



Pergamon

Journal of Insect Physiology 49 (2003) 591–601

Journal  
of  
Insect  
Physiology

www.elsevier.com/locate/jinsphys

# Proline can be utilized as an energy substrate during flight of *Aedes aegypti* females

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Received 9 September 2002; received in revised form 19 February 2003; accepted 19 February 2003

## Abstract

In order to determine whether proline can be utilized as fuel during flight of *Aedes aegypti*, proline, alanine, and glutamine concentrations were monitored at 0, 30 and 60 min after flight using sugar-fed males and females, and blood meal-fed females. In sugar-fed and blood meal-fed females, flight lead to a significant decrease in proline and a significant increase in glutamine concentration in both hemolymph and thorax. Only during flight after a blood meal was a significant increase in the alanine concentration observed in hemolymph. After flight, the proline alanine and glutamine levels in the hemolymph and thorax from males did not change significantly. In addition, activities of enzymes related to amino acid metabolism were assayed in homogenates of cephalothorax and thorax from both sexes, and in fat body and midgut from females. In both sexes, the activities of all the enzymes studied were significantly higher in thorax than in cephalothorax. The levels of the enzymes involved in proline oxidation were higher in thorax than in fat body and midgut. These results suggest that proline can be used as an energy substrate for flight muscle of *Ae. aegypti* females. However, the elevation in glutamine levels observed in hemolymph and thorax after flight has not been reported in other insects that fuel flight using proline and may suggest an additional mechanism for shuttling ammonia between flight muscle and fat body is present in mosquitoes.

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**Keywords:** Amino acid; Thorax; Midgut; Fat body

## 1. Introduction

Insect flight muscles use different fuels to power flight. It is widely accepted that Diptera and Hymenoptera mainly utilize carbohydrate as the energy supply during flight, whereas Lepidoptera and Orthoptera utilize carbohydrate in combination with lipid (Beenakkers et al., 1984). Many authors have suggested that, in mosquitoes, the energy for flight comes from sugar oxidation with glycogen serving as the principal flight substrate (Clements, 1955, 1992; Nayar and Van Handel, 1971; Nayar and Sauerman, 1973; Foster, 1995; Briegel et al., 2001a,b). However, it has not been demonstrated that sugar is directly transferred from glycogen stores in fat body to flight muscle. In mosquitoes, proline is the most

abundant free amino acid in hemolymph (Henn et al., 1998; Uchida et al., 1990; Goldstrohm et al., 2003) and we wondered whether proline might play a role in moving oxidizable substrate from the fat body to the flight muscle.

Several studies in a variety of insect species suggest that amino acids, especially proline, can be used as an energy substrate in flight muscle. Proline is known to be the main fuel utilized during flight of tsetse fly *Glossina morsitans* (Bursell, 1963, 1975) and the Colorado potato beetle *Leptinotarsa decemlineata* (de Kort et al., 1973; Mordue and de Kort, 1978; Brouwers and de Kort, 1979; Weeda et al., 1979). The blowfly *Phormia regina* (Sacktor and Childress, 1967), the Japanese beetle *Popillia japonica* (Hansford and Johnson, 1975), the African fruit beetle *Pachnoda sinuata* (Zebe and Gäde, 1993; Auerswald et al., 1998a,b; Auerswald and Gäde, 1999, 2002; Gäde and Auerswald, 2002), the blister beetle *Decapotoma lunata* (Auerswald and Gäde, 1995) are some examples of insects that can also use proline dur-

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ing flight. Micheu et al. (2000) have also indicated utilization of proline during flight of honeybee *Apis mellifera carnica*.

The function of proline in flight is to transport acetyl units derived from acetyl-CoA from fat body to flight muscle (Fig. 1). Thus, acetyl-CoA derived from either fatty acid, glucose or amino acid metabolism is first converted to  $\alpha$ -ketoglutarate via the citric acid cycle in fat body. The  $\alpha$ -ketoglutarate is then converted to proline in the fat body and the proline is transported to the flight muscle. In flight muscle, proline is converted back to  $\alpha$ -ketoglutarate, which then enters the citric acid cycle to be oxidized to produce ATP. Simply measuring the decrease in glycogen stores or even the respiratory quotient cannot prove whether the oxidizable substrates are moved directly from the fat body to flight muscle as glucose or trehalose, or as proline.

Insects that fuel flight using proline as a shuttle to move acetyl units from fat body to flight muscle are characterized by the following: 1) a high concentration of proline in hemolymph of resting insects, which decreases significantly during flight with a concomitant increase in alanine concentration; 2) a distinctive pattern of enzymes in both flight muscle and fat body, which includes high levels of alanine aminotransferase (enzymes 1, Fig. 1); a high level of NAD-linked 'malic' enzyme (3) compared to malate dehydrogenase (2), which is required to convert malate to pyruvate in flight muscle (enzymes 3 and 2, Fig. 1); and the presence of pyruvate carboxylase (enzyme 4, Fig. 1) in fat body,

which is required to convert pyruvate to oxaloacetate. The presence of pyruvate carboxylase has been reported in fat body and flight muscle in *Ae. aegypti* (Tu and Hagedorn, 1997).

There are no studies in the literature that preclude proline as playing a role in energy production in flight muscle in *Ae. aegypti*. Therefore, the present study was designed to investigate whether proline can be utilized as fuel during flight of *Ae. aegypti* females and males.

## 2. Materials and methods

### 2.1. Insects

*Aedes aegypti* (NIH-Rockefeller) were maintained at 28 °C, 70–80% relative humidity and a light: dark cycle of 16 h: 8 h. Larvae were maintained on a diet consisting of rat chow (Sunburst Pet Foods, Phoenix, AZ), lactalbumin hydrolysate (USB, Cleveland, OH) and yeast hydrolysate (USB, Cleveland, OH). Adults were kept at 28 °C, 70–80% relative humidity, and a photoperiod of 16:8 (L: D) h and allowed ad libitum access to pads soaked in 3% sucrose in water until 24 h before the experimental feeding. For all experiments, we used three or four day old adults.

Some adults were fed on 3% sucrose to repletion and 2 h after feeding they were used for enzymatic assays and for flight experiments. Other adults were fed on cow

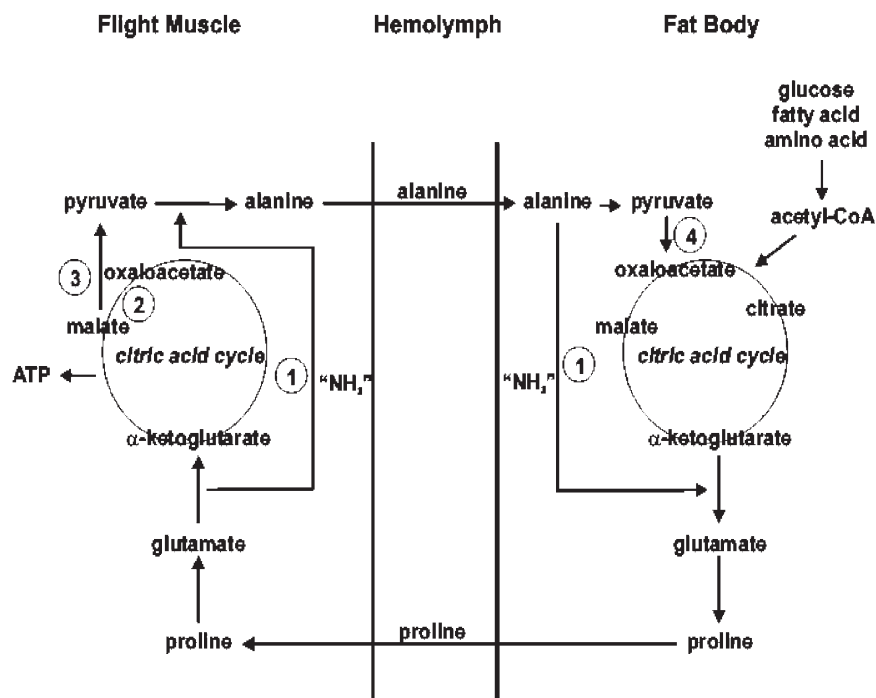


Fig. 1. Schematic representation of the role of proline as a shuttle in moving acetyl units from the fat body to flight muscle. Key enzymes are 1 = Alanine aminotransferase; 2 = Malate dehydrogenase; 3 = NAD - linked 'malic' enzyme; 4 = pyruvate carboxylase.

blood to repletion and 24, 48 and 72 h after feeding they were used in flight experiments.

## 2.2. Flight experiments

Mosquitoes were tethered at the anterior part of the proboscis with a 18 cm fine wire attached to a crossbar. The experiments were conducted in a constant temperature room at 30 °C and 30% relative humidity. Air currents were provided by a ceiling fan and the tarsal reflex was used to maintain flight. If an individual stopped flying it was gently prodded to reinitiate flight but if the mosquito did not reinitiate flight, it was discarded. The duration of flight was 0, 30 and 60 min. At the end of the flight the insects were dissected immediately.

In sugar fed males and females, the hemolymph was isolated and prepared for amino acid analysis as described by Goldstrohm et al. (2003). Each sample contained hemolymph from five adults. After a blood meal, the hemolymph of five females was collected according to the procedure of Pennington et al. (1996) with the modification of using water as the flushing fluid instead of *Aedes* saline. The sample was then prepared for amino acid analysis by the addition of 2 µl of 50% sulfosalicylic acid. The sample was mixed and centrifuged at 13,800 x g for 5 min and the supernatant was used for amino acid analysis.

Thoraces were dissected out under a stereoscopic microscope. Each sample represented a pool of thoraces from five adults. Tissue samples were homogenized in 30 µl PBS (50 mM Na<sub>2</sub>HPO<sub>4</sub>, 150 mM NaCl, pH 7.2) using a hand held pestle. The suspension was centrifuged at 13,800 x g for 3 min and the supernatant collected. Two microliters of 50% Sulfosalicylic acid and 20 µl of water were added to each sample. The tubes were capped, vortexed, and centrifuged at 13,800 x g for 5 min. The supernatant was collected and analyzed at The Arizona Research Laboratories Proteomics Core Facility using a Beckman 7300 (post-column, ninhydrin method) dedicated Amino Acid Analyzer. All amino acid composition data are expressed as pmol/animal.

## 2.3. Preparations of extracts for enzyme assays

Pupal cephalothoraxes and adult thoraxes, fat bodies (abdomen free of gut, ovaries and Malpighian tubules) and midguts were separately 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 suspension was centrifuged at 8000 x g for 20 min. The supernatant was used immediately for protein determinations and to determine the activities of alanine aminotransferase, malate dehydrogenase, NAD-linked 'malic' enzyme, glutamate dehydrogenase, and aspartate aminotransferase.

To measure proline dehydrogenase, the cephalothor-

aces, thoraces, fat bodies and midguts were separately homogenized in a Dounce all-glass homogenizer, with 5 parts of a 250 mM sucrose solution containing 1 mM EDTA and adjusted to pH 7.4 (1:5 W:V). Each homogenate was centrifuged for 5 min at 400 x g and the supernatant was used for enzyme assays and protein determinations.

## 2.4. Enzyme assays

All the methods were adjusted to achieve optimal conditions of pH and substrate concentrations. The final assay volume was 1 ml for all enzymes. Enzyme activities were determined in the supernatant by following the changes in absorbance in a spectrophotometer at 25 °C. In all the assays the reaction was started by addition of the enzyme preparation, diluted to give an absorbance change between 0.050 and 0.150 per min and absorbance was read every 25 s during 3 min. Controls were performed in all determinations by omitting the substrate in the assay mixture. The molar extinction coefficient of 6.22 cm<sup>2</sup>/µmol at 340 nm was used to calculate NADH oxidation or NAD<sup>+</sup> reduction. For 2, 6-dichloroindophenol (Cl<sub>2</sub>Ind), the molar absorption coefficient of 21.5 cm<sup>2</sup>/µmol at 600 nm was used. One unit of enzyme is the amount that utilizes 1 µmol of substrate per min under the assay conditions and enzyme activity is expressed as U per g of wet tissue (U/g) and as specific activity (U/mg protein).

Alanine aminotransferase (AlaAT, EC 2.6.1.2) was assayed according to Auerswald and Gäde (1995). The assay medium contained 50 mM Triethanolamine/HCl (TRA-HCl) buffer, pH 7.4, 0.3 mM NADH (disodium salt), 4 mM oxoglutarate (monosodium salt), 50 mM L-alanine and 5 U/ml lactate dehydrogenase.

Malate dehydrogenase (MDH, EC 1.1.1.37) was assayed according to Auerswald and Gäde (1995). The assay mixture contained 100 mM Tris-HCl buffer pH 9.4, 1 mM NAD<sup>+</sup>, 50 mM L-malate (disodium salt).

NAD-linked 'malic' enzyme (NAD-ME, EC 1.1.1.39) was assayed as described by Zebe and Gäde (1993). The assay medium was composed of 50 mM TRA-HCl pH 7.6, 5 mM EDTA, 8 mM Mg<sup>2+</sup>, 0.5 mM NAD<sup>+</sup>, 2 mM L-malate (disodium salt) and 2.5 U/ml of malate dehydrogenase.

Glutamate dehydrogenase (GluDH, EC 1.4.1.2) was assayed using a modification of the method described by Cazzulo et al. (1977). The assay medium contained 40 mM Tris-HCl pH 7.6, 100 mM NH<sub>4</sub>Cl, 0.9 mM EDTA, 0.17 mM NADH (disodium salt), 3 mM oxoglutarate (monosodium salt), and 1.40 mM ADP (potassium salt).

Aspartate aminotransferase (AspAT, EC 2.6.1.1) was assayed using a modification of the method of Bergmeyer and Bernt (1974). The assay medium was 80 mM phosphate buffer pH 7.4, 12 mM oxoglutarate (monosodium salt), 50 mM L-aspartate (monopotassium

salt), 0.18 mM NADH (disodium salt) and 0.6 U/ml malate dehydrogenase.

Proline dehydrogenase (PDH, EC 1.5.99.8) was assayed using a modification of the method of Sakuraba et al. (2001). The reaction mixture was composed of 500 mM potassium phosphate buffer, pH 7.5, 600 mM proline, 0.05 mM  $\text{Cl}_2\text{Ind}$ . The  $\text{Cl}_2\text{Ind}$  was previously dissolved in 50 mM potassium phosphate buffer.

### 2.5. Total protein

The amount of protein was determined according to the method of Hartree (1972) using bovine serum albumin as the standard.

### 2.6. Statistical analyses

Data are presented as mean  $\pm$  standard error of the mean for three different samples. One way analysis of variance (ANOVA) was used. In all cases, *P* values less than 0.05 were considered as significant.

## 3. Results

### 3.1. Amino acid concentration changes in the hemolymph and thorax during flight

Proline and glutamine showed significant changes during flight in females. After a sucrose meal, there was a marked decrease in the proline content and a significant increase in glutamine content in both hemolymph and thorax from females 30 min after flight, but the alanine concentration did not change significantly (Fig. 2). After 60 min of flight, the proline content of hemolymph increased slightly, but in thorax there was not a further significant decrease. The glutamine concentration did not change after 60 min of flight in either hemolymph or thorax. There was not a significant change in alanine content in either hemolymph or thorax at 60 min of flight. In contrast, the proline, alanine and glutamine levels in hemolymph and thorax from males did not change significantly during 30 or 60 min of flight (Fig. 3).

Twenty-four hours after a blood meal, when there is still significant protein digestion occurring in the midgut (Clements, 1992), the proline and alanine content from female hemolymph remained constant throughout flight (Fig. 4A), but the hemolymph concentration of glutamine increased significantly (Fig. 4D). However, 48 hours after a blood meal, when most of the protein has been digested, 30 min of flight lead to a significant decrease in proline content in hemolymph and a significant increase in alanine (Fig. 4B) and glutamine (Fig. 4D) levels in hemolymph. After 60 min of flight the proline content did not change, while the alanine content returned to preflight levels but the glutamine levels

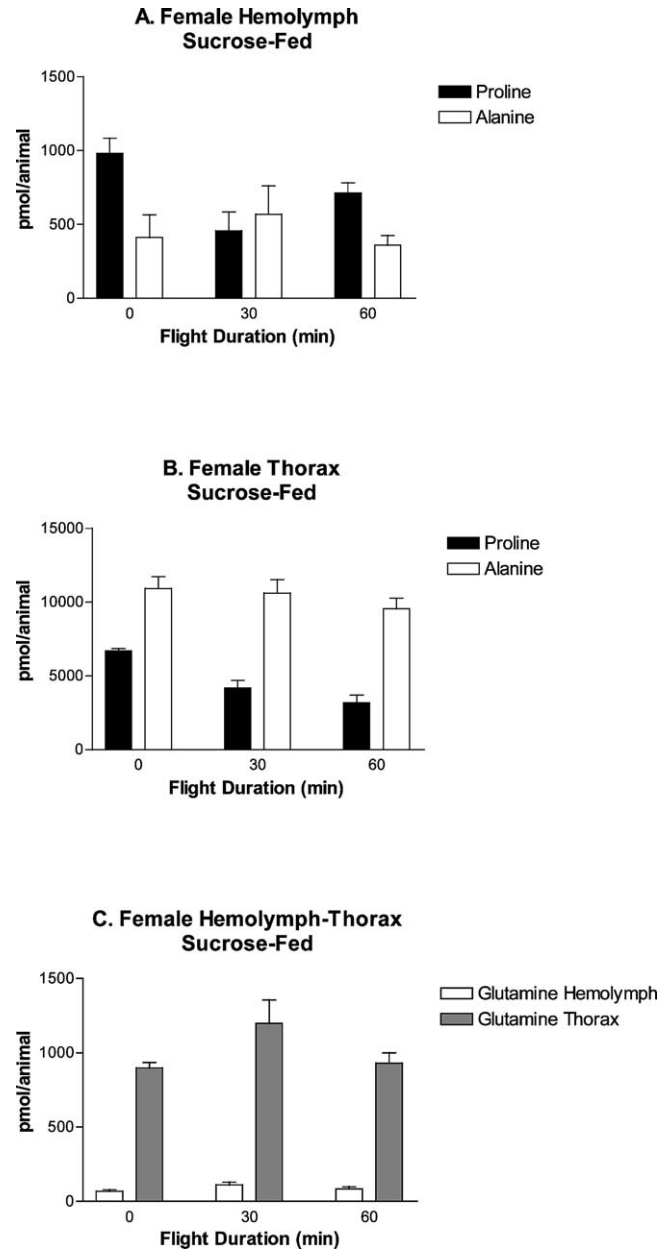


Fig. 2. Concentrations of proline and alanine in hemolymph (A) and thorax (B), and glutamine in hemolymph and thorax (C) from *Ae. aegypti* females after a sucrose meal at 0, 30 and 60 min of flight. Values represent mean  $\pm$  SEM.

remained elevated. A similar pattern was observed 72 h after a blood meal (Fig. 4C, D).

Fig. 5 shows the effect of flight on the concentrations of proline, alanine and glutamine in thorax 24, 48 and 72 h after a blood meal. In all cases, the proline level dropped significantly after 30 min of flight, but only at 24 h did the proline content change further between 30 and 60 min of flight (Fig. 5 A, B, C). In all cases there was not a significant change of alanine content after 30 min of flight, and only after 60 min of flight, 24 h after a blood meal, was there a significant decrease of alanine content (Fig. 5 A, B, C). In thorax, 24 h after a blood

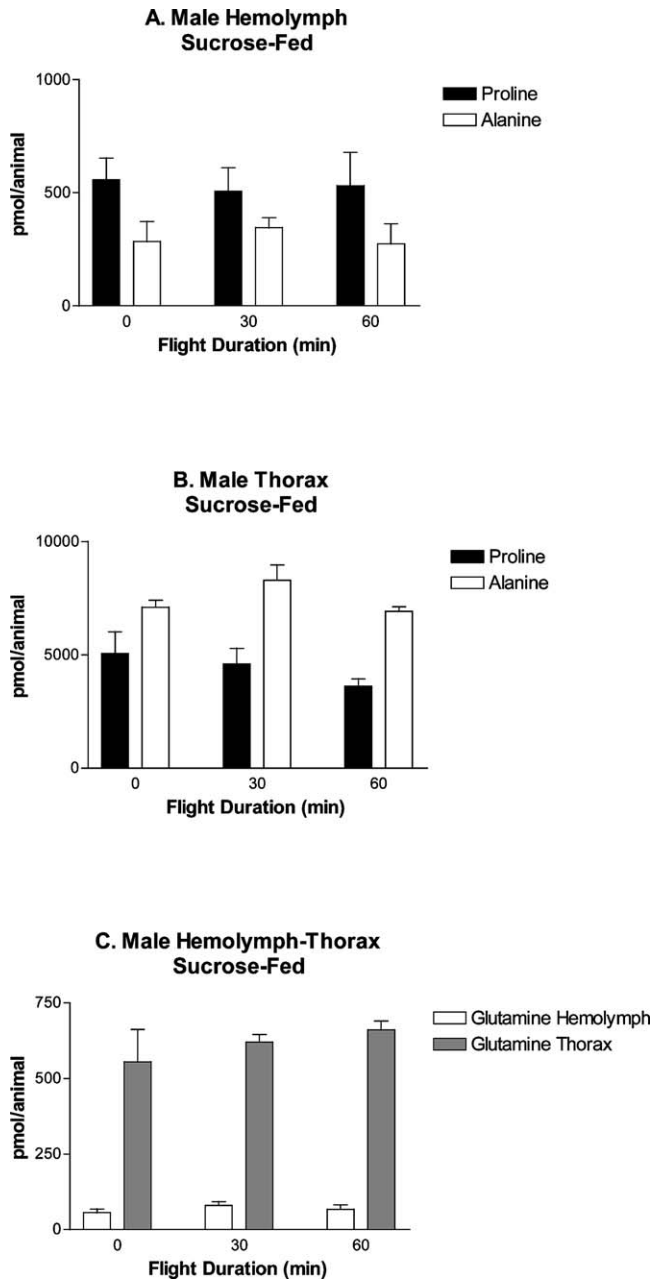


Fig. 3. Concentrations of proline and alanine in hemolymph (A) and thorax (B), and glutamine in hemolymph and thorax (C) from *Ae. aegypti* males after a sucrose meal at 0, 30 and 60 min of flight. Values represent means  $\pm$  SEM.

meal the glutamine content of thorax was quite high and did not change during flight, but at both 48 and 72 h after a blood meal the glutamine level increased after 30 min of flight and did not change significantly at 60 min of flight.

### 3.2. Enzymatic activities

The activity of all enzymes investigated in thorax from females and males was much higher in adults than in early and late cephalothorax from pupae (Tables 1

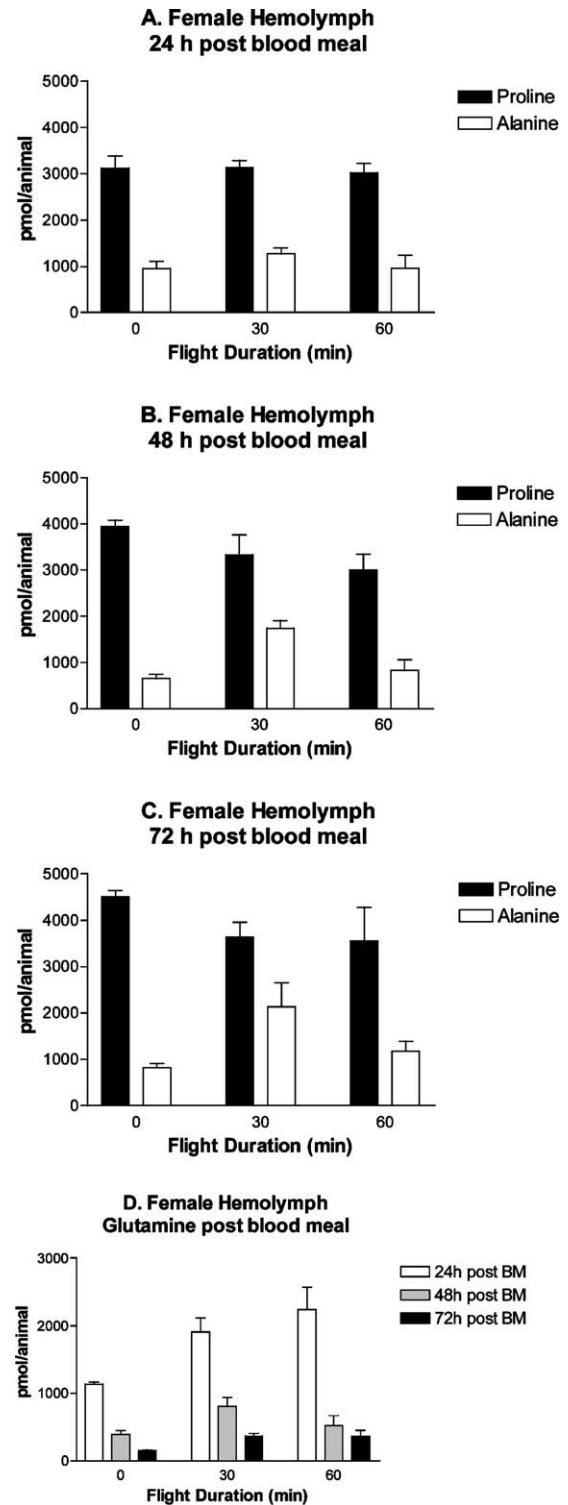


Fig. 4. Concentrations of proline and alanine in hemolymph 24 (A), 48 (B) and 72 (C) h after a blood meal at 0, 30 and 60 min of flight. Concentrations of glutamine in hemolymph 24, 48 and 72 h after a blood meal at 0, 30 and 60 min of flight (D). Values represent means  $\pm$  SEM.

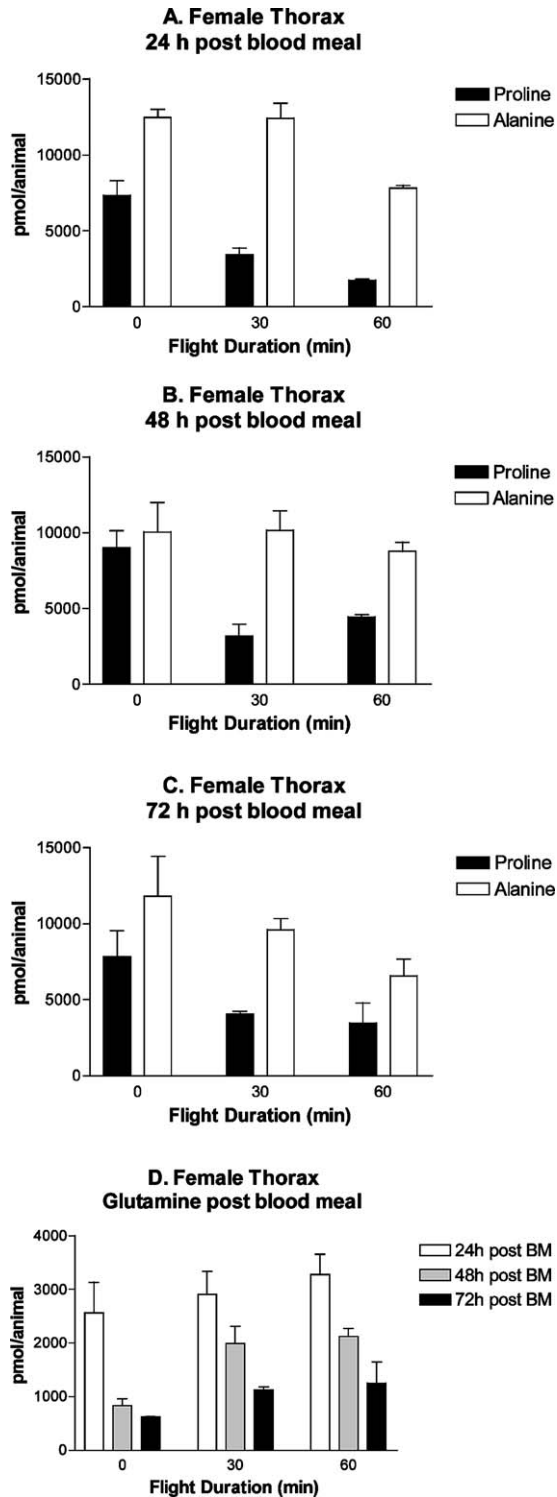


Fig. 5. Concentrations of proline and alanine in thorax 24 (A), 48 (B) and 72 (C) h after a blood meal at 0, 30 and 60 min of flight. Concentrations of glutamine in thorax 24, 48 and 72 h after a blood meal at 0, 30 and 60 min of flight (D). Values represent means  $\pm$  SEM.

and 2). The developmental pattern was similar in males and females. The enzymatic activities assayed in homogenates of adult female thorax, fat body and midgut are shown in the Table 3. The highest activities of AlaAT, MDH, NAD-ME and PDH were found in homogenates of thorax. GluDH showed similar activities in thorax and midgut, but AspAT activity was lower in thorax than in midgut and fat body. Activities of AlaAT and MDH were of the same order of magnitude in fat body and midgut, while NAD-ME, GluDH and PDH were lower in fat body than in midgut.

#### 4. Discussion

In this paper we present evidence that proline can be utilized as a fuel for the flight muscle of *Ae. aegypti* females. Consistent with the criteria given in the introduction, we reported that: 1) the concentration of proline in hemolymph and thorax always decreased during flight experiments using females, except in the hemolymph 24 h after a blood meal; and 2) the activities of the enzymes involved in proline metabolism in fat body and flight muscle are consistent with proline serving as a shuttle to move acetyl units from the fat body to flight muscle. However, there were some unexpected results: 1) the concentration of alanine did not always change; 2) there was a significant change in glutamine concentration under most conditions, an observation not reported in other insects that use proline to shuttle acetyl units from fat body to flight muscle; and 3) although males have the requisite enzymatic properties, no evidence could be found to show that they used proline during flight.

Several insects, such as the tsetse fly, *G. morsitans* (Bursell, 1963; Olembo and Pearson, 1982), the Colorado potato beetle *L. decemlineata* (Weeda et al., 1979), and several other species of beetle (Gäde and Auerswald, 2002) have a higher proline concentration in hemolymph and flight muscle than found in hemolymph and thorax from *Ae. aegypti*. However, significant decreases in proline levels occurred during 30 min of flight in hemolymph and thorax of *Ae. aegypti* females. This change was observed whether the females were sugar-fed or 48 and 72 h after blood feeding. Twenty-four hours after a blood meal, there was not a change of proline concentration in hemolymph after flight, most likely because there was still significant amino acid entry into the hemolymph from proteolysis in the midgut. For technical reasons, related to fixing the mosquitoes to the flight apparatus and the time required to collect hemolymph, attempts to measure hemolymph amino acid levels after shorter times of flight gave very erratic and inconsistent results, but this does not detract from the fact that significant changes in proline concentration were found at longer time of flight.

Enzymes related to amino acid metabolism in *Ae.*

Table 1  
Activities of enzymes related to amino acid metabolism in adult thorax and pupal cephalothorax from male *Aedes aegypti*<sup>a</sup>

Enzymes <sup>b</sup>	Thorax			EP <sup>c</sup> Cephalothorax			LP <sup>c</sup> Cephalothorax			Ratios		
	U/g tissue	U/mg Protein	U/g tissue	U/mg Protein	U/g tissue	U/mg Protein	U/g tissue	U/mg Protein	Th/EPC		Th/LPC	
									U/g	U/mg P	U/g	U/mg P
AlaAT	204.83 ± 29.39	1.384 ± 0.112	9.34 ± 1.68	0.101 ± 0.014	20.40 ± 0.50	0.212 ± 0.005	21.93	13.70	10.04	6.53		
MDH	302.13 ± 51.36	2.054 ± 0.234	31.10 ± 1.13	0.339 ± 0.004	41.39 ± 0.43	0.449 ± 0.004	9.71	6.06	7.30	4.57		
NAD-ME	36.96 ± 4.37	0.252 ± 0.031	2.18 ± 0.12	0.024 ± 0.001	3.33 ± 0.28	0.039 ± 0.003	16.95	10.50	11.10	6.46		
GluDH	41.52 ± 6.24	0.280 ± 0.028	6.02 ± 0.34	0.066 ± 0.004	5.75 ± 0.22	0.063 ± 0.002	6.90	4.24	7.22	4.44		
AspAT	35.55 ± 6.17	0.243 ± 0.052	6.80 ± 0.32	0.074 ± 0.002	6.81 ± 0.05	0.075 ± 0.001	5.23	3.28	5.22	3.24		
PDH	9.01 ± 0.82	0.057 ± 0.000	1.08 ± 0.11	0.008 ± 0.002	1.46 ± 0.08	0.011 ± 0.001	8.34	7.12	6.17	5.18		

<sup>a</sup> Values are the means of three determinations ± SEM.

<sup>b</sup> AlaAT = Alanine aminotransferase, MDH = Malate dehydrogenase, NAD - ME = NAD - linked 'malic' enzyme, GluDH = Glutamate dehydrogenase, AspAT = Aspartate aminotransferase, PDH = Proline dehydrogenase

<sup>c</sup> EP = early pupae; LP = late pupae; Th = thorax.

Table 2  
Activities of enzymes related to amino acid metabolism in adult thorax and pupal cephalothorax from female *Aedes aegypti*<sup>a</sup>

Enzymes <sup>b</sup>	Thorax			EP <sup>c</sup> Cephalothorax			LP <sup>c</sup> Cephalothorax			Ratios		
	U/g tissue	U/mg Protein	U/g tissue	U/mg Protein	U/g tissue	U/mg Protein	U/g tissue	U/mg Protein	Th <sup>c</sup> /EPC	Th/LPC	U/g	U/mg P
									U/g	U/mg P	U/g	U/mg P
AlaAT	181.33 ± 13.56	1.745 ± 0.092	10.45 ± 0.01	0.122 ± 0.005	19.29 ± 1.61	0.211 ± 0.021	17.35	14.30	9.40	8.27		
MDH	235.00 ± 17.33	2.265 ± 0.175	24.12 ± 0.01	0.282 ± 0.012	40.19 ± 2.68	0.439 ± 0.022	9.74	8.03	5.85	5.16		
NAD-ME	35.67 ± 1.33	0.376 ± 0.014	1.85 ± 0.08	0.022 ± 0.001	3.85 ± 0.01	0.042 ± 0.001	19.28	17.09	9.26	8.95		
GluDH	49.67 ± 4.22	0.480 ± 0.020	6.04 ± 0.01	0.071 ± 0.003	6.23 ± 0.20	0.068 ± 0.003	8.22	6.76	7.97	7.06		
AspAT	19.33 ± 1.11	0.186 ± 0.014	6.99 ± 0.02	0.082 ± 0.004	7.62 ± 0.65	0.083 ± 0.008	2.77	2.27	2.54	2.24		
PDH	12.65 ± 2.11	0.072 ± 0.004	1.16 ± 0.06	0.008 ± 0.001	1.73 ± 0.06	0.013 ± 0.001	10.91	9.00	7.31	5.54		

<sup>a</sup> Values are the means of three determinations ± SEM

<sup>b</sup> AlaAT = Alanine aminotransferase, MDH = Malate dehydrogenase, NAD - ME = NAD - linked 'malic' enzyme, GluDH = Glutamate dehydrogenase, AspAT = Aspartate aminotransferase, PDH = Proline dehydrogenase

<sup>c</sup> EP = early pupae; LP = late pupae; Th = thorax

Table 3

Activities of enzymes related to amino acid metabolism in adult *Aedes aegypti* female thorax, fat body and midgut<sup>a</sup>

Enzymes <sup>b</sup>	Thorax		Fat Body		Midgut	
	U/g tissue	U/mg Protein	U/g tissue	U/mg Protein	U/g tissue	U/mg Protein
AlaAT	181 ± 13.56	1.745 ± 0.092	48 ± 5.33	0.471 ± 0.036	41 ± 0.67	0.384 ± 0.010
MDH	235 ± 17.33	2.265 ± 0.175	36 ± 4.67	0.352 ± 0.032	44 ± 1.78	0.405 ± 0.018
NAD-ME	36 ± 1.33	0.376 ± 0.014	0.1 ± 0.01	0.001 ± 0.001	0.4 ± 0.04	0.003 ± 0.001
GluDH	50 ± 4.22	0.480 ± 0.020	5 ± 0.67	0.050 ± 0.005	52 ± 4.44	0.480 ± 0.046
AspAT	19 ± 1.11	0.186 ± 0.014	27 ± 3.78	0.250 ± 0.033	40 ± 2.00	0.371 ± 0.047
PDH	13 ± 2.11	0.072 ± 0.004	0.7 ± 0.01	0.005 ± 0.001	3.5 ± 0.33	0.027 ± 0.002

<sup>a</sup> Values are the means of three determinations ± SEM<sup>b</sup> AlaAT = Alanine aminotransferase, MDH = Malate dehydrogenase, NAD - ME = NAD - linked 'malic' enzyme, GluDH = Glutamate dehydrogenase, AspAT = Aspartate aminotransferase, PDH = Proline dehydrogenase

*aegypti* females and males undergo significant changes in activity levels after the molt from pupa to adult, showing that the capacity to metabolize proline is not fully developed in pupal stages. These changes are necessary to support the use of proline as a shuttle for acetyl units from fat body to flight muscle in adults. The profile of enzyme activities responsible for proline metabolism in thorax from *Ae. aegypti* females exhibits a similar pattern to that found in flight muscle from insects which use proline as fuel during flight, and it contrasts with the profile found in those insects that do not use proline to power flight (Crabtree and Newsholme, 1970; Hansford and Johnson, 1975; Pearson et al., 1979; Norden and Matanganyidze, 1979; Weeda et al., 1980; Auerswald and Gäde, 1995, 1999, 2002).

Alanine aminotransferase (AlaAT) and NAD-linked "malic" enzyme (NAD-ME) activities from *Ae. aegypti* thorax are similar to those found in flight muscle of *Popillia japonica* (Hansford and Johnson, 1975), *G. morsitans* (Pearson et al., 1979), and *D. lunata* (Auerswald and Gäde, 1995). In flight muscle of *P. sinuate*, Auerswald and Gäde (1999, 2002) reported values of AlaAT, malate dehydrogenase (MDH), expressed as U/g tissue, close to those in thorax of *Ae. aegypti*. Also, in flight muscle of *G. morsitans* (Norden and Matanganyidze, 1979) and *L. decemlineata* (Weeda et al., 1980) the levels of NAD-ME and MDH are similar to those found in flight muscle of *Ae. aegypti*.

The use of proline as fuel for flight by muscle from three species of Coleoptera has been proposed by Pearson et al. (1979) based on the high activities of AlaAT, NAD-ME (in *Catharsius sp.*, *Heliocopriss dilloni* and *Scarabaeus sp.*) and proline dehydrogenase (PDH) (in *Heliocopriss dilloni*). It is important to note that MDH, NAD-ME, aspartate aminotransferase (AspAT) and

PDH are all more active in thorax of *Ae. aegypti* than in that of *H. dilloni* (Pearson et al., 1979). In addition, it is noteworthy that the activities of all enzymes involved in the proline metabolic pathway proposed by Bursell (1963) are higher in thorax from *Ae. aegypti* females than in fat body and midgut. The quantitative differences in the activity of enzymes involved in the metabolic cycling (Fig. 1) of proline between thorax, fat body and midgut is also consistent with the suggestion that mosquito flight muscle has the capacity to use proline as a substrate in supplying energy for flight.

In the resting state of other insects that use proline to fuel flight the proline content in thorax is higher than the alanine content, and during flight the proline content decreases sharply while the alanine content generally increases (Bursell, 1963; Olembo and Pearson, 1982; Sacktor and Wormser-Shavit, 1966; Zebe and Gäde, 1993; Auerswald and Gäde, 1995; Auerswald et al., 1998 a,b). This increase in alanine is necessary in order to shuttle the amino group derived from the conversion of proline to  $\alpha$ -ketoglutarate in flight muscle back to fat body where it is used to convert  $\alpha$ -ketoglutarate to proline (Fig. 1). This ensures that there is no net utilization of proline, which is required if proline is to act as a shuttle. But in sugar fed females, the alanine concentration in hemolymph and thorax did not change throughout flight; however there was a significant increase in glutamine content. The alanine concentration was also constant during flight in hemolymph of females 24 h after a blood meal, but again there was an increase in glutamine content. However, 48 and 72 h after a blood meal, the concentration of alanine in hemolymph increased significantly after flight, as did the concentration of glutamine. Taken together these data suggest that glutamine and/or alanine could be involved in

shuttling amino groups from flight muscle to the fat body. When there is more proline to use as a substrate for shuttling acetyl units from fat body to flight muscle, as after a blood meal, the role of both alanine and glutamine in shuttling amino groups is apparent. However, under sugar-fed conditions, the shuttling of amino groups seems to be carried out predominately by glutamine. At this moment it is unclear exactly how glutamine acts in this cycle and this is under investigation.

The levels of the enzymes assayed in flight muscle from *Ae. aegypti* males suggests males should also be able to oxidize proline, but we could find no evidence that it occurs to a significant extent based on changes in proline levels during flight. We have no explanation for this observation.

While it has been clearly demonstrated that carbohydrates are an important fuel during flight of mosquitoes (Clements, 1955, 1992; Nayar and Van Handel, 1971; Nayar and Sauerman, 1973; Foster, 1995; Briegel et al., 2001a,b), the levels of enzyme activities assayed in thorax and the marked decreased in proline concentration during flight in hemolymph and thorax support the hypothesis that proline can serve as a shuttle in moving acetyl units from fat body to flight muscle in *Ae. aegypti* females during flight.

## Acknowledgements

We thank Wallace Clark from The University of Arizona Division of Biotechnology Proteomics Core Facility for his excellent work on the amino acid analysis. Thanks to James Pennington for his critical review of the manuscript. This work was supported by NIH grant AI46541.

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