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Changes in Amounts of Total Salivary Gland Proteins of *Lutzomyia longipalpis* (Diptera: Psychodidae) According to Age and Diet

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ABSTRACT Saliva plays important roles in facilitation of a bloodmeal, lubrication of mouthparts, and parasite transmission for some vector insects. Salivary composition changes during the lifetime of an insect, and differences in the salivary profile may influence its functions. In this report, the amount and profile of salivary gland protein of the American visceral leishmaniasis vector *Lutzomyia longipalpis* (Lutz & Neiva, 1912) were analyzed at different times of insect development and diet. Protein content from unfed female sand flies increased significantly with age, and a significant difference was observed in sugar-fed females during the first 10 d of adult life. Salivary protein content sharply decreased 1 d after blood feeding, with gradual increase in concentration the following days. SDS-polyacrylamide gel electrophoresis analysis revealed that most polypeptides present in the saliva of sugar-fed also were present in the saliva of blood-fed females. Understanding changes in sand fly's saliva contents at distinct days after emergence and the influence of a bloodmeal in this aspect may reveal the role played by saliva during leishmaniasis transmission.

KEY WORDS *Lutzomyia longipalpis*, salivary gland, saliva, SDS-polyacrylamide gel electrophoresis

Saliva of hematophagous arthropods plays important roles in lubrication of mouthparts, solubilization and initial digestion of food, and protection against infection (Marinotti et al. 1990, Moreira-Ferro et al. 1998, Moreira-Ferro et al. 1999). In addition, this saliva contains substances with antihaemostatic, antiinflammatory, and immunomodulatory properties (Ribeiro and Francischetti 2003, Andrade et al. 2005).

In general, female insects need to ingest sugar to meet the energy demands of basal metabolism and flight, and they need to ingest blood for egg development. Saliva is elemental in both types of feeding, playing different roles because it contains different sets of enzymes for blood and sugar feeding (Marinotti et al. 1990). The physiological state of the insects has been shown to be an important factor in salivary protein amount and composition. Reduction in total salivary gland protein from four anopheline vectors of human malaria blood-fed on human volunteers and hamsters has been observed previously (Golenda et al. 1995). In sand flies, it has been demonstrated that enzyme activities of the salivary glands decrease after a bloodmeal, indicating that salivary compounds were injected during blood feeding (Charlab et al. 1999, 2000; Katz et al. 2000; Ribeiro et al. 2000a, b; Ribeiro and Modi 2001). Additionally, differences in the composition of salivary proteins in sand flies are found according to species and age (Volf et al. 2000).

Lutzomyia longipalpis (Lutz & Neiva, 1912) (Diptera: Psychodidae) is the main vector of *Leishmania infantum chagasi*, the etiological agent of American visceral leishmaniasis (Soares and Turco 2003). The role of their salivary gland proteins in inflammation and immunomodulation has been described previously (Barral et al. 2000, Gomes et al. 2002, Costa et al. 2004, Silva et al. 2005, Teixeira et al. 2005). Despite the importance of saliva, the salivary gland protein profile of sand flies at diverse ages and physiological conditions has only been studied in Old World species of phlebotomines. The aim of this study was to show the protein profile of saliva from *L. longipalpis* obtained at different times of development and diet.

Materials and Methods

Sand Fly Rearing and Diet. Adult *L. longipalpis* captured in Cavunge (Bahia, Brazil) were reared at the Laboratório de Imunoparasitologia/Centro de Pesquisas Gonçalo Moniz (CPqGM)/FIOCRUZ (Bahia, Brazil) as described previously (Silva et al. 2005). After emergence, adult insects were divided into three

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groups. The first consisted of unfed females that were used throughout the whole period of experimentation. The second group included female sand flies that were fed in cotton containing a sucrose solution throughout the whole period. A third group of female sand flies was maintained with sucrose solution up to the third day after emergence and blood-fed once in anesthetized hamsters. Salivary glands were dissected at different times after a bloodmeal. The experimental procedures were approved by the Animal Care and Use Committee of FIOCRUZ–Bahia.

Salivary Gland Dissection. Salivary glands were obtained from female sand fly groups as described above. From unfed sand flies, salivary glands were collected at days 1, 3, and 5 after insect emergence. From sugar-fed sand flies, salivary glands were collected at days 1, 3, 4, 5, 6, 7, 8, and 10 after insect emergence. Finally, from blood-fed sand flies, salivary glands were obtained at days 1, 3, 5, and 7 after insect blood feeding. In this group, sand flies 1 d after a bloodmeal were 4 d old, 3 d after a bloodmeal were 6 d old, 5 d after a bloodmeal were 8 d old, and 7 d after a bloodmeal were 10 d old. Salivary glands were dissected under a stereoscopic microscope (Stemi 2000, Carl Zeiss, Jena, Germany) in 1×phosphate-buffered saline (PBS), pH 7.4, by using entomological needles. Salivary glands were placed in a tube containing 1×PBS and stored at −70°C until use. Glands were sonicated (Sonifier 450, Branson, Danbury, CT) and centrifuged at 10,000 rpm at 4°C for 5 min. The supernatants, or salivary gland homogenates (SGH), were used for experiments.

Protein Quantification. SGH total protein content was determined using the Bio-Rad Assay kit (Bio-Rad, Hercules, CA), based on a bovine serum albumin (BSA) standard curve as described previously (Bradford 1976). There were six replicates for each treatment, consisting of protein quantification from three pooled sand fly salivary glands. Two independent protein determinations were performed.

SDS-polyacrylamide gel electrophoresis (PAGE). Salivary glands from a different group of sugar-fed and blood-fed female sand flies dissolved in Tris-glycine SDS sample buffer (Invitrogen, Carlsbad, CA) were resolved under nonreducing and reducing conditions by SDS-PAGE in a 16% Tris-glycine gel by using the Invitrogen–Novex precast proteins, and then they were stained with Coomassie Blue. For each experimental group, five pairs of pooled salivary glands were applied to the gels. From sugar-fed sand flies, salivary glands were collected at days 1, 3, 5, and 7 after insect emergence; and from blood-fed sand flies, salivary glands were obtained at days 1, 3, 5, and 7 after insect blood feeding. In this group, sand flies 1 d after a bloodmeal were 8 d old, 3 d after a bloodmeal were 10 d old, 5 d after a bloodmeal were 12 d old, and 7 d after a bloodmeal were 14 d old.

Statistical Analysis. Statistical analyses were performed using GraphPad Prism software (GraphPad Software Inc., San Diego, CA). The mean and SEM were calculated for total salivary gland protein for six replicates of pooled salivary glands. A Kruskal–Wallis nonparametric test was used to evaluate significance among properties from different time points, and the Dunns' post test was used for multiple comparisons.

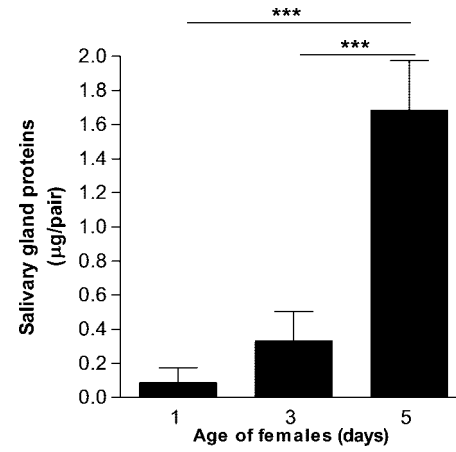

Fig. 1. Salivary gland protein content from unfed *L. longipalpis* females. After emergence, females were kept without any meal source, and their salivary glands were obtained at different days after emergence (1, 3, and 5). Values represent mean ± SEM of six pools, containing three pairs of salivary glands each, used for each treatment. Results represent an average of two independent dosages. Asterisks indicate significant difference calculated by Kruskal–Wallis nonparametric test. ***, $P < 0.001$ days 1 and 3 versus day 5.

Results

The total protein content of unfed female sand fly glands increased significantly from the first or third day to the fifth day of adult sand fly development (Fig. 1). In sugar-fed female sand flies, the total protein content was quantified during the first 10 d of adult life. There was already a significant increase in the amount of total salivary gland proteins at day 3, and this was maintained throughout the 10th day (Fig. 2). When *L. longipalpis* females were blood-fed on hamsters and their salivary glands were dissected at different times after meal for protein quantification, there was a significant reduction (≈95%) in total salivary gland protein on the first day after the bloodmeal compared with sugar-fed females of the same age (Fig. 2). However, salivary protein content increased in blood-fed females over subsequent days, being significant on days 5 and 7 after a bloodmeal, corresponding to the eighth and 10th day after emergence of adult insect, respectively.

SDS-PAGE analysis of proteins present in the salivary glands of sugar-fed or blood-fed *L. longipalpis* females is shown in Fig. 3A and B. Under nonreducing conditions, all experimental groups showed a similar protein profile, revealing the presence of 12 polypeptides bands with molecular weights ranging from 6 to 130 kDa. However, 1 d after emergence of sugar-fed females, it was not possible to observe the 6-, 24-, 27-, and 130-kDa bands using Coomassie stain. The same phenomena occurred 1 d after blood feeding, where it was not possible to observe the 6-, 16-, 27-, 32-, 37-, 49-,

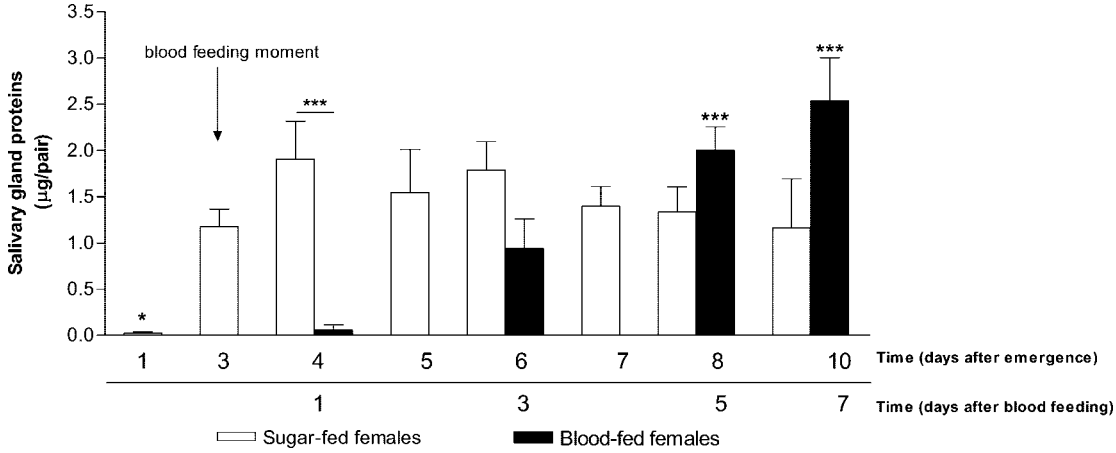

Fig. 2. Salivary gland protein content from sugar-fed and blood-fed *L. longipalpis* females. Open bars represent salivary protein from sugar-fed females at different times after emergence. In this group, insects were maintained on a sugar diet and salivary glands were obtained at different days after emergence (1, 3, 4, 5, 6, 7, 8, and 10). Closed bars represent salivary protein from blood-fed females. In this group, insects were maintained on a sugar diet until blood feeding on the third day after emergence. Salivary glands were obtained on different days after blood feeding (1, 3, 5, and 7). Values represent mean ± SEM of six pools, containing three pairs of salivary glands each, used for each treatment. Results represent an average of two independent dosages. Asterisks indicate significant differences calculated by Kruskal–Wallis nonparametric test. *, $P < 0.05$ day 1 after emergence compared with all sugar-fed females group. ***, $P < 0.001$ day 4 after emergence versus day 1 after a bloodmeal and days 5 and 7 after a bloodmeal versus day 1 after a bloodmeal.

61-, and 130-kDa bands (Fig. 3A). Under reducing conditions the 130-, 27-, 17-, and 16-kDa bands were not observed (Fig. 3B).

Discussion

Results presented in this study indicate that total salivary proteins of *L. longipalpis* depend on physiological factors such as age and diet. The protein content from unfed sand flies increased 94% from the first to the fifth day after emergence. Such variation can be related to the synthesis of important enzymes for meal ingestion and initial digestion. Although at different rates, the increase in the amount of protein in female salivary glands during insect development has been described in other blood-sucking dipterans, such as *Culex quinquefasciatus* Say, *Culex pipiens* L., and *Armigeres subalbatus* (Coquillett) (Nascimento et al. 2000, Siriyasatien et al. 2005a, b , Siriyasatien et al. 2005b).

Unfed sand fly females experience massive death at the fifth day of emergence, precluding long observations. However, sugar feeding allows longer survival and was used up to the 10th day. In this case, there was an early significant increase of protein amount on the third day that was maintained until the 10th day of insect development. Sugar meal is a source of longevity of insects (Schlein and Jacobson 1999). This meal was probably an important factor for survival of the *L. longipalpis* females. In general, salivary glands of hematophagous insects become mature at the third or fifth day after emergence; at this time, there are substances in the saliva that are important to hematophagy (al-Ahdal et al. 1990, Marinotti et al. 1990, Brennan et al. 2000). In *L. longipalpis*, we observed that sugar-fed females presented a significant increase in salivary protein values on the third day after emergence.

Using SDS-PAGE, we observed polypeptides bands with similar molecular masses as some of the previously reported most abundant secreted proteins from the salivary glands of *L. longipalpis* (Valenzuela et al. 2004). Proteins seem to begin being secreted into salivary gland after the first days of adult emergence, because from 12 polypeptide bands observed on SDS-PAGE, the 6-, 24-, 27-, and 130-kDa bands could not be observed on days 1 and 3 after emergence. It is possible that the absence of some polypeptide bands is due to their small amounts, below the threshold of detection of Coomassie stain. Only at the fifth day after emergence could we detect all 12 polypeptide bands in these insects, in contrast to other species of dipterans like the Old World sand fly *Phlebotomus papatasi* Scopoli, where the full protein profile was reached on day 3 (Volf et al. 2000). It has been previously described that variability in the salivary content can occur among different groups of arthropods and individuals of the same species (Warburg et al. 1994, Ribeiro and Francischetti 2003). Regarding the two SDS-PAGE conditions performed, the only differences observed were found in four polypeptide bands that are present on nonreducing conditions. This indicates that those proteins are generated by the interaction of different polypeptides.

Hematophagous insects also require vertebrate host blood for nutrition, egg development, and survival (Andrade et al. 2005). Several works have demonstrated that proteins are lost from the salivary glands of insects during blood feeding, indicating that the flow of saliva is continuous throughout blood feeding. In this work, we quantified the decrease in total sal-

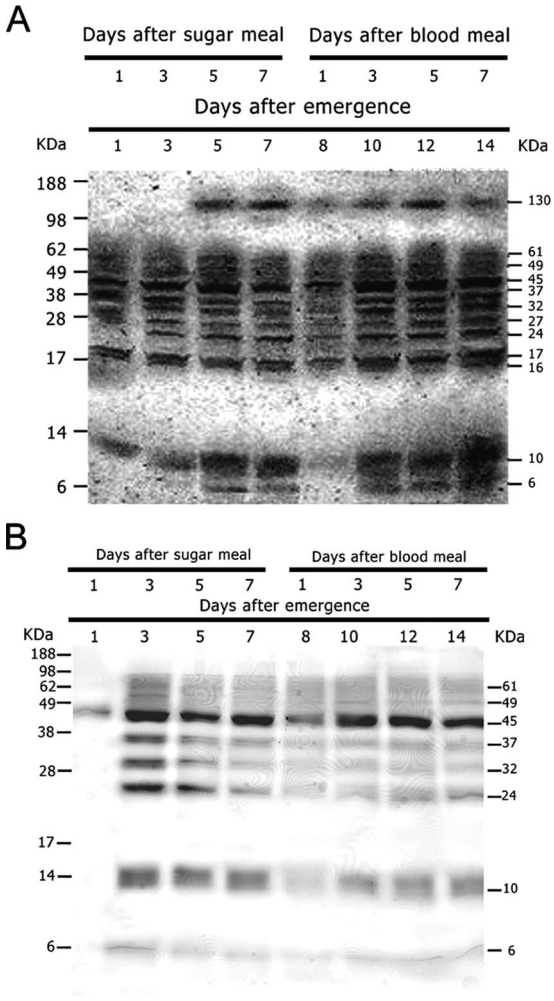

Fig. 3. Electrophoretic profile of salivary gland polypeptides from *L. longipalpis* females. Polypeptides from five pairs of salivary glands dissected at different days after emergence and after blood feeding were separated under nonreducing conditions (A) and reducing conditions (B) by SDS-PAGE in a 16% polyacrylamide gel stained with Coomassie Blue. Molecular weights in kilodaltons are indicated.

ivary protein, and we found that on the first day after blood feeding a depletion of total protein content from salivary glands of *L. longipalpis* occurred. However, the protein content increased over subsequent days returning to similar values observed for sugar-fed sand flies before to blood sucking. The decrease in salivary protein amount after a bloodmeal has been previously demonstrated in mosquitoes, but not sand flies (Soliman et al. 1999, Volf et al. 2000).

When we observed the electrophoretic pattern of salivary proteins from 1-d blood-fed *L. longipalpis* females, we could not detect proteins with similar molecular masses to some secreted proteins already described in the salivary gland of this species of sand fly (6, 16, 27, 32, 49, and 61 kDa) (Valenzuela et al. 2004). The electrophoretic pattern of salivary proteins from blood-fed sand flies also showed that all polypeptides present in glands of sugar-fed sand flies are also present in blood-fed flies, except on the first day after a bloodmeal, perhaps because these polypeptides are present in small amounts and cannot be detected by Coomassie stain.

In summary, our results suggest that the salivary protein composition of *L. longipalpis* depends on factors such as age and diet, indicating that there is a dynamic in the process of salivation that mainly occurs during blood feeding by these insects. These results expand possibilities for future studies characterizing which of these proteins are specifically present at different physiological conditions and which mechanisms are involved in secretion and salivation of this New World leishmaniasis vector.

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