Published in final edited form as:

J Proteome Res. 2015 May 1; 14(5): 2331–2347. doi:10.1021/acs.jproteome.5b00159.

Developmental Changes for the Hemolymph Metabolome of Silkworm (*Bombyx mori* L.)

Lihong Zhou^{†,‡,¶}, Huihui Li[‡], Fuhua Hao[‡], Ning Li[‡], Xin Liu[†], Guoliang Wang[¶], Yulan Wang^{‡,⊥}, and Huiru Tang^{*,§,‡}

[†]College of Life Science and Technology, Huazhong University of Science and Technology, Wuhan 430074, China

[‡]Key Laboratory of Magnetic Resonance in Biological Systems, State Key Laboratory of Magnetic Resonance and Atomic and Molecular Physics, National Center for Magnetic Resonance in Wuhan, Wuhan Institute of Physics and Mathematics, Chinese Academy of Sciences, Wuhan 430071, China

[§]State Key Laboratory of Genetic Engineering, Collaborative Innovation Center for Genetics and Development, Metabonomics and Systems Biology Laboratory, School of Life Sciences, Fudan University, Shanghai 200433, China

[⊥]Collaborative Innovation Center for Diagnosis and Treatment of Infectious Diseases, Zhejiang University, Hangzhou 310058, China

[¶]College of Life Sciences, Jiangnan University, Wuhan 430056, China

Abstract

Silkworm (*Bombyx mori*) is a lepidopteran-holometabolic model organism. To understand its developmental biochemistry, we characterized the larval hemolymph metabolome from the third instar to prepupa stage using ¹H NMR spectroscopy whilst hemolymph fatty acid composition using GC-FID/MS. We unambiguously assigned more than 60 metabolites, among which tyrosine-*o*- β -glucuronide, mesaconate, homocarnosine, and picolinate were reported for the first time from the silkworm hemolymph. Phosphorylcholine was the most abundant metabolite in all developmental stages with exception for the periods before the third and fourth molting. We also found obvious developmental dependence for the hemolymph metabolome involving multiple pathways including protein biosyntheses, glycolysis, TCA cycle, the metabolisms of choline amino acids, fatty acids, purines, and pyrimidines. Most hemolymph amino acids had two elevations during the feeding period of the fourth instar and prepupa stage. Trehalose was the major blood sugar before day 8 of the fifth instar, whereas glucose became the major blood sugar after spinning. C16:0, C18:0 and its unsaturated forms were dominant fatty acids in hemolymph.

*Corresponding Author huiru_tang@fudan.edu.cn. Phone: +86-(0)21-51630725..

The authors declare no competing financial interest.

Supporting Information

Developmental stages for silkworms. Developmental changes of silkworm hemolymph pH values. PCA scores plots from the ¹H NMR data of silkworm hemolymph at different developmental stages. OPLS-DA results from the ¹H NMR data of silkworm hemolymph at different developmental stages. The levels of silkworm hemolymph metabolites at different developmental stages derived from their ¹H NMR signal integrals. This material is available free of charge via the Internet at <http://pubs.acs.org>.

The developmental changes of hemolymph metabonome were associated with dietary nutrient intakes, biosyntheses of cell membrane, pigments, proteins, and energy metabolism. These findings offered essential biochemistry information in terms of the dynamic metabolic changes during silkworm development.

Keywords

silkworm hemolymph; development; metabonomics; pigments; protein biosynthesis

INTRODUCTION

Silkworm (*Bombyx mori*) has been economically important with its essential roles in silk production¹ for at least 5000 years in China.² This insect can also be used to produce recombinant proteins like proteinaceous drugs and as a source of biomaterials.³ In addition, the silkworm has now become an insect model, second only to the fruitfly (*Drosophila melanogaster*),⁴ for developmental and genetic studies with their ease of rearing, mutant availabilities with about 400 visible phenotypes,⁵ and data on their biology.⁶ Consequently, many studies have been carried out on the growth process of the silkworm.

The entire life cycle of *B. mori* spans through about 45–55 days with four distinctive developmental stages, namely egg, larva, pupa, and adult stages (Figure S1), among which larval stage is by far the longest.⁷ Normally, eggs take about 10–14 days to hatch into larvae (in the form of black caterpillars), which eat mulberry leaves almost constantly for 4–6 weeks until pupation. Most strains of larvae go through the five instar phases intervened by four times of ecdysis, during which they stop eating. When larvae are in mature stage around day 8–10 of the fifth instar, they stop eating, and their bodies become slightly yellow with their skin becoming tighter. They then enclose themselves in a cocoon spun from raw silk protein produced by their salivary glands in a process that takes two or more days, during which larvae are also considered as being in the prepupa stage. *B. mori* then turns into a brown-shelled pupa and into an adult moth within about 3 weeks. The moth then reproduces and dies within about 5 days with the female normally laying 200–500 yellowish eggs that will eventually turn black.

The silkworm is now regarded as one of the best characterized models for biochemical, molecular biology, and genomic studies of Lepidopteran insects.^{8,9} Since many important physiological processes of insects are conserved through evolution, further detailed studies

of silkworms will further benefit elucidation of gene function and understandings of insect endocrinology, reproduction, immunity, and domestication. This insect is also a potentially good model for developmental biology, environmental exposomic effects,¹⁰ and drug developments.^{11,12} With the completion of its genome sequencing,^{13,14} systems approaches have been employed to understand the developmental biology of this insect.

The first silkworm transcriptome study has been reported using high-throughput RNA sequencing technology with a database constructed for the integration of the silkworm transcriptome and genome data.¹⁵ The results have indicated that the silkworm transcriptome is much more complex than previously anticipated.¹⁵ A large-scale gene screening revealed that 106 miRNAs were expressed in all stages, whereas 248 miRNAs were egg- and pupa-specific, which indicated the significant roles that insect miRNAs played in embryogenesis and metamorphosis.¹⁶ Most silkworm miRNAs were found to be conserved in insects with a small number of silkworm miRNAs having orthologs in mammals and nematodes.¹⁶

Proteomics results showed that silkworm protein expressions varied with the insect's developmental processes. About 241 protein spots were detected in larva hemolymph on day 1 of the fifth instar with three-fourths of them having the molecular mass of 35–90 kDa. In contrast, about 300 protein spots were observed in larva hemolymph on day 7 of the fifth instar including 57 newly expressed spots.¹⁷ These proteins were related to silk protein biosynthesis or enhanced biosynthesis of carbohydrate and fatty acids for the larva-to-pupa metamorphosis.¹⁷ Marked hemolymph proteomic changes were also observed for silkworm developmental process with 34 identified proteins involved in metamorphosis, metabolisms, programmed cell death, nutrient storage, and transportations.

During days 4–5 of the fifth instar, the silkworm larvae were fast-growing with outstanding changes observed for a group of 30 kDa proteins in hemolymph, four of which were obviously up-regulated.¹⁸ One-hundred and eight hemolymph proteins were identified with cellular functions including development, metabolism, nutrient transportation, and defense responses.¹⁸ Furthermore, applications of these techniques have now been extended to molecular aspects of silkworm phenotypes. For instance, both proteomics and transcriptomics techniques were employed to investigate the mechanistic aspects of the low fibroin production for the ZB silkworm strain.¹⁹ Significant enhancements were observed in this strain for proteasome pathway, glycolysis/gluconeogenesis, and TCA cycle, indicating the enhanced protein degradation and energy metabolism.¹⁹

Silkworms have an open circulatory system with hemolymph consisting of blood and tissue fluids. Hemolymph is considered as a depository of nutrients, energy, and metabolic intermediates for all organs and cells of this insect.^{7,20} It is conceivable that the metabolite composition (metabonome) of silkworm hemolymph varies with the silkworm development and growth as in the case of hemolymph proteome.^{17,18,20} Characterizing the developmental dependence of metabonomic features for silkworm hemolymph is therefore of great importance for understanding the silkworm metabolism and for investigating its responses to environmental changes.

A number of classical studies reported some metabolic information for the silkworm hemolymph. Silkworm hemolymph had pH value of 6.45–6.57 and contained various amino acids and sugars, whose levels were developmentally dependent.²¹ The major sugar in larval hemolymph was trehalose,²² which was synthesized together with glycogen in the silkworm fat bodies by utilizing sucrose, glucose, fructose, maltose, cellobiose, and sorbitol from food intakes.^{23,24} The concentration of hemolymph trehalose was as high as 20 mM for larvae at the middle fifth instar,²⁴ and the dynamic level changes for trehalose were closely related to molting, metamorphosis, and diapause,^{25,26} which correlated with the developmental dependence of the sorbitol-6-phosphate level controlled by ecdysteroid.²⁶ The trehalose level varied from larval to pupal blood²² with a reduced trehalose synthesis²⁴ and an increase in glycogen synthesis²⁷ in fat body during metamorphosis. Some other studies were focused on the silkworm's metabolism related to its growth and development in terms of insect hormones.²⁸ More recently, ¹H–¹³C HSQC 2D NMR approaches were employed to identify 56 metabolites in silkworm hemolymph,²⁹ and the development-associated metabolic changes were related to energy and nitrogen metabolism.²⁹ These studies undoubtedly have proven that many silkworm metabolic processes such as glycolysis, protein biosynthesis, and degradation are closely associated with the insect developmental processes. However, the hemolymph metabonomic features of the silkworm are far from completely characterized, and many metabolic pathways such as TCA, purine, and pyrimidine metabolisms remain to be reported in details in terms of silkworm development and growth. Most of the compositional data available for the silkworm hemolymph metabolites were obtained from very old techniques (e.g., paper chromatographic and chemical methods) with the data quality requiring further clarification and improvement with more modern technologies.

NMR-based metabonomic analysis approaches ought to be suitable to characterize the silkworm hemolymph metabonome and its dynamic changes associated with the insect growth and development. This is because metabonomics systemically characterizes the metabolite composition of integrated biological systems and its dynamic responses to both endogenous and exogenous changes.^{30,31} As a systems biology approach, metabonomics approaches were successfully applied in investigating the developmental dependence of the tissue metabolism of mammalian gastrointestinal tracts³² and their contents,³³ molecular aspects of pathogenesis and progression of metabolic diseases^{34,35} and infectious diseases.^{36,37} A recent metabonomic study using GC–MS analysis was reported on important glycine roles in silk synthesis.³⁸ Metabonomics approaches have also been successfully used in characterizing the metabolic phenotypes of the larval and pupal hemolymph of the tobacco hornworms (*Manduca sexta*) in different developmental stages.³⁹ These studies undoubtedly indicated the potentials for metabonomics approaches to reveal the development-associated metabonomic features for this important insect. To the best of our knowledge, however, there are no reports published so far on metabonomics of *B. mori* in terms of its systematic development.

In this study, we systematically analyzed the silkworm hemolymph metabonome and its dynamic changes from the third instar to the end of the fifth instar (prepupa) using ¹H NMR spectroscopy in conjunction with multivariate data analysis. We further analyzed the

composition of fatty acids in silkworm hemolymph throughout these developmental periods using GC–FID/MS techniques. The goals of these endeavors are to define the metabonomic feature of the silkworm hemolymph and its dynamic changes associated with the growth and development processes of silkworm larvae.

MATERIALS AND METHODS

Chemicals

Analytical grade thiourea, $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$, hexane, K_2CO_3 , and NaCl were obtained from Shanghai Sinopharm Chemical Reagent Co. Ltd. (China). Sodium azide (NaN_3) was from Tianjin FuChen company (China). Methyl tricosanate (99.0%), methyl heptadecanoate, acetyl chloride, and D_2O (99.9% D) were bought from Sigma-Aldrich. A mixed standard consisting of methyl esters of 37 fatty acids and 3,5-di-*tert*-butyl-4-hydroxytoluene (BHT) were obtained from Supelco (Bellefonte, PA). Phosphate buffer (0.045M, pH7.41) was prepared from NaH_2PO_4 and K_2HPO_4 containing 50% D_2O and used as adding solvent for NMR analysis of hemolymph samples with good low-temperature stability of this buffer.⁴⁰

Animal Experiments and Sample Collection

About 6000 disease-free eggs of silkworm (*Bombyx mori* strain P50) were acquired from the Sericultural Research Institute, Chinese Academy of Agricultural Sciences. They were hatched and reared on mulberry leaves at 28 °C with a relative humidity of 90% for the first instar of the silkworm, followed with 1 °C temperature decrease and 5% humidity decrease in each instar. Photoperiod was set to have 12 h of light and 12 h of darkness. Silkworms were weighed before and after they molted from the first instar to the fourth instar. In the fifth instar, they were weighed on day 1, 3, 5, 7, 8, and after spinning (prepupal stage), respectively. We used male and female larvae together since they were not readily distinguishable. Hemolymph samples were collected into microcentrifuge tubes individually by cutting an abdominal leg of silkworm. Approximately 4 μL of thiourea (0.2M) was added immediately to every hemolymph sample tube as antioxidant to inhibit tyrosinase activity to prevent sample blackening. The silkworm hemolymph was not collected before the third instar due to quantity limitation but was collected on day 3 in the third instar (3d3I) (right before the third ecdysis), day 1 in the fourth instar (1d4I) (newly ecdysed), day 4 in the fourth instar (4d4I) (before the fourth ecdysis), day 1 in the fifth instar (1d5I) (newly ecdysed), day 3 in the fifth instar (3d5I), day 5 in the fifth instar (5d5I), day 7 in the fifth instar (7d5I), day 8 in the fifth instar (8d5I) (mature larvae or wandering stage), and last the prepupa (pP) (after cocooning). Each sample was collected from approximately three silkworms for these before the fifth instar and from only one silkworm after the fourth ecdysis. After centrifugation for 10 min (4000 g, 4 °C), serum samples ($n = 15$) were obtained, snap-frozen with liquid nitrogen, and then stored at –80 °C until further analysis. Tissue and fecal samples were also collected, snap-frozen with liquid nitrogen, and stored at –80 °C for future analysis.

Samples Preparation for NMR Analysis

Hemolymph samples were prepared individually by mixing about 30 μ L of hemolymph with 30 μ L of phosphate buffer (0.045 M, pH 7.41). After 10 min of centrifugation (4000 g, 4 $^{\circ}$ C), 55 μ L of supernatant was transferred into 1.7 mm NMR tubes for ^{1} H NMR analysis. For 2D NMR, 10 samples from different developmental stages were pooled into a 5 mm NMR tube to obtain good sensitivity.

NMR Spectroscopy Measurements

One-dimensional ^{1} H NMR spectra of silkworm hemolymph were obtained on a Bruker Avance II 500 MHz NMR spectrometer (500.13 MHz for proton frequency) with an inverse detection probe (Bruker Biospin, Germany) at 298 K. ^{1} H NMR spectra of hemolymph were acquired employing CPMGPR1D pulse sequence [RD-90 $^{\circ}$ -(τ -180 $^{\circ}$ - τ) n -ACQ] to obtain signals from small molecule metabolites with water signal suppressed during the recycle delay (RD, 2 s). Ninety degree pulse length was set to about 10 μ s for every sample, n to 100, and τ to 350 μ s. Two-hundred fifty-six transients were collected into 32768 data points over a spectral width of 20 ppm.

For assignment purposes, a set of 2D NMR spectra was recorded and processed for some selected samples with procedures and parameters as described previously,^{41–43} including ^{1} H- ^{1} H correlation spectroscopy (COSY), ^{1} H J-resolved spectroscopy (JRES), ^{1} H- ^{1} H total correlation spectroscopy (TOCSY), ^{1} H- ^{13} C heteronuclear multiple bond correlation spectroscopy (HMBC), and ^{1} H- ^{13} C heteronuclear single quantum correlation spectroscopy (HSQC).

NMR Data Processing and Multivariate Data Analysis

All the NMR spectra were processed with Topspin software (V3.0, Bruker Biospin). All free induction decays were applied with an exponential function with a line-broadening factor of 1 Hz and zero-filled to 128 k prior to Fourier transformation (FT). The phase and baseline of all spectra were corrected manually with chemical shift referenced to trehalose (δ 5.189 for ^{1} H). The spectral region δ 0.55–9.60 was integrated into bins with width of 0.002 ppm (1 Hz) using AMIX package (v3.8, Bruker Biospin). The region at δ 4.65–5.10 was discarded to eliminate the water suppression effects. The areas of all these bins were normalized to the volume of hemolymph samples to get absolute metabolite-proton concentration (as the peak area per unit volume).

Multivariate data analysis was performed with software SIMCA-P⁺ (v12.0, Umetrics, Sweden). Principal component analysis (PCA) was done with the mean-centered data to obtain the overall groupings and to find possible outliers. After careful examination of the spectra, potential outliers were removed prior to further data-mining. The orthogonal projection to latent structure discriminant analysis (OPLS-DA) was done with a seven-fold cross-validation and unit-variance scaling. OPLS-DA model qualities were appraised with R^2 X representing the explained variations and Q^2 for the model predictabilities. All OPLS-DA models were further assessed by CV-ANOVA tests for significant intergroup differentiations (at the level of $p < 0.05$).

Loadings from OPLS-DA models were back-transformed⁴⁴ and plotted with correlation coefficients, which were color-coded, using an in-house developed Matlab script (v7.1, the Math-works, MA) so as to find these significantly changed metabolites. In the loading plots, the warm-colored (e.g., red) variables (metabolites) contributed more significantly toward intergroup differentiation than the cool-colored metabolites (e.g., blue). Cutoff values for correlation coefficients (depending on the sample numbers) were used for the statistical significance based on the test for the significance of the Pearson's product-moment correlation coefficients for discrimination significance⁴⁴ at the level of $p < 0.05$.

Analysis of Fatty Acid Composition Using GC–FID/MS

Fatty acids in hemolymph samples were measured in the forms of their methyl esters using an optimized method reported previously^{45–47} with some modifications. In brief, 40 μL of each silkworm hemolymph sample (after NMR analysis) was mixed in a Pyrex tube with 20 μL of internal standard (containing 0.1 mg/mL methyl heptadecanoate, 0.05 mg/mL methyl tricosanoate, 0.2 mg/mL of BHT) and then added with 1 mL of methanol–hexane (4:1). After these tubes were cooled above a liquid-nitrogen bath (without touching liquid nitrogen) for 15 min, 100 μL of acetyl chloride was carefully added to the tubes. After the mixture was stored (airtight, 25 °C) in the dark for 24 h, the reaction mixture in an ice bath was slowly added with 2.5 mL of K_2CO_3 (6%) with gentle shaking. Another 200 μL hexane was then added to the mixture to extract lipids followed with a 10 min centrifugation (800 g, 4 °C). The organic layer was transferred into a fresh glass sample vial. The extraction process was repeated twice, and the resultant supernatants were combined followed with evaporation to dryness. Each extract was then redissolved in 25 μL of hexane and immediately used for GC–FID/MS analysis.

Methylated fatty acids from silkworm hemolymph were analyzed on a Shimadzu GC2010Plus GC–MS spectrometer (Shimadzu Scientific Instruments) equipped with a flame ionization detector (FID) and a mass spectrometer. A DB-225 capillary GC column (from Agilent with a length of 10 m, film thickness of 0.1 μm , and ID of 0.1 mm) was used with helium as the carrier and makeup gas. The above methylated samples (1 μL) were injected with a splitter (1:60) with the injection port and detector temperature set to 230 and 250 °C, respectively. The column temperature was set to 155 °C first and ramped to 205 °C (25 °C/min) and kept for 3 min. The temperature was then increased to 225 °C (10 °C/min) and maintained for 3 min. Mass spectra were acquired with an electron impact (EI) (70 eV) and a full scan mode (m/z 45–650). Identities of the methylated fatty acids were confirmed with 37 known standards and their mass spectral data. Furthermore, methylated fatty acids were quantified with their FID signal integrals and internal standards. Final results were expressed as μmol fatty acids per liter of hemolymph. The molar percentages were calculated for total fatty acids (ToFA), saturated fatty acids (SFA), unsaturated fatty acids (UFA), and polyunsaturated fatty acids (PUFA), respectively. The ratio of n3- and n6-type PUFA was also calculated.

RESULTS

Phenotypes of Silkworms

The average body weight of silkworms increased steadily (from 0.39–15 mg) before the third instar (Figure 1). Their weights rose from 15 to about 100 mg during the third instar (Figure 1, inset), whereas from about 120 to 400 mg during the fourth instar. During the fifth instar, silkworms showed marked growth with body weight increased from about 400 mg on day 1 (1d5I) to 2000 mg on day 7 (7d5I) followed with a sharp decrease to about 1000 mg around prepupa stage (Figure 1). This growth curve broadly agrees with what has been reported for a hybrid (N134×C135) silkworm,⁴⁸ although the latter generally has a much bigger size. Silkworm hemolymph was found to be acidic and had a moderate pH increase (from 6.71 to 6.96) during larva growth (Figure S2, Supporting Information).

Metabolites in Silkworm Hemolymph

Hemolymph metabolites for our silkworm strain (P50) at various developmental stages (Figure 2) were unambiguously assigned (Table 1) based on the literature data,^{45,49,50} publically accessible (MMCD), and in-house databases. These assignments were further confirmed individually for each metabolite using a series of 2D NMR spectra of hemolymph samples in the manner as reported previously.^{33,41} Over 60 metabolites were assigned in the silkworm hemolymph (Table 1), including organic acids (quinic, lactic, acetic, pyruvic), amino acids (Val, Ala, Gly), carbohydrates (glucose, trehalose, glycogen, N-acetylglucosamine), organic amines (putrescine, dopamine, trimethylamine), TCA cycle intermediates (citrate, 2-ketoglutarate, succinate, malate), betaine, ascorbate, nicotinamide, and metabolites of choline, purines, and pyrimidines.

Level differences in silkworm hemolymph metabolites were observable at different development stages. For example, hemolymph on 3d5I (day 3 of the fifth instar) had much lower levels for branched-chain amino acids but higher citrate levels than on 3d3I and prepupa stage. Trehalose level was outstandingly higher on 3d3I and 3d5I. It is particularly interesting to note that some metabolites observed in silkworm hemolymph at prepupa (pP) stage were not visible in that of other stages such as N-acetylglucosamine, methionine-sulfoxide, mesaconate, tyrosine- β -glucuronide, and hydroxykynurenine (Figure 2, Table 1). To obtain the details about the metabolic changes related to development processes, we conducted multivariate data analyses on the NMR data of silkworm hemolymph at different developmental stages (from the third instar to prepupa).

Metabonomic Changes of Silkworm Hemolymph Associated with Development Process

PCA scores plot (Figure S3, Supporting Information) showed that silkworm hemolymph metabonome had obvious developmental dependence from 3d3I to prepupa stages. OPLS-DA models were constructed to further reveal the significant changed metabolites during the developmental process (Figure S4). Changed metabolites with their correlation coefficients between two adjacent instars were extracted and tabulated in Table 2.

During the third molting (from 3d3I to 1d4I) and fourth one (from 4d4I to 1d5I), the levels of Val, Ile, Leu, Ala, Thr, Lys, Arg, Asn, Tyr, and Trp declined significantly, whereas

quinate and phosphorylcholine (PC) levels showed significant elevation (Figure S4, Table 2). The brain-specific dipeptide N-(4-aminobutyl)-histidine (i.e., homocarnosine) had significant level decrease during both moltings together with two TCA cycle intermediates, 2-ketoglutarate, and succinate (Figure S4, Table 2). In contrast, some amino acids including Glu, Met, His, and Phe showed significant decline together with glycogen, UDP-glucose, glucose, pyruvate, choline, betaine, and CMP during the third molting, although these metabolites had no changes during the fourth molting (Figure S4, Table 2). Significant level elevation for Gly, Pro, putrescine, cytidine, and xanthosine were observed during the fourth molting together with significant decline for trehalose, fumarate, and malate. However, no changes were observable for these metabolites during the third molting. Citrate was elevated during the third molting but decreased during the fourth molting (Figure S4, Table 2).

In the fourth instar (from 1d4I to 4d4I), significant elevations were observable for Val, Ile, Leu, Ala, Gly, Thr, Gln, Lys, Arg, Met, His, homocarnosine, hydroxykynurenine, putrescine, UDP-glucose, 2-ketoglutarate, citrate, malate, succinate, fumarate, uridine, β -alanine, CMP, lipids, choline, and betaine, whereas levels declined for PC, xanthosine, ascorbate, 1-deoxynojirimycin and quinate. No significant changes were observed for Asp, Pro, Tyr, Trp, Phe, glycogen, glucose, and metabolites from glycolysis (Figure S4, Table 2).

In the fifth instar, three branch-chain amino acids (Leu, Ile, Val), Arg, Tyr, 2-ketoglutarate, and uridine decreased continually from day 1 to day 3, whereas quinate changed in the opposite direction (Figure S4, Table 2). In the fourth molting, Thr, Ala, Lys, Asn, His, homocarnosine, and trehalose showed significant elevation, whereas Pro, PC, putrescine, cytidine, and xanthosine had significant declines. Additionally, Gln, Met, β -Ala, Phe, glycogen, pyruvate, betaine, and CMP had a decline, whereas His, dopamine, and ascorbate showed elevations (Figure S4, Table 2). From 3d5I to 8d5I, Ile, Leu, Val, Thr, Ala, Phe, Trp, homocarnosine, hydroxykynurenine, Glc, lactate, uridine, and TMA showed no changes. However, Lys, Arg, Gln, Asn, Met, putrescine, β -Ala, choline, PC, citrate, malate, and fumarate were elevated, whereas Gly, Pro, Arg, Gln, Tyr, quinate, glycogen, UDP-glucose, pyruvate, 2-ketoglutarate, succinate, betaine, dopamine, ascorbate, cytidine, 1-deoxynojirimycin and xanthosine showed some level declines (Figure S4, Table 2). Trehalose level was elevated from 1d5I to 5d5I substantially but drastically decreased from 5d5I to 8d5I. Significant increases were seen for malate and fumarate, but decreases for 2-ketoglutarate were observed from 3d5I to 5d5I; the levels of these metabolites changed little afterward (Figure S4, Table 2).

Marked metabonomic changes were detectable from 8d5I to prepupa stage in which larvae went through the spinning process.

Significant increases were detected for the levels of Val, Ile, Leu, Ala, Gly, Thr, Pro, Arg, Met, Phe, Tyr, tyrosine-o- β -glucuronide, Trp, His, homocarnosine, dopamine, hydroxykynurenine, putrescine, Glc, UDP-glucose, pyruvate, lactate, choline, PC, betaine, lipids, NMN, nicotinamide, xanthosine, IMP, CMP, uridine, β -Ala, and methionine-sulfoxide, whereas Lys, Asn, trehalose, citrate, malate, and fumarate were significantly decreased (Figure S4, Table 2).

Fatty Acid Compositional Changes for Silkworm Hemolymph Associated with Development

To obtain the detailed changes of silkworm fatty acids during silkworm development, we further analyzed the fatty acid composition of silkworm hemolymph using GC-FID/MS methods (Figure 3). The results showed that silkworm hemolymph mainly contained more than 10 fatty acids ranging from 16–22 carbons. Among them, C16:0, C18:0, C18:1, C18:2n6, and C18:3n3 accounted for more than 95% (mol), amongst which C18:3n3 accounts for about 45%. About 65% of them were unsaturated fatty acids (UFA), and over 56% were polyunsaturated fatty acids (PUFA), among which the n3-to-n6 ratio was about 3.6 (Table 3).

During the third and fourth molting stages, the levels of most hemolymph fatty acids decreased significantly except for C20:5n3, which showed the opposite trend. In contrast, the levels of all hemolymph fatty acids increased significantly in the prepupa stage. In the fourth instar (from 1d4I to 4d4I), most fatty acids showed significantly level elevations (Table 3). However, during the fifth instar (from 1d5I to 8d5I), they had only some moderate changes.

DISCUSSION

When developing from eggs to pupa, silkworms go through five larval instars, in which there are four molting processes between the instars, during which they generally stop food intakes. Our results showed that this developmental process of silkworms (*Bombyx mori*) was closely associated with the hemolymph metabonomic changes including glycolysis, TCA cycle, metabolisms of fatty acids, amino acids, choline, purines, and pyrimidines. These changes were related to nutrient absorption, membrane and energy metabolism, biosyntheses of cuticle pigments together with the cuticle, body, and silk proteins (Figure 5).

¹H NMR Spectroscopic Analysis of Silkworm (*Bombyx mori*) Hemolymph

Metabonomic analysis of silkworms is surprisingly less common as far as studying silkworm developmental biochemistry is concerned, although the NMR-based metabonomic studies have been widely conducted for a wide range of invertebrate biofluids and tissue extracts.^{51–55} Here we have detected a variety of endogenous metabolites from silkworm hemolymph, many of which are also commonly observable in human blood plasma.⁴⁹ Some metabolites such as trehalose were observed in both silkworm^{22,24,29,56} and other insects such as tobacco hornworm (*Manduca sexta*),^{39,57} whereas putrescine was not reported in the former but in the latter. In fact, many metabolites detected here have not been reported in silkworm hemolymph such as tyrosine-o- β -glucuronide, methionine-sulfoxide, mesaconate, 1-methylhistidine, homocarnosine, and picolinate. Phosphorylcholine is an abundant metabolite of silkworm hemolymph in all its developmental stages with exceptions for the periods right before the third and fourth molting. We also noticed that many metabolites, which were identified using ¹H–¹³C HSQC method from the hemolymph extracts of silkworm (from the fourth to fifth instars) fed with ¹³C-labeled glucose and amino acids,²⁹ were not observable in our silkworm hemolymph especially those phosphorylated sugars and coenzymes (CoAs). Close inspections of our own ¹H–¹³C HSQC spectra led to the same

conclusion. In fact, dozens of metabolites detected in our samples (such as quinate, methionine-sulfoxide, *cis*-aconitate, citrate, 2-ketoglutarate, succinate, fumarate, tyrosine- α -glucuronide, N-acetylglucosamine, dopamine, hydroxykynurenine, and ascorbate) were also not reported in the aforementioned HSQC study.²⁹ Such discrepancy may result from the NMR-sensitivity enhancement by ¹³C-labeling in the reported study or strain differences (with silkworm strains not being stated in the reported study).

Our results for the levels of silkworm hemolymph metabolites also differed largely from those obtained using chromatographic methods in some classical literature.^{21,22} From 3d3I to 5d5I, for example, paper chromatographic results showed Gln as the most prolific amino acid in silkworm hemolymph,^{21,22} whereas our results showed that Gly, His, Val, Ala, and Gln were high in their levels from 3d3I to 5d5I. Such differences probably resulted from inaccuracy of classic chromatographic methods, although differences in silkworm strains used were another possibility. Our results also differed obviously from the classical data in terms of developmental associated level changes. Citrate, trehalose, malate, and quinate were by far the most abundant four metabolites from the third to the end of the fifth instars, while phosphorylcholine levels also became prominent during the prepupa stage. In contrast, detailed levels for these metabolites at different developmental stages were not systematically reported, and associated metabolic pathways were not sufficiently discussed in previous studies.

Developmental Changes Associated with Skin Pigment Biosynthesis

Silkworms undergo five major metamorphosis processes including four ecdyses and a pupa stage before finally developing into adults, during which biosynthesis of skin pigments is one of the major biochemical events. Significant decline (50–80%) for Tyr, Trp, and 3-hydroxykynurenine (Figure 4) during the third and fourth ecdyses indicated a promotion of biosynthesis of skin pigments⁵⁸ since silkworms had to shed the old transparent skin after the third instar and to synthesize new brownish colored skin (Figure 5). From 8d5I (prespinning stage) to prepupal (after spinning) stage, significant elevation in the levels for metabolites related to skin pigment synthesis (Tyr, Phe, Trp, dopamine, and 3-hydroxykynurenine, Figure 4) was probably due to degradation of larval pigments to synthesize new pigments for the pupa. In contrast, during the feeding period of the fourth instar (from 1d4I to 4d4I), 3-hydroxykynurenine level was increased probably due to metabolizing tryptophan from mulberry leaves⁵⁹ to prepare enough precursors for pigment biosynthesis during the fifth molting. During the feeding period of the fifth instar (from 1d5I to 8d5I), steady level decline for Tyr and Phe and minor fluctuation of dopamine level (Figure 4) indicated that these amino acids were mainly used for biosynthesis of proteins (for both body growth and preparation of silk) since pigment biosynthesis was no longer in high demand after the fourth molting. This notion is supported by a steady level decline for quinate, and changes for dopamine since dopamine is a Tyr metabolite used for epidermis and melanin synthesis,⁶⁰ although quinate is from silkworm diet and the precursor for Tyr and Phe in mulberry leaves. Dopamine elevation from 1d5I to 3d5I was probably due to reduced demand for epidermis and melanin synthesis after molting, whereas its level decrease from 3d5I to 7d5I was due to diversion of its precursor (Tyr) to silk and skin

protein biosynthesis. This is further supported by the elevation of Ala, which is one of main amino acids in silk protein.

Developmental Changes of Amino Acids Related to Cuticle Proteins

During two ecdysis periods (from 3d3I to 1d4I and from 4d4I to 1d5I), the level decline (Table 2, Figure 4) for amino acids associated with skin proteins such as Ala, Val, Lys, Tyr, Thr, Leu, Ile, and Phe was probably associated with the demands for biosynthesis of cuticle proteins. During these periods, silkworms naturally molt to shed old skin and to develop new skin, which contains about 50% proteins. Gly level had different behavior here probably due to silkworms' capability to convert Ala, Thr, and Ser into Gly.^{61,62}

During the feeding period of the fourth instar (from 1d4I to 4d4I), silkworms grew quite rapidly (Figure 1) and absorbed fair amounts of nutrients from mulberry leaves, stockpiling on the hemolymph amino acids such as Gly, Ala, Val, and Thr for biosynthesis of skin, body, and storage proteins.⁶³ During the feeding period of the fifth instar, silkworm larvae grew more rapidly in size (Figure 1). Such growth undoubtedly required even more biosynthesis of skin and body proteins leading to the level declines for hemolymph amino acids related to skin proteins such as Val, Pro, Leu, Ile, Met, Phe, and Tyr (Figure 4) in early period of the fifth instar (from 1d5I to 3d5I).⁶³ On the other hand, elevation of Ala, Thr, His, and Asn (Figure 4) indicated that larvae were preparing amino acids for biosynthesis of silk protein in later period of this instar.⁶⁴ From 4d5I to 8d5I, silkworm larvae kept the momentum of rapid growth, and the biosynthesis of both storage and silk proteins became essential demands for many amino acids. This was supported by level decreases for two amino acids essential for silk proteins⁶⁵ (Table 2, Figure 4) given the low Gly content in mulberry leaves.⁶⁶ We did not observe systematic level declines for many other hemolymph amino acids, in all likelihood due to compensation of these amino acids from diet. Nevertheless, during the spinning period (from 8d5I to prepupa), tissue remodeling became the dominant biochemical event with concurrent degradation of skin proteins and excretion of silk protein leading to elevation for most amino acids in hemolymph (Figure 4) so as to prepare for synthesizing new cuticle proteins of the pupa. Asn experienced a level decline for its high abundance in silk protein but not in skin proteins.

Histidine was one of the most abundant amino acids in our silkworm hemolymph. Its level was increased steadily especially after the fourth instar due to high His content in mulberry leaves⁶⁷ and increased diet intakes for silkworms during these feeding periods. In fact, silkworms cannot efficiently synthesize histidine nor metabolize it, and histidine from mulberry leaves can only be stockpiled in circulation and excreted through feces and urine.⁶⁷ Larvae stopped eating during ecdyses and wandering stage (from 7d5I to 8d5I) so that His level was expected to decline as observed (Figure 4). Further rise of histidine level in the spinning stage (from 8d5I to prepupal) observed here probably resulted from degradation of skin and body proteins required by the tissue remodeling to prepare for metamorphosis.

Asn level is high in mulberry leaves so that it is readily available to be converted into Asp. The latter is one of the main amino acids in sericin (but not in skin proteins) and can readily be fed into TCA cycle via oxaloacetate. Proline is an energy source in insects, and it can be

synthesized in fat body of silkworms (through fatty acid derived acetyl-CoA and alanine) entering hemolymph⁶⁸ apart from its important role in biosynthesis of skin proteins. Therefore, the level changes for both Asn and Pro ought to be considered with a view of complex metabolic network, although ¹³C labeled diet can provide vitally important information on metabolic fluxes of these amino acids.

Developmental Changes in Glycolysis and TCA Cycle

Significant level declines for glycogen, glucose, pyruvate, lactate, succinate, and 2-ketoglutarate (Table 2, Figure 4) during the third ecdysis (from 3d3I to 1d4I) suggested up-regulation of glycolysis and TCA cycle to prepare energy and amino acids for biosynthesis of skin and body proteins.⁶⁹ The elevation of citrate and concurrent decreases of most fatty acids (Table 3) also indicated contribution of citrate biosynthesis from acetyl-CoA generated via the up-regulation of fatty acid catabolism. Similar metabolic changes were observable for the fourth ecdysis (from 4d4I to 1d5I) with significant level declines for trehalose, lactate, 2-ketoglutarate, citrate, succinate, fumarate, and malate (Table 2, Figure 4).

During the feeding period of the fourth instar (from 1d4I to 4d4I), silkworms became physically active and experienced an increased diet consumption. Significant elevations of citrate, 2-ketoglutarate, succinate, fumarate, and malate observed during this period (Table 2, Figure 4) indicated that increased energy production from TCA cycle was strengthened probably from nutrients. During the early feeding period of the fifth instar (1d5I to 5d5I), similar observation was apparent for the level elevation of citrate, malate, and fumarate (Table 2, Figure 4) probably for the same reasons. However, significant trehalose elevation together with the declines for glycogen seemed to suggest that silkworms were stockpiling trehalose (at the expenses of nutrients) as well during this period probably to prepare for silk protein synthesis afterward. This notion is further supported by trehalose consumption and little changes for glucose and glycogen from 5d5I to 8d5I (Table 2). This is not surprising since trehalose concentration is about 10–30 times higher than the glucose concentration thus, by far the most excessive blood sugar for silkworms during the fifth instar (Figure S5). From wondering to prepupal stages, drastic trehalose depletion together with the significant elevation of glucose and glycogen (Table 2, Figure 4, and Figure S5) indicted a switch from larva sugar (trehalose) to pupa sugar (glycogen and glucose).⁷⁰ However, relativity of the NMR signal intensities of these sugars indicated that not all trehalose was converted into glucose and glycogen²² with some consumed via glycolysis to generate energy for the uses of silk-spinning and metamorphosis since larvae stopped taking in nutrients during this period. Level declines for citrate, malate, and fumarate accompanied by the elevation of pyruvate and lactate (Table 2, Figure 4) further supported such notion since larvae minimized their movements for pupation during this period.

Our observation of the slight decrease in trehalose level during the third ecdysis (from 3d3I to 1d4I) differed from what was reported for the male silkworms of two hybrid races (Gunpo × Shugyoku and Kinshu × Showa), which showed more than 50% trehalose decrease during this molting.²⁶ An abrupt decrease (ca. 60%) for trehalose during the fourth ecdysis (from 4d4I to 1d5I) was broadly consistent with previous observations though for different strain of silkworms.^{24,26,70} These were followed by some moderate increases (from 1d4I to 4d4I)

that rose significantly from 1d5I to 5d5I probably from nutrient followed with a drastic decline from 5d5I to prepupal stage as discussed earlier. This trend was broadly consistent with what was observed previously,^{24,26,70} although different strains used in literature had fairly different durations especially for the fifth instar. This indicated that the level changes of this principal hemolymph sugar were developmentally dependent and probably regulated by balancing trehalose synthase and trehalase activities.^{24–26,70} Since trehalose is produced in fat body,⁷¹ biosynthesis and release of trehalose are therefore expected to be closely coupled to fatty acid mobilization and oxidation as well.

Developmental Changes of Fatty Acids in Silkworm Hemolymph

Limited data for hemolymph fatty acids have been reported so far and only for silkworms on 5d5I.⁷² We therefore analyzed the developmental changes of the silkworm hemolymph fatty acids systematically. Our data showed that between the 3d3I and the prepupal stage, C18:0 and its unsaturated forms together with C16:0 accounted for over 95% (mol) of all fatty acids in silkworm hemolymph (Table 3). This is broadly consistent with what has been reported⁷² and probably due to the high contents of C16:0, C18:0, C18:2n6, and C18:3n3 in mulberry leaves with these two unsaturated fatty acids as essential fatty acids for silkworms.⁷² However, C16:0 and C18:2n6 levels were much lower, while C18:0 was more apparent in our samples than what had been reported in a different strain. During both the third and fourth ecdysis, most fatty acids had significant level decreases (Table 3) because of the demands (of new skin synthesis) for energy and cell membrane biosynthesis without diet supplies. During the feeding period of the fourth instar (from 1d4I to 3d4I), almost all fatty acids had significant level elevations (Table 3) probably due to absorption from diet. During the feeding period of the fifth instar, in contrast, fatty acids showed moderate changes (Table 3). Only C18:1 and C18:2n6 showed significant level declines from 1d5I to 3d5I, and C18:1 was elevated from 5d5I to 7d5I (Table 3). This probably indicated the balance of fatty acid absorptions from food and the requirement of fatty acids for silkworm body growths given the fact that the levels of fatty acids had some obvious increases in larval body.⁷² During spinning period, the levels of all fatty acids rose significantly, although there was no food consumption probably due to fat dispositions into hemolymph resulting from the tissue remodeling.

Developmental Changes of Choline Metabolism

Significant PC elevation during the third and fourth ecdyses and the spinning period (Table 2) probably resulted from degradation of the old cuticle cell membrane with PC as an essential membrane fragment.⁷³ Decreases of choline and betaine levels during the third ecdysis were probably due to lack of choline ingestions from food. During feeding period of the fourth instar, PC was used to synthesize cell membrane for new skin and rapid body growth leading to its level decline. During the same period, increased food intake resulted in elevation of choline and betaine, which could also be further turned into glycine for the biosynthesis of skin proteins. From 3d5I to 7d5I, choline level was elevated probably also due to food intakes. Choline was further converted into PC and led to significant level increase for PC (from 5d5I to 8d5I), which was used to synthesize phosphatidylcholines for egg or sperm development. Significant level decline of betaine in the fifth instar was probably due to its conversion into glycine for silk protein biosynthesis.

CONCLUSIONS

This study showed that dynamic metabonomic changes in silkworm hemolymph were closely associated with the silkworm larva development involving multiple metabolic pathways. Such changes were dominated by degradation of the old skins and synthesis of new skins during moulting processes (i.e., ecdyses) and metamorphosis. This was accompanied by biosynthesis of pigments by using tyrosine and tryptophan metabolites. In the fifth instar, biosyntheses of silk protein and body proteins were also some outstanding metabolic events. The other dominant changes were related to trehalose metabolism including its biosynthesis and conversion into glucose for energy generation involving glycolysis and TCA cycle. The main “blood sugar” for larvae, trehalose, had a sharp level decrease during wondering period, and then glucose became an abundant blood sugar after spinning period. Hemolymph quinate generated by mulberry leaves (i.e., silkworm's sole diet) for biosynthesis of aromatic amino acids can be a good marker for dietary intakes and probably silkworm health. The hemolymph levels of both fatty acids and choline were directly affected by diet intakes facilitating biosynthesis of phosphorylcholine for cell membrane. C18:0 and its unsaturated forms together with C16:0 accounted for 95% of hemolymph fatty acids, among which C18:3n3 accounted for more than 40% of all fatty acids; n3-to-n6 ratios for fatty acids were about 3:1 and 4:1. These provided detailed information on silkworm hemolymph metabonome and its dynamic changes at various stages of larval development from the third instar to prepupal period. Future studies are necessary to obtain metabolic information for silkworm before the third instar and its adulthood.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

We acknowledge the Ministry of Science and Technology of China (2010CB912500 and 2012CB934000) and NSFC (91439102, 21175149, 20825520 and 21221064) for the financial support. This work utilized the MMC Database supported by NIH Grants R21 DK070297 and P41 RR02301.

ABBREVIATIONS

NMR	nuclear magnetic resonance
PCA	principal component analysis
OPLS-DA	orthogonal projection to latent structure with discriminant analysis
CPMG	Carr–Purcell–Meiboom–Gill
GPC	glycerophosphorylcholine
PC	phosphorylcholine
ToFA	total fatty acid
SFA	saturated fatty acid

UFA	unsaturated fatty acid
PUFA	polyunsaturated fatty acid
MUFA	monounsaturated fatty acid
TCA cycle	tricarboxylic acid cycle
TMA	trimethylamine
GC–FID/MS	gas chromatography–flame ionization detector/mass spectrometry
3d3I	day 3 in the third instar
pP	prepupal stage

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Figure 1.

Developmental changes in the average body weight of silkworms ($n = 15$) with inset data for silkworms before 1d4I *, $p < 0.05$; **, $p < 0.001$ when comparing with silkworm weights at the adjacent previous time point. For stage labels, the first number denotes the day while second number the instar (e.g., 4d4I means day 4 in the fourth instar); pP, prepupa.

Figure 2.

Average ^1H NMR spectra of silkworm hemolymph (strain P50) on 3d3I (day 3 in the third instar), 4d4I (day 4 in the fourth instar before ecdysis), 3d5I (day 3 in the fifth instar), and pP (prepupa stage after cocooning). The spectral regions marked with the dash-line boxes were vertically expanded to reveal small signals. Metabolite keys are in Table 1.

Figure 3. GC-FID spectra of methylated fatty acids in silkworm hemolymph (strain P50) on 3d3I (day 3 in the third instar), 4d4I (day 4 in the fourth instar before ecdysis), 3d5I (day 3 in the fifth instar), and pP (prepupa stage after cocooning). The region of 7.75–10.5 min was vertically expanded for 40 times. Methyl heptadecanoate (C17:0) was used as internal standard.

Figure 4.

Ratios of changes for silkworm hemolymph metabolites at different developmental stages against these on 3d3I (day 3 in the third instar). C_t , level of metabolites at different developmental stages; C_{3d3I} , levels of metabolites on day 3 in the third instar. For stage labels, the first number denotes the day while second number the instar (e.g., 4d4I means day 4 in the fourth instar); pP, prepupa.

Figure 5. Schematic representation of some developmental-associated changes in metabolic pathway for silkworm hemolymph especially biosyntheses of skin-pigments, skin, and silk proteins.

Table 1NMR Data for Metabolites Assigned in Hemolymph of Silkworms (*Bombyx mori*)

no.	metabolites	moieties	$\delta^1\text{H}$ (ppm) (multiplicity ^a)	$\delta^{13}\text{C}$ (ppm)
1	isoleucine	δCH_3	0.93(t)	13.7
		$\beta\text{CH}_3'$	1.00(d)	17.4
		γCH_2	1.25(m)	27.6
		$\gamma\text{CH}_2'$	1.46(m)	27.0
		βCH	1.98(m)	39.0
		αCH	3.67(d)	62.1
		COOH		176.1
2	leucine	δCH_3	0.94(d)	23.7
		$\delta\text{CH}_3'$	0.96(d)	24.9
		γCH	1.69(m)	29.0
		βCH_2	1.72(m)	42.0
		αCH	3.73(t)	57.7
		COOH		176.3
3	valine	γCH_3	0.98(d)	19.7
		$\gamma\text{CH}_3'$	1.03(d)	20.7
		βCH	2.26(m)	32.2
		αCH	3.60(d)	65.1
		COOH		176.0
4	threonine	γCH_3	1.32(d)	22.1
		αCH	3.59(d)	63.8
		βCH	4.26(q)	69.1
		COOH		176.9
5	lactate	βCH_3	1.32(d)	22.3
		αCH	4.11(q)	69.9
		COOH		185.8
6	alanine	βCH_3	1.47(d)	19.23
		αCH	3.78(q)	53.8
		COOH		179.0
7	lysine	γCH_2	1.43(m)	24.7
		$\gamma\text{CH}_2'$	1.49(m)	24.7
		δCH_2	1.71(m)	29.1
		βCH_2	1.90(m)	32.68
		ϵCH_2	3.01(t)	41.7
		αCH	3.75(t)	57.6
		COOH		178.2
8	arginine	γCH_2	1.67(m)	27.1
		βCH_2	1.91(m)	30.0

no.	metabolites	moieties	$\delta^1\text{H}$ (ppm) (multiplicity ^a)	$\delta^{13}\text{C}$ (ppm)
9	putrescine	δCH_2	3.23(t)	43.0
		αCH	3.76(t)	56.9
		C=NH		159.8
		COOH		174.9
		CH_2	1.75(m)	26.7
		NCH_2	3.04(t)	41.7
10	quininate	5-CH	4.14(q)	73.0
		4-CH	3.54(dd)	77.8
		3-CH	4.01(m)	69.7
		2- CH_2	1.86(dd)	43.2
		2- CH_2'	2.05(dd)	43.2
		6- CH_2	1.95(dd)	40.1
		6- CH_2'	2.05(dd)	40.1
		1-C		78.1
11	acetate	COOH		184.8
		CH_3	1.91(s)	26.1
		COOH		184.3
12	α -N-acetyl-D-glucosamine	7-CH	2.05(s)	25.1
		4-CH	3.46(m)	72.2
		3-CH	3.75(m)	73.4
		6- CH_2	3.79(m)	63.9
		5-CH	3.84(m)	73.7
		6- CH_2'	3.84(m)	63.9
		2-CH	3.86(m)	56.6
		1-CH	5.20(d)	93.6
13	β -N-acetyl-D-glucosamine	7-CH	2.05(s)	25.1
		5-CH	3.45(m)	78.6
		4-CH	3.46(m)	72.5
		3-CH	3.52(m)	76.2
		2-CH	3.66(m)	62.3
		6-CH	3.75(m)	63.6
		6- CH'	3.90(m)	63.6
		1-CH	4.71(d)	97.7
14	glutamate	βCH_2	2.06(m)	30.0
		γCH_2	2.34(m)	36.0
		αCH	3.75(t)	57.0
		αCOOH		176.2
		COOH		185.0
		βCH_2	2.13(m)	29.0
15	glutamine	γCH_2	2.45(m)	33.0
		αCH	3.77(t)	57.2

no.	metabolites	moieties	$\delta^1\text{H}$ (ppm) (multiplicity ^a)	$\delta^{13}\text{C}$ (ppm)
16	methionine	COOH		177.2
		C=O		180.1
		S-CH ₃	2.12(s)	16.7
		βCH_2	2.16(m)	33.0
		$\beta\text{CH}_2'$	2.19(m)	33.0
		γCH_2	2.63(t)	30.0
		αCH	3.85(t)	57.0
17	methionine-sulfoxide	COOH		177.0
		βCH_2	2.30(m)	27.1
		δCH_2	2.74(s)	39.2
		γCH_2	3.00(m)	51.0
		$\gamma\text{CH}_2'$	3.04(m)	51.0
18	pyruvate	αCH	3.88(t)	56.2
		COOH		176.1
		CH ₃	2.36(s)	29.0
		C=O		207.9
19	succinate	CH ₂	2.39(s)	37.0
		COOH		185.4
20	2-ketoglutarate	CH ₂ COOH	2.43(t)	33.0
		CH ₂ C=O	3.00(t)	38.0
		COOH		184.2
		C=O		208.4
21	citrate	CH ₂	2.70(d)	46.9
		CH ₂ '	2.54(d)	46.9
		C-OH		78.4
		CH ₂ COOH		181.6
		C-COOH		184.4
22	β -alanine	αCH_2	2.54(t)	36.4
		βCH_2	3.17(t)	39.5
		COOH		181.0
23	trimethylamine	CH ₃	2.86(s)	45.0
24	asparagine	βCH_2	2.85(dd)	37.0
		$\beta\text{CH}_2'$	2.94(dd)	37.0
		αCH	4.00(m)	54.1
		COOH		176.9
25	choline	C=O		177.1
		N(CH ₃) ₃	3.19(s)	56.7
		NCH ₂	3.51(dd)	70.7
		OCH ₂	4.05(ddd)	58.4

no.	metabolites	moieties	$\delta^1\text{H}$ (ppm) (multiplicity ^a)	$\delta^{13}\text{C}$ (ppm)
26	phosphorylcholine (PC)	$\text{N}(\text{CH}_3)_3$	3.21(s)	56.6
		NCH_2	3.58(m)	69.0
		OCH_2	4.16(m)	60.7
27	betaine	$\text{N}(\text{CH}_3)_3$	3.25(s)	55.8
		CH_2	3.89(s)	69.7
		COOH		172.1
28	glycine	CH_2	3.55(s)	44.0
		COOH		175.0
29	serine	βCH_2	3.97(m)	63.2
		αCH	3.84(dd)	59.1
		COOH		176.2
30	proline	γCH_2	1.99(m)	26.6
		βCH_2	2.34(m)	31.0
		$\beta\text{CH}_2'$	2.58(m)	31.0
		δCH_2	3.33(m)	49.0
		$\delta\text{CH}_2'$	3.41(m)	49.0
		αCH	4.12(m)	64.0
		COOH		176.1
31	malate	αCH_2	2.38(dd)	45.0
		$\alpha\text{CH}_2'$	2.67(dd)	45.0
		βCH	4.30(dd)	73.0
		βCOOH		181.4
		αCOOH		183.7
32	ascorbate	γCH_2	3.73(d)	65.0
		βCH	4.00(m)	72.0
		αCH	4.51(d)	81.0
		$\text{C}=\text{O}$		115.8
		$\text{C}-\text{COH}$		178.4
		$\alpha\text{CH}-\text{COH}$		180.3
33	β -glucose	2-CH	3.24(dd)	77.1
		4-CH	3.39(dd)	72.6
		5-CH	3.46(m)	78.6
		3-CH	3.48(t)	78.6
		6-CH ₂	3.71(dd)	63.4
		6-CH ₂ '	3.89(dd)	63.4
		1-CH	4.64(d)	98.8
34	α -glucose	4-CH	3.40(dd)	72.6
		2-CH	3.53(dd)	74.3
		3-CH	3.71(dd)	75.3
		5-CH	3.83(m)	73.7

no.	metabolites	moieties	$\delta^1\text{H}$ (ppm) (multiplicity ^a)	$\delta^{13}\text{C}$ (ppm)
35	trehalose	6-CH	3.82(m)	63.5
		1-CH	5.22(d)	94.8
		4-CH	3.44(dd)	72.4
		2-CH	3.64(dd)	73.7
		5-CH	3.82(m)	74.9
		3-CH	3.84(m)	75.3
		6-CH ₂	3.86(m)	63.2
36	glycogen	6-CH ₂ '	3.75(m)	63.2
		1-CH	5.18(d)	95.9
		4-CH	3.42(m)	75.5
37	UDP-glucose	1-CH	5.40(d)	102.1
		Glc-1CH	5.61(dd)	97.8
		C=OCH	5.94(d)	105.1
38	<i>cis</i> -aconitate	N-CH	7.98(d)	144.0
		CH	5.70(s)	127.0
39	xanthosine	4-CH(ribose)	4.28(m)	68.9
		3-CH(ribose)	4.39(m)	73.2
		2-CH(ribose)	5.85(d)	90.1
		N ₂ -CH(ring)	7.86(s)	139.0
40	uridine	3-CH(ribose)	4.23(dd)	69.0
		1-CH(ribose)	5.97(d)	91.0
		6-CH(ring)	5.97(d)	105.0
		5-CH(ring)	7.93(d)	144.0
41	cytidine	1-CH(ribose)	5.89(d)	93.0
		4-CH(ring)	6.05(d)	99.1
		5-CH(ring)	7.82(d)	147.0
42	mesaconate	CH ₃	1.92(s)	17.5
		CH	6.46(s)	132.3
		C-COOH		132.2
		COOH		180.2
43	fumarate	CH	6.51(s)	138.1
		COOH		177.5
44	dopamine	4-CH(ring)	6.72(dd)	124.0
		6-CH(ring)	6.82(d)	119.0
		3-CH(ring)	6.89(d)	118.0
		1,2-C-OH(ring)		146.0
45	3-hydroxykynurenine	5-CH(ring)	6.68(t)	118.0
		4-CH(ring)	7.02(d)	121.0
		6-CH(ring)	7.43(d)	125.2
46	tyrosine	3,5-CH	6.88(d)	118.0
		2,6-CH	7.18(d)	133.0

no.	metabolites	moieties	$\delta^1\text{H}$ (ppm) (multiplicity ^a)	$\delta^{13}\text{C}$ (ppm)
47	l-methylhistidine	N-CH ₃	3.68(s)	ND
		2-CH	7.02(s)	120.0
		4-CH	7.67(s)	140.0
		1-C		133.0
48	histidine	αCH_2	3.15(dd)	30.9
		$\alpha\text{CH}_2'$	3.24(m)	30.9
		βCH	3.98(dd)	57.1
		2-CH(ring)	7.09(d)	119.7
		4-CH(ring)	7.87(d)	138.7
49	phenylalanine	2,6-CH	7.32(dd)	132.0
		4-CH	7.37(m)	130.6
		3,5-CH	7.42(dd)	131.7
50	tryptophan	7-CH	7.19(m)	122.3
		8-CH	7.27(m)	121.0
		2-CH	7.31(s)	127.0
		5-CH	7.52(d)	114.0
		6-CH	7.72(d)	121.0
51	homocarnosine	N-CH=C	7.04(d)	119.0
		N-CH-N	7.99(d)	137.0
52	niacinamide	5-CH	7.59(m)	126.0
		6-CH	8.25(m)	139.0
		4-CH	8.69(m)	154.0
		2-CH	8.95(t)	149.0
53	guanosine	CH-OH	4.36(m)	73.0
		N-CH-O	5.97(d)	90.0
		N=CH-N	7.99(s)	139.0
54	AMP ^c	2-CH	6.12(d)	ND
		12-CH	8.25(s)	ND
		7-CH	8.59(s)	ND
55	CMP	2-CH	5.97(d)	ND
		10-CH	6.11(d)	ND
		11-CH	8.04(d)	ND
56	inosine 5'-phosphate (IMP)	2-CH	6.13(d)	ND
		12-CH	8.22(s)	ND
		7-CH	8.56(s)	ND
57	nicotinamide ribotide	5-CH	8.33(t)	ND
		4-CH	8.99(d)	ND
		6-CH	9.33(d)	ND
		2-CH	9.57(s)	ND
58	lipids	CH ₃	0.81(m)	16.6
		CH ₃	0.88(m)	16.7

no.	metabolites	moieties	$\delta^1\text{H}$ (ppm) (multiplicity ^a)	$\delta^{13}\text{C}$ (ppm)
59	L-tyrosine-O- β -glucuronide	CH ₂	1.21(m)	33.3
		CH ₂ CH ₂ CO	1.53(m)	27.6
		CH ₂ C=C	1.97(m)	30.1
		C=CCH ₂ C=C	2.76(m)	29.2
		CH=CH	5.26(m)	130.0
		3-CH ₂	3.02(m)	38.0
		2-CH ₂	3.26(m)	58.0
		4-CH(glucuronide)	3.49(m)	72.0
		2-CH(glucuronide)	3.57(m)	72.0
		3,5-CH(glucuronide)	3.61(m)	79.0
		6-CH ₂ '(glucuronide)	3.74(m)	63.0
		6-CH ₂ (glucuronide)	3.83(m)	63.0
		2-CH ₂ '(glucuronide)	4.04(m)	58.0
		1-CH(glucuronide)	5.13(d)	103.0
		6,8-CH	7.12(d)	119.0
60	picolinate	5,9-CH	7.28(d)	133.0
		4-C		132.0
		7-C		159.0
		4-CH	7.48(m)	ND ^b
		2-CH	7.90(d)	ND
61	1-deoxynojirimycin		8.52(d)	ND
		1-CH(ring)	2.70(m)	50.7
		5-CH(ring)	2.86(m)	63.2
		2-CH(ring)	3.31(q)	50.7
		3-CH(ring)	3.42(dd)	72.0
		6-CH	3.64(m)	72.6
		1-CH'(ring)	3.75(q)	63.1
62	U1	6-CH'	3.86(dd)	63.1
		4-CH	3.96(m)	72.1
		5-CH	4.25(m)	73.2
		1-CH	5.54(m)	97.3
63	U2		8.27(m)	130.2
		3.82(m)	ND	
			3.96(m)	ND
			3.58(m)	ND
			4.75(m)	ND
			5.51(m)	97.3
64	U3		8.34(m)	ND
			3.11(s)	55.5
65	U4		3.61(m)	78.0

no.	metabolites	moieties	$\delta^1\text{H}$ (ppm) (multiplicity ^a)	$\delta^{13}\text{C}$ (ppm)
66	U5		5.07(d)	103.0
			5.64(m)	110.0
			4.32(m)	ND
			4.71(m)	ND
67	U6		5.25(m)	63.1
			6.89(m)	ND

^a s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; dd, doublet of doublets.

^b ND, not determined.

^c AMP, adenosine monophosphate; CMP, cytidine monophosphate.

Table 2 Correlation Coefficients for the Significantly Changed Metabolites in Silkworm Hemolymph^a

keys	metabolites	1d4I/3d3I	4d4I/1d4I	1d5I/4d4I	3d5I/1d5I	5d5I/3d5I	7d5I/5d5I	8d5I/7d5I	pP/8d5I
1 ^b	isoleucine	−0.985 ^c	+0.899	−0.885	−0.900				+0.938
2	leucine	−0.981	+0.796	−0.806	−0.940				+0.981
3	valine	−0.962	+0.958	−0.961	−0.674				+0.937
4	threonine	−0.952	+0.884	−0.877	+0.538				+0.694
6	alanine	−0.651	+0.615	−0.915	+0.878				+0.654
7	lysine	−0.941	+0.907	−0.916	+0.752			+0.714	−0.604
9	putrescine		+0.843	+0.858	−0.905	+0.579		+0.775	+0.803
30	proline			+0.675	−0.910		−0.730	−0.930	+0.989
10	quinat	+0.876	−0.730	+0.769	+0.659	−0.861	−0.911		
15	glutamine	−0.683	+0.878		−0.682	−0.891			
8	arginine	−0.949	+0.906	−0.601	−0.927	−0.673			+0.914
16	methionine	−0.803	+0.816		−0.756		+0.705		+0.94
20	2-ketoglutarate	−0.60	+0.88	−0.52	−0.611	−0.823			
21	citrate	+0.70	+0.72	−0.89		+0.682	−0.74	+0.80	−0.94
31	malate		+0.89	−0.90		+0.877			−0.53
19	succinate	−0.55	+0.92	−0.91			−0.78		
43	fumarate		+0.60	−0.97		+0.78			−0.72
32	ascorbate	+0.91	−0.88		+0.79	−0.94		−0.65	+0.65
24	asparagine	+0.96		−0.65	+0.70		+0.54		−0.73
22	β-alanine		+0.92		−0.63		+0.68		+0.91
28	glycine		+0.603	+0.598		−0.838	−0.781		+0.545
51	homocarnosine	−0.92	+0.89	−0.79	+0.76				+0.95
48	histidine	−0.630	+0.889		+0.544	+0.773	+0.632	−0.541	+0.641
44	dopamine				+0.581	−0.850	−0.796		+0.872
45	hydroxykynurenine	−0.956	+0.547	−0.520					+0.775
46	tyrosine	−0.965		−0.611	−0.901	−0.913			+0.984
49	phenylalanine	−0.580			−0.798				+0.963
50	tryptophan	−0.910		−0.624					+0.817
5	lactate	−0.542		−0.558					+0.814
33	glucose	−0.756							+0.901
35	trehalose			−0.673	+0.895	+0.704	−0.585	−0.614	−0.962
36	glycogen	−0.572			−0.911	−0.842			
37	UDP-glucose	−0.74	+0.71			−0.68			+0.93
18	pyruvate	−0.54			−0.77	−0.80	−0.78		+0.92
25	choline	−0.870	+0.575			+0.630	+0.579		+0.820
26	phosphorylcholine	+0.957	−0.966	+0.977	−0.569		+0.881	+0.690	+0.981
27	betaine	−0.674	+0.777		−0.889	−0.745		−0.863	+0.956
40	uridine	−0.73	+0.84	−0.66	−0.79				+0.98
41	cytidine			+0.66	−0.78	−0.53			

keys	metabolites	1d4I/3d3I	4d4I/1d4I	1d5I/4d4I	3d5I/1d5I	5d5I/3d5I	7d5I/5d5I	8d5I/7d5I	pP/8d5I
39	xanthosine		-0.75	+0.90	-0.73	-0.65			+0.87
55	CMP	-0.54	+0.52		-0.52				+0.92

^aThese coefficients were obtained from OPLS-DA results.

^bMetabolite keys are in Table 1.

^cPositive and negative signs indicate the elevation and decrease of the metabolites. Only these with significant difference were tabulated in this Table. For stage labels, the first number denotes the day while second number the instar (e.g., 4d4I means day 4 in the fourth instar); pP, prepupa.

Table 3 Developmental Changes for Silkworm Hemolymph Fatty Acids ($\mu\text{mol/L}$)^{ab}

FAs	3d3I	1d4I	4d4I	1d5I	3d5I	5d5I	7d5I	8d5I	pP
C16:0	1.07±0.10	0.68±0.03	1.32±0.05	0.69±0.04	0.62±0.05	0.59±0.03	0.72±0.01	0.64±0.11	1.79±0.07
<i>p</i> ^a		(0.032)	(0.000)	(0.014)	(0.248)	(0.579)	(0.009)	(0.501)	(0.000)
C17:1	0.02±0.00	0.03±0.00	0.03±0.00	0.03±0.00	0.02±0.00	0.02±0.00	0.02±0.00	0.01±0.00	0.02±0.00
<i>p</i>		(0.207)	(0.457)	(0.071)	(0.149)	(0.386)	(0.083)	(0.009)	(0.048)
C18:0	0.73±0.07	0.38±0.01	0.95±0.04	4.52±0.01	0.48±0.03	0.47±0.02	0.53±0.01	0.58±0.057	1.91±0.03
<i>p</i>		(0.012)	(0.000)	(0.000)	(0.365)	(0.811)	(0.096)	(0.455)	(0.000)
C18:1n9	0.45±0.05	0.27±0.01	0.65±0.12	0.56±0.01	0.22±0.01	0.24±0.01	0.36±0.02	0.39±0.07	1.66±0.08
<i>p</i>		(0.010)	(0.034)	(0.516)	(0.000)	(0.234)	(0.006)	(0.683)	(0.000)
C18:2n6	0.65±0.07	0.31±0.01	0.59±0.03	0.35±0.01	0.20±0.01	0.20±0.01	0.24±0.01	0.22±0.03	1.03±0.03
<i>p</i>		(0.004)	(0.000)	(0.000)	(0.000)	(0.764)	(0.078)	(0.672)	(0.000)
C18:3n3	2.34±0.26	1.03±0.70	3.06±0.15	1.20±0.05	1.21±0.09	1.38±0.14	1.68±0.04	1.46±0.16	4.41±0.09
<i>p</i>		(0.011)	(0.000)	(0.000)	(0.933)	(0.386)	(0.248)	(0.286)	(0.000)
C20:0	0.04±0.00	0.03±0.00	0.05±0.00	0.04±0.00	0.03±0.00	0.03±0.00	0.03±0.00	0.02±0.00	0.07±0.00
<i>p</i>		(0.011)	(0.001)	(0.002)	(0.102)	(0.122)	(0.219)	(0.112)	(0.000)
C20:3n3	0.01±0.00	0.003±0.00	0.01±0.00	0.004 ±0.00	0.01±0.00	0.01±0.00	0.01±0.00	0.01±0.00	0.03±0.00
<i>p</i>		(0.029)	(0.000)	(0.000)	(0.067)	(0.235)	(0.021)	(0.086)	(0.000)
C20:5n3	0.01±0.00	0.02±0.00	0.01±0.00	0.02±0.00	0.02±0.00	0.01±0.00	0.01±0.00	0.01±0.00	0.02±0.00
<i>p</i>		(0.002)	(0.115)	(0.022)	(0.902)	(0.150)	(0.296)	(0.162)	(0.015)
C22:0	0.01±0.00	0.01±0.00	0.01±0.00	0.01±0.00	0.02±0.00	0.01±0.00	0.01±0.00	0.01±0.00	0.02±0.00
<i>p</i>		(0.734)	(0.385)	(0.773)	(0.023)	(0.161)	(0.734)	(0.032)	(0.000)
ToFA ^b	5.35±0.53	2.76±0.04	6.70±0.21	3.36±0.07	2.84±0.16	2.98±0.21	3.61±0.05	3.36±0.43	10.95±0.21
<i>p</i>		(0.012)	(0.000)	(0.000)	(0.250)	(0.386)	(0.083)	(0.587)	(0.000)
SFA	1.85±0.17	1.10±0.041	2.34±0.05	1.20±0.05	1.16±0.08	1.11±0.05	1.29±0.02	1.25±0.16	3.78±0.09
<i>p</i>		(0.020)	(0.000)	(0.014)	(1.000)	(0.624)	(0.022)	(0.833)	(0.000)
UFA	3.49±0.38	1.65±0.07	4.36±0.20	2.17±0.05	1.68±0.11	1.87±0.16	2.32±0.03	2.11±0.26	7.17±0.13
<i>p</i>		(0.013)	(0.000)	(0.000)	(0.008)	(0.564)	(0.083)	(0.464)	(0.000)
PUFA	3.01±0.33	1.36±0.08	3.68±0.17	1.57±0.06	1.44±0.11	1.60±0.16	1.94±0.05	1.70±0.20	5.49±0.11
<i>p</i>		(0.011)	(0.000)	(0.000)	(0.298)	(0.386)	(0.248)	(0.331)	(0.000)
n3/n6	3.62±0.04	3.43±0.16	5.26±0.23	3.53±0.09	6.222±0.14	6.81±0.22	7.07±0.20	6.75±0.32	4.34±0.10
<i>p</i>		(0.071)	(0.001)	(0.001)	(0.000)	(0.066)	(0.419)	(0.461)	(0.000)

^a *P*-values were obtained from the content of fatty acids of silkworm hemolymph compared with the previous adjacent time point. Red colored values indicate significant increases, whereas blue ones mean significant decreases, and black ones indicate no significant change.

^b ToFA, SFA, UFA, and PUFA were abbreviation of total fatty acids, saturated fatty acids, unsaturated fatty acids, and polyunsaturated fatty acids, respectively. n3/n6 was the ratio for n3- and n6-type PUFA. For stage labels, the first number denotes the day while second number the instar (e.g., 4d4I means day 4 in the fourth instar); pP, prepupa.