Differential ammonia metabolism in *Aedes aegypti* fat body and midgut tissues

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ARTICLE INFO

ABSTRACT

In order to understand at the tissue level how *Aedes aegypti* copes with toxic ammonia concentrations that result from the rapid metabolism of blood meal proteins, we investigated the incorporation of ^{15}N from ^{15}NH$_4$Cl into amino acids using an *in vitro* tissue culture system. Fat body or midgut tissues from female mosquitoes were incubated in an *Aedes* saline solution supplemented with glucose and ^{15}NH$_4$Cl for 10–40 min. The media were then mixed with deuterium-labeled amino acids, dried and derivatized. The ^{15}N-labeled and unlabeled amino acids in each sample were quantified by mass spectrometry techniques. The results demonstrate that both tissues efficiently incorporate ammonia into amino acids, however, the specific metabolic pathways are distinct. In the fat body, the ^{15}N from ^{15}NH$_4$Cl is first incorporated into the amide side chain of Gln and then into the amino group of Gln, Glu, Ala and Pro. This process mainly occurs via the glutamine synthetase (GS) and glutamate synthase (GltS) pathway. In contrast, ^{15}N in midgut is first incorporated into the amino group of Glu and Ala, and then into the amide side chain of Gln. Interestingly, our data show that the GS/GltS pathway is not functional in the midgut. Instead, midgut cells detoxify ammonia by glutamate dehydrogenase, alanine aminotransferase and GS. These data provide new insights into ammonia metabolism in *A. aegypti* mosquitoes.

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1. Introduction

Aedes aegypti mosquitoes are vectors of medical importance. During blood feeding, *A. aegypti* females are able to transmit viruses that produce serious infectious diseases such as Dengue fever and Yellow fever causing significant morbidity and mortality worldwide. In recent years, the *A. aegypti* populations have been spread significantly increasing the risk of pathogens transmission in the human population. The availability of the genome sequence of *A. aegypti* (Nene et al., 2007) constitutes a valuable tool for investigating molecular mechanisms that are present in these vectors (Waterhouse et al., 2008). However, a better understanding of the multiple metabolic processes that operate in mosquitoes is still necessary for the discovery of new targets that leads to a successful control of mosquito populations.

Certainly, *A. aegypti* females are a remarkable biological system. In spite of the vast amount of blood meal proteins that females ingest in a short time, and the tremendous amino acid oxidation that occurs during blood metabolism (Zhou et al., 2004), *A. aegypti*

females are able to utilize dietary nutrients and efficiently remove toxic compounds and excess nitrogen. It is of interest to uncover the metabolic mechanisms that *A. aegypti* female mosquitoes use to avoid toxic accumulation of ammonia in the tissues, which result from massive amino acid deamination, in order to potentially develop new strategies for vector control. In this article, the term "ammonia" refers to both NH$_3$ and NH$_4^+$, or a combination of the two.

The strategies animals use to eliminate excess dietary nitrogen depend on several factors including the habitat (Wright, 1995; von Dungern and Briegel, 2001a,b; Singer, 2003; O'Donnell, 2009). Proline is the most abundant amino acid in *A. aegypti* hemolymph and can serve as a temporary sink for nitrogen during blood digestion (Goldstrohm et al., 2003; Scaraffia et al., 2005). In earlier studies, we showed that when proline is oxidized during mosquito flight, the ammonia is removed through the synthesis of alanine and glutamine, which acts to shuttle ammonia between flight muscles and the fat body (Scaraffia and Wells, 2003). Moreover, based on mass spectrometry methods using ^{15}NH$_4$Cl as a source of ^{15}N, we were able to measure the kinetics of ^{15}N incorporation into several labeled amino acids using intact *A. aegypti* mosquitoes (Zhang et al., 2005; Scaraffia et al., 2006). The results indicated that ammonia is mainly removed through the synthesis of glutamine and proline, via the glutamine synthetase and glutamate synthase (GS/GltS) pathway (Scaraffia et al., 2006), and by excretion of uric acid, allantoin, allantoic acid and urea (Scaraffia et al., 2008).

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The GS/GltS pathway was first discovered in the 1970s in bacteria (Tempest et al., 1970) and plants (Lea and Miflin, 1974). It has been studied extensively in several organisms especially bacteria and plants, but seldom in insects (van den Heuvel et al., 2004; Vanoni and Curti, 2008). According to molecular and biochemical studies, the fat body of *A. aegypti* seems to be the main tissue involved in ammonia detoxification (Scaraffia et al., 2005). However, it is unknown how the mosquito fat body metabolizes high levels of ammonia, or if any other tissues respond to an ammonia overload. Therefore, we undertook this metabolic labeling study to further understand how the blood feeding female mosquito is able to cope with acute toxic levels of ammonia.

2. Materials and methods

2.1. Insects

The *A. aegypti* (NIH-Rockefeller) colony was reared under standard conditions (Scaraffia and Wells, 2003). Adults were allowed to feed on 3% sucrose. Females 3 or 4 days old were utilized for *in vitro* experiments.

2.2. Reagents

The reagents utilized were the same as those described previously for studying ammonia metabolism in *A. aegypti* whole body (Scaraffia et al., 2006).

2.3. Sample preparation

Fat body (FB) or midgut (MG) tissues were dissected from 12 female mosquitoes and transferred to a 96-well microplate (four FB or four MG/well) containing *Aedes* saline solution (Hagedorn et al., 1977) supplemented with either glucose (Glc) and $^{15}NH_4Cl$, or Glc and $[^{15}N]$-glutamine labeled at different positions in a final volume of 50 μl. In some experiments, $[^{14}N]$-Glu or inhibitors such as DL-methionine DL-sulfoximine (Eisenberg et al., 2000; Meister, 1985) or O-Diazoacetyl-L-serine usually known as azaserine (Miflin and Lea, 1977; Doverskog et al., 2000) were also added to the media. In all the cases, the final concentration of each compound added ($^{15}NH_4Cl$, Glc, labeled glutamine, $[^{14}N]$-Glu or inhibitors) was 1 mM. Tissues used in this study were obtained from non-blood-fed females in order to facilitate the monitoring of (a) the kinetics of incorporation of ^{15}N from labeled compounds into ^{15}N-amino acids, and (b) the changes in the metabolism of pre-existing amino acids in the tissues during an ammonia challenge. *A. aegypti* fat body was prepared by isolating the abdomen from the thorax, removing the contents from the abdomen (complete gut, Malpighian tubules and ovaries), and then opening the abdomen by cutting along the distendable portion of the abdomen on one side. The fat body remained attached to the inner wall of the abdomen cuticle. The fat body preparation was floated on top of the incubation medium in each well with cuticle side up. The fat body or midgut tissues were maintained at 30 °C for 10, 20 or 40 min. Immediately after incubation, the tissues were removed from the incubation media. The incubation medium contained in each well was pooled and constituted an independent sample. For the quantification of unlabeled and labeled Gln, Glu and Ala, 50 μl of the incubation media was mixed with 5 μl of 1 mM deuterium-labeled amino acids as internal standards ($[^2H_5]$-Gln, $[^2H_5]$-Glu and $[^2H_4]$-Ala). For the quantification of proline, 50 μl of the incubation media was mixed with 5 μl of 1 mM $[^2H_7]$-Pro. All of the samples were then dried and derivatized as dimethylformamidine isobutyl esters or isobutyl esters, as described previously for amino acid analysis in mosquito whole body (Zhang et al., 2005; Scaraffia et al., 2006).

2.4. Electrospray ionization tandem mass spectrometry (ESI/MS/MS)

ESI/MS/MS analyses were carried out on a Finnigan TSQ-700 triple stage quadrupole mass spectrometer (San Jose, CA) equipped with a nanoelectrospray ion source operating in the positive mode and controlled by Finnigan ICIS software operated on a DEC alpha work station. The derivatization products were dissolved at 10–20 μM in methanol:H_2O:acetic acid (50:50:1). The solutions were then sprayed into the ESI source with a Harvard syringe pump at a rate of 3–5 μl/min. The needle voltage used was 1.2–1.6 kV and the skimmer was at ground potential. The capillary temperature was maintained at 200 °C. Collision-induced dissociation was performed with Argon (3 mTorr) using a collision energy of 20 eV. The instrument was tuned to unit mass resolution and the mass spectra were acquired in profile mode. Data were transferred and processed with the Xcalibur program (Xcalibur software version 1.4; Thermo Electron Corporation, San Jose, CA) using the peak area
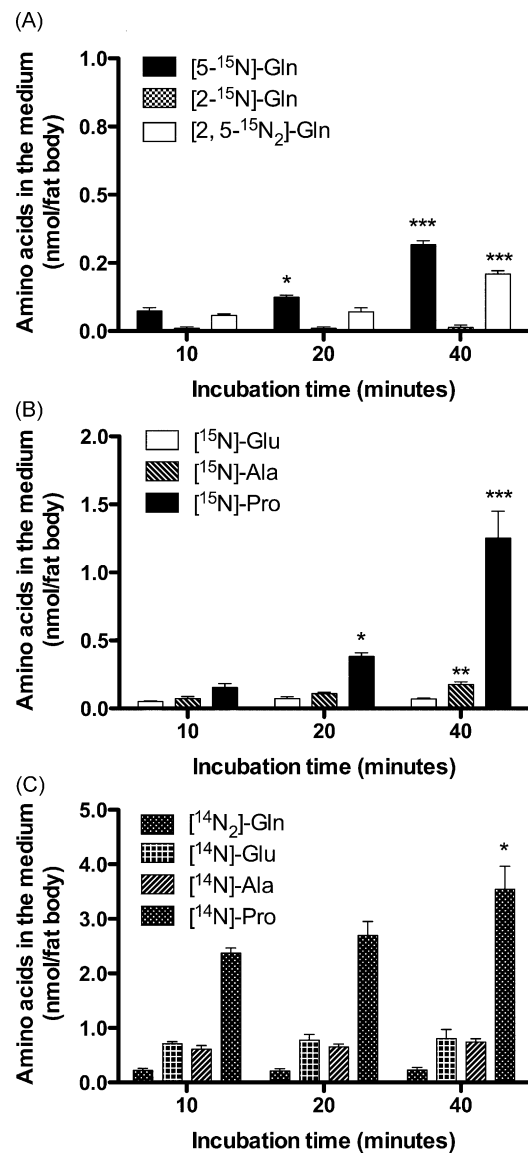

Fig. 1. Effect of 1 mM $^{15}NH_4Cl$ on *A. aegypti* fat body. (A) Time course of $[5-^{15}N]$-Gln, $[2-^{15}N]$-Gln and $[2,5-^{15}N_2]$-Gln in the media. (B) Time course of $[^{15}N]$-Glu, $[^{15}N]$-Ala and $[^{15}N]$-Pro in the media. (C) Time course of $[^{14}N_2]$-Gln, $[^{14}N]$-Glu, $[^{14}N]$-Ala and $[^{14}N]$-Pro in the media. Fat body was incubated in presence of a saline solution supplemented with 1 mM $^{15}NH_4Cl$ and 1 mM Glc for 40 min (see Section 2 for details). Data are presented as mean ± standard error of three to six independent samples. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.0001$ when compared with 10 min by ANOVA.

for the quantification of each amino acid. The identification and quantification of unlabeled and labeled amino acids were performed by multiple-reaction monitoring scans as previously described (Zhang et al., 2005; Scaraffia et al., 2006).

2.5. Statistical analyses

Student's t-test and one-way ANOVA followed by Dunnett's Multiple-Comparison test were used. Data are presented as mean $\pm$ standard error of three to six independent samples. P value <0.05 was considered to reflect statistically significant difference between groups of data. All the statistical analyses were carried out using GraphPad Prism 4.0 software (San Diego, CA, USA).

3. Results

3.1. Effect of 1 mM $^{15}NH_4Cl$ on A. aegypti fat body

Based on its essential role in blood meal metabolism, the fat body is the most likely tissue to carry out the bulk of ammonia detoxification. Therefore, we first incubated fat body tissue with 1 mM $^{15}NH_4Cl$ and measured the level of ^{15}N-labeled Gln, Glu, Ala

and Pro. As shown in Fig. 1A and B, fat body tissue efficiently and quickly converted ammonia into [^{15}N]-Pro and [5-^{15}N]-Gln. Levels of [5-^{15}N]-Gln, followed by [2,5-$^{15}N_2$]-Gln, both increased over the time course, whereas [2-^{15}N]-Gln levels remained low throughout the time course (Fig. 1A). The [^{15}N]-Glu concentration was constant throughout the time course, whereas the [^{15}N]-Ala level reached the highest concentration at 40 min. The [^{15}N]-Pro level increased markedly over the time course, peaking at 40 min post-incubation (Fig. 1B). With the exception of [^{14}N]-Pro, the concentrations of the other three unlabeled amino acids ([$^{14}N_2$]-Gln, [^{14}N]-Glu and [^{14}N]-Ala), were not altered significantly during this same time period (Fig. 1 C). When fat body was incubated in the absence of $^{15}NH_4Cl$, the [^{14}N]-amino acid concentrations did not change significantly over the time course (data not shown).

To evaluate the role of the GS/GltS pathway in fat body during an ammonia challenge, the isolated fat body tissue was incubated with 1 mM $^{15}NH_4Cl$ and specific inhibitors of GS or GltS. As shown in Fig. 2, in the presence of the GS inhibitor DL-methionine DL-sulfoximine (MX), the [5-^{15}N]-Gln and [2,5-$^{15}N_2$]-Gln levels decreased significantly, whereas [2-^{15}N]-Gln was not detected (Fig. 2A). Moreover, the [^{15}N]-Pro levels increased significantly, with the [^{15}N]-Glu and [^{15}N]-Ala levels decreasing (Fig. 2B). With
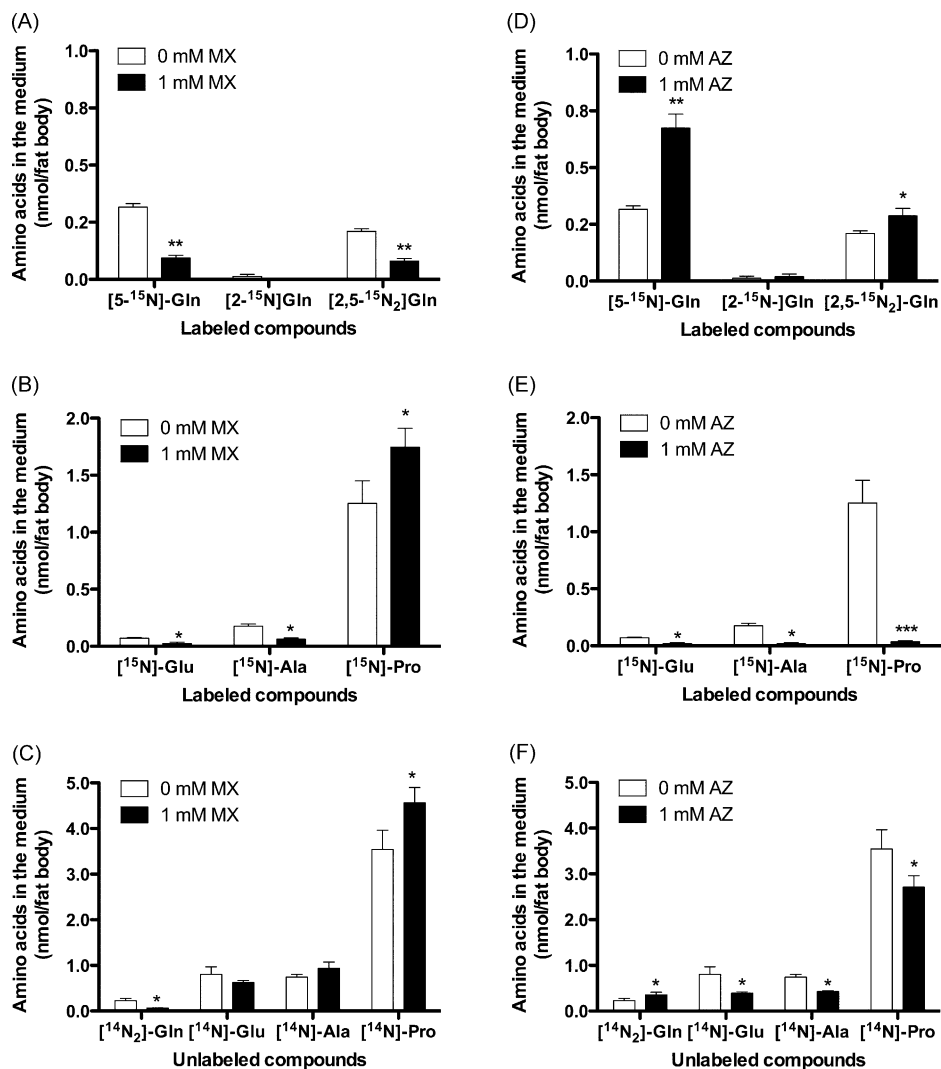

Fig. 2. Effect of $^{15}NH_4Cl$ and GS or GltS inhibitors on fat body. (A–C) Concentrations of labeled and unlabeled amino acids in presence of 1 or 0 mM D,L-methionine D,L-sulfoximine (MX), a GS inhibitor. (D–F) Concentrations of labeled and unlabeled amino acids in presence of 1 or 0 mM azaserine (AZ), a GltS inhibitor. Amino acid concentrations in the media were measured at 40 min after incubating fat body in a saline solution supplemented with 1 mM $^{15}NH_4Cl$, 1 mM Glc, 1 or 0 mM of inhibitors. Data are presented as mean $\pm$ standard error of three to six independent samples. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.0001$ when compared with 0 mM MX or 0 mM AZ by Student's t-test.

regard to the level of unlabeled amino acids, the concentrations of [^{14}N]-Glu and [^{14}N]-Ala remained constant. However, the [^{14}N]-Pro concentration increased and the [^{14}N$_2$]-Gln level decreased in the presence of the GS inhibitor (Fig. 2C). Addition of the GltS inhibitor azaserine (AZ), to the incubation media, led to increased [5-^{15}N]-Gln and [2,5-^{15}N$_2$]-Gln levels (Fig. 2D), whereas, [^{15}N]-Glu, [^{15}N]-Ala and [^{15}N]-Pro concentrations decreased markedly (Fig. 2E). In addition, we observed that the [^{14}N$_2$]-Gln level increased, whereas, the rest of the [^{14}N]-amino acid concentrations decreased (Fig. 2F). These results indicate that in fat body, the GS/GltS pathway plays a key role in ammonia detoxification.

3.2. Effect of incubating A. aegypti fat body with 1 mM labeled glutamine

To verify that glutamine serves as a precursor for Pro synthesis in the fat body, the tissue was incubated with 1 mM of ^{15}N-glutamine labeled at different positions. We observed that after incubating fat body with 1 mM [2,5-^{15}N$_2$]-Gln, the levels of [5-^{15}N]-Gln, [2-^{15}N]-Gln, [^{15}N]-Glu and [^{15}N]-Ala in the media did not change significantly over the time period tested. The level of [2,5-^{15}N$_2$]-Gln decreased, whereas the concentration of [^{15}N]-Pro

increased markedly (Fig. 3A and B). Concentrations of unlabeled amino acids did not change significantly at 40 min after incubation (Fig. 3C). Fat body tissue incubated with 1 mM [5-^{15}N]-Gln (Fig. 3D–F), or 1 mM [2-^{15}N]-Gln (data not shown), produced similar effects as those observed with [2,5-^{15}N$_2$]-Gln (Fig. 3A–C). However, the levels of [^{15}N]-Ala and [^{15}N]-Pro quantified in the presence of glutamine labeled at one position were almost two times lower than seen with [2,5-^{15}N$_2$]-Gln. As shown in Fig. 3D, the concentration of [5-^{15}N]-Gln decreased significantly, whereas the levels of [2,5-^{15}N$_2$]-Gln and [2-^{15}N]-Gln remained at very low levels after 40 min post-incubation with 1 mM [5-^{15}N]-Gln. Lastly, although the levels of [^{15}N]-Glu nor [^{15}N]-Ala were not altered, the [^{15}N]-Pro level increased significantly (Fig. 3E). The profile of [^{14}N]-amino acids was similar to that reported in Fig. 3C, except that the [^{14}N]-Pro concentration increased significantly at 40 min post-incubation (Fig. 3F). These data are well correlated with the fact that each [2,5-^{15}N$_2$]-Gln molecule catalyzed by GltS produces two [^{15}N]-Glu molecules, whereas each [5-^{15}N]-Gln catalyzed by GltS produces one [^{15}N]-Glu molecule and one [^{14}N]-Glu molecule.

When fat body was incubated in the presence of [2,5-^{15}N$_2$]-Gln and AZ for 40 min, we observed an increase in the concentrations of [5-^{15}N]-Gln, [2,5-^{15}N$_2$]-Gln, and [2-^{15}N]-Gln (Fig. 4A). Moreover,
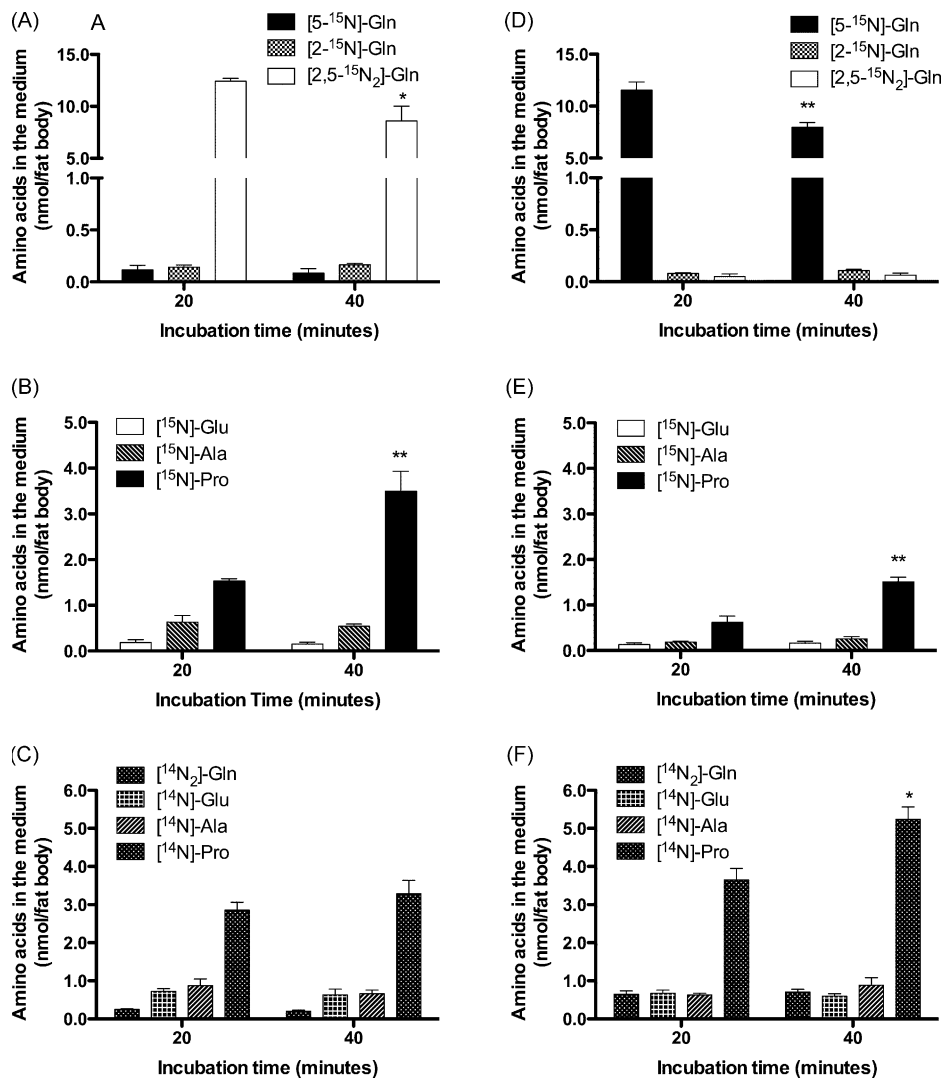

Fig. 3. Effect of [2,5-^{15}N$_2$]-Gln or [5-^{15}N]-Gln on fat body. (A–C) Concentrations of labeled and unlabeled amino acid in presence of [2,5-^{15}N$_2$]-Gln. (D–F) Concentrations of labeled and unlabeled amino acids in presence of [5-^{15}N]-Gln. Amino acid concentrations in the media were measured after incubating fat body in a saline solution supplemented with 1 mM [2,5-^{15}N$_2$]-Gln or [5-^{15}N$_2$]-Gln and 1 mM Glc. Data are presented as mean ± standard error of four independent samples. $^{*}P < 0.05$ and $^{**}P < 0.01$ when compared with 20 min by Student's t-test.
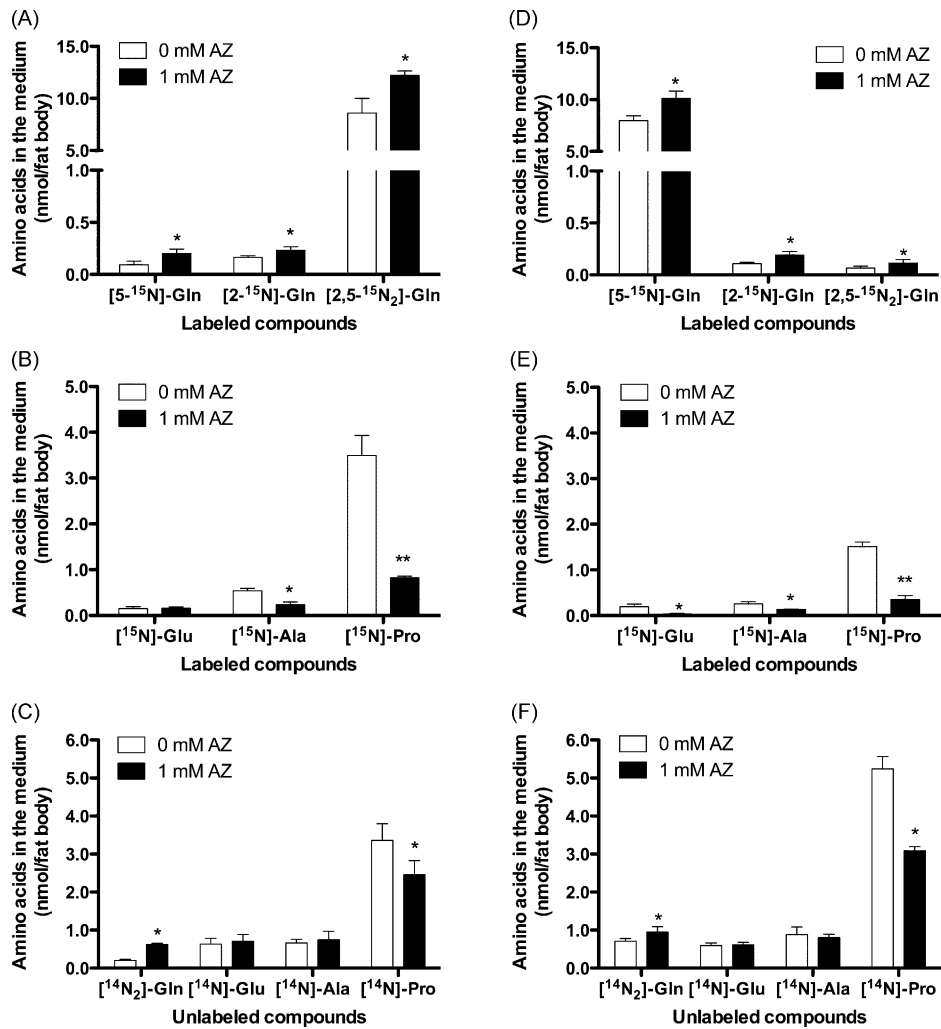
Fig. 4. Effect of $[2,5-^{15}N_2]$-Gln, $[5-^{15}N]$-Gln and GltS inhibitor (AZ) on fat body. (A–C) Concentrations of labeled and unlabeled amino acids in presence of $[2,5-^{15}N_2]$-Gln and AZ. (D–F) Concentrations of labeled and unlabeled amino acids in presence of $[5-^{15}N]$-Gln and AZ. Amino acid concentrations in the media were measured after incubating fat body in a saline solution supplemented with 1 mM $[2,5-^{15}N_2]$-Gln or $[5-^{15}N_2]$-Gln, 1 mM Glc and 1 or 0 mM of AZ. Data are presented as mean ± standard error of four independent samples. $^*P < 0.05$; $^{**}P < 0.01$ when compared with 0 mM by Student's *t*-test.

this treatment did not alter the $[^{15}N]$-Glu level, whereas the $[^{15}N]$-Ala and $[^{15}N]$-Pro levels decreased significantly (Fig. 4B). The concentrations of unlabeled $[^{14}N]$-Glu and $[^{14}N]$-Ala remained constant, however, $[^{14}N_2]$-Gln levels increased and $[^{14}N]$-Pro levels decreased significantly in the presence of AZ (Fig. 4C). Addition of AZ to the media containing $[5-^{15}N]$-Gln led to an increase in the levels of $[5-^{15}N]$-Gln, $[2-^{15}N]$-Gln and $[2,5-^{15}N_2]$-Gln (Fig. 4D), and a decrease in the concentrations of $[^{15}N]$-Glu, $[^{15}N]$-Ala and $[^{15}N]$-Pro concentrations (Fig. 4E). The profile of unlabeled amino acids (Fig. 4F) was similar to that reported in Fig. 4C. The results demonstrate that in fat body, glutamine acts as precursor for proline synthesis through a GltS catalyzed reaction.

3.3. Effect of incubating A. aegypti fat body with 1 mM $^{15}NH_4Cl$ and 1 mM glutamic acid

In order to have further evidence that the ^{15}N from labeled ammonia is first incorporated into the amide group of glutamine and then into the amino group of the other amino acids, the incubation medium was supplemented with unlabeled glutamic acid. As is shown in Fig. 5A and B, the $[5-^{15}N]$-Gln level rapidly increased over the time course evaluated and it was the most abundant labeled amino acid observed in the media followed by $[^{15}N]$-Pro, which is consistent with the role of GS/GltS pathway in

fixation and assimilation of ammonia. The concentrations of $[2,5-^{15}N_2]$-Gln increased gradually during 40 min, whereas the levels of $[^{15}N]$-Glu and $[^{15}N]$-Ala increased at the end of the time course. The concentration of $[2-^{15}N]$-Gln remained low and constant over the time course. The profile of some unlabeled amino acids changed significantly. The level of $[^{14}N]$-Glu decreased, whereas the $[^{14}N]$-Pro concentration increased significantly during the incubation period. No significant changes were observed in the $[^{14}N_2]$-Gln and $[^{14}N]$-Ala concentrations over the time course (Fig. 5C). GS or GltS inhibitors produced effects similar to that reported in Fig. 2 (data not shown). Taken together, these data confirm that ^{15}N from labeled ammonia is first incorporated into the amide group of glutamine and then into the amino group of the other amino acids.

3.4. Effect of 1 mM $^{15}NH_4Cl$ on A. aegypti midgut

We have previously found that GS and GltS genes are differentially expressed in fat body and midgut tissues (Scaraffia et al., 2005). Therefore, the results we obtained from ^{15}N-labeling of fat body tissue may not apply to mechanisms of ammonia metabolism in the midgut. To test this possibility, midgut tissue was incubated with 1 mM $^{15}NH_4Cl$ and the amino acids were quantified using the same methods as done previously with fat

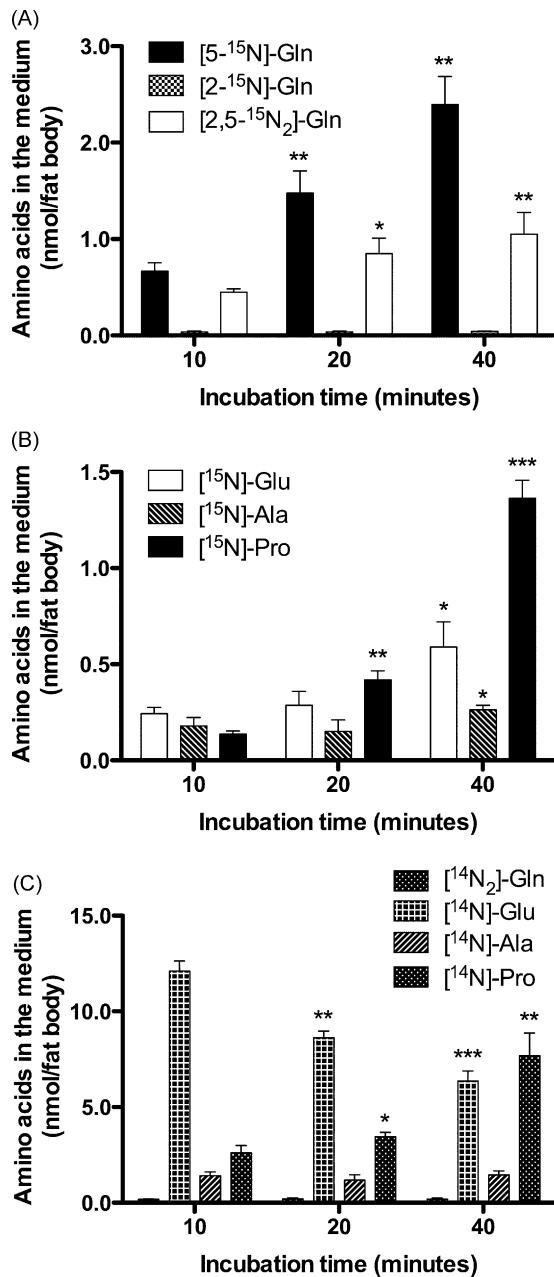**Fig. 5.** Effect of 1 mM $^{15}NH_4Cl$ and 1 mM [^{14}N]-Glu on fat body. (A) Time course of [5-^{15}N]-Gln, [2-^{15}N]-Gln and [2,5-$^{15}N_2$]-Gln in the media. (B) Time course of [^{15}N]-Glu, [^{15}N]-Ala and [^{15}N]-Pro in the media. (C) Time course of [$^{14}N_2$]-Gln, [^{14}N]-Glu, [^{14}N]-Ala and [^{14}N]-Pro in the media. Fat body was incubated in presence of a saline solution supplemented with 1 mM $^{15}NH_4Cl$, 1 mM Glc and 1 mM [^{14}N]-Glu for 40 min (see Section 2 for details). Data are presented as mean ± standard error of four independent samples. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.0001$ when compared with 10 min by ANOVA.

Fig. 6. Effect of 1 mM $^{15}NH_4Cl$ on *A. aegypti* midgut. (A) Time course of [5-^{15}N]-Gln, [2,5-$^{15}N_2$]-Gln and [^{15}N]-Ala in the media. (B) Time course of [$^{14}N_2$]-Gln, [^{14}N]-Glu, [^{14}N]-Ala and [^{14}N]-Pro in the media. Midgut was incubated in presence of a saline solution supplemented with 1 mM $^{15}NH_4Cl$ and 1 mM Glc for 40 min (see Section 2 for details). Data are presented as mean ± standard error of four independent samples. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.0001$ when compared with 10 min by ANOVA.

body tissue. As shown in Fig. 6A, [^{15}N]-Ala, followed by [5-^{15}N]-Gln and [2,5-$^{15}N_2$]-Gln were the most abundant ^{15}N-amino acids observed in the media. In contrast, [2-^{15}N]-Gln, [^{15}N]-Glu and [^{15}N]-Pro were not detected, suggesting that ammonia metabolism in fat body and midgut tissues is different. The levels of [^{14}N]-amino acids did not change significantly during the time course in presence (Fig. 6B) or absence of $^{15}NH_4Cl$ (data not shown).

The effect of GS or GltS inhibitors on ammonia metabolism in *A. aegypti* midgut tissue is shown in Fig. 7. In the presence of MX, [5-^{15}N]-Gln and [2,5-$^{15}N_2$]-Gln concentrations decreased, whereas the [^{15}N]-Ala level increased (Fig. 7A). In the presence of AZ, [5-^{15}N]-Gln, [2,5-$^{15}N_2$]-Gln and [^{15}N]-Ala levels were not modified

significantly (Fig. 7B). The addition of GS or GltS inhibitors to the incubation media did not significantly change the [^{14}N]-amino acid concentrations (data not shown). The results evidence that metabolic pathways that detoxify ammonia in midgut are distinct to fat body.

To verify that midgut depends on the provision of glucose to synthesize high levels of [^{15}N]-Ala during an ammonia challenge, the tissue was incubated in a medium with 1 mM $^{15}NH_4Cl$ and 0 or 1 mM glucose. As is shown in Fig. 8A, the [5-^{15}N]-Gln and [2,5-$^{15}N_2$]-Gln increased, whereas the [^{15}N]-Ala level decreased significantly in absence of glucose. [2-^{15}N]-Gln, [^{15}N]-Glu and [^{15}N]-Pro were not detected. The levels of [^{14}N]-Gln and [^{14}N]-Glu were not modified significantly. However, [^{14}N]-Ala and [^{14}N]-Pro levels declined when the medium was deprived of glucose (Fig. 8B). These data indicate that in mosquito midgut, glucose plays an important role in providing carbon skeleton for alanine synthesis during an ammonia challenge.

4. Discussion

A. aegypti mosquitoes utilize carbon derived from blood meal proteins primarily for oxidative metabolism, and to a lesser extent, for gluconeogenesis and lipogenesis (Zhou et al., 2004). This metabolic response to blood feeding also produces large amounts of ammonia as a side product. The ammonia is generated by numerous enzymes including glutamate dehydrogenase (through oxidative deamination of glutamic acid), aspartate ammonialyase, asparaginase, glutaminase, adenosine deaminase and glucosa-
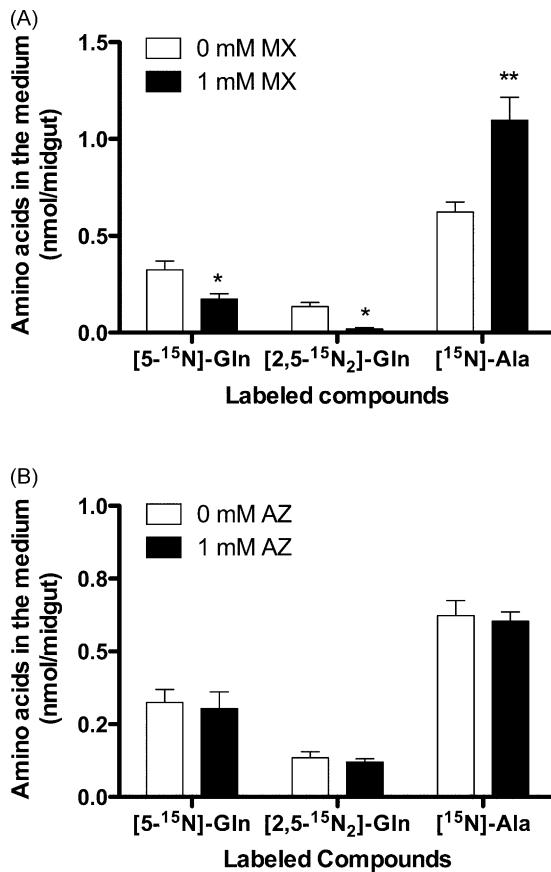
Fig. 7. Effect of $^{15}NH_4Cl$ and D,L-methionine D,L-sulfoximine (MX) or azaserine (AZ) on midgut. (A) Amino acid concentrations in the media were measured at 40 min after incubating midgut in a saline solution supplemented with 1 mM $^{15}NH_4Cl$, 1 mM Glc and 1 or 0 mM MX. (B) Amino acid concentrations in the media were measured at 40 min after incubating midgut in a saline solution supplemented with 1 mM $^{15}NH_4Cl$, 1 mM Glc and 1 or 0 mM AZ. Data are presented as mean ± standard error of four independent samples. *$P < 0.05$; **$P < 0.01$ when compared with 0 mM MS or AZ by Student's t-test.

Fig. 8. Effect of 1 mM $^{15}NH_4Cl$ on midgut in a medium deprived of glucose. (A) Labeled amino acids. (B) Unlabeled amino acids. Amino acid concentrations in the media were measured at 40 min after incubating midgut in a saline solution supplemented with 1 mM $^{15}NH_4Cl$ and 1 or 0 mM of Glc. Data are presented as mean ± standard error of four independent samples. *$P < 0.05$; **$P < 0.01$ when compared with 1 mM Glc by Student's t-test.

mine-6-phosphate deaminase. We had previously analyzed several key aspects of ammonia metabolism in *A. aegypti* and found that mosquitoes have efficient mechanisms to avoid ammonia intoxication (Scaraffia and Wells, 2003; Scaraffia et al., 2005, 2006, 2008). In the studies performed here, we have used an *in vitro* tissue culture system and ^{15}N-labeled compounds to show that *A. aegypti* fat body and midgut tissues can efficiently metabolize up to 1 mM $^{15}NH_4Cl$ using different metabolic pathways.

The results reveal that by incubating *A. aegypti* fat body with $^{15}NH_4Cl$, ammonia metabolism in the fat body requires the GS/GltS pathway as summarized in Fig. 9. Most of the ammonia in fat body tissue is converted to $[5-^{15}N]$-Gln and $[^{15}N]$-Pro. This is primarily done by fixing $^{15}NH_3$ into $[^{14}N]$-Glu via GS, which leads to a rapid increase of in the levels of $[5-^{15}N]$-Gln and $[^{15}N]$-Pro. Our analysis also shows that the $[5-^{15}N]$-Gln is mainly metabolized by GltS to yield $[^{14}N]$-Glu and $[^{15}N]$-Glu. Although, $[^{14}N]$-Glu or $[^{15}N]$-Glu can react with pyruvate to turn into $[^{14}N]$-Ala or $[^{15}N]$-Ala and α-ketoglutarate through alanine aminotransferase (ALAT), most of $[^{14}N]$-Glu and $[^{15}N]$-Glu are utilized for $[^{14}N]$-Pro and $[^{15}N]$-Pro synthesis via reactions catalyzed by pyrroline-5-carboxylate synthase (P5CS) and pyrroline-5-carboxylate reductase (P5CR). The $[^{15}N]$-Glu produced by GltS can also be used through GS to fix other labeled ammonia and produce $[2,5-^{15}N_2]$-Gln, which can become $[^{15}N]$-Glu via GltS (Fig. 9). The $[5-^{15}N]$-Gln, that is not metabolized by GltS, follows other metabolic fates such as
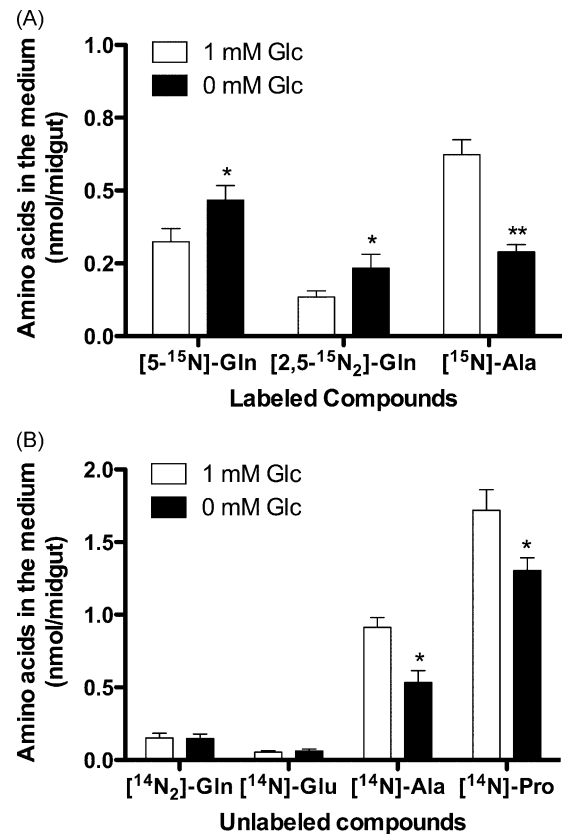
synthesis of $[^{15}N_2]$-uric acid (Scaraffia et al., 2006, 2008). The low levels of $[2-^{15}N]$-Gln in the media are expected since its synthesis requires $[^{15}N]$-Glu and unlabeled ammonia, which must be reduced in the tissue under a load of external labeled ammonia.

It was reported in *A. aegypti* fat body that blood feeding affects the pattern of expression of several enzymes (Scaraffia et al., 2005) involved in ammonia-metabolizing pathways shown in Fig. 9. For example, the transcription levels of two genes encoding GS (AaGS1 and AaGS2) were dramatically induced in fat body at 3 h post-blood meal (PBM) compared to control females. The gene encoding AaGDH was also up-regulated in response to a blood meal. However, the peak of the gene expression was detected at 12 h PBM. Certainly, the release of ammonia during the process of blood digestion stimulates fat body to a rapid fixation of ammonia into glutamic acid by GS, which is in agreement with the data presented here. Transcript levels corresponding to the gene encoding glutamate synthase (AaGltS) were significantly increased at 36 and 48 h PBM. This pattern of expression is consistent with the proposed role of the GS/GltS pathway in the fixation and assimilation of ammonia in fat body. The expression pattern of AaP5CS and AaP5CR1/2/3 in fat body were found to be regulated by blood meal intake, which are also in line with the biochemical results reported here.

Our data also show that inhibition of GS with DL-methionine DL-sulfoximine modified the functioning of the GS/GltS pathway. However, under these specific conditions, fat body was able to detoxify ammonia by fixing labeled ammonia into α-ketoglutarate through GDH. The $[^{15}N]$-Glu produced by this reaction was further utilized for $[^{15}N]$-Pro synthesis via P5CS and P5CR. The experi-
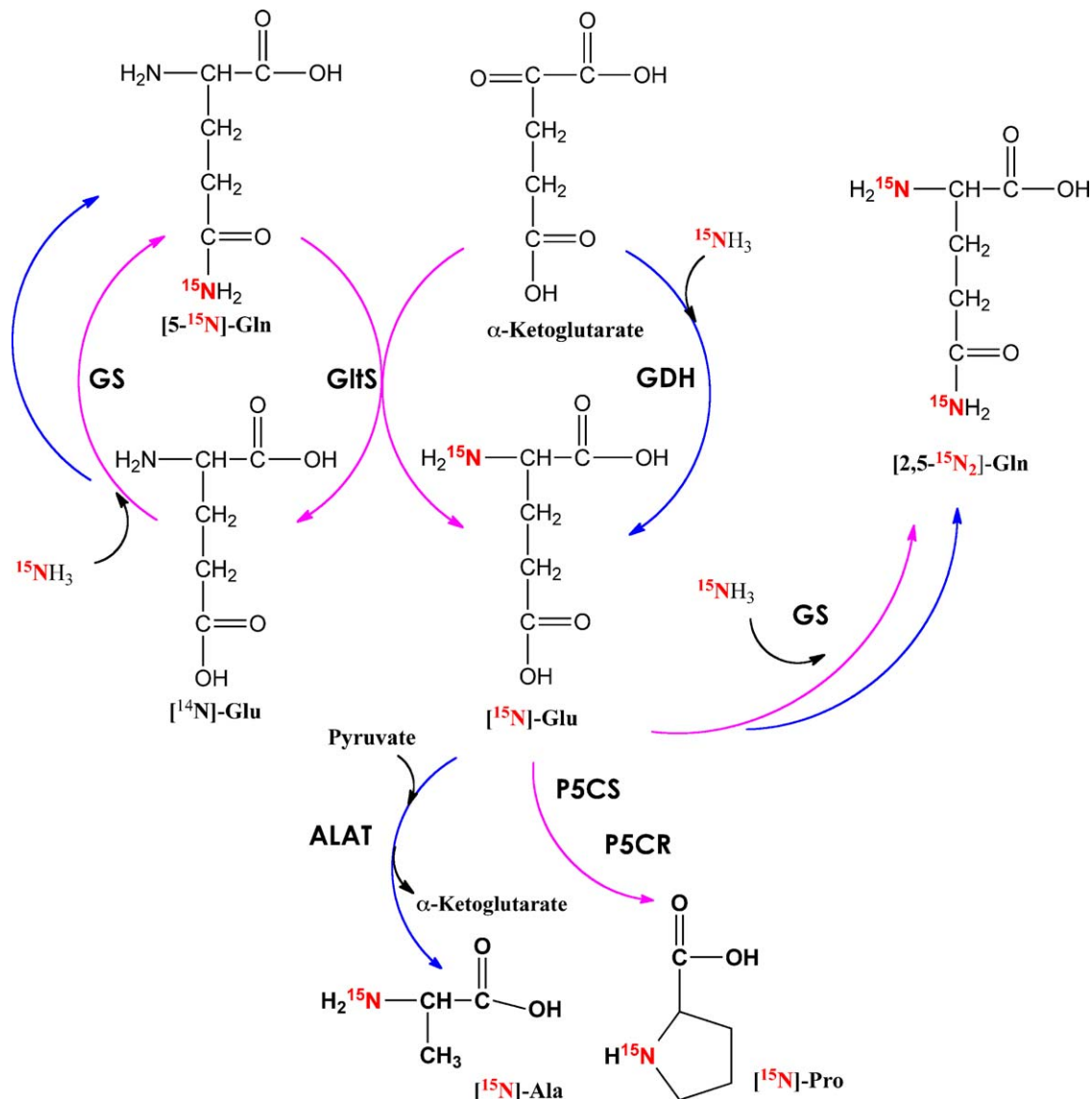
Fig. 9. Schematic representation of the metabolic pathways for ammonia metabolism in *A. aegypti* females. Different colors are used to highlight the main enzymatic reactions that operate during ammonia detoxification in fat body (pink) and midgut (blue) tissues (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article).Adapted from Scaraffia et al. (2006).

ments in which fat body tissue was incubated with $[2,5\text{-}^{15}N_2]$-Gln or $[5\text{-}^{15}N]$-Gln (Fig. 3), demonstrated that at least part of glutamine was utilized for proline synthesis via GltS. The participation of GltS in the metabolism of glutamine, and its role in proline synthesis, was confirmed when in presence of a GltS inhibitor, the substrate of GltS was accumulated and the $[^{15}N]$-Pro concentration reduced significantly (Fig. 4). These data are in agreement with those results reported previously in the hemolymph (Scaraffia et al., 2005) and whole body of *A. aegypti* females (Scaraffia et al., 2006).

In insects, fat body is the major tissue for metabolism and storing of nutrients. The remarkable functions performed by this tissue are similar to those of vertebrate adipose tissue and liver (Haunerland and Shirk, 1995; Liu et al., 2009; Arrese and Soulages, 2010). In spite of the absence of a functional urea cycle in mosquito, our ^{15}N-labeling studies demonstrate that the presence of a functional GS/GltS pathway in fat body plays a unique role in ammonia metabolism in *A. aegypti* females.

The midgut of *A. aegypti* mosquitoes also responds efficiently to high levels of ammonia. Following incubation with 1 mM $^5NH_4Cl$, we found significant $[^{15}N]$-Ala, $[5\text{-}^{15}N]$-Gln and $[2,5\text{-}^{15}N_2]$-Gln

levels in the midgut media throughout the incubation period. As shown in Fig. 9, we propose that this occurs mainly by reactions catalyzed by GDH, ALAT and GS as GDH fixes $^{15}NH_3$ at α-ketoglutarate and leads to $[^{15}N]$-Glu. ALAT transfers the amino group from $[^{15}N\text{-}Glu]$ to pyruvate and produces $[^{15}N]$-Ala and α-ketoglutarate. $^{15}NH_3$ that is fixed to $[^{14}N]$-Glu generates $[5\text{-}^{15}N]$-Gln, whereas the $^{15}NH_3$ that is fixed to $[^{15}N]$-Glu turns into $[2,5\text{-}^{15}N_2]$-Gln by reactions catalyzed by GS. Interestingly, in this study, neither $[2\text{-}^{15}N]$-Gln, $[^{15}N]$-Glu nor $[^{15}N]$-Pro were observed in the midgut tissue media. These results indicate that the unlabeled ammonia was reduced and that most of $[^{15}N]$-Glu was rapidly utilized for $[^{15}N]$-Ala and $[2,5\text{-}^{15}N_2]$-Gln synthesis. In agreement with these biochemical findings, genes encoding AaGS1 and AaGDH in *A. aegypti* midgut showed a similar profile of expression during blood meal digestion. Both genes were regulated by blood feeding, reaching the highest peak of expression between 6 and 12 h PBM. In contrast, the mRNA levels of AaGS2, AaGltS, AaP5CS, and AaP5CR1/3 in the midgut were found to be low, with little change in expression profile in response to a blood meal (Scaraffia et al., 2005).

The deprivation of glucose in the midgut cultures resulted in increased [5-^{15}N]-Gln and [2,5-^{15}N$_2$]-Gln labeling via GS and GDH-GS respectively. The decrease we observed in the [^{14}N]-Pro level suggests that this amino acid is mainly utilized for supplying [^{14}N]-Glu. The [^{14}N]-Ala and [^{15}N]-Ala levels were found to be reduced significantly in the absence of glucose, which highlights the importance of the glycolytic pathway in providing pyruvate for the reaction catalyzed by ALAT (Fig. 9).

It was reported that the presence of high ammonia concentrations in mammals and insect cells resulted in an increased alanine production, which is dependent on the availability of glucose (Häggström et al., 1996). Oba et al. (2005) proposed that net alanine synthesis by ruminant gut tissues is an important pathway for ammonia disposal. The ammonia-^{15}N incorporation into alanine by duodenal mucosal cells increased with the addition of glucose to the incubation media, suggesting that glucose is a primary source of the carbon skeleton for alanine synthesis (Oba et al., 2005). It was also reported that the addition of ammonia to isolated honeybee retinal cells stimulates the formation of alanine, as well as glycolysis (Tsacopoulos et al., 1997). Moreover, *Spodoptera frugiperda* insect cells have been shown to accumulate alanine as a metabolic by-product (Bedard et al., 1993; Ohman et al., 1995; Drews et al., 2000) with high levels of glucose consumption, which is indicative of an active glycolytic pathway (Neermann and Wagner, 1996; Raghunand and Dale, 1999). The presence of a GS inhibitor in *A. aegypti* midgut cultures decreased the levels of [5-^{15}N]-Gln and [2,5-^{15}N$_2$]-Gln but increased the concentration of [^{15}N]-Ala as regards controls (Fig. 7). These data indicate that the labeled ammonia that cannot be metabolized by GS, is fixed into α-ketoglutarate to produce [^{15}N]-Glu via GDH. The [^{15}N]-Glu is further converted into [^{15}N]-Ala by ALAT (Fig. 9). Doverskog et al. (2000) reported that in *S. frugiperda* insect cells, the [5-^{15}N]-Gln serves as precursor for [^{15}N]-Ala synthesis. By using NMR techniques, the authors reported that the nitrogen from [5-^{15}N]-Gln was selectively incorporated into α-ketoglutarate forming [^{15}N]-Glu via glutamate synthase and that the enzyme activity was completely inhibited by 1 mM azaserine (Doverskog et al., 2000).

In mosquito midgut, glutamine and alanine were found to be synthesized through reactions catalyzed by GS, GDH and ALAT (Fig. 9). The changes detected in the amino acid concentrations post-incubation with ^{15}NH$_4$Cl are consistent with the role that those enzymes play in the ammonia disposal. When *A. aegypti* midgut was incubated with 1 mM GltS inhibitor, no significant changes were observed in the levels of amino acids studied (Fig. 7). These data agree with the fact that GltS was not detected in mosquito midgut (Scaraffia et al., 2005) and confirm that neither GS/GltS pathway is functional in this tissue. The absence of a GS/GltS pathway does not minimize the role of the midgut in the ammonia metabolism. On the contrary, it indicates that this tissue can efficiently metabolize ammonia by using other metabolic routes. Our results also show that *A. aegypti* midgut can play a key role during blood digestion by removing toxic ammonia levels through the synthesis of specific amino acids, which can be immediately released to the hemolymph for diverse metabolic fates.

In summary, stable labeled isotopes and mass spectrometry techniques have proved to be powerful tools to investigate the ^{15}N-flux in mosquito tissues using an *in vitro* system. The results presented in this paper reveal that *A. aegypti* fat body and midgut are both involved in the ammonia detoxification. However, each tissue uses different metabolic pathways. As a result of ammonia metabolism, the tissues produce non-toxic molecules that can act as a nitrogen carrier. Indeed, fat body appears to mainly synthesizes glutamine and proline, whereas midgut mainly synthesizes glutamine and alanine (Fig. 9). The

conversion of excess ammonia to these amino acids would allow mosquitoes to temporarily store ammonia in a neutral form. It is important to point out, that in addition to the multiple roles that the mosquito midgut has in blood meal digestion (Terra, 1990; Sanders et al., 2003; Kato et al., 2006; Hegedus et al., 2009), we found that midgut tissue is also involved in ammonia metabolism. The participation of midgut in ammonia detoxification may reduce the ammonia load on the fat body during blood digestion.

Taken together, these findings allow us to gain new insights into the roles that both fat body and midgut tissues have in the functioning of whole body mosquito metabolism. In addition to a differential expression pattern of certain genes related to ammonia metabolism in *A. aegypti* fat body and midgut (Scaraffia et al., 2005), we know now that there is also a differential ammonia metabolism in these tissues. Clearly, the process of fixation, assimilation and excretion of ammonia is absolutely critical to the blood-fed females (Scaraffia et al., 2005, 2006, 2008). By disrupting or blocking this metabolic process, the female mosquitoes could fail to maintain the ammonia levels below lethal concentrations in the tissues with the eminent risk of dying. Therefore, targeting specific enzymes involved in ammonia-metabolizing pathways in mosquito tissues could provide a novel strategy for control mosquito population.

Acknowledgments

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