MANUEL ALEJANDRO IX BALAM

STRUCTURE OF ABDOMINAL AND MANDIBULAR EXOCRINE GLANDS IN DUNG BEETLES (COLEOPTERA: SCARABAEINAE)

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Entomologia, para obtenção do título de *Doctor Scientiae*.

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José Eduardo Serrão
(Coorientador)

Júlio Neil Cassa Louzada

Luanda Medeiros Santana

Morgana Maria Fonseca Porto

Eraldo Rodrigues de Lima
(Orientador)

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pais, Natalia e Luis Amado, e
meus avós, Célia e Manuel, por
sempre acreditarem em mim.

"If you want to understand an insect,
observe its behavior"
Modified from Albert Einstein

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Contents

ABSTRACT

Ix-Balam, Manuel Alejandro, D.Sc., Universidade Federal de Viçosa, May, 2018. **Structure of abdominal and mandibular exocrine glands in dung beetles (Coleoptera: Scarabaeinae)**. Adviser: Eraldo Rodrigues de Lima. Co-adviser: José Eduardo Serrão.

Chemical communication is the exchange of information between individuals through semiochemicals produced by exocrine glands. The study of the morphology and localization of the exocrine glands is important to understand the chemical communication in insects. Chemical communication plays an important role in dung beetles. However, different morphological aspects of semiochemical producing glands and the chemical nature of secretions are still little known. The dung roller beetle *Deltochilum furcatum* (Scarabaeinae) forms food balls using excrements and carrion, which are rolled to safe sites for feeding or nesting. During the rolling, *D. furcatum* marks the food ball with semiochemicals that inhibit the oviposition of competing flies. However, the origin and chemical composition of the secretions are unknown. Here, we examined the structural and histochemistry organization of exocrine glands associated with the chemical communication of *D. furcatum*. In the first study, we analyzed regions of the beetle body that are in frequent contact with the food ball during rolling. Also, we proposed a hypothetical model that describes the process of production and impregnation of the secretion in the food ball. We have demonstrated that *D. furcatum* presents bicellular and multicellular exocrine glands in the thorax, abdomen and pygidium. The secretory cell of the gland synthesized protein, lipids,

and carbohydrates compounds. The conformation of these exocrine glands in *D. furcatum* is distinct from that reported in other species of Scarabaeinae. In the second study, we analyzed the mandibles of *D. furcatum*. Associated with mandibles there are mandibular and intramandibular glands, the latter described by the first time in a dung beetle. The mandibular gland has bicellular secretory units connected to a wrinkled reservoir that opens at the base of the mandible. The intramandibular glands were formed of: (I) bicellular secretory units that open directly to the mandible surface; (II) epithelial glands with reservoirs formed of grouped modified epidermal cells forming the coating of an invaginated reservoir where the secretions are stored and released to the mandible surface; and (III) epithelial glands without reservoir that are hypertrophied epidermal cells in the conjunctive and the molar lobe of the mandible, releasing secretions by diffusion through the cuticle. The mandibular and intramandibular glands were rich in proteins and carbohydrates. The epithelial glands with reservoirs also have lipids. The structural characteristics of these mandibular glands of *D. furcatum* are similar to those of social insects, such as bees and ants, which have a complex chemical communication system.

RESUMO

Ix-Balam, Manuel Alejandro, D.Sc., Universidade Federal de Viçosa, maio de 2018. **Estrutura de glândulas exócrinas abdominais e mandibulares em besouros do esterco (Coleoptera: Scarabaeinae)**. Orientador: Eraldo Rodrigues de Lima. Coorientador: José Eduardo Serrão.

Comunicação química é a troca de informações entre indivíduos através de semioquímicos produzidos por glândulas exócrinas. O estudo da morfologia e localização das glândulas exócrinas é importante para entender a comunicação química em insetos. A comunicação química desempenha um papel importante nos besouros do estrume. No entanto, diferentes aspectos morfológicos das glândulas produtoras de semioquímicos e a natureza química das secreções são pouco conhecidos. O besouro rolador do esterco *Deltochilum furcatum* (Scarabaeinae) forma bolas de alimento utilizando fezes e carcaças, as quais rola até lugares seguros para alimentação ou reprodução. Durante a rolagem, *D. furcatum* marca a bola de alimento com semioquímicos que inibem a oviposição das moscas competidoras do alimento. No entanto, a origem e composição química das secreções são desconhecidas. Nesta tese, examinamos a organização estrutural e histoquímica de glândulas exócrinas associadas à comunicação química de *D. furcatum*. No primeiro estudo, analisamos regiões do corpo do besouro que entram em contato frequente com a bola de alimento durante a rolagem. Além disso, propusemos um modelo hipotético que descreve o processo de produção e impregnação da secreção na bola de alimento. Demonstramos que *D. furcatum* apresenta glândulas exócrinas bicelulares e multicelulares no tórax, abdome e pigídio. A célula

secretora da glândula sintetizou compostos proteicos, lipídicos e carboidratos. A conformação destas glândulas exócrinas em *D. furcatum* é distinta à reportada em outras espécies de Scarabaeinae. No segundo estudo analisamos as mandíbulas de *D. furcatum*. Associadas às mandíbulas, existem glândulas mandibulares e intramandibulares, sendo a última descrita pela primeira vez em um besouro do esterco. A glândula mandibular possui unidades secretoras bicelulares conectadas a um reservatório rugoso que se abre na base da mandíbula. As glândulas intramandibulares consistem em: (I) unidades secretoras bicelulares que se abrem diretamente para a superfície da mandíbula; (II) glândulas epiteliais com reservatório consistente em agrupamento de células epidérmicas modificadas, formando o revestimento de um reservatório invaginado, onde as secreções são armazenadas e liberadas na superfície mandibular; e (III) glândulas epiteliais sem reservatório que são células epidérmicas hipertrofiadas na conjuntiva e no lóbulo molar da mandíbula, liberando as secreções por difusão através da cutícula. As glândulas mandibular e intramandibular são ricas em proteínas e carboidratos. As glândulas epiteliais com reservatórios também possuem lipídios. As características estruturais destas glândulas mandibulares de *D. furcatum*, são semelhantes às dos insetos sociais, como abelhas e formigas, que possuem um complexo sistema de comunicação química.

General Introduction

Chemical communication is the exchange of information between individuals through chemical substances called semiochemicals. This communication is widespread in insects, particularly those living in social organizations (Ali & Morgan, 1990). The semiochemicals transmit information that acts at the intraspecific level (pheromones) or interspecific (allelochemics) (Nordlund & Lewis, 1976; Ali & Morgan, 1990). The semiochemicals are produced by exocrine glands that vary in their morphological structure, functional complexity and chemical components of their secretions (Noirot & Quennedey, 1974, 1991). The exocrine glands are located in specific regions or distributed throughout the body of the insect (Noirot & Quennedey, 1974). The study of the morphology and localization of the exocrine glands is important to understand the chemical communication in insects (Pluot-Sigwalt, 1995), and essential for the realization of experimental studies (Pluot-Sigwalt, 1988). Exocrine glands have been most studied in groups such as Hymenoptera (da Cruz-Landim *et al.*, 2011; Billen, 2009; Stökl & Herzner, 2016). However, little is known about these glands in Coleoptera, particularly in dung beetles.

The dung beetles (Coleoptera, Scarabaeidae) constitute a diverse group with more than 6000 species around the world (Schoolmeesters, 2017). These beetles use excrements and carrion as food resources and for nesting (Halffter & Edmonds, 1982; Hanski & Cambefort, 1991; Scholtz *et al.*, 2009). These food resources are ephemeral and rapidly lose their nutritional properties, causing an intense intraspecific and interspecific competition for the food resource (Hanski & Cambefort, 1991). Dung beetles have developed food relocation behaviors for diminution of the competition (see Halffter & Edmonds, 1982). To re-locates the food to safe sites, the dung roller beetles (Scarabaeinae) cut a fragment of food, ball-shaped, which is rolled to certain distances and then it is buried.

Pluot-Sigwalt (1982, 1983, 1986, 1988, 1991, 1995) described exocrine glands associated with different lifestyles and the complex reproductive behaviors of dung beetles. The pioneering works of Pluot-Sigwalt constitute the most important studies on comparative morphology of the exocrine glandular system of Scarabaeinae and other Scarabaoidea. The structural organization of these glands is based on the morphological variations of the segments that make up the cuticular duct of secretory units and the distribution of these ducts in the internal integument (Fig. 1). However, different morphological aspects of these integumentary glands and the chemical nature of the secretions are still little known.

In the Scarabaeinae, the secretions of these exocrine glands are impregnated in the food ball with diverse functions (Bellés & Favila, 1983; Favila, 1988; Ix-Balam, 2014). For example, males of the Scarabaeinae *Canthon cyanellus cyanellus* LeConte impregnate the food ball with attractive chemicals for the female (Favila, 1988), but repulsive against *Calliphora* fly larvae (Bellés & Favila, 1983). Food balls rolled by the copro-necrophagous beetle *Deltochilum furcatum* Castelnau receive less number of eggs of flies compared with balls that were not rolled (Ix-Balam, 2014). There fact suggests a chemical labeling of the ball by the beetles, which inhibit the oviposition of the flies, and which leads to less food competition. However, the origin and chemical composition of the secretions was not studied in *D. furcatum*.

The main objective of this work was to describe the morphology and histochemistry of exocrine glands associated with the chemical communication of the dung roller beetle *D. furcatum*. We analyze regions of the beetle body that come into frequent contact with the food ball during rolling. The mandibular structures were also analyzed as they are active elements during the consumption, cutting, and formation of the food ball in dung beetles. This work represents the first morphological study of these glands in dung beetles using techniques of scanning electron microscopy, histology, and histochemistry.

Overview of Chapter 1

It has been hypothesized that the beetle *D. furcatum* (Scarabaeinae) releases chemicals that are impregnated in the food ball during rolling. However, the origin and chemical nature of these secretions are unknown. In chapter 1 of this thesis, we selected regions of the beetle body that are in frequent contact with the food ball during rolling, and analyzed the gland structures and the chemical nature of their secretions. Also, we proposed a hypothetical model that describes the process of production and impregnation of the secretion in the food ball. As main results, we show that *D. furcatum* has a integumentary glandular system associated to the thorax, abdomen, and pygidium. This integumentary glandular system is made up of bicellular and multicellular glands. The bicellular gland consists of a large secretory cell and a small duct cell, and the multicellular ones have various bicellular subunits that connect trough the small cuticular ducts to a common cuticular duct. The secretory cell synthesized proteins, lipids, and carbohydrates,

which are transferred to a cuticular duct in the bicellular gland or to the common duct in the multicellular gland. These ducts transport the secretions to the exterior for a pore positioned in the integument. One or more glands can be associated with one internal pore. Using these morphological data, together with the reported for other Scarabaeinae species (Pluot-Sigwalt, 1982, 1983, 1986, 1988, 1991, 1995), we propose a hypothetical model that describes the process of secretion and impregnation of the chemical compounds to the food ball by the glandular units in *D. furcatum*. In the description of our model, we mentioned that the secretions are synthesized in the cytoplasm of the secretory cells. In the bicellular glands, the secretions are transferred to the folds that surround the cuticular duct. The secretions become fine as they pass through the folds until they become a homogeneous secretion that accumulates in the cuticular duct. The cuticular duct transports and releases the filament-shaped secretion. In multicellular glands, the secretory granules present in the secretory cells release their content into the cuticular duct that transfer it to the common cuticular duct. Finally, secretion is impregnated in the food ball during its formation and rolling.

Overview of Chapter 2

Regions such as mouthparts can produce and release compounds associated with the chemical communication. In this chapter, we analyze the structural organization and chemical nature of the secretions released of the exocrine glands associated with the mandible of the Scarabaeinae *D. furcatum*. As main results, we show that associated with mandibles there are mandibular and intramandibular glands, the latter described by the first time in a dung beetle. The mandibular gland has bicellular secretory units connected to a wrinkled reservoir that opens at the base of the mandible. The intramandibular glands were formed of secretory units and epithelial glands with and without reservoirs. The intramandibular secretory units are bicellular and open directly to the mandible surface. The epithelial glands with reservoirs are grouped of modified epidermal cells forming the coating of an invaginated reservoir where the secretions stored and released to the mandible surface. The epithelial glands without reservoir are hypertrophied epidermal cells in the conjunctive and the molar lobe of the mandible, releasing the secretions by diffusion through the cuticle. The mandibular and intramandibular glands were rich in proteins and carbohydrates

contents. The epithelial glands with reservoirs also have lipid. The structural characteristics of these glands are similar to that of social insects like bees and ants, who have a complex chemical communication system.

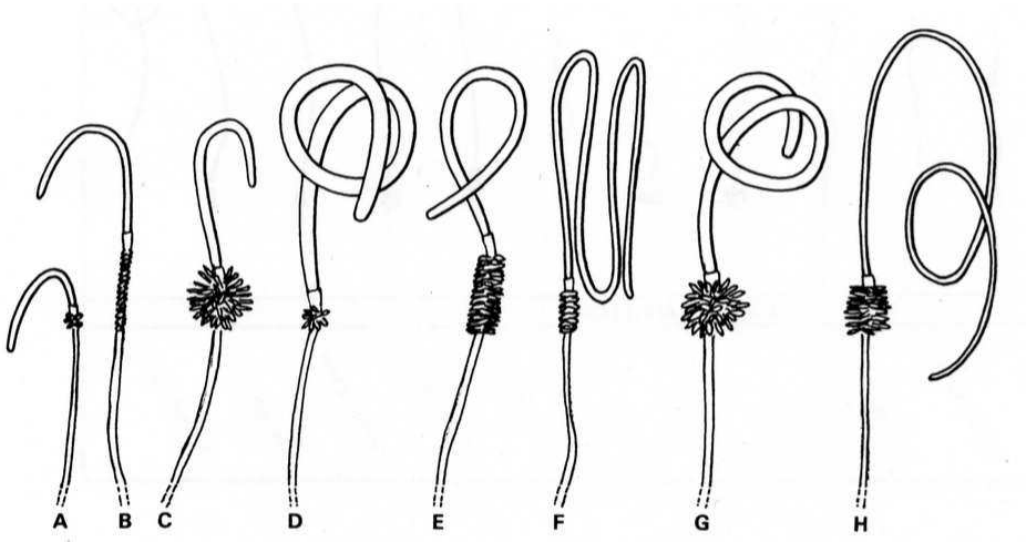

Figure 1. Main types of cuticular ducts found by Pluot-Sigwalt (1986) in Scarabaeidae. A, B, C, D and E, scattered glandular units. F, sternal glands of the female. G, sternal glands of the male. H, pygidial glands. .

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Chapter 1

The abdominal, metasternal and pygidial glands in the Neotropical dung roller beetle *Deltochilum furcatum* (Coleoptera: Scarabaeinae)

The abdominal, metasternal and pygidial glands in the Neotropical dung roller beetle *Deltochilum furcatum* (Coleoptera: Scarabaeinae)

Manuel Ix-Balam[1], Karen Salazar [2], José Eduardo Serrão[3] and Eraldo Lima[4]

Abstract

It is hypothesized that the dung roller beetle *Deltochilum furcatum* (Scarabaeinae) release chemicals that are impregnated into the food ball during rolling. However, the origin and chemical nature of these secretions are unknown. Here, we selected regions of the beetle body that are in frequent contact with the food ball during rolling, and analyzed the gland structures and the chemical nature of their secretions. Besides, we proposed a hypothetical model that describes the process of production and impregnation of the secretion in the food ball. The integumentary glandular system associated to the thorax, abdomen and pygidium of *D. furcatum* consists of bicellular and multicellular glands. The bicellular gland consists of a large secretory cell and a small duct cell, and the multicellular ones have various bicellular subunits that connect trough the small cuticular ducts to a common cuticular duct. The secretory cell synthesized proteins, lipids, and carbohydrates, which are transferred to a cuticular duct in the bicellular gland or to the common duct in the multicellular gland. These ducts transport the secretions to the exterior for a pore positioned in the integument. One or more glands can be associated to one internal pore. We discuss the morphologic differences of the secretory structures and the possible biological functions of the secretions.

Keywords: Coleoptera, integumentary glands, morphology, Scarabaeidae, semiochemicals

[1]Departamento de Entomologia - Universidade Federal de Viçosa, manuelbalam1786@gmail.com
[2]Departamento de Entomologia - Universidade Federal de Viçosa
[3]Departamento de Biologia Geral - Universidade Federal de Viçosa
[4]Prof. Associado IV - Departamento de Entomologia - Universidade Federal de Viçosa

1 Introduction

The dung beetles (Coleoptera, Scarabaeidae) constitute a diverse group with more than 6000 species around the world (Schoolmeesters, 2017). These beetles use excrements and carrion as food resources and for nesting (Halffter & Edmonds, 1982; Hanski & Cambefort, 1991; Scholtz *et al.*, 2009). These food resources are ephemeral and rapidly lose their nutritional properties, causing a strong intraspecific and interspecific competition for the food resource (Hanski & Cambefort, 1991). Dung beetles have developed food relocation behaviors for diminution of the competition (Halffter & Edmonds, 1982). To re-locates the food to safe sites, the dung roller beetles cut a fragment of food, ball-shaped, which is rolled to certain distances and then it is buried. Thus, these beetles assure a portion of the food (Scholtz *et al.*, 2009). The food ball seems to act as an important intermediary in the chemical communication of the dung beetles (Halffter, 1997). The chemical communication plays an important role in the behavior and the intra and interspecific interactions in the dung beetles (Bellés & Favila, 1983; Favila, 1988; Favila & Díaz, 1996; Favila, 2001; Cortez *et al.*, 2012; Burger, 2014). The rolling of the food ball for the Scarabaeinae has also been associated with the impregnation of semiochemicals to the ball (Bellés & Favila, 1983; Favila, 1988; Favila & Díaz, 1996; Ix-Balam, 2014). Males of *Canthon cyanellus cyanellus* LeConte impregnate the food ball with chemical substances with repulsive properties against larvae of flies (Bellés & Favila, 1983), but attractive for the female of *C. cyanellus* (Favila, 1988). Different integumentary exocrine glands have been proposed in the Scarabaeinae as responsible for semiochemicals production (Pluot-Sigwalt, 1982, 1983, 1986, 1991, 1995), but the chemical analysis of these secretions has been little studied, and some morphological aspects of these integumentary glands are poorly known.

Food balls rolled by the copro-necrophagous beetle *Deltochilum furcatum* Castelnau receive less number of eggs of flies compared with balls that were not rolled (Ix-Balam, 2014). This fact suggests a possible chemical labeling of the ball by the beetles, which inhibit the oviposition of the flies, and which leads to less food competition. However, the origin and chemical composition of the secretions has not been studied in *D. furcatum*. The objective of this study was describe the morphology of the secretory structures, the chemical nature of the secretions and some

possible aspects of the production and transport process of the glandular secretions, and their impregnation in the food ball by *D. furcatum*.

2 Methods and Materials

2.1 Biological material

Adult males and females of *D. furcatum* (Fig. 1A) were collected at the Reserva Florestal Mata do Paraíso (20°45'22"S/42°51'44"W), municipality of Viçosa, Minas Gerais State, Brazil. To capture of the beetles was used fresh meat baited pitfall traps. They were separated by sex and individually reared at room temperature in plastic boxes of 1000 mL containing humid soil as substrate, being fed with ground meat once a week (Ix-Balam, 2014).

2.2 Scanning electron microscopy (SEM)

2.2.1 Glandular pores, ducts and secretions

The body regions analyzed were selected due to the frequent contact of these body regions during the formation and rolling of the food ball (Favila, 2001, also personal observations) (Fig. 1 B-D). Two individuals of each sex were placed in 70% ethanol by 24 h to remove impurities attached to their bodies. The pygidium, abdominal sternites and metasternum were dissected, transferred to 5% KOH at 100°C for 5 minutes and washed with distilled water. After, the samples were dehydrated in graded ethanol series, transferred to hexamethyldisilazane (HMDS) for 10 minutes and dried at room temperature. Samples were attached to aluminum stubs, gold covered (20 nm thick) and analyzed in a scanning electron microscope LEO VP1430 (SEM). To observe the secretions in the glandular pores some samples were not treated with KOH (Pluot-Sigwalt, 1986).

2.2.2 Gland cells

Two individuals of each sex were cryoanesthetized at -4°C for 5 min. After, pygidium, abdominal sternites and metasternum were dissected in saline solution (0.1 M NaCl, 0.1 M KH_2PO_4, 0.1 M Na_2HPO_4), and transferred to Stefanini fixative solution pH 7.3 (Stefanini *et al.*, 1967) at 5°C for 24 h. Then, the samples were dehydrated in a graded ethanol series,

transferred to HMDS and air dried. Subsequently, the samples were gold covered and observed with SEM (as described above).

2.3 Light microscopy

Two individuals of each sex were cryoanesthetized. Next, the pygidium, abdominal sternites and metasternum were dissected and transferred to Stefanini fixative solution at 5°C for 24 h. Samples were washed with phosphate buffer saline (PBS) 0.1 M, pH 7.2, dehydrated in graded ethanol series, and embedded in JB4 historesin. Histological semi-thin sections (3 μm in thick) were obtained with a microtome Leica RM 2255 with glass knife. The samples were stained with Harris hematoxylin (Merck, Darmstadt, Germany) and eosin (Sigma-Aldrich, USA). Histological sections were submitted to mercury-bromophenol blue (MBB) to detection of total proteins, periodic Acid-Schiff (PAS) to detection polysaccharides and glycoconjugates, and 1% osmium tetroxide for lipid identification (Pearse, 1985; Bancroft & Gamble, 2008).

2.4 Morphometry

The morphometric data (diameter and density of glandular pores, length and width of ducts) were performed with the software Image-Pro Plus version 4.5.0.29 (Media Cybernetics, Inc.). The density of glandular pores was calculated as the number of pores present in an area of integument equivalent to 100 μm^2 on each side.

2.5 Terminology

The terminology used in the description of the glandular system and ducts were in agreement with Noirot & Quennedey (1974, 1991) and Pluot-Sigwalt (1986), respectively.

3 Results

3.1 Glandular pores and secretions

Circular glandular pores were observed in the external surface of pygidium, sternites, and metasternum of males and females of *D. furcatum*. These pores had similar diameter and density between sexes (Fig. 2). These pores crossed whole the integument. The glandular pores were separated in pygidium and sternites and separated or grouped in two or three in metasternum (Fig. 3A-C). The pores were distributed between cuticular depressions resembling discs surrounding a bristle in the pygidium and lateral regions of the sternites. The central regions of the sternites IV-VI had an internal depression in the cuticle, where there were grouped pores with an approximate diameter of 0.5 μm forming a cribellum (Fig. 3D). The glandular secretions were found released from pores, accumulating in the internal part of the pores with cribellum (Fig. 3E-G).

3.2 Glands units associated to the pores

The internal analysis of the integument of the pygidium, sternites and metasternum showed that the glandular pores described above were associated with glandular units with secretory gland cells and cuticular ducts. The glandular units were close to each other uniformly covering the entire inner surface of the integument (Fig. 4A). The gland cells were globular-shaped with variable size and smooth surface (Fig. 4B-C). Each gland cell was associated with a long and narrow cuticular duct, which extended from the inside of the gland cells to the glandular pore (Fig. 4D-E).

3.3 Cuticular ducts

Cuticular ducts were found in the pygidium, sternites and metasternum in both sexes of *D. furcatum*. These ducts ranged from 30 μm to 120 μm in length (pygidium and sternites: 63 $\pm$ 3 μm and 77 $\pm$ 2.7 μm in females and males, respectively, and 54.5 $\pm$ 2.4 μm in metasternum of both sexes). The duct had three successive and differentiated segments called conductive, apical receptor and basal receptor. The anterior part of the conductive segment extended until the pore

in the integument, and its posterior part end in the apical receptor. This segment had a uniform tubular aspect with length from 20 μm to 36 μm and 0.7 μm width. The apical receptor segment is the shorter segment (3.0 to 4.0 μm in length) with cuticular expansions from 2.0 to 3.0 μm in length and 2.0 μm in width and connect with the basal receptor by a small constriction. The basal receptor segment had a uniform tubular aspect with 26 μm to 39 μm in length and 1.0 μm in width. This segment had a small expansion in the tip (Fig. 5A, B). The cuticular ducts opened in the pores on the internal surface of the integument individually or in groups of two or more ducts (Fig. 5C-D). Dense groups of ducts with different size were found opening in the same glandular pore in the sternites IV-VI (Fig. 5E).

3.4 Glands

The pygidium, sternites and metasternum of both sexes of *D. furcatum* had bicellular and multicellular glands. The bicellular glands are closely arrayed and had a secretory cell and the duct cell, the latter forming the cuticular duct (Fig. 6A-C). The basal and apical receptor segments of the cuticular duct were placed inside the secretory cell (Figs. 6B-C). The secretory cell showed many folds surrounding the cuticular duct (Fig. 6B-C). The cell nucleus was placed in the cell periphery and the cytoplasm was filled with small secretory granules (Fig. 6C). The multicellular glands were formed by many bicellular subunits, each with a secretory cell and a duct cell. Each subunit connecting trough the small internal cuticular ducts to a common enlarged cuticular duct that opens in a pore on integument (Figs. 7A-C). The secretory cells had a small nucleus in the periphery and the cytoplasm was rich in large secretory granules (Fig. 7D-F). The limits between adjacent gland cells were difficult to be recognized (Fig. 7A-D). The cytoplasm granules of both bicellular and multicellular glands were positive for protein, lipid and carbohydrate (Fig. 7D-F).

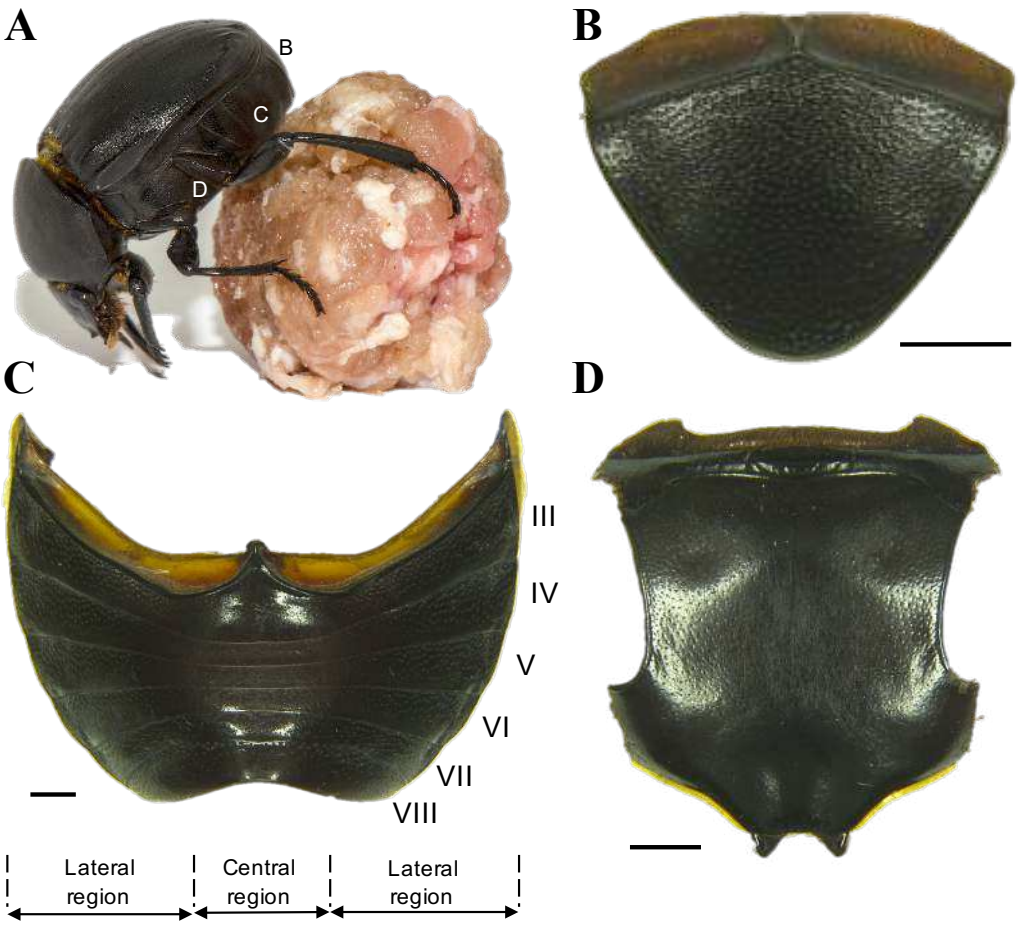

Figure 1. Individual the *Deltochilum furcatum*. A, female rolling a food ball, the letters indicate the parts of the body that come in frequent contact with the ball during the rolling; B, pygidium (dorsal view); C, abdominal sternites (ventral view) showing their segments (III-VIII) and regions; D, metasternum (ventral view). Scale bars: 1 mm (B-D).

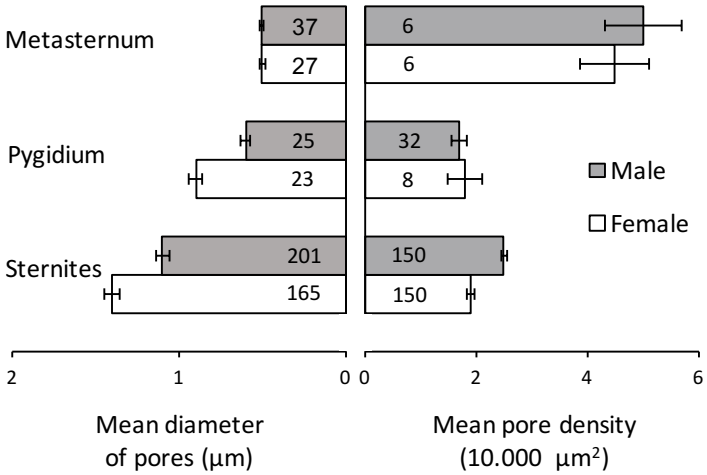

Figure 2. Average diameter and average density of pores on the integument of regions of the body of males and females of *Deltochilum furcatum*. The horizontal bars represent the mean values ± standard error. The numbers inside the bars represent the number of pores and quadrants measured to obtain the data.

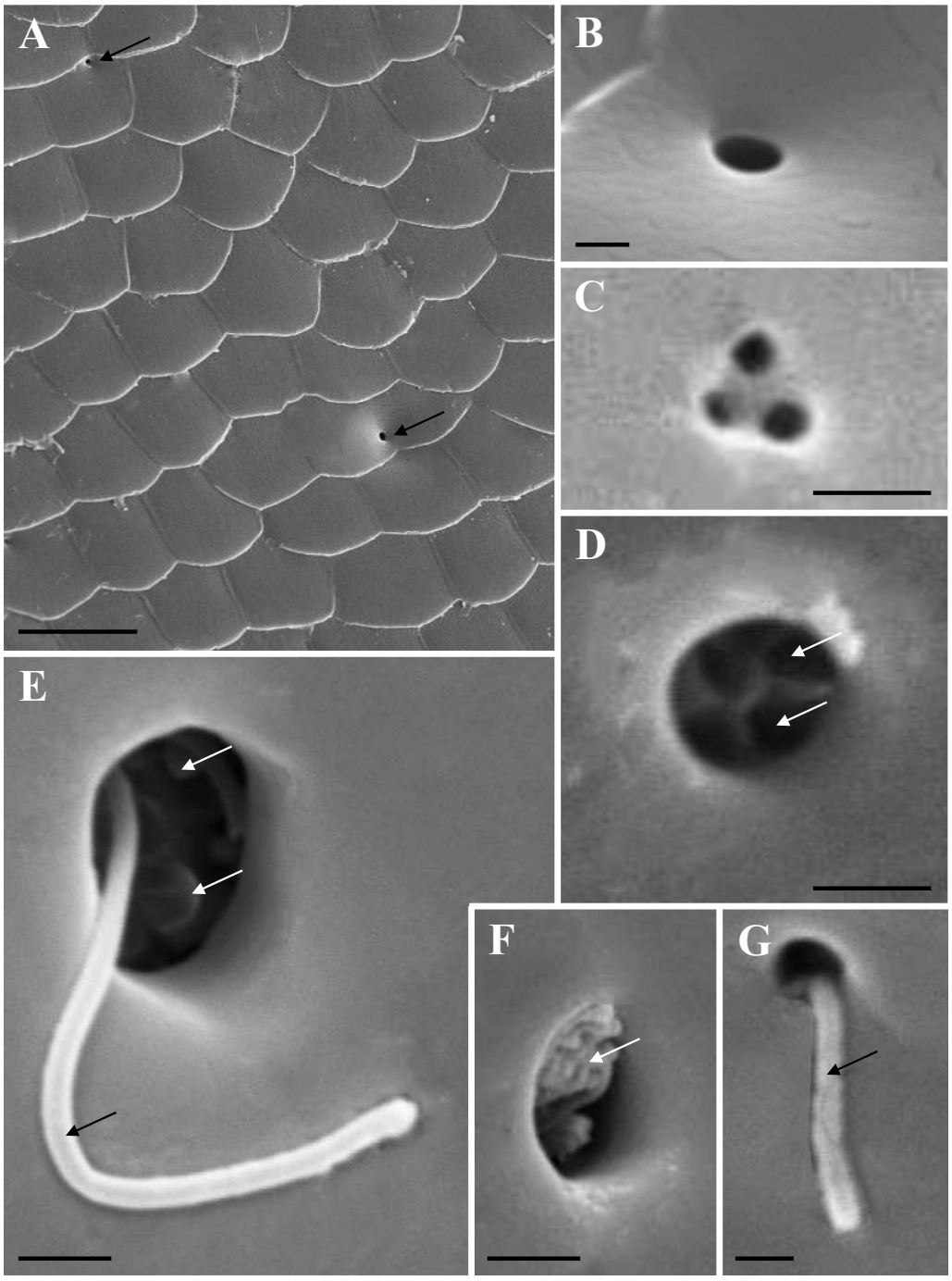

Figure 3. Glandular pores and secretions in the external integument of sternites, pygidium and metasternum of *Deltochilum furcatum* under SEM. A, two pores in the sternite (arrows); B, detail of one pore of Fig 3A; C, group of three pores in the metasternum; D-F, pore with an internal cribellum in IV-VI sternites. In (D) pointing to the cribellum pores (white arrows), in (E-F) showing the released glandular secretion (black arrows), and accumulation lar secretion in the internal cavity of the pore (white arrows); G, glandular secretions from one pore (black arrows). Scale bars: 10 μm (A); 1 μm (B-D, F); 2 μm (E); 0.5 μm (G).

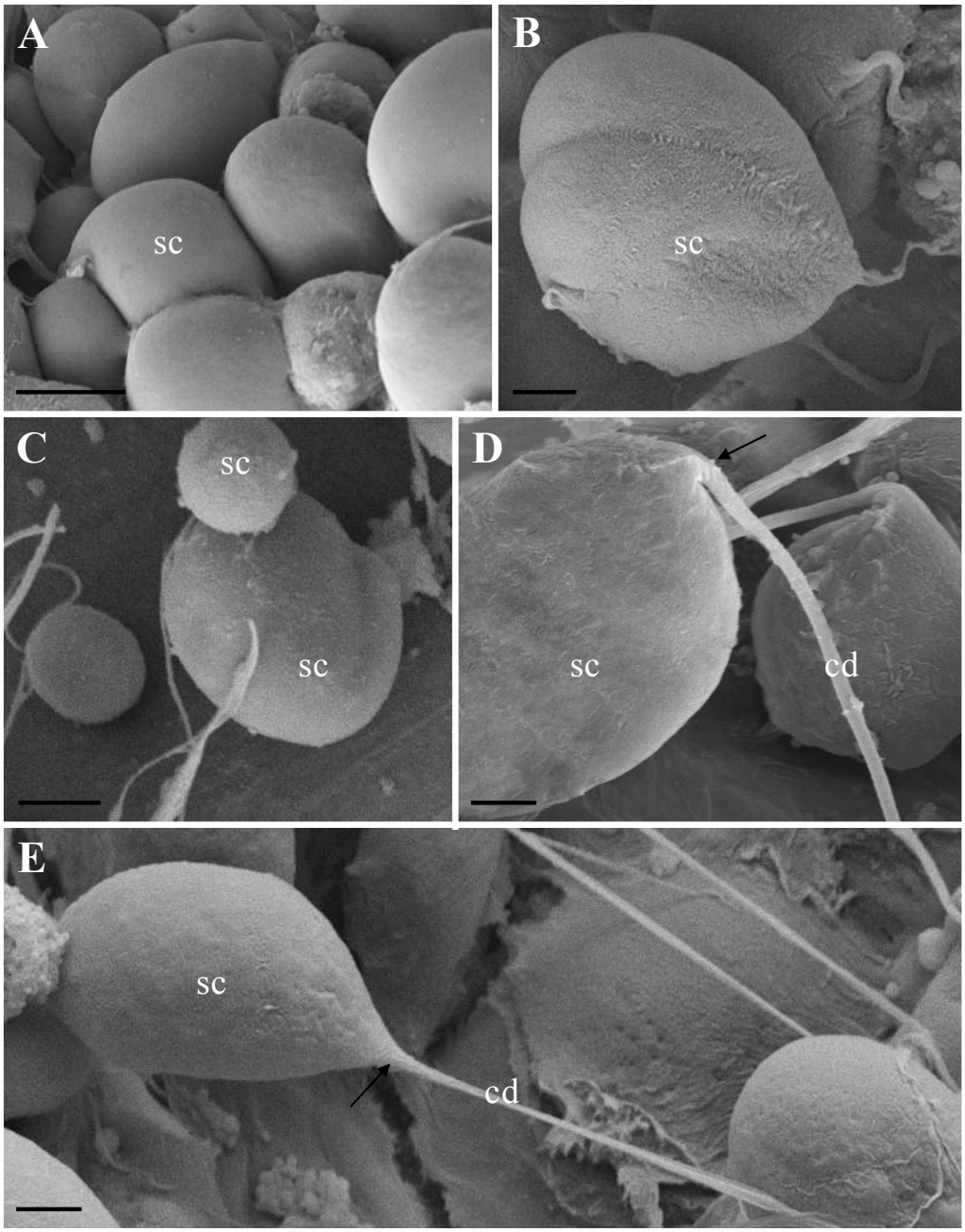

Figure 4. Glandular units that cover the inner surface tegument of the pygidium, sternites and metasternum of *Deltochilum furcatum* under SEM. A-C, secretory cell (sc) with variable size and globular-shaped; D, E, showing of the cuticular duct (cd) associated to a sc. Note where the cd joining with the sc (black arrows). Scale bars: 10 μm (A); 5 μm (B-E).

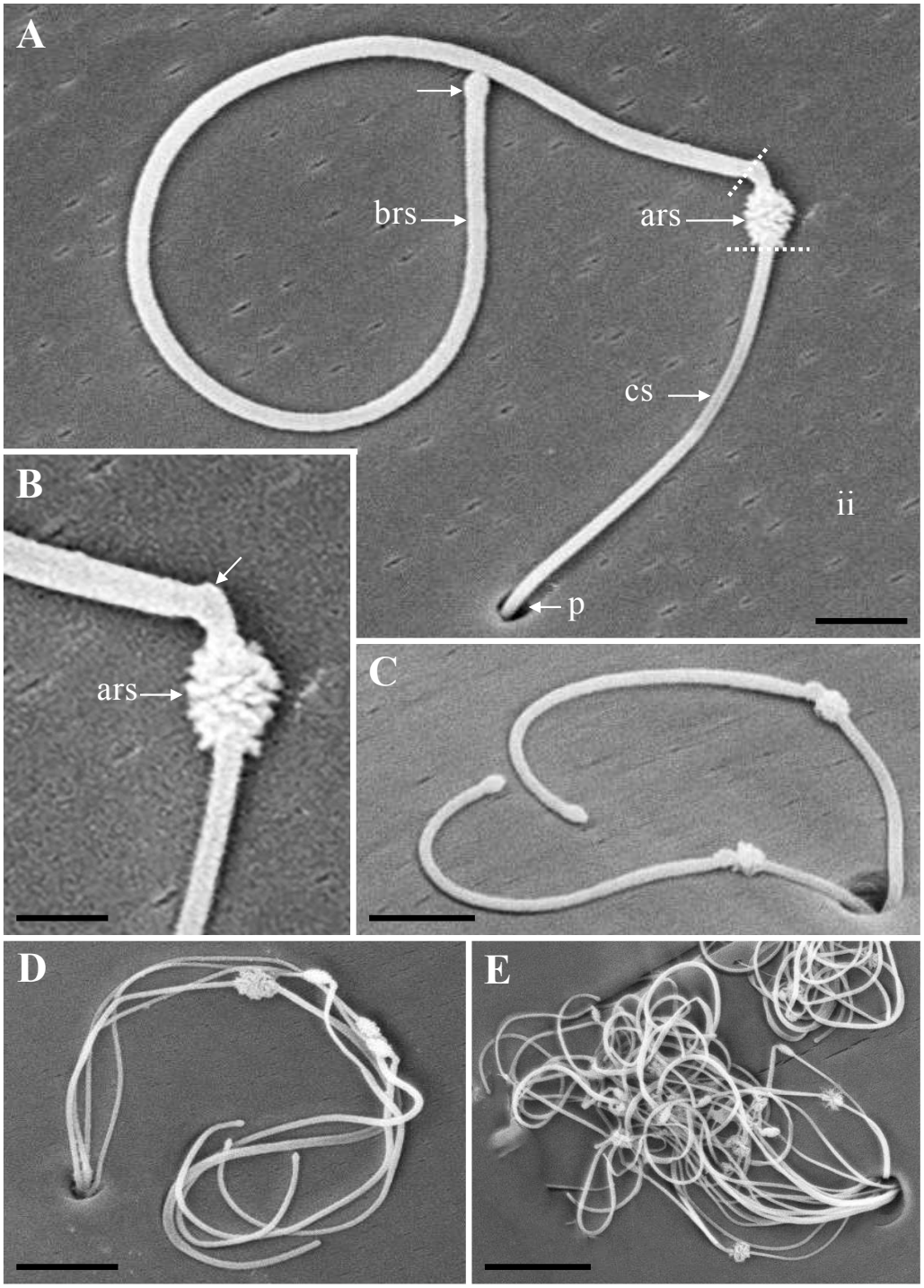

Figure 5. Cuticular ducts of the glandular units of the pygidium, sternites and metasternum in *Deltochilum furcatum* under SEM. A, segments of the cuticular duct. Note the expansion in the distal end of the basal receptor segment (brs) (arrow) and the limits between each segment (dotted lines). B, cuticular expansions of the apical receptor segment (ars) and constriction (arrow) that separates it from the brs. C-E, group of ducts opening to the same pore (p) of the internal integument (ii). Note that the numbers and length of ducts varies. cs: conductive segment. Scale bars: 5 μm (A,D); 3 μm (B); 10 μm (C); 20 μm (E).

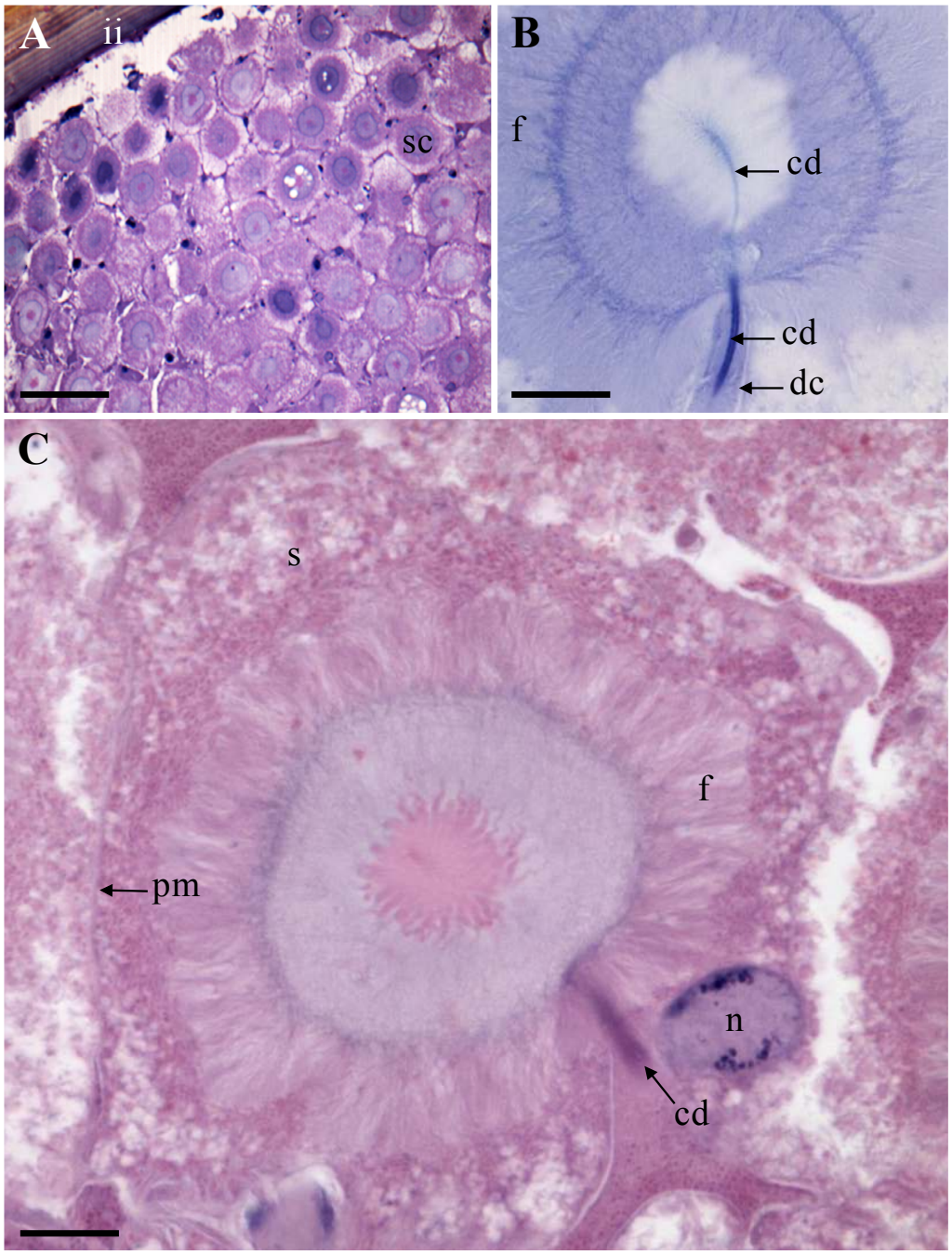

Figure 6. Gland bicellular present in pygidium, sternites and metasternum of *Deltochilum furcatum*. A, general view of a group of bicellular glands; B, C, details of secretory cell (sc) and duct cell (dc). Note the cuticular duct (cd) inside and outside the sc, the glandular secretions (s) in the cytoplasm, and folds (f) around the cd. ii: internal integument; n: nucleus; pm: plasma membrane of sc. Scale bars: 50 μm (A); 10 μm (B,C).

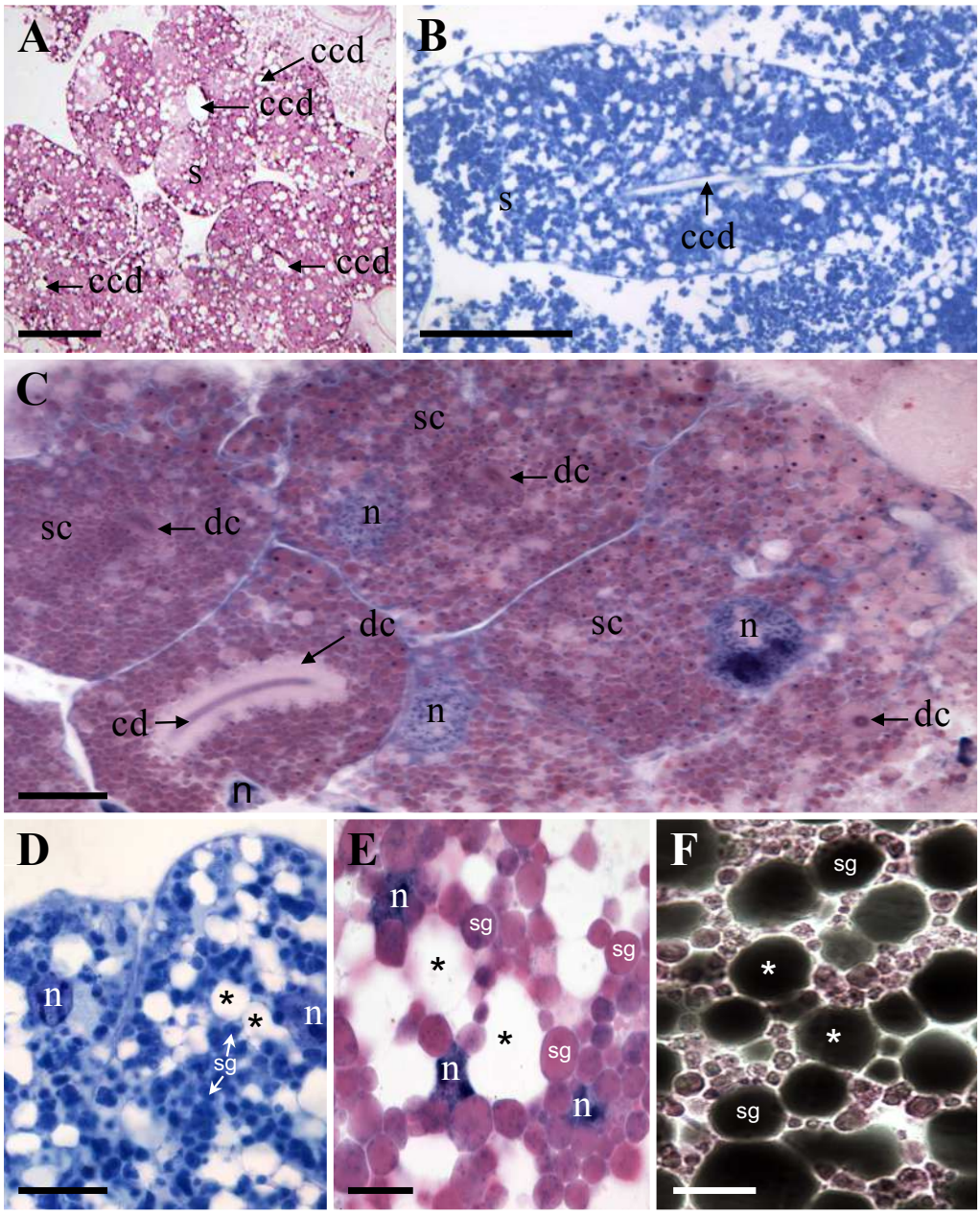

Figure 7. Multicellular gland present in pygidium, sternites and metasternum of *Deltochilum furcatum*. A, general view of a group of multicellular glands (transverse section); B, general view of a multicellular gland (longitudinal section) showing the common cuticular duct (ccd); C, bicellular subunits with a secretory cell (sc) and a duct cell (dc). Note the dc and nucleus (n) of each bicellular subunit; D-F, cytoplasm of the secretory cell showing secretion granules (sg) of proteins, carbohydrates and lipids, respectively. Scale bars: 100 μm (A,B); 10 μm (C,E,F); 20 μm (D).

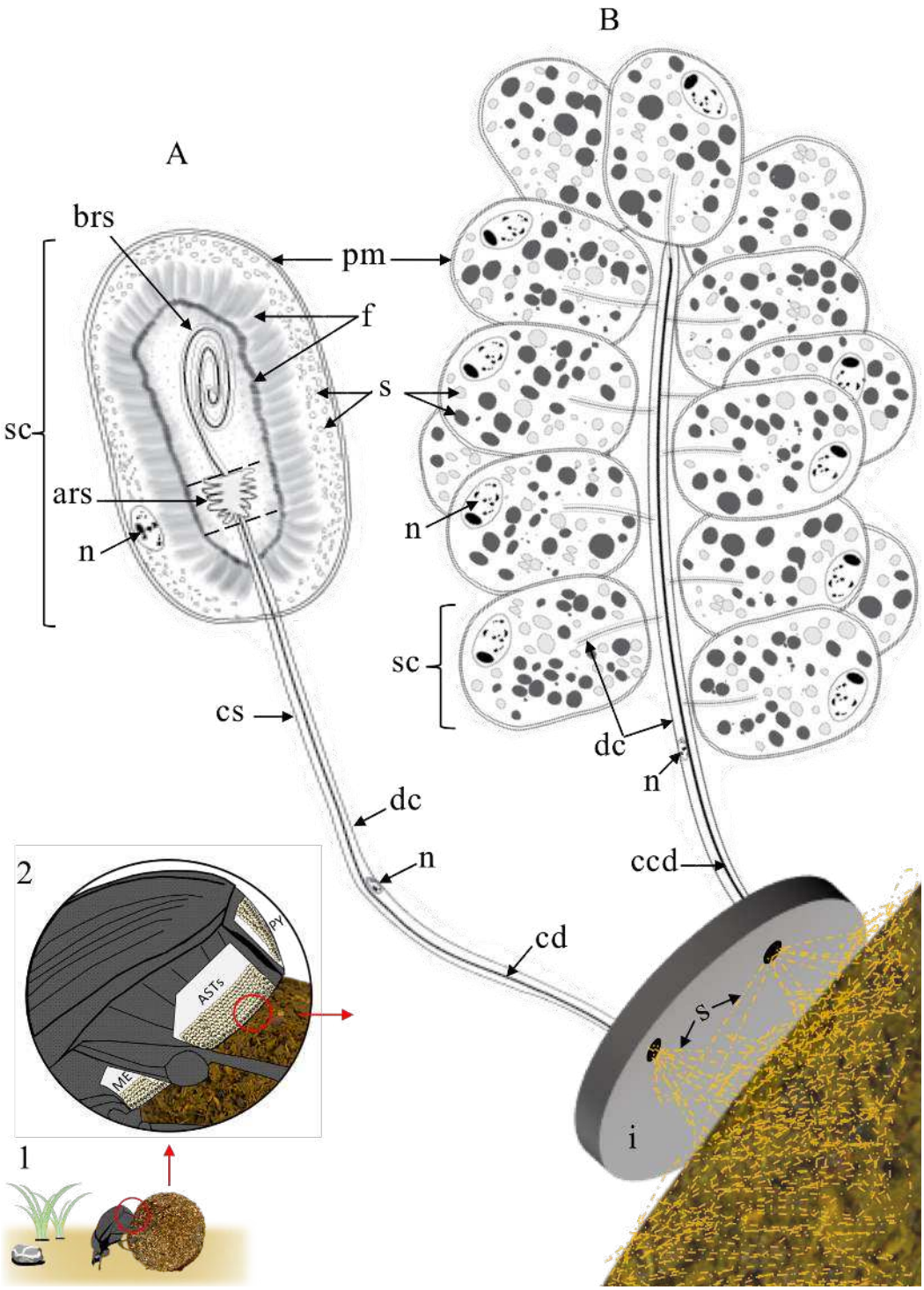

Figure 8. Schematic representation of integumentary bicellular and multicellular glands in *Deltochilum furcatum* and process of production and impregnation of the secretion in the food ball. The beetle make and rolling food balls (1). During this process, pygidium (PY), abdominal sternites (ASTs) and metasternum (ME) come into frequent contact with the food ball (2). These regions have bicellular (A) and multicellular glands (B). The secretions (s) are synthesized in the cytoplasm of the secretory cell (sc). Note that in A the s becomes homogenous after crossing folds (f) that surround the cuticular duct (cd). In B the secretion is transported to the duct of the bicellular subunit and then to a common cuticular duct (ccd). The cd and ccd release the secretion in the form of filament through pores that pass through the integument (i), being the secretion impregnated in the food ball. ars: apical receptor segment; brs: basal receptor segment; cs: conducting segment; dc: duct cell; n: nucleus; pm: plasma membrane.

4 Discussion

The beetle *D. furcatum* has integumentary bicellular and multicellular glands distributed throughout the pygidium, abdominal sternites and metasternum. The secretions of these glands have proteins, carbohydrates and lipids.

Both bicellular and multicellular glands of *D. furcatum* are classified as glands of class III according to Noirot and Quennedey (1974, 1991). This gland class is characterized by having a secretory cell and a duct cell that carries secretions. Class III glands have been described in different insects, particularly in social insects producing compounds with diverse functions, such as defense and chemical communication (Noirot & Quennedey, 1974, 1991; Billen, 1991; Da Cruz-Landim *et al.*, 2006; Costa-Leonardo *et al.*, 2015; Nijs & Billen, 2015). In Coleoptera, similar glands occur in *Tenebrio molitor* Linnaeus (Delachambre, 1973) and *Semiadalia undecimnotata* Schneider (Coccinellidae) (Barbier *et al.*, 1992). In the Scarabaeinae integumentary glands have been described using the distribution of the cuticular ducts on the internal integument (Pluot-Sigwalt, 1982, 1983, 1986, 1991, 1995). However, this is the first morphological description of these glands in a Scarabaeinae using SEM and histology.

In *D. furcatum* the gland pores in the body surface are separated or grouped at pygidium, sternites and metasternum and release the glandular secretions. This distribution of glandular pores in the integument occurs in different Coleoptera species (Delachambre, 1973; Pluot-Sigwalt, 1988; Bellés & Favila, 1983; Barbier *et al.*, 1992). The ladybird *S. undecimnotata* has integumentary pores in different parts of the body grouped in a deep depression of the cuticle, the cribriform plate (Barbier *et al.*, 1992). These cribriform plates are similar to the pores with internal cribellum found in sternites of *D. furcatum*. However, Barbier et al. (1992) have pointed out the absence of secretions in this cuticular depression, such as in *D. furcatum*. The storage of secretion may be associated with the sexual maturity, which affects the glandular content in insects (Noirot & Quennedey, 1974; Favila, 1988; Da Cruz-Landim *et al.*, 2006; Scholtz *et al.*, 2009). The Scarabaeinae *C. cyanellus* and *C. indigaceus chevrolati* Harold have integumentary glandular pores similar to *D. furcatum* (Bellés & Favila, 1983; Pluot-Sigwalt, 1988). In these species, the cribellum present in the sternites are formed by dozens of contiguous pores located in a shallow depression of the cuticle. Glandular secretions may accumulate on the cribellum

of *Kheper lamarcki* MacLeay (Scarabaeinae) that releases abundant filamentous glandular secretions which accumulate in the form of cotton on the sternites being later dispersed by the male (Tribe, 1975; Burger, 2014).

The characteristics of the glands of *D. furcatum* contrast with those found in the bee *Scaptotrigona postica* Lepeletier, with spherical secretory cells, individual, and narrow excretory duct (Da Cruz-Landim *et al.*, 2006). The analysis of the cuticular duct of *D. furcatum* shows that they lead into the pores of the integument individually or in groups. The clusters of cuticular ducts are denser and more frequent in the sternites compared to the pygidium and metasternum. This pattern is similar to other Scarabaeinae, such as *C. cyanellus* (Pluot-Sigwalt, 1988) and *C. indigaceus chevrolati* (Pluot-Sigwalt, 1995), with dense aggregations of cuticular ducts in the sternites.

In *D. furcatum*, the pygidium, sternites and metasternum have two morphologically distinct integumentary glands. This is repeated in *S. undecimnotata* with an integumentary system with unicellular and bicellular glands (Barbier *et al.*, 1992). However, as in bees, morphologically similar glands can secrete different substances according to the age and physiological state of the individual (Francke *et al.*, 1983; Da Cruz-Landim *et al.*, 2006).

The three segments that make up the cuticular duct of the glandular units in *D. furcatum* are also present in the cuticular duct of the glandular units of dung beetles (Pluot-Sigwalt, 1995). In contrast to Scarabaeidae, based on the comparative analysis of different class III gland cells of Coleoptera, it was proposed that these glands in this beetle family is tricellular (Pluot-Sigwalt, 1986). However, this tricellular model of the glandular unit differs from the bicellular and multicellular glands found in *D. furcatum*, since both glands have a secretory cell and a duct cell.

The high quantity of cytoplasm granules in both glands of *D. furcatum* shows that these glands are active. The chemical composition of the secretory granules includes proteins, carbohydrates and lipids based in the histochemical tests. The behavioral studies in other species of rolling beetles indicates that the secretions have multiple biological functions, being important in intra and interspecific chemical communication (Tribe, 1975; Bellés & Favila, 1983; Favila, 1988; Favila & Díaz, 1996; Burger, 2014; Cortez *et al.*, 2015). The presence of lipids and carbohydrates in the secretion of the glands of *D. furcatum* suggests that these secretions are probably involved in

the formation of volatile substances, as sexual pheromones of rolling beetle species of the genus *Kheper* (Burger & Petersen, 2002; Burger *et al.*, 2002, 2008; Burger, 2014) and *C. cyanellus* (Halffter, 1997).

Kheper spp. males release filamentous secretions with high concentrations of proteins in the abdominal sternite (Tribe, 1975; Burger *et al.*, 1990; Burger, 2014). These proteins in the secretions have been associated to function in the chemical stability of volatile compounds (Burger *et al.*, 1990). Proteins are a solid support that maintaining the integrity of the volatiles for a longer time and over longer distances (Burger, 2014). The proteins also carrier, transport and control the diffusion of volatile sex pheromones (Burger *et al.*, 1990; Burger, 2014). *Deltochilum furcatum* perform a similar behavior (unpublished data) to the release behavior of the solid substance described in *Kheper* spp. (Tribe, 1975; Burger, 2014). This behavior, the abandonment of the food ball when the female lays the egg (Halffter, 1977) and the conservation of the food ball during hatching and larval development, suggest that *D. furcatum* impregnates the food ball with stable semiochemicals of the glands in the pygidium, sternites and metasternum like *Kheper* spp. males. Then these proteins secretions in *D. furcatum* might act in the same manner. Other biological functions such as antimicrobial peptides for the conservation of the food ball in *Nicrophorus* (Silphidae) (Hall *et al.*, 2011) cannot be ruled out.

Based in our morphological data together with the reported for other Scarabaeinae species (Pluot-Sigwalt, 1982, 1983, 1986, 1991, 1995), we propose a hypothetical model that describes the process of secretion and impregnation of the chemical compounds to the food ball by the glandular units in *D. furcatum*. The secretions are synthesized in the cytoplasm of the secretory cells. In the bicellular glands, the secretions are transferred to the folds that surround the cuticular duct. The secretions become fine as they pass through the folds, until they become a homogeneous secretion that accumulates in the cuticular duct. The cuticular duct transports and releases the filament-shaped secretion. In multicellular glands, the secretory granules present in the secretory cells release their content in to the cuticular duct that transfer it the common cuticular duct. Finally, secretion is impregnated in the food ball during its formation and rolling (Fig. 8). This model is limited to the secretory structures with cuticular ducts. But it is possible that other glands present in other regions of the body or different gland types with or without cuticular

ducts produce also substances of biological importance (Pluot-Sigwalt, 1988, 1997; Barbier *et al.*, 1992).

Our results show that *D. furcatum* has two types of glands in pygidium, sternites and metasternum. These glands synthesize and release protein, lipid and carbohydrate, which might play multiple biological roles.

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Chapter 2

Exocrine glands associated with the mandible of the dung roller beetle *Deltochilum furcatum* (Coleoptera: Scarabaeinae)

Exocrine glands associated with the mandible of the dung roller beetle *Deltochilum furcatum* (Coleoptera: Scarabaeinae)

Manuel Ix-Balam[1], Bruna de Souza Ribas [2], José Eduardo Serrão[3] and Eraldo Lima[4]

Abstract

Chemical communication plays an important role in the dung roller beetles (Scarabaeinae). However, the origin and composition of chemical substances are little known. We analyzed the structural organization and chemical nature of the secretions released of the exocrine glands associated with the mandible of the Scarabaeinae *Deltochilum furcatum*. Associated with mandibles there are mandibular and intramandibular glands, the latter described by the first time in a dung beetle. The mandibular gland has bicellular secretory units connected to a wrinkled reservoir that opens at the base of the mandible. The intramandibular glands were formed of secretory units and epithelial glands with and without reservoirs. The intramandibular secretory units are bicellular and open directly to the mandible surface. The epithelial glands with reservoirs are grouped of modified epidermal cells forming the coating of an invaginated reservoir where the secretions stored and released to the mandible surface. The epithelial glands without reservoir are hypertrophied epidermal cells in the conjunctive and the molar lobe of the mandible, releasing the secretions by diffusion through the cuticle. The mandibular and intramandibular glands were rich in proteins and carbohydrates contents. The epithelial glands with reservoirs also have lipid. We discuss the glandular structure and the possible biological functions of the secretions.

Keywords: chemical communication, dung beetle, intramandibular gland, mandibular gland, morphology

[1]Departamento de Entomologia - Universidade Federal de Viçosa, manuelbalam1786@gmail.com
[2]Departamento de Entomologia - Universidade Federal de Viçosa
[3]Departamento de Biologia Geral - Universidade Federal de Viçosa
[4]Prof. Associado IV - Departamento de Entomologia - Universidade Federal de Viçosa

1 Introduction

Chemical communication plays an important role in insects, particularly those living in social organizations (Ali & Morgan, 1990). The semiochemicals are the chemical substances responsible for this communication (Ali & Morgan, 1990; Nordlund & Lewis, 1976). The semiochemicals are produced by exocrine glands located in specific regions or distributed throughout the body of the insect (Noirot & Quennedey, 1974; Billen, 2009). The exocrine glands vary in their morphological structure, functional complexity and chemical components of their secretions (Noirot & Quennedey, 1974, 1991). The study of the morphology and localization of the exocrine glands is important to understand the chemical communication in insects (Pluot-Sigwalt, 1995). Exocrine glands have been most studied in groups such as Hymenoptera (da Cruz-Landim *et al.*, 2011; Billen, 2009; Stökl & Herzner, 2016). However, little is known about these glands in Coleoptera.

Dung roller beetles (Scarabaeinae) use excrement and carrion to form food balls that are rolled to safe sites for feeding and nesting (Halffter & Edmonds, 1982; Hanski & Cambefort, 1991). The ecological scenario in which dung beetles develop has favored their chemical communication (see Hanski & Cambefort, 1991; Favila, 2001). For example, males of the Scarabaeinae *Canthon cyanellus cyanellus* LeConte impregnate the food ball with attractive chemicals for the female (Favila, 1988), but repulsive against competitors *Calliphora* fly larvae (Bellés & Favila, 1983). The copro-necrophagous Scarabaeinae *Deltochilum furcatum* Castelnau marks the food ball with semiochemicals that inhibit the oviposition behavior of food-competing flies (Ix-Balam, 2014). The origin of these semiochemicals is associated with exocrine glands located in regions of the body with frequent contact with the food ball during rolling, such as pygidium and sternites (Pluot-Sigwalt,1995; Ix-Balam, et al., in prep.). Regions such as mouthparts can produce and release compounds associated with chemical communication. Cambefort (1984) points out that beetles *Gymnopleurus coerulescens* Olivier (Scarabaeinae) recognize each other by touching mouthparts. This behavior is also reported in *C. c. cyanellus* (Favila, 2001) and *D. furcatum* (personal observations). Within mouthparts, the mandibles are active elements during the consumption, cutting, and formation of the food ball in dung beetles (Hata & Edmonds, 1983). Edmonds (1972) reported that *Phanaeus* spp. have glands at the base of the mandibles. Subsequently, Pluot-Sigwalt (1988), using the structure of the cuticular ducts of the secretory units,

describes the mandibular glands in two species of *Canthon* (Pluot-Sigwalt, 1986). However, the morphological structure of mandibular glands, as well as the chemical nature of the secretions, are still unknown. The objective was to describe the morphology and histochemistry of exocrine glands associated with the mandible of *D. furcatum*.

2 Methods and Materials

2.1 Biological material

Adults of *D. furcatum* were collected at the Reserva Florestal Mata do Paraíso (20°45'22"S/ 42°51'44"W), municipality of Viçosa, Minas Gerais State, Brazil. To the capture of the beetles was used fresh meat baited pitfall traps. The beetles were individually reared at room temperature in plastic boxes of 1000 mL containing humid soil as the substrate. They were fed with ground meat once a week (Ix-Balam, 2014).

2.2 Scanning electron microscopy

Two individuals of each sex were cryoanesthetized at -4°C for 5 min. After, the mandibles were dissected in saline solution (0.1 M NaCl, 0.1 M KH_2PO_4, 0.1 M Na_2HPO_4), and transferred to Stefanini fixative solution pH 7.3 (Stefanini *et al.*, 1967) at 5°C for 24 h. Then, the samples were transferred to 5% KOH at 100°C for 5 minutes, washed in distilled water and dehydrated in a graded ethanol series. After, the samples were transferred to hexamethyldisilazane for 10 minutes and air dried. Samples were attached to aluminum stubs, gold covered (20 nm thick) and analyzed in a scanning electron microscope (SEM) LEO VP1430.

2.3 Light microscopy

Three individuals of each sex were cryoanesthetized. The mandibles were dissected and transferred to Stefanini fixative solution at 5°C for 24 h. Subsequently, the samples were washed with 0.1 M phosphate buffer saline (PBS) 0.1 M, pH 7.2, and dehydrated in graded ethanol series (70, 80, 90, 95°). After, the samples were embedded in JB4 historesin for 24h at 5°C. Histological sections (3 μm in thick) were obtained with a microtome Leica RM 2255 with glass knife. The samples were stained with Harris hematoxylin and eosin. Some histological sections were submitted to the following histochemical tests: mercury-bromophenol blue to detection of total proteins, periodic Acid-Schiff (PAS) to detection polysaccharides and glucoconjugates, and 1% osmium tetroxide for lipid identification (Pearse, 1985; Bancroft & Gamble, 2008).

2.4 Terminology

The terminology used in the description of the mandibular regions were in agreement with Miller (1961) and Edmonds (1972). The cell types of glands follow classification of Noirot and Quennedey (1974).

3 Results

The mandibles were located dorsally below the clypeus and labrum, with the half apical membranous and the bases strongly sclerotized. Both sexes of *D. furcatum* had mandibular and intramandibular glands (Figs. 1 and 4).

3.1 Mandibular gland

The mandibular gland was located in a cuticular receptacle at the base of the mandible and extended posteriorly between the molar lobe and the adductor muscle (Fig. 2A). The cuticular receptacle had muscular tissues associated to its cuticular wall. The mandibular gland had numerous superimposed secretory units forming a mass of cells surrounding the abductor muscle. The secretory units were globular-shaped with variable size and smooth surface with a long and narrow cuticular duct of approximately 1 μm of diameter (Fig. 2B). The secretory units of the mandibular gland were distributed near the external base of the incisor lobe (Fig. 3A). The secretory unit were bicellular, with a secretory cell and a ductile cell. The secretory cell had small granules in the cytoplasm and one or two spherical well-developed nucleus of approximately 24 μm in diameter (Fig. 3B, C). The ductile cell extended into the secretory cell, surrounding the cell nucleus. The cytoplasm granules were transferred to the ductile cell and then to the cuticular duct (Fig. 3D). The cuticular duct spread out of the secretory cell to open in a wrinkled reservoir. The reservoir had a layer of flattened epithelial cells with small nucleus of 4 μm in diameter. The inner wall of the reservoir was lined by a thin cuticle (Fig. 3E). The enlarged reservoir opens at the base of the mandible.

3.2 Intramandibular glands

The exocrine intramandibular glands were divided into three types according to their organization: secretory units (I), epithelial glands with reservoir (II) and epithelial glands without reservoir (III) (Fig. 4).

3.2.1 Secretory units (I)

They were located in the incisor lobe of the mandible and the proximal end of the molar lobe (Fig. 4). The secretory unit was bicellular with a voluminous secretory cell and a poorly visible ductile cell due to the nearness of the secretory unites, that do difficult to recognize the limits between adjacent secretory cells (Figs. 4 and 5A). The secretory cell had small granules in the cytoplasm and a nucleus of approximately 18 μm in diameter. The cuticular duct with an approximate 1 μm in diameter extended into the secretory cell (Fig. 5B). The cuticular ducts open individually in pores in the mandibular cuticle (Fig. 2C).

3.2.2 Epithelial glands with reservoir (II)

These multicellular glands were concentrated near the apex of the mandible and less frequently in the lateral margin and base of the incisor lobe (Fig. 4). Despite being close, the limits between cells were visible. These glands were globular-shaped with diameters varying from 40 to 130 μm (Fig. 5C). The gland was integrated by numerous modified secretory epidermal cells with a nucleus of 6.5 μm in diameter. The secretory epidermal cells formed the coating of an invaginated reservoir where the synthesized secretions were stored. The reservoir was connected to one pore crossing the mandible cuticle (Figs. 2C and 5D).

3.2.3 Epithelial glands without reservoir (III)

They were hypertrophied epidermal cells located in the mandibular conjunctive and the distal end of the molar lobe (Figs. 4 and 7). The secretory cells were columnar with nucleus of approximately 7 μm in diameter. The secretory cells had not differentiated structures for the storage or transport of the secretions outside the mandible (Figs. 6 and 7A). In the mandibular conjunctive, the thickness of the cuticle is reduced. The glandular epithelium, which forms a large exocrine structure, releases the secretions in a striated border at the base of the setae that cover the surface of the conjunctive (Fig. 6). In the molar lobe, the secretions crossed the thick cuticle wall through spaces and interstitial ducts; the secretions accumulated in cavities located between molar ridges that had slots in the distal end where the secretion was released (Fig. 7B, C).

3.3 Histochemistry

The epithelial glands with reservoir were positive for proteins, lipids and carbohydrates, whereas in the epithelial glands without reservoir, intramandibular secretory units and mandibular glands lipids were not detected.

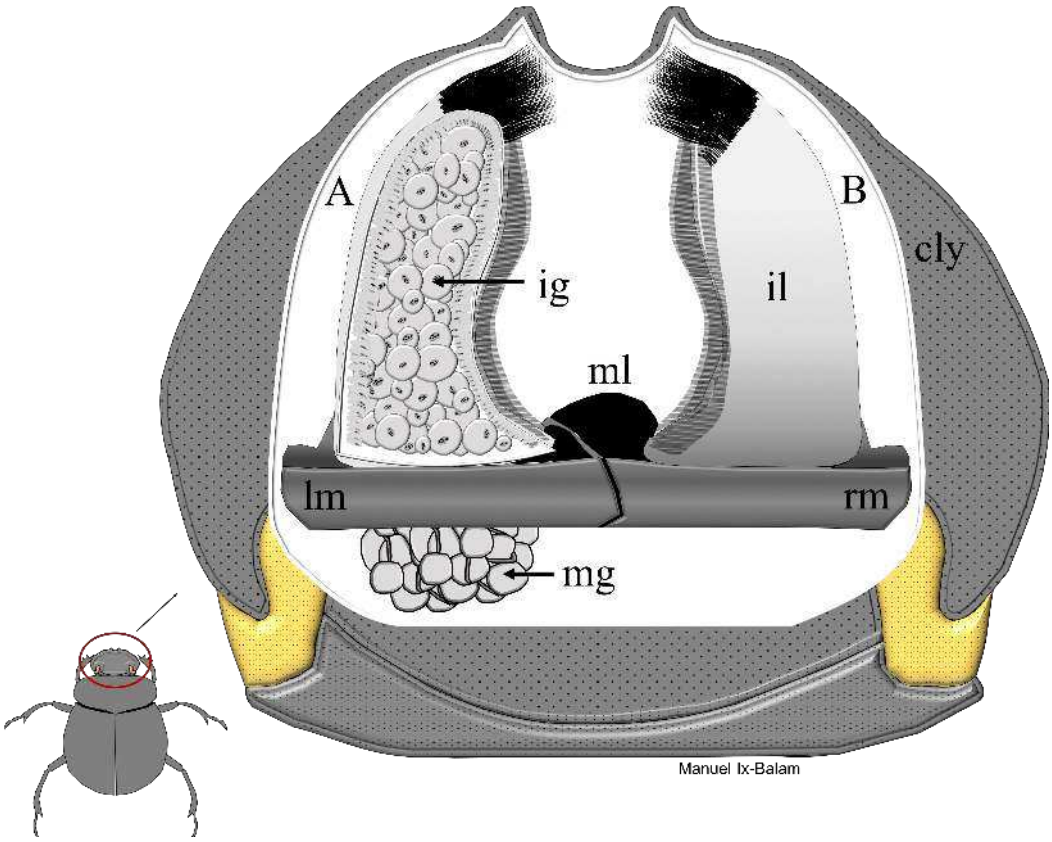

Figure 1. Representation of the mandibular and intramandibular glands of *Deltochilum furcatum* (dorsal view) (without scale). A, the position of the mandibles inside the cephalic capsule and position of the glands in the mandible is shown. B, regions of the mandible. be, basal end of the incisor lobe; cil, comb of incisor lobe; cly, clypeus; ig, intramandibular gland; il, incisor lobe; lm, left mandible; mg, mandibular gland; ml, molar lobe; rm, right mandible.

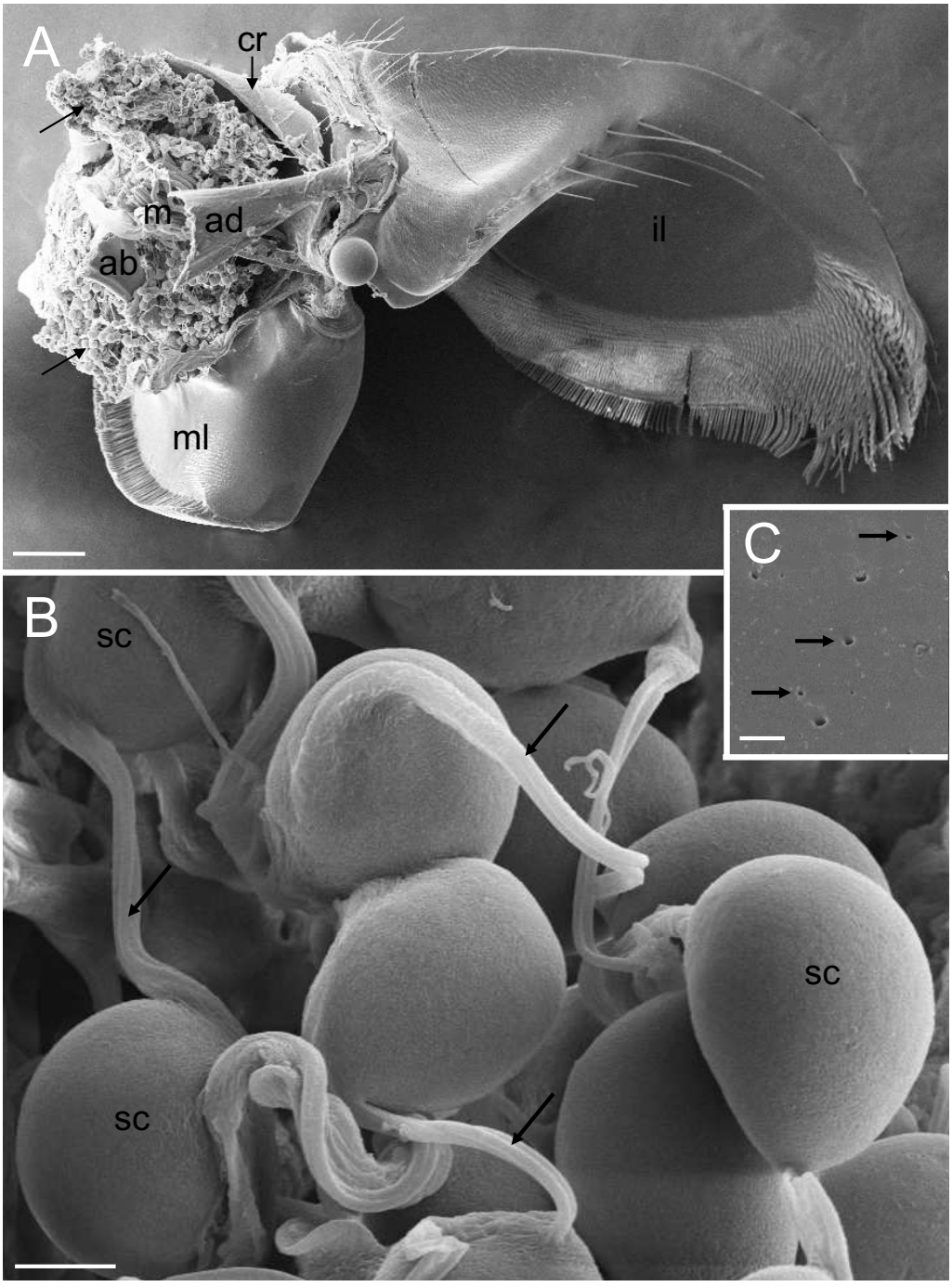

Figure 2. Mandibular gland of *Deltochilum furcatum* under SEM. A, mandible (ventro-lateral view). Note the cuticular receptacle (cr) where the secretory units are located (black arrows). B, details of the secretory units. Note the secretory cell (sc) and cuticular duct (black arrows) associated with the sc. C, glandular pores on the surface of the mandible. Note that the pores have different diameters (black arrows). ab, abductor muscle; ad, adductor muscle; ml, molar lobe; il, incisor lobe; m, muscle tissue. Scale bars: 200 μm (A); 10 μm (B); 30 μm (C).

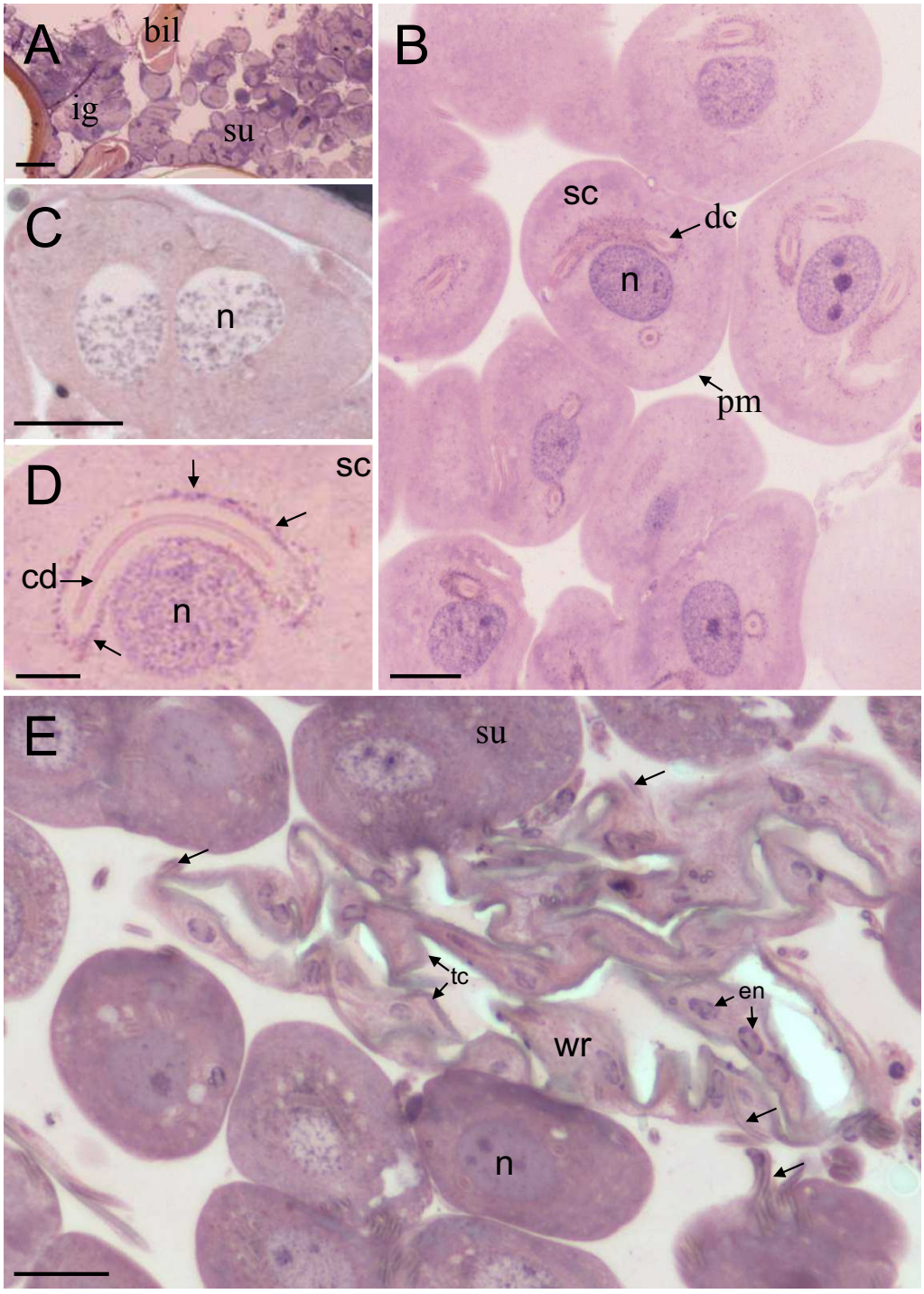

Figure 3. Secretory units of the mandibular gland of *Deltochilum furcatum*. Hematoxylin and eosin stained (Fig. A-D) and osmium tetroxide (Fig. E). A, limit between secretory units (su) and intramandibular glands (ig) at the base of the mandible. B, details of the secretory units. Note the ductile cell (dc) and secretory cell (sc). C, secretory unit binuclear. D, details of the ductile cell. Note the cuticular duct (cd) inside the ductile cell and granules (black arrows) around the ductile cell. D, details of the wrinkled reservoir. Note the cuticular ducts of the secretory units opening in the reservoir (black arrows). bil, incisor lobe; en, epithelial cell nucleus; n, nucleus of the secretory cell; tc, thin cuticle; wr, wrinkled reservoir. Scale bars: 100 μm (A); 20 μm (B, C, E); 10 μm (D).

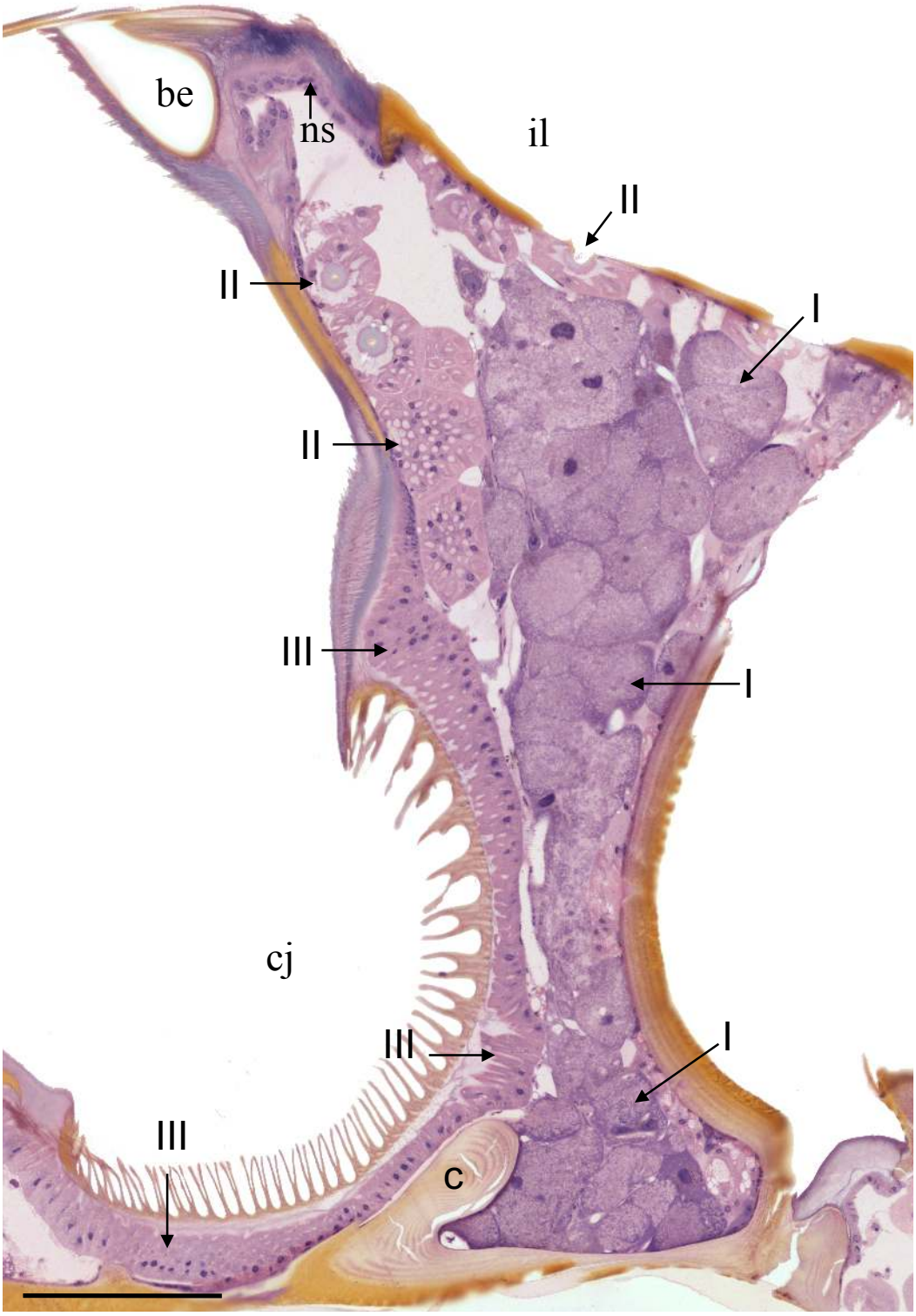

Figure 4. Histological section of the intramandibular glands of *Deltochilum furcatum*. Hematoxylin and eosin stained. Note the types of glands (I-III) and regions of the mandible. Note that the cut is oblique. I, secretory units; II, epithelial gland with reservoir; III, epithelial gland without reservoir; be: basal end of the incisor lobe; c, cuticle; cj, conjunctive; il, incisor lobe; ns, non-secretory epidermal cells. Scale bar: 200 μm.

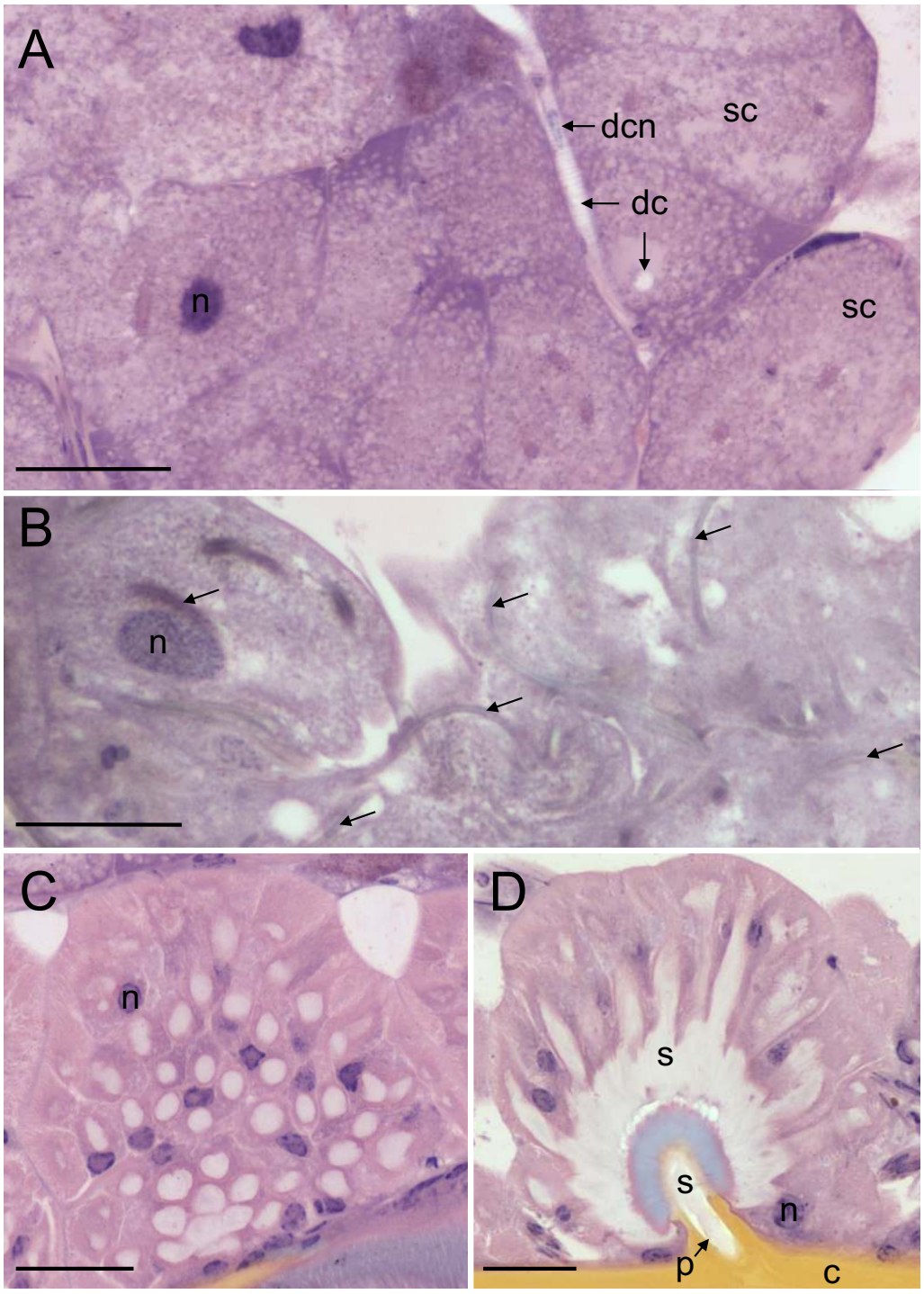

Figure 5. Intramandibular glands of *Deltochilum furcatum*. Hematoxylin and eosin stained (Fig. A,C,D) and osmium tetroxide (Fig. B). A-B, secretory units. Note tightly clustered secretory cells (sc), ductile cell (dc), cuticular ducts (black arrows) and nuclei (n). C, epithelial gland with reservoir (EGR). Note that each nucleus corresponds to a modified epidermal cell. D, Details of the invaginated reservoir of the EGR. Note the secretions (s) are released in the pore (p) that goes through the cuticle (c). dcn, ductal cell nucleus. Scale bars: 50 μm (A); 30 μm (B,C,D,).

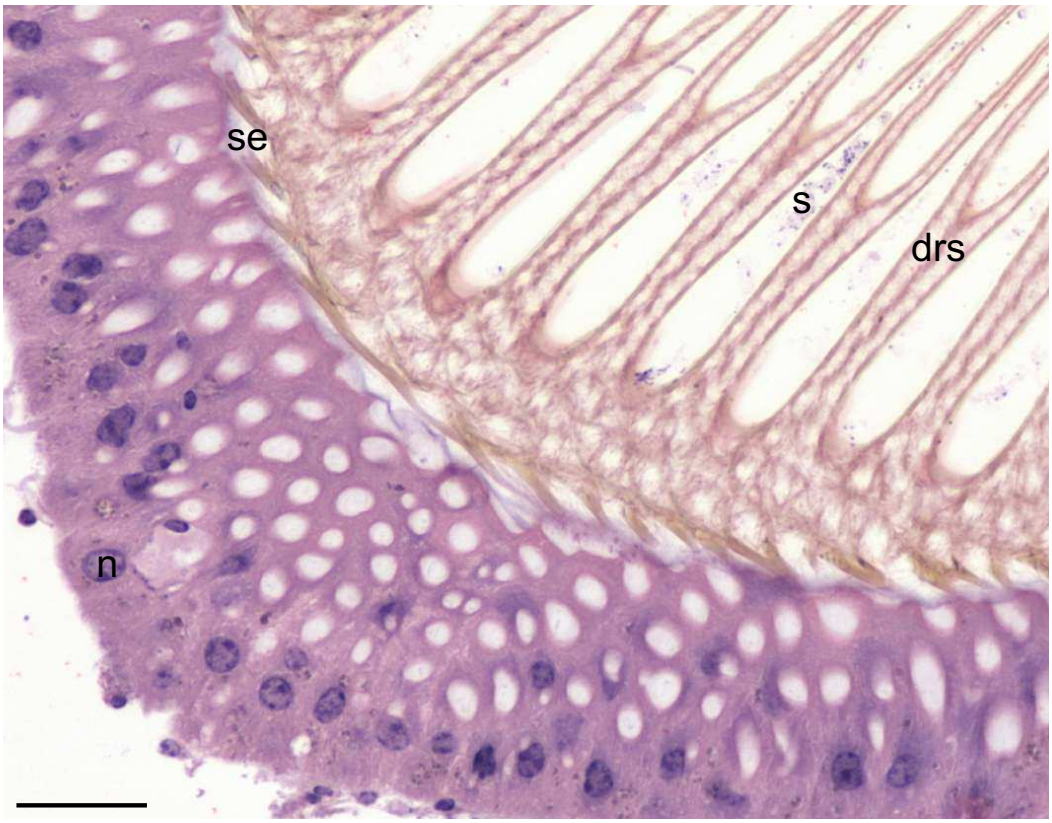

Figure 6. Epithelial glands without reservoir of the mandibular conjunctive of *Deltochilum furcatum*. Hematoxylin and eosin stained. Note that the cut is oblique and each nucleus (n) corresponds to a secretory epidermal cell. Striated edge (se), secretions (s), double rows of setae (drs). Scale bar: 30 μm.

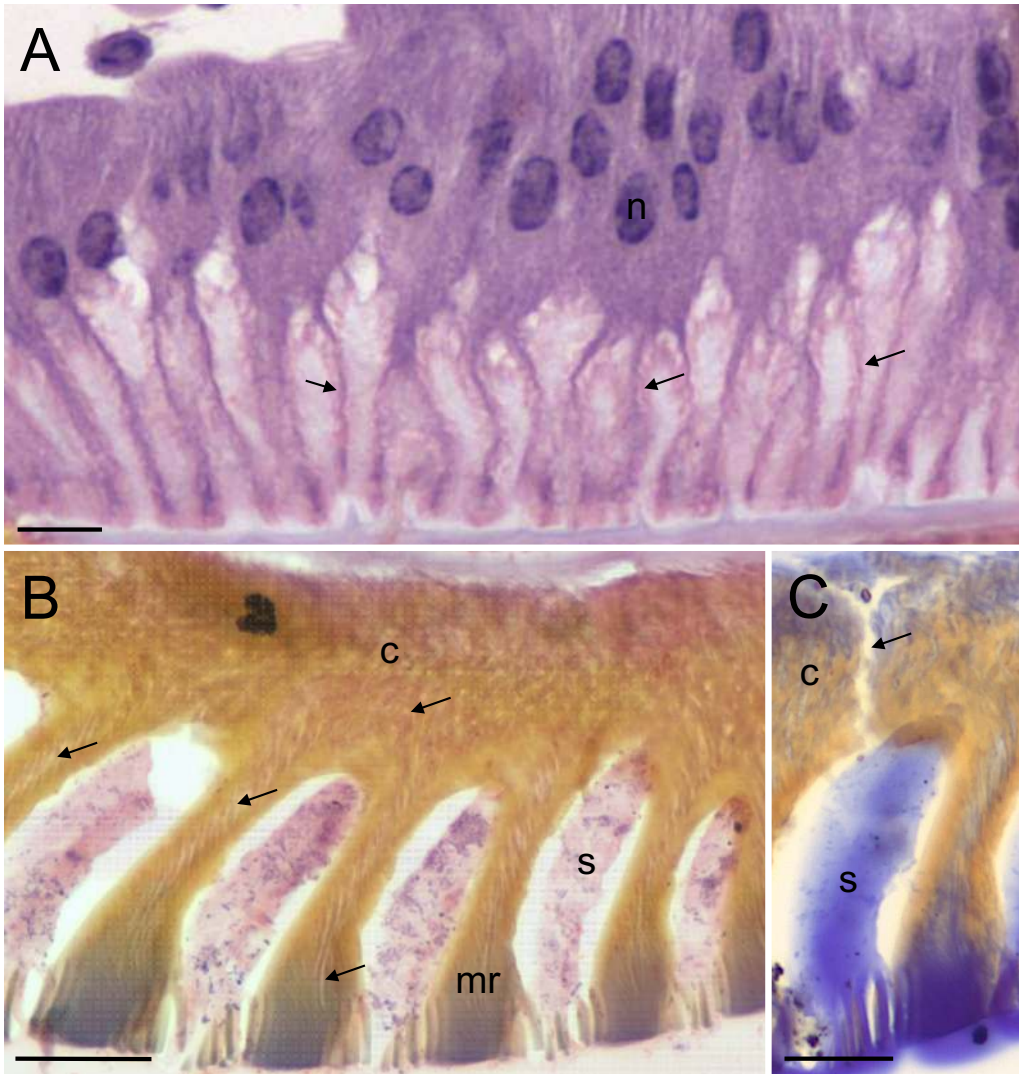

Figure 7. Epithelial glands without reservoir of the molar lobe of *Deltochilum furcatum*. Hematoxylin and eosin stained (Fig. A,B), Mercury-bromophenol blue (Fig. C). A, secretory cells with their nuclei and structures reminiscent of microvilli (arrows). B, details of the cuticle of the molar lobe. Note the secretions (s) crossing the thick cuticular wall (c) of the molar lobe through interstitial spaces (arrows) and accumulation of secretions in cavities between the molar ridges (mr). C, details of an interstitial duct (arrow) (the blue coloration indicates the presence of proteins in the secretions). Scale bars: 10 μm (A); 30 μm (B); 20 μm (C).

4 Discussion

We demonstrate the presence of mandibular and intramandibular glands in both sexes of *D. furcatum*. The mandibular gland of *D. furcatum* consists of bicellular secretory units around a reservoir. Each secretory unit is composed of a secretory cell and a ductile cell, the latter with a cuticular duct connected to the reservoir. The secretory units belong to class III glands according to Noirot and Quennedey (1974, 1991). *Melipona scutellaris* Latreille (Apidae), *Polyergus rufescens* Latreille (Formicidae), and the parasitoid *Leptopilina heterotoma* Thomson (Figitidae) have a structural organization of the mandibular gland similar to that found in *D. furcatum* (Grasso *et al.*, 2004; Santos *et al.*, 2015; Stökl & Herzner, 2016). All the species mentioned above have bicellular secretory units associated with a reservoir that releases the secretions at the base of the mandible. The mandibular reservoir of *D. furcatum* is surrounded by secretory units, without associated muscles, suggesting a mechanical release of secretions during the mandibular opening as a result of the increasing pressure of head hemolymph (Grasso *et al.*, 2004). However, the muscles adhered to the wall of the cuticular receptacle might also generate movements of the receptacle that help the release of the secretions (Edmonds, 1972). In Coleoptera, mandibular glands are reported in *Calochroa sexpunctata* Fabricius (Cicindelidae) and *Stenocentrus ostricilla* Newman and *Syllitus grammicus* Newman (Cerambycidae) (Moore & Brown, 1971; Pluot-Sigwalt, 1997). However, details of its morphology are poor.

In dung beetles, mandibular glands are reported or described in species of *Phanaeus*, *Canthon* and *Onthophagus* (Edmonds, 1972; Pluot-Sigwalt, 1988, 1997). In all cases, the mandibular gland is located in a cuticular receptacle at the base of the mandible. However, only in *Canthon* and *Onthophagus* are the presence of class III secretory units openin into the reservoir. *Deltochilum furcatum* has bicellular secretory unit that differ from tricellular ones (two secretory cells and a ductile cell) found in *C. c. cyanellus* and *Canthon indigaceus chevrolati* Harold (Pluot-Sigwalt, 1988). However, the tricellular conformation is based on the segments that make up the cuticular duct of secretory units described in Coleoptera species not belonging to Scarabaeidae (Pluot-Sigwalt, 1986).

Secretory cell of the mandibular gland of *D. furcatum* has large nucleus and cytoplasm with secretions. The large nucleus of secretory cells and secretions in the cytoplasm show metabolic

activity and production of compounds from the secretory units (Stökl & Herzner, 2016). These secretory units synthesize and release compounds associated with proteins and carbohydrates. The active role of the mandibles during feeding (Hata & Edmonds, 1983), suggests that these secretions may be associated with digestive enzymes or energy-supplying molecules, such as glycogen (Santos *et al.*, 2015).

On the other hand, in social insects the functions of mandibular glandular secretions are also associated with the lifestyle of the individual. For example, in *Solenopsis invicta* Buren (Formicidae) the mandibular secretion acts as a pheromone for mating flight initiation and as alarm pheromones (Vander-Meer *et al.*, 2010; Choi & Vander-Meer, 2015). A role in sex attraction and male aggregation activity has also been suggested (Choi & Vander-Meer, 2015). In virgin queens of *P. rufescens* secretion acts as sex pheromones (Grasso *et al.*, 2003). In ants of the genera *Crematogaster* and *Pseudomyrmex* the secretions are associated with pheromones of alarm and defense (Wood *et al.*, 2002; Wood, 2005). In workers of *Calomyrmex* sp. (Formicidae) secretion shows inhibitory and antimicrobial activity against soil microorganisms (Brough, 1983). In *Trigona hyalinata* Lepeletier (Apidae), it acts as a recruitment pheromone (Nieh *et al.*, 2003). Even in the parasitoid *L. heterotoma*, the secretion acts as allomona against predatory ants (Stökl *et al.*, 2012; Stökl & Herzner, 2016). In Coleoptera Cerambycidae *Stenocentrus ostricilla* Newman and *Syllitus grammicus* Newman, mandibular glands are associated with a defensive function (Moore & Brown, 1971). In dung beetle, for example in *Canthon* species is emphasized that the mandibular secretions can be impregnated to the food ball during the cutting and formation of the ball (Pluot-Sigwalt, 1988; Favila, 2001). The friction of the incisor lobe with the labrum setae and serous brush of the galeae and laciniae during mandibular movements (Hata & Edmonds, 1983), could facilitate the dispersion of these semiochemicals to the food ball and at close range, as in the dispersion of sexual pheromones of *Kheper nigroaeneus* Boheman (Tribe, 1975). In Diptera, the function these secretions would have on the food ball is associated with allomones against *Calliphora* larvae (Bellés & Favila, 1983; Favila, 2001), although it could also act against adults of *Lucilia cuprina* Wiedemann (Ix-Balam, 2014). Attractive effect at close range on the female of *C. c. cyanellus* and antimicrobial activity and conservation of the food ball, are also suggested (Favila1988; see Favila, 2001). The behavioral observations in dung beetles *G. coerulescens*, *C. c. cyanellus* and *D. furcatum* also suggest a role in intraspecific and

interspecific recognition (personal observations; Cambefort, 1984; Favila, 2001). A preliminary analysis of the mandibular secretions of *C. c. cyanellus* revealed the presence of carboxylic acids and phenolic acids, such as benzoic acid and phenylacetic acid respectively (Favila, 2001). These two compounds also occur in the pygidial secretions of *C. c. cyanellus* with functions associated with defense and antimicrobial (Cortez *et al.*, 2015).

The intramandibular gland of *D. furcatum* consists of secretory units and epithelial glands with and without reservoirs. To our knowledge, this is the first study to report intramandibular glands in a dung beetle. The intramandibular glands belong to class I (epithelial glands) and class III (secretory units) according classification of Noirot and Quennedey (1974, 1991). The intramandibular glands of bees *Plebeia emerina* Friese, *Melipona quadrifasciata* Lepeletier and *M. scutellaris* are also of classes I and III glands (Santos *et al.*, 2009; da Cruz-Landim *et al.*, 2011; Santos *et al.*, 2015). The simultaneous presence of class I and III glands indicates an independent action of these glands and the release of different secretions at different times (da Cruz-Landim *et al.*, 2011; Santos *et al.*, 2015). The conformation of the intramandibular secretory units of *D. furcatum* is similar to that of ants, with a voluminous secretory cell and a ductile cell opening directly to the outside (Schoeters & Billen, 1994; Grasso *et al.*, 2004). The absence of a membranous reservoir associated with the secretory units in *D. furcatum*, also occurs in ants and bees (Schoeters & Billen, 1994; Santos *et al.*, 2009; da Cruz-Landim *et al.*, 2011; Grasso *et al.*, 2004; Santos *et al.*, 2015).

The epithelial glands with reservoirs are made up of modified secretory epidermal cells that form the lining of an invaginated reservoir that empties into a pore. Martins et al (2015a) describe in the ant *Atta laevigata* Smith intramandibular glands formed by epidermal cells that surround a reservoir space. However, Billen and Al-Khalifa (2016) suggest that the microscopic images of said glands correspond to sensillar structures. Epithelial glands with invaginated reservoir also occurs in legs of ants (Billen, 2009). In *D. furcatum*, the epithelial glands without reservoirs form the glandular epithelium of the conjunctive and molar lobe. In *Brachyponera sennaarensis* Schmidt and Shattuck (Formicidae), the intramandibular glandular epithelium is conformed of cylindrical cells with nuclei of 8-10 μm in diameter (Billen & Al-Khalifa, 2016). In *M. quadrifasciata*, the glandular epithelium is composed of hypertrophied flat cells only in certain regions (da Cruz-Landim *et al.*, 2011). Likely in the molar glandular epithelium of *D. furcatum*,

in *B. sennaarensis* and *M. quadrifasciata* release the secretions of the glandular epithelium occurs through interstitial ducts that cross the thick cuticle.

The secretions of the intramandibular glands are associated with proteins and carbohydrates. Also, the epithelial glands with reservoirs presented lipids. In *A. laevigata* the presence of lipids in the intramandibular glands indicates a release of volatile compounds (Martins *et al.*, 2015a). In *Neoponera villosa* Fabricius (Formicidae) the secretions of the intramandibular glands are associated with alarm and trail pheromones (Martins *et al.*, 2015b). The compounds of the intramandibular secretory units of *M. scutellaris* and *P. rufescens* are associated with a lubricating function of the mandible (Grasso *et al.*, 2004; Santos *et al.*, 2015). In *M. quadrifasciata* the epithelial gland releases compounds associated with cuticular hydrocarbons (da Cruz-Landim *et al.*, 2011), which play an important role in social insects acting in intraspecific recognition (Ginzel & Blomquist, 2016). In *D. furcatum*, the location of the conjunctive and molar lobe in the oral cavity, as well as the role of these structures in the feeding (Miller, 1961; Hata & Edmonds, 1983), suggest a digestive function and lubrication of the secretions of its glandular epithelia.

In conclusion, we demonstrate the presence of mandibular and intramandibular glands in *D. furcatum*. The structural characteristics of these glands are similar to that of social insects like bees and ants, who have a complex chemical communication system. The glands in this dung beetle synthesize and release substances associated with proteins, carbohydrates and lipids with multiple possible biological functions.

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General Conclusions

This work show that *Deltochilum furcatum* has exocrine glands in regions of the body associated with the formation-manipulation of the food ball. We demonstrate that *D. furcatum* has bicellular and multicellular glandular units in regions of the body that come into frequent contact with the food ball during rolling. These glands synthesize and release protein, lipid, and carbohydrate. These chemical compounds are impregnated into the food ball during rolling and which might play multiple biological roles, as sex pheromones and allomones. *Deltochilum furcatum* has mandibular and intramandibular glands, the latter described by the first time in a dung beetle. The mandibular gland has bicellular secretory units connected to a wrinkled reservoir that opens at the base of the mandible. The intramandibular glands were formed of secretory units and epithelial glands with and without reservoirs. The simultaneous presence of glands of different conformation indicates an independent action of these glands and the release of different secretions at different times. Among the possible functions of mandibular secretions are compounds associated with food preservation, couple attraction at close range and sexual recognition, among others. These results, together with the proposed model, contribute to the morphological and production-release understanding of secretions of these exocrine glands. It also allows to understand the behavioral and chemical ecology aspects of dung beetles.