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MORPHOLOGY OF THE ILEUM, COLON AND RECTUM OF SILKWORM (LEPIDOPTERA: BOMBYCIDAE)

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ABSTRACT

Silkworm is an insect whose cocoon is used as raw material in the textile industry. For the production of quality silk, a proper functioning of the digestive system is essential. This study examined the morphology of the hindgut chambers, with details of the area of the anal canal, which has been little studied in this important holometabola. The functional dynamism of the intestinal chambers can obscure important morphological characteristics. For this, segments of the hindgut were processed using light microscopy and scanning electron microscopy. The analysis showed that the hindgut chambers were formed by a squamous epithelium lining that was supported on a basal lamina. Adjacent to the epithelium, there were multinucleated striated muscles arranged in longitudinal and circular layers. The anterior region of the rectum composes the cryptonephric system. The posterior rectum revealed an epithelium that continued to the integument; the associated musculature showed insertion points in the intima, establishing a dividing boundary with the anterior rectum. These muscles are responsible for the movements that lead to the removal of fecal pellets, and therefore, the posterior rectum constitutes the anal canal. This morphological knowledge provides basic information for future studies of this holometabolous, which is used in Brazilian sericulture.

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INTRODUCTION

Bombyx mori Linnaeus, 1758 (Lepidoptera: Bombycidae) is an important holometabola used in the sericulture industry in Brazil, and other developing countries; its cocoons are used to produce different silk threads and materials. The insect has also been used as a model in scientific studies, mainly due to its interesting biological characteristics (Brancalhão *et al.,* 2009; Matsumoto *et al.,* 2011). The commercial breeding of *B. mori* features the cross-breeding of two pure breeds (Chinese and Japanese), which produces hybrids that are better developed and adapted to Brazilian soils and climate, enabling greater productivity and resistance to disease (Fernandez *et al.,* 2005). Productivity is based on the fact that the cocoons have a high level of silk, protein that is obtained from a diet of mulberry leaves (*Morus* sp.). The ingested leaves are processed in the alimentary canal of the larvae, which, as in other phytophagous insects, is a long tube extending from the mouth to the anus, divided into the foregut, midgut and hindgut; and their transitions cardia, between foregut and midgut, and piloro, between midgut and hindgut (Snodgrass and Eickwort, 1993; Chapman, 1998; Cifuentes and Sohn, 1998). The foregut is the first region of the alimentary canal and it is responsible for the passage of food into the midgut, which is the main site of digestion and absorption of nutrients. The final stages of the digestive process occur in the hindgut (Chapman, 1998; Gullan and Cranston, 2010), which, in many Lepidoptera, is subdivided into pylorus, ileum, colon and rectum. The pylorus regulates the flow of food and prevents a backflow of food from the hindgut into the midgut. The ileum,

colon and rectum promote the absorption of water and mineral salts from digested food, leading to the formation and subsequent disposal of fecal pellets(Judy and Gilbert, 1970; Reinecke *et al.,* 1973; Chi *et al.,* 1975; Eaton, 1982; Snodgrass and Eickort, 1993; Chapman, 1998; Barbehenn and Kristensen, 2003; Levy *et al.,* 2004; Gillott, 2005; Levy *et al.,* 2008; Gullan and Cranston, 2010; Azuma, 2012). The hindgut is usually diversified in various insects, which hinders the use of a constant terminology; in addition, the functional dynamism of this intestinal region makes it difficult to identify anatomical areas and to describe them, a fact that must be considered in morphological studies. Thus, this article describes the morphology of the ileum, colon and rectum of fifth instar *B. mori* larvae, which is a very active phase of the life cycle of the insect, which accumulates energy reserves for metamorphosis and for the production of the raw material needed to build the cocoon. This article also analyzes the anal canal; the rectal area, which has been little studied in the literature and of which there is scarce morphological information due to the difficulties in collecting and handling, as observed by Levy et al. (2004).

MATERIALS AND METHODS

Hybrid *B. mori* larvae, at the third instar of development, were obtained from the BRATAC S.A sericulture company, which produces larvae for economic use in the state of Paraná, Brazil. The larvae were bred in polyethylene boxes in conditions similar to the natural environment, and were fed three times a day with fresh mulberry leaves until the beginning of the fifth instar. For the histological preparation, twenty-five, fifth instar larvae were randomly selected, anesthetized with ether, and dissected through a longitudinal opening of the integument of the anal region up to the head, under a stereoscopic microscope. The segments of the hindgut were placed in individually identified vials containing Dubosq Brazil fixer (Beçak and Paulete, 1976), for 24 hours at 4 °C. After fixing, the intestinal segments followed the routine steps for histological paraffin embedding, according to the protocol established by Brancalhão(2002). The 7 µm thick sections were stained with hematoxylin and eosin - HE (Junqueira and Junqueira, 1983) for analysis of the morphology of the epithelium. Periodic acid-Schiff (PAS) was used to identify the basal lamina (Prescock, 1994). The material obtained was analyzed and microphotographed using a BX60 microscope. The segments of the hindgut were fixed in a 1:1 mixture of 4% glutaraldehyde solution and PBS buffer pH 7.0 and then postfixed in 2% osmium tetroxide diluted in the same buffer. The intestinal segments were subsequently rinsed in PBS buffer pH 7.0, dehydrated in ascending series of acetone 30% to 100% (Scipio *et al.,* 2008) and brought to a critical point (Bal-Tec CPD 030) for final drying. The samples were placed in a stub and then coated with carbon ribbon for metalization by the Shimadzu machine for 3 minutes, covering the sample with 10 nm of gold dust, followed by analysis in a SS-550-Shimadzu scanning electron microscope.

RESULTS AND DISCUSSION

The presence of three distinct compartments or chambers was observed in the hindgut of fifth instar *B. mori* larvae: the ileum and colon, of approximate sizes and separated by a muscular ring, and the rectum, which was longer and delimited, relative to the colon by another muscular ring (the rectal valve), and in relation to the external environment through the anal sphincter (Fig. 1). The muscular rings were composed of several layers of muscles arranged circularly, and the rectal valve was more developed (Fig. 2).

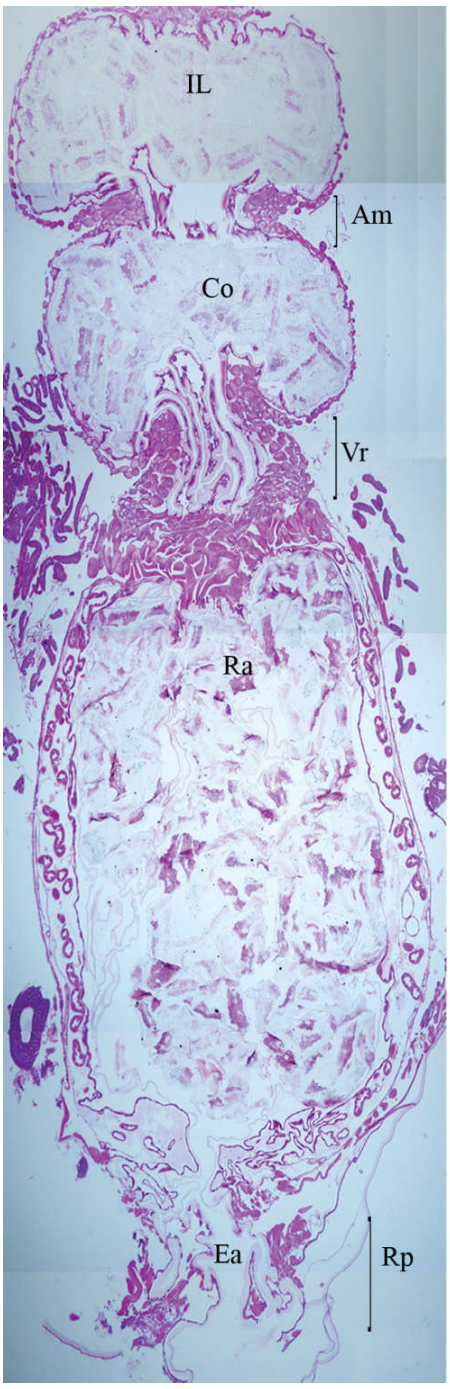
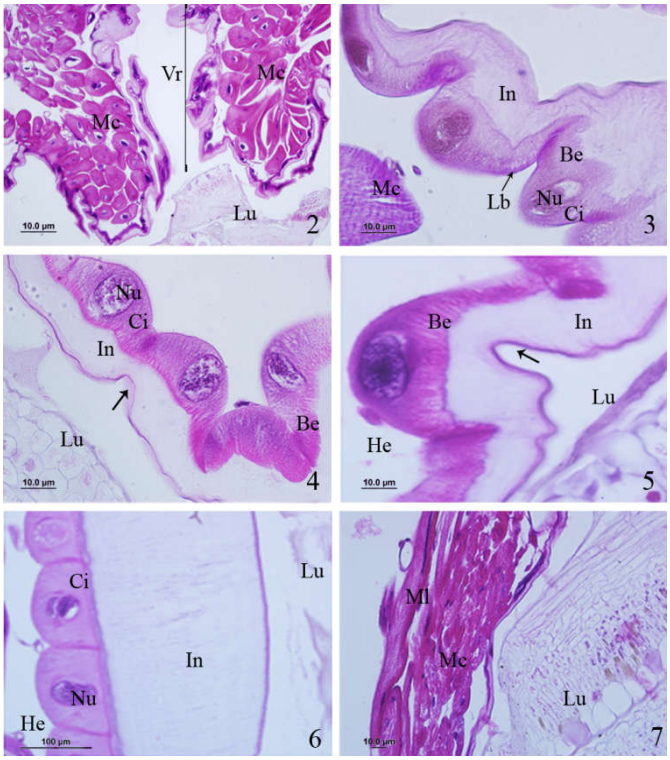


Figure 1. Montage of microphotographs of ileum (IL), colon (Co), anterior rectum (Ra), posterior rectum or anal canal (Rp) and their transitions; muscular ring between the ileum and colon (Am), rectal valve (Vr) and anal sphincter (Ea) of fifth instar *B. mori* larvae, longitudinal section, HE staining

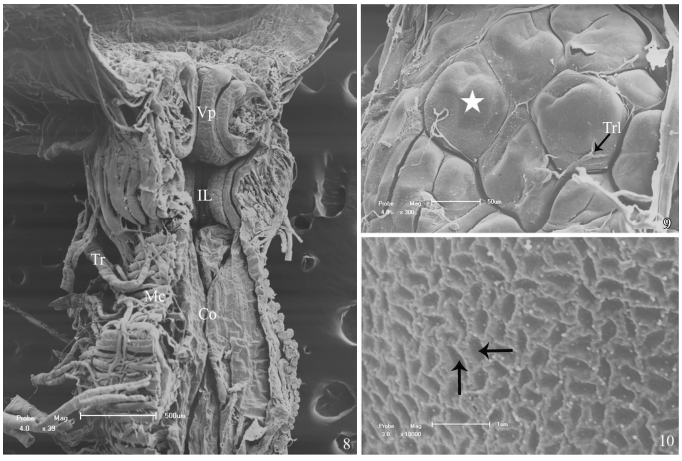
The morphological analyses of the ileum, colon and rectum showed an epithelium with a simple, continuous coating that rested on a basal lamina and was covered on the luminal surface by an intima (Figs. 3-6). The muscles surrounding the epithelium were well developed and characterized by the presence of circular muscles, generally internal, and longitudinal externally (7-12), which, in many regions, were inserted between the circular musculature. The fibers were multinucleated, with central and peripheral nuclei (Fig. 11).

The ileum, which was separated from the pylorus by the pyloric valve (Fig. 8), and the colon, presented an epithelium that was formed of polygonal cells that were squamous and with defined boundaries (Fig. 9). The nuclei were centralized, single, globular and bulky (Figs. 3 - 6). The apical surface of the epithelial cells showed a border with a distinct brush (Figs. 3, 4 and 5) and on the luminal surface the intima showed regular depressions at intervals along its length (Figs. 4, 5 and 10). In the ileum, it was possible to verify the intima of different thicknesses (Figs. 4 and 6), with the thicker regions called ileonodos, which showed a high cuboidal epithelium (Fig. 6).

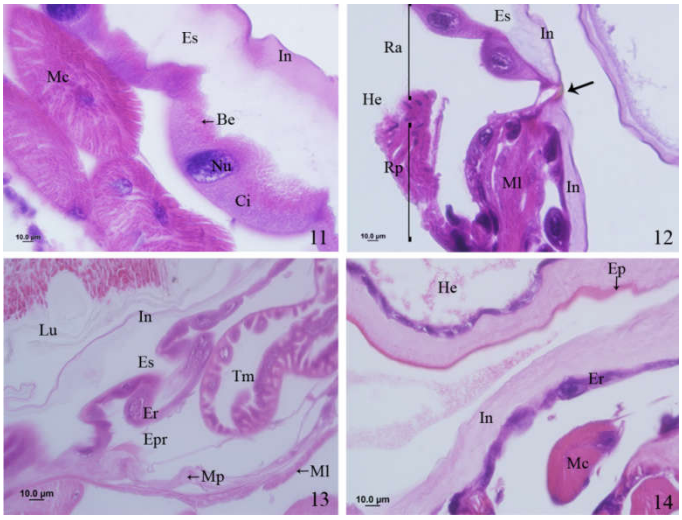


Figures 2-7. Microphotographs of the hindgut of fifth instar *B. mori* larvae, longitudinal section, HE staining: brush border (Be), cytoplasm (Ci), nucleus (Nu), basal lamina (Lb), intima (In), lumen (Lu), hemocoel (He), circular muscles (Mc) and longitudinal muscles (Ml). (2) detail of rectal valve with numerous layers of circular muscles (Mc). (3) basal lamina (Lb). (4-5) squamous epithelium of colon with brush border (Be) and depressions in the intima (black arrow). (6) detail of ileonodos cells. (7) organization of the muscular wall

In the rectum, the epithelial cells maintained a squamous shape, with variations in certain regions of length and width; these were polymorphic nuclei. On the apical surface, the border with a brush was apparently lower than in the ileum and the colon (Figs. 5 and 11), and it was not observed in the posterior rectum (Fig. 12). The epithelium of the anterior rectum showed a detached intima (Figs. 11-13), which would make up the cryptonephric system, while in the posterior rectum the intima was adhered (Figs. 13 and 14). This epithelium varied from simple and squamous to the cuboidal type, and it was continuous with the integument, which was characterized as a cuboidal epithelium (Fig. 14). In this posterior region of the rectum, the intima was continuous up to the cuticle of the integument, which displayed a sharp epicuticle (Fig. 14). Points of insertion of the musculature, directly into the intima, were also found in this region (Fig. 12). Branches of the tracheal system penetrated and profusely branched off along the hindgut (Figs. 8 and 9).



Figures 8-10. Electron microphotographs of hindgut of fifth instar *B. mori* larvae. (8) image obtained by montage of ileum (IL), colon (Co), pyloric valve (Vp), trachea (Tr) and circular muscle (Mc). (9) polygonal cells of the colon (white star), tracheoles (Trl) penetrating the tissues. (10) detail of the depressions in the intima that lines the colon (black arrow)



Figures 11-14. Microphotographs of the hindgut of fifth instar *B. mori* larvae, longitudinal section HE staining. (11) Detail of polygonal cells of the anterior rectum, detached intima (In), nucleus (Nu), cytoplasm (Ci), brush border (Be), subcuticular space (Es) and circular muscle (Mc). (12) Transition between the anterior rectum (Ra) with detached intima and the posterior rectum (Rp) with adhered intima (In), subcuticular space (Es), longitudinal muscle (Ml) and hemocoel (He). (13) detail of the cryptonephric system, rectal epithelium (Er), subcuticular space (Es), perinephric space (Epr), Malpighian tubule (Tm), detached intima (In), rectal or perinephric membrane (Mp), longitudinal muscle (Ml), hind gut lumen (Lu). (14) Transition between posterior rectum and integument, limited by epitcutícula (Ep), rectal epithelium (Er), intima (In), circular muscle (Mc) and hemocoel (He)

The cryptonephric system, or rectal complex, was identified in the anterior rectum (Fig. 13); a structure formed by the rectal epithelium and associated intima, which was detached, as previously stated, and which forms the subcuticular space. The rectal, or perinephric, membrane was a thin membrane composed of flattened cells, which created a perinephric space, where the distal portions of the Malpighian tubules and branches of the tracheal system were located. This membrane was found in association with the longitudinal musculature. The hindgut of larvae of fifth instar *B. mori* larvae showed the presence of the chambers of the ileum, colon and rectum.

These have also been noted in *Manduca sexta* Linnaeus, 1763 (Lepidoptera: Sphingidae) (Reinecke *et al.,* 1973), *Heliothis zea* Boddie, 1850 (Lepidoptera: Noctuidae) (MacGown and Sikorowski, 1982) and in *Anticarsia gemmatalis* Hübner, 1818(Lepidoptera: Noctuidae) (Levy *et al.,* 2008). However, Byers and Bond (1971), in studies of *Amathesc-nigrum* Linnaeus(1758)and *Pseudaletia unipuncta* Haworth (1809) and Chapman(1998), do not distinguish between the ileum and the colon, and consider these regions to be a single chamber, which increases the difficulty of using a single terminology.

The absorption of water and electrolytes occurs along these chambers, as well as the production and disposal of fecal pellets that are initially formed in the ileum. These pellets are transferred to the colon, where their compaction continues, following in a regulated manner to the rectum. Muscular rings control this act of transferring the pellets along the chambers, avoiding reflux, according to data from the literature (Levy *et al.,* 2004; Gillott 2005; Gullan and Cranston, 2010). The finalization of the absorptive process occurs in the rectum, with the participation of the cryptonephric system, which results in fecal pellets that are more compact and which are eliminated by the movements of contraction of the anal sphincter (Eaton, 1982; Levy *et al.,* 2004; Gullan and Cranston, 2010). The epithelial morphology of the intestinal chambers of *B. mori* resembles that of other Lepidoptera (Levy *et al.,* 2004), with particular respect to cell boundaries defined in the ileum and colon, which are not visible in *Leucinodes orbonalis* Guenée, 1854 (Lepidoptera: Pyraustidae) (Srivastava, 1959). In *Hyalophora cecropia* Linnaeus (1758) (Lepidoptera: Saturniidae), the shape of the nucleus is elongated and may be divided into lobes (Judy and Gilbert, 1970); however, this is not present in *B. mori.* Furthermore, in silkworm within the rectal epithelial cells the nucleus showed no definite shape, a reflection of the variations evident in the morphology of the cells of this chamber, which, in the opinion of Byers and Bond (1971), are the result of its extremely dynamic functional state. The brushed border present in the intestinal epithelium of the ileum, colon and rectum are apical microvilli, whose underlying cytoplasm is rich in mitochondria that are responsible for providing energy for the active absorption of salts and water (Chapman, 1998; Levy *et al.,* 2004). The differences observed in wall thicknesses, i.e. thicker in the ileum and colon compared to the anterior rectum, may be related to the development of a complex system of reabsorption of water (the cryptonephric system in this chamber) since larger microvilli might jeopardize its functionality, which is characterized by the transport of fluids, specially designed for the recycling of water, as noted by Azuma et al.(2012), which follows the intestinal lumen path, subcuticular space, transcellular transport by retal epithelium and cryptonephric space.

The basal lamina acts in support of the epithelium and regulates the passage of molecules between the epithelial cells and the hemolymph (Ryerse and Reisner, 1985); it is found in other tissues of invertebrates, such as nerves, fat, and also in the Malpighian tubules (Reddy and Locke, 1990). Another striking epithelial feature is the chitinous intima on the apical face that acts to protect against the abrasive action of food and the entry of pathogens, as well as being a filter to regulate the passage of molecules between cells and the intestinal lumen (Srivastava, 1959; Byers and Bond,1971; Reinecke *et al.,* 1973; Chi *et al.,* 1975; MacGown and Sikorowski, 1982; Levi *et al.,* 2004; Correia *et al.,* 2009). Furthermore, the depressions present in this structure are specializations that dynamize the absorptive capacity of this region, as discussed by Byers and Bond (1971). Differences in the thickness of the intima of the ileum, which are highly thickened in the ileonodos were also observed in *M. sexta* (Reinecke *et al.,* 1973). Functionally, the ileonodos are characterized as an area that is more rigid than the epithelium, which, together with the intestinal wall muscles, are involved in the production of the grooves in the fecal pellets as they pass through this intestinal segment (Reinecke *et al.,* 1973;Barbehem and Kristensen, 2003). The organization of the musculature in the longitudinal and circular layers allows peristalsis to occur, and this is observed throughout the digestive tract, promoting the movement and ejection of the bolus (Byers and Bond, 1971; Reinecke *et al.,* 1973; Levy *et al.,* 2004). Chapman (1998) calls this musculature "visceral", differentiating it from the skeletal, which is adhered to the exoskeleton. The organization of the internal circular musculature and the external longitudinal musculature was also observed by Srivastava(1959) in *L. orbonalis*. Levy et al. (2008) defined the positioning of the inner longitudinal musculature and the circular outer musculature for *A. gemmatalis*. With regard to the characteristics of the muscular fiber, Chapman (1998) states that they are uninucleate; different from those observed in *B. mori* larvae, whose cells form long fibers, many of which are from other intestinal regions (Reinecke et al. 1973) and which, therefore, result from the fusion of uninucleate cells, constituting the multinucleated pattern that was observed. Multinucleated fibers were also found in the hindgut of larvae of fifth instar *M. sexta* and six other species of the Sphingidae family (Reinecke and Adams**,** 1977).

The organization of the cryptonephric system in *B. mori* corroborates the data of Azuma (2012) and is the same as described for other Lepidoptera (Ramsay, 1976). This complex system in the rectum of larvae promotes the recycling of water and ions absorbed from the intestinal lumen into the hemolymph or Malpighian tubules. According to Azuma et al. (2012), the transport of water is carried out by two aquaporins, AQP-Bom1 and AQP-Bom3, which are expressed primarily in the apical and basal cell membrane of the colon and rectum. The presence and positioning of these proteins in the rectal epithelial cells and the Malpighian tubules directs the transport of water in order to avoid unnecessary loss and protects the larvae against dehydration. Thus, this system controls the water balance, which is essential to maintain the life of terrestrial insects (Ramsay, 1976; Levy *et al.,* 2008; Azuma *et al.,* 2012). Its operation, and the organ as a whole, also needs a large supply of oxygen, which is supplied by a well-developed tracheal system (Chapman, 1998). In the posterior rectum, the insertion of the visceral musculature in the intima made it possible, for the first time, to establish a clear dividing line with the anterior rectum, coupled with the presence of the intima, which was adhered to the rectal epithelium (Fig. 12). Levy et al. (2004) attributed the difficulties of identifying this region to the intense muscular adhesion, which makes it difficult to perform dissections, and because of this there have been few morphological descriptions. In *B. mori*, the posterior rectum and adjacent musculature form the anal canal that continues to the integument, and which is distinguishable by the presence of the epicuticle and the epithelial morphology, as can be seen from the results. In this area, the circular musculature that is developed forms the anal sphincter, which is involved in the elimination of fecal pellets. From the foregoing, it appears that the regions of the ileum, colon and

rectum of *B. mori* larvae resemble other Lepidoptera, although there are some differences in the organization of the musculature and epithelium, which respond to the functional status of the organ. It is noteworthy that this study presents morphological details of the posterior rectum or anal canal, a region that is difficult to prepare because of its anatomical features, especially related to the transition between the digestive tract and the integument. This morphological knowledge provides basic information for future studies of this holometabolous, which is used in Brazilian sericulture.

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