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The effect of choline and cystine on the utilisation of methionine for protein accretion, remethylation and trans-sulfuration in juvenile shrimp *Penaeus monodon*

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Abstract

This 35-d feeding experiment examined in juvenile shrimp *Penaeus monodon* (3·3 g initial body weight) the effects of methionine (Met), choline and cystine on protein accretion and the activity of two key enzymes of remethylation (betaine–homocysteine methyltransferase; BHMT) and trans-sulfuration (cystathionine β-synthase; CBS). The interaction between Met and choline was tested using semi-purified diets either adequate or limiting (30 or 50 %) in total sulphur amino acid (SAA) content with a constant cystine:Met ratio. The diets contained either basal or excess choline (3 *v.* 7 g/kg feed). Cystine was added to two other 30 and 50 % Met-limiting diets to adjust the SAA supply to that of the control diet in order to evaluate the interaction between Met and cystine. As expected, N accretion was significantly lower with the SAA-limiting diets but increased back to control levels by the extra choline or cystine, demonstrating their sparing effect on Met utilisation for protein accretion. We show, for the first time, the activities of BHMT and CBS in shrimp hepatopancreas. Only BHMT responded to the SAA deficiencies, whereas the extra choline and cystine did not stimulate remethylation or down-regulate trans-sulfuration. Our data also suggest the capacity of *P. monodon* to synthesise taurine, being significantly affected by the cystine level in the 30 % SAA-limiting diets. Further research is warranted to better understand the metabolic regulation of taurine synthesis in shrimp and of the observed Met-sparing effects.

Key words: Crustaceans: Sulphur amino acids: Methionine utilisation: Methionine sparing: Taurine

The marine black tiger shrimp *Penaeus monodon* is the world's second most cultured crustacean species[1]. For crustacean shrimp, as for farmed finfish, plant protein sources are increasingly included in feeds in order to reduce the reliance on wild-caught marine protein sources. However, the replacement of fish or shrimp meal by plant protein sources changes the amino acid (AA) profile of the diet, with methionine (Met) as one of the first-limiting essential AA[2,3]. In *P. monodon* fed an optimal crude protein level, we have previously noted that a 30 % Met deficiency diminished protein accretion[4] and increased deamination[5], suggesting a change in AA catabolism in shrimp receiving an imbalanced dietary Met supply. In *P. monodon*, or crustacean species in general, not much is known on the metabolic utilisation of Met besides its need for protein synthesis. In contrast, the importance of Met as a methyl-group donor for methylation reactions and as a precursor of other sulphur-containing compounds, such as cysteine or taurine, is well recognised in vertebrates[6–10]. As such, homocysteine (Hcy), at the branch point of the three major pathways of Met metabolism (transmethylation, remethylation and trans-sulfuration), is often regarded as a regulatory component of Met metabolism since Hcy can be either trans-sulfurated for cysteine production (catalysed by cystathionine β-synthase; CBS) or remethylated into Met. In the rat liver, the remethylation of Hcy into Met occurs by two different pathways using either the folate–vitamin B_{12}-dependent enzyme methionine synthase (MS) or the betaine–homocysteine methyltransferase (BHMT)[9], which appear to contribute equally to the regeneration of Met[11].

We have recently evaluated the Met requirement for maximal protein gain in *P. monodon* to be 0·56 g/kg body weight

Abbreviations: AA, amino acids; BHMT, betaine–homocysteine methyltransferase; CBS, cystathionine β-synthase; CC, choline chloride; CTL, control diet; Cyss, cystine; DEF30, 30 % Met- or sulphur amino acid-limiting diet; DEF50, 50 % Met- or sulphur amino acid-limiting diet; DGC, daily growth coefficient; Hcy, homocysteine; Met, methionine; MS, methionine synthase; SAA, total sulphur amino acids.

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per d, corresponding to a dietary level of 0·8 % Met (% DM)[4]. This value is close to the Met requirement value of 0·9 % found for post-larval *P. monodon*[12], but lower than the 1·3–1·4 % Met requirement values reported for juvenile *P. monodon*[13] or kuruma shrimp *Marsupenaeus japonicus*[14,15]. The total sulphur amino acid (SAA) requirements estimated at 1·1 % (0·8 % Met) by us in an earlier study[4] and 1·3 % (0·9 % Met) by Millamena *et al.*[12] included 0·3 and 0·4 % of cystine, respectively. Little attention has been paid to the interaction between dietary Met and cyst(e)ine when determining Met requirements in shrimp, despite the ample evidence of the Met-sparing effect of cystine in vertebrates such as teleosts[2,16,17], birds[18,19] or mammals[20,21], where 50 % or more of the requirement for Met can be covered by a dietary supply of cystine.

Dietary choline has been found to improve growth, providing free methyl groups[22–24], thus exerting a sparing effect on Met utilisation[15,23,25]. As BHMT is directly involved in the remethylation of Met through betaine, this enzyme is suggested to have a dual role: in the catabolism of choline (betaine) and/or the conservation of Met depending on the dietary level of Met[25].

For crustaceans, there is no information on the nutritional regulation of enzymes involved in SAA metabolism. In the present study, we examined the potential sparing effect of dietary choline and cystine on the utilisation of Met for protein accretion in juvenile shrimp *P. monodon* and their effect on the activity of two enzymes of Met metabolism involved in remethylation (BHMT) and trans-sulfuration (CBS).

Materials and methods

We investigated in shrimp the Met-sparing effect (i) of dietary choline when Met was either adequate (control diet; CTL) or limiting (30 and 50 %) in the diet (diets CTL, CTL + choline chloride (CC), 30 % Met- or sulphur amino acid-limiting diet (DEF30), DEF30 + CC, 50 % Met- or sulphur amino acid-limiting diet (DEF50) and DEF50 + CC) and (ii) of cystine added to the 30 and 50 % Met-limiting diets (diets DEF30, DEF30 + cystine (Cyss), DEF50 and DEF50 + Cyss). The first series of the diets was formulated to contain decreasing levels of SAA with a constant cystine:Met ratio. The second series contained similar levels of total SAA by modifying the cystine:Met ratio.

Experimental diets

We formulated eight semi-purified isonitrogenous diets to supply three dietary SAA levels and two choline levels (Tables 1 and 2). The dietary crude protein level was based on our previous study with juvenile *P. monodon*[4] and formulated to be 35 % crude protein as fed (38 % diet DM). N was supplied by casein and a crystalline AA blend at a ratio of 43:57 (Table 1). The diets were manufactured by Institut National de la Recherche Agronomique at the experimental facility of Donzacq (Donzacq, France). The crystalline AA blend was coated with 2 % agar dissolved in warm water (pH 7, 40°C). Glucosamine and fish protein-soluble concentrate (CPSP 90) were added to improve feed palatability. Casein, cholesterol, soyabean lecithin, sodium alginate, cellulose, CPSP 90, starch, glucosamine, minerals and vitamins were first mixed together and homogenised before adding the coated AA, fish oil and CC. After thorough mixing, feed was pelleted (3 mm), dried at 40°C and shipped to the experimental facility in Madagascar, where it was stored at 4°C.

Table 1. Formulation of the experimental semi-purified diets fed to juvenile *Penaeus monodon* for 5 weeks

Ingredients (g/kg diet)	Diets
Basal mixture*	621
Casein†	160
AA mixture	212
Arg HCl‡	31·7
His HCl‡	3·4
Ile‡	6·4
Leu‡	12·5
Lys§	7·5
DL-Met§	0·1–4·4‖
Phe‡	6·9
Thr‡	7·2
Try‡	2·4
Val‡	5·8
Ala†	16·2
Asp‡	25·6
Cyss‡	1·2–7·2‖
Glu‡	24·5
Gly†	23·9
Pro‡	20·5
Ser‡	4·8
Tyr‡	5·2
Choline chloride†¶	0–7

AA, amino acids.

* Basal mixture supplied (in g/kg diet as fed): gelatinised starch, 310; fish oil, 59; soya lecithin, 20; sodium alginate, 49; cellulose, 20; agar, 20; stabilised cholesterol, 15; fish protein concentrate (CPSP 90), 20; D-glucosamine 98 % HCl, 8; mineral mixture, 50; vitamin mixture, 50. Mineral and vitamin mixtures were as presented in Richard *et al.*[4,5] and supplied 167 g/kg mixture of choline chloride (60 %).

† Acros (Illkirch, France); 95 % pure casein (CAS 9000-71-9).

‡ Jerafrance (Jeufosse, France).

§ Eurolysine (Paris, France).

‖ The crystalline AA mixture supplied methionine (Met) at 4·5, 2·3 and 0·1 g/kg feed and cystine (Cyss) at 2·9, 2·0 and 1·2 g/kg feed in the control, 30 and 50 % Met-limiting diets, respectively. For the Cyss-enriched diets, the AA mixture supplied 5·1 and 7·2 g/kg feed in the 30 and 50 % Met-limiting diets, respectively. An increase in the amount of non-essential AA compensated for varying Met and Cyss levels.

¶ Choline chloride (99 %) was supplemented at 7 g/kg feed in the choline-enriched diets. Gelatinised starch (in basal mixture) compensated for the choline addition.

Study of the methionine-sparing effect of choline. We formulated three diets to be adequate (diet CTL) or 30 or 50 % limiting in Met (diets DEF30 and DEF50, respectively). In these diets (CTL, DEF30 and DEF50), the ratio of cystine:Met was kept constant at 0·4, so that total SAA levels decreased according to the Met level (from 1·2 to 0·6 g/100 g DM; Table 2). The adequate Met level (0·8 g/100 g DM) corresponds to the requirement value determined in our previous study[4]. We formulated three supplemental diets (CTL + CC, DEF30 + CC and DEF50 + CC) to supply choline in excess (7000 mg/kg dry feed). CC, an efficient choline source in shrimp[15], was used in the diets. The basal level of choline in the diet was 3000 mg/kg DM, as in our previous study[4].

Study of the methionine-sparing effect of cystine. We used the same two diets as described previously (DEF30 and

Table 2. Analysed chemical composition of the experimental semi-purified diets fed to juvenile *Penaeus monodon* for 5 weeks

	Diets							
	CTL	CTL + CC	DEF30	DEF30 + CC	DEF50	DEF50 + CC	DEF30 + Cyss	DEF50 + Cyss
DM (% diet)	89·6	90·1	90·6	90·2	90·2	90·1	90·5	90·1
Crude protein (N × 6·25, % DM)	37·5	38·4	37·7	38·3	38	38·1	37·8	38·7
Crude fat (% DM)	8·5	8·9	10·3	9·7	9·9	9	9·1	8·7
Ash (% DM)	5·9	6·0	5·9	6·0	5·9	5·9	5·8	6·0
Gross energy (kJ/g DM)	19·8	19·8	19·8	19·7	19·9	19·8	19·8	19·7
Choline (% DM)	0·34	0·75	0·29	0·74	0·34	0·71	0·28	0·21
Amino acids (g/kg DM)								
Arg	46·4	45·6	43·5	43·9	44·1	46·6	43·3	48·2
His	7·1	5·7	6·0	5·9	6·3	7·0	6·1	7·0
Ile	14·5	14·5	12·9	13·5	12·7	14·7	13·3	14·5
Leu	26·1	25·9	23·0	23·7	23·8	26·5	23·6	26·6
Lys	22·9	22·7	20·2	21·1	21·0	22·4	20·5	22·0
Met	8·3	8·3	5·6	5·9	4·2	4·4	5·8	4·5
Phe	14·8	14·5	11·8	13·4	13·5	14·9	12·9	15·0
Thr	15·6	15·3	13·9	14·2	14·3	15·2	14·0	15·5
Val	16·3	15·7	16·6	14·8	13·4	15·3	15·1	15·5
Ala	23·3	22·7	21·5	22·0	22·5	23·6	20·3	23·4
Asp	37·0	35·6	34·7	34·8	35·5	36·9	32·7	35·5
Cyss	3·4	3·2	2·6	2·7	1·8	1·7	5·4	7·2
Glu	55·7	54·2	51·2	52·3	54·0	56·9	50·3	54·6
Gly	27·4	27·4	26·3	26·3	26·5	28·0	25·9	27·8
Pro	38·7	37·6	33·6	34·0	35·8	38·4	33·3	39·1
Ser	13·0	12·9	12·2	12·6	12·7	13·5	12·1	13·0
Tyr	14·2	14·0	12·4	12·9	12·6	13·8	13·6	16·0
Cyss:Met ratio	0·4	0·4	0·5	0·5	0·4	0·4	0·9	1·6
Total SAA supply (% DM)	1·2	1·1	0·8	0·9	0·6	0·6	1·1	1·2

CTL, control diet adequate in Met or sulphur amino acid (SAA); CC, choline chloride; DEF30, 30 % Met- or SAA-limiting diet; DEF30 + CC, DEF30 with excess CC; DEF50, 50 % Met- or SAA-limiting diet; DEF50 + CC, DEF50 with excess CC; DEF30 + Cyss and DEF50 + Cyss, with extra Cyss in order to adjust the SAA level to that in CTL; Cyss, cystine.

DEF50). In two other diets, Cyss was added in excess (DEF30 + Cyss and DEF50 + Cyss) in order to adjust the total SAA supply to that of the diet CTL (1·1–1·2 g/100 g DM). In these diets, the cystine:Met ratios were 0·9 for diet DEF30 + Cyss and 1·6 for diet DEF50 + Cyss.

Animal husbandry

Juvenile *P. monodon* (3·3 (0·1) g; Aqualma hatchery, Madagascar) were reared in circular 150 litre fibreglass-covered tanks (fifteen shrimp per tank) for 5 weeks, following a 7 d adaptation period during which they were fed the CTL diet at 3·5 % biomass/d. For each diet, four replicate groups were used. Average temperature, oxygen concentration, pH and salinity of the water were, respectively, 29·8 ± 1·2°C, 6·1 (SD 0·4) mg/l, 8·0 (SD 0·1) and 35·0 (SD 0·5) g/l. Water was exchanged on a daily basis, at a minimum of 40 % for each tank.

The shrimp were fed *ad libitum* four times per d (08.00, 13.00, 18.00 and 23.00 hours) using three circular trays in each tank. After 2 h, feed was removed, and the left-over was carefully collected and stored at −20°C. Weekly apparent DM intake was calculated after drying the uneaten feed to a constant weight (48 h, 90°C). Every week, biomass of each tank was weighed. Mortality was checked daily, and dead shrimp were removed and weighed. Shrimp in ecdysis were not considered for final sampling. A representative sample of 12 h feed-deprived shrimp was taken at the start (twenty-five shrimp) and the end of the experiment (five shrimp from each of the four replicate tanks per dietary treatment) for whole-body composition analysis. All samples were kept at −20°C before analyses. Feed and whole carcasses were analysed for DM (105°C, 24 h), ash (550°C, 12 h) and crude protein (N × 6·25, Kjeldahl Nitrogen Analyser 2000; Fison Instruments, Milan, Italy). Feed samples were analysed for choline content at the laboratory IPL (Bordeaux, France). Daily growth coefficient (DGC, %) was calculated as: $(W_f^{0.3333} - W_i^{0.3333})/\Delta t) \times 100$, where W_i and W_f are the mean initial and final body weights (g), respectively, and Δt is the duration of the growth trial (35 d). N gain (mg/shrimp per d) was calculated as $((N_f \times W_f) - (N_i \times W_i))/\Delta t \times 1000$, where N_i and N_f are the N content of shrimp at the start and the end of the experiment (g/100 g fresh matter).

Enzyme activities

Tissue sampling and preparation. At sampling days, the shrimp were allowed to eat for 1 h before the excess feed was removed from the tank. At 3 h after feed removal, shrimp were sampled; hepatopancreas were quickly dissected out, immediately weighed and frozen in dry ice before stocking at −80°C. Frozen tissues were homogenised in ten volumes of ice-cold phosphate buffer (0·04 M, pH 7·4, 1 mM-EDTA, 1 mM-dithiothreitol) with Ultra-Turrax (16 000 rpm). The homogenates were centrifuged at 1000 ***g*** (4°C for 10 min). The supernatant fraction was then centrifuged at 45 000 ***g*** (4°C for 20 min). Samples were analysed for BHMT (*EC* 2.1.1.5) and CBS (*EC* 4.2.1.22) activities. Protein content of the hepatopancreas was determined by the Bradford

method with bovine serum albumin as the standard. From the preliminary results, we determined the optimal protein concentration to be 1 mg protein per tube for the determination of both BHMT and CBS activities, and the sample volume to be added in each tube was adjusted to this protein concentration.

Determination of the enzyme activity. Measurements of BHMT activity were based on Finkelstein & Mudd[26] and Lambert *et al.*[27]. The following compounds were incubated for 90 min at 37°C in a volume of 467·5 µl: 37·5 µl of 466·6 mM-potassium phosphate buffer (pH 7·4); 35 µl of 100 mM-DL-Hcy (Sigma-Aldrich, Saint-Quentin Fallavier, France); 55 µl of 59 mM-betaine $^{14}CH_3$ (ARC, Isobio, Fleurus, Belgium) at 2500 Bq. The volume of each tissue extract was adjusted according to its protein concentration and homogenisation buffer (0·04 mM-potassium phosphate buffer, pH 7·4, 1 mM-EDTA and 1 mM-dithiothreitol) was added to compensate the volume if necessary. After incubation, the reaction was stopped by adding 62·5 µl of cold water and 280 µl of the mixture were pipetted into a 10 ml polypropylene column (0·8 × 4 cm; Biorad, Marnes-la-Coquette, France) containing 2·5 ml of Dowex® ion exchange resin (1 × 4 (Cl^-), 200–400 mesh; Sigma Aldrich). The non-converted betaine was eluted with 10 ml of water, and the labelled products were eluted with 10 ml of 1 M-acetic acid (from the preliminary tests, acetic acid gave a 85 % product recovery).

Measurements of CBS activity were based on Mudd *et al.*[28] and Lambert *et al.*[27]. The following compounds were incubated for 120 min at 37°C in a volume of 400 µl: 100 µl of mix reagent (buffer with 1·2 M-Tris–HCl, pH 8·3, 20 mM-EDTA, 500 mM-DL-Hcy and 0·6 mM-pyridoxal phosphate); 20 µl of 50 mM-L-serine 3-^{14}C (ARC) at 2500 Bq. The added volume of each tissue extract was adjusted according to its protein concentration, and homogenisation buffer (0·03 mM-potassium phosphate buffer, pH 6·9, 1 mM-EDTA and 1 mM-dithiothreitol) was added to compensate the volume if necessary. After incubation, the reaction was stopped by adding 400 µl of 10 % cold TCA, and all samples were centrifuged for 5 min at 6500 rpm. The supernatant fraction (500 µl) was pipetted into 20 ml of water. The mixture was eluted to the column containing Dowex® ion exchange resin (50Wx4 (H^+), 200–400 mesh; Sigma Aldrich) by rinsing consecutively with 18 ml of water, 35 ml of 0·4 M-HCl, 10 ml of water and 4 ml of 2 M-NH_4OH. Preliminary tests indicated a recovery of 54 % with HCl and NH_4OH. Radioactivity of samples was measured in a Packard Tri-Carb liquid scintillation counter after addition of 10 ml of Ultima-Gold™ reagent (Perkin Elmer, Les Ulis, France). All analyses were done in duplicate, and a blank value obtained from incubation of a heat-inactivated extract was subtracted for each sample. BHMT and CBS activities are expressed as unit (U)/mg protein, where 1 unit is defined as 1 nmol of betaine and serine transformed in 90 and 120 min reaction, respectively.

Amino acid analyses

Feed protein-bound amino acids. Feed (100 mg) was hydrolysed (23 h, 110°C) with 25 ml of 6 M-HCl and 12·5 ml of 2-mercaptoethanol. For the analysis of cystine (through cysteic acid measurement), mercaptoethanol was replaced by a solution of 1 % phenol and 0·2 % sodium azide. After dilution (1:20), 10 µl of the hydrolysed sample were derivatised by adding 70 µl of AccQ.Tag buffer (Waters, Guyancourt, France) and 20 µl of AccQ.Fluor reagent (6-aminoquinolyl-*N*-hydroxysuccinimidyl carbonate) in a 0·5 ml microtube. A diluted (1:25) standard solution of 17 AA (Sigma Aldrich)) was derivatised in the same way. Each sample (5 µl) was then analysed by HPLC (column Symmetry C18, 5 µm, 3·9 × 150 mm) using three mobile phases (AccQ.Tag buffer, 100 % acetonitrile and water, respectively) with a total elution time of 45 min. The AA separations were done using a flow rate of 1 ml/min, and the control temperature was set at 37°C. The excitation and emission wavelengths in the fluorescence detector were 250 and 395 nm, respectively.

Haemolymph free amino acids. Haemolymph of six shrimp per treatment (from those sampled for the determination of enzyme activity) was collected by puncture between the cephalothorax and the first pair of pleopods using a 1 ml syringe (needle 25G 1·59 cm, 0·5 × 16 mm). Plasma was obtained following immediate centrifugation (5 min, 5000 ***g***) and stored at −20°C. Each plasma sample (100 µl) was then centrifuged (1 h 30 min, 2000 ***g***, 15°C) followed by a filtration (filters Amicon-Microcon, YM 100 kd) to eliminate protein. Samples were then derivatised following the AccQ.Tag method (Waters) and injected into the HPLC column as described previously. AA concentrations are expressed as µmol/l of haemolymph.

Statistical analysis

The effects of dietary Met and choline on metabolism and performances were analysed by a two-way ANOVA using Met (adequate, 30 % limiting and 50 % limiting) and choline (adequate or excess) as independent factors (six diets). The effect of Met and cystine dietary levels was analysed by a two-way ANOVA using the Met levels (30 and 50 % limiting) and cystine levels (normal or excess) (four diets). For the amino acid and enzyme analyses, outliers (detected by a scatterplot with box plots) were excluded from the analysis. Outlier and extreme points are described as follows:

$$\text{Outliers : value} > \text{UBV} + \text{oc} \times (\text{UBV} - \text{LBV}) \text{ and value} < \text{LBV} - \text{oc} \times (\text{UBV} - \text{LBV}),$$

$$\text{Extremes : value} > \text{UBV} + 2\text{oc} \times (\text{UBV} - \text{LBV}) \text{ and value} < \text{LBV} - 2\text{oc} \times (\text{UBV} - \text{LBV}),$$

where LBV is the lower value of the box plot (25th percentile), UBV is the upper value of the box plot (75th percentile) and oc is the outlier coefficient (constant 1·5).

For body composition and performances, four replicates per treatment were considered in the analysis, except for diet DEF30 (*n* 3). Because the dietary treatments did not affect the hepatic somatic index and hepatic protein concentration, the enzyme activities were compared in term of units/mg

protein. All analyses were performed using STATISTICA 5.0 software (StatSoft, Inc., Tulsa, OK, USA). Data were analysed by Duncan's multiple range test in the case of a significant effect ($P<0·05$).

Results

Performances

The survival (91–98%) of the shrimp was not affected by the dietary treatments ($P>0·05$; Tables 3 and 4). Growth parameters and feed efficiency were significantly higher in shrimp fed the excess dietary choline compared with those fed the basal choline diets ($P<0·05$; Table 3). The 30% Met-limiting diet significantly reduced the DGC (%) and feed efficiency of shrimp compared with the CTL diet (Table 3). Surprisingly, growth parameters of shrimp fed the 50% Met-limiting diets were intermediate between those of shrimp fed the CTL and 30% Met-limiting diets. We observed a significant interaction between Met and choline for individual N gain ($P<0·05$; Fig. 1). This interaction should be attributed to the fact that the extra choline improved N gain in shrimp fed the Met-limiting diets but not in shrimp fed the Met-adequate CTL diet. The addition of cystine significantly improved the DGC and feed efficiency of shrimp, irrespective of the level of the Met limitation ($P<0·05$; Table 4). N gains were significantly increased by the extra dietary cystine at both Met levels (30 and 50% limiting) ($P<0·05$; Fig. 1).

Methionine metabolism

Both BHMT and CBS were found to be active in the hepatopancreas of *P. monodon* shrimp with high intra-treatment variability. BHMT activity was significantly affected by the dietary Met or SAA level (Table 3). The DEF30-fed shrimp had a significantly lower BHMT activity compared with those fed the CTL diet (17 *v.* 33 U/mg protein, respectively). No significant difference in BHMT activity was found between shrimp fed the CTL and DEF50 diets (33 *v.* 36 U/mg protein, respectively). Surprisingly, the addition of dietary choline did not significantly affect BHMT activity, although there was a tendency ($P=0·126$) for lower BHMT activity in shrimp fed the excess choline level at the adequate (CTL) and 30% Met-limiting levels (DEF30) (Table 3). CBS activity was not affected by either the Met or choline level in the diets ($P>0·05$; Table 3). The activity in hepatopancreas varied from 3 to 7 U/mg protein, respectively, for DEF30 + CC- and DEF50-fed shrimp. No significant effect of extra dietary cystine could be detected on BHMT or CBS activity ($P>0·05$; Table 4).

Free Met, Hcy and serine concentrations in the haemolymph were not significantly affected by any of the dietary treatments, although free Met slightly decreased with the Met-limiting diets (11 and 10 *v.* 14 μmol/l in the CTL diet) and free serine tended to decrease in the haemolymph of shrimp fed the 30% Met-limiting diet ($P=0·072$; Table 3). Cysteine was not detected by the HPLC technique, which might reflect its very low concentration. Cystine, cysteic acid and taurine concentrations were significantly affected by the dietary Met level, but not by the dietary choline level (Table 3). Concentrations of cystine and cysteic acid were significantly higher in the haemolymph of shrimp fed the CTL diet compared with those fed the DEF30 diet (3·2 *v.* 2·1 μmol/l for cystine and 8·5 *v.* 4·5 μmol/l for cysteic acid). Haemolymph taurine concentration was significantly higher with the DEF50 diet compared with the DEF30 diet (635·5 *v.* 521·0 μmol/l). The addition of cystine to the DEF30 diet increased haemolymph cystine and taurine concentrations in shrimp (2·2 *v.* 3·7 and 523·4 *v.* 732·3 μmol/l, respectively, for cystine and taurine). However, no increase was observed at the 50% Met deficiency level (Table 4).

Discussion

Both choline and cystine have a methionine-sparing effect on protein accretion

Due to size-related differences in specific growth rate, with small animals having a higher specific growth rate than the larger ones[29], some authors proposed to use DGC in order

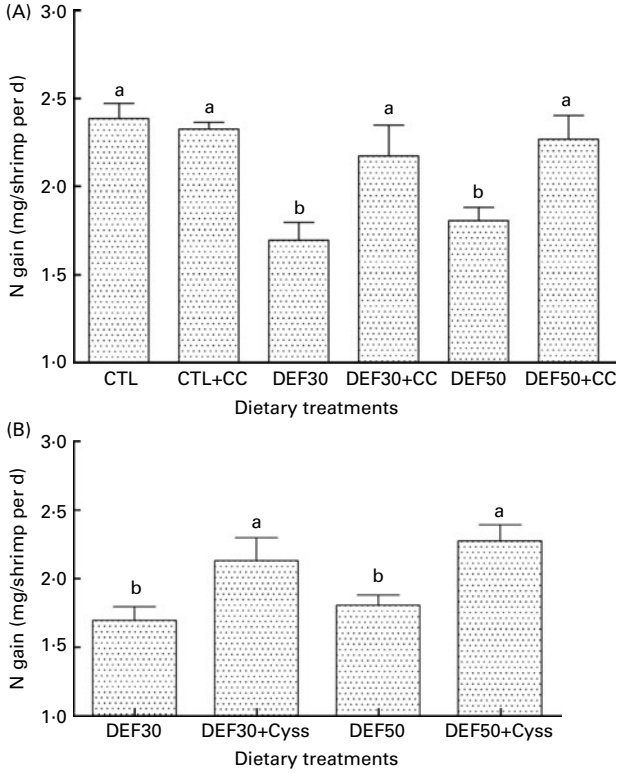

Fig. 1. Effect of dietary levels of choline (A) and cystine (Cyss) (B) on daily individual N gain (mg/shrimp per d) of juvenile *Penaeus monodon* fed semi-purified diets adequate (control; CTL) or limiting (30 or 50%) in sulphur amino acids (SAA; Met + Cyss) during 5 weeks. CC, choline chloride; DEF30, 30% Met or SAA-limiting diet; DEF50, 50% Met or SAA-limiting diet. Values are means (n 4 per treatment, except DEF30 where n 3), with standard errors of the mean represented by vertical bars. [a,b] Mean values with unlike letters are significantly different ($P<0·05$; one-way ANOVA). P values of the two-way ANOVA (Met × choline) are as follows: MET, $P=0·025$; choline, $P=0·023$; Met × choline, $P=0·031$. P values of the two-way ANOVA (Met × Cyss) are as follows: Met, $P=0·215$; Cyss, $P=0·036$; Met × Cyss, $P=0·313$.

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Table 3. Effect of methionine and choline on growth and metabolic parameters of juvenile *Penaeus monodon* fed the experimental diets during 35 d

(Mean values with their standard errors)

	Diets												*P*		
	CTL		CTL + CC		DEF30		DEF30 + CC		DEF50		DEF50 + CC				
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Met	CC	Met × CC
Performances*															
Survival (%)	91·7	3·2	96·7	3·3	91·1	4·4	95	3·2	96·7	3·3	98·3	1·7	0·373	0·197	0·864
Final body weight (g)	5·7	0·1	5·9	0·1	5·1	0·1	5·6	0·3	5·4	0·1	5·8	0·2	0·092	0·020	0·704
Daily growth coefficient (%)†	0·82	0·05	0·93	0·01	0·62	0·04	0·79	0·05	0·73	0·04	0·84	0·06	0·009	0·003	0·786
Apparent feed intake (mg/shrimp per d)‡	276	8	258	3	245	18	257	20	253	16	303	18	0·248	0·249	0·093
Feed efficiency	0·24	0	0·29	0	0·2	0	0·25	0	0·23	0	0·23	0	0·006	0·003	0·062
Enzyme activities (U/mg protein)§															
BHMT	42	8	26 (7)	5	22	5	13	4	36 (8)	9	37 (7)	3	0·012	0·126	0·363
CBS	5 (5)	1	5 (4)	1	5	2	3 (5)	1	7	3	4 (4)	1	0·626	0·419	0·867
Free haemolymph amino acids (μmol/l)§															
Methionine	13·6	1·1	11·9	2	10·9 (5)	1·5	7·8	1	9·7	2·4	10·0 (5)	1·6	0·105	0·281	0·622
Homocysteine	3·5	0·8	3·8	0·5	3·6	0·6	4·1	1·2	4	0·8	3·7	0·8	0·957	0·833	0·897
Serine	46·8	8	49·7	11	36·8	4·8	29·1	2·3	49	4·7	48·4 (5)	11	0·072	0·769	0·772
Cysteine	ND		ND	ND	ND	ND	ND	ND	ND	ND	ND	ND			
Cystine	3·1 (5)	0·2	3·2	0·3	2·2	0·2	2·0 (5)	0·5	2·5	0·2	2·7	0·3	0·001	0·961	0·787
Cysteic acid	9·8 (5)	1·3	7·3	1·4	5·1	0·7	3·8	0·7	4·4	1·4	4·1	1·6	0·002	0·177	0·693
Taurine	584	69	629·3	37	523·4	30	518·6	36	597·1	20	673·9	48	0·031	0·270	0·633

CTL, control diet; CC, choline chloride; DEF30, 30 % Met- or sulphur amino acid (SAA)-limiting diet; DEF50, 50 % Met- or SAA-limiting diet; BHMT, betaine–homocysteine methyltransferase; CBS, cystathionine β-synthase; ND, not detected.

* Values are mean values with their standard errors of four replicate tanks, with exception of treatment DEF30 where only three replicate tanks are considered.

† Daily growth coefficient (%): $(W_f^{(1/3)} - W_i^{(1/3)}/d) \times 100$, where W_i and W_f are the mean initial and final body weights (g), respectively.

‡ Apparent feed intake: $((FG - FU) \times (100 - \text{leaching \%}))/(N \times 35\,d)$, where FG is the total feed gift (g DM), FU is the total amount of uneaten feed (g DM), leaching % is the percentage of feed loss after 2 h immersion in water (salinity 30 parts per thousand) N is the average number of shrimp per tank over 35 d.

§ Values are means of six shrimp haemolymph samples unless noted otherwise (in parentheses).

Table 4. Effect of methionine and cystine on growth and metabolic parameters of juvenile *Penaeus monodon* fed the experimental diets during 35 d

(Mean values with their standard errors)

	Diets								*P*		
	DEF30		DEF30 + Cyss		DEF50		DEF50 + Cyss				
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Met	Cystine	Met × Cystine
Performances*											
Survival (%)	91·1	4·4	95	1·7	96·7	3·3	96·7	1·9	0·231	0·509	0·509
Final body weight (g)	5·1	0·1	5·7	0·2	5·4	0·1	5·7	0·3	0·606	0·055	0·608
Daily growth coefficient (%)†	0·62	0·04	0·84	0·04	0·73	0·04	0·84	0·05	0·236	0·002	0·226
Apparent feed intake (mg/shrimp per d)‡	245	18	269	4	253	16	284	25	0·539	0·148	0·832
Feed efficiency	0·2	0·01	0·25	0·02	0·23	0·01	0·24	0·01	0·57	0·03	0·132
Enzyme activities (U/mg protein)§											
BHMT	22	5	31 (5)	13	36 (8)	9	20 (5)	9	0·906	0·756	0·2
CBS	5	2	7	1	7	3	9 (4)	1	0·368	0·227	0·991
Free haemolymph amino acids (μmol/l)§											
Methionine	10·9 (5)	1·5	11·7 (5)	1·3	9·7	2·4	11·6	2·2	0·747	0·522	0·789
Homocysteine	3·6	0·6	4·4	1	4	0·8	3·6	0·7	0·778	0·835	0·48
Serine	36·8	4·8	36·3	4·5	49	4·7	31·2 (5)	5·9	0·48	0·079	0·096
Cysteine	ND		ND		ND		ND				
Cystine	$2·2^{c}$	0·2	$3·7^{a}$(5)	0·2	$2·5^{bc}$	0·2	$2·9^{b}$	0·2	0·334	0	0·009
Cysteic acid	5·1	0·7	5·1	0·9	4·4	1·4	2·2	0·5	0·069	0·246	0·268
Taurine	$523·4^{b}$	30·2	$732·3^{a}$	33·2	$597·1^{b}$	20·2	$576·9^{b}$ (5)	42·1	0·211	0·007	0·002

DEF30, 30 % Met- or sulphur amino acid (SAA)-limiting diet; Cyss, cystine; DEF50, 50 % Met- or SAA-limiting diet; BHMT, betaine–homocysteine methyltransferase; CBS, cystathionine β-synthase; ND, not detected.

[a,b,c] Mean values within a row with unlike superscript letters were significantly different ($P<0·05$).

* Values are means of four replicate tanks (SE), with exception of treatment DEF30, where only three replicate tanks are considered.

† Daily growth coefficient (%): $(W_f^{(1/3)} - W_i^{(1/3)}/d) \times 100$, where W_i and W_f are the mean initial and final body weights (g), respectively.

‡ Apparent feed intake: ((FG − FU) × (100 − leaching %))/(N × 35 d), where FG is the total feed gift (g DM), FU is the total amount of uneaten feed (g DM), Leaching% is the percentage of feed loss after 2 h immersion in water (salinity 30 parts per thousand) and *N* is the average number of shrimp per tank over 35 d.

§ Values are means of six shrimp haemolymph samples unless noted otherwise (in parentheses).

to compare growth data between studies with fish[30] or crustaceans[31]. The present DGC values (0·82–0·93 in Met adequate treatments) are similar to those found in other studies with shrimp fed semi-purified diets, in *P. monodon* (maximum DGC of 0·76–0·86)[32–34] or *M. japonicus* (maximum DGC of 0·68–0·89)[35,36]. The growth rate as observed in the present study was also similar to that of *P. monodon* (recalculated DGC of 0·75–1·18 %) having, as in the present study, a relatively high initial body weight (approximately 3 g) and being fed a practical diet[37].

As anticipated from our previous study on the determination of Met requirements in juvenile *P. monodon*[4], the 30 % SAA (Met and cystine) limitation significantly decreased protein accretion. This reduction in protein accretion (−29 %) due to the SAA limitation as well as the daily N gains of the control group is similar to data obtained in our previous study[4]. However, it remains unclear why N gain did not further decrease by the 20 % extra deficiency (DEF50 *v.* DEF30). This contrasts with findings in other growing animals such as chickens[38], pigs[39] or fish[40–42], where a severe Met deficiency induces a higher N loss than a marginal Met deficiency.

As with vertebrates, shrimp have a choline requirement for maximal weight gain, which seems to vary among shrimp species, being eight times superior in *P. monodon* compared with *M. japonicus* (0·47 *v.* 0·06 %, respectively)[43]. Since Met and choline share a common role as methyl donor, the requirement for Met is influenced by that for choline and vice versa[23,41]. The interactive effect of choline and Met on growth has been examined in only one penaeid shrimp, *M. japonicus*[15]. Using a 2 × 3 design with two Met concentrations (0·30 and 1·65 %) and three choline levels (0·03, 0·08 and 0·14 %), the foresaid authors have found that choline in excess (0·14 %) improved the weight gain of *M. japonicus* only when fed the low-Met diet. The present results suggest a similar interaction between choline and Met since extra choline significantly improved protein deposition when added to both Met-limiting diets, but not when added to the Met-adequate CTL diet. The latter observation not only validates that the basal Met supply (0·8 %) was appropriate for normal growth in *P. monodon*, but also suggests that the basal choline supply (about 0·3 %) fulfilled the requirements. This choline level is, however, lower than the requirement value (0·47 %) previously reported in *P. monodon*[43], possibly because the authors used a low Met level (0·49 %) close to that of the present 50 % Met-limiting diet.

The dietary supplementation of cystine, at both limiting Met levels, increased the N gain of the shrimp up to that obtained with the CTL. In other words, keeping the total SAA level constant at 1·1 % of the diet, while exchanging (on a weight basis) 30 or 50 % of Met by cystine, resulted in a similar protein accretion. Although the sparing effect of cyst(e)ine on Met requirements for protein accretion has not been examined before in shrimp, our data agree with studies in other animal species, showing that cystine can replace about 50 % of Met in growing pigs[21], chicks[18,19] or rainbow trout[16,17]. The observation that the 1·1 % SAA diets with the cystine:Met ratio of 0·4, 0·9 and 1·6 led to equivalent N gain in juvenile *P. monodon*, as well as recent results in fish, which reported no reduction in protein accretion when feeding diets low in Met but not in cyst(e)ine (e.g. Atlantic salmon)[6,44], underline the importance of estimating requirements for protein gain in terms of total SAA rather than for Met alone.

Regulation of methionine metabolism by methionine, cystine and choline

In crustaceans, so far, no study has examined the functionality of the pathways of Met metabolism and the dietary regulation of remethylation (BHMT or MS) and trans-sulfuration (CBS and cystathionase) enzyme activities, both being well documented in mammalian vertebrates[9]. The present study is thus the first ever to examine the ability of shrimp to regulate Met utilisation at the biochemical level.

Using the classical methodology developed for mammals[26–28], we demonstrated the presence of enzyme activity for both BHMT and CBS in *P. monodon* hepatopancreas, the major site for digestive enzyme secretion and nutrient absorption. In rats, activity of BHMT is principally detected in the liver[9], and that of CBS mostly in the liver and pancreas[28]. The specific BHMT activities in the present study varied between 13 and 42 U/mg protein (90 min reaction) and are within the range of those observed in cattle (17–22 U/mg protein per 90 min)[27], but two- to eightfold lower than in rats[7,45,46] or chickens[47]. Specific CBS activity (3–9 U/mg protein) was slightly below the hepatic CBS activity in growing cattle (15–20 U/mg protein per 60 min)[27] but more than tenfold lower than in the rat liver (with or without Met supply)[26].

The present results show a significant effect of the dietary SAA content on BHMT remethylation activity, being the lowest in shrimp fed the 30 % SAA-limiting diet and back to control levels with the 50 % SAA-limiting diet. Whereas BHMT activity has been reported to be unaffected by a reduction in dietary Met in pigs[24], comparable quadratic BHMT responses have been observed in the liver of cattle[27] and rats[46], following more extreme variations in Met supply (from excessive to quasi-absent), than in the present study (from adequate to highly limiting). Remethylation of Hcy to Met occurs by two pathways, using either BHMT or MS as the catalysing enzyme. Although the relative contribution of both enzymes has been suggested to be equal in the rat liver[11], recent data from broilers indicate that Hcy fluxes through BHMT and MS change according to the dietary SAA level, with an almost equal contribution at adequate SAA supply and a lower relative contribution of BHMT (compared with MS) at excess and limiting SAA supply[48]. The latter observation possibly explains the reduced BHMT activity observed in the present study with the 30 % SAA-limiting diet compared with SAA-adequate CTL. Regarding choline, several studies in mammals[24,45] and birds[47] have found that hepatic BHMT activity increases by adding choline to diets either adequate or limiting in Met, consistent with the use of choline-derived methyl groups for remethylation. In contrast, BHMT activity in the present study tended (P=0·126) to decrease in shrimp fed the extra choline at the

adequate or 30 % limiting SAA supply. That BHMT activity may not be as responsive to dietary choline as concluded previously has recently been underlined in a study with broilers following similar observation as in the present study[22]. Moreover, analysis of both remethylation pathways in terms of Hcy fluxes showed that excess choline in chicks (regardless of the dietary SAA level) decreases the relative contribution of BHMT to remethylation while increasing that of MS[48,49]. Several hypotheses may explain the apparent inconsistencies in the effect of choline on the regulation of Hcy remethylation[48]. First, an excess of methyl groups may inhibit BHMT due to an excess of dimethylglycine, a by-product of BHMT reaction[9,11,49]. Also, serine may be used for the production of more 5-methylenetetrahydrofolates, increasing remethylation through folate-dependent MS[48].

Following the addition of cystine in both SAA-limiting diets, shrimp did not respond by increasing BHMT remethylation or decreasing CBS activity, which catalyses the first step of trans-sulfuration. This was surprising, as the effect of cysteine on hepatic Met metabolism was earlier found to be related to dietary Met level, with a down-regulation of CBS only in rats fed a low-Met diet with extra cysteine[7,8]. Based on these results, we expected the CBS activity to decrease with the addition of dietary cystine, in line with the enhanced protein accretion observed in these shrimps. Hence, the constant low CBS activity, irrespective of the cystine or Met supply, seems to reflect a poor trans-sulfuration, with Met being directed mostly towards protein synthesis. However, metabolic fluxes have previously been reported to be affected by the catalytic constant rate, the enzyme concentration or the substrate concentration[50]. This was already observed in the rat liver in which the flow through the BHMT reaction increased while enzyme activity decreased[11]. In the present study, the absence of change in haemolymph concentrations of Met and Hcy implies that the pathways controlling intracellular Met/Hcy metabolism were effectively regulated in juvenile *P. monodon*. These observations emphasise the need to undertake complementary studies on the effect of dietary SAA levels on the flux of Hcy through remethylation and trans-sulfuration.

Apart from being needed for protein synthesis, Met and cyst(e)ine are precursors of biologically important S-containing molecules such as glutathione or taurine[9]. Among its many physiological functions, taurine plays a major role in cellular osmoregulation[51], which is of particular importance in *P. monodon* and other euryhaline animals living in intertidal ecosystems. In terrestrial vertebrates, taurine is synthesised mostly via cysteine sulphinic acid, involving cysteine dioxygenase and cysteinesulphinate decarboxylase[51,52]. However, the capacity for taurine synthesis appears to be species-dependent as illustrated by the dietary taurine requirement in cats, due to a lack of cysteinesulphinate decarboxylase[53]. Among teleost species, some debate remains regarding the capacity of some marine fish to synthesise taurine[6,52,54–57]. This field of research has importance especially in the context of replacing the fish and shrimp meal (rich in taurine) by plant protein sources (lacking in taurine) in the feeds. Literature on taurine synthesis capacity of crustacean species is scarce. While the freshwater prawn *Macrobrachium rosenbergii* appears able to cover its taurine requirement by biosynthesis[58], the enhanced growth of *P. monodon* following the addition of taurine to purified diets (4–8 g/kg diet) suggests limited taurine synthesis in *P. monodon*[59]. Nevertheless, in the present study, the growth of the shrimp fed diets that were devoid of crystalline taurine was not lower than that of shrimp receiving semi-purified diets with 5 g/kg diet of taurine as part of the attractant blend[4]. Furthermore, the taurine concentrations in the haemolymph of shrimp in the present study were within the range of values reported before in *P. monodon* reared at a salinity of 30 g/l[60], even with the 50 % SAA-limiting diet. Moreover, the increase in haemolymph taurine concentration following cystine addition to the 30 % Met-limiting diet suggests that *P. monodon* has a capacity to regulate taurine synthesis in relation to dietary cystine levels. However, the role of taurine and the regulatory pathways of taurine synthesis in *P. monodon,* and aquatic crustacean species in general, needs further investigation.

Conclusions

Our data demonstrate the sparing effect of choline and cystine on Met requirements for protein accretion in juvenile *P. monodon*, and suggest that growth requirements should be expressed in terms of total SAA rather than Met alone. The present study shows for the first time the existence and functionality of enzymes involved in Met metabolism (i.e. BHMT and CBS) located in shrimp hepatopancreas. BHMT, but not CBS, which had an overall low activity, was found to respond to the dietary SAA deficiency. The addition of choline and cystine to the SAA-limiting diets did not stimulate remethylation by BHMT nor did they down-regulate CBS trans-sulfuration activity. However, the constant Met and Hcy concentrations in the haemolymph, independent from dietary treatment, suggest that the shrimp were able to maintain remethylation/trans-sulfuration equilibrium. Finally, our data suggest the capacity of shrimp to synthesise taurine. However, further research is needed to better understand the sparing effects of choline and cystine on Met requirements and to characterise the pathways regulating taurine synthesis.

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