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Effect of black soldier fly (*Hermetia illucens*) larvae meal on lipid and glucose metabolism of Pacific white shrimp *Litopenaeus vannamei*

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Abstract

The present study investigated the effect of black soldier fly (*Hermetia illucens*) larvae meal (BSF) on haemolymph biochemical indicators, muscle metabolites as well as the lipid and glucose metabolism of Pacific white shrimp *Litopenaeus vannamei*. Four diets were formulated in which the control diet contained 25 % of fishmeal (FM) and 10 % (BSF10), 20 % (BSF20), and 30 % (BSF30) of FM protein were replaced with BSF. Four hundred and eighty shrimp (0·88 ± 0·00 g) were distributed to four groups of three replicates and fed for 7 weeks. Results showed that growth performance of shrimp fed BSF30 significantly decreased compared with those fed FM, but there was no significant difference in survival among groups. The whole shrimp crude lipid content, haemolymph TAG and total cholesterol were decreased with the increasing BSF inclusion. The results of metabolomics showed that the metabolite patterns of shrimp fed different diets were altered, with significant changes in metabolites related to lipid metabolism, glucose metabolism as well as TCA cycle. The mRNA expressions of *hk*, *pfk*, *pk*, *pepck*, *ampk*, *mcd*, *cpt-1* and *scd1* in hepatopancreas were downregulated in shrimp fed BSF30, but mRNA expression of *acc1* was upregulated. Unlike BSF30, the mRNA expressions of *fas*, *cpt-1*, *fbp* and *6pgd* in hepatopancreas were upregulated in shrimp fed BSF20. This study indicates that BSF20 diet promoted lipid synthesis and lipolysis, while BSF30 diet weakened *β*-oxidation and glycolysis as well as affected the unsaturated fatty acids synthesis, which may affect the growth performance and body composition of shrimp.

Key word: ***Litopenaeus vannamei*: Black soldier fly: Fishmeal replacement: Lipid metabolism: Glucose metabolism**

The Pacific white shrimp *Litopenaeus vannamei* has become the world's most productive shrimp species because of its fast growth rate, high survival rate and good economic benefits[1,2]. With fishmeal (FM) accounting for more than 25 % of shrimp diet formula, increasing demand and rising cost for FM have brought attention to possible alternatives[3,4]. During the last two decades, insect proteins are considered a potentially sustainable alternative to FM[5,6]. As a scavenger insect produced from organic waste, the black soldier fly (*Hermetia illucens*) larvae meal (BSF) typically contains 30–58 % crude protein (CP) and 11 %-40 % crude lipid (CL)[7]. Both BSF and defatted BSF have been studied in aquatic animals. Previous studies have shown that the dietary replacement of FM with BSF can reach 75 % in African catfish (*Clarias gariepinus*)[8], 19·5 % in European seabass (*Dicentrarchus labrax*)[9] as well as 68 % in yellow catfish (*Pelteobagrus fulvidraco*)[10] without negative effects on the growth performance. Furthermore, replacing 50 % of FM with BSF in diets improved the intestinal microbiota of rainbow trout (*Oncorhynchus mykiss*)[11] and replacing 100 % of FM with BSF in diets did not impair the intestinal health of Atlantic salmon (*Salmo salar*)[12]. In terms of defatted BSF, previous studies have also suggested that there were no negative effects on the growth performance when 50 % dietary FM was replaced with defatted BSF in rainbow trout[13] and Jian carp (*Cyprinus carpio var.* Jian)[14]. In Japanese seabass (*Lateolabrax japonicus*), 64 % dietary FM could be replaced by defatted BSF to achieve the same results[15]. Also, BSF has been evaluated on *Litopenaeus vannamei* in previous studies. In shrimp diets, dietary BSF that

Abbreviations: AMPK, 5′-AMP-activated protein kinase; BSF, black soldier fly larvae meal; CL, crude lipid; CP, crude protein; G6p, glucose-6-phosphate; OPLS-DA, orthogonal partial least-squares discriminant analysis; T-CHO, total cholesterol; qPCR, quantitative PCR.

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replaced 25 % of FM had no adverse effect on growth, while defatted BSF, replacing 60 % of FM, can achieve the same result[16,17].

Previous study indicated that lipid content of BSF may differ substantially from that of FM[18]. Compared with FM, the fatty acid composition of BSF is higher in lauric acid (C12:0), oleic acid (C18:1*n*-9) and linoleic acid (C18:2*n*-6), but lower in arachidonic acid (ARA, C20:4*n*-6) and remarkably lower in DHA (C22:6*n*-3) and EPA (C20:5*n*-3) content. As a novel ingredient, it is necessary to investigate how the composition of BSF affects the growth and physiological status of shrimp. Lipid functions in maintaining the structural integrity of cell membranes and β-oxidation to provide energy for molting and growth[19–21]. Closely related to lipid metabolism, glucose metabolism also serves as the main energy provider in the organism. Glycolysis is an important way for animals to use glucose to produce energy and is usually activated when shrimp are lacking energy[22]. Nutritional metabolomics have revealed that several metabolic pathways including glycolysis, lipolysis and lipid synthesis were affected by dietary protein[23–25] and lipid sources[26–28].

In addition to some classical parameters used to evaluate the effects of a novel ingredient on metabolism, metabolomics provides greater insight into the effects of such ingredients on animals through the identification and screening of small-molecule metabolites. Combining metabolomics tools with classical biological techniques such as real-time quantitative PCR (qPCR) analysis seems to decipher the metabolic mechanisms involved in the phenotypic response[29]. The use of metabolomic approaches to study the effects of dietary BSF on aquatic animals is rarely reported. A previous study has shown that BSF protein extracts provide high levels of free amino acids and peptides, which increase glutamate and glutamine levels in the liver, reduce the accumulation of essential free amino acids in muscle and alanine cycling, and provide substrates for protein anabolism and associated energy requirements in rainbow trout[29]. However, the study of dietary BSF on metabolism of *Litopenaeus vannamei* has not been reported until now. Therefore, the present study was conducted to investigate the effects of FM replacement by BSF on the lipid and glucose metabolism of *Litopenaeus vannamei*.

Material and methods

Ethics statement

This study was carried out following the recommendations of Care and Use of Laboratory Animals in China, Animal Ethical and Welfare Committee of China Experimental Animal Society. The protocol was approved by the Animal Ethical and Welfare Committee of Guangdong Ocean University (Guangdong, China).

Diet preparation

FM, soyabean meal and peanut meal were used as the main protein sources, and fish oil, soyabean oil and soya lecithin were used as the main lipid sources to formulate the basal diets (FM), and then 10 % (BSF10), 20 % (BSF20) and 30 % (BSF30) of the FM protein in the basal diets were replaced by BSF to formulate the three experimental diets. The BSF used in this study was provided by Guangzhou Fishtech Biotechnology Co., Ltd, containing 35·17 % of CP and 32·60 % of CL. Fish oil, soyabean oil and soya lecithin levels in the diets were adjusted to balance the CL content of each diet. Several essential amino acids were supplemented to the BSF diets to obtain a similar amino acid composition as FM diet. The ingredients are ground through an 80-mesh screen, weighed and mixed with a mixer (M-256, South China University of Technology), then oil and distilled water are added to the mixture in sequence. The 1·0- and 1·5-mm diameter pellets were produced by a pelletizer (Institute of Chemical Engineering, South China University of Technology) and then heated at 60°C for 30 min. After air-drying to a moisture content of about 10 %, the feed is stored in a –20°C refrigerator before use. The formulation of experimental diets was shown in Table 1.

Table 1. Formulation and proximate composition of experimental diets (% DM)

	Diets			
Ingredients	FM	BSF10	BSF20	BSF30
Brown FM*	25·00	22·50	20·00	17·50
Soyabean meal	25·00	25·00	25·00	25·00
Peanut meal	10·00	10·00	10·00	10·00
Wheat flour	24·05	22·93	21·82	20·70
Beer yeast	3·00	3·00	3·00	3·00
Shrimp shell meal	5·00	5·00	5·00	5·00
Black soldier fly larvae meal†		4·75	9·50	14·25
Fish oil	2·00	1·45	0·90	0·35
Soyabean oil	2·00	1·45	0·90	0·35
Soyabean lecithin	1·00	0·85	0·70	0·55
Choline chloride	0·30	0·30	0·30	0·30
Vitamin and mineral premix‡	1·00	1·00	1·00	1·00
Calcium monophosphate	1·50	1·50	1·50	1·50
Vitamin C	0·10	0·10	0·10	0·10
Methionine§		0·04	0·07	0·11
Lysine§		0·08	0·16	0·24
Threonine§				0·01
Ethoxyquin	0·05	0·05	0·05	0·05
Proximate composition				
DM (%)	92·01	92·47	92·71	92·62
Crude protein (%)	41·42	41·95	42·51	42·31
Crude lipid (%)	7·79	7·11	7·04	7·29
Ash (%)	10·73	11·01	11·10	11·67
Cholesterol (%)	0·12	0·10	0·09	0·07
Phospholipid (%)	1·42	1·49	1·56	1·75

FM, fishmeal; BSF, black soldier fly larvae meal.
* Brown fishmeal: 68·21 % crude protein, 9·00 % crude lipid, bought from Zhanjiang HaiBao Feed Co. Ltd.
† Black soldier fly larvae meal: 35·17 % crude protein, 32·60 % crude lipid, provided by Guangzhou Fishtech Biotechnology Co., Ltd.
‡ Vitamin and mineral premix was provided by Beijing Enhalor Biotechnology Co., Ltd.
§ Amino acids were bought from Shanghai Sanjie Biotechnology Co., Ltd.

Shrimp rearing and experimental conditions

Obtained from the nursery base of Guangdong Yuehai Seed Co., Ltd (Zhanjiang, China), juvenile *Litopenaeus vannamei* were fed commercial feed (Guangdong HAID Group, CP 48·0 %, CL 8·0 %) and acclimatised for 1 week. At the beginning

of the experiment, 480 shrimp with an average body weight of 0·88 g were equally distributed to four groups with triplicate cylindrical fibreglass tanks (300 l) per group and 40 shrimp in each tank. Shrimp were fed different diets four times daily at 7:20, 11:20, 16:00 and 21:00 with 5–8 % of body weight to apparent satiation. During the 7-week feeding trial, the water temperature fluctuated from 27 to 31°C and salinity was in a range of 25–30 ‰.

Sample collection and analysis

After a 7-week feeding trial, shrimp were fasted for 24 h before sampling. The shrimp in each tank were counted and weighted. Four shrimp were collected from each tank and stored at –20°C to analyse the whole shrimp composition. Haemolymph was collected from twelve shrimp using a 1-ml sterile syringe and placed in 1·5-ml tubes and then stored at 4°C for 16 h; the supernatant was collected by centrifugation (8000 rpm/5867 g, 5 min, 4°C) and stored at –80°C to determine haemolymph biochemical parameters. Hepatopancreas from three shrimp per tank were collected intact and immediately frozen at –80°C for RNA extraction. Samples of muscle (eight samples per tank and two shrimp per sample) were washed with PBS, blotted dry, and immediately placed in liquid N_2 and stored at –80°C before the ultra high performance liquid chromatography (UHPLC)-Q/time of flight (TOF)-MS analysis.

The CP was determined by the Dumas Nitrogen method with a Primacs100 analyzer (Skalar): after full combustion of the feed, the nitrogen oxides are reduced to nitrogen (CP = total-N × 6·25). The CL was determined by the ether extraction method with an XT15 extractor (Ankom): weight reduction of feed after extraction by petroleum ether. The moisture was determined by oven drying at 105°C: weight reduction of feed after drying. Amino acids, fatty acids, cholesterol and phospholipid contents of different diets were determined with the help of Willtest Technology Co. Ltd, Sichuan, China. Briefly, the amino acids profiles of diets were measured by an automatic amino acid analyzer 433D (Sykam) after acid hydrolysis in 6 M HCl for 24 h at 110°C. The fatty acid composition of the diets was analysed by GC (7890B GC System, Agilent) after the following processes: the lipid in the feed was extracted through hydrolysis in 8·3 M HCl and extraction by diethyl ether/petroleum ether (1:1, v/v), and the lipid was saponified by 2 % sodium hydroxide methanol solution and methyl esterified under alkaline conditions. The cholesterol contents of diets were measured by GC (UltiMate™ 3000 RSLCnano System, Thermo) using nitrogen as the mobile phase after chloroform extraction of cholesterol from the sample. The phospholipid contents of diets were measured by a colorimetric method: phospholipids in the diets were extracted by chloroform/methanol (2:1, v/v), cauterised and then dissolved by 6 M HCl to phosphoric acid. The phosphoric acid was reduced to molybdenum blue by 0·015 % hydrazine sulphate after reaction with 2·5 % sodium molybdate. The molybdenum blue products were measured at a wavelength of 650 nm, and the phospholipid content was calculated by a standard curve. The proximate composition of the diets is shown in Table 1, the fatty acid composition of the diets is shown in Table 2 and the amino acid composition of the diets is shown in Table 3.

Table 2. Fatty acid compositions of different diets (% of total fatty acids)

	Diets			
Fatty acids	FM	BSF10	BSF20	BSF30
12:0	0·06	3·59	6·99	10·20
14:0	2·19	2·86	3·57	3·87
15:0	0·24	0·25	0·25	0·25
16:0	16·60	17·90	19·20	20·60
18:0	5·01	4·77	4·58	4·43
20:0	0·44	0·39	0·35	0·32
ΣSFA	24·54	29·76	34·95	39·68
16:1*n*-7	2·98	2·98	3·03	2·98
18:1*n*-9	14·70	16·40	17·90	19·60
22:1*n*-9	0·11	0·10	0·06	0·04
ΣMUFA	17·79	19·48	20·99	22·62
18:2*n*-6	29·20	27·20	25·40	24·00
20:4*n*-6	0·78	0·70	0·59	0·50
ΣPUFA *n*-6	29·98	27·90	25·99	24·50
18:3*n*-3	3·94	3·52	3·02	2·68
20:5*n*-3	11·30	8·94	6·60	4·20
22:6*n*-3	8·95	7·23	5·52	3·73
ΣPUFA *n*-3	24·19	19·69	15·14	10·61
n-3/*n*-6	0·81	0·71	0·58	0·43

FM, fishmeal; BSF, black soldier fly larvae meal.

Table 3. Amino acid composition of different diets (% DM)

	Diets			
Amino acids	FM	BSF10	BSF20	BSF30
Aspartic acid	3·79	3·91	3·78	3·60
Threonine	1·55	1·61	1·54	1·44
Serine	1·75	1·84	1·76	1·66
Glutamic acid	6·89	7·14	6·87	6·48
Glysine	1·92	1·97	1·91	1·87
Alanine	1·98	2·05	1·99	1·96
Cystine	0·46	0·46	0·47	0·43
Valine	1·87	1·98	1·84	1·83
Methionine	0·64	0·66	0·63	0·60
Isoleucine	1·58	1·64	1·57	1·50
Leucine	2·87	2·98	2·84	2·73
Tyrosine	1·03	1·13	1·27	1·15
Phenylalanine	1·78	1·85	1·73	1·69
Lysine	2·42	2·44	2·45	2·41
Histine	1·01	0·98	0·95	0·96
Arginine	2·59	2·65	2·71	2·49
Proline	1·75	1·90	1·81	1·83

FM, fishmeal; BSF, black soldier fly larvae meal.

Haemolymph biochemical parameters

TAG (Item No. A110–1–1), total cholesterol (T-CHO, Item No. A111–1–1), LDL-cholesterol (Item No. A113–1–1) and HDL-cholesterol (Item No. A112–1–1) contents were detected by commercial kits (Nanjing Jiancheng Bioengineering Institute) and glucose were quantified by a kit (Item No. 361 510) (Shanghai Rongsheng Biotech Co., Ltd) following the instructions, through monitoring the absorbance changes by a full-wavelength microplate reader (Thermo, Multiskan GO 1510).

UHPLC-Q/TOF-MS analysis

Muscle samples of FM, BSF20 and BSF30 were selected for the metabolomics analysis. The process was performed on ice. 100 mg of muscle samples were ground with 1000 µl of cold

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methanol and adequately vortexed. The lysate was homogenised by MP homogenizer (24 × 2, 6·0 M/S, 60 s, twice) and sonicated at low temperature (4°C, 30 min/once, twice). The mixture was centrifuged for 20 min (17 500 rpm/14000 g, 4°C). The supernatant was dried in a vacuum centrifuge and re-dissolved in 100 µl of acetonitrile/water (1:1, v/v) solvent for LC-MS analysis. Analyses were performed using a UHPLC (1290 Infinity LC, Agilent Technologies) coupled to a quadrupole time-of-flight (AB Sciex TripleTOF 6600) in Shanghai Applied Protein Technology Co., Ltd. For hydrophilic interaction chromatography separation, samples were analysed using a 2·1 mm × 100 mm ACQUIY UPLC BEH 1·7 µm column (Waters). In both electron spray ionization (ESI)-positive and -negative modes, the mobile phase contained A = 25 mM of ammonium acetate and 25 mM of ammonium hydroxide in water and B = acetonitrile. The gradient was 85 % B for 1 min and was linearly reduced to 65 % in 11 min, and then was reduced to 40 % in 0·1 min and kept for 4 min, and then increased to 85 % in 0·1 min, with a 5-min re-equilibration period employed. For reversed-phase liquid chromatography separation, a 2·1 mm × 100 mm ACQUIY UPLC HSS T3 1·8 µm column (Waters) was used. In ESI-positive mode, the mobile phase contained A = water with 0·1 % formic acid and B = acetonitrile with 0·1 % formic acid; and in ESI-negative mode, the mobile phase contained A = 0·5 mM ammonium fluoride in water and B = acetonitrile. The gradient was 1 % B for 1·5 min and was linearly increased to 99 % in 11·5 min and kept for 3·5 min. Then it was reduced to 1 % in 0·1 min and a 3·4 min of re-equilibration period was employed. The gradients were at a flow rate of 0·3 ml/min, and the column temperatures were kept constant at 25°C. A 2-µl aliquot of each sample was injected. The ESI source conditions were set as follows: Ion Source Gas1 (Gas1) as 60, Ion Source Gas2 (Gas2) as 60, curtain gas (CUR) as 30, source temperature: 600°C, IonSpray Voltage Floating (ISVF) ± 5500 V. In MS only acquisition, the instrument was set to acquire over the m/z range 60–1000 Da, and the accumulation time for TOF MS scan was set at 0·20 s/spectra. In auto MS/MS acquisition, the instrument was set to acquire over the m/z range 25–1000 Da, and the accumulation time for product ion scan was set at 0·05 s/spectra. The product ion scan is acquired using information-dependent acquisition with high sensitivity mode selected. The parameters were set as follows: the collision energy was fixed at 35 V with ± 15 eV; declustering potential, 60 V (+) and –60 V (−); exclude isotopes within 4 Da, candidate ions to monitor per cycle: 10.

The raw MS data were converted to MzXML files using ProteoWizard MS Convert version 3.0.6458 before importing into freely available XCMS software. For peak picking, the following parameters were used: centWave m/z = 25 ppm, peakwidth = c (10, 60) and prefilter = c (10, 100). For peak grouping, bw = 5, mzwid = 0·025 and minfrac = 0·5 were used. In the extracted ion features, only the variables having more than 50 % of the non-zero measurement values in at least one group were kept. Compound identification of metabolites by MS/MS spectra with an in-house database established with available authentic standards. After normalised to total peak intensity, the processed data were uploaded before importing into SIMCA-P (version 14.1, Umetrics), where it was subjected to multivariate data analysis, including orthogonal partial least-squares discriminant analysis (OPLS-DA). The sevenfold cross-validation and response permutation tests (n 200) were used to evaluate the robustness of the model. The variable importance in the projection (VIP) value of each variable in the OPLS-DA model was calculated to indicate its contribution to the classification. Metabolites with the VIP value > 1 were further applied to Student's t test at a univariate level to measure the significance of each metabolite, and the P-values less than 0·05 were considered as statistically significant.

Total RNA extraction, reverse transcription and real-time quantitative PCR

Hepatopancreas samples were used to study the metabolism-related gene expression in shrimp fed FM, BSF20 and BSF30 diets. The method of RNA extraction, reverse transcription and real-time qPCR were described as Yang *et al.*[30]. Total RNA was extracted from hepatopancreas using TransZol Up Plus RNA kit (Item No. ER501, TransGen) following the instruction. Spectrophotometric analysis (Nanodrop 2000) was used to assess RNA quality and concentration. cDNA was synthesised using an *Evo M-MLV* RT Kit with gDNA Clean for qPCR II (Item No. AG11711, Accurate Biotechnology Hunan Co., Ltd), according to the manufacturer's instructions. Briefly, gDNA was firstly removed by gDNA clean reagent and gDNA clean buffer, in which 1000 ng of RNA was used in a total of 10-µl reaction volume. The total reaction solution was incubated at 42°C for 2 min and stored at 4°C. Then, *Evo M-MLV* RTase Enzyme Mix, RT Primer Mix, RTase Reaction Buffer Mix and RNase free water were added to the reaction solution according to the given volume, and the reverse transcription process was completed through the process of 37°C for 15 min, 85°C for 5 s and 4°C for incubation.

Real-time PCR for the target genes (Table 4) was performed using a SYBR® Green Premix *Pro Taq* HS qPCR Kit II (Item No. AG11702, Accurate Biotechnology Hunan Co., Ltd) and quantified on the LightCycler 480 (Roche Applied Science) using the following program: 0·5 µM of forward and reverse specific primers, 5 µl of 2 × SYBR® Green *Pro Taq* HS Premix II, 10 ng of cDNA template and nuclease-free water to a final volume of 10 µl, denaturation step at 95°C for the 30 s, followed by forty amplification cycles of 5 s denaturation at 95°C, 30 s annealing at 60°C, followed by a melt–curve analysis and cooling to 4°C. Elongation factor 1-α (*ef-1α*) was used as the reference gene based on the previous studies[31,32]. The relative quantification method was conducted to analyse the genes expression levels using the $2^{-\Delta\Delta Ct}$ method as described by Livak and Schmittgen[33].

Calculations and statistical analysis

The growth performance was calculated as follows:

Weight gain (WG, %) = 100 × (final body weight – initial body weight)/initial body weight

Specific growth rate (SGR, % d^{-1}) = 100 × (Ln final body weight – Ln initial body weight)/t

Survival (%) = 100 × (final number of shrimp)/(initial number of shrimp)

Feed conversion ratio (FCR) = feed consumed (g)/(final body weight – initial body weight)

Table 4. Primers used for quantitative real-time PCR

Gene name	Sequence of primer (5′-3′)	GenBank no.	Product size
ampk	CCAACAGCCATCAGAGGAGG AGCCCGAGGTCTAATAGGCA	KP272117·1	100bp
srebp	ACTGAGCTCAACACCTTCCG TGCTGGTGAAGAGCTGTCTG	MG770374·1	99bp
acc1	TGCATAGAAACGGCATTGCG TTTGACACCTGAGCCAGACC	XM_027360190·1	134bp
fas	CAGGTGGAGATGCTCCTCGTGTT GGTGACTAGCTCGGCTACATGGTT	HM595630·1	126bp
scd1	TGTCTTACACCTTATCAATGGC CGTTCGTATGTTCCTCTTCGTC	XM_027374708·1	154bp
cpt-1	CAACTTCTACGGCACTGAT GTCGGTCCACCAATCTTC	XM_027361886·1	110bp
mcd	AAGACCACAGGAAGGGACCA GACACTTGAGATGCCACCCA	XM_027376735·1	114bp
hk	GGACATAGAATGGGGTGCC CCTGCGTATCTAACTTGCCG	XM_027378214·1	221bp
pfk	CTGCTGCTCAAGAAGGGTGA CGATAGTCATGTCCGTGCCA	EF102107·1	118bp
pk	CGGTCTGTAGAGATGCTGG GGCCAGTACGAATTTCAGG	EF102105·1	202bp
6pgd	GTAGTCCTTGTCGTGAGCGG TTTCCCATCCTGAACGGAGC	XM_027353839·1	94bp
pepck	ACTGAACCTCGCTTTCCAGG TCGACGAACGAACGAACCTT	AJ250829·1	87bp
fbp	ACGGACTCCATGACGTTGAC TGGCCTTGACAGCAGTTTGA	KP057246·1	112bp
pc	GGAGACGAGTTGGACACAGG TCAGCATTGCCGGACTTCAT	XM_027368506·1	125bp
g6p	GATTCACTGGAGCTGCCCTT GACCCCCAGCAACATGTGTA	XM_027351517·1	104bp
ef-1α	GTATTGGAACAGTGCCCGTG ACCAGGGACAGCCTCAGTAAG	GU136229·1	143bp

ampk, 5′-AMP-activated protein kinase; *srebp*, sterol regulatory element-binding protein; *acc1*, acetyl-CoA carboxylase 1; *fas*, fatty acid synthase; *scd1*, stearoyl-CoA desaturase; *cpt-1*, carnitine palmitoyl transferase 1; *mcd*, malonyl-CoA decarboxylase; *hk*, hexokinase; *pfk*, phosphofructokinase; *pk*, pyruvate kinase; *6pgd*, 6-phosphogluconate dehydrogenase; *pepck*, phosphoenolpyruvate carboxykinase; *fbp*, fructose 1,6-bisphosphatase; *pc*, pyruvate carboxylase; *g6p*, glucose-6-phosphatase; *ef-1α*, elongation factor 1-alpha (reference gene).

where t is the experimental duration in days.

The results were presented as the mean and standard error of the mean. The data were firstly tested for normality (Shapiro–Wilk test) and homogeneity (Levene's test). Then all data were subjected to one-way ANOVA followed by Tukey's honestly significant difference (HSD) test to determine significant differences among treatments using SPSS 21.0 (SPSS). Probability value of $P < 0{\cdot}05$ was deemed to be statistically significant.

Result

Effects of BSF on growth performance, whole body composition of shrimp

As shown in Table 5, final body weight, weight gain (WG) and specific growth rate (SGR) decreased with the increasing BSF substitution, and final body weight, WG as well as SGR of shrimp fed BSF30 diet was significantly lower than those fed FM diet ($P < 0{\cdot}05$). Feed conversion ratio (FCR) was significantly higher in shrimp fed BSF30 diet than those fed BSF20 ($P < 0{\cdot}05$) but was not significantly different from that of shrimp fed FM ($P > 0{\cdot}05$). There was no significant difference in survival among the four groups ($P > 0{\cdot}05$).

In terms of whole body composition, there were no significant changes in moisture, CP and ash among shrimp fed four diets ($P > 0{\cdot}05$). However, the CL content of whole shrimp significantly decreased with the increasing BSF substitution ($P < 0{\cdot}05$) and the CL contents of shrimp fed BSF20 and BSF30 diets were significantly lower than those fed FM diet ($P < 0{\cdot}05$).

Effect of BSF on haemolymph biochemical parameters of shrimp

As shown in Fig. 1, the glucose content of shrimp fed BSF10 diet was significantly higher than those fed FM and BSF20 diets ($P < 0{\cdot}05$), and the glucose content of shrimp fed BSF30 diet was significantly higher than those fed BSF20 diet ($P < 0{\cdot}05$). TAG contents showed a decreasing trend with the increasing BSF substitution ($P = 0{\cdot}081$). T-CHO contents significantly decreased with the increasing BSF substitution and T-CHO of shrimp fed BSF30 diet was significantly lower than those fed FM diet ($P < 0{\cdot}05$). The LDL-cholesterol contents were higher in shrimp fed BSF10 compared with those fed FM and BSF30 ($P < 0{\cdot}05$). However, the HDL-cholesterol contents were significantly lower in shrimp fed three BSF diets compared with those fed FM diet ($P < 0{\cdot}05$).

Muscle metabolites profiling of shrimp

The metabolites in muscle samples were detected in the positive (ESI+) and negative (ESI-) modes using the OPLS-DA. The score

Table 5. Growth performance and whole body composition of shrimp fed different diets (Mean values and standard errors for the mean)

	FM		BSF10		BSF20		BSF30	
Parameter	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
IBW (g)	0·88	0·001	0·88	0·002	0·88	0·001	0·88	0·002
FBW (g)	7·76^{b}	0·09	7·66^{ab}	0·19	7·55^{ab}	0·13	7·06^{a}	0·16
WG (%)	776·38^{b}	10·30	767·58^{ab}	25·00	758·11^{ab}	13·51	698·35^{a}	18·95
SGR (% d^{-1})	4·34^{b}	0·02	4·32^{ab}	0·05	4·29^{ab}	0·03	4·15^{a}	0·04
Survival (%)	93·75	1·25	96·66	3·11	94·16	8·24	100·00	0·00
FCR	1·62^{ab}	0·04	1·60^{ab}	0·03	1·55^{a}	0·00	1·70^{b}	0·04
Proximate composition								
Moisture (%)	75·82	1·80	74·24	0·37	75·21	0·66	74·93	0·45
CP (%)	18·59	1·20	18·85	0·31	17·92	0·46	18·31	0·32
CL (%)	2·13^{c}	0·07	2·06^{bc}	0·06	1·74^{ab}	0·16	1·62^{a}	0·05
Ash (%)	12·90	1·68	13·05	0·35	14·37	0·98	14·23	0·32

FM, fishmeal; BSF, black soldier fly larvae meal; IBW, initial body weight (g); FBW, final body weight (g); WG, weight gain (%); SGR, specific growth rate (% d^{-1}); FCR, feed conversion ratio; CP, crude protein; CL, crude lipid.
Data represent mean ± SEM of three replicates (n 3). Values in the same row with different letters are significantly different ($P < 0·05$) based on the Tukey' HSD test. The lack of superscript letter indicates no significant differences among treatments.

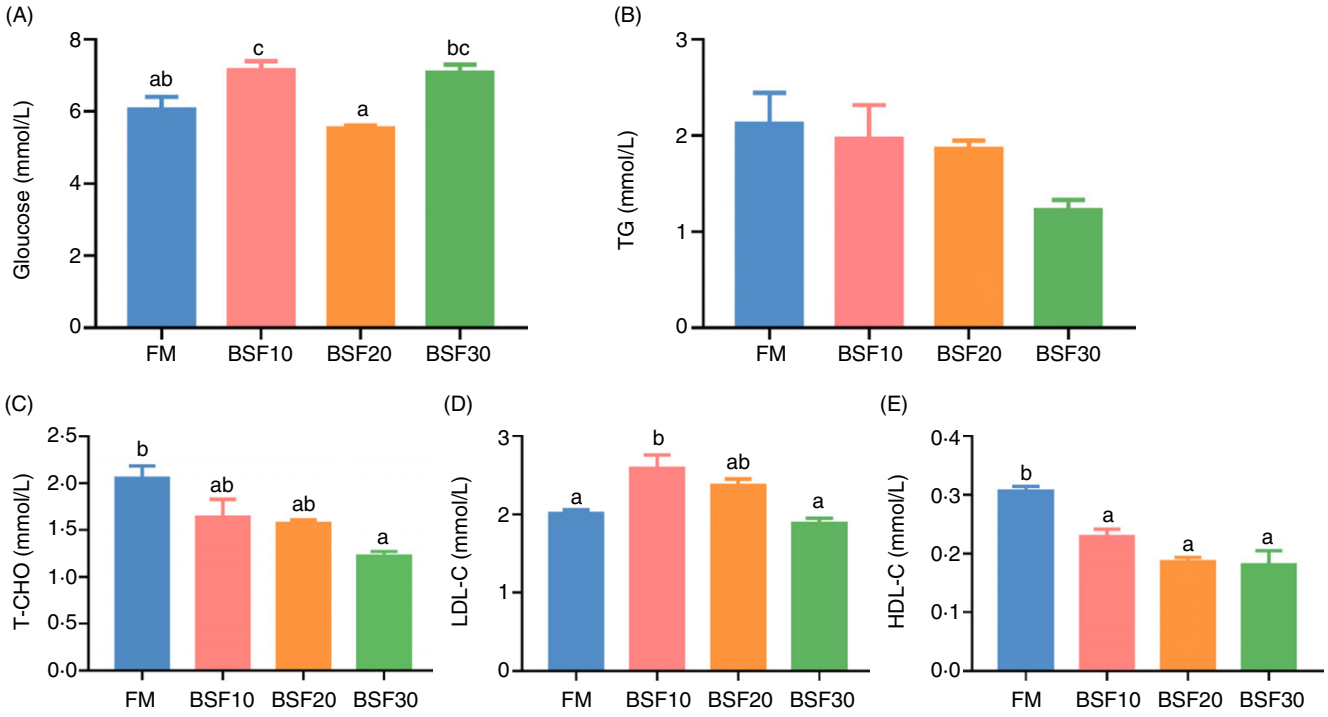

Fig. 1. Effect of black soldier fly larvae meal (BSF) on serum biochemical parameters: (a) glucose; (b) TAG; (c) T-CHO, total cholesterol; (d) LDL-cholesterol and (E) HDL-cholesterol in serum of *L. vannamei*. Vertical bars represent the mean ± SEM (n 3). Data marked with different letters differ significantly ($P < 0·05$) among groups based on the Tukey' HSD test.

plots of OPLS-DA and results of the permutation tests (n 200) are shown in Supplementary Fig. S1. The goodness of fit ($R2Y$) and goodness of prediction ($Q2$) were greater than 0·6, indicating that the OPLS-DA model had strong explanatory power for the data. The clustering effect of the samples within the same group and the separation between different groups under different patterns were good, indicating that the metabolic patterns of shrimp muscles changed significantly after feeding different diets. The $Q2$ in the permutation tests are less than 0·05, indicating that the model is robust and not overfitting. The VIP values, P-values, fold change, m/z (mass-to-charge ratio) and rt (retention time) of differential metabolites in ESI+ and ESI- were shown in Table S1 and Table S2, respectively. Heatmap based on the significantly different metabolites showed clear differences among shrimp fed FM, BSF20 and BSF30 diets (Fig. 2 and Fig. 3).

Venn diagrams demonstrated that the differential metabolites between BSF20 *v.* FM and BSF30 *v.* FM were not identical and have their characteristics (online Supplementary Fig. S2A). According to the judgement condition (VIP > 1 and P-value < 0·05), there were twenty-seven overlapped differential metabolites between BSF20 *v.* FM and BSF30 *v.* FM. Fifty-eight unique differential metabolites were identified in the BSF20 *v.* FM, while

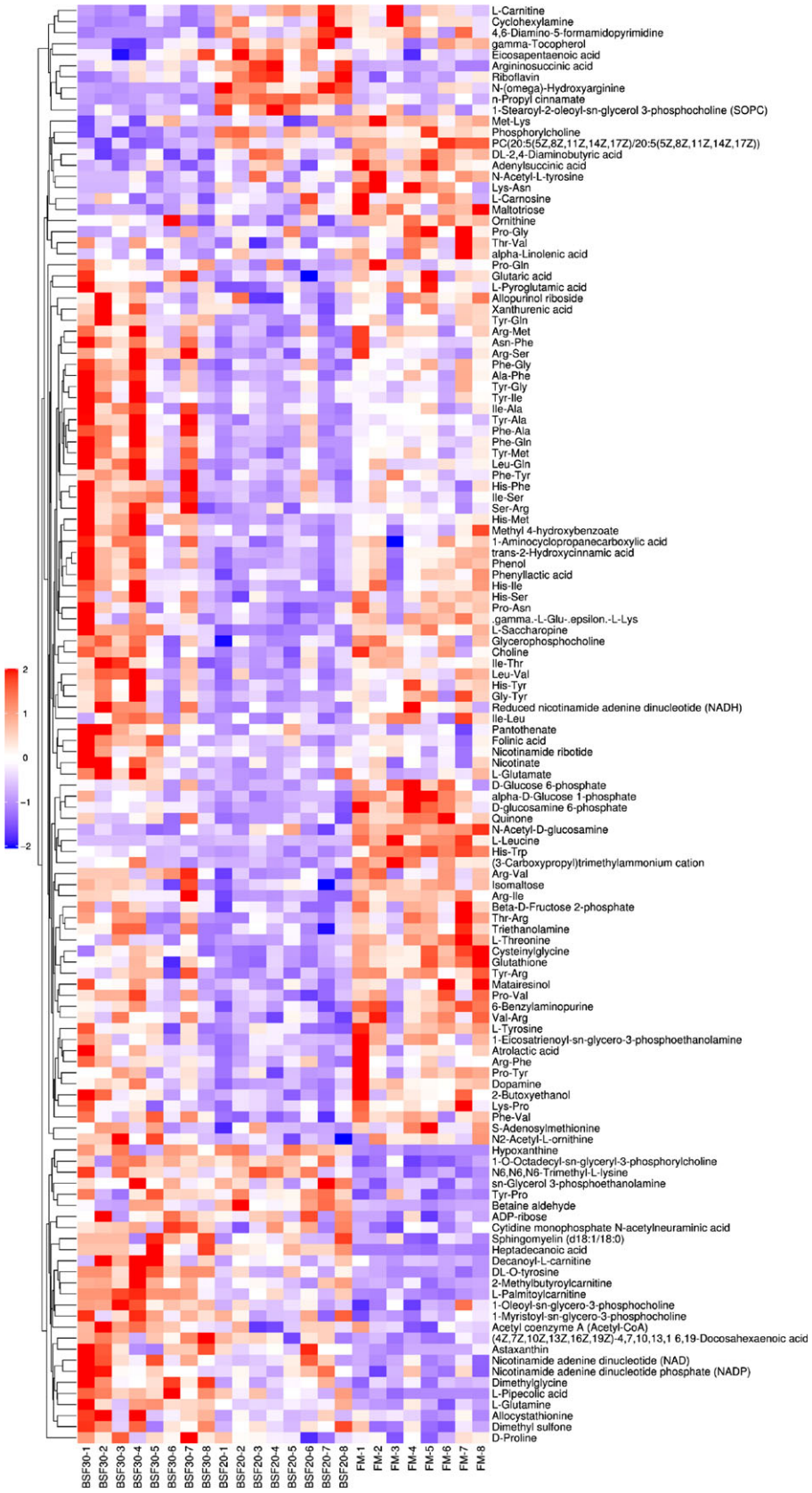

Fig. 2. The heatmap of the differential metabolites among shrimp fed FM, BSF20 and BSF30 diets in ESI+ mode. FM, fishmeal; BSF, black soldier fly larvae meal. 2; 1; 0; −1; −2.

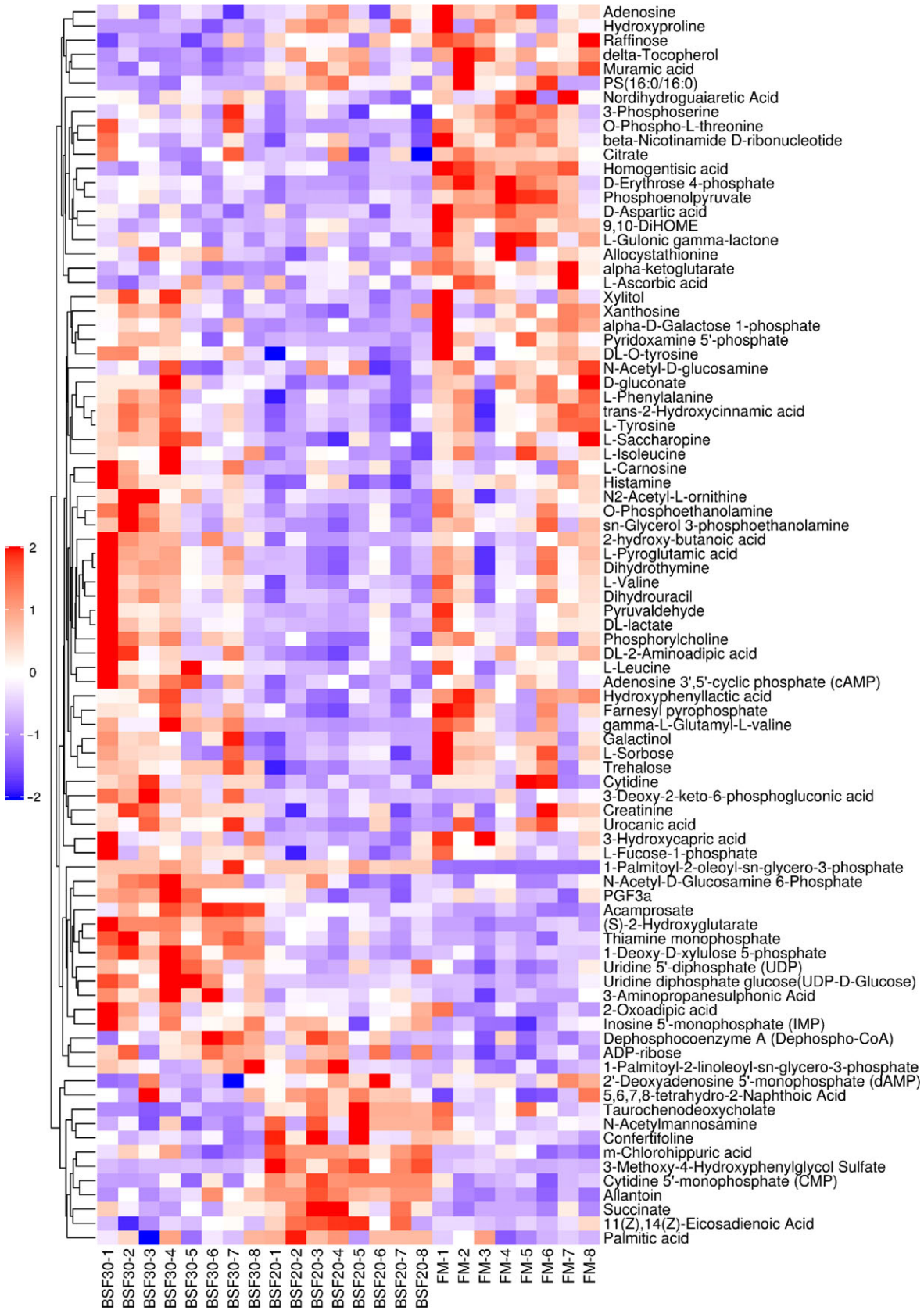

Fig. 3. The heatmap of the differential metabolites among shrimp fed FM, BSF20 and BSF30 diets in ESI- mode. FM, fishmeal; BSF, black soldier fly larvae meal. 2; 1; 0; −1; −2.

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twenty-eight unique differential metabolites were identified in the BSF30 *v.* FM. The KEGG pathway analysis of differential metabolites among FM, BSF20 and BSF30 groups was shown in Supplementary Fig. S2B.

The differential metabolites related to glucose metabolism and lipid metabolism were shown in Fig. 4. The normalised abundance of glucose-6-phosphate (G6P) and citrate of shrimp fed BSF20 significantly decreased compared with FM ($P < 0{\cdot}05$). The normalised abundance of phosphoenolpyruvate and α-ketoglutarate significantly decreased in shrimp fed BSF20 and BSF30 diets compared with FM diet ($P < 0{\cdot}05$). Normalised abundance of L-malic acid and α-linolenic acid significantly decreased in shrimp fed BSF30 diet compared with those fed FM diet, while the succinate and palmitic acid significantly increased in shrimp fed BSF20 diet compared with those fed FM diet ($P < 0{\cdot}05$).

Effect of BSF on lipid and glucose metabolism-related gene expression of shrimp

As shown in Fig. 5, there was no significant change in mRNA levels of *srebp* among the three groups ($P > 0{\cdot}05$). The mRNA levels of *ampk* and *mcd* significantly decreased with the increasing BSF inclusion ($P < 0{\cdot}05$). However, the mRNA levels of *acc1* significantly increased with the increasing BSF inclusion ($P < 0{\cdot}05$). The mRNA levels of *fas* and *cpt-1* were significantly higher in shrimp fed BSF20 diet than those fed FM and BSF30 diets ($P < 0{\cdot}05$), while the mRNA levels of *cpt-1* were significantly lower in shrimp fed BSF30 diet than those fed FM and BSF20 ($P < 0{\cdot}05$). The mRNA levels of *scd1* significantly decreased in shrimp fed BSF30 compared with those fed FM and BSF20 ($P < 0{\cdot}05$).

As shown in Fig. 6, mRNA levels of *hk*, *pk*, *pfk* and *pepck* significantly decreased in shrimp fed BSF30 diet compared with those fed FM ($P < 0{\cdot}05$). The mRNA levels of *6pgd* and *fbp* significantly increased in shrimp fed BSF20 diet compared with those fed FM diet ($P < 0{\cdot}05$). However, there were no significant differences in *g6p* and *pc* among the three groups ($P > 0{\cdot}05$).

Discussion

Research about the use of BSF as an ingredient in aquatic animals has increased, but most attention has focused on the effects of dietary insect proteins on growth and immunity[34]. In Pacific white shrimp, it was reported that most growth responses could be obtained if replacement of FM by BSF (CP 52·0 % and CL 15·1 %) was limited below 25 %, but higher replacement would impair the growth response[35]. Additionally, the growth was promoted when the proportion of BSF (CP 40·8 % and CL 31·2 %) replacing FM in the feed was less than 30 %, and the best growth performance was achieved at a substitution amount of 15 % in *Litopenaeus vannamei*[16]. However, dietary defatted BSF (CP 42·2 % and CL 9·1 %) could replace 60 % of FM without negative effects on growth performance and immunity of *Litopenaeus vannamei*[17]. The appearance of different results may be due to the different pre-treatment methods of BSF in different studies and also suggested that the lipid content and composition of BSF may be a key factor limiting its use in shrimp diets. In the present study, the lipid content of BSF was 32·60 %, which was higher than the previous studies and the BSF was not subjected to processing steps such as lipid extraction. Our results revealed that replacement of 20 % FM with BSF (9·5 % of the diet) had no negative effects on the growth performance, but replacement of 30 % FM with BSF (14·25 % of the diet) would reduce the growth responses of shrimp, which was consistent with the study of Hu *et al.*[16].

With the increasing replacement levels of FM, the CL contents of whole shrimp were decreased, which is consistent with the results reported by Cummins *et al.*[35] and also found in rice field eel (*Monopterus albus*)[36]. Moreover, TAG, T-CHO, LDL-cholesterol and HDL-cholesterol are important haemolymph biochemical parameters of lipid metabolism status[21]. In the present study, TAG, T-CHO and HDL-cholesterol contents decreased with the BSF inclusion, which was consistent with the CL of the whole shrimp. In contrast, LDL-cholesterol content increased in shrimp fed BSF10. On the one hand, under the present experimental conditions, the content of SFA and MUFA in the diets increased and the content of PUFA decreased with the increasing BSF inclusion levels, which may affect the growth and lipid utilisation of shrimp. A previous study showed that elevated SFA content in diets did reduce CL in whole shrimp and decreased growth performance and survival[19]. The increase in SFA, especially lauric acid, may increase the use of fatty acids for oxidative breakdown rather than storage. In a previous study, dietary BSF reduced the liver TAG in *Salmo salar*, possibly due to the rapid oxidation and low deposition of lauric acid[37]. In addition to lauric acid, palmitic acid also makes up about one-fifth of the lipid composition in BSF and is likely to enter the mitochondria and break down to produce energy in *Penaeus monodon* and *Litopenaeus vannamei*, which may affect the lipid content and composition[38,39]. Actually, the oil extracted from black soldier fly larvae has also been studied on rainbow trout and Jian carp[40,41] and reduces abdominal lipid deposition and decreasing adipocyte size in carp by upregulating PPARα expression, which further implied that dietary BSF may affect the lipid metabolism. On the other hand, because of the extremely high lipid content of BSF, the addition of fish oil, soyabean oil and soya lecithin to the diets had to be reduced in order to keep overall lipid levels similar in each diet, which might result in a deficiency of cholesterol for the shrimp. In contrast, when soya lecithin addition was reduced, the phospholipid content of the diets increased with higher BSF inclusion, suggesting that BSF is a rich source of phospholipids. A previous study showed that the phospholipid content of the oil extracted from BSF was about 5·78 %[42]. Phospholipids and cholesterol are cell membrane components that function in maintaining cell function and structure. Moreover, phospholipid is a source of second messenger for cellular signal transduction and an important intermediate in lipid metabolism, and cholesterol is the precursor of steroid hormones and the major steroid in shrimp[43]. Previous studies have concluded that shrimp cannot *de novo* synthesise cholesterol[44], and the phospholipids synthesised *in vivo* generally do not meet the demand of shrimp[45]. Phospholipids and cholesterol have significant interactions in shrimp, and higher amount of phospholipids may compensate the cholesterol deficiency in shrimp. At 1·5 % and 3 % dietary phospholipids, the dietary cholesterol requirements of shrimp are 0·14 % and 0·13 %, respectively[43].

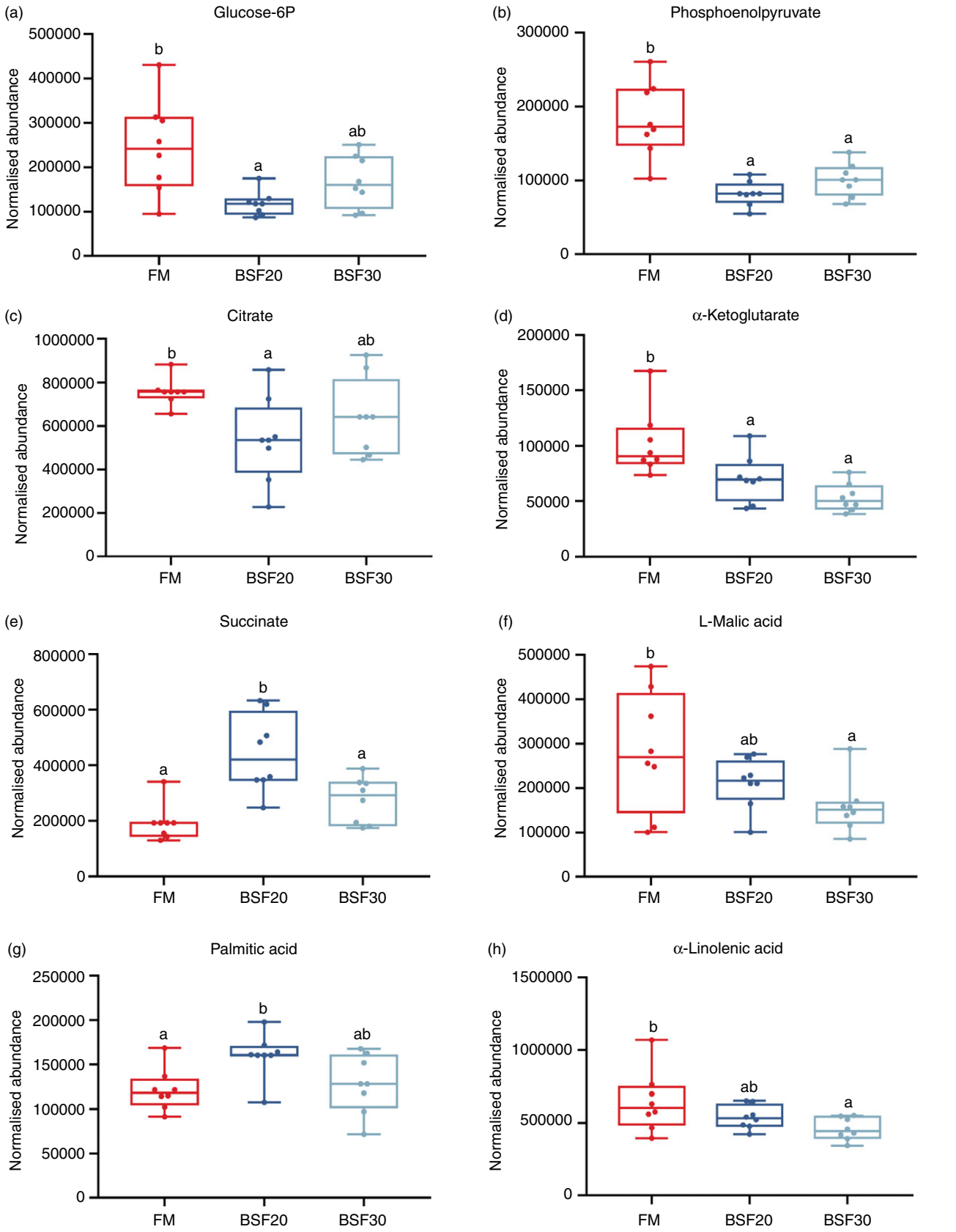

Fig. 4. Effect of black soldier fly larvae meal (BSF) on the normalised abundance of metabolites related to glucose and lipid metabolism: (a) glucose-6-phosphate, (b) phosphoenolpyruvate, (c) citrate, (d) α-ketoglutarate, (e) succinate, (f) L-malic acid, (g) palmitic acid and (h) α-linolenic acid. The *P*-values between any two groups are labelled above the box plot. *P*-values less than 0·05 represent significant differences (n 8) based on the Tukey' HSD test.

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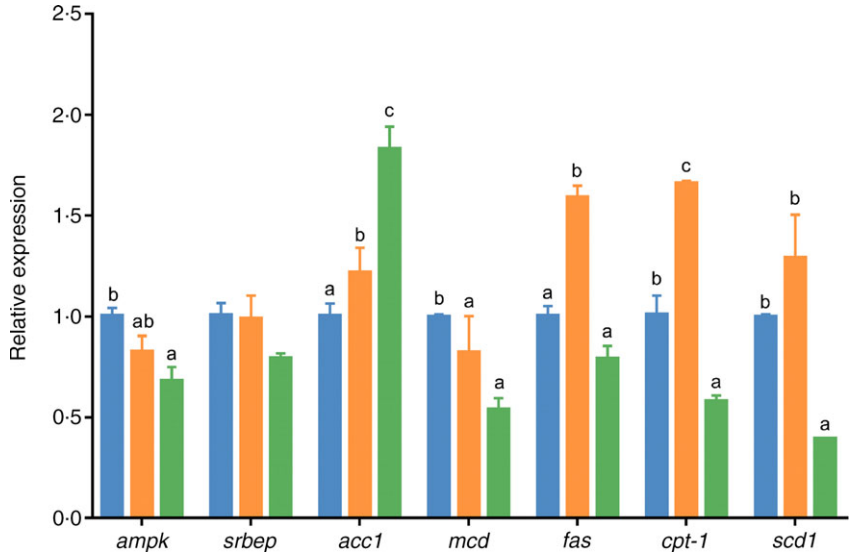

Fig. 5. Effect of black soldier fly larvae meal (BSF) on lipid metabolism gene expressions in hepatopancreas of *L. vannamei*. Vertical bars represent the mean ± SEM (n 3). Data marked with different letters differ significantly ($P < 0{\cdot}05$) among groups based on the Tukey' HSD test. FM, fishmeal; *ampk*, 5′-AMP-activated protein kinase; *srebp*, sterol regulatory element-binding protein; *acc1*, acetyl-CoA carboxylase 1; *mcd*, malonyl-CoA decarboxylase; *fas*, fatty acid synthase; *cpt-1*, carnitine palmitoyl transferase 1; *scd1*, stearoyl-CoA desaturase. ■, FM; ■, BSF20; ■, BSF30.

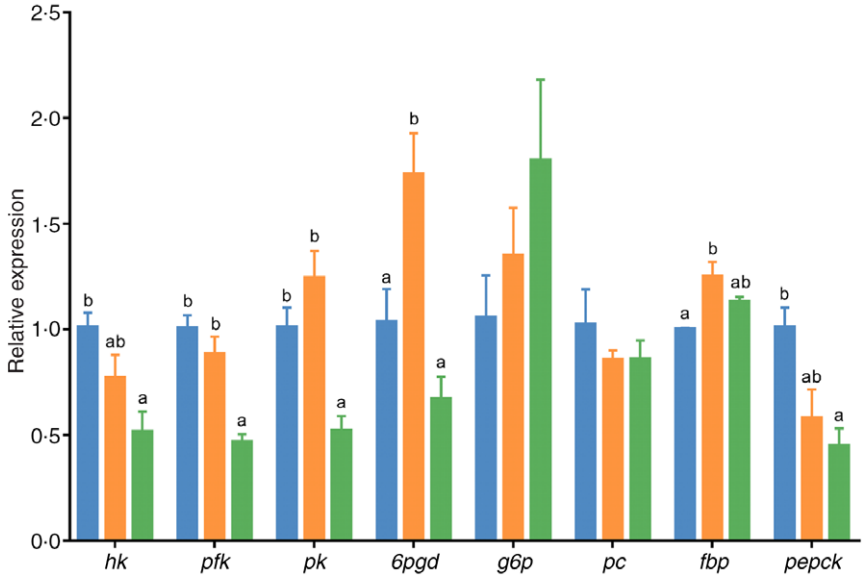

Fig. 6. Effect of black soldier fly larvae meal (BSF) on glucose metabolism gene expressions in hepatopancreas of *L. vannamei*. Vertical bars represent the mean ± SEM (n 3). Data marked with different letters differ significantly ($P < 0{\cdot}05$) among groups based on the Tukey' HSD test. FM, fishmeal, *hk*, hexokinase; *pfk*, phosphofructokinase; *pk*, pyruvate kinase; *6pgd*, 6-phosphogluconate dehydrogenase; *g6p*, glucose 6-phosphatase; *pc*, pyruvate carboxylase; *fbp*, fructose 1,6-bisphosphatase; *pepck*, phosphoenolpyruvate carboxykinase. ■, FM; ■, BSF20; ■, BSF30.

In the present study, with increasing BSF inclusion, the deficiency of cholesterol may lead to a reduction of CL in whole shrimp as well as TAG and T-CHO in the haemolymph[46]. It is also reported that cholesterol deficiencies may affect lipoprotein formation in crustaceans[47]. HDL-cholesterol plays an important role in the transport of lipids such as cholesterol, and its reduced levels may weaken the ability of shrimp to absorb and transport lipids[48], ultimately reducing TAG and T-CHO levels. Future studies would benefit in testing BSF with cholesterol supplementation.

In FM replacement experiments, the optimum replacement can help to reduce the cost of feed while achieving better growth performance, thus improving economic efficiency of farming. However, when the replacement amount exceeds the optimum value, the growth performance and physiological condition may be adversely affected. Based on the growth performance, metabolomics approaches and RT-qPCR were conducted to further investigate the effects of dietary BSF on lipid and glucose metabolism in shrimp fed FM, BSF20 and BSF30 diets (Fig. 7). Results showed that shrimp fed BSF30 exhibited a higher

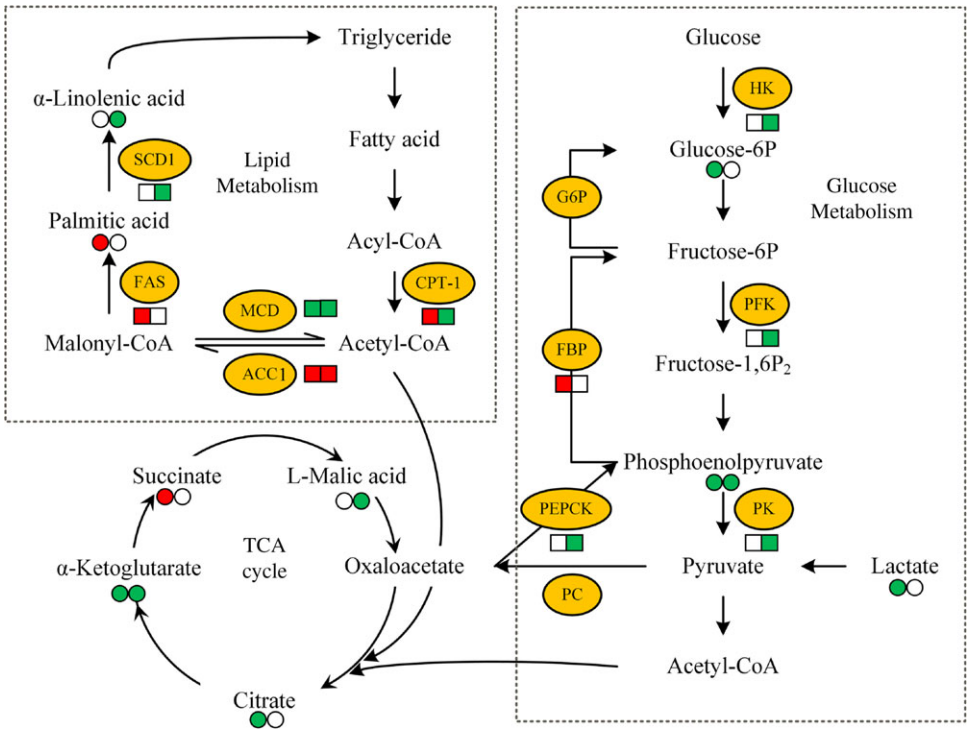

Fig. 7. Different glucose metabolism and lipid metabolism patterns of shrimp fed different diets. The squares represent comparisons of BSF20-FM and BSF30-FM in hepatopancreas gene expressions. The circles represent comparisons of BSF20-FM and BSF30-FM in differential metabolites; ○□, no significant change; ●■, decrease significantly; ●■, increase significantly. FM, fishmeal; BSF, black soldier fly larvae meal.

expression of *acc1* and lower expressions of *ampk*, *cpt-1*, *mcd* and *scd1*; however, unlike BSF30 diet, the mRNA expression of *cpt-1*, *scd1*, *fas* and *6pgd* exhibited an increasing trend in shrimp fed BSF20 diet. AMPK (5′-AMP-activated protein kinase) is associated with glucose and lipid metabolism[49,50]. In case of energy deficiency, AMPK activates fatty acid oxidation (related to *cpt-1*)[51] and glycolysis (related to *mcd*) to produce energy[52], inhibiting fatty acids synthesis (related to *acc1*, *scd1*, *6pgd* and *fas*)[53–55], whereas when sufficient dietary energy is acquired, AMPK mediates the opposite process[56]. Previous studies showed that dietary black soldier fly larvae oil increased the SFA (lauric acid and palmitic acid) in the muscles of Jian carp[41]. In addition to elevated lauric acid content, a decrease in PUFA was also found in the whole fish of zebrafish (*Danio rerio*)[57] as well as the liver of rainbow trout[58] when they were fed diets containing black soldier fly prepupae. Similar to the fatty acid composition of BSF, coconut oil is rich in lauric acid, and dietary coconut oil significantly increased the expression level of hepatic *cpt-1* gene in large yellow croaker (*Larimichthys crocea*), orange-spotted grouper (*Epinephelus coioides*) and blunt snout bream (*Megalobrama amblycephala*)[59–61] as well as upregulated the gene expressions of lipid metabolism in rat[62]. Therefore, it is speculated that the synthesis of SFA such as palmitic acid as well as the lipid oxidative catabolism is promoted in shrimp fed BSF20. On the other hand, a high amount of BSF in BSF30 diet may hinder the synthesis of MUFA and PUFA in shrimp, as verified by the changes in palmitic and α-linolenic acid contents in the metabolite results.

In addition to lipid metabolism, with increasing BSF addition, the down-regulation of *ampk* may affect the sensitivity of shrimp to glucose metabolism and energy regulation[63]. Glycolysis is the major pathway of glucose catabolism in all organisms[64]. The regulation of the glycolytic pathway is largely dependent on the activities of several rate-limiting enzymes: *hk*, *pfk* and *pk*[65–67]. In the present study, mRNA levels of *hk*, *pfk* and *pk* in the hepatopancreas decreased with increasing BSF supplementation, suggesting that the glycolytic process may be inhibited in shrimp fed with dietary BSF, which was also proved by the significant decrease of G6p and phosphoenolpyruvate in the metabolomics results. Inhibition of the glycolytic pathway may affect the downstream of TCA cycle and thus have an impact on energy production. The TCA cycle is one of the most important metabolic pathways in all aerobic organisms[68]. In the present study, a variety of intermediate metabolites associated with the TCA cycle were detected to show a decreasing trend, such as citrate, α-ketoglutarate, succinate and L-malic acid, indicating that dietary BSF disturbed the TCA cycle in shrimp. In addition, lactate is the end product of anaerobic metabolism of glucose[69,70]. DL-lactic acid was significantly reduced in the metabolomic results of shrimp fed BSF20, indicating that anaerobic metabolism of glucose was reduced. Thus, glucose catabolism, which generates energy through glycolysis and the TCA cycle, appears to be inhibited in shrimp fed BSF due to increased lipolysis. On the other hand, the role of carbohydrates may be overlooked despite the consistent CP and CL content of the feed. The reduction of wheat flour content in the feed may affect the

availability of glucose and thus the TCA cycle. Moreover, gluconeogenesis plays a major role in maintaining glucose homeostasis by regulating the synthesis of glucose from non-carbohydrate carbon substrates[54]. This process is regulated by several rate-limiting enzymes: pyruvate carboxylase (PC), phosphoenolpyruvate carboxykinase (PEPCK), fructose 1,6-bisphosphatase (FBP) and G6P[71]. In the present study, the mRNA levels of *fbp* were upregulated in shrimp fed BSF20 diet, while the mRNA levels of *pepck* were downregulated in shrimp fed BSF30. However, there were no significant differences in mRNA levels of *g6p* and *pc*. Previous studies suggested that the activity of the gluconeogenic pathway in shrimp was inversely proportional to the carbohydrate content of the feed, that is, the activity of the gluconeogenic pathway was lower in shrimp fed feeds containing higher carbohydrates[72]. Therefore, the elevated mRNA level of *fbp* is to be expected. The down-regulation of *pepck* in BSF30 is considered to be a deficiency of substrates, such as phosphoenolpyruvate and pyruvate, or possibly due to a discordance between the transcriptional and translational levels. More evidence is needed on how two enzymes affected the gluconeogenic pathway.

In conclusion, the results of gene expression, enzyme activity assays and metabolomics verified that the BSF20 diet promoted the lipid metabolism of shrimp. However, possibly due to cholesterol deficiency, a reduction in β-oxidation and glycolytic processes was found in shrimp fed BSF30 diet, as well as an effect on unsaturated fatty acid synthesis, which further affected the growth performance and body composition. Therefore, when using BSF as a replacement for large amounts of FM in shrimp diets, it must be taken into account that the high lipid content of non-defatted BSF may lead to significant changes in the composition of fatty acids in the feed. On the other hand, using defatted BSF instead of BSF may avoid disturbing the fatty acid composition of the feed in order to maximise its inclusion in shrimp diets. In addition, some essential nutrients, such as cholesterol, may also be insufficient to meet the demands of shrimp due to the reduction of FM or fish oil.

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Y. C designed the study, carried out the study, analysed the data, and drafted and revised the article. S. C assisted in the correction and developed the questions. S. Z. designed the experiment. X. D. designed the experiment and assisted in the correction. Q. Y. designed the experiment, assisted in the correction and developed the questions. H. L. designed the experiment. B. T. approved the final version to be published and agreed to be accountable for all aspects of the work. S. X. designed the experiment, revised the manuscript critically for important intellectual content and approved the final version to be published. All authors read and approved the final version of the manuscript.

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Supplementary material

For supplementary material/s referred to in this article, please visit https://doi.org/10.1017/S0007114521004670

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