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# Perimicrovillar membranes promote hemozoin formation into *Rhodnius prolixus* midgut

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**Abstract**

*Rhodnius prolixus* is a hematophagous insect that ingests large quantities of blood in each blood-feeding session. This ingested blood provides important nutrients to sustain the insect's oogenesis and metabolic pathways. During the digestive process, however, huge amounts of heme are generated as a consequence of the hemoglobin breakdown. Heme is an extremely dangerous molecule, since it can generate reactive oxygen species in the presence of oxygen that impair the normal metabolism of the insect. Part of the hemoglobin-derived heme can associate with the perimicrovillar membranes (PMM) in the gut lumen of *R. prolixus*; in this study we demonstrate the participation of the PMM in a heme detoxification process. These membranes were able to successfully induce heme aggregation into hemozoin (Hz). Heme aggregation was not dependent on the erythrocyte membranes, since the contribution of these membranes to the process was negligible, demonstrating that the ability to induce heme aggregation is a feature of the PMM, possibly representing a pre-adaptation of the hemipterans to feeding on blood.


*Keywords:* Perimicrovillar membranes; Heme; Hemozoin; *Rhodnius prolixus*

## 1. Introduction

Insects represent a very successful group with regard to alimentary sources. An important factor that has made the adaptation to different fonts of diet possible was the development of appropriate mouthparts and midgut adaptations (Terra, 1990; Law et al., 1992). The hematophagous insects, in particular, present some crucial adaptations that enable them to successfully feed on blood. One of the challenges which the hematophagous organisms have to deal with is the presence of high concentrations of heme released in the gut lumen during the digestion of hemoglobin (Wigglesworth, 1943; Oliveira et al., 2000a; Pascoa et al., 2002; Oliveira and Oliveira, 2002). Wigglesworth (1943) studied the fate of hemoglobin in *R. prolixus* during blood digestion and, at that time, stated that hemoglobin was broken down, in the gut lumen, to a protohematin that was excreted unchanged. Heme is a toxic molecule since it may catalyze reactive oxygen species formation that can damage many biomolecules (Vincent, 1989). Moreover, heme can associate with phospholipid membranes, interfering with their physical integrity and leading to cell disruption (Schmitt et al., 1993). Hence, hematophagous organisms have developed strategies to protect themselves against heme toxicity. In the midgut of the cattle tick, *Boophilus microplus*, heme is sequestered in a cellular structure named the hemosome (Lara et al., 2003). In the hemolymph, some organisms are able to synthesize heme-binding proteins such as Help, a heme lipoprotein from the cattle tick, *B. microplus* (Maya-Monteiro et al.,

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2004), and RHBP, a heme-binding protein from *R. prolixus* (Oliveira et al., 1995; Dansa-Petretski et al., 1995), which can bind heme, playing a role as an antioxidant protein.

Other antioxidant mechanisms have also been described for *R. prolixus* (Graça-Souza et al., 1997; Paes et al., 2001; Paiva-Silva et al., 2006; Silva et al., 2006). Oliveira et al. (1999) showed that the *R. prolixus* sequesters the heme generated in the gut lumen in a dark-brown pigment named hemozoin (Hz). This is an insoluble and less reactive heme aggregate that represents the first line of defense against heme toxicity in the midgut of *R. prolixus* (Oliveira et al., 2000a, 2002). This mechanism represents one of the major heme detoxification pathway for hematophagous organisms (Oliveira et al., 2000a, b, 2002, 2004; Chen et al., 2001; Pisciotta et al., 2005). Despite the many descriptions of Hz, little is known about the process of Hz formation. Some evidence suggests lipids to be catalysts in this process by increasing the heme solubility in acidic conditions (Oliveira et al., 2004, 2005; Bendrat et al., 1995; Dorn et al., 1998; Fitch et al., 1999, 2000; Hempelmann et al., 2003; Jackson et al., 2004; Pandey et al. 2003; Egan et al., 2001). However, the participation of proteins in this process is also argued, since Sullivan et al. (1996) showed that histidine-rich protein-II (HRP-II) is capable of promoting heme aggregation into Hz. Another report has indicated that Hz formation by HRP-II correlates with aspartate and not with histidine in pH values of around 6 (Lynn et al., 1999).

In this study, we present data to show that Hz formation in *R. prolixus* is dependent on the perimicrovillar membranes (PMM), an unusual extracellular lipoprotein membrane that sheaths the microvilli of midgut cells in all Hemiptera (Silva et al., 2004). Lane and Harrison (1979) were the first to recognize this unusual cell surface modification as a second membrane along the *R. prolixus* midgut epithelia, where they are present throughout the life of the animal both before and after a blood meal. These membranes play critical roles in amino acid absorption in sap sucker insects (Terra, 1988), compartmentalizing the digestive process in higher Heteroptera with luminal digestion (Houseman and Downe, 1983; Billingsley and Downe, 1983, 1985, 1988; Ferreira et al., 1988; Terra, 1990; Terra and Ferreira, 1994; Terra et al, 2006) and immobilizing enzymes in a large area to avoid excretion (Cristofoletti et al., 2003). Here, we show that these membranes present an additional function, playing a role in heme detoxification in the gut lumen of *R. prolixus*.

## 2. Material and methods

### 2.1. Insects

A colony of *R. prolixus* was kept at 28 °C and 80% relative humidity. The *Rhodnius* females were fed every 21 days with rabbit blood or plasma, using an artificial feeder described by Garcia et al. (1975). Insects fed on plasma were first fed twice on blood in order to guarantee that they were physiologically mature. For experiments, only adult female insects were used.

### 2.2. Transmission electron microscopy

The intestines of five adult female insects, at 7 days after a blood meal, were dissected in their own hemolymph. The material was fixed in 2.5% glutaraldehyde, 4% paraformaldehyde and 5 mm $CaCl_2$ in 0.1 M sodium cacodylate buffer, pH 7.2. Samples were then rinsed with 0.1 M cacodylate buffer, pH 7.2. After rinsing, material was post-fixed in 1% osmium tetroxide and 0.8% potassium ferrocyanide for 30 min and rinsed with 0.1 M sodium cacodylate buffer, pH 7.2. The material was dehydrated in graded acetone at room temperature and embedded in Spurr resin for 12 h at 70 °C. Ultrathin sections were cut, using an ultramicrotome (Reichert Ultracuts), and collected on copper grids and stained with uranyl acetate and lead citrate. Samples were observed with a Zeiss 900 transmission electron microscope, operated at 80 kV.

#### 2.3.1. Heme-peroxidase activity

A protocol was followed, according to Pascoa et al. (2002) and Silva et al. (2006): Intestines of five adult female insects, at 5 days after a blood meal, were dissected and fixed overnight at room temperature in 1% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2. After the fixation step, the material was rinsed with 0.1 M sodium cacodylate buffer, pH 7.2, and incubated in 2.5 mM diaminobenzidine (DAB) in 0.1 M sodium cacodylate buffer, pH 6.5, for 60 min at 37 °C. After this period, the tissue was transferred to the same solution containing 0.03% $H_2O_2$ for 60 min. Following the incubation, the material was rinsed with 0.1 M sodium cacodylate buffer, pH 6.5, post-fixed in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer, pH 7.2, and processed for TEM, as already described. Controls were obtained by incubation in 0.1 M sodium cacodylate buffer, pH 7.2, without DAB, and transferred to the same solution containing 0.03% $H_2O_2$ for 60 min.

### 2.4. Erythrocyte membranes preparation

Rabbit blood was collected in tubes containing anticoagulant heparin. One mL of blood was centrifuged for 10 min at room temperature and $1500 \times g$. The plasma supernatant and the top layer of the sediment were then discarded. The erythrocyte sediment was lysed in 10 mL of cold 10 mM tris-HCl, pH 8.0, after which the membranes were centrifuged for 20 min at 4 °C and $20{,}000 \times g$. The supernatant was discarded and the sediment washed 10 times in the same buffer. The amount of protein in the EM was measured (Lowry et al., 1951).