

Metabolic fate of [^{14}C]-labeled meal protein amino acids in *Aedes aegypti* mosquitoes

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Abstract

We developed a method to follow the metabolic fate of [^{14}C]-labeled *Euglena gracilis* protein amino acids in *Aedes aegypti* mosquitoes under three different adult nutritional regimes. Quantitative analysis of blood meal protein amino acid metabolism showed that most of the carbon of the amino acids was either oxidized to CO_2 or excreted as waste. Under the three different adult nutritional regimes, no significant differences in the metabolism of amino acids were found, which indicated that the female *A. aegypti* mosquitoes possess a substantial capacity of maintaining metabolic homeostasis during a gonotrophic cycle. The amount of maternal glycogen and lipid after egg laying were significantly lower in the mosquitoes that underwent a partial starvation before a blood meal and/or starvation after the blood meal. The content of egg lipid or protein or the number of eggs laid did not show a significant difference among the three different regimes, which indicates that stable fecundity of *A. aegypti* under the partial starvation before a blood meal and/or starvation after the blood meal seemed to result from a trade-off between current fecundity and future survival after the eggs laid. The methods described in this paper can be applied to a wide range of questions about the effects of environmental conditions on the utilization of blood meal amino acids.

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1. Introduction

The evolution of hematophagy in anautogenous mosquitoes led to a rigid dependence on vertebrate blood for reproduction (Briegel, 1985). Anautogenous mosquitoes can also accumulate energy reserves (lipid and glycogen) for survival and flight from a blood meal (Nayar and Sauerman, 1975a; Clements, 1992). The partitioning of blood meal protein amino acids between reproduction and energy storage can be impacted by many factors, including (1) teneral body size, which is dependent on larval nutrition (Briegel, 1990a,b; Briegel and Timmermann, 2001; Briegel et al., 2001, 2002; Takken et al., 1998; Naksathit and Scott, 1998; Naksathit et al., 1999a,b), (2) adult nutrition,

which can include sugar feeding or starvation (Nayar and Sauerman, 1971a, 1975a,b; Nayar and Pierce, 1977; France and Judson, 1979; Briegel, 1990b; van Handel, 1991, 1992, 1993; Andersson, 1992; Day et al., 1994; Foster, 1995; Scott et al., 1997; Naksathit and Scott, 1998; Ziegler, 1997; Ziegler and Ibrahim, 2001; Naksathit et al., 1999a; Briegel et al., 2001; Harrington et al., 2001; Gary and Foster, 2001), (3) gonotrophic cycle (Briegel et al., 2002), (4) source of the blood meal (Harrington et al., 2001), (5) mating status (Klowden, 1993), and (6) age (Naksathit et al., 1999a).

Usually, sugar feeding is considered as an important source of nutrients for mosquitoes. Sugar is the only food consumed by male mosquitoes and is an important food for building energy reserves for survival and flight in female mosquitoes (van Handel, 1965a; Nayar and Sauerman, 1971b, 1974, 1975c; Naksathit et al., 1999c) and can spare the blood meal protein amino acids for egg production (Nayar and Sauerman, 1975b;

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van Handel, 1992, 1993; Gary and Foster, 2001; Briegel et al., 2001). Sugar feeding is routinely used for mosquito colony maintenance in most laboratories, although Harrington et al. (2001) maintain their colony solely by blood feeding. Andersson (1992) concluded that the initial sugar meal was very important for longevity in female *Aedes communis* and that one sugar meal, one blood meal and several sugar meals during oogenesis was the optimal diet for this species. Therefore, sugar feeding could be one of the extremely important factors affecting the fate of blood meal protein amino acids.

However, all of the above-mentioned studies have depended on static measurements of the mass of components such as proteins, lipids and glycogen, etc. using either the whole mosquito or selected mosquito tissue(s). A deeper understanding of the fate of blood meal protein amino acids can be gained by following the time-course for the metabolism of the amino acids. Using a dynamic method for a detailed analysis of metabolism of amino acids derived from blood meal proteins can provide new insights into the physiology and biochemistry of the mosquito.

The present study was designed to obtain a dynamic overview of the metabolic fate of amino acids derived from dietary proteins in *Aedes aegypti* mosquitoes with different adult nutritional regimes by using [^{14}C]-labeled meal protein and following the fate of the labeled amino acid carbons after a blood meal.

2. Materials and methods

2.1. Mosquito

A. aegypti (Rockefeller) were used. Larvae were maintained on a diet consisting of equal proportions of rat chow (Sunburst Pet Foods, Phoenix, AZ), lactalbumin hydrolysate (USB, Cleveland, OH) and yeast hydrolysate (USB, Cleveland, OH) in batches of 2000 in a 13-cm deep pan measuring 142×76 cm, containing about 4-cm of water. Female pupae were separated from males using a mosquito separator. Adult mosquitoes were routinely maintained at 28°C , 70–80% relative humidity and a photoperiod of 16:8 h (L:D) on 3% sucrose ad libitum for 3 days after eclosion.

2.2. Preparation of [^{14}C]-labeled proteins from *Euglena gracilis*

In preliminary experiments, we found that [^{14}C]-labeled free amino acids added to a protein meal were metabolized very rapidly compared to the rate at which the protein in the meal was hydrolyzed. Within about 8 h, all the labeled free amino acids were either oxidized to CO_2 or incorporated into midgut proteins and there

was no further metabolism during the remainder of the gonotrophic cycle. Thus, the metabolic fate of the free amino acids did not reflect the metabolism of the meal protein amino acids because they were completely metabolized long before significant hydrolysis of the meal protein occurred.

Thus, it was necessary to use [^{14}C]-labeled proteins to follow the fate of meal protein amino acids. Because there are no commercially ready-made [^{14}C]-labeled protein products, we needed to prepare one. The following protocol for [^{14}C]-amino acid labeled proteins is based on the procedure of Krzywicha and Wagner (1975). *E. gracilis* were grown in neutral phototrophic mineral medium (pH 6.8) (Cramer and Myers, 1952; Edmunds, 1965) at room temperature with shaking and constant illumination by a 60-W soft light white bulb. Green cells from a mass culture during the exponential growth phase were transferred into a sterilized 25-ml incubation flask with a red rubber sleeve and a polypropylene suspended center well (Kontes, Vineland, NJ) (Fig. 1). Before assembly, the center well was wetted with 80 μl of water to trap the electrostatic BaCO_3 and then 0.3 mCi of $\text{Ba}^{14}\text{CO}_3$ (NEN[®] Life Science Products, MA) was added. The center well was inserted into the airtight cap and placed into the flask. Sufficient HCl was injected through the stopper into the center well to convert all the $\text{Ba}^{14}\text{CO}_3$ into $^{14}\text{CO}_2$. The $^{14}\text{CO}_2$ served as the sole carbon source for the *E. gracilis* culture. The culture was statically incubated for 7 days under illumination from a 60-W soft light white bulb. Prior to opening the flask, the center well was filled with NaOH to remove any unincorporated $^{14}\text{CO}_2$, which was trapped as Na_2CO_3 .

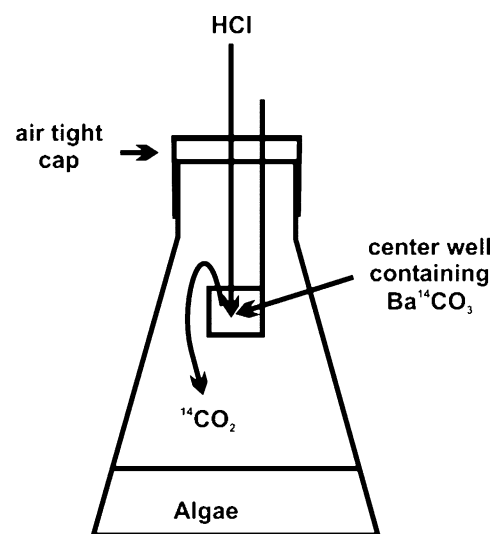


Fig. 1. Diagram of the apparatus used to prepare [^{14}C]-labeled *Euglena gracilis* proteins. $\text{Ba}^{14}\text{CO}_3$ was placed in the center well and converted to $^{14}\text{CO}_2$ by injecting HCl. The *Euglena* was grown phototrophically with the labeled CO_2 as the sole carbon source.

The [^{14}C]-labeled cells were harvested by centrifugation at 4 °C and 3000×g for 15 min and resuspended in mosquito feeding buffer (100 mM NaHCO_3 , 150 mM NaCl , pH 7.0). The suspension was rapidly frozen and thawed twice to disrupt the cell pellicles. The thawed mixture was sonicated for 5 min at 4 °C with a Branson Sonifier (pulse sonication, 12% duty cycle, and 3.2 on the output control) and centrifuged at 4 °C and 7700×g for 15 min to pellet cellular debris. The supernatant was dialyzed against freshly prepared feeding buffer at 4 °C for 8 h through the Spectra/Por[®]3 Membrane (MWCO 3500, 1.0 ml/cm, Spectrum Medical Industries Inc., CA) to remove free amino acids and some small peptides. The dialysis efficiency was determined by running a small sample of [^{14}C]-*Euglena* protein through a HPLC column (Synchropak GPC 100 250 × 4.6 mm I.D., SynChrom, Inc., IN, flow rate: 0.5 ml/min using a Programmable Solvent Module 126, Scanning Detector Module 167, Beckman System Gold, Beckman Instruments, Inc., CA) and showing that all the radioactivity eluted with the protein peak. The resulting [^{14}C]-protein concentration was measured (Lowry et al., 1951), and its radioactivity was determined (LS 6000 IC Series, Beckman Instruments, Inc.). The [^{14}C]-proteins were aliquoted into fractions containing 25 μCi /tube and stored at -80 °C. The yield was about 28.8 mg of protein with a specific activity of about 28 μCi /mg. The labeling efficiency was about 60%.

2.3. [^{14}C]-protein meal feeding and sampling of mosquitoes and their metabolic products

Three groups of experiments were carried out with 3-day-old female *A. aegypti* mosquitoes, which were maintained ad libitum on 3% sucrose. *Experiment 1*: Sugar-fed females were fed with the blood meal containing [^{14}C]-labeled protein, and then maintained with 3% sucrose ad libitum. This group was designated as dietary sequence sugar–blood–sugar or *s–b–s*. *Experiment 2*: Sugar-fed females were maintained with water only after the [^{14}C]-protein meal. This group was designated as dietary sequence sugar–blood–water or *s–b–w*. *Experiment 3*: Three-day-old sugar-fed females were starved for 1 day with access to water only before the [^{14}C]-protein meal and then, as in experiment 2, were maintained with water only after [^{14}C]-protein meal. This group was designated as dietary sequence sugar–water–blood–water or *s–w–b–w*.

A critical aspect of developing a reproducible procedure for measuring [^{14}C]-protein amino acid metabolism was the incubation conditions, which needed to take into consideration both radioisotope safety and the appropriate environment for maintaining mosquitoes fed the [^{14}C]-protein meal. We maintained the mosquitoes in a convection incubator at 28 °C (inside

dimension 43 × 46 × 69 cm, Precision Scientific, Inc., IL), which contained a pan of water for maintaining the relative humidity at 80%, a 4-W cool burning night light bulb (Ace Hardware Corp, IL) connected to a timer (SB111, Intermatic TIME-ALL, Intermatic Incorporated, IL) for adjusting the photoperiod to 16:8 h (L:D), and several 50 ml beakers containing 1 N NaOH for trapping $^{14}\text{CO}_2$. The temperature and humidity were measured with a digital hygrometer/thermometer (VWR, Scientific Products, Phoenix, AZ). The normal thermometer hole at the top of the incubator was plugged with a rubber stopper holding a glass tube (inside diameter: 15 mm) containing a glass wool plug and fresh 8–20 mesh Ascarite II[®] (EM Science) for trapping any residual $^{14}\text{CO}_2$.

The [^{14}C]-labeled protein meal consisted of 25 μCi of [^{14}C]-labeled *Euglena* proteins and 1 ml of pig blood containing 5 mM ATP on an artificial feeder as described by Kogan (1990). About 100 female mosquitoes were fed on the [^{14}C]-labeled protein meal for 1 h in each experiment. The mosquitoes were then treated as follows:

1. Five fed mosquitoes were frozen immediately after feeding for determination of the total amount of radioactivity consumed.
2. Five fed mosquitoes were used to measure cumulative $^{14}\text{CO}_2$ at 1, 3, 6, 12, 18, 24, 36, 48 and 60 h post-feeding, as described in Section 2.4.
3. Nine scintillation vials were marked as 1, 3, 6, 12, 18, 24, 36, 48 and 60 h, respectively, and five fed females were transferred into each of them, immediately after [^{14}C]-protein meal feeding. The vials were maintained in the incubator in a plastic box with a few 50 ml beakers containing 1 N NaOH . At each of the times indicated above, the mosquitoes were collected and analyzed for the incorporation of [^{14}C] into body proteins, lipids, amino acids, glycogen and sugars, as described in Section 2.7 and the contents of the vial analyzed for [^{14}C]-waste, as described in Section 2.5.
4. Five fed mosquitoes were placed in a 20-ml scintillation vial contained a small piece of cotton soaked in 3% sucrose or water and maintained until eggs had been laid. After the eggs were laid, the mosquitoes were analyzed for the incorporation of [^{14}C] into mosquito body proteins, lipids, amino acids, glycogen and sugars and the eggs were analyzed for [^{14}C] incorporation into proteins and lipids, as described in Section 2.8.

2.4. Collection and measurement of $^{14}\text{CO}_2$ respired from the fed mosquitoes

Five fed mosquitoes were placed in a 20-ml scintillation vial containing a small piece of cotton soaked in

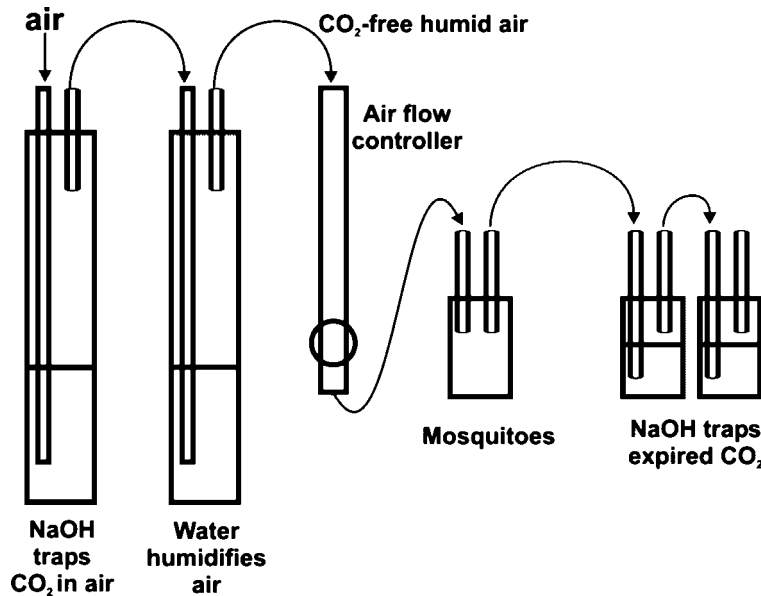


Fig. 2. Diagram of a system for collecting $^{14}\text{CO}_2$ respired from the five ^{14}C -proteins fed mosquitoes. Before the five fed mosquitoes were placed in a 20-ml scintillation vial, a small piece of cotton soaked in 3% sucrose or water was squeezed to remove excess sucrose or water and then placed on the bottom of the vial. At appropriate time intervals, the vials containing 1N KOH were replaced with fresh vials (refer to Section 2.4 for more details).

3% sucrose or water (Fig. 2). The cap of the vial had two small holes drilled through it, through which 18 gauge hypodermic needles were passed and then glued to make an airtight seal. CO_2 -free humid air was produced by passing air through a 1 N NaOH solution and then water and then passed through the mosquito-containing vial at a flow rate of about 25 ml/h. An airflow controller was used to ensure that the rate of airflow through each vial was the same. The air exiting the vial was passed through two scintillation vials containing 1 N KOH, which traps the $^{14}\text{CO}_2$ produced by the mosquitoes. At appropriate time intervals (as mentioned above), the vials containing the KOH were replaced with fresh vials. One milliliter of the KOH solution was put into a scintillation vial and 10 ml of counting cocktail added. The radioactivity was not counted until the vial containing the mixture of KOH and counting cocktail had been stored in darkness at room temperature for 40 h to improve subsequent counting efficiency.

2.5. Collection and measurement of excretory waste from mosquitoes

At the appropriate time, the five females in one of the nine labeled vials were removed from the vial and frozen at -80°C for microseparation (Section 2.7). The vial containing the accumulated excretory waste was stored at 4°C for counting. To each vial 20 ml of counting cocktail were added and the vial was shaken several times to completely disperse the waste and then counted.

2.6. Determination of the amount of radioactivity consumed

The five fed females, which were frozen immediately after ^{14}C -protein feeding, were transferred into a 2-ml glass homogenizing tube (Cole-Parmer Instrument Company, Vernon Hills, IL) containing 2 ml of PBS (phosphate-buffered saline, 150 mM NaCl, 50 mM Na_2HPO_4 , pH 7.2) and homogenized with a Teflon[®] Pestle (diameter: 7.8 mm) (Cole-Parmer Instrument Company, Vernon Hills, IL) and a Wheaton Overhead Stirrer (Wheaton Instruments, Millville, NJ). The homogenate was transferred into a 15-ml conical tube. The glass tube and pestle were rinsed with 3 ml of PBS and the total volume of the homogenate was adjusted to 5 ml. A small aliquot of the mixture was mixed with 10 ml of cocktail and counted.

2.7. Determination of ^{14}C incorporation in mosquito proteins, lipids, glycogen, amino acids and sugars

In order to measure incorporation of ^{14}C into the various nutrient classes, we modified the microseparation procedure described by van Handel (1965b) (Fig. 3). Two critical modifications were made: (1) the fraction containing total lipids was passed through a column containing silicic acid to remove phospholipids from the triacylglycerols, which are the storage lipids; (2) The combined aqueous extracts, which contain the sugars (glucose, trehalose and sucrose, but not glycogen) and the amino acids, were mixed with cation-exchange

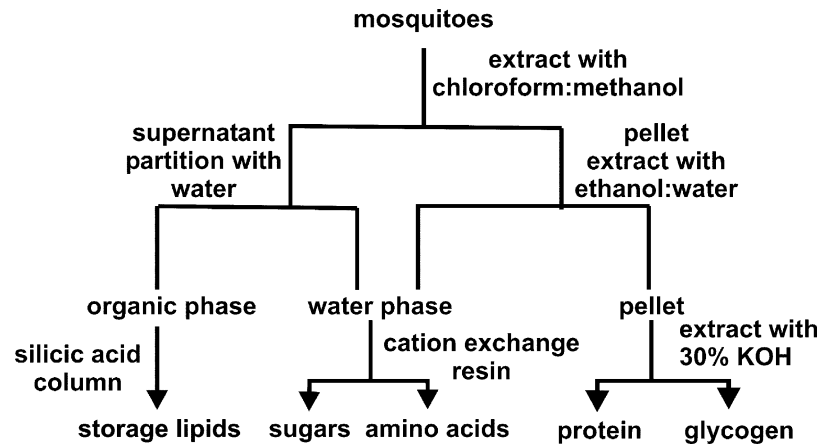


Fig. 3. Flow chart of microseparation of proteins, amino acids, glycogen, sugars, and storage lipids in mosquito bodies. Prior to this procedure, five mosquitoes were placed in a disposable glass tube and dried at 100 °C in a heating block for 1 h (refer to van Handel (1965b) and Section 2.7 in this paper for more details).

resin to separate amino acids and sugars. The detailed modified procedures are as follows.

Five mosquitoes were placed in a 12 × 75 mm disposable glass culture tube labeled *Tube A*, and dried at 100 °C for 1 h. After cooling, 100 µl of saturated Na₂SO₄ in H₂O and 200 µl of methanol were added and the mosquitoes were crushed well with a glass rod. The rod was rinsed with 100 µl of water and then with 1 ml of methanol:chloroform (1:1 v/v). If two layers formed, 100 µl of methanol were added and the contents mixed. The one phase mixture was centrifuged at 800×g for 5 min. The supernatant was transferred into *Tube B₁*. The pellet in *Tube A* was extracted again with 1 ml of methanol:chloroform (1:1 v/v) and the resulting supernatant transferred into *Tube B₁*. *Tube A* was placed on ice until ready for further extraction. The volume of the contents of *Tube B₁* was brought to 5 ml with chloroform and 0.5 ml of water added. The contents were mixed well and centrifuged at 800×g for 5 min. The lower organic phase was transferred into *Tube B₂*. The aqueous phase in *Tube B₁* was placed on ice. Sufficient chloroform was added to *Tube B₂* to bring the volume to 5 ml and 1 ml of water added. The contents were mixed well and centrifuged at 800×g for 5 min. A white precipitate formed between the organic and aqueous layers. The lower organic phase, which contains all the lipids, was collected, being careful to leave the precipitate in *Tube B₂*, and transferred to a clean tube. To remove phospholipids from this fraction, it was passed through a silicic acid column (Dittmer and Wells, 1969) prepared by packing 200 mg of 100-mesh silicic acid (Sigma Chemical Co, MO) that had been dried overnight and suspended in chloroform in a Pasteur pipette column containing a glass wool plug. The neutral lipids, mainly triacylglycerol, were eluted from the column with 8 ml of chloroform into

Tube B₃ and chloroform was removed under N₂ gas and the dried lipids were stored until counting.

Tube B₂, containing the aqueous phase and the white precipitate, was placed on ice and the aqueous phase from *Tube B₁* was added. The contents were mixed well and passed through a glass wool plugged Pasteur pipette into *Tube C*. Water, 0.5 ml, was added to *Tube C*, which was placed on ice. One milliliter of 66% ethanol saturated with Na₂SO₄ was added into *Tube A*. The contents were mixed and centrifuged at 800×g for 5 min and the supernatant was transferred into *Tube C*. The contents remaining in *Tube A* were extracted again with 0.5 ml of 66% ethanol saturated with Na₂SO₄ and the supernatant was transferred into *Tube C*. *Tube A* was then gently warmed at 55 °C for 5 min to remove ethanol and 0.5 ml of 30% KOH added and the mixture heated at 100 °C for 20 min. After cooling, 1 ml of 95% ethanol was added, the contents mixed well and centrifuged at 1150×g for 15 min. The supernatant was transferred into *Tube D*. The pellet in *Tube A* was extracted again with 0.5 ml of water and 1 ml of 95% ethanol and the supernatant added to *Tube D*.

Tubes A, B₃, C and D contained glycogen, lipids, a mixture of amino acids and sugars, and proteins, respectively. To *Tube A*, 2 ml of water was added and the contents mixed well; the volume of *Tube D* was adjusted to 3 ml with water and the contents mixed well; and the contents of *Tube B₃* were dissolved in 3 ml of chloroform. Samples (200 µl) were taken from *Tube A, D and B₃*, and counted. The total volume of solution in *Tube C* was measured (V_1) and 200 µl were counted (R_1). The pH of the remaining fraction C was adjusted to 3.0–4.0 with 0.1 N HCl, as indicated by pH paper, and 1 g of AG[®] 50W-X8 Resin (BIO-RAD, 200–400 mesh, hydrogen form) was added. The contents were mixed and centrifuged at 800×g for 2 min.

The supernatant, which contained sugars—the amino acids were bound to the resin and removed from the solution, as confirmed using radioactive amino acids—was transferred to a 15-ml plastic tube (*Tube C₁*), the volume measured (V_2), and 200 μ l of C_1 were counted (R_2). The radioactivity of fraction C represents [14 C]-sugars plus [14 C]-amino acids, while the radioactivity of fraction C_1 represents [14 C]-sugars only. Thus, the amount of [14 C]-sugars was calculated as $[R_2 \times V_2 \times V_1] / [200(V_1 - 200)]$ and the amount of [14 C]-amino acids as $[(R_1 \times V_1 / 200) - (R_2 \times V_2 \times V_1) / [200(V_1 - 200)]]$, respectively.

2.8. Separation of lipids and proteins from the eggs laid by the spent mosquitoes

It was difficult to grind the five mosquito eggs with a glass rod as described by van Handel (1965b) without significant loss. Instead, the eggs were transferred into a 2-ml glass homogenizing tube (Cole-Parmer Instrument Company, Vernon Hills, IL) containing 100 μ l of saturated Na_2SO_4 in H_2O , 200 μ l of methanol, 100 μ l of water and 1 ml of methanol:chloroform (1:1, v/v), and homogenized with a Teflon® Pestle (diameter: 7.8 mm) (Cole-Parmer Instrument Company, Vernon Hills, IL) and a Wheaton Overhead Stirrer (Wheaton Instruments, Millville, NJ) for 2–3 min. The homogenate was transferred into a 12 \times 75 mm disposable glass culture tube. The homogenizing tube and pestle were rinsed with 100 μ l of water and 1 ml of methanol:chloroform (1:1, v/v), successively. The rinse solution was also transferred into the above-mentioned culture tube. The extract was mixed and centrifuged at $800 \times g$ for 5 min. The supernatant was transferred into another clean tube. This extraction was repeated with 1 ml of methanol:chloroform (1:1, v/v). The resulting supernatant contained egg lipids, and the precipitate egg proteins. They were individually mixed with scintillation cocktail and counted.

2.9. Data analysis

Data were converted to dpm/female to correct for counting efficiency and then expressed as the percentage of original radioactivity converted to the various biochemical products. Each group of experiments was repeated four or six times to determine the reproducibility. Because one-way ANOVA ignores the fact that the time points come in order (i.e. it does not recognize trends), we used linear or nonlinear regression to fit the data to a model (e.g. quadratic, cubic, or exponential curve model, etc.) that would describe the time-course of the percentage of radioactivity in proteins, amino acids, lipids, glycogen, sugar, CO_2 and waste following the [14 C]-protein meal. The repeated measures multivariate ANOVA (MANOVA) was used to compare the

time-courses of [14 C]-labeled proteins, amino acids, lipids, glycogen, sugars, CO_2 and waste among the different nutritional regimes. One-way ANOVA with post hoc Newman–Keuls multiple comparison tests was used for comparison of maternal proteins, amino acids, lipids, glycogen and sugars as well as egg proteins and lipids after the eggs were laid among the different nutritional regimes. The significance level, α , was 0.05. All the statistical analysis were carried out by SPSS for Windows (v11.5) (SPSS Inc., IL) and all the statistical graphs were created using GraphPad Prism 3 (GraphPad Prism Software, Inc., San Diego, CA).

3. Results

3.1. Time-courses of label distribution in all biochemical classes following a [14 C]-protein meal in the female *A. aegypti*

3.1.1. Labeling pattern of proteins and amino acids

The labeling pattern of proteins in the female mosquito followed an exponential curve model (Fig. 4A and Table 1), which showed that the percentage of radioactivity in proteins decreased more rapidly during the first 24 h than after 24 h. The time-course of [14 C]-amino acids fit a second half of a quadratic curve model (Fig. 4B and Table 1), which demonstrated that [14 C]-amino acids quickly reached a relative plateau, accounting for 5% of the total ingested [14 C]-*Euglena* proteins at the end of 1 h post-blood meal, and then showed a slow decrease over time until 36 h post-blood meal and then significantly declined to 2% at 48 h and 1% at 60 h post-blood meal.

3.1.2. Labeling pattern of glycogen, sugars and lipids

The time-course of radioactivity incorporation into glycogen followed the first two-thirds of a cubic curve model (a kind of elongated sine-shaped curve) (Fig. 4C and Table 1), which showed that the level of [14 C]-glycogen quickly reached about 2% of ingested label at 1 h, increased to a maximum of about 4% at 18 h, and then decreased to about 2% at 60 h post-blood meal. The truncated first one-third of a quadratic curve model was developed to fit the distribution pattern of [14 C]-sugar percentage (Fig. 4D and Table 1), which demonstrated that the [14 C]-sugars quickly reached a level of about 3% at 1 h, and then fluctuated around 3.0–3.7% before 36 h, and then significantly decreased to 1.5% at 48 h and to 1% at 60 h post-blood meal. The time-course of [14 C]-lipids was represented by the first half of cubic curve model (Fig. 4E and Table 1), which showed that the level of [14 C]-lipids reached 2.5% at 1 h, and then increased with time and reached a peak of 16% at 36 h, and then declined at a slow rate and reached 12% at 60 h post-blood meal.

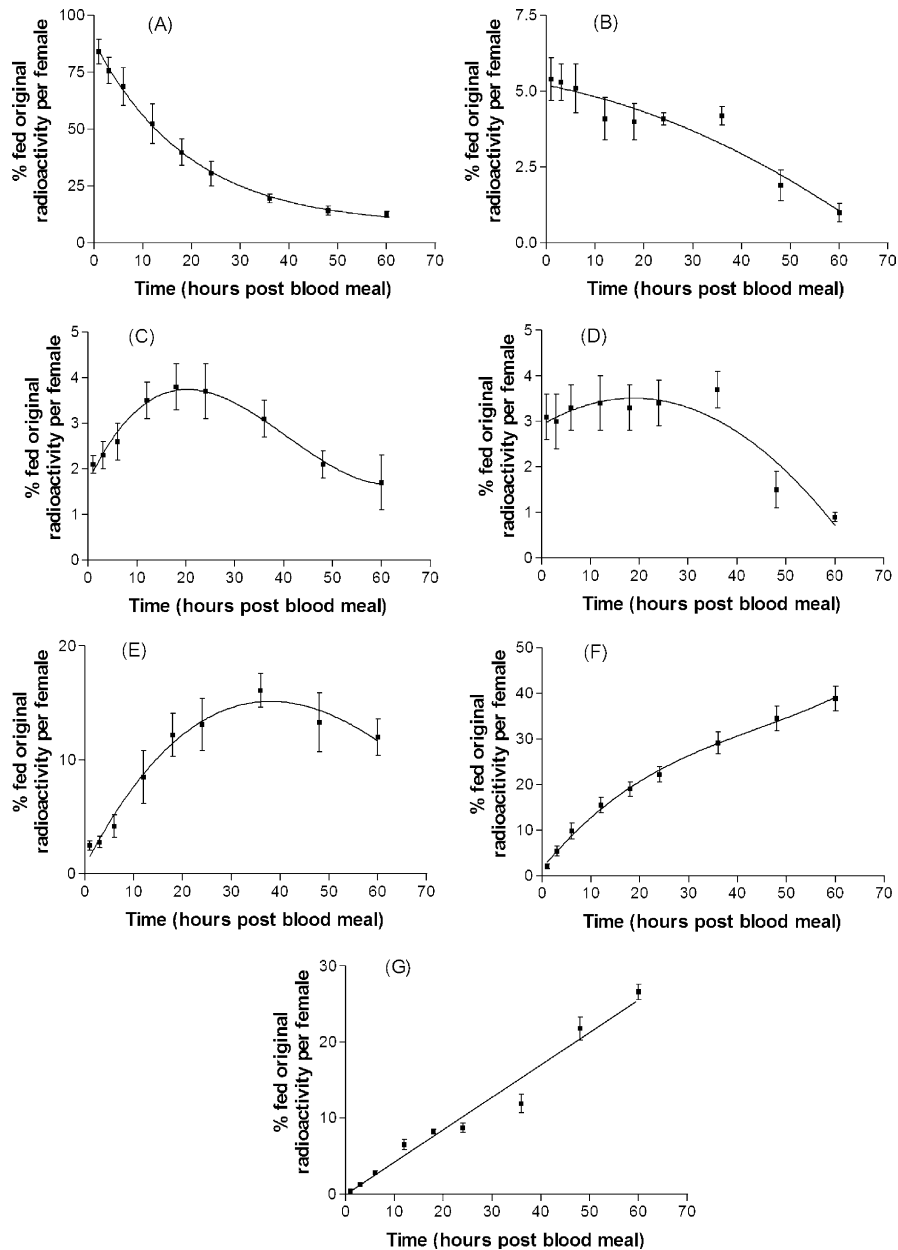


Fig. 4. The curve-fitting results for all time-courses of radioactivity percentage in proteins (A), amino acids (B), glycogen (C), sugars (D), lipids (E), CO_2 (F) and waste (G) in the female *A. aegypti* mosquitoes with the dietary sequence sugar–blood–sugar after a ^{14}C -protein meal. The proteins, amino acids, sugars, glycogen and lipids from the five fed females at nine time points were extracted and counted, respectively, as described in Section 2.7. The results were expressed in the percentage of original fed radioactivity converted to the various biochemical products per female. Data from each point represent the mean \pm SD of four independent experiments.

The level of carbohydrate reserves, including sugars and glycogen derived from the meal protein amino acids, quickly reached about 5% (3% of sugars plus 2% of glycogen) at 1 h, while the level of lipid reserve reached about 2.5% at this time point. The maximal conversion of amino acids to sugars plus glycogen (7%) was smaller than the conversion to lipid (16%). The ^{14}C -sugars decreased more significantly than ^{14}C -glycogen after 36 h post-blood meal.

3.1.3. Labeling pattern of CO_2 and waste

The time-course of $^{14}\text{CO}_2$ production followed the first one-third of cubic curve model (Fig. 4F and Table 1), which showed that the level of $^{14}\text{CO}_2$ successively increased with time, and that the rate of radioactivity in CO_2 production was faster during the first 12 h than at later times. The ^{14}C -waste distribution pattern presented a simple linear increase with time (Fig. 2G and Table 1).

Table 1

Summary of curve-fitting results for time-courses of labeling of proteins, amino acids, glycogen, sugars, lipids, CO₂, and waste under the dietary sequence sugar–blood–sugar^a. They were obtained by running two processes including curve estimation (two-stage least squares method) and non-linear regression (iteration method) in SPSS for Windows (v11.5) (SPSS Inc.) (refer to Section 2.9)

Compound	Curve model	R ²	Regression equation	F value	Significance of F
[¹⁴ C]-proteins	Exponential	0.9933	$y = 86.2760 \times (0.9603)^t$	240.9730	0.0000
[¹⁴ C]-amino acids	Quadratic	0.9006	$y = 5.2155 - (0.0299 \times t) - (0.0007 \times t^2)$	27.1893	0.0010
[¹⁴ C]-glycogen	Cubic	0.9801	$y = 1.7033 + (0.2271 \times t) - (0.0074 \times t^2) + (0.00006 \times t^3)$	82.0129	0.0001
[¹⁴ C]-sugars	Quadratic	0.8663	$y = 2.9389 + (0.0620 \times t) - (0.0017 \times t^2)$	19.4305	0.0024
[¹⁴ C]-lipids	Cubic	0.9763	$y = 0.6898 + (0.8351 \times t) - (0.1380 \times t^2) + (0.00005 \times t^3)$	68.7886	0.0002
[¹⁴ C]-CO ₂	Cubic	0.9968	$y = 1.6984 + (1.3005 \times t) - (0.0205 \times t^2) + (0.0002 \times t^3)$	522.9338	0.0000
[¹⁴ C]-waste	Linear	0.9709	$y = 0.0532 + (0.4268 \times t)$	233.7032	0.0000

^a The experiment under the dietary sequence sugar–blood–sugar was independently repeated four times.

3.2. Effect of different adult nutritional regimes on the metabolism of blood meal amino acids

3.2.1. Time-courses of label distribution in all biochemical classes following a [¹⁴C]-protein meal in female *A. aegypti* mosquitoes with different adult nutritional regimes

Because there is a certain extent of correlation between measured values of a given biochemical index at two time points, the repeated measures MANOVA, in which each time point was considered as a variable besides the treatment factor variable (i.e. different nutritional regime), was selected to compare the time-courses of all biochemical indexes among the female *A. aegypti* mosquitoes with different adult nutritional regimes. The results showed that all biochemical indexes under the different adult nutritional regimes were a function of time and there were no significant differences in all time-courses of label percentage in all biochemical products except for amino acids: $P > 0.05$ for protein, lipid, glycogen, sugar, CO₂, and waste, and $P < 0.05$ for amino acids in time \times regime variance (Table 2).

3.2.2. Fate of blood meal protein amino acids at the end of the gonotrophic cycle

Table 3 summarizes the distribution of radioactivity in maternal and eggs components at the end of the gonotrophic cycle. The female retains 7–8% of the fed radioactivity as proteins regardless of the adult diet. The small amount of radioactivity present as amino acids and sugars is also not affected by the adult diet. However, the amount of glycogen and lipid retained by the female is significantly lowered by starvation of the adult. In contrast, no significant differences were detected

in the amount of label in egg protein or egg lipid or in the number of eggs laid among the three groups.

4. Discussion

A number of technical problems needed to be solved before reproducible results could be obtained. Some of the more critical ones are discussed briefly below: (1) A reliable source of [¹⁴C]-labeled protein was essential for these experiments. Obtaining such material from a commercial source proved impossible. After many considerations, we chose to use *Euglena* to produce the labeled proteins starting from Ba¹⁴CO₃. This proved to be a cost-efficient and reproducible method. In addition, it has been shown that *Euglena* proteins have

Table 2

Repeated measures multivariate tests (Pillai's trace method) for comparison of time-courses among the different adult nutritional regimes

Source of variance	Type of time-course	Significance
Time	Proteins	0.000
	Amino acids	0.000
	Lipids	0.000
	Glycogen	0.000
	Sugars	0.000
	CO ₂	0.000
	Waste	0.000
	Time \times regime	Proteins
	Amino acids	0.016**
	Lipids	0.346
	Glycogen	0.794
	Sugars	0.217
	CO ₂	0.142
	Waste	0.674

** $P < 0.05$.

Table 3

Fate of meal protein amino acids at the end of the gonotrophic cycle in *A. aegypti* under the three different nutritional regimes^a

	Group 1, sugar–blood–sugar, (mean ± SD)	Group 2, sugar–blood–water, (mean ± SD)	Group 3, sugar–water–blood– water, (mean ± SD)
Maternal proteins (%/♀)	8.1 ± 0.5	7.1 ± 1.0	7.1 ± 1.6
Maternal amino acids (%/♀)	0.9 ± 0.1	1.0 ± 0.1	0.9 ± 0.1
Maternal glycogen (%/♀) ^b	1.2 ± 0.1	0.6 ± 0.1	0.6 ± 0.2
Maternal sugar (%/♀)	0.6 ± 0.2	0.6 ± 0.03	0.7 ± 0.1
Maternal lipids (%/♀) ^c	7.5 ± 0.9	2.7 ± 1.4	3.2 ± 1.1
CO ₂ at 60 h PBM ^d (%/♀)	38.9 ± 2.7	33.3 ± 2.0	29.6 ± 2.6
Waste at 60 h PBM (%/♀)	26.6 ± 1.0	25.3 ± 0.9	24.1 ± 2.1
Egg proteins (%/♀)	3.3 ± 1.1	4.6 ± 1.3	2.3 ± 0.7
Egg lipids (%/♀)	6.2 ± 2.0	7.5 ± 2.7	5.4 ± 1.3
No. of eggs laid (eggs/♀)	46 ± 7	54 ± 10	44 ± 7

^a Data from each cell represent the mean ± SD of four (in Group 1 and 2) or six (in Group 3) independent experiments.^b Group 1 vs. Group 2 or 3, $P < 0.05$, one-way ANOVA with post hoc Newman–Keuls multiple comparisons.^c Group 1 vs. Group 2 or 3, $P < 0.05$, one-way ANOVA with post hoc Newman–Keuls multiple comparisons.^d PBM means post-blood meal.

a high nutritional value in vertebrates (Antonian et al., 1985) and we observed that unlabeled *Euglena* proteins supported normal egg production when fed to *A. aegypti* as the sole protein source in an artificial blood meal (Kogan, 1990) (data not shown). (2) Finding an appropriate location in which to carry out the experiments that provided for protection against contamination by radioactive CO₂ and an optimal environment for mosquito culture required several trials. Of particular importance was maintaining both temperature and humidity. (3) In setting up the CO₂ collection train, it was critical to use CO₂-free air, otherwise the NaOH in the collecting vials became saturated with CO₂ from the air and radioactive CO₂ was lost; to use water-saturated air, otherwise the mosquitoes became desiccated rapidly; and to maintain a constant flow rate of air.

It has been reported that in *A. aegypti*, trypsin activity was first observed 3 h after blood feeding (Felix et al., 1991), and that a low level of aminopeptidase activity is already present in the midgut of non-blood fed females and increases rapidly after blood feeding (Graf and Briegel, 1982; Clements, 1992). Thus, the protein and amino acid distribution patterns observed during the first hour in our experiment could not be caused by trypsin hydrolysis of meal proteins, but likely by some peptides in the labeled *Euglena* proteins, which provided substrates for aminopeptidase immediately after feeding. On the other hand, the basic pattern of blood digestion in mosquitoes proceeded almost simultaneously over the entire surface of the meal bolus (Lehane, 1991). The meal bolus has a larger surface in the beginning, which can provide more substrates for protease in earlier stages of digestion than in later stages. These physiological and biochemical mechanisms may explain the distributions of [¹⁴C]-protein and [¹⁴C]-amino acids observed during digestion of the labeled protein meal.

Koller and Raikhel (1991) reported that the rate of protein synthesis in the ovaries of *A. aegypti* increased threefold within 30 min of feeding. Vitellogenin mRNA was observed in the fat body 1 h post-blood meal (Racioppi et al., 1986; Raikhel and Lea, 1983), while vitellogenin was first detected by immunofluorescence 1 h after feeding and was maximal between 18 and 24 h (Raikhel and Lea, 1983; Raikhel, 1986, 1987a, b). Thus, there are two competing processes, which affect the level of labeled proteins: protein catabolism (meal protein digestion) and protein synthesis (vitellogenesis).

There are several pathways for metabolism of the meal protein amino acids, such as meal protein degradation, yolk protein synthesis, amino acid deamination and oxidation for gluconeogenesis, lipogenesis or energy production (see the following discussion). Amino acids from the blood meal digestion are not only utilized for yolk protein synthesis but also have the potential to initiate and promote mosquito oogenesis (Uchida et al., 1992, 2001; Uchida, 1993, 1998). That the [¹⁴C]-amino acids quickly reached a relative high level and then very slowly decreased before 36 h post-blood meal may be significant in maintaining normal physiological and biochemical processes.

The carbohydrate and lipid reserves of insects can be derived from ingested carbohydrate, and also from ingested amino acids, which constitute an important source when supplies of dietary carbohydrate are limited (Clements, 1992). Our results showed that the sugar-fed female *A. aegypti* mosquitoes still need to obtain additional energy reserves from the meal proteins to meet the need for physiological processes, including reproductive activities and survival after the first gonotrophic cycle.

Our results also showed that the level of carbohydrate reserves, including sugars and glycogen derived from the meal protein amino acids, quickly reached about 5% (3% of sugars plus 2% of glycogen) at 1 h,

while the level of lipid reserve reached about 2.5% at this time point. This suggested that gluconeogenesis from meal protein amino acids is a rapidly responsive metabolic pathway, compared with lipogenesis. On the other hand, the maximal conversion of amino acids to sugars plus glycogen (7%) was smaller than the conversion to lipid (16%). This suggests that lipogenesis must meet the needs of both maternal triacylglycerol (TAG) reserves and egg lipids (see below).

In addition, the [^{14}C]-sugars decreased more significantly than [^{14}C]-glycogen after 36 h post-blood meal, which suggested that there may be a substantial amount of [^{14}C]-sugar metabolism at this time. Whether the sugar is oxidized or converted to glycogen or lipid is unknown.

Although the synthesis and utilization of energy reserves in mosquitoes have been extensively studied, the question that how much of the respired CO_2 is directly derived from the meal proteins during a gonotrophic cycle still remains unsettled. Most researchers in this field estimated energy reserves by measuring the lipid, sugar and glycogen content of a single mosquito, and have expressed them in terms of energy values, using calories or joules as the unit. In the current study, we designed a system for directly collecting $^{14}\text{CO}_2$ respired from the oxidation of the [^{14}C]-protein-fed mosquitoes. As shown in Fig. 4F and Table 1, the rate of radioactive CO_2 production was faster during the first 12 h than at later times. Probably, the initiation of diuresis, meal protein digestion, gluconeogenesis, lipogenesis, and vitellogenesis or oogenesis during the first several hours post-blood meal requires extra energy, much of which is derived from amino acid oxidation leading to a rapid increase of CO_2 production by aerobic metabolism.

As soon as protein digestion starts, nitrogen waste (uric acid and urea) excretion also starts. After digestion has been completed (about 36 h post-feeding) (Briegel and Lea, 1975), the residual substances including some undeaminated histidine, arginine and glycine in the midgut are voided (Clements, 1992), which could explain why the time-course of [^{14}C]-waste production presented a simple linear increase with time (Fig. 2G and Table 1).

As mentioned above, sugar feeding is one of the important factors determining the fate of blood meal protein amino acids. Foster (1995) reviewed in detail the effect of sugar feeding or starvation on the utilization of meal protein by female mosquitoes. However, how starvation before and/or after the blood meal quantitatively affects the fate of meal protein amino acids in mosquitoes had not been investigated. Therefore, we performed experiments to observe the effects of different adult nutritional regimes on metabolism of blood meal amino acids. The three nutritional regimes were: (1) s–b–s—sugar-fed females were fed blood and then maintained with 3% sucrose; (2) s–b–w—sugar-fed

females were maintained with water only after the blood meal. Compared with the first regime, the second one was characterized by starvation (i.e. no sugar feeding) after the blood meal; and (3) s–w–b–w—3-day-old sugar-fed females were starved for 1 day with access to water only before the blood meal and then were maintained with water only. Originally, we wanted to observe the situation in which 3-day-old water-fed females were maintained with water or sugar only after the blood meal. However, because some of the 3-day-old water-fed females died during CO_2 and waste collection, as well as, egg production post the blood meal, we had to change the protocol into the current regime, i.e. s–w–b–w. Compared with the second regime, the third one was characterized by a little more limitation in sugar feeding, i.e. starvation after a blood meal plus a partial starvation before the blood meal. Multivariate analysis for comparison of the time-courses of all biochemical indexes among the female *A. aegypti* mosquitoes with different adult nutritional regimes suggested that the metabolism of amino acids derived from meal proteins may be more sensitive to the different nutritional regimes, compared with the other biochemical parameters. However, we must also consider other possibilities such as the larger variation in the [^{14}C]-amino acid data among different individuals. More data would be required to confirm whether amino acid metabolism is affected by adult nutrition. Curve fitting for each of the three [^{14}C]-amino acid time-courses under the three different adult nutritional regimes showed that all curves were best fitted to the second half of a quadratic curve ($r^2 = 0.9006$, regression equation: $y = 5.2155 - (0.0299 \times t) - (0.0007 \times t^2)$, $F = 27.1893$, $P < 0.05$ for the dietary sequence s–b–s; $r^2 = 0.9641$, regression equation: $y = 5.3120 + (0.0287 \times t) - (0.0016 \times t^2)$, $F = 80.5983$, $P < 0.05$ for the dietary sequence s–b–w; $r^2 = 0.6902$, regression equation: $y = 4.799 + (0.0752 \times t) - (0.0019 \times t^2)$, $F = 6.6848$, $P < 0.05$ for the dietary sequence s–w–b–w), which at least suggested different adult nutritional regimes did not significantly affect the general trend of [^{14}C]-amino acids utilization. Overall, our results suggested that female *A. aegypti* mosquitoes possess a substantial capacity of maintaining metabolic homeostasis during their gonotrophic cycle, even in the face of an environmental stress, e.g. starvation (no sugar feeding) after a blood meal even plus a partial starvation before the blood meal. This may be the result of a long-term physiological adaptation of this mosquito species to its domestic environment, which is characterized by poor sugar and rich blood (Harrington et al., 2001).

Over all the experiments $33.9 \pm 3.3\%$ of the fed label was expired as CO_2 and $25.3 \pm 0.8\%$ of the fed label was excreted as waste and about $9.8 \pm 1.6\%$ was stored in the eggs. Briegel (1990a) reported that in the smal-

lest females examined (wing length 2.4 mm) only about 2% of the dietary protein was converted to yolk protein, while in the largest females (wing length 3.4 mm) about 20% was converted to yolk protein. In our experiment, the wing length of the females was about 3.1–3.3 mm. It seems that the conversion of the meal protein amino acids to egg proteins is somewhat lower in our experiment than in Briegel's. However, it must be noted that ovarian nitrogen, which was expressed as a percentage of blood meal nitrogen, was used in evaluating the utilization of the blood proteins in Briegel's experiment, while in our experiment we measured the percentage of meal protein [^{14}C]-labeled amino acid carbon skeleton used. Obviously, a direct comparison between these two experimental results cannot be carried out due to the different nitrogen content of different amino acids.

The female retains 7–8% of the fed radioactivity as proteins regardless of the adult diet and the small amount of radioactivity present as amino acids and sugars is also not affected by the adult diet, while the amount of glycogen and lipid retained by the female is significantly lowered by starvation of the adult, which suggests that sugar feeding post-blood meal may be important for maintaining the energy reserves of the female after egg laying. In contrast, no significant differences were detected in the amount of label in egg protein or egg lipid or in the number of eggs laid among the three groups, which indicates that starvation after a blood meal, even with a partial starvation before the blood meal, did not influence the fecundity of *A. aegypti*. As described in section above, *A. aegypti* is domestic, and in the domestic environment, low sugar is available, while blood sources are rich. Thus, the females seldom feed on sugar (Edman et al., 1992) and tend to take supplementary blood meals to improve their energy reserves in the field (Scott et al., 1993a,b; Foster, 1995). Day et al. (1994) concluded that *A. aegypti* females live longer in the laboratory if they are provided a source of carbohydrate, but the increased survival associated with sugar feeding does not increase reproductive success and there is no fitness disadvantage associated with multiple blood feeding or the utilization of blood as the sole energy source for flight and maintenance, as well as, reproduction. Scott et al. (1997) further suggested that frequent feeding by *A. aegypti* on human hosts, without imbibing carbohydrates, confers a reproductive (fitness) advantage according to the life table and survival experiment. Thus, at the biochemical level, our experimental results provide important quantitative evidence for the above conclusions. In addition, these results also suggest that during a gonotrophic cycle with the nutritional stress, there seems to be a trade-off between fecundity and survival in this mosquito species. In other words, the unchangeable fecundity could

be at the expense of reducing maternal survival. Obviously, such an adaptive strategy of reproduction priority in the face of nutritional stress could be important to regeneration and flourishing of mosquito populations. At the same time, such a result also verifies the conclusion that in natural selection the trait selected is always reproductive success (Stearns, 1999).

We must also point out that sugar feeding before a blood meal not only directly contributes to egg proteins and egg lipids, but also plays an extremely important role in energy production and survival post-egg laying; furthermore, 3-day-old sugar-fed females still maintain a substantial amount of larval stage derived lipid reserves, which also contribute to egg lipid and energy production (Zhou and Wells, unpublished observations). In addition, under nutritive stress (i.e. starvation for 4–6 days), female *A. aegypti* mobilized 54–84% of the teneral lipids, almost all total carbohydrates (less than one fifth of the total calories) and 14–31% of proteins (Briegel, 1990a). Obviously, these can provide a reasonable explanation for our preliminary result about the mortality of 3-day-old water-fed females during CO_2 and waste collections as well as egg production after the blood meal.

5. Conclusions

- (1) Quantitative analysis of blood meal protein amino acid metabolism showed that most of the carbon of the amino acids was either oxidized to CO_2 or excreted as waste.
- (2) Under three different adult nutritional regimes, no significant differences in the metabolism of amino acids were found, which indicated that the female *A. aegypti* mosquitoes possess a substantial capacity of maintaining metabolic homeostasis during a gonotrophic cycle.
- (3) The amount of maternal glycogen and lipids after egg laying were significantly lower in the mosquitoes that were not fed sugar after the blood meal.
- (4) Adult starvation during a gonotrophic cycle did not significantly affect the content of egg lipids or proteins or the number of eggs laid, which indicated that a partial starvation before a blood meal and/or starvation after the blood meal did not influence the fecundity of *A. aegypti*.
- (5) The methods described in this paper can be applied to a wide range of questions about the effects of environmental conditions on the utilization of blood meal amino acids. We are currently repeating these experiments using *Anopheles albimanus* and hope to explore other species and to compare laboratory and wild strains.

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