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REVIEW

The amazing complexity of insect midgut cells: types, peculiarities, and functions

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Abstract

The insect midgut epithelium represents an interface between the internal and the external environment and it is the almost unique epithelial tissue by which these arthropods acquire nutrients. This epithelium is indeed able to produce digestive enzymes and to support vectorial transport of small organic nutrients, ions, and water. Moreover, it plays a key role in the defense against pathogenic microorganisms and in shaping gut microbiota. Another important midgut function is the ability to produce signaling molecules that regulate its own physiology and the activity of other organs. The two main mature cell types present in the midgut of all insects, i.e., columnar and endocrine cells, are responsible for these functions. In addition, stem cells, located at the base of the midgut epithelium, ensure the growth and renewal of the midgut during development and after injury. In insects belonging to specific orders, midgut physiology is deeply conditioned by the presence of unique cell types, i.e., goblet and copper cells, which confer peculiar features to this organ. This review reports current knowledge on the cells that form the insect midgut epithelium, focusing attention on their morphological and functional features. Notwithstanding the apparent structural simplicity of this organ, the properties of the cells make the midgut a key player in insect development and homeostasis.

Keywords Insect midgut · Midgut lumen pH · Columnar cell · Stem cell · Endocrine cell · Goblet cell · Copper cell

Introduction

Insects are the dominant form of animal life, both in terms of species richness and abundance. The evolutionary success of these organisms is demonstrated by the number of terrestrial ecological niches that they have colonized and the variety of food substrates they are able to exploit. The exceptional variety of feeding habits is reflected in the morphofunctional adaptations of the alimentary canal that characterize this taxon (Terra 1988).

The ancestral insect was likely somewhat similar to a cockroach, thus a generalist feeder with chewing mouthparts and a tubular, slightly convoluted gut. Feeding differentiation and specialization in respect to the basal feeding habit were accompanied with a concurrent evolution of mouthparts, as well as gut morphology and physiology (Nation 2008; Chapman 2013). This evolution was driven by food texture, with gut of liquid feeders that tends to be more long, narrow, and convolute in contrast with a short, wide, and straight alimentary canal often provided with a peritrophic matrix (PM) of solid feeders (Lehane 1997). The nutritional quality of the food is another important issue that led to the specialization of the insect gut. Animal-based diets are nutritionally well-balanced but often irregularly available; thus, insects generally have guts with large capacity and structures (e.g., the crop) to store the ingested food that is thereafter slowly digested. Although plant food is available without limitation, its unbalanced nutrient composition forces insects to feed frequently and abundantly and the gut does not fulfill storage functions. A case in point of extreme morphofunctional specialization is represented by the filter chamber present in some hemipteran insects that feed on plant sap (Billingsley and Lehane 1996). The arrangement of their gut allows the intimate contact of the anterior and posterior part of the alimentary canal, the effective removal of the excess of water intake, and the concomitant concentration of nutrients and ions (Hubert et al. 1989; Billingsley and Lehane 1996; Le Caherec et al. 1997).

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Regardless of the variety in general morphology, the insect gut is a single layer of epithelial cells supported by a basal lamina surrounded by muscles. The gut is organized in three main regions with different features, functions, and embryonic origin (i.e., the foregut, the midgut, and the hindgut) (see Fig. 1 for an overview on the organization and functions of the insect gut) (Nation 2008; Chapman 2013).

The midgut is the functional core of the alimentary canal since it accomplishes food digestion and nutrient absorption (Nation 2008; Chapman 2013) (Fig. 1). The enzymes responsible for food digestion are secreted in the lumen by columnar cells, the major cell type present in the midgut of all insects, or are membrane proteins located in the apical membrane of these cells (Giordana et al. 1982; Terra 1988). Lateral diverticula, called gastric caeca, may arise in the initial part of the midgut, and more rarely along the midgut. In some cases, they are the major site of digestion and absorption (Nation 2008). In some insects, midgut cells are also responsible for the production of the PM, a gel-like structure that lines the midgut of most insects and (i) protects cell microvilli from the abrasion by food particles, (ii) delimits the site of action of different classes of hydrolytic enzymes, and (iii) avoids the contact between pathogens and the midgut epithelium (Terra 1988; Lehane 1997; Hegedus et al. 2009). Hemipterans (and some other paraneopterans) lack PM and evolved a lipoprotein membrane, i.e., the perimicrovillar membrane, that covers like a glove the microvilli of midgut cells (Silva et al. 2004; Chapman 2013). As the PM, this structure acts as a mechanical barrier and protects the intestinal epithelium from physical abrasion and toxic compounds but, in addition, confers to the insect the capacity to absorb the extremely diluted nutrients in the diet, i.e., amino acids (Silva et al. 2004; Chapman 2013).

The midgut represents an effective barrier for the entrance of pathogens into the insect body cavity and it is also the

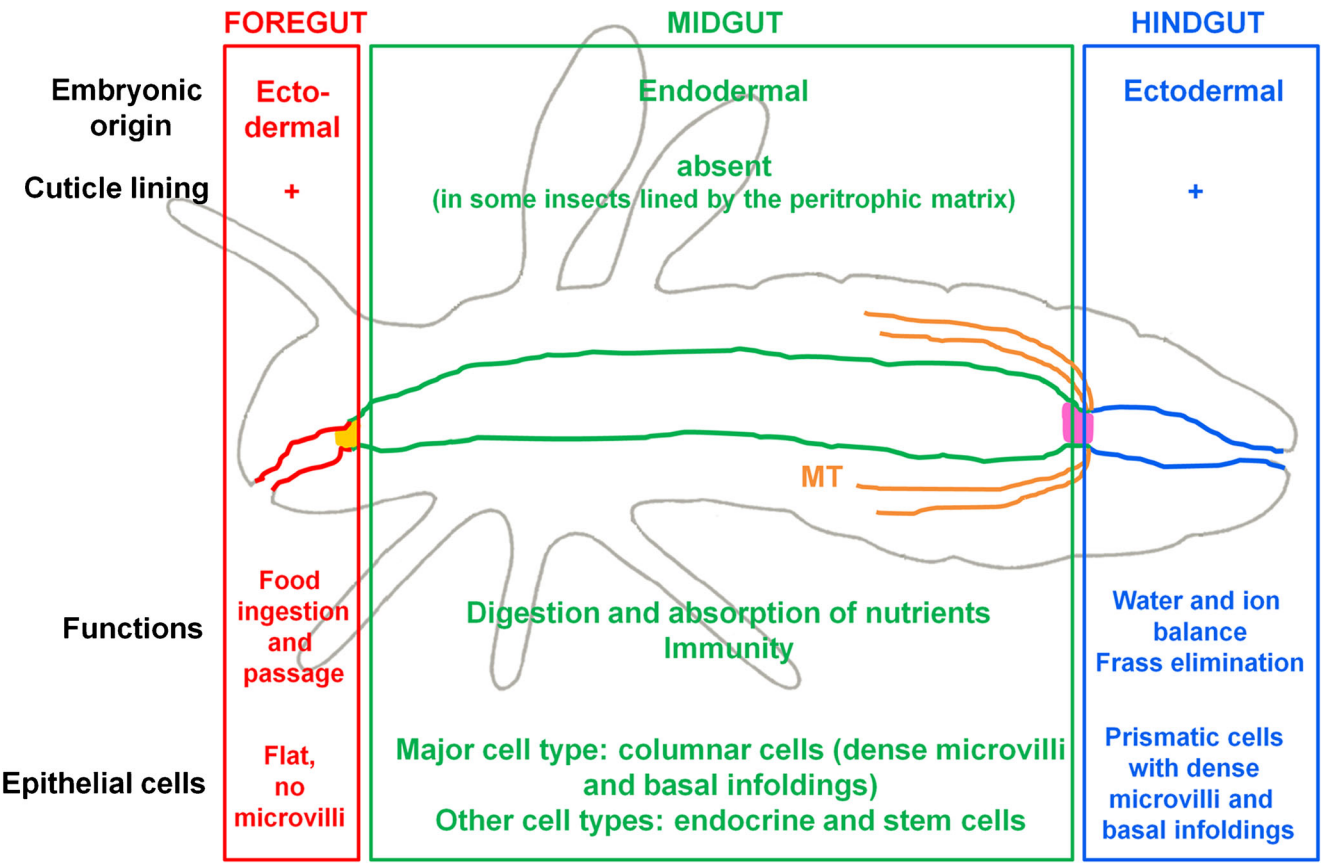

Fig. 1 Overview of common and general features of insect alimentary canal. The gut is a tube formed by a monolayered epithelium running from the mouth to the anus, and it starts with the foregut. The latter, lined by an unsclerotized cuticle called intima, is concerned with food passage to the midgut. In some insects, this region is assigned to mechanical disruption of the food and/or modified to form diverticula for food storage (e.g., the crop). The cardiac sphincter (in yellow), of variable tightness, separates the foregut from the midgut. This region of the gut is protected in some insects by the peritrophic matrix, a gel-like acellular structure produced by midgut cells or specialized cells in the cardiac region and composed by chitin and proteins with variable glycosylation level. The midgut ends with the pyloric sphincter (in pink), followed by the hindgut. Apart from some exceptions, the hindgut functions together with Malpighian tubules (MT, thin and blind tubules that collect the primary urine and pour it into the hindgut just after the pyloric valve) as excretory organ. The hindgut modifies the primary urine, thus contributing to insect ion and water balance. The mixture of modified urine and feces is removed through the anus

second more active immunological organ after the fat body, in reason of the production of antibiotics (e.g., antimicrobial peptides (AMPs) and reactive oxygen species) and the management of resident microbiota avoiding dysbiosis (Ryu et al. 2010; Douglas 2015; Huang et al. 2015; Wu et al. 2018). For these reasons, it has been the object of a number of studies aiming to clarify the pathways involved in AMP production, analyze the effects of the diet on AMP synthesis, and isolate new AMPs (Vizioli et al. 2001; Ursic-Bedoya et al. 2011; Mylonakis et al. 2016; Vogel et al. 2018).

In many insects, the midgut is characterized by a complex morphofunctional regionalization. In some flies (i.e., non-hematophagous brachycerous Diptera), where this specialization is particularly evident, the different midgut regions (anterior, middle, and posterior) are characterized by peculiar features, such as columnar cell morphology, presence of atypical cell types, expression of genes encoding for cell proteins and digestive enzymes, luminal pH, and even microbiota load and composition (Terra et al. 1988; Lemos and Terra 1991; Buchon et al. 2013a, b; Broderick et al. 2014; Buchon and Osman 2015; Pimentel et al. 2018; Bonelli et al. 2019; Bruno et al. 2019a).

Insect midgut has emerged as an interesting model to investigate tissue recovery after injury by pathogens (Castagnola and Jurat-Fuentes 2016; Dubovskiy et al. 2016; Janeh et al. 2017). Studies in *Drosophila melanogaster* have indeed individuated major highlights of midgut stem cell–mediated response to bacteria-induced tissue damage (Nászai et al. 2015; Bonfini et al. 2016; Jiang et al. 2016) and, importantly, the key role of midgut cell proliferation for the establishment of insect vector competence (Baton and Ranford-Cartwright 2007; Janeh et al. 2017; Taracena et al. 2018). Furthermore, midgut tissue remodeling has been studied in detail in holometabolous insects that, during metamorphosis, undergo profound changes, including the complete renewal of the alimentary canal (Hakim et al. 2010; Franzetti et al. 2012; Romanelli et al. 2016; Malta et al. 2017; Tettamanti and Casartelli 2019; Tettamanti et al. 2019).

The insect midgut is the target for economically important entomopathogens used in pest control (e.g., *Bacillus thuringiensis* and baculoviruses) and, as such, it has been intensively studied within the framework of the fine characterization of the mode of action of these microbes and their potent virulence factors (Clem and Passarelli 2013; Pardo-López et al. 2013; Lacey et al. 2015). On the other hand, the midgut is a barrier for a number of promising bioinsecticidal molecules, individuated so far and yet to be discovered, that have their target in insect body cavity (i.e., the hemocoel) and that, after ingestion, may be unable to cross the gut epithelium and reach the hemocoel in an unaltered form and/or in sufficient quantity to exert their activity (Whetstone and Hammock 2007; Hughes et al. 2012; Bonning and Chougule 2014). For this reason, and due to the pressing need to enrich the poor repertoire of commercially available bioinsecticides, this issue has greatly stimulated not only the research on suitable methods for the delivery of bioinsecticides with hemocoelic target (mainly proteinaceous molecules), but also the study of the complex and intriguing phenomenon of protein absorption in insect midgut (Caccia et al. 2007; Casartelli et al. 2005, 2007, 2008; Whetstone and Hammock 2007; Jeffers and Roe 2008; Cermenati et al. 2011; Hughes et al. 2012; Bonning and Chougule 2014).

This review aims to offer a concise, but comprehensive, overview on insect midgut at the cellular level, describing the cell types that characterize the midgut of all insects and those peculiar cells that are restricted to a few insect orders or even to a specific developmental stage, but represent uniqueness in nature and significantly condition the physiology of the midgut (Fig. 2). Along with cell morphology, we report here a synopsis of the functions for each cell type, to provide the reader a broad picture of this complex organ.

Major cell types in the insect midgut

Columnar cells

Columnar cells (CCs) are the predominant cell type in the insect midgut and are responsible for digestive enzyme production and absorption of nutrients (Cioffi 1984; Billingsley and Lehane 1996; Nation 2008; Holtof et al. 2019). Since *D. melanogaster* is widely used as a model organism for studying human diseases, metabolic dysfunctions, development, morphogenesis, behavior, and aging, the CCs of this insect are commonly called "enterocytes," which is the term used to define the absorptive cells in mammalian small intestine. Nevertheless, the term "enterocyte" is formally and substantially incorrect when used to indicate the main cell type of insect midgut, since enterocytes are essentially involved in transport of organic solutes, ions, and water, unlike CCs that are also responsible for digestive enzyme secretion. CCs are characterized by a central nucleus (Figs. 2 and 3a) and a deeply folded apical membrane, which forms microvilli (Fig. 3b) with an internal actin cytoskeleton (Bonfanti et al. 1992). The portion of basolateral membrane that lays on the basal lamina usually presents well-developed infoldings (Fig. 3c), and the apical third of the lateral domain of the membrane is involved in forming junctional complexes with the neighboring cells (Billingsley and Lehane, 1996) (Fig. 3d, e). Although these general morphological features are common to CCs observed in all insects, peculiar ultrastructural features can be recognized in CCs located in the different regions of the midgut, according to their main function and the insect feeding habits (Billingsley, 1990; Billingsley and Lehane, 1996; Azevedo et al. 2009; Shanbhag and Tripathi 2009; Monteiro et al. 2014; Rost-Roszkowska et al. 2017; Santos et al. 2017;

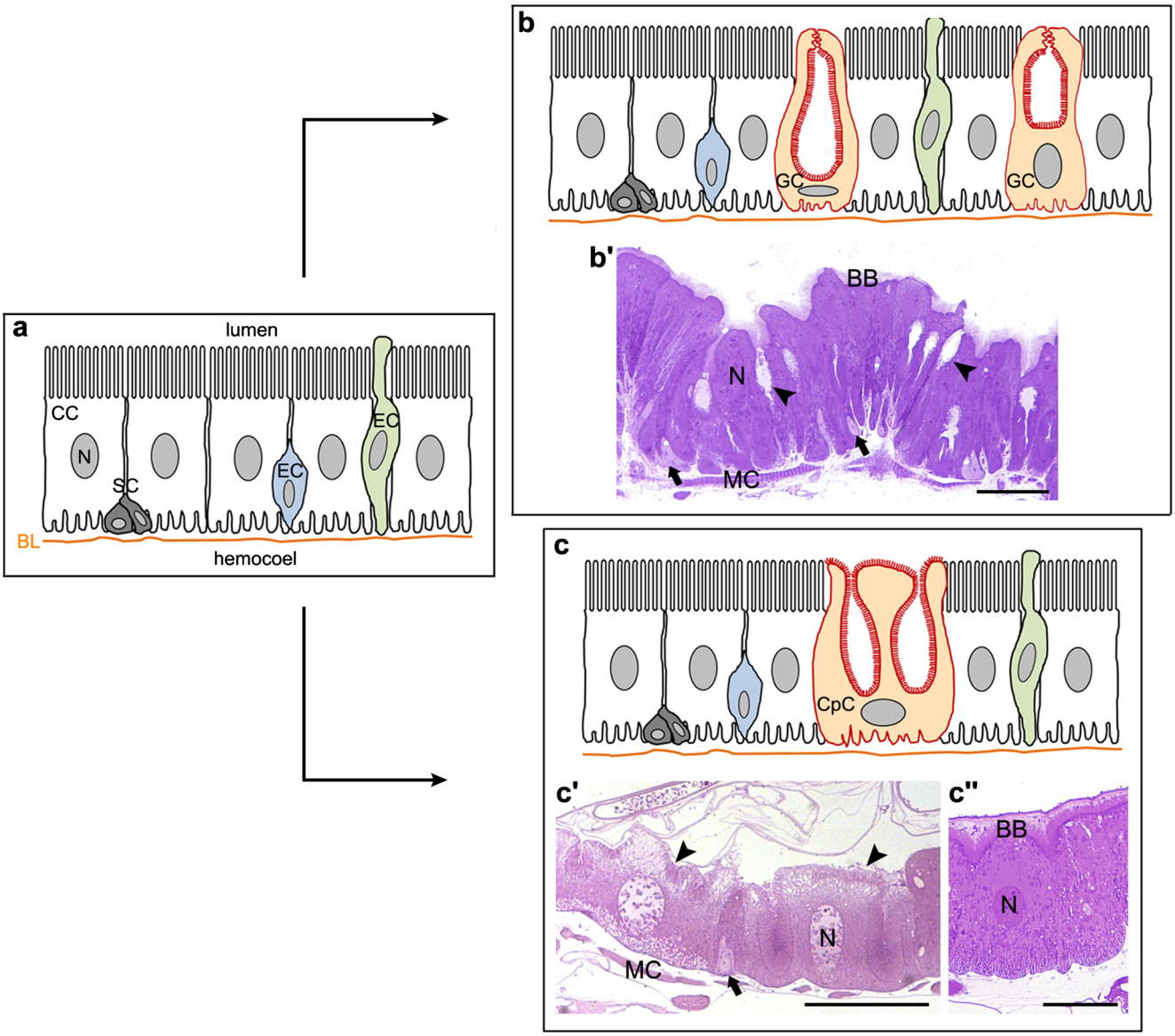

Fig. 2 Schematic representation of cell types that form insect midgut epithelium (**a**). Endocrine cells (EC) are intercalated to columnar cells (CC), the major cell type. A closed EC and an open EC are shown in blue and green, respectively. Stem cells (SC) are located at the base of the epithelium, isolated or grouped depending on the insect. (**b**) In the lepidopteran larval midgut, goblet cells (GCs, in orange) are present between CCs (in **b′** GC cavity is indicated by arrowheads in the cross-section of *Bombyx mori* midgut). GCs located in the anterior/middle portion of the midgut has an extended cavity (GC on the left) compared with GCs in the posterior midgut. (**c**) In the midgut of brachycerous Diptera, the peculiar cell type is the copper cell (CpC, in orange). In the cross-section in **c′**, CpCs in *Hermetia illucens* larval midgut are indicated by arrowheads, whereas in **c″** the cross-section shows CCs in the same epithelium. Arrows in cross-sections indicate stem cells (**b′**, **c′**, **c″**). BB brush border, BL basal lamina, MC muscles, N nucleus. Bars, 100 μm (**a**), 50 μm (**b**, **c**)

Bonelli et al. 2019). A pure absorptive or secretory function of specific CCs has never been demonstrated. CCs mainly involved in secretion display a variable number of granules in their apical cytoplasm, an abundant endoplasmic reticulum, and many lysosomes, whereas those mainly responsible for absorption possess long microvilli and elaborated basolateral infoldings, contain storage products (e.g., glycogen and lipid droplets) and spherites, and show little secretory activity. The different ultrastructural properties of CCs can be frequently appreciated in different midgut regions since digestion and absorption of nutrients can be differentially achieved along the midgut and one function can predominate over the other in the different regions, as described in the examples hereafter. In hematophagous insects, that ingest large amount of blood at intermittent intervals, the anterior midgut often shows ultrastructural specializations that ensure rapid absorption of water from the lumen to the hemolymph. Indeed, in the dipteran *Stomoxys calcitrans*, CCs in this region possess extensive basal infoldings that appear tightly apposed one to the other before feeding, but become separated after the blood meal.

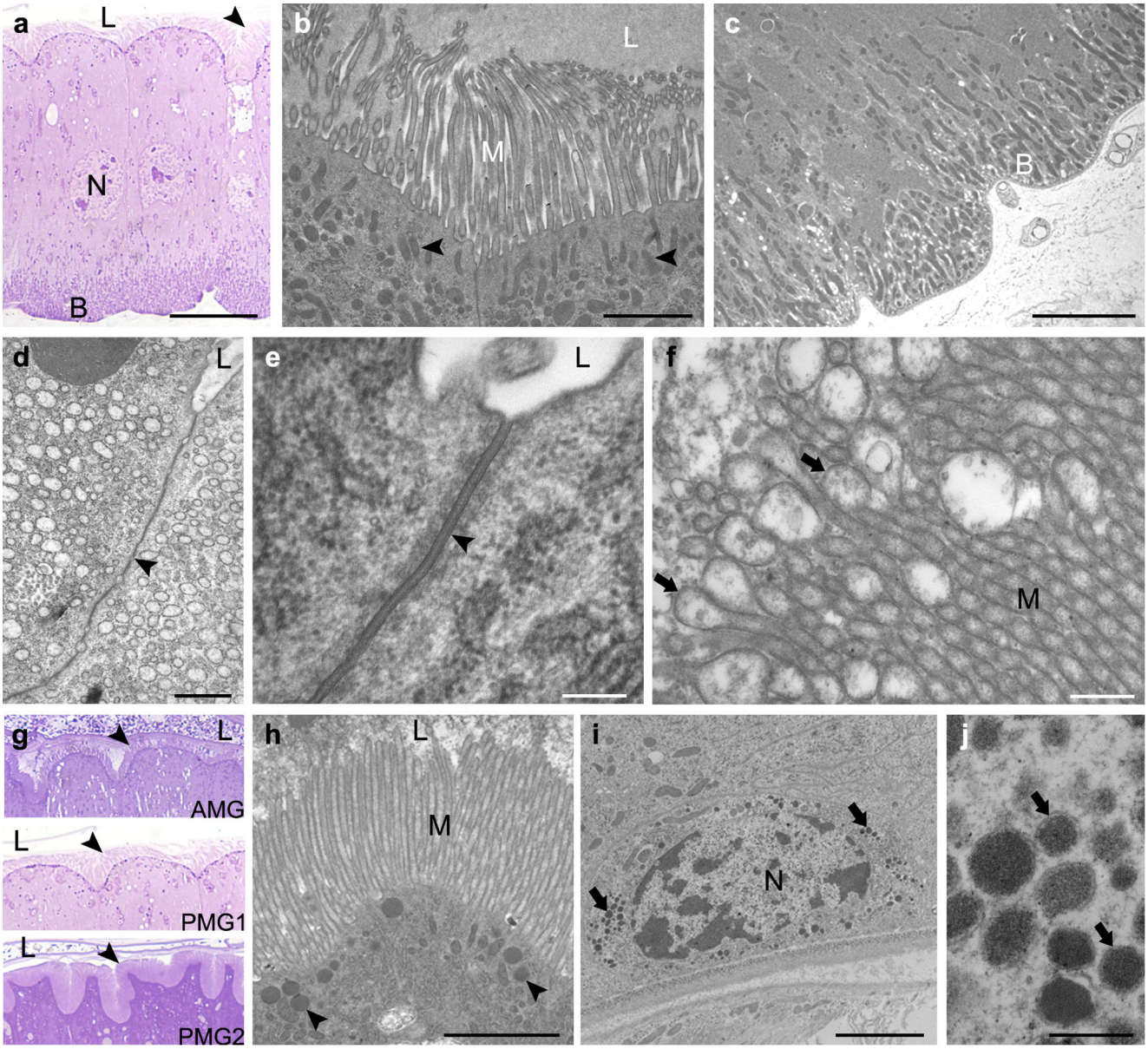

Fig. 3 (**a**) Cross-section of insect midgut (from *Hermetia illucens* larva) where columnar cells are visible; the arrowhead indicates the brush border. (**b-f**) TEM micrographs. (**b**) Detail of the deeply folded apical membrane of columnar cells, which forms microvilli (M); mitochondria (arrowheads) are visible in the cytoplasm. (**c**) Infoldings (B) of the basal membrane of columnar cells. (**d**) Junctional complex that joints two neighboring midgut cells. (**e**) Detail of the junctional complex. (**f**) Vesicles of microapocrine secretion (arrows) pinching off from at the apex of microvilli (M). (**g**) Cross-sections of the anterior midgut (AMG), first part of the posterior midgut (PMG1), and second part of the posterior midgut (PMG2) of *H. illucens* larvae, apical brush border is indicated by arrowheads. (**h**–**j**) TEM micrographs. (**h**) Secretory vesicles appear as electron-dense bodies (arrowheads) in the apical part of columnar cells of *H. illucens* larvae, under the microvilli (M), here reported in a micrograph of the AMG tract. (**i**) A closed-type endocrine cell with electron-dense granules in the cytoplasm (arrows). (**j**) Detail of the electron-dense granules (arrows). L lumen, N nucleus, B basal infoldings. Bars, 25 μm (**a**), 2 μm (**b**, **h**, **i**), 5 μm (**c**), 1 μm (**d**), 200 nm (**e**, **j**), 500 nm (**f**)

The movement of water is promoted by primary active transport of sodium out of the cells across this membrane domain, supported by the numerous mitochondria present in the basal region of these cells (Billingsley and Lehane, 1996). After this region, a short tract has CCs that are mainly involved in the synthesis and secretion of digestive proteases, as demonstrated by the numerous secretory vesicles in their apical cytoplasm and the abundant rough endoplasmic reticulum (Billingsley and Lehane, 1996). It must be highlighted that the accumulation of a large amount of vesicles containing digestive enzymes and their controlled release immediately after feeding observed in *S. calcitrans* are not common features in insects, even among those species that occasionally ingest food (Billingsley and Lehane, 1996). Indeed, constitutive secretion is thought to be the major route for the release of digestive enzymes into the midgut lumen in

insects as only few vesicles are usually observed in CCs. The last region of the midgut in hematophagous insects is mainly involved in absorption, as demonstrated by CCs with a large surface area of microvilli and the presence of many lipid vesicles in their cytoplasm (Billingsley and Lehane, 1996). In the bed bug *Cimex hemipterus*, CCs of the anterior midgut, which has a sac-like structure, are mainly involved in absorption of water, ions, and organic nutrients, as demonstrated by well-developed basal infoldings, the presence of a large amount of spherites, and glycogen and lipid inclusions (Azevedo et al. 2009). CCs of the middle midgut contain secretory granules, many lysosomes, and abundant rough endoplasmic reticulum, features that indicate an involvement of these cells in extra- and intra-cellular blood digestion (Azevedo et al. 2009). In the posterior midgut, CCs ultrastructure demonstrates their intense absorption activity, even though characteristics of secretory cells are also present (Azevedo et al. 2009).

Cioffi (1979) performed one of the first detailed morphological and ultrastructural analysis on the insect midgut. The author examined the midgut of a lepidopteran larva and observed that the fine structure of CCs gradually changes from the anterior to the posterior end of the midgut. In particular, in the anterior region of the midgut, the microvilli are irregular and vesicles form from their membrane; in the posterior region, the microvilli consist of long, thin, and regular projections of the apical membrane. Although the transport of nutrients across the apical membrane takes place all along the midgut of these insects (Giordana et al. 1998; Casartelli et al. 2001), these features suggest that CCs present in the anterior region of the midgut play a major role in the secretion of digestive enzymes. The latter can be secreted into the midgut lumen by classical exocytosis and by apocrine or, more frequently, microapocrine (Fig. 3f) secretion, or be associated to the apical membrane of CCs (Terra and Ferreira 1994; Billingsley and Lehane 1996). The enzymes associated to the apical membranes are involved in the final phase of digestion (e.g., exopeptidases and maltases), whereas those present in the lumen are responsible for the first step of digestion (e.g., endopeptidases and amylases) (Terra and Ferreira 1994; Holtof et al. 2019). Enzymes produced by these cells as well as the region of the midgut where they are active depend on dietary habits and phylogeny (Dow 1986; Terra and Ferreira 1994). Significant advances in the comprehension of the regionalization of digestion processes in the insect midgut have been reached in recent years thanks to molecular studies in *D. melanogaster* (Buchon et al. 2013b; Marianes and Spradling 2013; Buchon and Osman 2015). Moreover, a comprehensive description of morphological and functional regionalization of the insect midgut has been recently performed in the larvae of the dipteran *Hermetia illucens* (Bonelli et al. 2019). Concerning CCs, those in the anterior midgut (Fig. 3g, AMG) are characterized by the presence of many secretory vesicles in their apical portion that appear as electron-dense granules (Fig. 3h) and are responsible for the production and secretion of amylases, lipases, and lysozyme, whose activity has been recorded in the lumen of this region. Similar features (i.e., electron-dense vesicles in the apical cytoplasm, Fig. 3h) are present in CCs in the first part of the posterior midgut (Fig. 3g, PMG1), where molecular data demonstrated the expression of serine proteases, which are responsible for the initial phase of protein digestion that takes place in the lumen of midgut terminal end. On the contrary, in CCs present in the second part of the posterior midgut (Fig. 3g, PMG2), ultrastructural features related to secretory activity are less evident and microvilli are longer and denser than in other regions, indicating a main role of these cells in nutrient absorption (Bonelli et al. 2019).

Since digestive enzymes are directly responsible for nutrient availability, it is not surprising that their synthesis and activity are tightly regulated. Different factors such as nutrient composition and quantity, endocrine and neuronal signals, and gut-associated bacteria are involved in this modulation (Sakai et al. 2006; Clissold et al. 2010; Amcheslavsky et al. 2014; Chng et al. 2014; Erkosar et al. 2014).

CCs are also responsible for the absorption of the final products of nutrient digestion (Holtof et al. 2019). Transport proteins located in the apical and basolateral membrane domains accomplish this function, and their distribution along the midgut can vary according to the regionalization of this organ (Giordana et al. 1998; Leonardi et al. 1998; Casartelli et al. 2001; Buchon et al. 2013b; Miguel-Aliaga et al. 2018). A complete functional characterization of nutrient absorption in insect midgut epithelium has never been performed, although a fairly comprehensive picture has been obtained in the hymenopterous parasitoid *Aphidius ervi* (Caccia et al. 2005, 2007; Fiandra et al. 2010). Larval stages of this insect develop inside the host body, an aphid, which represents the feeding substrate of the juveniles that do not kill the host until pupation, when aphid tissues are completely consumed (de Eguileor et al. 2001). The midgut of these larvae is fully equipped for sugar and amino acid absorption, that occurs through the transcellular pathway, implying the presence of specific transporters in the apical and basolateral membrane of CCs. Hexoses are transported inside these cells from the midgut lumen by a SGLT1-like (SLC5A1-like) Na^+/glucose cotransporter and a GLUT5-like (SLC2A5-like) facilitative glucose transporter located in apical membranes, then a GLUT2-like (SLC2A2-like) facilitative transporter mediates their exit through the basolateral membrane. Na^+/K^+-ATPase, which generates the sodium electrochemical gradient to energize apical cotransporters, is also present in this membrane domain (de Eguileor et al. 2001; Caccia et al. 2005, 2007). In addition, the inducible expression of apical GLUT2-like transporters makes this model surprisingly similar to sugar absorption mechanism in mammalian gut (Caccia et al. 2007). In CC apical membrane, a Na^+/amino acid cotransporter, functionally similar to the B_0 broad-spectrum transport system (SLC1A5), internalizes neutral amino acids

that are then transported into the hemolymph by uniporters located in the CC basolateral membrane (Fiandra et al. 2010). The latter also hosts an obligatory Na^+-independent amino acid exchanger that may be crucial for essential amino acid movement as well as for gradient onset to promote the movement of amino acids (Fiandra et al. 2010).

Lepidopteran larval midgut has always attracted great attention due to its peculiar features. Indeed, the functional activity of this epithelium is dominated by specialized cells, the goblet cells, which are responsible for the secretion of potassium ions into the lumen, the high pH values of this environment, and the generation of a high electrical potential difference across the apical membrane of midgut cells (see the "Unique cell types and extreme pH conditions in the midgut lumen" section for details). CCs of these insects host unique cotransport systems located in their apical membranes that, in these extreme conditions, efficiently meet the high demand of amino acids that support the huge and rapid increase in body size of lepidopteran larvae. Interestingly, unlike the absorbing epithelia of other animals, the basolateral membrane of CCs lacks Na^+/K^+ ATPase. Thus, apical secondary active cotransporters do not exploit Na^+ as driving ion, but use the electrochemical gradient of K^+ generated by goblet cells to transport amino acids deriving from protein digestion from the lumen to the cell cytoplasm (Giordana et al. 1982, 1989, 1994, 1998; Parenti et al. 1992; Wolfersberger 1996; Casartelli et al. 2001; Leonardi et al. 2006).

In addition to their involvement in nutrition, CCs play a crucial role in the interaction with microorganisms present in the gut lumen. Lysozyme secreted by these cells shows a particularly high activity in the acidic middle region of the midgut of many Diptera (Lemos et al. 1993; Padilha et al. 2009; Bonelli et al. 2019) and it appears to be involved, together with the extreme pH value of this district, in shaping microbiota load and composition along the different regions of this organ (Bruno et al. 2019a). Moreover, in CC apical membrane of *D. melanogaster*, an enzyme (i.e., Doux) with antimicrobial activity has been identified (Ha et al. 2005). Duox is an oxidase able to generate reactive oxygen species and, after silencing its gene, adult flies show marked mortality in response to ingestion of microbe-contaminated food. CCs also release humoral immune factors, including AMPs. Most research in this field has been performed in *D. melanogaster* and in particular on immune deficiency (IMD) pathway, which is finely regulated (Lemaitre and Miguel-Aliaga 2013). For instance, resident microbiota does not trigger IMD pathway activation, which instead is boosted to produce AMPs by pathogenic bacteria that enter the midgut with the food (Buchon et al. 2013a).

D. melanogaster CCs have been recently demonstrated to produce and secrete the signaling protein Hedgehog (Hh) into the hemolymph (Rodenfels et al. 2014). In particular, circulating Hh released by CCs acts as a link between nutritional availability, lipid metabolism, and development. Under starvation, Hh causes the mobilization of fat body triacylglycerol stores, and inhibits ecdysteroid production in the prothoracic glands, by reducing the transcription of genes encoding for enzymes involved in this biosynthetic pathway, thus delaying pupation. Therefore, CCs can also coordinate the response of multiple tissues to nutrient availability. Coupling nutrition to growth and development is fundamental for animal survival and different organs communicate to coordinate this process in insects (i.e., fat body, prothoracic gland, and central nervous system) (Colombani et al. 2005; Mirth et al. 2005; Geminard et al. 2009; Delanoue et al. 2010). The evidence reported by Rodenfels et al. (2014) highlights the importance of the midgut, and in particular of CCs, as another crucial point in this interorgan signaling network.

Endocrine cells

Even though the main function of the insect midgut concerns food digestion and nutrient absorption, this tract of the alimentary canal also plays an important regulatory activity that is fundamental not only for midgut functionality, but also for insect homeostasis. Endocrine cells (ECs) are responsible for this function thanks to the production and secretion of bioactive peptides.

ECs, which are scattered among the other midgut cells, were identified in insects belonging to different orders more than 30 years ago, when also their peptidergic nature was revealed (Fujita et al. 1981; Iwanaga et al. 1981; Nishiitsutsuji-Uwo and Endo 1981; Schols et al. 1987; Andriès and Tramu 1985). ECs are characterized by two different morphologies (Sehnal and Žitňan 1996) (Fig. 2). Open-type ECs are elongated and in direct contact with the midgut lumen thanks to a narrow extension from the larger basal part of the cell. The basal membrane can directly rest on the midgut basal lamina (Sehnal and Žitňan 1996) or have a smaller projection extending towards it (Pabla and Lange 1999). Closed-type ECs (Fig. 3i) can be different in shape (e.g., ovoidal, spheroidal, or pyramidal), do not extend throughout the midgut epithelium, and, as a consequence, do not reach the lumen (Sehnal and Žitňan 1996; Bonelli et al. 2019) and the contact with the basal lamina may be limited (Andriès and Tramu 1985). The secretory nature of ECs is evidenced by many electron-dense granules in their cytoplasm (de Sousa and Conte, 2013; Bonelli et al. 2019) (Fig. 3i, j).

Until a few years ago, most studies focused attention on the morphological and ultrastructural features of ECs, as well as the immunohistochemical identification of the regulatory peptides produced. The recent advent of genomics, transcriptomics, and proteomics is allowing the elucidation of the important functional role of ECs in *D. melanogaster* physiology and in other few insects (Predel et al. 2010; Reiher et al. 2011; Wegener and Veenstra 2015). Even though some bioactive peptides widely occur across insects, none of the peptides

identified so far is present in all insect species (Wegener and Veenstra 2015). Moreover, ECs expressing specific peptides are generally present in specific regions of the midgut (Veenstra and Ida 2014; Chen et al. 2016) and their distribution can vary between the larval and the adult stages (Veenstra and Ida 2014). These peptides derive from protein precursors which are cleaved and post-translationally modified to produce the active forms. For example, ECs in the adult midgut of *D. melanogaster* produce nine major precursors that are processed into more than 20 active peptides (Reiher et al. 2011).

All the identified peptides produced by ECs are also released by neurons (Wegener and Veenstra 2015). In the central nervous system, some of them are secreted only by interneurons (Li et al. 2013; Shen and Cai 2001), and therefore only midgut ECs are responsible for the paracrine or hormonal effects of these peptides (i.e., only peptides released by ECs can affect neighboring midgut cells or, if these molecules reach the hemolymph, other organs). On the other hand, some peptides can also be secreted by neurohemal organs into the hemolymph and hormonally modulate target organs (Predel 2001). Therefore, complex regulation mechanisms can be set in motion and it is not simple to define and distinguish the effect on target cells of a peptide which is produced by midgut ECs but also released into the hemolymph by neurons. Nevertheless, thanks to the genetic tools developed in *D. melanogaster*, the systemic role of peptides produced by ECs in this dipterous has been assessed. Song et al. (2017) demonstrated that high-sugar diets administered to *D. melanogaster* larvae caused hyperglycemia, abnormally enhancing the adipokinetic hormone action in the fat body and thus impairing carbohydrate metabolism. This effect is mediated by activin-β, a peptide produced by ECs that binds to the transforming growth factor β receptor Baboon in fat body cells, increasing the expression of the adipokinetic hormone receptor. An interorgan communication between the midgut and the brain has also been suggested (Li et al. 2013). The authors demonstrated that open-type ECs producing CCHamide peptides are present in the midgut of *D. melanogaster* larvae and adults, where, at variance from the brain, their expression is consistent, and CHHamide-2 receptor gene is highly expressed in the brain, but absent in the gut. On the basis of these expression patterns, Li and coworkers (Li et al. 2013) suggest that CCHamide-producing ECs may sense food quality in the gut lumen and release these peptides into the hemolymph that thus reach the brain and bind to their receptors. This long-distance hormonal signaling may induce an adaptation in insect feeding behavior. In another study (Sano et al. 2015), it has been demonstrated that CCHamide peptides produced by the fat body and midgut ECs in *D. melanogaster* larvae act as a nutrient-dependent signal on insulin-like peptide–producing neurons, which express the specific receptor for these peptides. Thus, these peptides can play an important role in coordinating insect growth and nutritional availability. Although these data suggest the existence of a midgut-brain signaling, further investigations are necessary to definitely demonstrate that EC-derived peptides can regulate neurons in the central nervous system.

Peptides produced by ECs can have an important paracrine action on the midgut and play an essential role in its homeostasis. Even though gut motility is controlled by the stomatogastric nervous system, (Hartenstein 1997), also peptides released by ECs are involved in the regulation of midgut peristalsis, a fundamental process for the transit of food along the gut, therefore indirectly affecting digestion and absorption processes. In particular, ECs located at the junction of the anterior and the middle acidic region in *D. melanogaster* larval midgut play a key role in midgut motility (LaJeunesse et al. 2010). These cells produce diuretic hormone 31, a peptide that is necessary and sufficient to guarantee proper peristalsis within this region of the midgut. Another evidence of the paracrine role of EC peptides comes from the midgut of adult *D. melanogaster* (Song et al. 2014). Tachykinin peptides, the most abundant bioactive molecules produced by ECs in this insect (Veenstra et al. 2008; Veenstra 2009), regulate intestinal lipid metabolism by controlling lipid synthesis in CCs (Song et al. 2014). Ablation of tachykinin-producing ECs, as well as specific knockdown of EC tachykinin gene, cause a dramatic increase of neutral lipids in CCs by modulating lipogenesis and promote systemic lipid distribution (i.e., increase in hemolymphatic triglyceride titer and accumulation of neutral lipids in the fat body). Similar to previous observations that starvation causes tachykinin secretion by ECs in locust and cockroach midgut (Winther and Nässel 2001), Song et al. (2014) demonstrated that nutrient deprivation enhances tachykinin production in ECs and a diet rich in amino acids, but not in sugars or lipids, suppresses the production of these peptides, indicating that amino acids may directly affect tachykinin-secreting midgut ECs. Peptides produced by ECs are also involved in the regulation of digestion enzyme release in the midgut lumen. An inducible mechanism has been demonstrated in the cockroach *Periplaneta americana*: crustacean cardioactive peptide is synthesized in response to nutrient ingestion and, in turn, stimulates production and release of amylases and proteases (Sakai et al. 2006). However, CCs in *D. melanogaster scute* mutants, which lack midgut ECs, are able to secrete digestive enzymes, although at a reduced level compared with wild-type (Amcheslavsky et al. 2014). Indeed, other mechanisms can regulate the production of digestive enzymes, e.g., the transforming growth factor β ligand Dawdle (Chng et al. 2014). EC bioactive peptides are also involved in the regulation of stem cell proliferation by acting on muscle cells that surround the midgut. In *D. melanogaster* flies, the peptide Bursicon, produced by specific ECs in the posterior midgut, represses the production of Vein, a mitogen factor produced by visceral muscles that promotes midgut stem cell

proliferation, thus inducing their quiescence (Scopelliti et al. 2014). On the other hand, peptides produced by ECs are also responsible for midgut stem cell proliferation in newly eclosed flies. In fact, in *scute* mutants that lack midgut ECs, diet-stimulated midgut growth, that depends on the insulin-like peptide 3 expression in the surrounding visceral muscle (O'Brien et al. 2011), is defective (Amcheslavsky et al. 2014). In particular, the authors demonstrated that ECs act as an important link for this regulatory pathway by producing paracrine hormones, such as tachykinins, to upregulate insulin-like peptide expression and therefore stem cell proliferation.

The regulation of peptide release from ECs is still an unexplored topic. It is reasonable to hypothesize that the quality and the quantity of ingested nutrients are important factors that can be sensed by ECs, and a few studies, including the one reported above (Song et al. 2014), support this hypothesis. Park and Kwon (2011) demonstrated the expression of gustatory receptors in ECs of *D. melanogaster* adult midgut, and more recently the effect of various dietary conditions such as starvation, sugar, high fat, and protein on EC activation has been evaluated (Park et al. 2016). These authors identified a specific subpopulation of ECs in the posterior midgut which expresse diuretic hormone 31 and tachykinins that are activated by the presence of proteins and amino acids in the diet.

Even though *D. melanogaster* represents an excellent model to study regulation mechanisms in insect physiology, and the definition of the modulating function of some peptides produced by midgut ECs is an example, the regulative processes defined so far in this insect cannot be extended to other species without the risk of missing important peculiarities present in other insects. Therefore, to increase our knowledge in this field of research, molecular and functional studies in other insects are mandatory. A deep understanding of hormonal and paracrine regulations set in motion by the peptides produced by ECs and of the regulation of their production and release will help to complete the complex picture of midgut physiology and insect homeostasis.

Unique cell types and extreme pH conditions in the midgut lumen

Along with the ubiquitous cell types described above, cells with distinctive morphology and functions have been reported in the midgut of some insects, namely "goblet cells" (GCs) in larval Lepidoptera (although GCs have been also reported in insects belonging to different orders, these studies have never been corroborated by robust ultrastructural evidence) and "copper cells" (CpCs) in brachycerous Diptera (Cioffi 1984; Terra et al. 1996; Nation 2008; Shanbhag and Tripathi 2009). They are both characterized by the invagination of the apical membrane to form a large cavity (Figs. 2 and 4a, b) and their presence is accompanied by extreme pH values of the midgut lumen.

GCs are exclusively present in the midgut of lepidopteran larvae and are interspersed among CCs (Fig. 2) in a ratio that varies between 1:3 and 1:7, depending on midgut region and insect species, and are joined with them and each other by septate and gap junctions (Cioffi 1984; Baldwin and Hakim 1987, 1991). They possess a goblet shape as a result of the invagination of the apical membrane to form a large cavity (goblet cell cavity, GCC) lined by dense microvilli that are particularly extended in basal GCC. An apical complex structure, called valve, does not allow the free movement of solutes between the GCC and the midgut lumen (Fig. 4c). The morphology of these peculiar cells changes in the different midgut tracts (Cioffi 1979), being more pear-like in anterior and middle midgut, with a large cavity that almost reaches the base of the cell, and more cup-like in the posterior tract where the cavity is apical and smaller (Fig. 2). Basolateral membrane is poorly folded and usually the nucleus is basally located (Figs. 2 and 4a), although in GCs present in anterior and middle midgut, the invagination of the apical membrane is so pronounced that it almost reaches the basal pole of the cell and the remaining thin cytoplasmic layer cannot harbor the nucleus that may be positioned lateral to the cavity (Cioffi 1984; Gomes et al. 2013).

GCs greatly contribute in conferring the unique functional features that characterize the midgut of these insects. A vacuolar-type proton pump (V-H^+ ATPase) is located in the apical membrane of GCs and actively transports H^+ from the cytoplasm to the GCC against its electrochemical gradient, exploiting the energy derived from ATP hydrolysis (Wieczorek et al. 1989) (Fig. 4d). The pump is thus involved in the establishment of a transmembrane electrical potential across the apical membrane of midgut cells that exceeds 150 mV (Dow 1992). The pump is associated to a $K^+/2H^+$ antiporter that exchanges H^+ for K^+ leading to a net flux of K^+ from GC cytoplasm into the GCC (Wieczorek et al. 1991; Azuma et al. 1995) (Fig. 4d). The presence of a cavity protects and amplifies the driving force for K^+ secretion, allowing readily recapture of H^+ protons secreted by the V-H^+ ATPase into GCC by the $K^+/2H^+$ antiporter (Fig. 4d). The electrochemical gradient favorable for the K^+ movement from the GCC into the midgut lumen through the patent valve (Moffett and Koch 1992; Moffett et al. 1995), accompanied by the flux of HCO_3^- generated in the reaction catalyzed by GC carbonic anhydrase (Turbeck and Foder 1970; Dow 1992), allows the establishment of the extremely high pH value (up to 10–12) that characterizes the midgut lumen of lepidopteran larvae (Fig. 4d). This peculiarity evolved by reason of their feeding habits (Clark 1999; Nation 2008). These insects are predominately phytophagous and feed on plant material rich in toxic secondary metabolites such as tannins and polyphenols that may interfere with digestion by

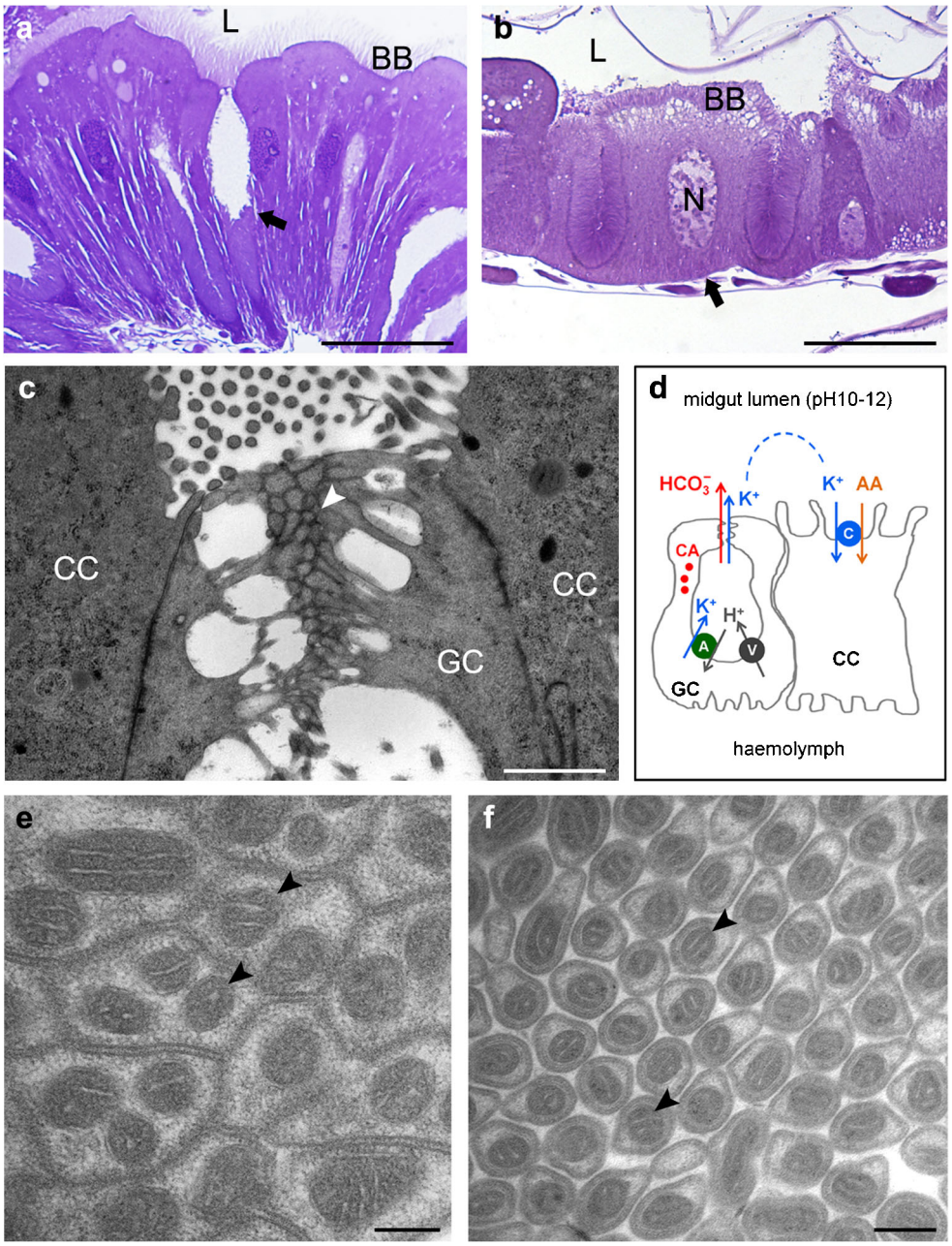

Fig. 4 Cross-sections of lepidopteran and dipteran larval midgut where peculiar goblet (**a**) and copper (**b**) cells (GC and CpC), respectively, are indicated (arrows). (**c**) TEM image shows a detail of the valve of GC (arrowhead) that separates the cell cavity from the midgut lumen. (**d**) Schematic representation of the mechanism of lumen alkalinization and potassium secretion by the GC of lepidopteran larval midgut. H^+ protons are pumped by a V-ATPase (V) into the GC cavity, and then removed by an antiporter (A) in exchange with K^+ cations (in a $K^+/2H^+$ ratio) that diffuse into the midgut lumen. Carbonic anhydrase (CA) associated to GC produces carbonic acid that dissociates into H^+ (captured by A) and bicarbonate that is eventually secreted into the lumen, determining the alkaline pH value. The high electrochemical gradient that drives luminal K^+ into cell cytoplasm is exploited by cotransporters (C) located in the apical membrane of columnar cells (CC) to internalize amino acids derived from luminal protein digestion into the cell. (**e**) and (**f**) TEM images show mitochondria (arrowheads) inside the apical microvilli of CpC and GC, respectively, respectively. BB brush border, L lumen, N nucleus. Bars, 25 μm (**a**, **b**), 1 μm (**c**), 200 nm (**e**, **f**)

decreasing the availability of food nitrogen by cross-linking with proteins and forming insoluble complexes. This behavior is prevented at alkaline pH, thus avoiding the binding of the metabolites to food proteins and digestive enzymes in the midgut lumen. On the other hand, the active secretion of K^+ in the midgut lumen serves as a driving force for secondary active amino acid uptake into adjacent CCs (see the "Major cell types in the insect midgut" section for details) (Fig. 4d).

Recent reports support the involvement of GCs in metal storage and likely in metal detoxification. In particular, the presence of electron-dense spherites in GCs and their release in the GCC have been observed in the lepidopteran *Anticarsia gemmatalis* (Gomes et al. 2012, 2013). In-depth analyses have revealed that these spherites are inorganic polyphosphate (polyP)–rich membrane-bounded organelles able to accumulate metals such as copper and zinc (Gomes et al. 2012, 2013). In other organisms, the acidity of these organelles is maintained by proton pumps, as V-H^+ ATPase, expressed on their membrane and *A. gemmatalis* is no exception as metal uptake of GC polyp–rich organelles was modulated by V-H^+ ATPase inhibitors (Gomes et al. 2012, 2013; Docampo 2016).

The presence of V-H^+ ATPase pump in the apical membrane also characterizes the second peculiar cell type, namely the CpCs in the midgut of brachycerous Diptera. CpCs were originally named "cuprophilic cells" because of their ability to accumulate copper and become fluorescent in copper-fed

Drosophila larvae (Filshie et al. 1971; Dubreuil 2004). Further studies clarified that copper accumulation is accompanied by the formation of cation-metallothionein fluorescent complexes that cause the blockage of normal physiological features of these cells (McNulty et al. 2001; Dubreuil 2004). For this reason, they are currently, and more correctly, named CpCs. As in GCs, the apical membrane of CpCs forms microvilli and is highly, deeply invaginated to form a cavity with variable forms and apertures on the midgut lumen, depending on the insect species (Fig. 4b) (Dubreuil 2004; Bonelli et al. 2019). The nucleus is located basally and the basolateral membrane domain of CpCs shows a few infoldings (Shanbhag and Tripathi 2009; Bonelli et al. 2019) (Fig. 4b).

The midgut of larval and adult brachycerous Diptera is characterized by a marked morphofunctional regionalization that consists in differences in tissue histology, gene expression pattern, digestive properties, immune response, and microbiota composition (Terra and Ferreira 1994; Terra et al. 1996; Buchon et al. 2013a, b; Marianes and Spradling 2013; Buchon and Osman 2015; Broderick 2016; Bonelli et al. 2019; Bruno et al. 2019a, b). Luminal pH value also differs in different midgut tracts, showing transitions between neutral or mildly alkaline values with the exception of a short middle portion that is strongly acidic (pH 2–4). CpCs are present in the initial tract of this portion (i.e., the CpC region) scattered among CCs. The latter, in this region, are called interstitial cells (ICs). CpCs are present in larval and adult stages (Figs. 2 and 4b) and are responsible for the low pH values observed in this portion of the midgut (Dubreuil 2004; Shanbhag and Tripathi 2009). The brachycerous *H. illucens* represents an exception since CpCs are present in the larva (Bonelli et al. 2019), whereas CpCs are absent in the adult midgut, where luminal pH never reaches extreme values (Bruno et al. 2019b). ICs alternate to CpCs in the CpC region and have an apically located nucleus, short microvilli, and an extensively folded basolateral membrane (Dubreuil 2004; Shanbhag and Tripathi 2009). In histological sections, they are clearly recognizable from CpCs because of their darker cytoplasm (Dubreuil 2004). It has been hypothesized that ICs and CpCs constitute a functional unit, since ICs may be implicated in supporting CpC secretion activity (Dubreuil 2004). The typical insect septate junctions seal cells also in this midgut tract (Shanbhag and Tripathi 2009; Furuse and Izumi 2017; Bonelli et al. 2019). Contrary to mammals, where H^+/K^+-ATPase is responsible for the acidification of the stomach lumen, in these insects, V-H^+ ATPase pumps H^+ in the cavity of CpCs that passively moves into the midgut lumen. Interestingly, recent experiments based on RNA interference (RNAi) have shown the contribution of ion transporters (namely proteins involved in H^+, Cl^-, K^+, and HCO_3^- movements) in the generation and maintenance of such pH values in this midgut tract of *Drosophila* larvae (Overend et al. 2016). According to most studies performed in *Drosophila* and other insects, the reason for this extreme pH condition in the CpC region is not linked to digestion, but rather lies in the regulation of the density and composition of the midgut flora (Dubreuil et al. 2001; Broderick 2016; Bruno et al. 2019a).

V-H^+ ATPase expressed in GCs and CpCs, which is highly conserved between eukaryotes, is ubiquitously present in acidic organelle membranes, where it is responsible for pH maintenance, and in the plasma membrane of different animal cell types (the first one was identified in apical membranes of GCs in the larvae of the lepidopteron *Manduca sexta*). V-H^+ ATPase consists of two subunits: a cytoplasmic V_1 complex uses the energy derived from ATP hydrolysis to drive protons into a V_0 transmembrane domain that extrudes the protons into the cavity (Wieczorek et al. 2009). The high ATP demand is locally supported by the presence, inside the microvilli of both GCs and CpCs, of elongated mitochondria (Harvey et al. 1981; Cioffi 1984; Shanbhag and Tripathi 2009; Gomes et al. 2013; Bonelli et al. 2019) (Fig. 4e, f). At an ultrastructural level, the pumps, densely packed in the microvilli, appear as spherical structures (approximately 10 nm in diameter) that stud the cytoplasmic side of the plasma membrane (Harvey et al. 1981; Cioffi 1984; Shanbhag and Tripathi 2009; Gomes et al. 2013; Bonelli et al. 2019). These small electron-dense bodies, named "portasomes" (Harvey 1980), are indeed responsible for the efflux of H^+ from the cytoplasm into the cavity (see Shanbhag and Tripathi 2009 and Bonelli et al. 2019 for ultrastructural details of portasomes).

A peculiar mechanism of regulation of V-H^+ ATPase activity (i.e., the reversible disassembly) was first observed in yeasts and *M. sexta* caterpillars (Kane 1995; Sumner et al. 1995) and was later described as an universal regulatory mechanism of V-H^+ ATPase activity to save energy (Wieczorek et al. 2000; Cotter et al. 2015). In *M. sexta* larvae, this phenomenon occurs during molting, when the insect stops feeding, or starvation and seems to be controlled by ecdysteroids (Sumner et al. 1995; Reineke et al. 2002). The disassembly consists on the reversible disassociation of the functional holoenzyme into inactive V_0 and V_1 domains and, in insects, is modulated by the phosphorylation state of a subunit in V_1 domain (Wieczorek et al. 2000; Voss et al. 2007; Cotter et al. 2015). In particular, holoenzyme disassembly leads to the migration of V_1 domain in the cytoplasm where, upon proper stimuli, PKA-dependent phosphorylation promotes the reassembly of the V_1 domain with the transmembrane domain and the reactivation of the pump (Wieczorek et al. 2000; Voss et al. 2007; Cotter et al. 2015). Interestingly, small non-coding RNA with complementary sequence to V_0 transcripts were detected in *M. sexta* midgut, suggesting a regulatory mechanism that may involve RNAi and act on V_0 domain (Wieczorek et al. 2000). However, much work remains to be performed in order to fully clarify this regulatory process at molecular level.

The forever young core of the midgut epithelium: the stem cell

As highlighted above, the digestive system of insects shows a precise functional and structural organization that is determined by feeding habits and development. In this context, peculiar functional features are guaranteed by the appropriate distribution of the different cell types. This pattern may dramatically vary during insect development, such as in holometabolous insects whose gut is significantly rearranged during metamorphosis due to a severe change in feeding habits between larval and adult stages. The overall integrity of the gut must be maintained to guarantee insect homeostasis, despite the challenging environment to which the digestive tract of insects is subjected. In fact, the gut must cope with mechanical food abrasion, interact with resident bacteria, and act as a barrier against ingested toxic compounds and pathogens (Huang et al. 2015). In this scenario, stem cells (SCs), also known as regenerative cells, play a key role.

SCs lay at the base of the gut epithelium among mature cells (Figs. 2 and 5a–c). They can be scattered as single cells along the gut as in Lepidoptera and Diptera, organized in small clusters or nidi as in Orthoptera and Odonata, or lie within regenerative crypts or pouches that project through the muscle layers that surround the gut epithelium as in Coleoptera (Wigglesworth 1972; Billingsley and Lehane 1996) (Figs. 2 and 5a–c). Like all undifferentiated cells, SCs are characterized by a blast-like morphology, with limited, dense cytoplasm, and few organelles (Billingsley and Lehane 1996) (Fig. 5b). The presence of glycogen granules and lipid droplets in the cytoplasm of SCs has been reported in the midgut epithelium of Lepidoptera and Diptera, although the role of these storage molecules is not clear yet (Tettamanti et al. 2007; Marianes and Spradling 2013; Franzetti et al. 2015). SCs can undergo asymmetric division (i.e., formation of a SC and a cell that terminally differentiates) to assure the maintenance of a constant number of SCs, or symmetric division that results in the generation of two SCs or two daughter mature cells (Gervais and Bardin 2017). SCs have been described in the gut of many insects (Wigglesworth 1972), but most of the knowledge on these cells derives from Holometabola, in particular the dipteran *D. melanogaster* and Lepidoptera. In addition, although the intervention of multipotent progenitors in maintaining tissue homeostasis has been reported in all the three gut regions (i.e., foregut, midgut, and hindgut), attention has been focused on midgut SCs.

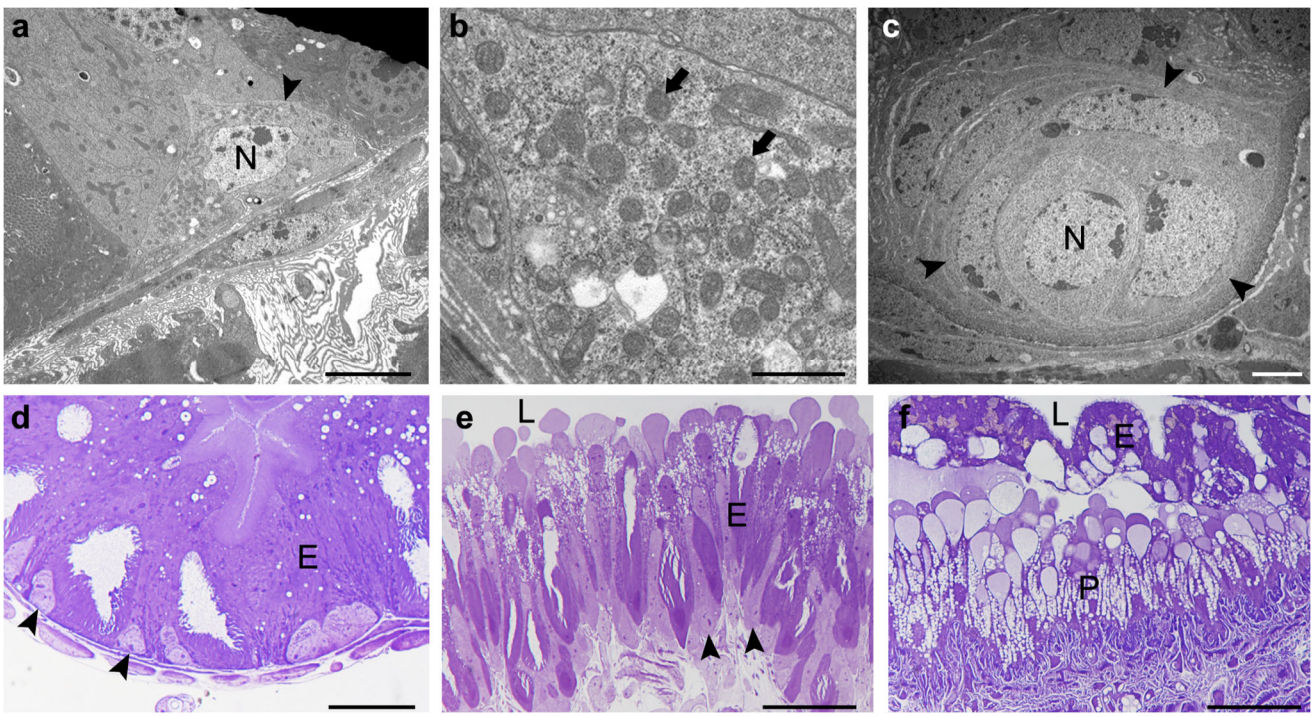

Fig. 5 (**a**–**c**) TEM images of stem cells. (**a**) Single stem cell (arrowhead) localized at the base of the midgut epithelium in the silkworm; (**b**) mitochondria (arrows) and ribosomes are visible in the cytoplasm; (**c**) nidi of stem cells (arrowheads) in the midgut epithelium of *Tribolium castaneum* larvae. (**d**–**f**) Cross-sections of the midgut epithelium of Lepidoptera at different developmental stages. While at last larval instar, stem cells (arrowheads) are sparse in the basal region of the epithelium (**d**), during larva-larva molt, they proliferate and differentiate into new cells that intercalate among columnar and goblet cells (**e**). At pupal stage, stem cells give rise to a new epithelium (P) that will differentiate into the adult midgut epithelium (**f**). E larval epithelium, L lumen, N nucleus. Bars, 5 μm (**a**), 1 μm (**b**), 2 μm (**c**), 25 μm (**d**), and 50 μm (**e**, **f**)

The remarkable capacity of SCs to proliferate is fundamental to ensure the growth of the midgut during larval development in Lepidoptera (Fig. 5d, e). Indeed, they are responsible for the 200-fold increase of the midgut cell number during the larval development of *M. sexta* (Baldwin and Hakim 1991). In this insect, it has been demonstrated that the growth of the larval epithelium is episodic and, at each molt, sparse SCs start to proliferate prior to the appearance of any external signs of molting, when the ecdysteroid peak occurs, and they continue to divide during early molting stage to increase in number (Baldwin and Hakim 1991). This timing is slightly different compared with the silkworm, *Bombyx mori*, in which the use of mitotic markers demonstrated that the highest proliferation activity of SCs is essentially limited to the first half of the molt period (Franzetti et al. 2016). Despite this difference, the process that leads to the differentiation of SCs into mature cell types in larval midgut recapitulates embryonic differentiation in both lepidopteran species.

During metamorphosis, the lepidopteran larval midgut is completely replaced by a newly forming epithelium that originates from the proliferation and differentiation of larval SCs (Tettamanti et al. 2007; Franzetti et al. 2015) (Fig. 5f). The pupal epithelial layer later becomes the adult midgut epithelium and absorbs molecules derived from the larval midgut cells that degenerate in the lumen. In the silkworm, a first pulse of mitotic activity in larval SCs is observed at spinning stage, as soon as the larva starts to spin the cocoon, then cell division is resumed at pupal stage. This biphasic pattern of SC division overlaps the two ecdysone peaks that occur during metamorphosis (Franzetti et al. 2015). SCs that act during pupal stage are substantially unipotent since they differentiate mostly into the major cell type, i.e., the CCs (and to a very minor extent into ECs) that will form the adult midgut epithelium. This raises the possibility that two different SC populations may exist in the larval midgut of Lepidoptera, the first one that is responsible for the formation of the mature cell types in the larva and the second in the pupa/adult. Alternatively, a single population of larval SCs could be subjected to differential regulatory signals that elicit different outcomes. During the differentiation process that brings to the formation of the adult midgut, these cells show two peculiar features in their cytoplasm: a high amount of spherites and the presence of a large vacuole that disappears as the cell differentiates. While spherites could be involved in detoxification or metabolic activities (Franzetti et al. 2015), the vacuole contains lysozyme and the release of this enzyme with antibacterial activity by the cells into the lumen of the newly forming epithelium likely protects the pupa and the adult from bacterial threats that may arise during the tissue remodeling activities occurring in this organ during metamorphosis (Russell and Dunn 1991; Tettamanti et al. 2007; Franzetti et al. 2015). Besides supplying new mature cells for the formation of the adult midgut, SCs in the pupa undergo self-renewal to form a population of SCs that is maintained in the midgut epithelium of the adult insect (Franzetti et al. 2015). Noteworthy, a limited proliferative activity was detected in the silkmoth gut; thus, these SCs could help the adult midgut to reach its final size. Notwithstanding, the potential role of adult SCs in repairing the epithelium following damage in this insect needs to be further investigated.

The knowledge on the signals that regulate SC proliferation and differentiation in the larval midgut of Lepidoptera comes from primary cell cultures. This powerful in vitro system allowed to demonstrate that *M. sexta* midgut SCs can proliferate and differentiate in response to the signals coming from the fat body and the midgut epithelium (Baines et al. 1994; Sadrud-Din et al. 1994). Among the signals that promote SC division, there are ecdysteroids, α-arylphorin, and bombyxin (Hakim et al., 2010). While ecdysone seems to be more effective in stimulating in vitro proliferation of SCs, 20-hydroxyecdysone (20E), that regulates larva-larva and larva-pupa transition, can induce their differentiation (Smagghe et al. 2005). Arylphorins are high molecular weight protein complexes, related to the storage proteins hexamerins, produced by the fat body that, after being released into the hemolymph, are taken up again by the fat body just before metamorphosis (Hakim et al. 2010). α-arylphorin isolated from pupal fat body has been demonstrated to elicit proliferation in larval midgut SCs of many lepidopteran species (Hakim et al. 2007). Finally, bombyxin is a peptide that belongs to the insulin family and might work in concert with 20E and α-arylphorin (Goto et al. 2005; Nijhout et al. 2007). Besides the various mitogens, four peptides, called midgut differentiation factors (MDF 1-4), promoting differentiation of SCs, have been isolated from the hemolymph or midgut cell–conditioned media (Loeb et al. 2004). The source of these factors is not clear and their apparent redundancy in regulating proliferation of SCs needs to be confirmed.

D. melanogaster is by far the most studied model insect and, not surprisingly, its midgut SCs have been the object of a large number of studies. In *Drosophila*, the growth of the larval midgut takes place by endoreplication (i.e., the cell undergoes multiple S phases without entering mitosis), followed by an increase in cell volume of individual cells (Takashima et al. 2011; Zielke et al. 2013). During the larval stage, a population of SCs (i.e., adult midgut progenitors) within the midgut epithelium serves as precursor for the adult midgut and proliferates extensively under the control of the epidermal growth factor (EGF) signaling pathway to form groups of islets. During early metamorphosis, these cell clusters form a pupal epithelium that intercalates between the larval and the adult epithelium that is formed by a second burst of differentiation and surrounds both larval and pupal epithelia (Takashima et al. 2011). The precise function of the pupal gut is still debated, although it does not seem to be endowed with digestive activity and could simply represent an

evolutionary vestige. However, the pupal midgut is a transient structure that becomes later sequestered, together with the larval epithelium, into the lumen of the adult midgut where both tissues are histolized (Takashima et al. 2011). A small number of undifferentiated cells remain as SCs in the adult midgut since, conversely to Lepidoptera, the midgut of adult *Drosophila* is subjected to a significant turnover and is renewed every 2 weeks under steady-state conditions (Ohlstein and Spradling 2006; Takashima et al. 2013). In the adult midgut, each SC asymmetrically divides and gives rise to a new SC and an immature cell (i.e., the enteroblast) that, in turn, can differentiate into a CC or EC (and possibly into a CpC in the CpC region) (Gervais and Bardin 2017). The striking regional diversification of *Drosophila* midgut along the anterior-posterior axis is accompanied by the presence of SCs that are characterized by different ultrastructure, gene expression profile, and rate of proliferation and are able to specifically differentiate into mature cell types associated to each subregion (Marianes and Spradling 2013). This pattern, established during the first 2 days after eclosion, is associated to the expression of key genes that are required for the patterning of the gut embryo, and is maintained throughout the insect life (Buchon et al. 2013b).

The coordinated action of SCs is needed to guarantee a basal, homeostatic cell renewal of the midgut epithelium during the 8-week lifespan of the fly. Moreover, in the last years, the study of the regulatory mechanisms underpinning SC proliferation has revealed an extreme plasticity of the midgut in response to physical-, chemical-, or pathogen-induced damage as long as in response to changes in nutritional condition, such as starvation, and during mating to support female reproductive success (Biteau et al. 2008; McLeod et al. 2010; Shim et al., 2013; Hudry et al. 2016; Regan et al. 2016; Mattila et al. 2018; Obniski et al. 2018).

The knowledge obtained on *Drosophila* SCs, especially adult SCs, has been obtained through multi-level approaches and it would be unfeasible to summarize that amount of information in a few pages. Thus, a general picture of SCs behavior during postembryonic development in *D. melanogaster* has been provided herein, although additional in-depth information on division and differentiation behavior during development and in the different midgut regions, as long as the involved molecular pathways, are reported in many research articles and reviews (see Dubreuil et al. 1998; Lin et al. 2008; Ohlstein and Spradling 2006; Lucchetta and Ohlstein 2012; Buchon and Osman 2015; Nászai et al., 2015; Guo et al. 2016; Li et al. 2016; Gervais and Bardin 2017).

Since information on SCs in other insect orders is fragmentary and the available studies have mainly evaluated the behavior of SCs in relation to environmental cues rather than in developmental settings (i.e., molting or metamorphosis), we included an overview on the most representative studies for the sake of completeness. In adult *Apis mellifera*, SCs are grouped into regenerative crypts. They differentiate in a synchronized manner, remaining organized in tetrads around the crypt from which they are derived. During differentiation, cells form intercellular bridges (i.e., fusomes) that are maintained in mature cells (Raes et al. 1994). A cell communication system that may synchronize SC differentiation has been observed also in the bee *Melipona quadrifasciata anthioides* (Martins et al. 2006), although in this case it is due to the presence of gap junctions, and in *Epilachna nylanderi* (Coleoptera, Coccinellidae) (Rost-Roszkowska et al. 2010a). The adult insect of this species feeds on the Ni-accumulating *Berkheya coddii* plant species. Consequently, the midgut is subjected to a continuous renewal to replace mature cells that are discarded due to the accumulation of high levels of nickel ingested. In this case, single SCs proliferate to form groups of 6–8 cells that progressively differentiate, keeping in contact through intercellular junctions (Rost-Roszkowska et al. 2010a). This continuous regeneration of the midgut epithelium through division and differentiation of SCs during the adult stage in response to external factors appears to be a common process in Coleoptera (Fig. 5c). In these insects, larval SCs differentiate into pupal/adult midgut epithelium at metamorphosis (Parthasarathy and Palli 2008), while some of them retain their SC status and become adult SCs organized in regenerative crypts (Nardi et al. 2010). A comparative study that considered 18 coleopteran species demonstrated a correlation among the presence and density of SCs in the adult midgut epithelium, the feeding habits of the beetle, the presence of the PM, and the turnover of the mature cells (Nardi and Bee 2012). Thus, few SCs are present in those species that have a PM as a protective barrier of the midgut epithelium, while in species lacking PM, the need to continuously replace midgut cells requires the presence of a high amount of SCs. Adult beetles that rarely, if ever, feed, do not have a PM, nor SCs, in their midgut epithelium.

Similar to other holometabolous insects, also in Culicidae, the larval midgut is subjected to metamorphic remodeling, thanks to the proliferation of SCs that initiates at the end of the larval period and proceeds during pupation (Ray et al. 2009; Fernandes et al. 2014). Interestingly, a significant variation in the behavior of SCs has been observed in the adult insect of different culicidian species. Thus, while in non-hematophagous mosquitoes, such as *Toxorhynchites theobaldi* that exclusively feed on sugar diets, no SC division can be observed in the midgut of the adult insect (Godoy et al. 2015), blood-feeding species need to counteract the deleterious effects of blood digestion. In fact, the digestion of vertebrate hemoglobin results in the production of a large amount of free haem, a cytotoxic molecule that can bring midgut cells to death (Okuda et al. 2007). In *Aedes aegypti*, PM protects the midgut epithelium by binding the haem (Pascoa et al. 2002) and the insect sequesters iron by increasing the synthesis of ferritin in the midgut (Dunkov et al. 2002): these protective

mechanisms justify the absence of SC proliferation both in the midgut of newly emerged (Fernandes et al. 2014) and blood-fed (Okuda et al. 2007) adults. Conversely, *Culex quinquefasciatus* is endowed with a restoration mechanism that, by differentiating pre-existing SCs, is able to replace apoptotic CCs that are lost after a blood meal (Okuda et al. 2007). This ability decreases after successive blood meals since the pool of SCs is not replenished by cell division. This cell replacement mechanism triggered by the midgut could represent a recent adaptation of this hematophagous insect and highlights how the dynamics of SC division and differentiation in mosquitoes need to be further investigated in the light of developmental stages and nutritional needs of the insect.

In Hemimetabola, SCs are clustered in nests similar to some holometabolous insects (Illa-Bochaca and Montuenga 2006; Teixeira et al. 2013). In the hemipteran *Podisus nigrispinus*, SCs divide along the whole post-embryonic phase that, starting from first instar nymphs, leads to the development of the adult insect (Teixeira et al. 2013). Cell proliferation is more consistent during the third and fourth nymphal instars and contributes to the growth of the midgut that, unlike holometabolous species, does not undergo extensive remodeling involving cell death. In the orthopteran *Acheta domesticus*, the production of mature cells from SCs appears to occur in a continuous manner, replacing degenerated epithelial cells (Rost-Roszkowska 2008). A detailed analysis of the proliferation and differentiation events of SCs in a hemimetabolous insect was performed in *Locusta migratoria* (Illa-Bochaca and Montuenga 2006). In this orthopteran, the authors describe the organization of SCs in a niche that is somewhat comparable with the intestinal villus crypt, and is able to generate both CCs and ECs. Also in Hemimetabola, SCs show a certain degree of plasticity. In fact, the cockroach *P. americana* is able to regulate the proliferation of SCs in response to changes in nutrient availability. In fact, in insects experiencing starvation, a reduction of SC division occurs (Park and Takeda 2008). Prolonged starvation even induces cell death, not only in CCs, but also in SCs (Park et al. 2009). This survival strategy reduces digestion activity at a minimum and maintains midgut cells viable. After this lag phase, that determines a general atrophy of the midgut, SC proliferation is restored at higher levels than in feeding condition, to re-establish the epithelial cell population (Park et al. 2009).

In Ametabola, the activity of SCs is generally related to the renewal of midgut cells that can occur in a cyclic manner, mainly in relation to the molt, or in a continuous manner (Rost-Roszkowska 2006a; Rost-Roszkowska et al. 2010b, c). However, the picture is rather variegated and marked differences have been observed even in closely related groups (Rost et al. 2005; Rost-Roszkowska 2006b; Rost-Roszkowska et al. 2007). In fact, SCs can simply differentiate into mature epithelial cells, and are thus not considered true SCs (Rost-Roszkowska 2006b), or undergo both proliferation and differentiation (Rost-Roszkowska et al. 2007, 2010b). In addition, they can be dispersed as single cells in the midgut epithelium (Rost-Roszkowska 2006b; Rost-Roszkowska et al. 2010d) or organized in nests that contain up to several dozens of cells (Rost-Roszkowska et al. 2007, 2010b, c, e), where they can be joined each other by junctions. In the latter case, inner cells usually proliferate and maintain the pool of SCs in the midgut epithelium, while outer cells undergo differentiation into CCs (Rost-Roszkowska et al. 2007, 2010b, c, e). According to this evidence, the hypothesis of a gradual increase in the evolution of SCs, i.e., starting from those Collembola species that lack SCs and regenerate epithelial cells by themselves (Jura 1958; Rost-Roszkowska and Undrul 2008), passing through single cells sparse in the epithelium, and ending up with SCs organized in nests, is questioned and further studies are necessary to fill the gap of knowledge, which is still fragmentary, on these Hexapoda.

Conclusions and future perspectives

This review provides a general picture of insect midgut cell structure and functions, even though the rich diversity observed in this taxon precludes a punctual description. Common features can be individuated, such as the general morphology of the alimentary canal, major cell types forming the midgut monolayered epithelium, and their main functional contribution to nutrient digestion and absorption and insect homeostasis. Nevertheless, excluding the insect model *D. melanogaster*, the fragmentation of the information that results from old and recent studies is evident. The ultrastructure of the gut has been investigated in detail in a few insect species and in the overwhelming majority of cases these studies were not complemented with functional nor molecular analyses. Further work is required to deepen and complete at multiple level characterizations already approached rather than starting fresh with studies on punctual aspects on new insect species.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with animals performed by any of the authors.

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