Comparative Proteomics and Expression Analysis of Five Genes in *Epicauta chinensis* Larvae from the First to Fifth Instar

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**Abstract**

Blister beetle is an important insect model for both medicinal and pure research. Previous research has mainly focused on its biology and biochemistry, but very little data is yet available in the molecular biology. This study uses differential proteomics technology to analyze the soluble proteins extracted from each of the 5 instars larvae of *Epicauta chinensis*. 42 of the differentially-expressed proteins were identified successfully by MALDI-TOF/TOF-MS. Some of these proteins’ function and their expression profiles are analyzed. Our analysis revealed dynamics regulation of the following proteins: Axin-like protein pry-1 (APR-1), dihydrolipoyl dehydrogenase (DLD), vitellogenin (Vg) and lysozyme C (Lmz-S). APR-1 negatively regulates the Wnt signaling pathway. Its overexpression could result in embryo, leg, eye and ovary ectopica or malformation. DLD catalyzes the pyruvate into acetyl-CoA, the latter is the starting material of juvenile hormone (JH) and ipsdienol biosynthesis through the MVA pathway in insects. While Vg synthesis can be regulated by JH and stimulated by food factors. So DLD may affect the synthesis of JH, ipsdienol and Vg indirectly. The activity of lysozyme is an indicator of the immunity. Nutrition/food should be taken into account for its potential role during the development of larva in the future. Among the five genes and their corresponding proteins’ expression, only hsc70 gene showed a good correspondence with the protein level. This reflects the fluctuating relationship between mRNA and protein levels.

**Introduction**

The blister beetle *Epicauta chinensis* Laporte (Coleoptera: Meloidae) produces the active monoterpene substance called cantharidin. In clinical practice, cantharidin is utilized in curing cancer by restraining the growth of cancer cells. In recent years, cantharidin has also been found to function in killing pests and weeds, as an antiviral and antibiotic, and useful in plant protection [1–3]. Cantharidin exists in all developmental stages: egg, larva, pupa and adult. Therefore, the Meloidae are undergoing more research attention.

*Epicauta chinensis* L. occurs with one generation a year in the northern area of China. They are polyphagous insects with adults mainly feeding on fresh soybean and Lucerne (alfalfa) leaves. Larvae feed on locust eggs, such as *Locusta migratoria*, *Oxya chinensis*, etc. or on the larvae and provisions of soil- or wood-nesting bees [4,5]. This insect undergoes hypermetamorphosis. There are 6 larval instars in *Epicauta chinensis*. The 1st instar is a “triungulin” that is slender, active and well-sclerotized. At this stage they encounter their food source, then excavate and eat the grasshopper eggs. The 2nd to 4th instars are the “first grubs” and are less mobile. These are the primary feeding stages. The 5th instar is a pseudo pupa that does not eat and does not move, but enters diapause. This state can endure about half a year in the natural world; this affects both artificial breeding in the laboratory and our ability to conduct research in a timely manner. The 6th instar, called the “second grub”, is the non-feeding state just prior to pupation [4–6]. Such larval development with such marked differences is referred to as hypermetamorphosis, and is perhaps best known in the Meloidae. However, what actually happens within the 5th instar larval body and what factors cause them enter diapause deserves further investigation.

Proteomics is the large-scale study of gene expression at the protein level that provides direct measurement of protein expression levels and insights into the activity of all relevant proteins [7]. Two-dimensional gel electrophoresis (2-DE) separates proteins based on charge and molecular weight. When combined with mass spectrum (MS) and bioinformatics approaches, it provides a powerful tool for understanding complicated organismal processes at the protein level. The techniques have been used successfully in separating insect proteins [8–10]. This has expanded research on the fruit fly, mosquito, silkworm, honeybee, and cotton bollworm involving insect immune regulation, physiogenesis, behavior, etc. [11–15]. Only a few studies of blister beetle developmental proteomics have become available [16].

This work extends proteomic investigations to larvae growth and development. Comparative analysis of the larval proteome was performed on the 1st to 5th instars. Our main purposes were to
(1) identify the most variable proteins within the blister beetle larvae under protein extraction conditions; (2) link the protein variations with major stages of larval growth and development on the basis of their physiological role; and (3) attempt to determine the factors influencing the growth and development of larvae, such as feeding stimulating factors, diapause induction factors, molting factors, and regulating factors for some key metabolic pathways, etc.

Materials and Methods

1. Blister Beetle Larvae

_Epicauta chinensis_ L. were raised in our laboratory at Northwest Agriculture & Forestry University, Yangling, Shaanxi, China. Adults were captured in the field in Suide, Shaanxi Province, on July, 2011, and bred indoors. The larvae were reared in plastic cups and maintained under appropriate conditions (30.0±0.5°C, 10.0±1.0% soil humidity, LD = 16:8) in incubators. After eclosion, male and female adults were raised in cages. Larvae were reared on _Locusta migratoria_ eggs and adults were reared on leaves of _Medicago sativa_ (alfalfa).

2. Ethics Statement

The blister beetles were not the endangered or protected species in our country and in the world. And the places where _Epicauta chinensis_ were captured were all public areas of China. Therefore, no specific permissions were required for our field studies within these public locations.

3. Sample Preparation

Larvae for our proteomic and mRNA studies were collected from the soil in cups. Their surfaces were cleaned with 75% alcohol which was then absorbed on filter papers. Samples were grinded to be ultrafines using a mortar and pestle in liquid nitrogen. The majority of this powder was used to extract protein and the remainder was used for RNA extraction.

Powder from the larvae (1 mg larval powder/10 µl buffer) was mixed in Phosphate Buffer Solution (PBS) containing 32.5 mM K2HPO4, 2.6 mM KH2PO4, 400 mM NaCl, and a cocktail of protease inhibitors, and placed on ice for 15 min. It was then centrifuged at 12000×g, 4°C for 10 min, and further centrifuged at 15000×g, 4°C for 10 min. The supernatant was removed to another centrifuge tube for further use. The precipitate was mixed with PBS (1 mg larvae/5 µl buffer), then centrifuged at 15000×g, 4°C for 10 min. Supernatant was transferred to another tube and used as a PBS-soluble protein extract. The pellets containing insoluble-proteins were mixed with a lysis buffer (LB) (8 M Urea (SANLAND, Los Angeles, USA), 2 M Thiourea (AMRESCO, Solon, USA), 4% CHAPS (AMRESCO, Solon, USA), 20 mM Tris-base (AMRESCO, Solon, USA), 30 mM DTT (Merck, Darmstadt, Germany), and 2% Bio-lyte (Bio-Rad Hercules, CA, USA), pH 3–10), then homogenized for 2 min on ice and centrifuged at 15000×g, 4°C for 10 min. The supernatant was removed to the tube containing the PBS-soluble proteins extract, and the residual was discarded. Trichloroacetic acid (TCA, Alfa Aesar, Lancaster, USA) was added to the supernatants to reach 10% of the final concentration, and then kept on ice for 10 min for protein precipitation and desalting. This mixture was centrifuged at 15000×g, 4°C for 20 min. Supernatant was discarded and the precipitated protein was washed three times in ice-cold acetone containing 0.2% (wt/vol) DTT, with vigorous disruption by a plastic rod between each wash, and then air-dried. This was then redissolved in the LB containing a cocktail of protease inhibitors (Sigma, Santa Clara, USA) and further ground to help it dissolve.

This was brought to room temperature for 2 h, then centrifuged at 15000×g, 4°C for 30 min. The protein concentration was determined according to the method developed by Bradford [17]. This mixture containing the protein extracts of _Epicauta chinensis_ larvae was subpackaged and stored at −80°C.

4. Two-dimensional Gel Electrophoresis (2-DE)

A total of 300 µg of protein sample was loaded on 17-cm linear IPG strips at pH 5–8 (Bio-Rad Hercules, USA). The final volume of the loading sample was 350 µl. After active rehydration for 16 h, isoelectric focusing (IEF) was carried out at 20°C (Protein IEF Cell, Bio-Rad Hercules, USA) using the following program: step 1: 300 V, 2 h; step 2: gradient 300 V, 2 h; step 3: gradient 1000 V, 4 h; step 4: gradient 8500 V, 5 h; and step 5: steep and hold 8500 V until 70000 Vh. Next, the strips were equilibrated for two intervals of 15 min in an equilibration buffer containing 6 M urea, 50 mM Tris-HCl, 0.07% SDS (AMRESCO, Solon, USA), and 30% glycerol, at pH 7.6. For the first equilibration step, 1% DTT (wt/vol) was added to reduce cystine bridges. Thereafter the proteins were carbamidomethylated with 4% (wt/vol) iodoacetamide. The second dimension was carried out using 12.5% T SDS polyacrylamide gel (1.00 mm).

Meanwhile, 10 µl of 2-DE marker was loaded onto a piece of filter paper, and was transferred adjacent to the acid tip of the strip when the filter paper was nearly dry. The protein II Xi Cell (Bio-Rad Hercules, USA) was used to run the second dimension at 20°C in the following steps: 1 W, 45 min; 7 W, 6 h. The gels were then fixed in a solution of 40% methanol and 10% acetic acid for at least 4 h, used Silver Nitrate Staining method and de-stained in water for 12 h prior to scanning.

5. Gel Analysis

Gels were scanned by a transparency mode scanner connected to a PC system, at 32-bit red-green-blue colors and 400 dpi resolution for documentation. Images were analyzed using PDQuest vers.7.3.0 (Bio-Rad Hercules, USA). Spots were selected as being differentially-expressed if they showed a >1.5-fold change in spot density and an ANOVA score of <0.05.

6. Mass Spectrometry (MS)

For MALDI-TOF/TOF-MS analysis, protein spots were excised and digested [18]. The chosen protein spots were picked manually from the preparative gels using a 1.5-mm picking pen (The Gel Company, San Francisco, USA) and put in Eppendorfs respectively. The excised fragments were washed twice in distilled water, 10 min each time. Ultrasound decoloration used a 1:1 mix of 50 mM ammonium bicarbonate (NH4HCO3); acetonitrile (ACN) until the blue faded. ACN was added to make the granule become completely white. This was vacuum-dried for 10 min. Dehydrated gel plugs were bathed in water at 56°C with 10 mM DTT (compounded by 10 µl 1 M DTT, 990 µl 25 mM NH4HCO3). After cooling to room temperature, this was dried and immersed in 55 mM IAM (55 µl M IAM, 945 µl 25 mM NH4HCO3) in a dark room for 45 min. The samples were washed three times (25 mM NH4HCO3 (2×10 min), 25 mM NH4HCO3+50% ACN (2×10 min), ACN (10 min) and then freeze-dried for 10 min in a vacuum concentrator. The 0.1 µg/µl stock solution of enzyme was diluted 10–20 multiples using 25 mM NH4HCO3. Then we added 2–3 µl to each Eppendorf, centrifuged, and placed it on ice for 30 min. After the solution was absorbed by gel plugs, 25 mM NH4HCO3 was added to make the total volume 10–15 µl. This was transferred to 37°C for overnight digestion. 1% TFA was added until a final concentration of 0.1% stopped the action; this was shaken to help blending, centrifuged
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and saved for MS analysis. Each sample (CBB Staining spot, 1 μl; two Silver Staining spots, 5 μl) was applied to a target plate (Applied Biosystems, Foster City, USA) and mixed with 0.1 μl matrix (α-cyano-4-hydroxycinnamic acid in 70% ACN/0.1% TFA) using the dried droplet method [19]. Mass spectrometry was performed using a 4700 MALDI-TOF/TOF Proteomics Analyzer (Applied Biosystems, Foster City, USA). Proteins were identified in the NCBI nr database through peptide mass fingerprinting by using MASCOT (Matrix Science, London) [20,21].

All MS data were obtained using the 4000 Explorer software (Version 3.6; Applied Biosystems).

7. RNA Extraction and Real-Time PCR

Total RNA was extracted using RNAiso Plus (TaKaRa, USA) and the resulting RNA concentration was measured using Infinite M200 PRO (TECAN, Switzerland). The cDNAs were synthesized individually by reverse transcription using Prime Script RT Reagent Kit (TaKaRa, USA) in 20 μl reaction liquid following the recommended protocol provided by the manufacturer. The gene sequences were obtained from the undisclosed transcriptome data of Epicauta chinensis, sequenced by the Institute of Microbiology at the Chinese Academy of Science in Beijing. The sequences of five target genes and one reference gene have been submitted to GenBank and their accession numbers are: KF986611 (pc), KF986612 (hsa70), KF986613 (if44), KF986614 (eno), KF986615 (ugdh), JQ764814 (β-actin). Primer sequences (Table S1) were designed according to their nucleotide sequences using free online primer design tool Primer 3 (http://www.simgene.com/Primer3) or OligoArchitect Online V3.0 (http://www.oligoarchitect.com/Login.jsp). Primers were synthesized by AuGCT (Beijing, China). The PCR reaction was performed on an IQ 5 Optical Module (BioRad, USA). β-actin was used as a reference gene.

8. Data Analysis and Processing

Correlation-measured based distances and the UPGMA algorithm were used for the analysis. Protein spot variations were submitted to SPSS 19.0. One-way ANOVAs were used to test the expression differences among five different proteins and their corresponding genes in the five instars larvae. They are presented as mean +/- SEM and differences are considered significant at the P<0.05 level. Figures and tables are produced by GraphPad software.

Results

1. Proteomic Comparison of Epicauta chinensis Larvae During Instars 1–5

Two-dimensional electrophoresis(2-DE) gels of the five different developmental stages were compared and stained with silver nitrate as shown in Figure 1. In the first dimension, the pH range of IPG strips is from 5 to 8 with most of proteins located between pH 6.0 and pH 7.5. Along the second dimension, proteins mainly distributed within the range of 20–70 kDa according to molecular weight (MW). There are prominent differences in body morphology throughout larval growth from the 1st to 5th instar. The total number of detected spots varied according to the growth stage. In total, about 453 spots, 365 spots, 341 spots, 329 spots, and 314 protein spots were separately detected in the corresponding 1st, 2nd, 3rd, 4th, and 5th larval bodies. On the basis of average intensity ratios of protein spots from 1st to 5th instar, protein spots with a ratio higher than 1.8 or lower than 0.55 were identified as differentially-expressed. 42 proteins were picked and submitted to MALDI-TOF/TOF-MS analysis from these differentially-expressed spots.

2. MS Identification of Differentially-expressed Proteins

Forty-two spots were significantly variable (ANOVA) as detected using PDQuest software. They were excised from 2-DE gels and subjected to in-gel trypsin digestion and subsequent MALDI-TOF/TOF-MS identification. The predicted MWs and pHS for a proportion of the identified proteins were generally consistent with the experimental data, as judged from the location of spots on 2-DE gels. However, there were some exceptions. For instance, spots 15, 24, 29, 31, 33, 36, 37, 38, 40 and 42 (Table 1 and Figure 1) had an obvious molecular mass greater than the corresponding identified protein, while spots 2, 4, 5, 19 and 28 had a molecular weight lower than the predicted value. Beyond that, some of the spots (spots 10, 14, 23, 35) distributed in different positions in the same gel were identified as the same protein glycerol-3-phosphate dehydrogenase (GAPDH) or similar to GAPDH which reflects a shift in protein MW or pH. These deviations in molecular weight or pH, may be caused by a variety of factors, including protein degradation, post-translation modification, partial synthesis of proteins during blister beetles larval development, protein homologues that probably are unique for larvae, or protein translation from selectively spliced mRNAs [22].

Data obtained for these spots are presented in Table 1 and spot positions are illustrated on the gels in Figure 1.

3. Bioinformatics Analysis of Differentially-Expressed Spots by Functional Classes

The identified proteins were submitted to http://wego.genomics.org.cn/cgi-bin/wego/index.pl for GO (gene ontology) annotation. The 42 proteins were classified into three groups as shown in Figure 2, including cellular components, molecular function and biological process. At the level of cellular component, most of the differentially-expressed proteins were involved in cells and cell parts. Among biological processes, cellular and metabolic processes comprised the largest proportion. Molecular functions include antioxidant, binding, catalytic, molecular transducer, structural molecule, transcription regulator and transporter. The highest proportion of proteins fell into binding and catalytic functions. A few of them showed stage specificity, for instance, some proteins expressed mostly in the 4th instar mainly participated in metabolic processes while some proteins that reached their highest expression level in the 5th instar had a catalytic activity.

4. Dynamics of Protein Networks during 1st and 5th Instar

To summarize the information contained in Table 1 and to cluster the proteins showing similar expression profiles during the larval growth and development, hierarchical clustering was applied to the 42 identified spots (Figure 3a). Spots were classified according to their pattern of volume variation from the 1st to 5th instar using the unweighted pair group method with the arithmetic method (UPGMA). The 42 spots were clustered into two groups. Cluster I was composed of 25 proteins whose overall abundance increased during larval growth. The first subcluster (from spots 35–38) comprised 10 spots. Among them, there were 8 spots (from 35 to 36) whose abundance increased from the 1st to 2nd instar, decreased in the 3rd and 4th and then increased dramatically in the 5th. The other two spots (spot 12 and 38) had a protein expression index that kept increasing and reached the highest level in the 5th instar. A second subcluster (from spots 6–15) comprised 6 spots whose abundance increased in the 2nd rather than 1st instar, then...
decreased in the 3rd, and declined to the lowest abundance in the 5th instar. The cluster I also included 9 spots (from 4 to 11) whose trend of protein expression increased from the 1st to 3rd instar, decreased at the 4th but increased again at the 5th instar, and had the largest expression amount at the 3rd instar.

Cluster II was comprised of 17 spots. Seven (spots 34–21) showed a higher level of abundance in the 1st instar, whereas the rest of the ten protein spots (spots 8–37) reached their maximal abundance in the 4th instar. Among these spots, it is worth noting that the important material of egg and larva growth - vitellogenin (spot 42, Vg), it was considerably expressed in the first, second and third instars, reaching a peak at the fourth instar, then decreased in the fifth instar.

As shown by the transposed cluster tree (Figure 3b), the age effect is the primary factor explaining spot variations. The five stages were clustered into three sub clusters which corresponded to 1st instar (three-jaw type), 2nd–3rd instars (melolonthoid) and 4th–5th instars (melolonthoid and pseudo pupa).

5. Translation and Transcription Level Expression

To assess the correspondence between protein expression abundance and transcriptional activity, 5 genes were selected for RT-PCR analysis (Figure 4b). The data showed that only one gene hsc70 was in full accord with what registered at protein level (Figure 4a). This extent of change was very significant, a change of up to 37-fold in hsc70 mRNA level only resulted in a 1.6-fold increase in hsc70 protein level in the 2nd over 1st instar. In some cases, the relationship between mRNA abundance and protein functionality is relatively straightforward, with apparent up-regulation of pc (propionyl-CoA carboxylase) and pc gene both in the 2nd and 5th instars. But there might be a discrepancy in, e.g. eIF4A (eukaryotic initiation factor 4AII), its expression in the mRNA level kept nearly in 1st, 3rd and 4th instars, while it down-regulated distinctly in the corresponding stages at the protein level; enolase (eno) down-regulated in the 4th instar at mRNA level yet enolase maintained a continuous increase from 1st to 4th instars at protein level. In addition, ugdh gene down-regulated in the 5th instar whereas its protein up-regulated obviously.

Discussion

1. Function Analysis of Some Differentially-expressed Proteins

In this study, we employed comparative proteomics and successfully identified 42 spots from the blister beetle larvae by using MALDI-TOF/TOF-MS. Most of them were found to be
Table 1. List of proteins identified from the 1st to 5th instar larvae of *Epicauta chinensis*.

| Spot no. | Protein name | Species | Protein ID | Accession no. | Database | Theoretical (pI/Mr)(kDa) | Calculated(2-D) (pI/Mr)(kDa) | Protein score | Sequence Coverage (%) |
|----------|--------------|---------|------------|---------------|----------|--------------------------|-----------------------------|---------------|----------------------|
| 1        | eukaryotic initiation factor 4AII | *Homo sapiens* | gi|485388 | BA063336 | NCBInr | 5.3/46.6 | 7.3/42.5 | 92 | 23 |
| 2        | actin homologue | *Ostrinia scapulalis* | gi|315433391 | BAJ9799 | NCBInr | 5.3/30.7 | 5.7/55.0 | 78 | 45 |
| 3        | G24228 | *Drosophila mojavensis* | gi|195108671 | XP_001998916 | NCBInr | 5.9/57.1 | 6.0/65.9 | 47 | 14 |
| 4        | PREDICTED: lysozyme C, spleen isozyme-like | *Cavia porcellus* | gi|348580423 | XP_004347978 | NCBInr | 10.6/20.6 | 6.0/59.2 | 53 | 33 |
| 5        | myosin heavy chain | *Pennatia argentata* | gi|9071579 | BAB12571 | NCBInr | 5.3/22.2 | 6.9/57.6 | 74 | 20 |
| 6        | PREDICTED: similar to voltage-dependent anion-selective channel isoform 2 | *Tribolium castaneum* | gi|91086623 | XP_976150 | NCBInr | 8.6/30.4 | 6.1/26.6 | 157 | 9 |
| 7        | Heat shock 70 kDa protein cognate 5 | *Harpegnothos saltator* | gi|307211659 | EFN87680 | NCBInr | 6.4/75.0 | 6.1/82.9 | 239 | 15 |
| 8        | PREDICTED: adenosyl homocysteinase | *Macaca mulatta* | gi|297259974 | XP_001104495 | NCBInr | 6.5/49.0 | 6.8/40.9 | 212 | 27 |
| 9        | Enolase | *Aedes aegypti* | gi|157121051 | XP_001653750 | NCBInr | 6.7/46.9 | 6.6/45.8 | 322 | 22 |
| 10       | PREDICTED: similar to glycerol-3-phosphate dehydrogenase | *Tribolium castaneum* | gi|91076880 | XP_975007 | NCBInr | 6.8/39.5 | 7.0/37 | 141 | 34 |
| 11       | Peroxiredoxin 1 | *Camponotus floridanus* | gi|307175821 | EFN65636 | NCBInr | 6.3/21.9 | 7.1/21.1 | 162 | 26 |
| 12       | UDP-glucose/GDP-mannose dehydrogenase family protein | *Loa loa* | gi|312076607 | XP_003140937 | NCBInr | 6.3/52.9 | 7.1/61.2 | 58 | 22 |
| 13       | beta-tubulin | *Bombyx mori* | gi|112983318 | O17449 | NCBInr | 4.8/50.6 | 6.5/46.9 | 182 | 48 |
| 14       | glyceraldehyde-3-phosphate dehydrogenase | *Pyrrhogyra crameri* | gi|269117589 | XP_001599507 | NCBInr | 6.5/24.3 | 7.0/23.3 | 115 | 26 |
| 15       | PREDICTED: similar to Myosin heavy chain CG17927-PF isoform 5 | *Tribolium castaneum* | gi|189239931 | XP001813815 | NCBInr | 5.8/224.7 | 5.5/84.9 | 152 | 15 |
| 16       | kinesin, putative | *Trypanosoma vivax* | gi|340055502 | CC49821 | NCBInr | 5.4/110.8 | 6.0/97.7 | 49 | 6 |
| 17       | NtpA | *Tribolium castaneum* | gi|270006640 | EFA02908 | NCBInr | 4.9/68.4 | 5.8/70.0 | 300 | 14 |
| 18       | elongation factor 2 (eEF-2) | *Tasmanophilus spinatus* | gi|34597242 | AAQ77196 | NCBInr | 5.8/79.5 | 5.8/78.9 | 83 | 31 |
| 19       | hypothetical protein TcasGA2_TCO10240 | *Tribolium castaneum* | gi|270016170 | EFA12618 | NCBInr | 7.2 /27.7 | 6.0/72.0 | 96 | 17 |
| 20       | PREDICTED: dihydrolipoyl dehydrogenase, mitochondrial-like | *Nasonia vitripennis* | gi|3454946470 | XP_001602610 | NCBInr | 8.4/56.5 | 5.3/55.0 | 86 | 22 |
| 21       | putative actin | *Diapharina citri* | gi|110456520 | ABG74719 | NCBInr | 5.5/24.7 | 7.1/21.8 | 154 | 62 |
| Spot no. | Protein name | Species | Protein ID | Accession no. | Database | Theoretical (pI/Mr) (kDa) | Calculated (2-D) (pI/Mr) (kDa) | Protein score | Sequence coverage (%) |
|---|---|---|---|---|---|---|---|---|---|
| 24 | Dihydrolipoyl dehydrogenase, mitochondrial (E1 component) | Camponotus floridanus | gi|307919323 | EN77426 | NCBInr | 5.9/55.4 | 6.3/55.4 | 119 |
| 25 | glyceraldehyde 3-phosphate dehydrogenase | Gadus morhua | gi|25989189 | AAL05892 | NCBInr | 6.9/36.2 | 6.3/54.5 | 25 |
| 26 | PREDICTED: dynein heavy chain 5, axonemal-like | Meleagris gallopavo | gi|326917138 | XP_003204868 | NCBInr | 5.9/55.4 | 6.3/55.4 | 119 |
| 27 | serum albumin | Bos indicus | gi|30794280 | NP_851335 | NCBInr | 6.1/71.3 | 6.3/55.4 | 119 |
| 28 | PREDICTED: mitochondrial import receptor subunit TOM22 homolog | Bombus impatiens | gi|350411102 | XP_003489240 | NCBInr | 4.6/27.1 | 6.3/55.4 | 119 |
| 29 | PREDICTED: cytoplasmic dynein 1 heavy chain 1 | Oryctolagus cuniculus | gi|108744087 | XP_002721533 | NCBInr | 6.0/55.4 | 6.3/55.4 | 119 |
| 30 | hypothetical protein | Tribolium castaneum | gi|270006944 | EF0A992 | NCBInr | 7.2/30.3 | 6.3/55.4 | 119 |
| 31 | scyllo-inositol phosphate 3-kinase | Drosophila melanogaster | gi|148095651 | COJ00682 | NCBInr | 5.8/27.1 | 6.3/55.4 | 119 |
| 32 | PREDICTED: similar to glycerol-3-phosphate dehydrogenase | Tribolium castaneum | gi|91070880 | XP_003570077 | NCBInr | 6.8/39.5 | 6.3/55.4 | 119 |
| 33 | unnamed protein product | Oikopleura dioica | gi|313228938 | CBY19090 | NCBInr | 5.7/25.8 | 6.3/55.4 | 119 |
| 34 | PREDICTED: similar to carboxylase: pyruvate/acetacetyl-CoA | Tribolium castaneum | gi|91080283 | EF0A992 | NCBInr | 6.9/25.8 | 6.3/55.4 | 119 |
| 35 | Protein Y71G12B.11, isoform a | Caenorhabditis elegans | gi|251403518 | NP_490886 | NCBInr | 5.8/25.8 | 6.3/55.4 | 119 |
| 36 | stat3 | Bubalus bubalis | gi|108744087 | XP_002721533 | NCBInr | 6.0/55.4 | 6.3/55.4 | 119 |
| 37 | focal adhesion kinase | Drosophila melanogaster | gi|148098551 | COJ00682 | NCBInr | 5.8/25.8 | 6.3/55.4 | 119 |
| 38 | vitellogenin | Spodoptera litura | gi|156481320 | ABU68426 | NCBInr | 6.6/49.3 | 6.3/55.4 | 119 |

**Table 1.** Cont.

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related with the processes of substance and energy metabolism, nutrient digestion and absorption, and innate immunity.

**Proteins Relevant to Substance and Energy Metabolism.** We found thirteen proteins were related to the metabolism of carbohydrate and energy production involved in glycolysis (spots 9, 10, 14, 23, 30, and 35), synthesis of fatty acids (spot 37), metabolic processes of fructose and mannose (spot 12), the synthesis of hub substances for energy and substance metabolism and processes of glycolysis and the citric acid cycle (spot 22), and ATP generation (spots 5, 8, 16 and 40).

GAPDH transforms 3-glyceraldehyde phosphate into 1,3-diphosphoglycerate in the glycolysis process. It acts as reversible metabolic switch under oxidative stress [23]. In addition, it has other physiological functions, such as initiating apoptosis [24], membrane fusion, vesicle trafficking, a chaperone of phosphotransferase, DNA repairing and transcriptional regulation [25].

Enolase is known to be a multifunctional protein, e.g. as a glycolytic enzyme, plasminogen-binding protein and heat-shock protein [26,27], participates in the regulation and control of transcription, apoptosis and cell differentiation [28–30]. Enolase functions as a virulence agent in early egg establishment at *Aphidius ervi* [31]. Its expression level increased gradually from 1st to 4th instar, then decreased back to near the level of the 1st instar when they were the 5th instar larvae. This may be associated with nutrition because the 1st and 5th instar larvae do not feed, while the 2nd, 3rd and 4th instars are the major feeding stages.

Dihydrolipoyl dehydrogenase (DLD), also known as dihydrolipoamide dehydrogenase is the compound enzyme which catalyzes pyruvate into acetyl-CoA. It is a type of pivotal carbohydrate during energy metabolism, e.g. the citric acid (TCA) cycle and mevalonic acid (MVA) pathway. The Juvenile hormones (JHs) which are a group of acyclic sesquiterpenoids can regulate development, reproduction, diapause, and polyphenisms of insect [32,33]. Its biosynthetic pathway in insects is divided into two main components: the early steps, acetyl-CoA up to farnesyl dipiphosphate (FPP), belong to the MVA pathway while the later steps effect the conversion of FPP into JH [34]. Besides, the male pine engraver *Ips pini* contains one type of monoterpenoid pheromone called ipsdieneol. It is synthesized in the males’ midgut by the MVA pathway [35]. As the starting material of the MVA pathway, acetyl-CoA is first catalyzed to generate the C5 isopentenyl dipiphosphate (IPP), which then undergoes two successive condensations to generate FPP [36]. In the case of enough precursor pyruvate, the activity of the catalyzing enzyme DLD determines acetyl-CoA’s production, thus influences the content of JH and ipsdieneol indirectly.

**Differentially-expressed Proteins Involved in Digestion and Absorption of Nutrients.** In this study, four proteins which were identified as Myosin heavy chain CG17927-PF isoform 5 (spot 15), axonemal-like (spot 24), Cytoplasmic Dynein 1 heavy chain 1 (spot 29) and Tropomyosin-1-like isoform 1 (spot 34) may function in digestion and absorption of nutrients (Figure 1 and Table 1). These proteins are related to the actin filament through driving myosin molecules to move along the actin filaments [37], stabilizing actin filaments [38] and inhibiting actin filament formation [39]. We found that the Myosin heavy chain CG17927-PF isoform 5 and Dynein Heavy chain 5 had the highest expression in the 2nd instar and then decreased gradually at later stages. Cytoplasmic Dynein 1 heavy chain 1 expressed the highest amount in the first instar, it then decreased gradually. The decreased expression of the Myosin heavy chain causes the destabilization of the apical brush border membrane; this will result in increased sensitivity to oral infection by bacterial pathogens [40]. While Tropomyosin-1-like isoform 1 expressed the highest amount in the first instar, it then decreased gradually. The decreased expression of Tropomyosin-1-like isoform 1 inhibits the formation of the actin filament and thereby
Proteomic Profiles of Epicauta chinensis Larvae

(a) Heatmap and dendrogram showing the proteomic profiles of Epicauta chinensis larvae.

(b) Heatmap showing the expression levels of different proteins in Epicauta chinensis larvae.

Legend:
- GAPDH
- beta-tubulin
- myosin heavy chain
- hypothetical protein
- unnamed protein product
- Protein Y71G12B.11, isoform
- anion-selective channel isoform
- PREDICTED: dynemin heavy chain
- Axin-like protein
- try 10
- Hsc 70
- Myosin heavy chain CG17927-PF isoform
- lysozyme C
- DLD
- cytoplasmic dynein 1 heavy chain 1
- serum albumin precursor
- NtpA
- Stat 3
- enolase
- Mitochondrial receptor subunit TOM 22
- Peroxiredoxin 1
- tropomyosin-1-like isoform
- DLD
- splicing factor 3a
- hypothetical protein TcasGA2_TC010240
- actin homologue
- eEF-2
- Actin homologue
- vitellogenin
- PC

Matrix of correlation coefficients:

- Correlation coefficient matrix showing the correlation between different proteins.

Cluster analysis:

- Cluster I
- Cluster II

Scale:
- Heatmap intensity scale from green to red.
weakens the contraction ability of the smooth muscles in the blister beetle midgut.

Proteins Related to Blister Beetle Larval Innate Immune System. Hsc70 apparently down-regulated in the fourth and fifth instars. It is in the HSP family. HSPs are produced as a protective response by cells to a variety of stress factors. The major physiological functions of the Hsp70 superfamily’s members are protein folding, unfolding and translocation [41]. Hsp70s are the most conservative and important subfamily and they are the most sensitive in responding to stress. They could protect cells from stimulation and injury as well as promote the repair of damaged cells as well as have anti-inflammatory and anti-apoptotic effects. They also provide attractive targets for immune responses towards pathogens [42,43]. Expression levels of Hsc70 are positively correlated to the level of tissue cells resistance to damage and their ability to protect themselves.

Vitellogenin (Vg) is the key component of insect eggs and is the main source of nutrition for early development of eggs and larvae. It has the function of carrying and transporting fat as well as having immunological properties [44]. Vg is produced in the fat body of insects [45,46]. Its synthesis could be stimulated by food factors [47]. For some species of the investigated insects, e.g. Drosophila melanogaster, Locusta migratoria, it has been documented that JH stimulates the transcription of the vitellogenin genes and the consequent control of vitellogenin production [48,49]. However, in some other insects such as Helicoverpa zea, Lymantria dispar, honeybee and so on, JH is proposed to act as a repressor of Vg synthesis [50–52]. A low JH titre in honey bee workers permits the onset accumulation of Vg in haemolymph, whereas high JH levels turn off Vg synthesis [53]. The Drosophila melanogaster and Locusta migratoria are attached to Diptera and Orthoptera respectively. While Helicoverpa zea and Lymantria dispar are members of Lepidoptera and honeybee belongs to Hymenoptera. The promotion and repression impact on Vg brought by JH may attribute to the different biosynthesis process of Vg and JH in different insects, or different insects’ diverse physiological processes or functions.

Lysozyme C (Lmz-S) is a type of alkaline enzyme which can hydrolyze mucoitin in pathogenic bacteria. It acts on the extraneous antigen that then makes it dissolve or decompose. This enzyme has anti-bacterial, anti-inflammatory and anti-viral activities and is also a cold adapted protein [54], so it has a role in defense and immunity. Lmz-S exhibits considerable catalytic activity at low temperatures and low activation energies [55]. Lysozyme activity can be detected by the resonance scattering spectra of the micrococcus method, lysodeikticus turbidimetric and colorimetric methods, etc. to assay the level of immunity [56]. The activity of Lysozyme develops in haemolymph of immature stages.

Figure 3. Hierarchical clustering analysis of the 42 identified spots. (a) spots listed in Table 1 were clustered according to their percentage of volume from 1st to 5th instar by the method of UPGMA. The spot number is indicated on the right of the heat plot, the protein name is on the left side. Two main clusters were formed. (b) Cluster according to the stages of larvae growth. Numbers “1”, “2”, “3”, “4”, “5” are used instead of the 1st, 2nd, 3rd, 4th and 5th instar. The five instars were clustered into three groups.

doi:10.1371/journal.pone.0089607.g003

Figure 4. Expression pattern at protein and mRNA level. (a) Differential expression profiles of the 5 identified proteins. The plots show the mean standardized log abundance derived from the three replicated gels (n = 3) among 1st, 2nd, 3rd, 4th and 5th instars larvae. Different letters indicate when the expression quantities were significantly different. Master spot number and abbreviation of the proteins’ names are indicated on top of each graph. (b) Gene expression patterns. Gene expression of candidate genes in blister beetles larvae of 1st, 2nd, 3rd, 4th and 5th instars, respectively. Expression levels are given relative to the 5th instar. Significant differences are indicated by different letters. ** The numbers 1, 2, 3, 4, 5 represent the 1st, 2nd, 3rd, 4th and 5th instar respectively.

doi:10.1371/journal.pone.0089607.g004
of *Bombyx mori* and *Galleria mellonella* after injection with microorganisms but not after saline injection [57].

It has been reported that dietary influences the immunity of an organism [50]. The immunity is stronger after feeding compared with those that have not fed [59]. And a dietary with supplementation improved the immunity of *Lambdocos terreris* [60]. So we conjecture that this may be relevant with blister beetles’ feeding. The sampled 1st instar larvae of blister beetles have not fed, so those immune system proteins’ levels are the lowest and their immunity is the lowest. The second and third instars have a longer feeding period thus promoting the expression levels significantly. The fourth stage larvae eat less, while the fifth stage larvae do not feed at all. Therefore most are down-regulated in the 4th and 5th instars to some extent, but still express higher than in the 1st instar.

**Proteins Related to Egg Development and Adult Limb Formation.** Axin-like protein pry-1 (APR-1) is a typical negative regulator of the Wnt/wingless signaling pathway and it regulates the stability of the Wnt/wingless pathway effector beta-catenin [61]. *Tribolium castaneum* female adults injected with *Tc-axin* dsRNA produced progeny phenotypes that ranged from mildly affected embryos with cuticles displaying a graded loss of anterior structures, to defective embryos that condensed at the posterior pole of the abdominal in the absence of serosa [62]. Ectopic expression of axin induces notches in the wing, generation of a supernumerary leg from the ventral side of the normal leg and loss of the sternite structure in the abdomen. Furthermore, overexpression of axin results in eye and ovary ectopia in *Drosophila melanogaster* [63].

**2. Gene-Nutrient/Food Interaction**

Many genes’ expression depends on the environment in which they survive and involves the food they take in. A nutrient is regarded as a vital environmental factor and there are extensive interactions between nutrients and genes [64,65]. In this study, we found that as one type of nutrient formed, diet could alter the expression of proteins related to carbohydrates and energy metabolism, immunity, digestion and absorption of nutrients. For example, lysozyme C, adenosyl homocysteinase, dihydrodipoyl dehydrogenase, mitochondrial import receptor subunit TOM 22 homolog, hypothetical protein, and vitellogenin may be affected by nutrient factors. Mitochondrial import receptor subunit TOM 22 homolog is the outer mitochondrial membrane transporter enzyme. Its function is to help the protein across the mitochondrial outer membrane, mediate the signal peptide of mitochondrial protein into the cavity between the mitochondrial membrane and help the proteins which have penetrated into the mitochondrial outer membrane. This protein spot has a lower expression in the 1st instar, but it accelerates suddenly in the 2nd instar after feeding. We infer that the activity of this outer mitochondrial membrane transporter enzyme is possibly connected with stimulation from food factors. As a consequence, nutrients affect growth and development as well as the resistance to pathogens in blister beetles.

**3. Comparation at Protein and mRNA Level**

According to comparative results of the five proteins and corresponding five genes expression profiles, we found there were discrepancies between the protein and mRNA levels. It may be attributed to the following reasons. First, the process of mRNA translating to proteins is downstream of gene expression and this process may generate variability e.g. post-transcriptional modification and regulation [66]. Second, when gene expression down-regulates but protein expression up-regulates, the gene has not been positively-regulated at the mRNA level, and there is a constitutively-expressed protein regulated by an intracellular activator that becomes the active form. Beyond that, the instability of mRNA is an important source of randomness in gene expression, especially when mRNA levels are low. Therefore a direct relationship between protein and mRNA levels cannot always be assumed or expected [67,60].

**4. Cantharidin Biosynthesis Pathway**

The same as JH and ipsdienol, cantharidin is also a kind of terpenoid. There are two pathways: the MVA pathway and the non-MVA pathway in which terpenoid biosynthesis in organism have been discovered by scientists. So far, the non-MVA pathway is mainly found in plants, protists and microorganisms [69]. While the MVA pathway exists in many viruses and in all higher eukaryotes including insects [35,70]. Researchers have not found the non-MVA pathway in animals up to now.

Because of cantharidin consists of 10 C atoms, at first it was thought synthesized by acetate through the polyketide (PK) pathway or MVA pathway, with the latter more likely. However, Guenther et al. [71] verified that cantharidin could not be synthesized by acetate through degradation experiment. McCormick, et al. [72] have demonstrated that cantharidin is produced by degradation of the farnesol carbon skeleton and suggested the possibility of cantharidin as a juvenile hormone metabolite by tracing the source of oxygen atoms in cantharidin using the isotope-labeling method. Peter et al. [73] also discovered farnesol takes part in cantharidin biosynthesis in beetles. Schlatter et al. [74] found all of the H atoms in cantharidin come from farnesol directly and derive from mevalonate except for the H atom of C6. The process, from acetyl-CoA to farnesyl diphasphate (FPP), belongs to the MVA pathway. A soluble form of phosphatase is capable of converting FPP into farnesol [75].

Though some kinds of terpenoids are synthesized by MVA pathway, however, due to they have different chemical construction and are attached to different orders and families of insects, so whether cantharidin is indeed biosynthesized by the MVA pathway in blister beetles, there is no definite evidence that can prove it so far.

**Conclusion**

In this work, we employed a comparative proteomic approach to investigate the proteomic differences of *Epicauta chinensis* larvae from first to fifth instar. 2-DE profiles showed variations in some spots’ abundance. 42 differentially-expressed proteins were identified successfully by MALDI-TOF/TOF. The identified differentially-expressed proteins Vg, DLD and lysozyme C take important roles in blister beetle larval development. Vg supplies nutrients for eggs and larvae growth and its biosynthesis can be regulated by JH and stimulated by food factors. DLD catalyzes the pyruvate into acetyl-CoA then further affects biosynthesis of JH, ipsdienol and Vg. Lysozyme activity is an indicator of the immunity. GO function and cluster correlation analysis results showed the differential proteins’ expression had stage characteristics. The identified proteins expressing higher in the 4th and 5th instars mainly participate in the metabolism of basic carbohydrates. The first and fifth instars are two stages which larvae do not feed. While the 2nd, 3rd and 4th instars are the active feeding stages, proteins responsible for their immune system, the nervous system and regulation of cell growth and development are active. We suggest that the proteins’ expression is related to the stimulation of food to some extent. In this study, only the heat shock family protein (hsc70) shows a good correspondence. There is no direct relationship between protein and gene expression. In general, further studies such as genomic function identification, systematic
Acknowledgments
We thank Xianchun Li (University of Arizona, USA) for his help in bioinformatics of the proteins, and Mr. Hongbing Li (Northwest A&F University, China) for his help with the 2-DE experiment and protein analysis using MALDI-TOF/TOF-MS. Thanks also to Ming Jiang for collecting blister beetles in the field and Hainan Shao, and Zhengwei Wu for their technical work in the experiment. We also give our sincere thanks to John Richard Schock (Emporia State University, USA) for proofreading our manuscript.

Author Contributions
Conceived and designed the experiments: QL DW SL YZ. Performed the experiments: QL. Analyzed the data: QL. Contributed reagents/materials/analysis tools: QL SL. Wrote the paper: QL YZ.

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