70 DEPs were detected in both the cuticle and haemolymph (Fig. 3C). Several of which are associated with colouration of the larvae of *A. pernyi* included

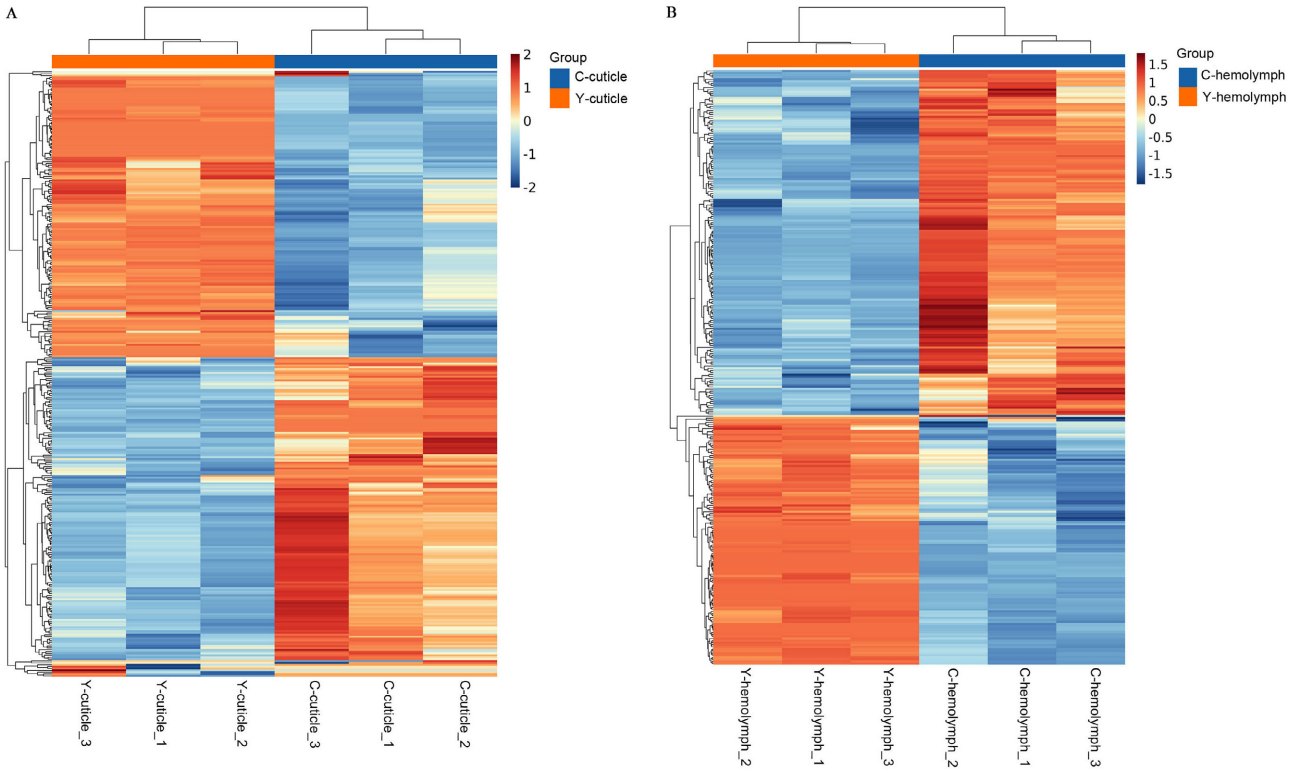

Fig. 4. Hierarchical clustering of the DEPs in the cuticle (A) and haemolymph (B) of the cyan and yellow strains of *A. pernyi*.

an ommochrome-binding protein (GWHPABGR006793), juvenile hormone esterase (GWHPABGR014261), protein yellow (GWHPABGR003184), L-dopachrome tautomerase yellow-f2 (GWHPABGR008874) and fumarylacetoacetase (GWHPABGR008261). GO enrichment analysis revealed that juvenile hormone esterase was enriched in carboxylic ester hydrolase activity and fumarylacetoacetase was enriched in L-phenylalanine catabolic process, in terms of the tyrosine catabolic process, fumarylacetoacetase activity and metal ion binding (Table 1). KEGG enrichment analysis indicated L-dopachrome tautomerase yellow-f2 was enriched in the tyrosine metabolism and fumarylacetoacetase in two pathways including tyrosine metabolism and styrene degradation (Table 2).

Ommochromes are tryptophan metabolites and some of the most common pigments localized in the eye and cuticle of insects, which range from yellow to brown, orange, or red (Oxford & Gillespie, 1998; Coates et al., 2005). A yellow coloured ommochrome-binding protein is secreted into the haemolymph during the late life of the larvae of *M. sexta*, which indicates its function in transporting the ommochromes pass through the haemolymph from their synthetic sites in the epidermis (Martel & Law, 1991, 1992). In *Drosophila melanogaster*, yellow-f2 has an essential role in melanisation and mutation of the *yellow* gene, which is involved in wing and cuticle pigmentation, results in a yellow-coloured cuticle rather than a black one (Han et al., 2002). In the present study, the higher expression levels of ommochrome-

Table 1. GO enrichment analysis of the candidate DEPs associated with larval colouration of different strains of *A. pernyi*.

Id	GO term	Category
GWHPABGR006793	Unknow	Unknow
GWHPABGR014261	Carboxylic ester hydrolase activity	Molecular function
GWHPABGR003184	Unknow	Unknow
GWHPABGR008874	Unknow	Unknow
GWHPABGR008261	L-phenylalanine catabolic process Tyrosine catabolic process	Biological process
	Fumarylacetoacetase activity Metal ion binding	Molecular function

Table 2. KEGG enrichment analysis of the candidate DEPs associated with larval colouration of different strains of *A. pernyi*.

Id	Pathway	Classification level 2	Classification level 1
GWHPABGR006793	Unknow	Unknow	Unknow
GWHPABGR014261	Unknow	Unknow	Unknow
GWHPABGR003184	Unknow	Unknow	Unknow
GWHPABGR008874	Tyrosine metabolism	Amino acid metabolism	Metabolism
GWHPABGR008261	Tyrosine metabolism Styrene degradation	Amino acid metabolism Xenobiotics biodegradation and metabolism	Metabolism

binding protein and yellow-f2 in the cuticle and haemolymph in yellow *A. pernyi* may cause the higher amount of the yellow coloured ommochrome, which may be an important factor determining the differences in body colour of the two strains. Juvenile hormone esterase is one of the key enzymes that degrade juvenile hormone regulating the overall black or green forms caused by differing environmental conditions in several kinds of larvae of Lepidoptera and adults of Orthoptera (Nijhout, 1999; Suzuki & Nijhout, 2006). Fumarylacetoacetase is a terminal rate limiting enzyme in the tyrosine catabolic pathway involved in the tanning and melanisation of the cuticle in insects. The difference in the level of expression of these two enzymes in the yellow and cyan strains of *A. pernyi* may indicate their potential roles contributing to the different colours of the two strains. In general, the results presented provide useful information for further investigations into mechanisms underlying the colouration of the larvae of *A. pernyi*.

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CONFLICT OF INTERESTS. The authors declare that there are no conflicts of interest.

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