

Ammonia metabolism in *Aedes aegypti*

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Received 14 October 2004; received in revised form 13 December 2004; accepted 26 January 2005

Abstract

We investigated the mechanisms by which *Aedes aegypti* mosquitoes are able to metabolize ammonia. When females were given access to solutions containing NH₄Cl or to a blood meal, hemolymph glutamine and proline concentrations increased markedly, indicating that ammonium/ammonia can be removed from the body through the synthesis of these two amino acids. The importance of glutamine synthetase was shown when an inhibitor of the enzyme was added to the meal causing the glutamine concentration in hemolymph to decrease significantly, while the proline concentration increased dramatically. Unexpectedly, we found an important role for glutamate synthase. When mosquitoes were fed azaserine, an inhibitor of glutamate synthase, the glutamine concentration increased and the proline concentration decreased significantly. This confirms the presence of glutamate synthase in mosquitoes and suggests that this enzyme contributes to the production of glutamate for proline synthesis. Several key enzymes related to ammonium/ammonia metabolism showed activity in homogenates of mosquito fat body and midgut. The mosquito genes encoding glutamate dehydrogenase, glutamine synthetase, glutamate synthase, pyrroline-5-carboxylate synthase were cloned and sequenced. The mRNA expression patterns of these genes were examined by a real-time RT-PCR in fat body and midgut. The results show that female mosquitoes have evolved efficient mechanisms to detoxify large loads of ammonium/ammonia.

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Keywords: Amino acids; Enzymes; Inhibitors; Metabolism; Pathways; Gene expression

1. Introduction

In *Aedes aegypti* mosquitoes a significant proportion of the amino acids derived from blood meal proteins are oxidized with concomitant production of ammonia (Briegel, 1986; Zhou et al., 2004). In this paper we will use ammonia to refer to both NH₃ and NH₄⁺ or a combination of the two (Campbell, 1997). It is well known that the production of ammonia is toxic to animal tissues; however, the molecular form excreted varies according to the physiology and habitat of each organism. It can be eliminated as ammonia, uric acid or urea. Although mosquitoes can make urea through the

action of arginase on arginine, they lack a complete urea cycle because they lack the ornithine carbamoyltransferase (EC: 2.1.3.3) gene (Zdobnov et al., 2002), and therefore can not dispose off ammonia via this pathway. Most terrestrial insects, reptiles, birds and some amphibians are uricotelic animals (excrete uric acid), and they eliminate the toxic ammonia without a huge loss of body water. The ureotelic animals include most adult amphibians, a few fishes, some reptiles and most mammals (Wright, 1995; Singer, 2003).

Although for many years it was thought that microorganisms, plants, and animals assimilate ammonia mainly by the combined action of glutamate dehydrogenase and glutamine synthetase, in the early 1970s it was shown that bacteria (Tempest et al., 1970; Brown et al., 1974) and plants (Lea and Mifflin, 1974) contain an alternative pathway which involves glutamine synthetase and glutamate synthase. The latter

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enzyme catalyzes the reductive transfer of the glutamine amide group to carbon 2 of oxoglutarate with the production of two molecules of L-glutamate (Raushel et al., 2003; Reitzer, 2003; Lea and Mifflin, 2003; van den Heuvel et al., 2004). In animal tissues, the presence of glutamate synthase has been reported only in *Bombyx mori* (Seshachalam et al., 1992; Hirayama et al., 1997,1998; Hirayama and Nakamura, 2002), *Samia cynthia* (Osanai et al., 2000), and *Spodoptera frugiperda* insect cells (Drews et al., 2000; Doverskog et al., 2000).

The mode of detoxification of ammonia in mosquitoes after a blood meal was suggested to be through synthesis of uric acid, urea and release of ammonia together with free amino acids and hemein (Briegel, 1986). Later it was suggested that in female mosquitoes the excess protein ingested with the blood meal is mainly catabolized by a uricotelic pathway (von Dungern and Briegel, 2001a,b) and we now know that urea synthesis cannot contribute to ammonia excretion, as described above. In our laboratory we have recently proposed the existence of a proline cycle in *Aedes aegypti* that allows temporary storage of ammonia derived from amino acid deamination in a non-toxic form (Goldstrohm et al., 2003). The ammonia can be recovered from proline for excretion and the carbon skeleton used for synthesis of different compounds or for energy production. It has also been observed that *Ae. aegypti* females are able to use proline as a fuel during flight, whereas alanine and glutamine seem to be involved in shuttling ammonia from flight muscle to the fat body (Scaraffia and Wells, 2003).

In this paper we investigated how mosquitoes handle a large load of ammonia after feeding on solutions containing NH_4Cl . We also analyzed the effects of inhibitors of enzymes related to ammonia metabolism. Concentrations of ammonia, uric acid and urea were analyzed in feces after feeding on NH_4Cl . In addition, we determined the activity of several enzymes involved in ammonia metabolism in homogenates of fat body and midgut and analyzed the pattern of gene expression of glutamate dehydrogenase, glutamine synthetase, glutamate synthase, pyrroline-5-carboxylate synthase and pyrroline-5-carboxylate reductase in fat body and midgut before and after blood meal.

2. Material and methods

2.1. Insects

Ae. aegypti (NIH-Rockefeller) were reared under standard conditions (Scaraffia and Wells, 2003). Adults were allowed to feed on 3% sucrose for the first 3–4 days and starved for 24 h before experimental feeding. Females were placed individually in 10-ml plastic scintillation vials covered with nylon mesh for feeding

unless otherwise stated. Mosquitoes were allowed to feed on either 3% sucrose solution (control) or 20 or 80 mM NH_4Cl in 3% sucrose for 1 h. Immediately after feeding, some insects were transferred and kept in groups of 10 in plastic scintillation vials to facilitate the collection of the feces. For blood feeding, groups of females were fed on cow blood supplemented with 5 mM ATP for 1 h. When the inhibitors were added to the meal, the concentration used is mentioned in the figure legends. Only mosquitoes that fed to repletion were used.

2.2. Amino acids analysis

The hemolymph was collected and prepared for amino acid analysis as described previously by Scaraffia and Wells (2003). The samples containing the hemolymph of 5 females were analyzed using a Beckman 7300 Amino Acid Analyzer (post column, ninhydrin method) at The University of Arizona Division of Biotechnology Proteomics Core Facility.

2.3. Determinations of ammonia, uric acid and urea

The presence of ammonia, uric acid and urea in the fecal samples was analyzed before and after feeding on 80 mM NH_4Cl in 3% sucrose. For ammonia determinations, the feces of 10 females were dispersed in a final volume of 1.0 ml water. For the determination of uric acid the feces of 10 females were dispersed in 1 ml of 0.1% of lithium carbonate whereas for urea determination the feces of 60 females were dispersed in 1 ml of water, dried using a vacuum centrifuge and then dissolved in water.

The analyses of ammonia and uric acid were conducted with Ammonia reagent set and Uric acid reagent set (Point Scientific, Inc.), whereas the determination of urea was performed using the Stanbio urea nitrogen kit (Stanbio laboratory).

2.4. Preparations of extracts for enzyme assay

Fat bodies (abdomen free of gut, ovaries and Malpighian tubules) from mosquitoes that fed on either sucrose or 80 mM ammonium chloride solution were dissected 1 h post-feeding; blood meal fed mosquitoes were dissected 24 h post-feeding. Since blood proteins present in the midgut lumen cannot be totally removed and interfere with the enzyme assays, only midgut samples from the mosquitoes that had fed on sucrose or sucrose- NH_4Cl solutions were assayed. Fat bodies and midguts were stored at -80°C until they were utilized.

The tissues were homogenized in an ice bath in 5 parts of 0.1 M triethanolamine-HCl buffer pH 7.6 (1:5 W:V) using a tissue disruptor and three 1-min pulses at 15-s intervals. The homogenates were centrifuged at 20,000 g

for 10 min, and the supernatant was used to determine enzymatic activities.

2.5. Enzyme assays

The enzyme assays were carried out at 25 °C. All the methods were adjusted to achieve optimal conditions of pH and substrate concentrations. The reactions were initiated by addition of enzyme (diluted to yield a linear reaction for 5 min). Controls were performed in all determinations by omitting the substrate in the assay mixture. The final assay volume was 1 ml for all enzymes, except for glutamine synthetase which was 0.5 ml. The enzymatic activities were expressed as *U* per g of wet tissue (*U/g*). One unit of enzyme is the amount that utilizes 1 μmol of substrate per min under the assay conditions.

Alanine aminotransferase (AlaAT, EC 2.6.1.2), aspartate aminotransferase (AspAT, EC 2.6.1.1) and glutamate dehydrogenase (GDH, EC 1.4.1.2) were assayed as previously described (Scaraffia and Wells, 2003).

Glutamine synthetase (GS, EC 6.3.1.2) was assayed using a modification of the method of Meister (1985). The assay medium was 100 mM imidazole-HCl pH 7.2, 20 mM MgCl₂, 25 mM 2-mercaptoethanol, 50 mM sodium L-glutamate pH 7.2, 125 mM hydroxylamine pH 7.2 and 10 mM ATP. The reaction was started with the addition of the enzyme extract, incubated for 5 min, and stopped by the addition of 0.75 ml of ferric acid reagent (0.37 M FeCl₃, 0.2 M trichloroacetic acid and 0.67 M HCl). After centrifugation, the absorbance of the supernatant was read at 535 nm against a reagent blank.

Glutamate synthase or glutamine (amide):2-oxoglutarate amidotransferase (GOGAT, EC 1.4.1.14) was determined according to Hirayama and Nakamura (2002). The assay mixture contained 0.1 M potassium phosphate pH 7.5, 2 mM 2-oxoglutarate, 0.2 mM NADH and 20 mM glutamine.

Pyrroline-5-carboxylate reductase (P5CR) was assayed using a modification of the method of Rossi et al. (1977). The reaction mixture was composed of 100 mM potassium phosphate buffer, pH 7.0, 3.5 mM pyrroline-5-carboxylate, 0.3 mM NADH. The pyrroline-5-carboxylate was isolated from its 2, 4-dinitrophenylhydrazone according to Mezl and Knox (1976).

2.6. Molecular cloning of mosquito gene and the pattern of gene expression

In order to identify genes encoding GDH, GS, GOGAT, pyrroline-5-carboxylate synthase (P5CS) and P5CR in mosquitoes, we searched the *Anopheles gambiae* and *Drosophila melanogaster* genome databases with the BLASTP algorithm for these protein homologs. Subsequently, several sets of degenerate oligonucleotide

primers were designed based on the conserved amino acid sequences between the two insects in order to clone and sequence homologous genes from *Ae. aegypti*. Standard molecular biological techniques, including PCR amplification, agarose gel electrophoresis, ligation into pCR4-TOPO vectors (Invitrogen), transformation and plasmid DNA purification were employed. Plasmid DNA was sequenced using T3 and T7 primers on ABI PRISM[®] 377 DNA Sequencers (Applied Biosystems) at the Genetic Analysis and Technology Core, the University of Arizona. After confirming the insert DNA as genes encoding those putative enzymes by NCBI Conserved Domain Search, gene-specific oligonucleotide primers were designed for each unique gene for expression studies.

The mRNA expression patterns of these genes were examined by quantitative real-time reverse transcriptase-polymerase chain reaction (QRT-PCR). Fat body and midgut, before and after a blood meal (3, 6, 12, 18, 24, 36, 48, 72, and 96 h post blood meal) were dissected. Isolated tissues were stored in RNAlater (Ambion) at –80 °C until use. Total RNA was extracted from the tissues using Trizol reagent (Invitrogen) according to the manufacturer's instructions and quantified by spectrophotometry (A260/280 ratio). Prior to synthesizing cDNA from each sample, 1.0 μg total RNA was first treated with RNase-free DNaseI to ensure complete degradation of potential genomic DNA contamination followed by the addition of 25 mM EDTA and heat denaturation of the enzyme. Reverse transcription was carried out using oligo-(dT)₂₀ primer and reverse transcriptase (NEB). To minimize potential variations in reverse transcriptase efficiency, all cDNA synthesis were carried out simultaneously.

QRT-PCR amplifications were carried out in the ABI PRISM 7700 Sequence Detection System (Applied Biosystems) in a 96-well microtiter plate with a 10.0 μl reaction volume containing 5.0 μl SYBR Green PCR Master Mix (Applied Biosystems), 3.0 μl of each primer set (0.5 μM final concentration), and 2.0 μl of cDNA templates. Negative controls without template were performed in each run. The standard curve consisted of purified PCR products of five 10-fold serial dilutions. Each sample was run in triplicate for each gene. Default PCR conditions were used: preincubation was performed for 10 min at 95 °C to denature the target DNA and activate AmpliTaq Gold DNA Polymerase. DNA was amplified for 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Data were analyzed using ABI Prism 7000 SDS Software (Applied Biosystems).

2.7. Statistical analyses

Data are presented as mean ± standard error of the mean for three independent samples. One way analysis of variance (ANOVA) followed by Dunnett's Multiple

Comparison Test, unpaired Student's *t*-test and non-linear regression were used. A *p*-value less than 0.05 was considered as significant. All the statistical analysis were carried out using GraphPad Prism 4.0 (GraphPad Prism 4.0 Software, Inc.).

3. Results

3.1. Effect of NH_4Cl or a blood meal on hemolymph amino acid concentrations

One hour after feeding 20 or 80 mM NH_4Cl in a sucrose solution, the levels of aspartate, asparagine, glutamate, glutamine, alanine and proline were monitored (Fig. 1). Glutamine and proline were the major hemolymph amino acids after an ammonium chloride meal. The levels of proline, before feeding and after feeding on 20 mM of ammonium chloride, were higher than glutamine, but at 80 mM NH_4Cl the concentration of the two amino acids was similar. The concentrations of aspartate, asparagine, glutamate and alanine did not change after NH_4Cl feeding (Fig. 1). The concentration of glutamine increased with time until 60 min after feeding on 80 mM ammonium chloride, and then decreased (Fig. 2).

After feeding on an ammonium chloride solution, mosquitoes excrete ammonia, uric acid and urea. However, the excretion of ammonia was notably higher than uric acid and urea, and among the three products excreted, urea was the lowest (Fig. 3).

After a blood meal, the increase in the concentration of alanine, glutamine and especially proline was more

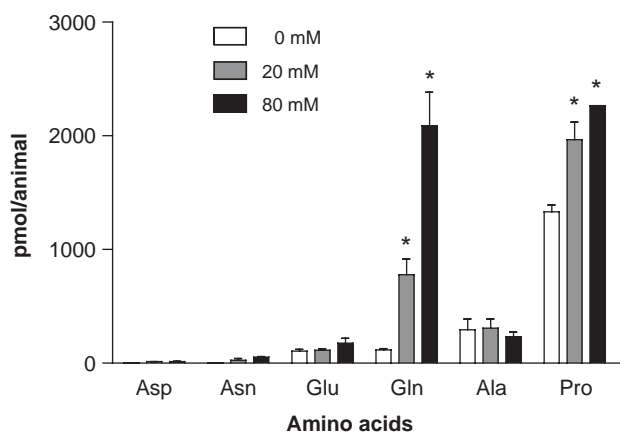


Fig. 1. Concentrations of aspartate (Asp), asparagine (Asn), glutamate (Glu), glutamine (Gln), alanine (Ala) and proline (Pro) in hemolymph from *Ae. aegypti* 1h after feeding on NH_4Cl in 3% sucrose. Concentrations of NH_4Cl were 20 and 80 mM. Control mosquitoes (0 mM) were fed only on 3% sucrose. Data are presented as mean \pm standard error of the mean for three independent samples. **p* < 0.05 when was compared to 0, by ANOVA.

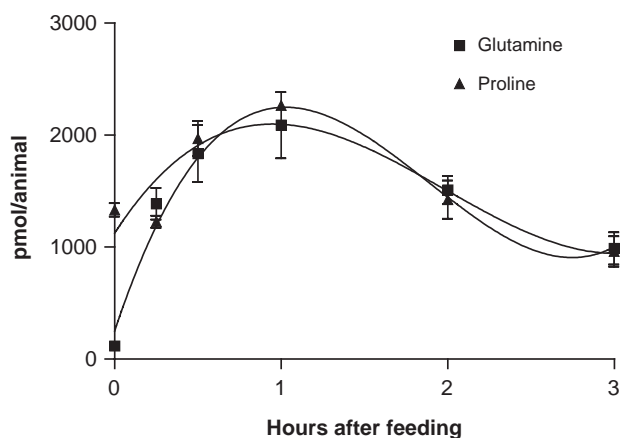


Fig. 2. Glutamine and proline concentrations in hemolymph from *Ae. aegypti*. Time course: 0, 0.25, 0.5, 1, 2 and 3 h after feeding on 80 mM NH_4Cl in 3% sucrose. Control mosquitoes (0) were fed only on 3% sucrose. Data are presented as mean \pm standard error of the mean for three independent samples. Glutamine and proline follow a third-order polynomial curve fitting model (R^2 is 0.82 for glutamine and 0.72 for proline).

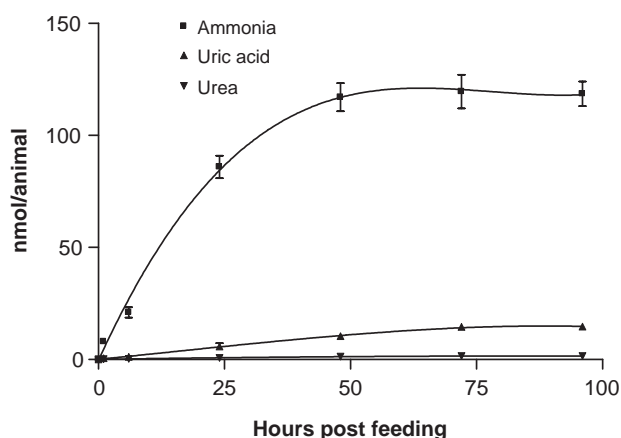


Fig. 3. Ammonia, uric acid and urea concentrations in feces of *Ae. aegypti*. Time course: 0, 1, 6, 24, 48, 72 and 96 h after feeding on 80 mM NH_4Cl in 3% sucrose. Control mosquitoes (0) were fed only on 3% sucrose. Data are presented as mean \pm standard error of the mean for three independent samples. Ammonia, uric acid and urea follow a third-order polynomial curve fitting model (R^2 is 0.98 for ammonia, 0.93 for uric acid and 0.92 for urea).

pronounced than that observed for the other amino acids (Fig. 4). Proline, glutamine and alanine reached a maximum level at about 18 h post feeding.

3.2. Effect of DL-methionine DL-sulfoximine and azaserine

The effect of inhibitors on the survival of mosquitoes is shown in Table 1. A significantly higher mortality was observed if methionine sulfoximine (inhibitor of glutamine synthetase) or azaserine (inhibitor of glutamate

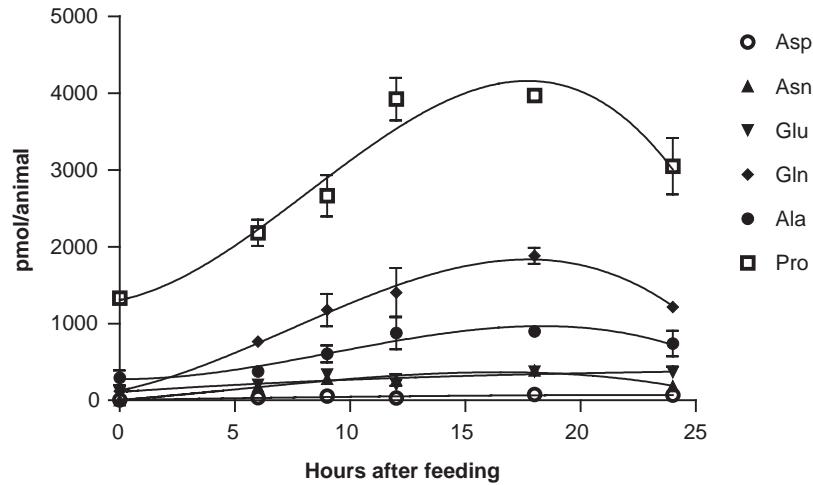


Fig. 4. Amino acids concentrations in hemolymph from *Ae. aegypti* after feeding on blood meal. Time course: 0, 6, 9, 12, 18, 24 h after feeding. Control mosquitoes (0) were fed only on 3% sucrose. Data are presented as mean \pm standard error of the mean for three independent samples. Asp, Asn, Glu, Gln, Ala and Pro follow a fourth-order polynomial curve fitting model: ($R^2 = 0.74, 0.76, 0.54, 0.84, 0.58$ and 0.85 , respectively).

Table 1
Effect of DL-methionine DL-sulfoximine (MS) and azaserine (AZ) on *Aedes aegypti* mortality (%)

MS inhibitor (mM)	Time after feeding (h)						AZ inhibitor (mM)	Time after feeding (h)							
	3	6	9	12	18	24		3	6	9	12	18	24		
11.1	A	0 \pm 0	34 \pm 11	79 \pm 2	90 \pm 0	93 \pm 4	96 \pm 4	11.1	A	16 \pm 5	63 \pm 2	97 \pm 2	98 \pm 1	100 \pm 0	100 \pm 0
	B	0 \pm 0	0 \pm 0	0 \pm 0	0 \pm 0	10 \pm 0	10 \pm 0	B	0 \pm 0	0 \pm 0	0 \pm 0	0 \pm 0	20 \pm 5	30 \pm 5	
5.5	A	0 \pm 0	11 \pm 1	31 \pm 1	39 \pm 1	43 \pm 2	49 \pm 5	5.5	A	0 \pm 0	2 \pm 1	26 \pm 1	51 \pm 7	92 \pm 2	97 \pm 2
	B	0 \pm 0	0 \pm 0	0 \pm 0	0 \pm 0	0 \pm 0	0 \pm 0	B	0 \pm 0	0 \pm 0	0 \pm 0	0 \pm 0	0 \pm 0	10 \pm 0	
2.7	A	0 \pm 0	3 \pm 1	6 \pm 1	9 \pm 2	10 \pm 3	11 \pm 3	2.7	A	0 \pm 0	1 \pm 1	4 \pm 2	7 \pm 1	25 \pm 2	45 \pm 1
	B	0 \pm 0	0 \pm 0	0 \pm 0	0 \pm 0	0 \pm 0	1 \pm 0	B	0 \pm 0	0 \pm 0	0 \pm 0	0 \pm 0	0 \pm 0	0 \pm 0	

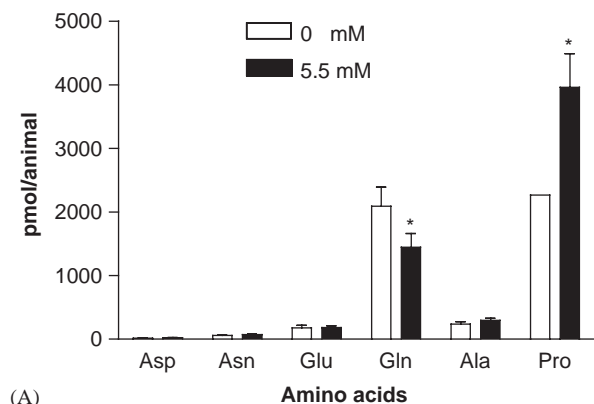
Females were fed either a blood meal plus inhibitor (A) or a solution composed of 80 mM NH₄Cl plus inhibitor in 3% sucrose (B) during 1 h. Number of animals tested in each concentration of inhibitor was 100. Data are presented as mean \pm standard error of the mean for three independent experiments.

synthase) were added to a blood meal, compared to addition to an ammonium chloride-sucrose solution. In all cases, mosquito death was preceded by suppression of locomotory activity.

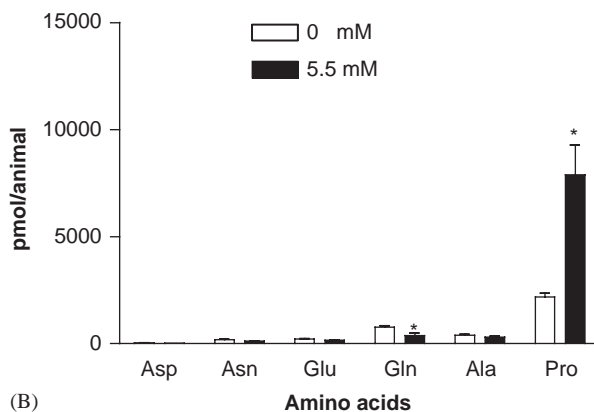
When the inhibitor—ammonium chloride—sucrose solution was diluted with a green food dye solution, we observed that the crop was full and midgut only partially full, unlike mosquitoes that have fed on a blood meal, in which the blood is directly placed into the midgut lumen without passing through the crop. Thus, the higher mortality observed when the inhibitors were added to a blood meal probably arises from the fact that all the inhibitor enters the midgut from which it can be absorbed. On the other hand, the inhibitor in the crop is not absorbed until the crop contents are delivered to the midgut. This delivery occurs over several hours, so the concentration of inhibitor in the midgut is always at a lower level than when it is present in a blood meal.

When 5.5 mM methionine sulfoximine was added to an ammonium chloride—sucrose solution or a blood meal, the glutamine content in the hemolymph decreased significantly, whereas the proline increased markedly (Figs. 5 A, B and C). Aspartate, asparagine, glutamate and alanine concentration did not change significantly, except at 18 h after blood meal in the presence of inhibitor where there was a small but significant decrease in the glutamate concentration.

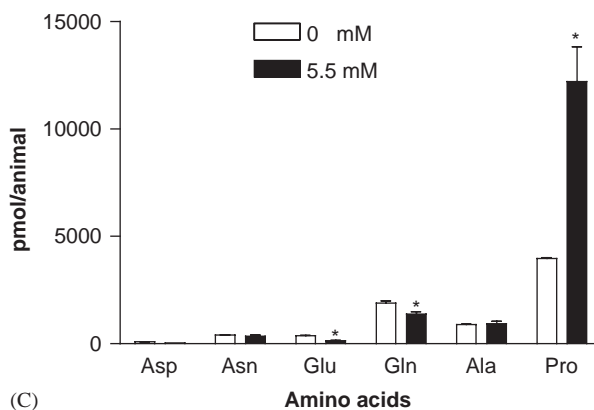
After feeding mosquitoes with an ammonium chloride—sucrose solution in the presence of azaserine, the aspartate and glutamine concentrations increased significantly, whereas the proline concentration decreased. The concentration of asparagine, glutamate and alanine did not change (Fig. 6A). Six and 18 h after a blood meal, the concentrations of glutamine increased dramatically (Figs. 6B and 6C), whereas the proline and alanine concentration decreased significantly only at



(A)



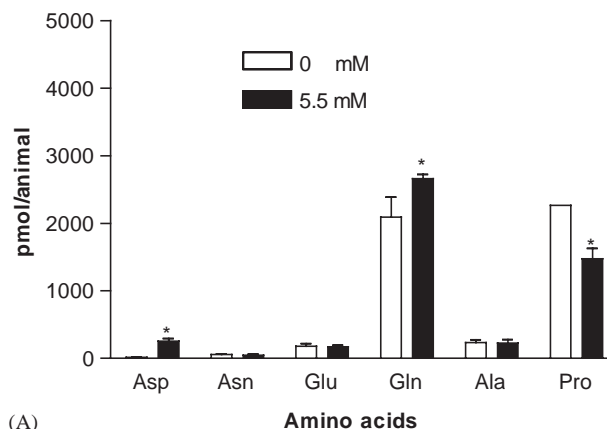
(B)



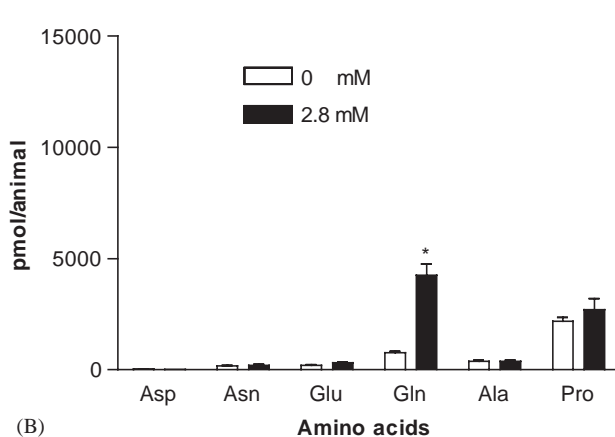
(C)

Fig. 5. Effect of 5.5 mM D,L- methionine D,L- sulfoximine on amino acids concentrations in *Ae. aegypti* hemolymph. Amino acid concentrations observed 1h after feeding on sucrose solution containing 80 mM NH_4Cl and inhibitor (A), and 6 (B) and 18 h (C) after feeding blood meal with inhibitor. Control mosquitoes (0) were fed with 80 mM NH_4Cl in 3% sucrose or blood meal. Data are presented as mean \pm standard error of the mean for three independent samples. * $p < 0.05$ when was compared to 0 by ANOVA.

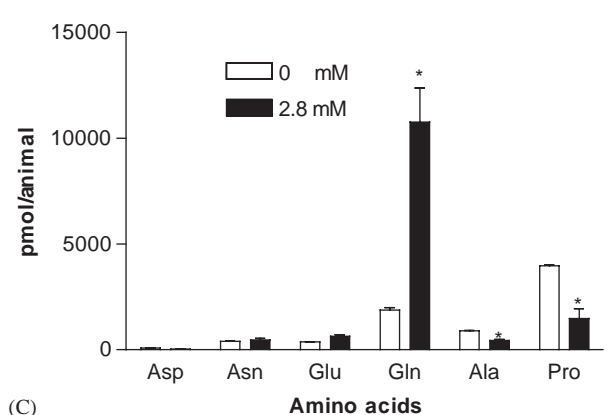
18 h post feeding (Fig. 6C). The aspartate, asparagine, and glutamate concentrations were maintained at steady state 6 and 18 h after feeding. Six hours post blood meal the glutamine concentration increased dramatically whereas the other amino acid concentrations did not change (Fig. 6B).



(A)



(B)



(C)

Fig. 6. Effect of azaserine on amino acid concentrations in *Ae. aegypti* hemolymph. Amino acid concentrations observed 1h after feeding on sucrose solution containing 80 mM NH_4Cl and 5.5 mM of inhibitor (A), and 6 (B) and 18 h (C) after feeding blood meal with 2.8 mM of inhibitor. Control mosquitoes (0) were fed on 80 mM NH_4Cl in 3% sucrose or blood meal. Data are presented as mean \pm standard error of the mean for three independent samples. * $p < 0.05$ when was compared to 0 by ANOVA.

3.3. Enzymatic activities

Alanine aminotransferase, aspartate aminotransferase, glutamate dehydrogenase, glutamine synthetase, and pyrrolidine-5-phosphate reductase showed activity in homogenates of fat body and midgut from *Ae. aegypti*

Table 2
Activities of enzymes related to ammonia metabolism in *Aedes aegypti*

Enzymes	Fat body				Midgut
	After SM	After AM	After BM	After SM	After AM
	U/g	U/g	U/g	U/g	U/g
AlaAT	48.02±2.00	48.62±4.34 ^b	56.11±4.37 ^b	40.98±3.60	41.67±5.04 ^b
AspAT	29.37±1.58	33.90±3.13 ^b	35.72±1.40 ^b	42.35±1.78	46.81±3.10 ^b
GS	3.95±0.28	4.88±0.52 ^b	5.42±0.10 ^a	2.05±0.18	2.79±0.77 ^b
GOGAT	1.78±0.11	2.22±0.15 ^b	2.54±0.26 ^b	ND	ND
GDH	6.74±1.16	8.08±0.57 ^b	16.03±3.14 ^a	65.00±0.33	64.34±5.55 ^b
P5CR	58.65±4.80	70.15±1.34 ^b	76.69±5.05 ^a	26.01±1.84	28.16±3.31 ^b

AlaAT = alanine aminotransferase; AspAT = aspartate aminotransferase; GS = glutamine synthetase; GOGAT = glutamate synthase; GDH = glutamate dehydrogenase; P5CR = Pyrroline-5-carboxylate reductase. After SM = after a sucrose meal (control); After AM = after an ammonium chloride meal; After BM = 24 h after a blood meal. The activity of the enzymes was not determined in the midgut after a blood meal due to the presence of the blood proteins. ND = not detected. Values are the means of three independent determinations±SEM.

^a $p < 0.05$ when compared to the control by Student's *t*-test.

^bnot significant.

females before and after an ammonium chloride meal and in fat body 24 h after a blood meal (Table 2). Glutamate synthase showed activity in fat body, but it was not detected in midgut. The activity of all enzymes studied did not change significantly in the homogenates of fat body or midgut after an ammonium chloride meal. Glutamine synthetase, glutamate dehydrogenase and pyrroline-5-phosphate reductase activities increased in fat body after feeding on a blood meal.

3.4. Gene expression

Evolutionally conserved functional domains are present in each enzyme examined above. To clone and sequence *Ae. aegypti* orthologous genes, PCR was performed with enzyme-specific degenerate primers designed based on the conserved deduced amino acid sequences (Table 3), resulting in the determination of partial nucleotide sequences encoding five enzymes in *Ae. aegypti*. The sequence identity between insect species and a copy number for each enzyme for *An. gambiae* and *D. melanogaster* are shown in Table 4. Because all 5 enzymes are encoded by either one or two genes in both species, the sequences obtained from *Ae. aegypti* are most likely orthologous to those of the two other species. Subsequently, gene-specific oligonucleotide primers were designed (Table 3).

To understand the importance of these gene products during ammonia metabolism in more detail, the pattern of their expression in fat body and midgut was examined by real-time PCR. Ingestion of a blood meal changed the level of gene expression during blood meal digestion (Fig. 7), whereas the ammonium chloride–sucrose meal did not influence the mRNA levels when compared with control mosquitoes (data not shown).

Gene expression for AaGDH in fat body is immediately up-regulated in response to a blood meal. The peak

of gene expression occurs at 12 h post blood meal (PBM), and the level gradually drops. At 36 h PBM, the transcript returns to the control level. The overall pattern of gene expression in midgut is similar to that of fat body, except that the peak of AaGDH expression is observed at 6 h PBM, and the level returns to that of control midgut at 18 h PBM.

The pattern of expression for the two genes encoding glutamine synthetase, AaGS-1 and AaGS-2, were examined using gene-specific primers. The transcription level of AaGS-1 in fat body is induced 5-fold at 3 h PBM compared to control females. The mRNA level gradually decreases over the next 33 h, and returns to control level 36 h PBM. In midgut, the mRNA is almost undetectable prior to feeding blood, but it dramatically increases in response to feeding, reaching the highest level between 6 and 12 h PBM. The level also gradually decreases for the next 24 h and becomes undetectable at 48 h PBM. The midgut pattern of gene expression for AaGS-1 shown by real-time PCR in this report is consistent with the previous results by Northern blot analysis (Smartt et al., 1998). The mRNA encoding AaGS-2 in the fat body has two peaks. The first high peak is observed at 3 h PBM followed by dramatic reduction of the level at 12 and 18 h PBM. The second small peak is present between 24 and 36 h PBM followed by a gradual decrease in the level. The overall AaGS-2 mRNA level in the midgut is five times lower than that of fat body. The mRNA abundance appears to be constitutively expressed before and during blood meal digestion with no dramatic changes in the level.

A single gene encoding glutamate synthase, AaGOGAT, was cloned and sequenced. In the fat body, the level of AaGOGAT mRNA is constantly present for the first 24 h PBM. However, a dramatic increase of the level of gene expression is observed between 36 and 48 h PBM, and then the level returns to the steady state after

Table 3
Oligonucleotide primers used in this study

Genes		Primer sequences (5' to 3')	PCR (bp)
<i>Degenerate primers used for cloning</i>			
AaGDH	Forward	TTYATHGTNCARGGNTTYGG	617
	Reverse	SWRTGNACDATRTCYTTYTC	
AaGOGAT	Forward	CAYGCNTTYGARCARGGNTGGAT	612
	Reverse	CCAYTGNGGCCANGGRTRTC	
AaGS-2	Forward	CARCCNTGGTTYGGNATHGA	672
	Reverse	ACNSWRTANGGRTRCARTT	
AaP5CR-1	Forward	GGYGGNAAYATGGCNTAYGC	705
	Reverse	GGCGARCANACYTCRTCYTT	
AaP5CR-2	Forward	GGCGAGAACCTGATGTACGA	300
	Reverse	GAGCAGGTGGTAGTTGGACT	
AaP5CS	Forward	GCNTGYAAAYGCNATGGARAC	464
	Reverse	AR DATCCAYTTSGTSGTNA	
<i>Gene-specific primers used for real-time PCR</i>			
AaGDH	Forward	GGCGAGAACCTGATGTACGA	300
	Reverse	GAGCAGGTGGTAGTTGGACT	
AaGOGAT	Forward	CTCCTACAATACGGCATTCCAAC	276
	Reverse	CGTGATCCGAGTAACTTCTTCTG	
AaGS-1	Forward	CCTCTATGCTGGAGTTGACT	237
	Reverse	CGCATCTGCTTGGTGGAGA	
AaGS-2	Forward	GAGGAGTTTGGCATCGTTG	181
	Reverse	GGTCGTAGGCTCGGATATG	
AaP5CR-1	Forward	GTGAACGAAGCACAGATGGAT	159
	Reverse	TGCAGCTCCCATAACTGTTTG	
AaP5CR-2	Forward	GCACGCGTCATCAGGGTCA	217
	Reverse	CCTCAATCATGGTGAACACATA	
AaP5CR-3	Forward	GAGGCTATCGGTACTTGTGAA	203
	Reverse	CCAGTATCACGAACCAGTTGA	
AaP5CS	Forward	GCGGGAAGGTGTTAAGATCA	207
	Reverse	GCAGCCGAATCATTCTCAGT	

Table 4
Sequence identity of enzymes among three insects

Protein functions	<i>Ae. aegypti</i>	GenBank Accession Nos.	<i>An. gambiae</i> ^a	<i>D. melanogaster</i> ^b
Glutamate dehydrogenase	AaGDH	AY623405	agCP14138 (91%)	CG5320 (84%/83%)
Glutamate synthase	AaGOGAT	AY623407	agCP6407 (86%)	CG9674 (77%/76%)
Glutamine synthetase	AaGS1	AAK76448 ^c	agCP4738 (86%)	CG2718 (68%/71%)
Glutamine synthetase	AaGS2	AY623406	agCP1592 (86%)	CG1743 (82%/80%)
Pyrroline-5-carboxylate reductase	AaP5CR1	AY623401	agCP1224 (59%)	CG6009 (57%/56%)
Pyrroline-5-carboxylate reductase	AaP5CR2	AY623402	agCP2142 (73%)	CG5840 (63%/64%)
Pyrroline-5-carboxylate reductase	AaP5CR3	AY623403	agCP2142 (79%)	CG5840 (64%/64%)
Pyrroline-5-carboxylate synthase	AaP5CS	AY623404	agCP1511 (87%)	CG7470 (80%/80%)

^aSequence identity between *Ae. aegypti* and *An. gambiae* homologs.

^bSequence identity between *Ae. aegypti* and *D. melanogaster*/*An. gambiae* and *D. melanogaster* homologs.

^cAaGS1 protein sequence was retrieved from Smartt et al. (2001).

72 h PBM. The relative mRNA abundance of AaGOGAT transcript in fat body is five times higher than that of midgut. The level of the transcript is high during blood meal digestion (12–36 h PBM) in the midgut, and the level returns to the steady state by 48 h PBM. Although the transcript is present, we could not detect

any enzyme activity in the midgut, perhaps due to the sensitivity of the assay.

P5CS is encoded by a single gene in mosquito. In the fat body, AaP5CS transcript is constitutively and constantly expressed at low level for the first 18 h PBM. However, the level of expression increases

gradually, reaching a maximum level at 48 h PBM, and then the level returns to the steady state. The level of gene expression observed in the midgut is significantly lower than that of fat body. The level of gene expression in midgut is not changed until 12 h PBM. The peak expression is observed at 36 h PBM, but it is 20 times lower than the peak expression level of fat body.

Three genes encoding P5CR were isolated and sequenced from *Ae. aegypti*. Real-time PCR shows that the level of AaP5CR-1 mRNA is constitutively expressed in the fat body throughout vitellogenesis. A high level of transcript is only observed in midgut isolated from unfed and those samples after completion of blood meal digestion (72 and 96 h PBM) with five time lower

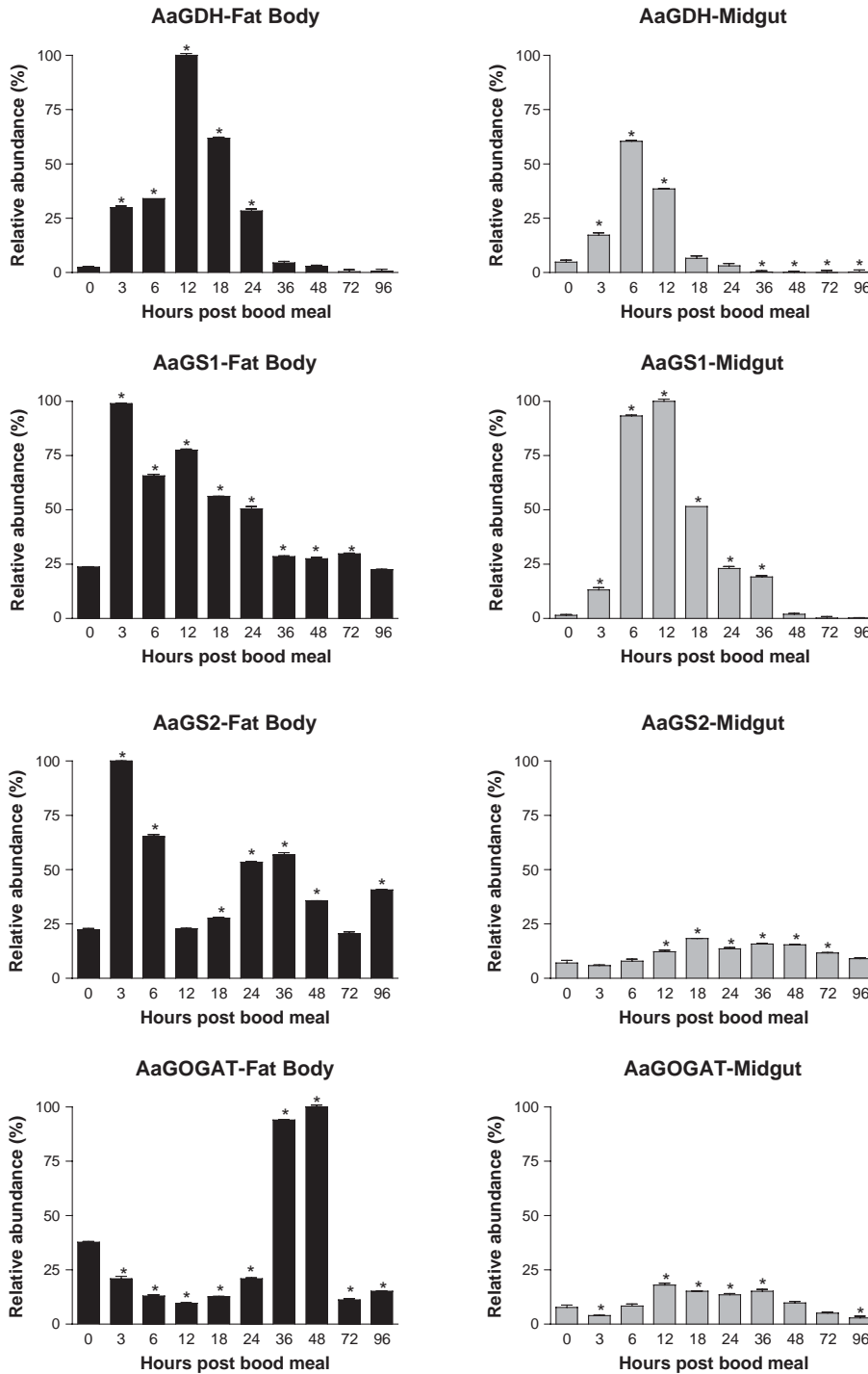


Fig. 7. The pattern of gene expression in *Ae. aegypti* females. Data are presented as mean \pm standard error of the mean for three independent samples. Control mosquitoes (0) were fed only on 3% sucrose. * $p < 0.05$ when was compared to 0 by ANOVA.

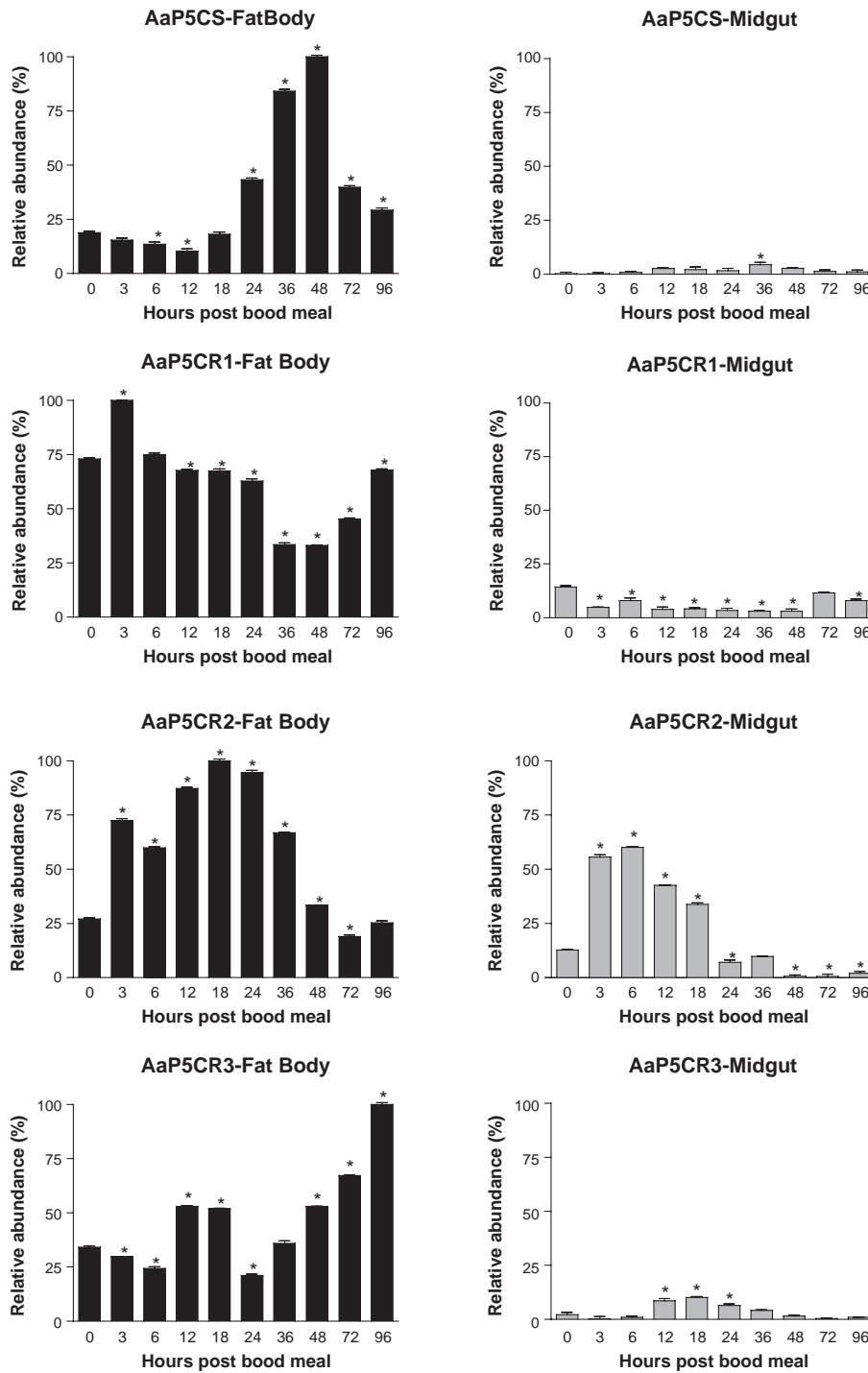


Fig. 7. (Continued)

mRNA abundance than fat body. In contrast to AaP5CR-1, the AaP5CR-2 mRNA abundance in fat body is regulated by blood meal intake. The level is immediately up-regulated three-fold in response to feeding, reaching a maximum level of gene expression at 18 h PBM. At 48 h PBM, the level of gene expression returns to that of the control. The level of gene expression is also markedly up-regulated in the midgut

with peak gene expression observed between 3 and 6 h PBM. At 24 h PBM the level returns to control. Among the three genes encoding P5CR in mosquitoes, AaP5CR-3 shows a unique pattern of gene expression. Two distinct peaks are observed for AaP5CR-3 in the fat body. The first lower peak is 12 and 18 h PBM, and the highest level of gene expression is observed toward the end of vitellogenesis (96 h PBM) with steady increase

of the mRNA. The AaP5CR-3 transcription level is induced upon feeding blood meal in the midgut, and the maximum level of gene expression is observed between 12 and 24 h PBM. However, the level of gene expression in the midgut is lower than that of fat body.

4. Discussion

In this paper we present evidence that glutamine and proline serve as nitrogen sinks that contribute to ammonia detoxification in *Ae. aegypti* females. Mosquitoes are able to handle a huge load of ammonia through the synthesis of these two amino acids coupled to a high rate of ammonia excretion in the feces, with lesser excretion of uric acid and urea. An increase of mRNA expression and activity of some key enzymes involved in metabolism of ammonia was observed in the fat body after a blood meal, suggesting that it is the main tissue involved in ammonia detoxification.

The amino acid concentration in the hemolymph is elevated in response to feeding an ammonium chloride–sucrose solution and/or a blood meal. When mosquitoes were fed on ammonium chloride, aspartate, asparagine, glutamate and alanine were maintained at a steady state, whereas the levels of proline and especially glutamine increased, indicating that part of the ammonia is removed from the body and stored through the synthesis of these amino acids. The fact that the level of glutamate in hemolymph was basically unaffected suggests that mosquitoes maintain a steady state level of glutamate despite significant metabolic turnover of this amino acid, which is involved in both glutamine and proline synthesis. During the digestion of a blood meal, the changes in hemolymph amino acid concentrations were similar to an ammonium chloride meal except that the increase of proline in the hemolymph was greater after a blood meal than the change in the concentration of glutamine. Also the concentration of alanine increased following a blood meal but did not change after an ammonium chloride meal. However, caution should be exercised when comparing the two situations— NH_4Cl and blood fed—because the physiology of the mosquito is quite different.

Both glutamine and proline reached maximum levels about 1 h after feeding on ammonium chloride, but the synthesis of glutamine responded more rapidly than the synthesis of proline. The presence of a detectable amount of uric acid in the feces 1 h after feeding on an ammonium chloride meal suggests release or mobilization of sequestered ammonia that had accumulated during the feeding. The glutamine and proline concentration in whole body is approximately ten times higher than that of hemolymph 1 h after feeding on an ammonium chloride meal (unpublished results).

It was reported that uric acid, urea and ammonia are excreted in the feces of *Ae. aegypti* females after a blood meal (Briegel, 1986; von Dungern and Briegel, 2001a). Xanthine dehydrogenase is involved in uric acid synthesis in mosquitoes and the main site of its activity seems to be fat body (von Dungern and Briegel, 2001a). Although arginase that catalyzes the hydrolysis of arginine and leads to the formation of urea and ornithine is active in several tissues in *Ae. aegypti* (von Dungern and Briegel, 2001b), the absence of a functional urea cycle in mosquitoes due to the lack of ornithine carbamyltransferase gene (Zdobnov et al., 2002) indicates that arginase is not part of the urea cycle. Thus, the small amount of urea observed in feces after feeding on an ammonium chloride meal most likely arises from arginine metabolism. The excretion of ammonia through the feces seems to be a rapid and metabolically inexpensive mechanism to eliminate ammonia. The storage of the ammonium chloride–sucrose solution in the crop and the release to the midgut allows the continuous excretion of nitrogen compounds after feeding on an ammonium chloride meal and makes the ammonia detoxification process more efficient.

The feeding of ammonium chloride had no effect on the activities of AlaAT, AspAT, GDH, GS, GOGAT and P5CR from homogenates of fat body or midgut. Additionally, the feeding of ammonium chloride did not up-regulate their corresponding mRNA transcripts (data not shown). In contrast, GDH, GS, and P5CR activities and their corresponding mRNA levels increased significantly in fat body post blood meal. It has been shown that in *Ae. aegypti* that the GS1 message is expressed in all development stages and is increased in midgut and fat body following a blood meal (Smartt et al., 1998, 2001; Niu et al., 2003). In the present study, one additional gene encoding glutamine synthetase, AaGS2, was identified. Two genes encoding GS have been also reported in *D. melanogaster* (Caizzi et al., 1990; Caggese et al., 1994).

In the presence of a GS inhibitor, the glutamine concentration decreased in the hemolymph, however mosquitoes with reduced GS activity were able to produce a large amount of proline in order to cope with the excess ammonia. Under these conditions, it is evident that the role of the GDH reaction in furnishing glutamate for proline synthesis becomes essential. In addition, the results obtained in the presence of a GS inhibitor highlight the importance of proline serving as nitrogen sink in the detoxification of ammonia and support the existence of a recently proposed proline cycle in mosquitoes (Goldstrohm et al., 2003). The synthesis of proline in higher eukaryotes is catalyzed by two enzymes, P5CS and P5CR (Willett and Burton, 2002). In *Ae. aegypti*, P5CR activity was observed in both fat body and midgut. Although P5CS enzymatic activity was not measured in this study, the level of the

corresponding mRNA transcripts suggests that the fat body is the main tissue synthesizing proline in the mosquito.

Notably, glutamate synthase (GOGAT) is present in the fat body of *Ae. aegypti*. The effects of the GOGAT inhibitor in a blood meal on mosquito mortality and the suppression of locomotory activity were comparable to that of the GS inhibitor, although the percentage mortality observed in the presence of azaserine were higher than with the GS inhibitor. A sucrose solution containing ammonium chloride with the GS or GOGAT inhibitors did not show the same effect on mosquitoes probably due to the compartmentalization of the food. Thus, the initial storage of the ammonium chloride solution in the crop and the gradual discharge of it into the midgut could reduce the concentration of ammonium chloride in the midgut and reduce mortality. These data indicate that both GS and GOGAT enzymes play a key role in ammonia detoxification in mosquitoes.

In hemolymph, the glutamine concentration increased significantly after feeding mosquitoes in the presence of azaserine whether in an ammonium chloride meal or a blood meal. Azaserine caused a decrease in the proline concentration after an ammonium chloride meal and 18 h post blood meal. The results support the role of GOGAT in the production of glutamate and show the importance of this enzyme in ammonia metabolism in mosquitoes.

It has been observed that GS/GOGAT pathway catalyzes the conversion of the glutamine amide nitrogen into the amino group of glutamate for silk synthesis in *B. mori* (Hirayama et al., 1997, 1998; Hirayama and Nakamura, 2002). It was also shown that GOGAT is the key enzyme which transfers the glutamine amide nitrogen to the amine position in glutamate from where it is transaminated to alanine in *S. cynthia ricini* (Osanaï et al., 2000) and *S. frugiperda* insect cells (Drews et al., 2000; Doverskog et al., 2000). According to our results, when ammonia is in excess in mosquitoes, it can be fixed mainly by converting glutamate to glutamine by GS. Glutamine and 2-oxoglutarate are then metabolized by GOGAT which catalyzes the transfer of the amide group of glutamine to 2-oxoglutarate in order to yield two molecules of glutamate. Some of the glutamate can be used as a substrate for further metabolic reactions, such as the synthesis of proline (Fig. 8) and some of the glutamate can be utilized to maintain the glutamate concentration in the hemolymph. Because GDH, AlaAT and AspAT are active in mosquitoes and they catalyze reversible reactions, they may be involved in the regulation of the glutamate, alanine and aspartate concentrations and maintain them at a steady state in the hemolymph.

Our results conclude that a high concentration of ammonia given externally or as the result of blood meal protein and amino acid catabolism leads to an increase

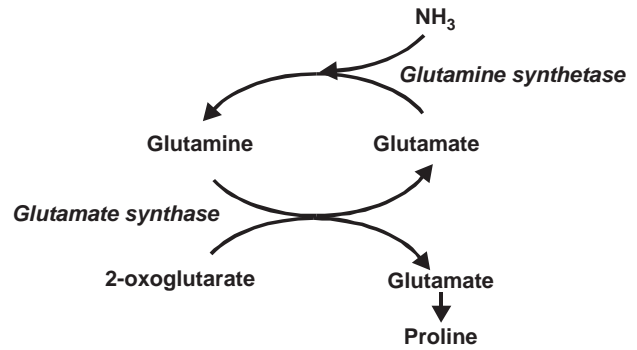


Fig. 8. Schematic representation of the GS/GOGAT pathway for the assimilation of ammonia in mosquitoes.

in glutamine and proline concentration in the *Ae. aegypti* mosquitoes. The conversion of excess ammonia to these amino acids would allow mosquitoes to store the toxic ammonia in a neutral form until both amino acids can be utilized in other pathways. The presence of GOGAT activity and the effect of GS and GOGAT inhibitors indicate that a GS/GOGAT alternative pathway for the assimilation of ammonia is highly active in mosquitoes. The activity and expression pattern of GDH, GS, GOGAT, P5CR and P5CS showed that fat body is the major tissue involved in detoxification of ammonia in mosquitoes.

Acknowledgements

We thank Ms. Robin K. Roche for rearing mosquitoes. We also thank Drs. Roger Miesfeld, Guoli Zhou and an anonymous reviewer for critical revision of the manuscript. This work was supported by National Institutes of Health Grant AI 46541.

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