Microscopy and Microanalysis (2020), 1–9
doi:10.1017/S1431927620024484

Microscopy AND Microanalysis

Original Article

Ultrastructure of the Bacteriocytes in the Midgut of the Carpenter ant *Camponotus rufipes*: Endosymbiont Control by Autophagy

Wagner G. Gonçalves[1], Kenner M. Fernandes[1], Ana Paula A. Silva[2], Danilo G. Gonçalves[3], Muhammad Fiaz[2] and José Eduardo Serrão[1]*

[1]Department of General Biology, Universidade Federal de Viçosa, Viçosa, Brazil; [2]Department of Entomology, Universidade Federal de Viçosa, Viçosa, Brazil and [3]Department of Basic and Life Sciences, Universidade Federal de Juiz de Fora, Governador Valadares, Brazil

Abstract

The carpenter ant *Camponotus rufipes* has intracellular bacteria in bacteriocytes scattered in the midgut epithelium, which have different amounts of endosymbionts, according to the developmental stages. However, there are no detailed data about the midgut cells in adult workers. The present work aimed to evaluate the morphology and cellular events that coordinate the abundance of endosymbionts in the midgut cells in *C. rufipes* workers. The midgut epithelium has digestive cells, bacteriocytes, and cells with intermediate morphology. The latter is similar to bacteriocytes, due to the abundance of endosymbionts, and similar to digestive cells, due to their microvilli. The digestive and intermediate cells are rich in autophagosomes and autolysosomes, both with bacteria debris in the lumen. These findings suggest that midgut cells of *C. rufipes* control the endosymbiont level by the autophagy pathway.

Key words: autophagy, *Blochmannia*, Formicidae, insect, symbiosis

(Received 18 May 2020; revised 20 July 2020; accepted 30 August 2020)

Introduction

Ants of the genus *Camponotus* have approximately 1000 species with worldwide distribution, and this success is attributed, in part, to the association with intracellular endosymbiont bacteria (Schröder et al., 1996; Wernegreen et al., 2009).

Endosymbionts are common in insects with specialized diets, supplanting nutritional deficiencies with the production of essential metabolites, such as amino acids and vitamins (Buchner, 1965; Skidmore & Hansen, 2017; Cossolin et al., 2020). However, the representatives of *Camponotus* have a generalist feeding habit and, in these ants, the endosymbionts have been claimed to provide essential amino acids (Zientz et al., 2006; Feldhaar et al., 2007), which contributes to immune defense and colony growth (Souza et al., 2009).

The presence of endosymbionts in *Camponotus* ants has been reported for more than 200 years (Bolton, 1996). Since then, the bacterial characterization, the function, and the abundance of endosymbionts in the different phases of ant development have been studied (Schröder et al., 1996; Peloquin et al., 2001; Sauer et al., 2002; Feldhaar et al., 2007).

The main endosymbiont in *Camponotus* spp. is the bacterium *Blochmannia* found in the midgut and ovary (Schröder et al., 1996). In the midgut, *Blochmannia* occurs in specialized cells, bacteriocytes, which are side-by-side with the digestive cells (Schröder et al., 1996). Unlike bacteriophytes, digestive cells are characterized by the non-accumulation of endosymbiont bacteria and morphology that suggest protein synthesis and nutrient absorption (Schröder et al., 1996).

In males and virgin queens of *Camponotus floridanus*, bacteriocytes have the abundance of bacteria, whereas in mature queens, bacteria are few or absent in the midgut, which indicates that the degree of endosymbiosis changes according to the reproductive status (Sauer et al., 2002). The amount of endosymbionts also varies according to the developmental stage in *C. floridanus*. Few bacteriocytes are found in the midgut of larvae, followed by a significant increase of these cells in pupae and decrease in adults (Stoll et al., 2010). Some genes associated with autophagy and lysosomes have been reported in the midgut of carpenter ants, suggesting a possible lysosomal pathway in the regulation of endosymbionts populations (Ratzka et al., 2013).

Those studies suggest some dynamics in the endosymbionts in the midgut of *Camponotus* spp., but little is known about the organization of the midgut epithelium and the cellular events that coordinate the abundance of endosymbionts. The present work aimed to describe the morphology of midgut cells in the carpenter ant *Camponotus rufipes*, so as to fill these important gaps and contribute to understand the symbiotic relationships in this ant.

Materials and Methods

Ants

Adult females of *C. rufipes* engaged in foraging activity were collected at the Universidade Federal de Viçosa, transferred to the

*Author for correspondence: José Eduardo Serrão, E-mail: jeserrao@ufv.br

Cite this article: Gonçalves WG, Fernandes KM, Silva APA, Gonçalves DG, Fiaz M, Serrão JE (2020) Ultrastructure of the Bacteriocytes in the Midgut of the Carpenter ant *Camponotus rufipes*: Endosymbiont Control by Autophagy. *Microsc Microanal.* doi:10.1017/S1431927620024484

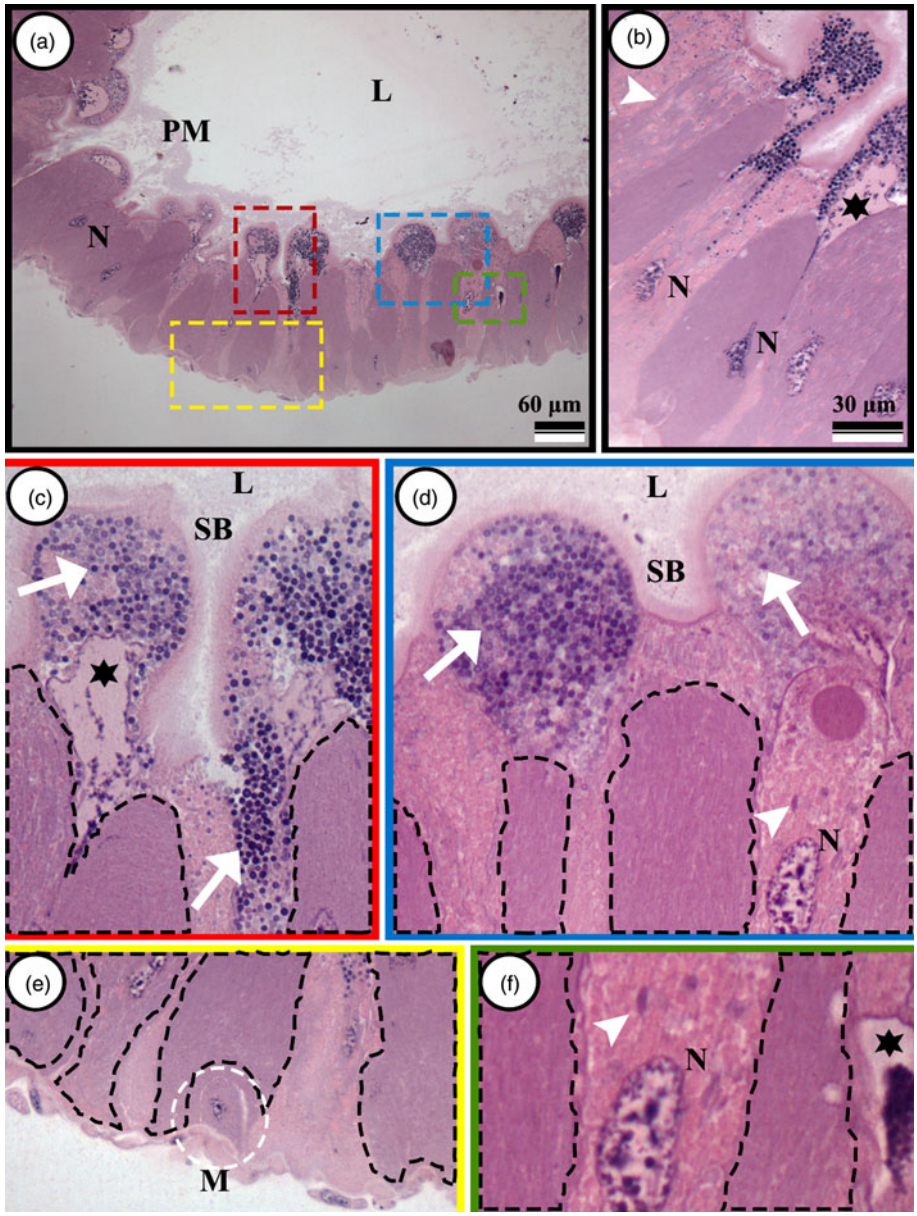

Fig. 1. Light micrographs of the midgut epithelium of *C. rufipes*. (**a**) General view of the midgut showing the nucleus (N), peritrophic matrix (PM), and lumen (L). (**b**) General view of midgut epithelium showing digestive cells with acidophilic cytoplasm (white arrowhead), nucleus (N), and amorphous vacuoles with basophilic granules (black star). (**c**) Detail of dotted red square in (**a**) showing protrusions of the digestive cells with basophilic granules (white arrows), amorphous vacuoles (black star), and striated border (SB). (**d**) Detail of dotted blue square in (**a**) showing digestive cells with apical protrusions-rich basophilic granules (white arrows) and well-developed striated border (SB). (**e**) Detail of dotted yellow square in (**a**) showing basal region of the epithelium with an isolated regenerative cell (white circle) and muscle (M). (**f**) Detail of dotted green square in (**a**) showing digestive cells with basophilic region (white arrowhead), amorphous vacuoles with basophic content (black star), and nucleus (N). Dotted lines show bacteriocytes.

laboratory and cold anesthetized at −5°C for 2 min. Then, the midguts were dissected in insect saline solution (0.1 M NaCl, 0.2 M KH_2PO_4, and 0.2 M Na_2HPO_4) for morphological analyses.

Light Microscopy

The midguts of 10 ants were transferred to Zamboni fixative solution (Stefanini et al., 1967) for 12 h. Subsequently, the samples were dehydrated in a graded ethanol series (70, 80, 90, and 95%) and embedded in JB4 historesin (Electron Microscopy Science, Hatfield, USA). Three micron thickness slices were stained with hematoxylin and eosin. Some sections were submitted to the histochemical tests of periodic acid Schiff test (PAS) and mercury-bromophenol blue for the detection of glycoconjugates and total proteins, respectively. Briefly, for the PAS test, slices were treated with 0.5% periodic acid for 20 min, washed in distilled water and placed in Schiff's reagent for 15 min at room temperature. After washing with distilled water, they were placed in mercury-bromophenol blue solution (Bancroft & Gamble, 2008) for 2 h and washed in tap water for 30 min at room temperature. Then, the samples were air-dried, mounted with Entellan (Sigma-Aldrich, Saint Louis, USA), analyzed and

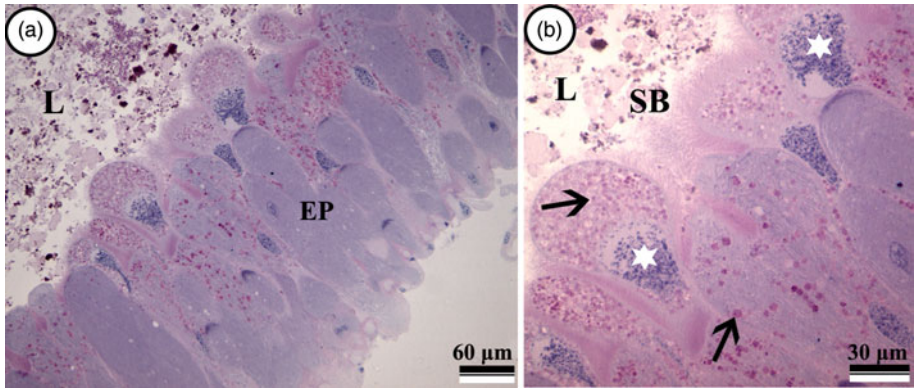

Fig. 2. Light micrographs of the midgut epithelium of *C. rufipes* submitted to PAS and mercury-bromophenol blue. (**a**) Midgut epithelium (EP) with different cells. (**b**) Digestive cells with apical protrusion reaching the lumen (L) with striated border (SB), PAS-positive granules (black arrows), and amorphous vacuoles positive for proteins (white stars).

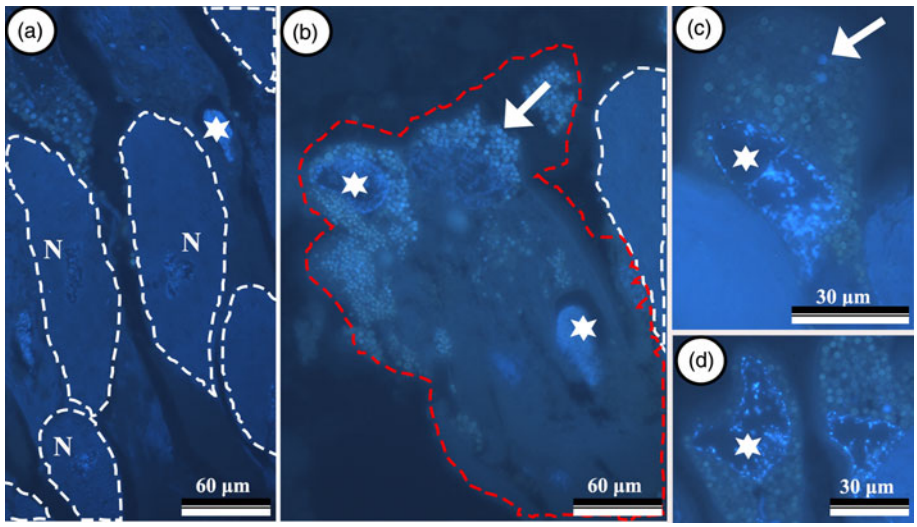

Fig. 3. Fluorescence micrographs of the midgut cells DAPI-stained of *C. rufipes*. (**a**) Cells with cytoplasm strongly labeled with DAPI (white dotted lines) closely associated with cells with cytoplasm weakly labeled with DAPI. (**b**) Detail of cell with weak DDAPI signal (red dotted line) showing a apical region with DAPI-positive granules (white arrow) and amorphous vacuoles (white stars). (**c,d**) Detail of DAPI-positive amorphous vacuoles (white star) and apical granules (white arrow) in the cells with weak fluorescence. N, nucleus.

photographed using a light microscope Olympus BX-60 (Olympus, Tokyo, Japan) coupled with a digital camera Q-Color, 3 Olympus (Olympus, Tokyo, Japan).

Fluorescence Microscopy

To identify the presence of nucleic acid, midgut sections were obtained as aforementioned for light microscopy, except staining, and were incubated with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI, Sigma-Aldrich, Saint Louis, USA), for 30 min. The samples were then washed in distilled water, mounted with 50% sucrose solution and analyzed under an epifluorescence microscope Olympus BX-60, (Olympus, Tokyo, Japan) with UV excitation filter and coupled digital camera Q-Color, 3 Olympus (Olympus, Tokyo, Japan).

Transmission Electron Microscopy

Six midguts were transferred to 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2 with 0.2 M sucrose, for 4 h. Then, the samples were post-fixed in 1% osmium tetroxide in the same buffer for 2 h, dehydrated in a graded ethanol series (70, 80, 90, and 99%) and embedded in LR White resin (Sigma-Aldrich, Saint Louis, USA). Ultra-thin sections were stained with 1% aqueous uranyl acetate, for 20 min, and lead citrate (Reynolds, 1963), for 10 min. The samples were analyzed and photographed in a Zeiss EM 109 transmission electron microscope (Carl Zeiss, Jena, Germany) at the Nucleus for Microscopy and Microanalysis, at the Universidade Federal de Viçosa.

Immunofluorescence

Six midguts were dissected as described and transferred to Zamboni's fixative solution for 2 h, for the detection of autophagy in the midgut cells. The samples were then washed in 0.1 M sodium phosphate buffer pH 7.2 (PBS) with 1% Tween-20 (PBST), for 2 h. Next, the samples were incubated for 12 h with antibody anti-LC3A/B (Abcam, Shangai, China) diluted in PBS (1:500). The samples were then washed in PBST and incubated for 12 h in secondary antibody anti-rabbit IgG-FITC conjugate

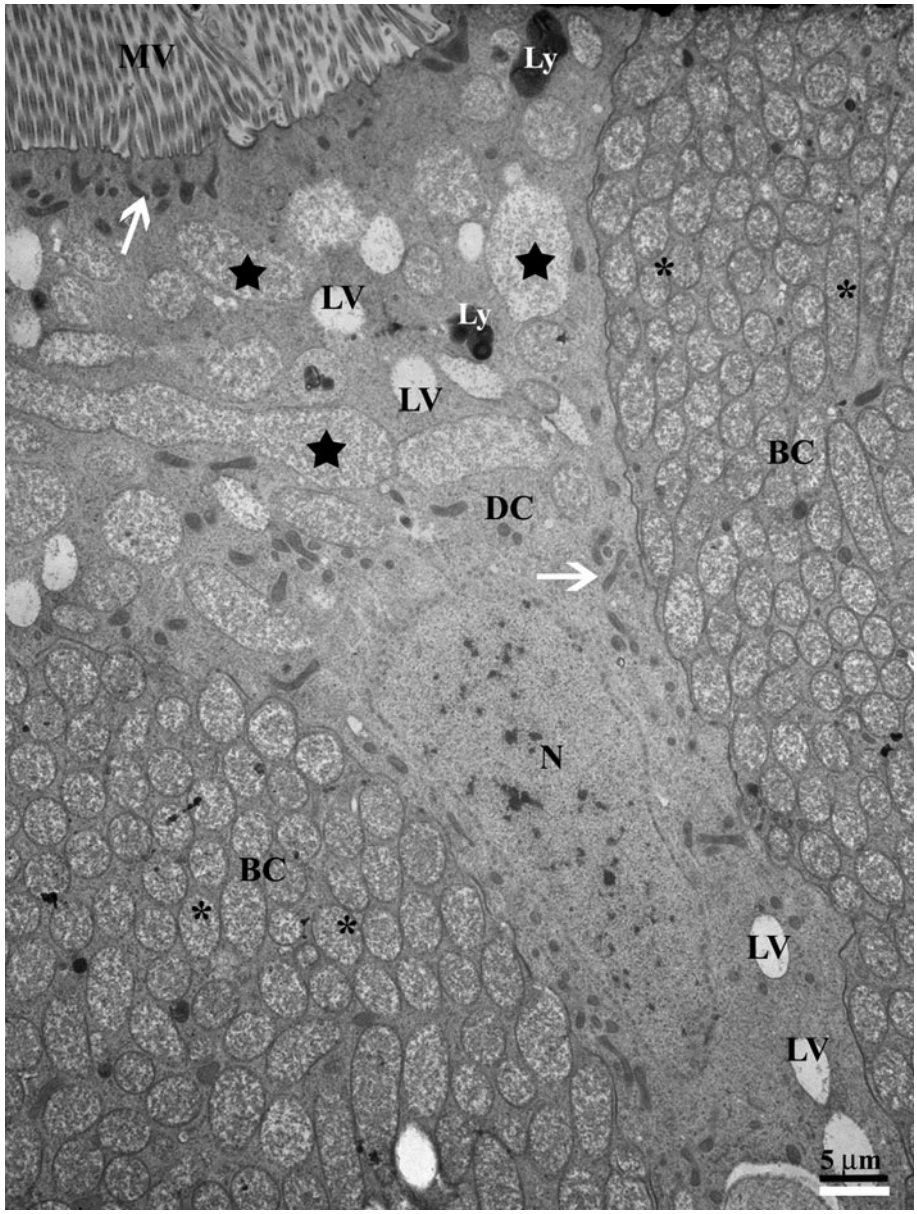

Fig. 4. Transmission electronic micrographs of midgut of *C. rufipes* showing digestive cell (DC) and bacteriocytes (BC). Note digestive cell with apical microvilli (MV), nucleus rich in decondensed chromatin (N) and cytoplasm with mitochondria (white arrows), amorphous vacuoles (black stars), electron-lucent vesicles (LV), and lysosomes (Ly). The cytoplasm of the bacteriocytes is filled with bacteria (asterisks).

(Sigma-Aldrich, Saint Louis, USA) diluted in PBS (1:500). Then, the samples were washed and incubated for 30 min with nucleus marker TO-PRO-3 iodide (Life Technologies) in PBS (1:1000). The samples were washed and mounted in Mowiol (Sigma-Aldrich, Saint Louis, USA) and analyzed with a Zeiss LSM-META (Carl Zeiss, Oberkochen, Germany) confocal microscope at the Nucleus for Microscopy and Microanalysis, at the Universidade Federal de Viçosa.

Negative controls were performed according to the procedure above, but incubation with anti-LC3A/B was omitted.

Results

The midgut epithelium of *C. rufipes* was formed by a single layer of cells with different staining intensities and nuclei positioned at different heights (Figs. 1a, 2a). Three cell types were found in the midgut: (i) digestive cells with cytoplasm showing basophilic granules, amorphous vacuoles reaching to the midgut lumen with a well-developed striated border (Figs. 1b, 1c, 1d), (ii) bacteriocytes with acidophilic cytoplasm not reaching to the midgut lumen (Figs. 1c, 1d), and (iii) few regenerative cells scattered in the basal region of the epithelium (Fig. 1e).

The digestive cells were characterized by apical protrusions to the midgut lumen, rich in basophilic granules (Figs. 1c, 1d), which were positive for glycoconjugates (Fig. 2a) and proteins (Fig. 2b). These cells had also amorphous vacuoles with some basophilic granules (Figs. 1b, 1c, 1f) which were positive for proteins (Fig. 2b).

Analyses with the fluorescence microscope of DAPI-stained slices showed that the cytoplasm of the midgut cells had different

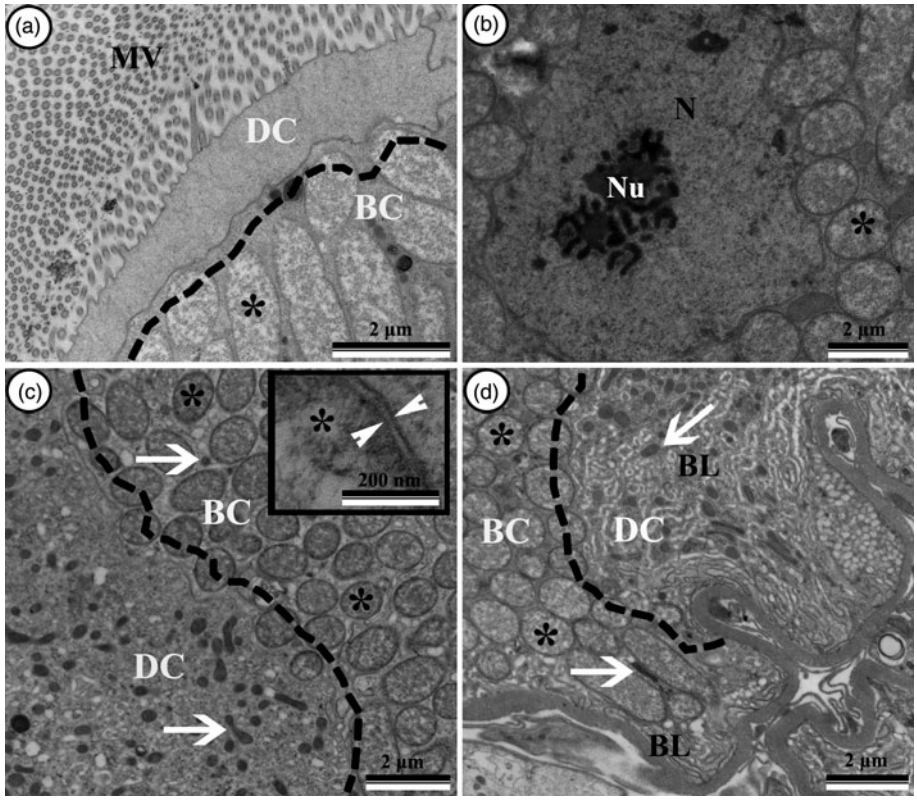

Fig. 5. Transmission electronic micrographs of midgut of *C. rufipes*. (**a**) Bacteriocyte (BC) with bacteria (asterisk) below the digestive cell (DC) with microvilli (MV). (**b**) Middle region of the bacteriocyte showing the nucleus (N) with decondensed chromatin and nucleolus (Nu) and cytoplasm with bacteria (asterisk). (**c**) Bacteriocyte (BC) and digestive cell (DC) with mitochondria (white arrows) and bacteria (asterisks). Insert: Bacteria (asterisk) with the double membrane (white arrowheads). (**d**) Basal region of the midgut showing bacteriocyte (BC) with short basal labyrinth (BL) and digestive cell (DC) with basal plasma membrane infoldings forming enlarged extracellular space as a basal labyrinth (BL) associated with mitochondria (white arrows). Dotted lines, cell boundary.

fluorescence signals (Figs. 3a, 3b). Cells with the weak fluorescence signals showed many granules (Figs. 3b–3d) and amorphous vacuoles with DAPI-positive content in the apical region (Figs. 3c, 3d).

The ultrastructure analysis of the midgut epithelium showed digestive cells closely associated with bacteriocytes (Fig. 4).

Bacteriocytes presented an apical surface that did not reach the midgut lumen and had no microvilli (Fig. 5a), and the cytoplasm was filled with double-membrane bacteria (Figs. 4, 5a–5c) with few mitochondria (Fig. 5d). The nucleus had a predominance of decondensed chromatin and well-developed nucleolus (Fig. 5b). The basal surface of these cells showed plasma membrane infoldings, forming a basal labyrinth with short extracellular spaces (Fig. 5d).

In the digestive cells, the apical surface presented well-developed microvilli (Figs. 4, 5a), cytoplasm rich in mitochondria (Figs. 4, 5c), large amorphous compartments with electron-lucent content, electron-lucent vesicles, and lysosomes (Fig. 4). In the basal cell region, the plasma membrane infoldings were long and formed a well-developed basal labyrinth of enlarged extracellular spaces associated with mitochondria (Fig. 5d).

In addition to the bacteriocytes and digestive cells, there were cells with different ultrastructure, characterized by apical microvilli, cytoplasm-rich mitochondria, and electron-lucent vesicles (Fig. 6a), such as digestive cells, but they had also many bacteria (Fig. 6a), such as bacteriocytes. These cells will, henceforth, termed as intermediate cells.

Both the digestive and intermediate cells had an apical cytoplasm with electron-lucent vesicles (Fig. 6a), lysosomes (Fig. 6b), autophagosome (Figs. 6c–6e), and residual bodies rich in membranous content (Fig. 6f). In both these cell types, the apical cytoplasm had abundant bacteria inside vesicles closely associated with autophagosomes (Figs. 7a, 7b). These vesicles with bacteria were found fused with autophagosomes (Figs. 7c, 7d).

To verify if the apical vesicles were autophagosomes, immunofluorescence detection to LC3A/B proteins revealed that they were undergoing autophagy (Fig. 8).

Discussion and Summary

Bacteriocytes in the midgut of *C. rufipes* do not reach the organ lumen and accumulate bacteria in the cytoplasm, similar to the findings for these cells in a previous study in *Camponotus* spp. (Schröder et al., 1996). These cells are strongly acidopholic and DAPI-positive, due to the presence of bacteria as revealed by the ultrastructural analyses. On the other hand, the midgut digestive cells of *C. rufipes* have some basophilic granules and well-developed microvilli reaching to the midgut lumen, such as reported for *C. rufipes*, *C. floridanus*, *C. herculeanus*, and *C. ligniperdus* (Schröder et al., 1996). *Camponotus floridanus* males have bacteria in the digestive cells (Sauer et al., 2002) and workers seem to have symbionts in these cells too (Stoll et al., 2010). This conflict in the occurrence of endosymbionts in digestive cells may be explained by the dynamics of the midgut cells since we demonstrate the occurrence of a midgut cell type with intermediate morphology, considering that they are similar to bacteriocytes

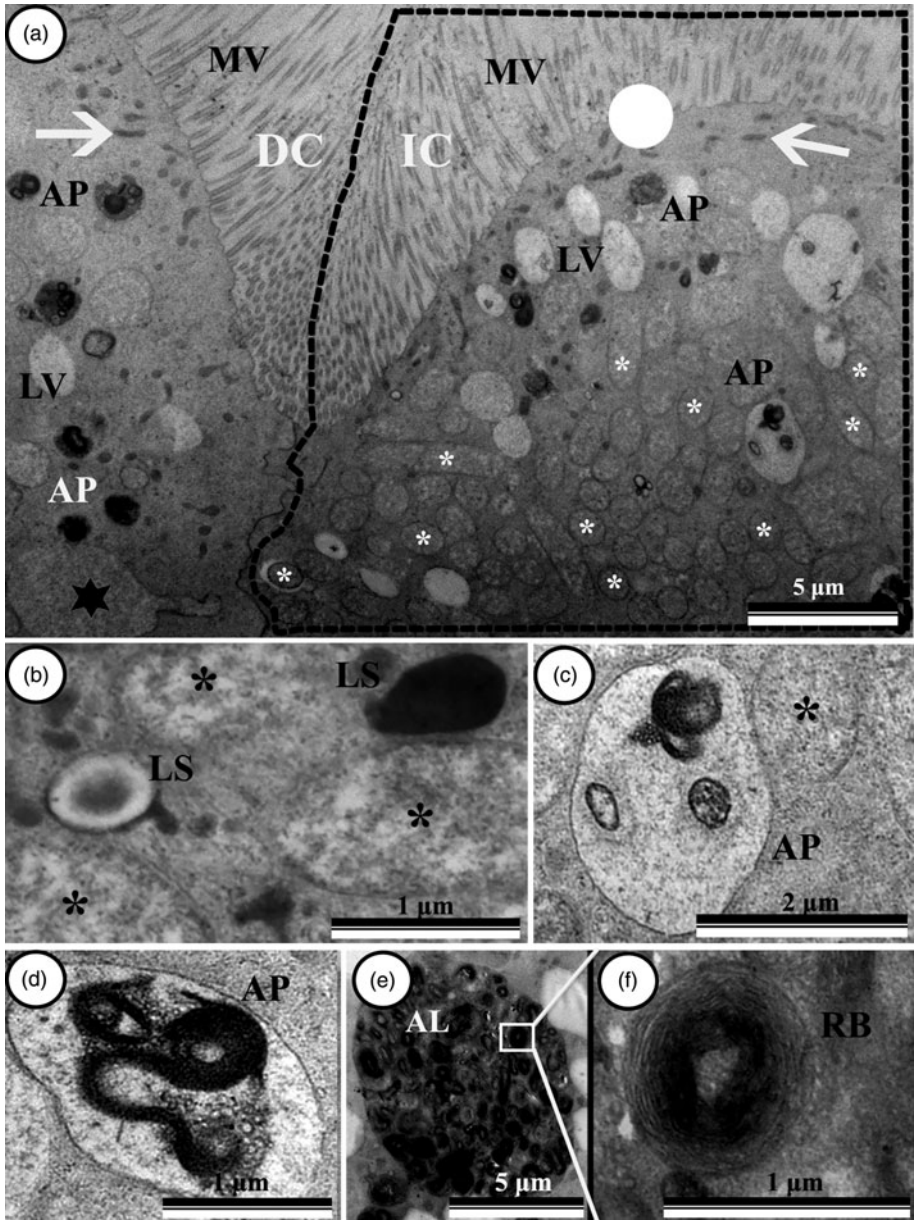

Fig. 6. Transmission electronic micrographs of the cells with intermediate morphology in the midgut of *C. rufipes*. (**a**) Apical region of the digestive cell (DC) in juxtaposition (dotted line) with intermediate cell (IC), both with microvilli (MV), mitochondria (white arrows), autophagosomes (AP), and electron-lucent vesicles (LV). Note the abundance of bacteria (asterisks) in the intermediate cell (IC) and amorphous compartment (black star) in the digestive cell (DC). (**b**) Detail of lysosome (LS) and bacteria (asterisks). (**c–e**) Autophagosomes (AP) and autolysosomes (AL) with different contents. (**f**) Detail of membrane fragments in the residual body (RB).

(cytoplasm with bacteria) as well as to digestive cells (presence of apical microvilli).

Regardless of the richness of bacteria, all cell types of the midgut of *C. rufipes*, excepting the regenerative ones, host endosymbionts. However, unlike the bacteriocytes, the digestive and intermediate cells are rich in lysosomes, autophagosomes, autolysosomes, and residual bodies, which indicates intracellular digestion (Deretic & Levine, 2009). In the aphid *Acyrthosiphon pisum* and the ant *C. floridanus*, acidic organelles and lysosomal-autophagic genes occur in bacteriocytes and digestive cells, suggesting a lysosomal pathway for degradation of endosymbionts (Nishikori et al., 2009*a*; Ratzka et al., 2013). Both digestive and intermediate cells of *C. rufipes*, here studied, reveal bacterial degradation with the presence of bacteria inside the vacuoles. In addition, there is evidence of bacterial disruption since the inner membrane may collapse into vesicles in the large amorphous compartments that are positive for autophagic LC3A/B protein. Although these autophagic pathways need further studies, our results suggest a degradation mechanism of endosymbionts in *C. rufipes*, similar to that reported in *Camponotus sericeiventris* (Wolschin et al., 2004), *A. pisum* (Nishikori et al., 2009*b*), and in the *Sitophilus* beetles (Vigneron et al., 2014).

The apical cytoplasm of the digestive cells shows granules and compartments with basophilic, PAS, protein, and DAPI-positive contents, which suggests the occurrence of glycoconjugates and nucleic acids and corroborates the hypothesis of endosymbiont degradation in *C. rufipes*, since granules and positive compartments for nucleic acids and glycoconjugates may result from

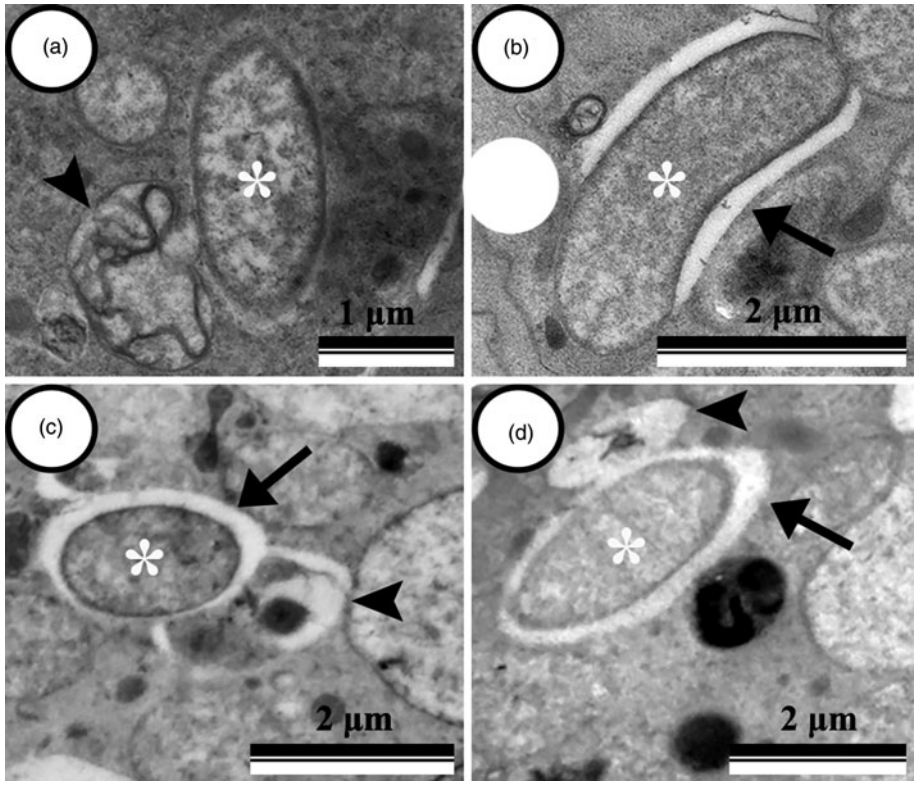

Fig. 7. Transmission electronic micrographs of intermediate cells in the midgut of *C. rufipes.* (**a**) Bacteria (asterisks) closely associated with autophagosome (black arrowhead). (**b**) Bacteria (asterisk) into a vacuole (black arrow). (**c**) Bacteria (asterisk) into a vacuole (black arrow) fused with autophagosome (black arrowhead). (**d**) Advanced fusion between autophagosome (black arrowhead) and bacteria (asterisk) into a vacuole (black arrow).

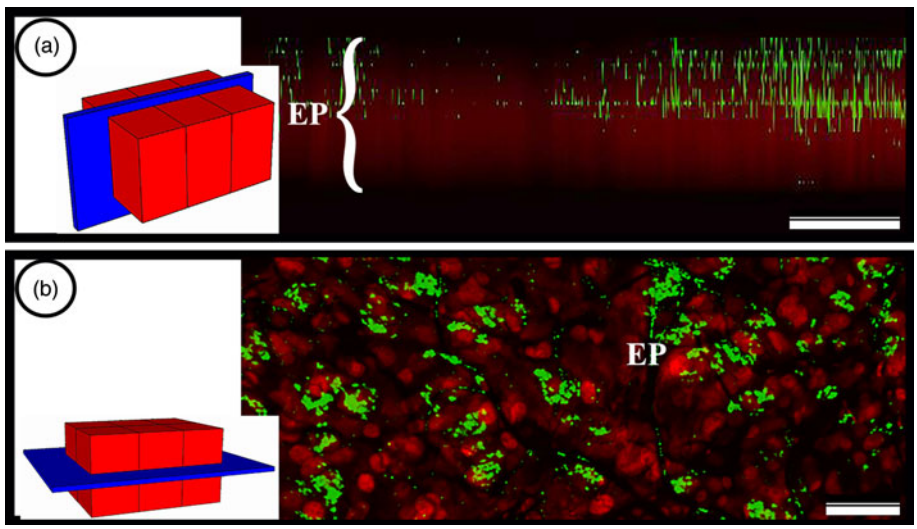

Fig. 8. Confocal fluorescence micrographs of the midgut cells of *C. rufipes* for immunolabeling of autophagosomes with antibody anti-LC3 A/B. (**a**) Epithelial cells (EP) in the longitudinal plane showing apical zone positive for autophagy (green). (**b**) Epithelial cells (EP) in the transverse plane showing cells rich in autophagosomes (green). Nuclei are in red (red).

bacterial digestion by autophagy. This is in agreement with the findings for *C. floridanus*, in which the abundance of vesicles positive for nucleic acids is associated with the degradation of endosymbionts (Stoll et al., 2010).

Bacteriocytes and digestive cells have already been reported in the midgut of *Camponotus* spp. (Schröder et al., 1996). However, in *C. rufipes*, we found a cell with intermediate morphology, suggesting a dynamic cellular dimorphism in the midgut of this ant. Although it is theoretical, we propose a model in which bacteriocytes increase in volume, reaching the midgut lumen and forming microvilli. Then, the degradation of endosymbionts occurs by the autophagy. These events result in cells with few bacteria, acquiring morphology similar to that of the digestive cells (Fig. 9). In representatives of *Camponotus*, evidence of

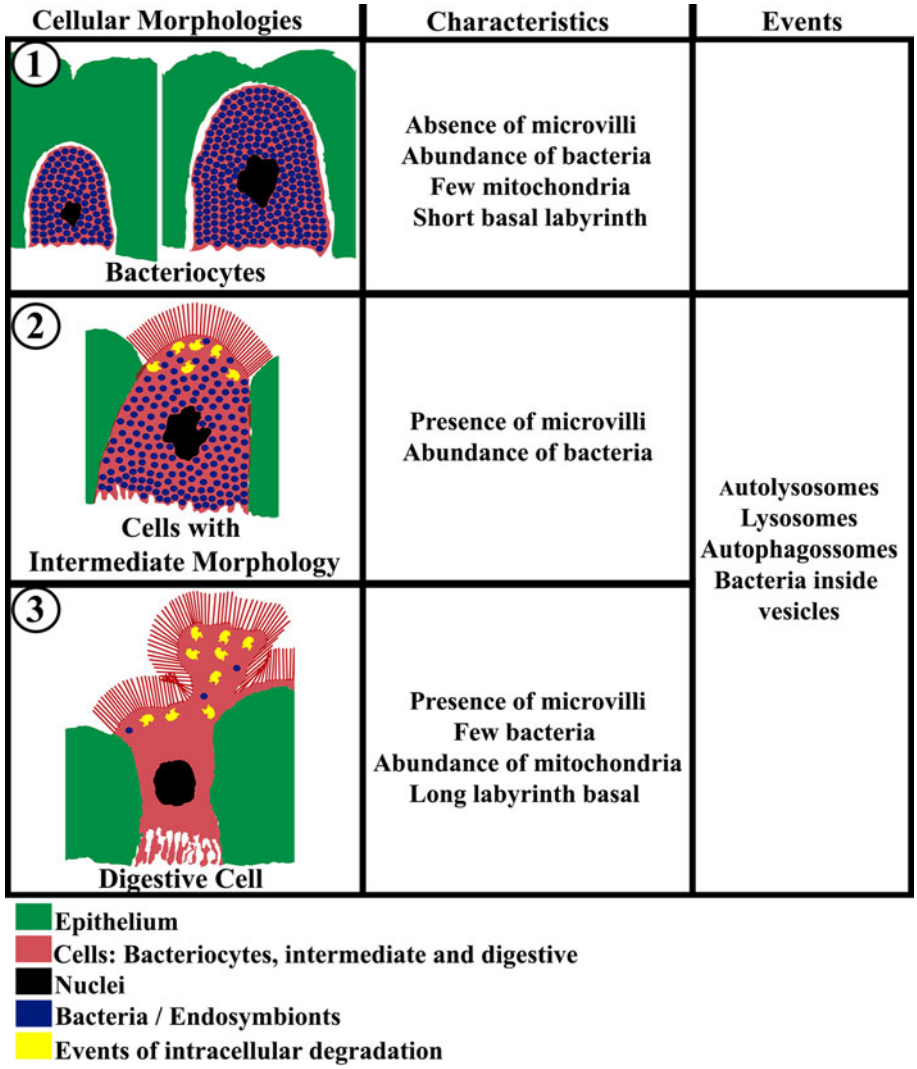

Fig. 9. Model for changes in the morphology of midgut cells of *C. rufipes* during the degradation of intracellular symbionts. [**1, 2, and 3**] Sequences of morphological changes and their associated characteristics and cellular events.

bacteriocytes in organs other than the midgut and a decreased amount of midgut bacteriocytes have been associated with intracellular degradation of endosymbionts (Wolschin et al., 2004; Stoll et al., 2010; Ratzka et al., 2013).

Overall, our study suggests that, in the midgut cells of *C. rufipes*, the amount of endosymbionts is controlled by the autophagy. Thus, these cells change from a specialized bacteriocyte storing endosymbiont to a digestive cell. Some gaps in relation to the evidence of endosymbionts in different midgut cell types and bacteriocyte decrease during the ant development can be better understood with our proposed model, which may contribute to the comprehension of the symbiosis relationships in carpenter ants.

Acknowledgments. We are grateful to the Nucleus of Microscopy and Microanalysis (NMM) of the Universidade Federal de Viçosa for the technical assistance. This study was supported by the National Council for Scientific and Technological Development (CNPq), Coordination for the Improvement of Higher Level Personnel (CAPES), and Minas Gerais State Research Agency (FAPEMIG).

Conflict of Interest. The authors declare no conflict of interest.

References

Bancroft JD & Gamble M (2008). *Theory and Practice of Histological Techniques*. London: Churchill Livingstone.

Bolton B (1996). *A New General Catalogue of the Ants of the World*. Cambridge: Harvard University Press.

Buchner P (1965). *Endosymbiosis of Animals with Plant Microorganisms*. New York: Intersciences Publishers Inc.

Cossolin JFS, Lopes DRG, Martinez LC, Santos HCP, Fiaz M, Pereira MJB, Vivan LM, Mantovani HC & Serrão JE (2020). Morphology and composition of the midgut bacterial community of *Scaptocoris castanea* perty, 1830 (Hemiptera: Cydnidae). *Cell Tissue Res*. https://doi.org/10.1007/s00441-020-03197-7

Deretic V & Levine B (2009). Autophagy, immunity, and microbial adaptations. *Cell Host Microbe* **5**, 527–549.

Feldhaar H, Straka J, Krischke M, Berthold K, Stoll S, Mueller MJ & Gross R (2007). Nutritional upgrading for omnivorous carpenter ants by the endosymbiont *Blochmannia*. *BMC Biol* **5**, 48.

Nishikori K, Kubo T & Morioka M (2009*b*). Morph-dependent expression and subcellular localization of host serine carboxypeptidase in bacteriocytes of the pea aphid associated with degradation of the endosymbiotic bacterium *Buchnera*. *Zool Sci* **26**, 415–420.

Nishikori K, Morioka K, Kubo T & Morioka M (2009*a*). Age- and morph-dependent activation of the lysosomal system and *Buchnera* degradation in aphid endosymbiosis. *J Insect Physiol* **55**, 351–357.

Peloquin JJ, Miller SG, Klotz SA, Stouthammer R, Davis Jr. LR & Klotz JH (2001). Bacterial endosymbionts from the genus *Camponotus* (Hymenoptera: Formicidae). *Sociobiology* **38**, 695–708.

Ratzka C, Gross R & Feldhaar H (2013). Gene expression analysis of the endosymbiont-bearing midgut tissue during ontogeny of the carpenter ant *Camponotus floridanus*. *J Insect Physiol* **59**, 611–623.

Reynolds ES (1963). Use of lead citrate at high pH as an electron-opaque stain in electron microscopy. *J Cell Biol* **17**, 208–212.

Sauer C, Dudaczek D, Hölldobler B & Gross R (2002). Tissue localization of the endosymbiotic bacterium "*Candidatus Blochmannia floridanus*" in adults and larvae of the carpenter ant *Camponotus floridanus*. *Appl Environ Microbiol* **68**, 4187–4193.

Schröder D, Deppisch H, Obermayer M, Krohne G, Stackebrandt E, Hölldobler B, Goebel W & Gross R (1996). Intracellular endosymbiotic bacteria of *Camponotus* species (carpenter ants): Systematics, evolution and ultrastructural characterization. *Mol Microbiol* **21**, 479–489.

Skidmore IH & Hansen AK (2017). The evolutionary development of plant-feeding insects and their nutritional endosymbionts. *Insect Sci* **24**, 910–928.

Souza DJ, Bézier A, Depoix D, Drezen JM & Lenoir A (2009). *Blochmannia* endosymbionts improve colony growth and immune defence in the ant *Camponotus fellah*. *BMC Microbiol* **9**, 29.

Stefanini M, Demartino C & Zamboni L (1967). Fixation of ejaculated spermatozoa for electron microscopy. *Nature* **216**, 173–174.

Stoll S, Feldhaar H, Fraunholz MJ & Gross R (2010). Bacteriocyte dynamics during development of a holometabolous insect, the carpenter ant *Camponotus floridanus*. *BMC Microbiol* **10**, 308.

Vigneron A, Masson F, Vallier A, Balmand S, Rey M, Vincent-Monégat C, Aksoy E, Aubailly-Giraud E, Zaidman-Rémy A & Heddi A (2014). Insects recycle endosymbionts when the benefit is over. *Curr Biol* **24**, 2267–2273.

Wernegreen JJ, Kauppinen SN, Brady SG & Ward PS (2009). One nutritional symbiosis begat another: Phylogenetic evidence that the ant tribe camponotini acquired *Blochmannia* by tending sap-feeding insects. *BMC Evol Biol* **9**, 292.

Wolschin F, Hölldobler B, Gross R & Zientz E (2004). Replication of the endosymbiotic bacterium *Blochmannia floridanus* is correlated with the developmental and reproductive stages of its ant host. *Appl Environ Microbiol* **70**, 4096–4102.

Zientz E, Beyaert I, Gross R & Feldhaar H (2006). Relevance of the endosymbiosis of *Blochmannia floridanus* and carpenter ants at different stages of the life cycle of the host. *Appl Environ Microbiol* **72**, 6027–6033.