



The digestive system of the adult *Hermetia illucens* (Diptera: Stratiomyidae): morphological features and functional properties

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Abstract

The larvae of the black soldier fly (BSF), *Hermetia illucens* (Linnaeus, 1758) (Diptera: Stratiomyidae), are considered an efficient system for the bioconversion of organic waste into usable products, such as insect protein for animal feed and bioactive molecules. Despite the great interest toward *H. illucens* and its biotechnological applications, information on the biology of this insect is still scarce. In particular, no data on the structural and functional properties of the digestive system of the adult insect are available and it is a common belief that the fly does not eat. In the present work, we therefore investigate the remodeling process of the BSF larval midgut during metamorphosis, analyze the morphofunctional properties of the adult midgut, evaluate if the fly is able to ingest and digest food and assess whether the feeding supply influences the adult performances. Our results show that the larval midgut of *H. illucens* is removed during metamorphosis and a new pupal-adult epithelium, characterized by peculiar features compared to the larval organ, is formed by proliferation and differentiation of midgut stem cells. Moreover, our experiments indicate that the adult insect possesses a functional digestive system and that food administration affects the longevity of the fly. These data not only demonstrate that the adult BSF is able to eat but also open up the possibility to manipulate the feeding substrate of the fly to improve its performances in mass rearing procedures.

Keywords Black soldier fly · Feeding habits · Insect digestive system · Metamorphosis · Tissue remodeling

Introduction

The larvae of the black soldier fly (BSF), *Hermetia illucens* (Linnaeus, 1758) (Diptera: Stratiomyidae), can convert low-

quality biomass, such as food waste, organic residues and byproducts of the agri-food transformation chain, into nutritionally valuable proteins and bioactive molecules (Cickova et al. 2015; Meneguz et al. 2018; Muller et al. 2017; Nguyen et al. 2015; Vogel et al. 2018). In particular, the high nutritional value of the larvae renders this insect useful for producing feedstuffs (Makkar et al. 2014; Wang and Shelomi 2017). Recently, European Commission Regulation No 2017/893 partially lifted the feed ban rules regarding the use of processed animal proteins from BSF and six other insect species for aquaculture (European Commission 2017). This change in the European legislative landscape will surely contribute to promoting the development of the insect industry for the feed sector, as demonstrated by the increasing number of insect companies in Europe (Ilkka Taponen's entomology database, <https://ilkkataponen.com/entomology-company-database/>). However, despite the great interest in BSF, data on the biology of this species remain scarce. In particular, a deep characterization of the morphology, physiology and development of the alimentary canal and especially of the midgut, which is responsible for food digestion and nutrient

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absorption, would not only increase the information on the biology of this insect but also provide data that could be exploited to improve the performances of the fly during mass rearing. Another aspect that has been neglected so far concerns the feeding habits of the adult insect. This lack of knowledge could represent a major obstacle to using BSF, since such information is correlated with the safety of the procedures for rearing this insect. In fact, it must be considered that the mouthparts of adult Diptera (Gullan and Cranston 2014), as well as some structures associated to the digestive system (Stoffolano and Haselton 2013), have been implicated in the transmission of pathogens, thus representing a potential risk factor in the use of BSF as feed (EFSA Scientific Committee 2015). The urgent need to investigate the feeding habits of the fly can be further appreciated if we consider that, according to the current literature but without any supporting experimental data, *H. illucens* does not need to eat or is even considered unable to eat in the adult stage and therefore it depends exclusively on reserves accumulated during the larval stage (Gobbi et al. 2013; Sheppard et al. 1994; Sheppard et al. 2002; Tomberlin et al. 2009; Tomberlin and Sheppard 2002; Tomberlin et al. 2002). Consequently, only the quality and quantity of food administered to the larvae are considered able to affect the growth, survival and biological traits of adult flies (Gobbi et al. 2013). For this reason, attention in past years has been focused on how the food ingested by the larva (Gobbi et al. 2013; Nguyen et al. 2013; Tomberlin et al. 2002), rearing temperature (Tomberlin et al. 2009), relative humidity (Holmes et al. 2012) and pupation substrate (Holmes et al. 2013) determine both the morphological and physiological development of the adults, while the alimentary behavior of the fly has been ignored so far. It is noteworthy that in most of these studies no food, or only water, was offered to the flies and, according to the results collected, this did not seem to be a limiting factor for successful reproduction (Sheppard et al. 2002; Tomberlin and Sheppard 2002; Tomberlin et al. 2002). Considering this evidence, the adult stage did not attract the attention of researchers and an accurate study of this developmental stage has never been performed. To our knowledge, only two studies have evaluated the longevity of adult BSF fed on different substrates but the functional properties of the digestive system were not considered (Bertinetti et al. 2019; Nakamura et al. 2016).

The alimentary canal of insects is organized in three main regions. In addition to the foregut and the hindgut that are involved in food ingestion, storage and grinding and water and ion absorption, respectively, the midgut represents the central part of the gut and is responsible for food digestion and nutrient absorption (Dow 1986). Notwithstanding its apparent simplicity (the insect midgut consists of only a single-layered epithelium that rests on a basal lamina and striated muscle fibers), it is characterized by a marked regionalization and cellular diversity. This organization is necessary to

optimize its function by enabling sequential activities ranging from digestive enzyme secretion to nutrient absorption and from endocrine signaling to regulation of midgut homeostasis (Dow 1986; Sehnal and Zitnan 1996; Terra 1990; Terra and Ferreira 1994; Terra et al. 1996a; Terra et al. 1996b). In particular, in the nonhematophagous brachycerous Diptera examined so far, the larval midgut presents three regions with differing luminal pH and morphofunctional properties (Dubreuil 2004; Lemos and Terra 1991; Pimentel et al. 2018; Shanbhag and Tripathi 2009; Terra et al. 1988b). Holometabolous insects undergo significant remodeling of the larval midgut during metamorphosis to fulfill changes in dietary requirements between the larval and the adult stage. The adult midgut of the common fruit fly, *Drosophila melanogaster* (Diptera: Drosophilidae), a model species among Diptera, is generated de novo during larva-adult transition. In fact, the larval midgut degenerates completely during metamorphosis and the adult midgut epithelium is formed by proliferation and differentiation of adult midgut precursors, i.e., stem cells that lie in the basal region of the larval epithelium (Lemaitre and Miguel-Aliaga 2013; Micchelli and Perrimon 2006; Ohlstein and Spradling 2006, 2007). The midgut of the adult *D. melanogaster* is characterized by regional variation; although it maintains a tripartite organization that roughly resembles that of the larval midgut, it presents a complex organization and different subregions have been identified according to gene expression patterns and anatomical and histological features (Buchon et al. 2013; Marianes and Spradling 2013). Another peculiar feature of the alimentary canal of the fly is the crop, a bag-like organ that is connected to the gut just before the midgut, where food is mixed, detoxified and stored (Stoffolano and Haselton 2013).

In the present study, we structurally and functionally characterize the midgut of *H. illucens* during the larva-pupa and pupa-adult transition, to investigate the remodeling process of this organ during metamorphosis. To this aim, we analyze the morphology of the midgut epithelium, the ability of stem cells to proliferate and differentiate into mature cells and the mobilization of long-term storage molecules. Moreover, we investigate the morphofunctional features of the midgut of the adult insect and its ability to ingest and digest food by means of vital stains and fluorescent molecules and measuring digestive enzyme activity.

Our results demonstrate that the larval midgut of *H. illucens* is completely removed during metamorphosis and a new pupal-adult epithelium, characterized by peculiar features, is progressively formed by the proliferation and differentiation of stem cells. Moreover, the feeding habits of the adult insect and the morphofunctional features of its digestive system demonstrate that *H. illucens* fly, at variance with most of the information reported in literature, can ingest and digest food and that this has an impact on its lifespan.

Materials and methods

Experimental animals

H. illucens larvae, pupae and adults (Table 1) used in this study were obtained from a colony established in 2015 at the University of Insubria (Varese, Italy) starting from larvae purchased from a local dealer (Redbug, Italy).

The larvae were reared on standard diet for Diptera (Hogsette 1992), composed of 50% wheat bran, 30% corn meal and 20% alfalfa meal mixed in the ratio 1:1 dry matter/water, as previously reported (Bonelli et al. 2019; Pimentel et al. 2017). After eclosion, the flies were maintained at 27.0 ± 0.5 °C and 70 ± 5% relative humidity. A 36 W/765 FLUO lamp (Osram, Munich, Germany) guaranteed a 12:12-h light/dark photoperiod. The insects were anesthetized on ice prior to dissection. In each experiment, the midgut isolated from at least three insects was examined, unless otherwise specified. As the preliminary investigation did not show any significant differences between the sexes, the midguts from both males and females were used for all the analyses on the flies.

Scanning electron microscopy

For three-dimensional scanning electron microscopy (SEM) imaging, head samples were fixed with 4% glutaraldehyde in 0.1 M Na-cacodylate buffer (pH 7.4) for 1 h at room temperature. After washes in Na-cacodylate buffer, specimens were postfixed in a solution of 1% osmium tetroxide and 1.25% potassium ferrocyanide for 1 h. Samples were then dehydrated in an increasing series of ethanol and washed twice (8 min each) with hexamethyldisilazane. Dried samples were mounted on stubs, gold-coated with a Sputter K250 coater and then observed with a SEM-FEG XL-30 microscope (Philips, Amsterdam, The Netherlands).

Light microscopy and transmission electron microscopy

The midgut was isolated from last instar larvae, pupae (days 4, 8 and 10) and flies (day 1) and immediately fixed in 4% glutaraldehyde (in 0.1 M Na-cacodylate buffer, pH 7.4) overnight at 4 °C. After postfixation in 2% osmium tetroxide for 1 h, samples were dehydrated in an ethanol series and

embedded in resin (Epon/Araldite 812 mixture). Semi-thin sections were stained with crystal violet and basic fuchsin and observed by using an Eclipse Ni-U microscope (Nikon, Tokyo, Japan) equipped with a TrueChrome II S digital camera system (Tucsen Photonics, Fuzhou, China). Ultra-thin sections were stained with uranyl acetate and lead citrate and observed by using a JEM-1010 electron microscope (Jeol, Tokyo, Japan) equipped with a Morada digital camera (Olympus, Tokyo, Japan).

Analysis of stem cell proliferation

Midguts were isolated from pupae (days 4, 8 and 10) and flies (day 1) and homogenized with a T10 basic ULTRA-TURRAX (IKA, Staufen im Breisgau, Germany) in 1 ml/0.14 g tissue of RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0), to which 1× protease inhibitor cocktail (Sigma-Aldrich, Saint Louis, MO, USA) was added. Homogenates were clarified by centrifugation at 15,000×g for 15 min at 4 °C and supernatants were denatured by boiling the samples in 4× gel loading buffer for 5 min. SDS-PAGE was performed on a 12% acrylamide gel by loading 60 µg protein per lane. After electrophoretic separation, proteins were transferred onto nitrocellulose membranes (Merck-Millipore, Burlington, MA, USA). Membranes were saturated with a solution of 5% milk in Tris-buffered saline (TBS) (50 mM Tris-HCl, 150 mM NaCl, pH 7.5) for 2 h at room temperature and subsequently incubated for 1 h at room temperature with anti-phospho-histone H3 (H3P) antibody (dilution 1:1000 in 2% milk in TBS; Merck-Millipore) and anti-GAPDH antibody (dilution 1:2500 in 5% milk in TBS; Proteintech, Rosemont, IL, USA) to ensure equal gel loading. Antigens were revealed with an anti-rabbit HRP-conjugated secondary antibody (diluted 1:7500; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) and immunoreactivity was detected with SuperSignal chemiluminescence substrates (Thermo Fisher Scientific, Waltham, MA, USA).

Histochemistry

Midgut samples were isolated from last instar larvae and pupae (days 4 and 8), embedded in polyfreeze cryostat embedding medium after dissection and stored in liquid nitrogen

Table 1 Definition and description of the developmental stages of *Hermetia illucens* used in this study

Stage	Larva	Prepupa	Pupa
Cuticle	Light brown color	Brown color	Dark brown color
Motility	High	High	Decreases along the stage
Lifespan	17 days	24–48 h	12 days
Feeding habits	Active feeding	No feeding	No feeding

Rearing conditions are reported in the “Materials and methods” section

until use. Subsequently, 7- μm -thick cryosections were obtained with a CM 1850 cryostat (Leica, Nussloch, Germany) and slides were immediately used or stored at $-20\text{ }^{\circ}\text{C}$. Sections were processed with staining kits for histology (Bio-Optica, Milano, Italy) to reveal lipid droplets (Oil red O staining) and glycogen (Periodic acid-Schiff, PAS, staining) in the midgut tissue. PAS reaction was also performed in combination with diastase (PAS-D), which breaks down glycogen, to confirm the presence of this polysaccharide. Stainings were performed according to the manufacturer's instructions.

DNA fragmentation analysis

Midguts were dissected from pupae (days 4, 8 and 10) and immediately frozen in liquid nitrogen. Genomic DNA was extracted from 15 mg of midgut tissue using the PureLink Genomic DNA kit (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. After spectrophotometric quantification, 200 ng of genomic DNA were loaded on 1% agarose gel to which EuroSafe (Euroclone, Pero, Italy) was added for DNA staining. Electrophoresis was performed at 100 V for about 45 min and the gel was then observed with a UV transilluminator.

Enzyme assays

Midguts with the enclosed luminal content were dissected from adults (day 4) fed ad libitum with banana pulp and immediately frozen in liquid nitrogen.

The total proteolytic activity was assayed with azocasein (Sigma-Aldrich) (Caccia et al. 2014; Charney and Tomarelli 1947; Vinokurov et al. 2006). Frozen samples of midgut were thawed at $4\text{ }^{\circ}\text{C}$ and homogenized in 1 ml/100 mg tissue of Universal buffer (UB) at pH 8.5 (Coch Frugoni 1957). Samples were then centrifuged at $15,000\times g$ for 10 min at $4\text{ }^{\circ}\text{C}$ and supernatant was collected. Protein concentration was determined by the Bradford method (Bradford 1976). Different volumes of homogenate were diluted to 100 μl with UB at pH 8.5, 200 μl of 1% (*w/v*) azocasein solution dissolved in the same buffer were added to the samples and the mixtures were incubated for 30 min at $45\text{ }^{\circ}\text{C}$. The reaction was stopped by adding 300 μl of 12% (*w/v*) trichloroacetic acid at $4\text{ }^{\circ}\text{C}$. The samples were maintained for 30 min on ice and then centrifuged at $15,000\times g$ for 10 min at $4\text{ }^{\circ}\text{C}$. An equal volume of 500 mM NaOH was added to the supernatant and the absorbance was measured at 440 nm. One unit (U) of total proteolytic activity with azocasein was defined as the amount of enzyme that causes an increase in absorbance by 0.1 unit per min per mg of proteins. α -amylase activity was assayed using starch as a substrate (Bernfeld 1955). A standard curve was determined through linear regression of the maltose absorbance at 540 nm. Frozen samples of the midgut were thawed at $4\text{ }^{\circ}\text{C}$ and homogenized in 1 ml/100 mg tissue of amylase buffer (AB) (20 mM NaH_2PO_4 ,

6.7 mM NaCl, pH 6.9). Samples were then centrifuged at $15,000\times g$ for 10 min at $4\text{ }^{\circ}\text{C}$ and supernatant was collected. Protein concentration was determined and different volumes of homogenate were diluted to 595 μl with AB. Then, 90 μl of 1% (*w/v*) soluble starch solution in AB was added to the samples. Controls without homogenate and controls without substrate were performed for each experiment. All samples were incubated for 30 min at $45\text{ }^{\circ}\text{C}$ and, after adding 115 μl of Color Reagent Solution (1 M sodium potassium tartrate, 48 mM 3,5-dinitrosalicylic acid, 0.4 M NaOH), were heated at $100\text{ }^{\circ}\text{C}$ for 15 min, then cooled in ice to $25\text{ }^{\circ}\text{C}$ and their absorbance was measured at 540 nm. One unit of α -amylase activity (U) was defined as the amount of enzyme necessary to produce 1 mg of maltose per min per mg of proteins.

The activity of aminopeptidase N (APN) was assayed using L-leucine p-nitroanilide (Sigma-Aldrich) as substrate (Franzetti et al. 2015) and measuring its degradation by release of p-nitroaniline (pNA). Frozen samples of the midgut were thawed at $4\text{ }^{\circ}\text{C}$ and homogenized in 1 ml/100 mg tissue of Tris-HCl 50 mM, pH 7.5. Samples were then centrifuged at $15,000\times g$ for 10 min at $4\text{ }^{\circ}\text{C}$ and supernatant was collected. Protein concentration was determined and different volumes of homogenate were diluted to 800 μl with the same buffer; then, 200 μl of 20 mM L-leucine p-nitroanilide were added. Samples were subjected to a continuous absorbance reading at 410 nm at $45\text{ }^{\circ}\text{C}$. One unit/mg protein (U/mg) of APN activity was defined as the amount of enzyme that releases 1 μmol of pNA per min per mg of proteins.

Sucrose hydrolysis in midgut samples was measured using the Invertase Activity Colorimetric Assay Kit (BioVision, Milpitas, CA, USA). Frozen samples of the midgut were thawed at $4\text{ }^{\circ}\text{C}$ and homogenized in Invertase Hydrolysis buffer provided with the kit (40 μl buffer/1 mg of the midgut sample). The homogenate was centrifuged at $15,000\times g$ for 10 min at $4\text{ }^{\circ}\text{C}$ and the supernatant was collected and processed according to the manufacturer's instructions.

RT-PCR

Midguts were collected from flies just after eclosion. Insects were dissected on ice and the tissues were immediately frozen in liquid nitrogen until use. Total RNA was isolated from 15 mg of frozen tissue using TRIzol reagent (Life Technologies) according to the manufacturer's instructions. RNA was treated with a TURBO DNA-free kit (Life Technologies) to remove possible genomic DNA contamination and its integrity was assessed by electrophoresis. RNA was retrotranscribed to cDNA using M-MLV reverse transcriptase (Life Technologies) (Montali et al. 2017). PCR was performed using GoTaq DNA polymerase (Promega, Madison, WI, USA) ($95\text{ }^{\circ}\text{C}$ for 30 s, $T_m - 2\text{ }^{\circ}\text{C}$ for 30 s, $72\text{ }^{\circ}\text{C}$ for 30 s, 35 cycles) and a MyCycler Thermal Cycler System (Bio-Rad, Hercules, CA, USA). The primers used for PCR are listed

in Table 2. The primers for *Hi α -glucosidase* were designed on conserved regions of this gene in other insect species and the sequence was checked by sequencing the PCR product.

qRT-PCR

Midguts were collected from 45 starved flies and 45 flies reared on sugar (see the “Fly longevity” section below). The total RNA was isolated and retrotranscribed as described above (see the “RT-PCR” section). qRT-PCR was performed with iTaq Universal SYBR Green Supermix (Bio-Rad) using a 96-well CFX Connect Real-Time PCR Detection System (Bio-Rad). The relative expression of the *Hi α -glucosidase* gene was calculated by using the $2^{-\Delta\Delta C_t}$ method, with *HiRPL5* (*Hermetia illucens* Ribosomal Protein L5) as a housekeeping gene. The primers used for *HiRPL5* (Table 2) were designed on conserved regions of RPL5 in other insect species and the sequence was checked by sequencing the PCR product.

Analyses of food transit along the alimentary canal

To demonstrate the food transit along the alimentary canal of the adult insect, flies were collected just after eclosion, divided into separate groups (10 flies/group) and kept in a 165 × 30 mm Petri dish. A filter paper (214 cm²) was put on the bottom of the Petri dish. The experimental groups were subjected to the following treatments: (1) no feeding (control) and (2) feeding ad libitum with banana + 2% (w/w) purple food coloring. The groups were monitored daily and the color and number of spots observed on the filter paper were recorded until the flies died. Each condition was performed in triplicate. Flies fed with banana and food coloring (treatment 2) for 4 days were also video recorded to evaluate the release of fecal spots. Video recordings were performed with a camera with a macro lens (Canon EOS 550D equipped with Canon EF-S 60 mm f/2.8 Macro USM, Canon Inc., Tokyo, Japan) fixed onto a tripod placed under a Petri dish containing six BSF adults. The bottom of the Petri dish was covered with a white sheet.

The following treatments were performed to directly visualize the food transit in the midgut of the adult insect: (1) no

feeding or feeding ad libitum with banana (controls), (2) feeding ad libitum with banana + 1.25% (w/w) FITC (Sigma-Aldrich) and (3) feeding ad libitum with banana + 3.75% (w/w) 25 nm gold-conjugated protein A (Electron Microscopy Sciences, Hatfield, PA, USA). For all treatments, flies were dissected every 24 h from 1 to 6 days after the beginning of the experiment. For the first and second treatments, the alimentary canal was mounted with Citifluor (Electron Microscopy Sciences) on a glass slide and observed under a fluorescent microscope (filter 488 nm). Presence of green fluorescence was also analyzed on the labella, dorsal and ventral translucent windows on the abdomen and on the spots released on the filter paper. For the third treatment, the alimentary canal was processed for TEM analysis as described in the “Light microscopy and transmission electron microscopy” section.

Fly longevity

Forty newly emerged flies (< 15 h after eclosion) were placed in 30 × 30 × 30-cm cages and maintained under the environmental conditions reported in the “Experimental animals” section. The flies were reared in different conditions: (1) no food or water (starved), (2) only water (water) and (3) water and sugar cube (sugar). Water was provided to the animals in a 50-ml plastic tube containing cotton. Each experimental condition was performed in triplicate. The survival of the flies under different conditions was recorded every day.

Statistical analyses were performed with the R-statistical software (ver. 3.3.2). One-way analysis of variance (ANOVA) with longevity as the dependent variable followed by Tukey’s test was performed. Statistical differences between groups were considered significant at *p* value ≤ 0.05.

Results

Morphological analysis of the fly mouthparts

The adult Diptera, depending on dietary habits, exhibit a great variety of modifications of the mouthparts but in all of them

Table 2 Sequence of primers used in this study

Gene name	Accession number	Primer sequences	Melting temperature (T _m) (°C)	PCR product size (bp)
<i>HiTrypsin</i>	HQ424575	F: ATCAAGGTCTCCCAGGTC R: GGCAAGAGCAATAAGTTGGAT	56	126
<i>HiChymotrypsin</i>	HQ424574	F: AGAATGGAGGAAAGTTGGAGA R: CAATCGGTGTAAGCAGAGACA	57	109
<i>Hiα-glucosidase</i>	–	F: GGCTTTCAGTTGCTCCGTTA R: AGGCTCGTTATTGATGTCGC	58	127
<i>HiRPL5</i>	–	F: AGTCAGTCTTCCCTCACGA R: GCGTCAACTCGGATGCTA	57	145

the food canal is formed between the apposed labrum and labium and the salivary canal runs through the hypopharynx. BSF showed typical sponging mouthparts. As common in nonhematophagous Diptera, the mandibles and maxillae were lacking and the distal part of the labium was expanded to form the labella (Fig. 1a) which were traversed by a series of grooves known as pseudotracheae (Fig. 1b). These structures were maintained open by cuticular ribs (Fig. 1c), giving them a superficial similarity to the tracheae and converged centrally on the distal end of the food canal. Prestomal tooth-like structures (Fig. 1b) could be used to scrape semi-solid feeding substrates. Similar teeth are present in other Brachycera, including the housefly, *Musca domestica* Linnaeus, 1758 (Diptera: Muscidae) (Giangaspero and Broce 1993; Kovacs et al. 1990). The mouthparts presented many sensilla protruding from the cuticle (Fig. 1b), which are probably involved in chemo- and mechano-reception.

The morphological features of BSF mouthparts indicated that the adult insect may ingest food and prompted us to investigate the digestive apparatus of the fly.

Modification of the alimentary canal during metamorphosis: degeneration of the larval midgut epithelium and differentiation of the pupal-adult midgut

In holometabolous insects, the remodeling of the larval midgut is a key process that occurs during metamorphosis;

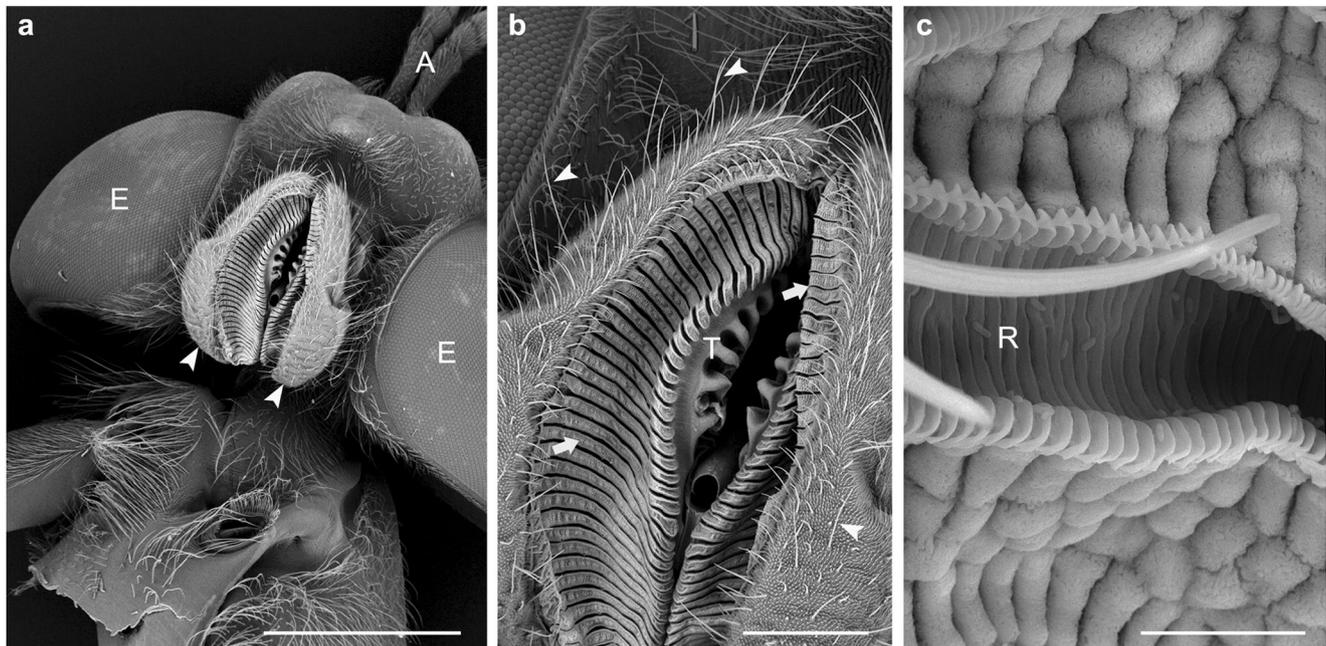


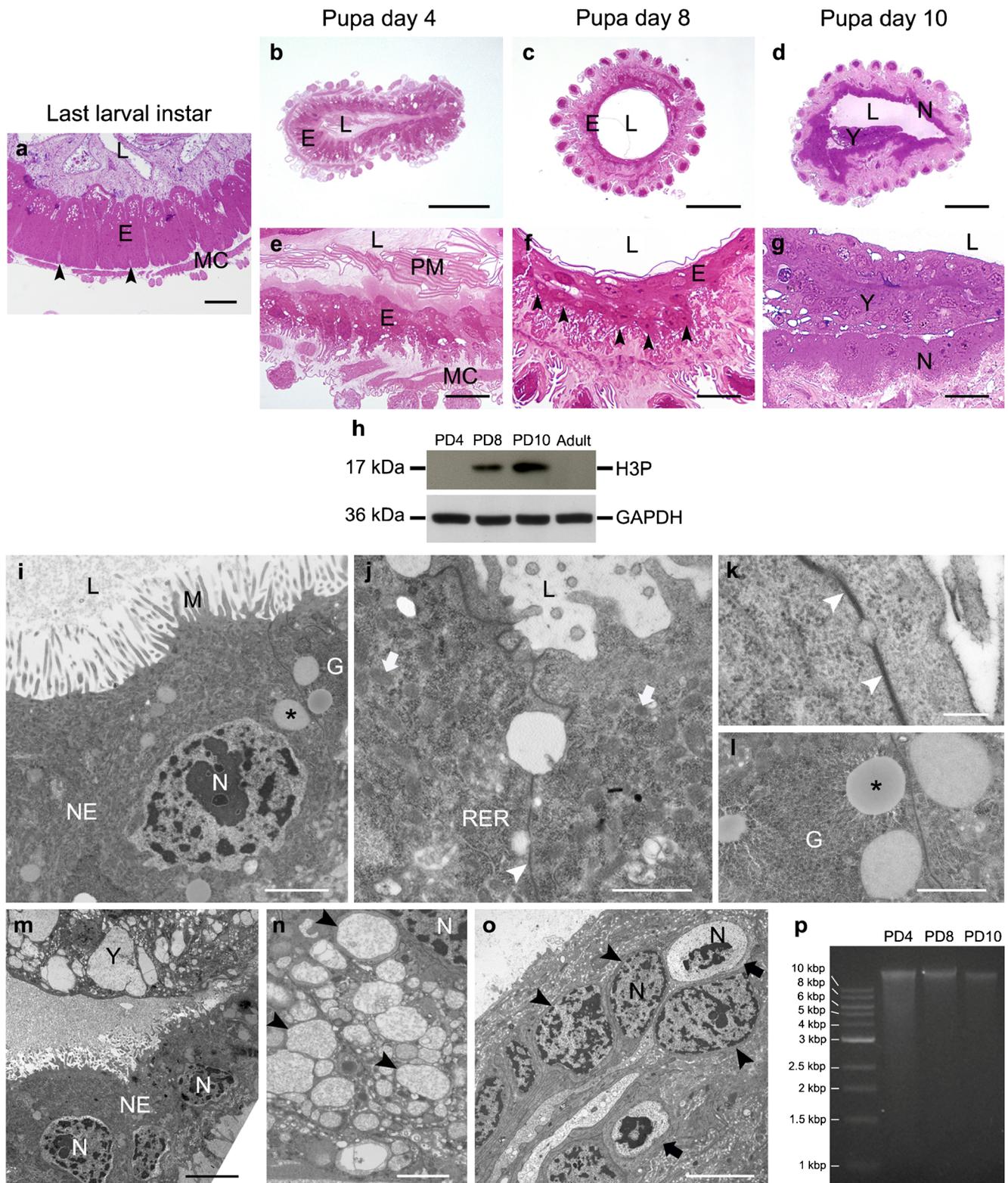
Fig. 1 SEM analysis of BSF adult mouthparts. **a** Ventral view of BSF head in which everted labella (arrowheads) are visible. **b** View of the sponging mouthparts where tooth-like structures (T), pseudotracheae

Fig. 2 Modification of the larval midgut during metamorphosis and ultrastructural analysis of pupal midgut. **a** Cross-section of the midgut epithelium of last instar larvae. Stem cells (arrowheads) are visible at the base of the epithelium. **b, e** Cross-sections of the midgut epithelium of day 4 pupae. **c, f** Cross-sections of the midgut epithelium of day 8 pupae. A high number of stem cells (arrowheads) are recognizable at the base of the epithelium. **d, g** Cross-sections of the midgut epithelium of day 10 pupae. The larval midgut detaches from the newly forming epithelium (N) and is pushed toward the lumen, forming the yellow body (Y). **h** Western blot analysis of phospho-histone 3 (H3P). **i–l** The newly forming midgut epithelium (NE) displays microvilli (M) in the apical membrane (**i**), abundant rough endoplasmic reticulum (RER) and mitochondria (arrows) in the cytoplasm (**j**). Smooth septate junctions (arrowheads) are present among columnar cells (**j, k**). Glycogen (G) and lipid droplets (asterisks) can be observed in the cytoplasm (**i, l**). **m** The newly forming midgut epithelium (NE) progressively detaches from the larval midgut, which forms the yellow body (Y). **n** Vacuoles (arrowheads) in the cytoplasm of yellow body cells. **o** Yellow body that contains a high number of cells with an intact nucleus (arrowheads) and some cells with pyknotic nuclei (arrows). **p** Ladder analysis of genomic DNA from midgut cells at pupa days 4, 8 and 10. **e, f, g** Details at higher magnification of **b, c, d**, respectively. *E* epithelium, *L* lumen; *MC* muscle cells, *N* nucleus, *PM* peritrophic matrix. Bars, 25 μm (**a, e–g**), 100 μm (**b–d**), 2 μm (**i, n**), 1 μm (**j, l**), 500 nm (**k**), and 5 μm (**m, o**)

therefore, we first investigated the morphology of the alimentary canal during the larva-pupa molt.

Although regional differentiation was observed along the anteroposterior axis of the larval midgut of *H. illucens* (Bonelli et al. 2019), this organ consisted of a monolayered epithelium, mainly formed by columnar cells, organized over a thin basal lamina and encircled by an extraepithelial layer composed of muscle fibers (Fig. 2a). Sparse stem cells were

(arrows) and sensilla on the labella (arrowheads) can be observed. **c** Detail of pseudotracheae characterized by cuticular ribs (R). *A* antennae, *E* compound eye. Bars, 1 mm (**a**), 200 μm (**b**), and 10 μm (**c**)



localized in the basal region of the epithelium (Fig. 2a). During the early pupal stage (up to day 4), the general morphology of the midgut epithelium was maintained and columnar cells could still be identified (Fig. 2b, e), while at pupa day

8 the presence of a large number of stem cells could be observed in the basal part of the epithelium (Fig. 2c, f). At late pupal stage (day 10), a newly forming epithelial layer was visible, while the larval cells were pushed in the lumen

(Fig. 2d, g). The proliferation of stem cells at pupa day 8 was confirmed by Western blot analysis of H3P, showing a high expression of this mitotic marker (Fig. 2h). The mitotic activity was also maintained at later stages (pupa day 10), close to the pupa-adult molt but no signal was detected in the adult midgut (Fig. 2h).

During the pupal stage (days 8–10), the newly forming midgut was characterized by features that are typical of a secretory/absorptive epithelium (Fig. 2i–l). In fact, the apical membrane formed microvilli (Fig. 2i), abundant rough endoplasmic reticulum and mitochondria could be observed in the cytoplasm (Fig. 2j) and the cells were linked by smooth septate junctions (Fig. 2j, k). Moreover, glycogen granules and lipid droplets were present in the cytoplasm (Fig. 2i, l). On the other hand, the old larval epithelium underwent a consistent remodeling during the pupal stage. In fact, the cells gave rise to a compact mass (called “yellow body”) that later detached from the newly forming epithelium (Fig. 2d, g, m). Although during the removal of the larval midgut epithelium clear signs of degeneration could be detected in some cells, i.e., vacuolization of the cytoplasm (Fig. 2m, n) and unusual organization of the nuclear chromatin (Fig. 2o), the large part of the cells showed an intact morphology (Fig. 2o) during pupal stage.

Given the presence of condensed chromatin in some yellow body cells, we investigated the occurrence of apoptosis in the midgut undergoing remodeling. Quite surprisingly, DNA ladder analysis did not show any DNA fragmentation in the degenerating larval tissue (Fig. 2p).

The histochemical analysis gave evidence that long-term storage molecules were mobilized during larva-pupa transition: in particular, a reduction of glycogen (Fig. 3a–c) and lipid (Fig. 3g–i) reservoirs was detected in the larval midgut epithelium at the early pupal stage (Fig. 3a, b, g, h) and no reactivity was present inside the yellow body (Fig. 3c, i). Specificity of the PAS reaction toward glycogen was confirmed by treatment with diastase (Fig. 3d–f).

Morphological analysis of the adult midgut

The new midgut epithelium observed in late pupae (day 10), just before the adult emerged, was retained in the fly, where it further differentiated. The midgut of the adult insect was subdivided into three morphologically distinct regions: while the anterior and posterior districts were thin and tubular structures, the middle region appeared as an enlarged compartment (Fig. 4a). A large crop was associated to the foregut (Fig. 4b). The regional organization of the central part of the alimentary canal of the fly was supported by the histological analysis showing a different organization of the epithelium along the midgut (Fig. 4c–h). While the anterior (Fig. 4c, f) and posterior (Fig. 4e, h) midgut were lined by a thick and infolded epithelium, a wide lumen was surrounded by a thin and

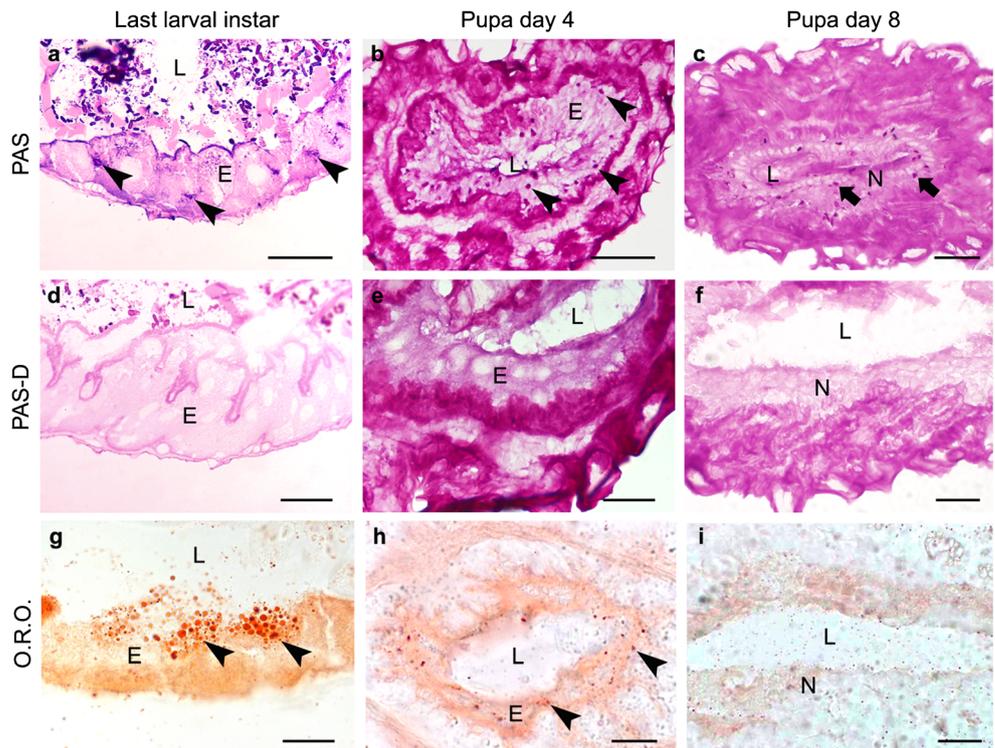
unfolded cell monolayer in the middle region (Fig. 4d, g). Columnar cells represented the main cell type found in all the three midgut districts. These cells, although characterized by a different thickness in the various districts, always showed a wide basal infolding (Fig. 5a) and apical microvilli (Fig. 5b) and were linked by smooth septate junctions (Fig. 5c). A great number of mitochondria was present in the apical region of the cells (Fig. 5b, d). Glycogen granules and abundant rough endoplasmic reticulum were visible in the cytoplasm (Fig. 5d). This evidence correlated well with a consistent secretory activity. In fact, we observed microvilli with an enlarged tip (Fig. 5e), a feature typical of microapocrine secretion and the release of secretory vesicles along the apical surface of the epithelium (apocrine secretion) (Terra and Ferreira 2005) (Fig. 5f). Interestingly, the peritrophic matrix was not present and the lumen content was in direct contact with the brush border (Fig. 5g, h). The midgut epithelium was supported by a thick basal lamina (Fig. 5i) and muscle (Fig. 5a, j). In all three midgut regions, endocrine cells localized in the basal region of the epithelium (Fig. 5j), containing electron-dense granules in the cytoplasm (encircled by a membrane) (Fig. 5k), were visible. Sparse, small round cells were localized in the basal region of the epithelium (Fig. 5l): due to their undifferentiated morphology and high nucleus-to-cytoplasm ratio, they could be classified as stem cells. Neither morphological analysis nor feeding assay with cupric chloride and pH lumen measurement indicated the presence of copper cells in the epithelium (Supplementary 1).

Some yellow body cells could be observed in the midgut lumen: similarly to the yellow body cells observed at the pupal stage (Fig. 2m, n), these cells showed a vacuolated cytoplasm but the nucleus appeared intact (Fig. 5m).

Analysis of ingested food transit in the adult midgut and its digestive capability

We first performed feeding experiments to evaluate the function of the alimentary canal in the fly (i.e., the ability of the adult insect to ingest food and move the bolus along the organ). Flies were fed *ad libitum* with banana and FITC and the midgut isolated from the insects was observed under a fluorescent microscope. Four days after the food substrate was administered, the whole midgut was characterized by a green fluorescence that could not be detected in the midgut of flies subjected to starvation or fed only with banana (controls) (Fig. 6a, a'). In addition, the analysis of the labella (Fig. 6b, b'), of the ventral (Fig. 6c, c') and dorsal (Fig. 6d, d') translucent windows on the abdomen and of the fecal spots (Fig. 6e, e') revealed green fluorescence only in animals fed with banana and FITC. These results confirmed that the fluorescent dye, after being ingested, passed through the midgut and was released by the anus.

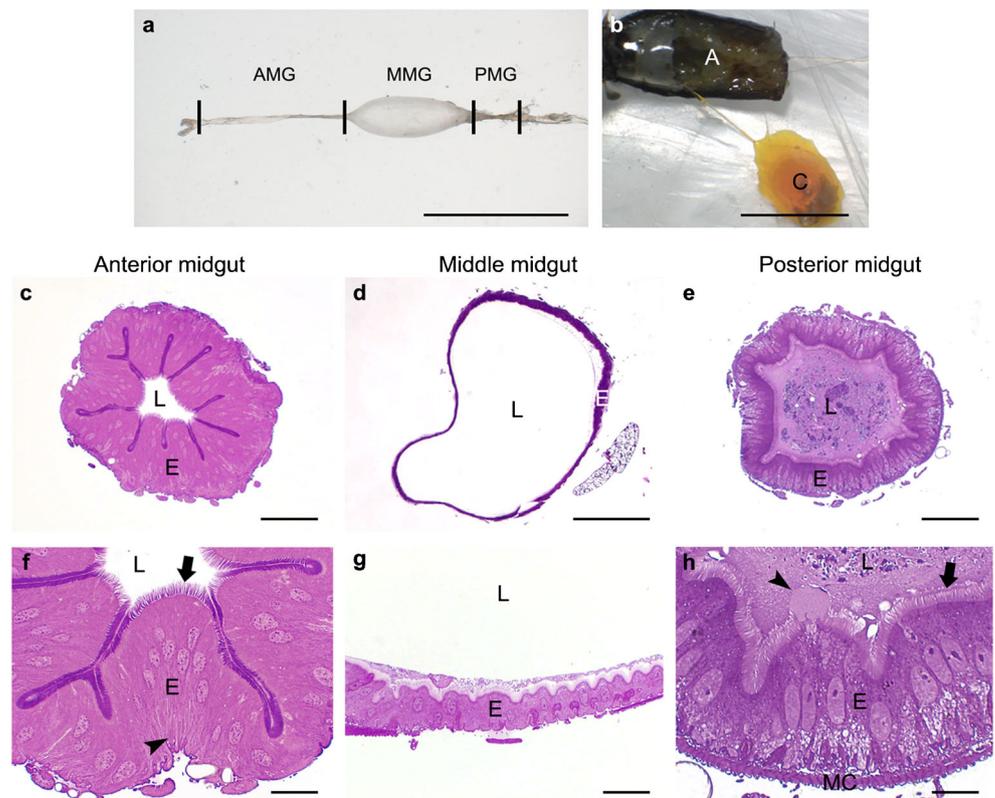
Fig. 3 Histochemical analysis of the midgut epithelium. **a–c** PAS. Staining for PAS reveals that glycogen deposits (purple, arrowheads) disappear in the larval midgut epithelium at the pupal stage. A signal is visible only in the newly forming midgut epithelium (arrows). **d–f** Glycogen deposits disappear after the treatment with diastase (PAS-D). **g–i** O.R.O. Staining for O.R.O. shows that lipid droplets (red, arrowheads) disappear in the larval midgut epithelium at the pupal stage. *E* larval epithelium, *L* lumen, *N* newly forming epithelium. Bars, 50 μ m (**a, b**), 20 μ m (**c–e, g, h**) and 10 μ m (**f, i**)



To better analyze the food transit along the digestive system, flies were fed with banana and purple food coloring and the fecal spots released by the insects on the filter paper on the

bottom of the Petri dish were analyzed and counted. As shown in Fig. 7(a–c), while in control insects (no food substrate administered) some spots were observed from day 1 to day 6, the

Fig. 4 Morphological analysis of the adult midgut. **a** General view of the adult midgut subdivided in anterior (AMG), middle (MMG) and posterior (PMG) midgut. **b** General view of the crop (C) associated to the gut. **c, f** Cross-section of the anterior midgut characterized by a thick epithelium with apical brush border (arrow) and developed basal infolding (arrowhead). **d, g** Cross-section of the middle midgut that shows a very thin epithelium. **e, h** Cross-section of the posterior midgut characterized by a thick epithelium with developed brush border (arrow) and apocrine secretion (arrowhead) in the apical membrane. **f, g, h** Details at higher magnification of **c, d** and **e**, respectively. *A* abdomen of the fly, *E* epithelium, *L* lumen, *MC* muscle cells. Bars, 5 mm (**a**), 3 mm (**b**), 50 μ m (**c, e**), 200 μ m (**d**) and 10 μ m (**f–h**)



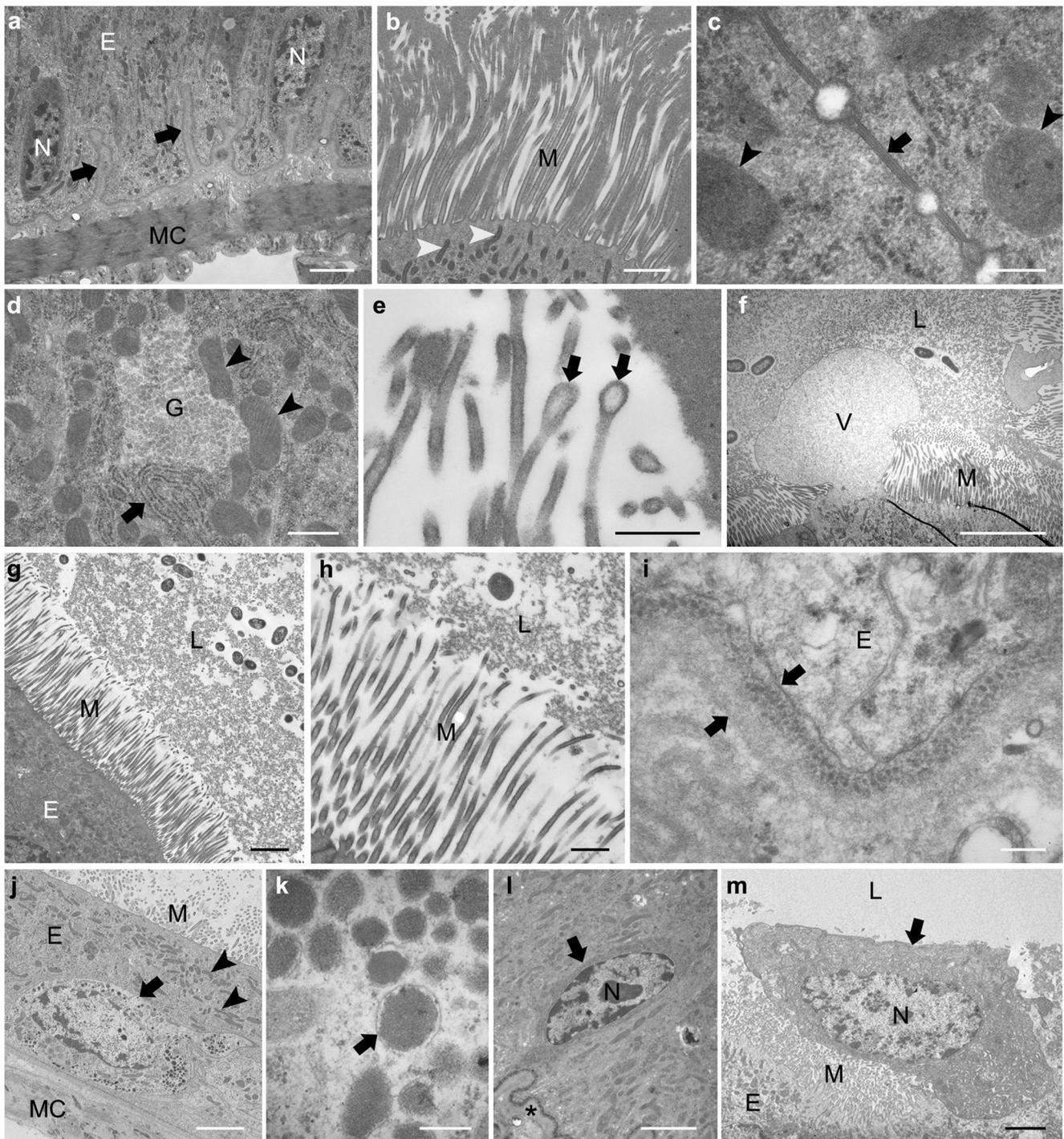


Fig. 5 Ultrastructural analysis of the adult midgut—TEM. **a** A thick muscle layer (MC) surrounds the epithelium, characterized by developed basal infoldings (arrows). **b** A high number of mitochondria (arrowheads) are present in the apical region of the cells, under the microvilli (M). **c** Detail of smooth septate junctions (arrow) between two columnar cells. **d** Glycogen granules (G), abundant rough endoplasmic reticulum (arrow), and mitochondria (arrowheads) are visible in the cytoplasm. **e** Apical microvilli show enlarged tips (arrows). **f** Secretion vesicles (V) can be observed in the apical membrane of the cells. **g, h** The lumen content (L) is in direct contact with the microvilli (M) due to the absence of a

peritrophic matrix. **i** A thick basal lamina (arrows) supports the midgut epithelium. **j, k** Endocrine cells (arrow) are located at the base of the epithelium (**j**). Their cytoplasm is filled with a high number of electron-dense granules encircled by a membrane (arrow) (**k**). **l** Detail of a stem cell (arrow) located at the base of the epithelium. Asterisk indicates the basal lamina. **m** Yellow body cell (arrow), localized in the midgut lumen close to the newly forming epithelium, characterized by an intact nucleus (N). Arrowheads mitochondria, E epithelium, L lumen, M microvilli, MC muscle cells, N nucleus. Bars, 2 μm (**a, g, h, j, l, m**), 1 μm (**b**), 500 nm (**d, e**), 200 nm (**c, i, k**) and 5 μm (**f**)

number of fecal spots rapidly increased from day 1 up to day 12 in animals fed with food coloring. It is noteworthy that, while in control flies the color of the spot was light brown (Fig. 7d), the spots were dark purple in fed animals (Fig. 7e), thus demonstrating the transit of the stain throughout the alimentary canal. Production of fecal spots could be clearly observed in the video (Fig. 7f–h' and Supplementary 2). To confirm that the adult insect was able to ingest food and the bolus transited along the alimentary canal, the midgut of flies fed with gold-conjugated protein A was isolated 6 days after administering the food substrate and analyzed at the ultrastructural level. TEM analysis demonstrated the presence of gold particles in the midgut lumen (Fig. 7i, j).

To better examine alimentary canal function, we investigated the digestive capabilities of the fly midgut by measuring the activity of enzymes that are involved in the initial and final phase of carbohydrate and protein digestion. As shown in Table 3, a significant total proteolytic activity was recorded, while no α -amylase activity was measured. We also recorded the activity of enzymes involved in the final phase of sugar and protein digestion, i.e., sucrase and aminopeptidase N (Table 3). Finally, we analyzed the expression of chymotrypsin- and trypsin-like proteases, the two most common endopeptidases involved in insect digestion and of α -glucosidase, which

hydrolyzes terminal, non-reducing (1 \rightarrow 4)-linked alpha-glucose residues to release glucose molecules, by RT-PCR. The amplification of a DNA band of the expected length for all the genes tested was obtained, thus demonstrating their expression in the adult midgut (Fig. 8).

Fly longevity under different nutrient conditions and effects of the diet on the expression levels of digestive enzymes

To evaluate whether and how nutrient administration affected the lifespan of *H. illucens* adults, flies were reared in the absence or in the presence of a food source. A remarkable mortality was recorded in starved flies 5 days after beginning the experiments and all the insects died about 1 week after eclosion (Fig. 9a). On the contrary, a higher survival rate was observed when water or water and a sugar cube were provided to flies (Fig. 9a). A significant difference in fly longevity was observed under the three different conditions, with the best performance obtained when water and a sugar cube were provided to insects (Fig. 9b). To evaluate whether the diet influenced the digestive physiology of the insect, we analyzed the expression levels of the gene coding for α -glucosidase in the midgut of flies grown on sugar, by using qRT-PCR. *Hia*-

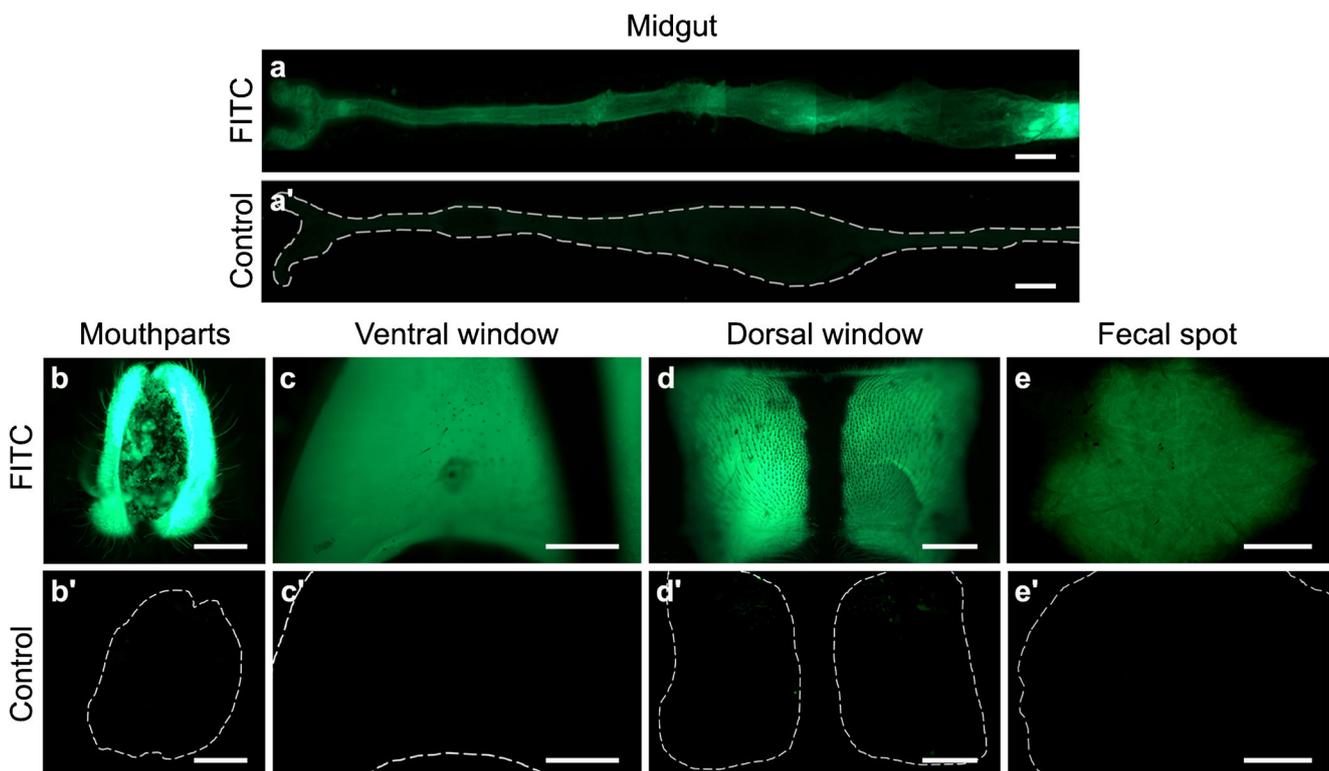


Fig. 6 Analysis of the transit of ingested food—FITC. **a–d** Midgut, mouthparts and ventral and dorsal translucent windows of flies fed with banana and FITC. **e** Fecal spot produced by flies fed with banana and FITC. **a'–e'** Midgut, mouthparts and ventral and dorsal translucent

windows and fecal spot of unfed flies or fed with banana (control). Dotted line indicates the profile of the midgut, mouthparts, translucent windows and fecal spot. Bars, 500 μ m (**a**, **a'**, **e**, **e'**) and 250 μ m (**b–d**, **b'–d'**)

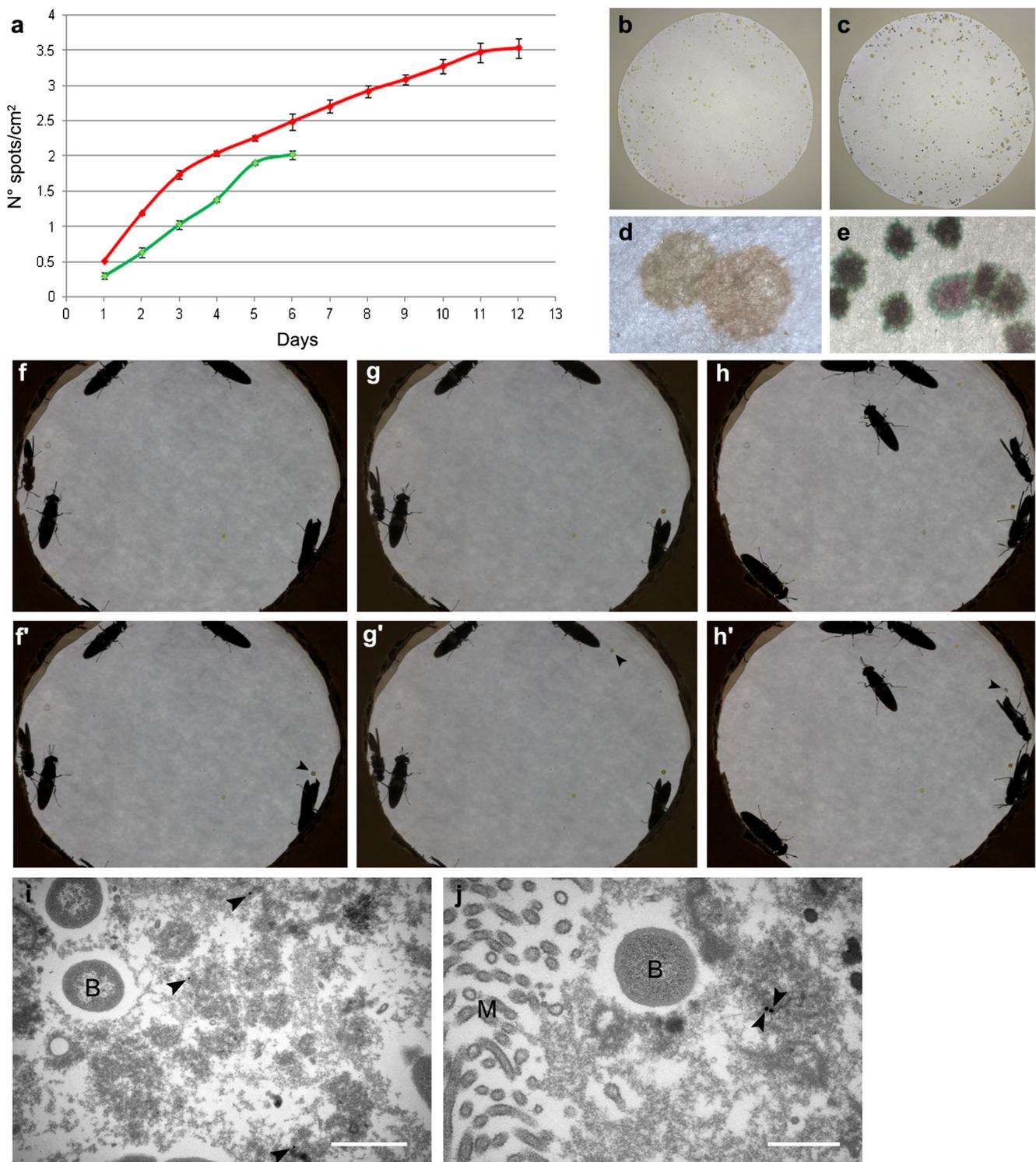


Fig. 7 Evaluation of the transit of ingested food—food coloring and gold-conjugated protein A. **a** Spot counting. Flies fed with banana to which purple food coloring was added are indicated in red; starved flies are indicated in green. **b**, **d** Filter paper showing spots produced by starved flies. **c**, **e** Filter paper showing spots produced by flies fed with banana added with purple food coloring. **f–h'** Video frames showing flies

immediately before **f–h** and after **f'–h'** the production of the spots (arrows). **i**, **j** The presence of gold particles (arrowheads) inside the midgut lumen of flies fed with gold-conjugated protein A can be appreciated in TEM micrographs. **d**, **e** Details at higher magnification of **b** and **c**, respectively. *B* bacteria, *M* microvilli. Bars, 1 μm (**i**) and 500 nm (**j**)

Table 3 Activity of digestive enzymes in the adult midgut homogenate

Total proteolytic activity	α -amylase activity	APN activity	Sucrase activity
(U)	(U)	(U/mg)	(U/ml)
1.64 \pm 0.46 (4)	Non-detectable	0.27 \pm 0.05 (4)	30.45 \pm 1.33 (3)

Mean \pm s.e.m., number of replicates in parentheses

glucosidase mRNA levels in sugar-fed flies were significantly higher than in starved flies (Fig. 9c), demonstrating that the diet was able to upregulate the transcription of genes coding for digestive enzymes in the midgut of *H. illucens* adults.

Discussion

The larvae of BSF represent a relevant option in the search for sustainable and alternative protein sources as they can convert low-quality biomass into nutritionally valuable proteins (Wang and Shelomi 2017). However, to support the growth of the emerging industrial sector of edible insects and their use in the feed market, important issues must be addressed, e.g., the safety of the production process, the production of high-quality insect meal and the exploitation of insects and their products for applications other than feed. In this scenario, a deep knowledge of the biology of the adult insect and in particular of its feeding habits, is necessary as this information

could not only improve mass rearing of BSF but also provide insights into the safety of using this insect for feed purposes. To fill in this gap of knowledge, we undertook the present work and investigated three specific aspects: (i) the remodeling process of the larval midgut during metamorphosis with particular attention on stem cells, (ii) the morphology and function of the adult midgut and (iii) the feeding habits of the fly.

Histolysis of larval organs is one of the key events that occurs in holometabolous insects and is necessary to remove larval cells that the adult insect no longer needs. Concomitantly, the growth and differentiation of adult tissues and organs take place (Franzetti et al. 2012; Hakim et al. 2010; Romanelli et al. 2016). Our results demonstrate that, in *H. illucens*, the larval midgut is completely replaced during metamorphosis by a new functional epithelium that is maintained up to the adult stage. During the pupal stage, midgut stem cells actively proliferate, as confirmed by the expression of H3P, a marker of cells undergoing mitosis. Western blot analysis showed that proliferation activity continues until pupa day 10 when the differentiation of these cells leads to the formation of a well-organized epithelium that will become the midgut of the fly. The larval cells are pushed toward the lumen, form the yellow body and then degenerate. This coordinated series of events, which is responsible for the remodeling of the BSF larval midgut, is generally conserved among Diptera (Lee et al. 2002; Takashima et al. 2011), Coleoptera (Parthasarathy and Palli 2008) and Lepidoptera (Franzetti et al. 2012; Li et al. 2018; Tettamanti et al. 2007, 2008, 2011). In *Bombyx mori*, it has been previously demonstrated that the complete digestion of larval midgut cells by autophagy, followed by apoptotic cell death and the release of their content in the lumen of the newly forming epithelium can contribute to trophically sustain the adult insect, which is unable to feed (Franzetti et al. 2015; Romanelli et al. 2016). Our results show that intact/viable yellow body cells apparently persist until the late pupal stage in *H. illucens* and some of them are still observable in the lumen of the midgut of the adult insect. This peculiar characteristic could be related to the fact that the fly is able to feed and process food, as demonstrated by our data. Thus, although long-term storage molecules, i.e., glycogen and lipids, are progressively reduced in the larval midgut epithelium during the early phase of metamorphosis, a trophic supply from degenerating larval cells might not be mandatory for the adult insect. Conversely, the reserves accumulated in the larval fat body could be sufficient to trophically sustain the insect during

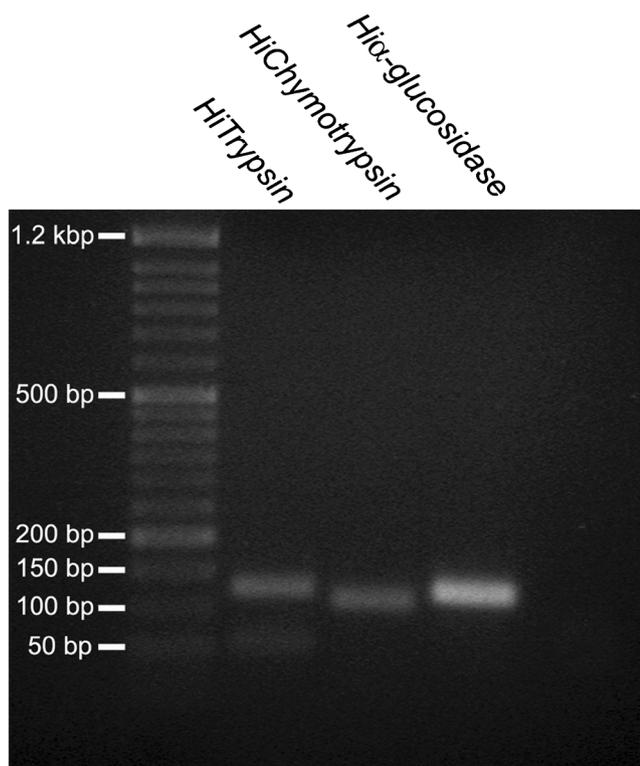


Fig. 8 Gene expression of digestive enzymes. RT-PCR analysis of the expression of *HiTrypsin*, *HiChymotrypsin*, and *Hi α -glucosidase* in the midgut of adult BSF

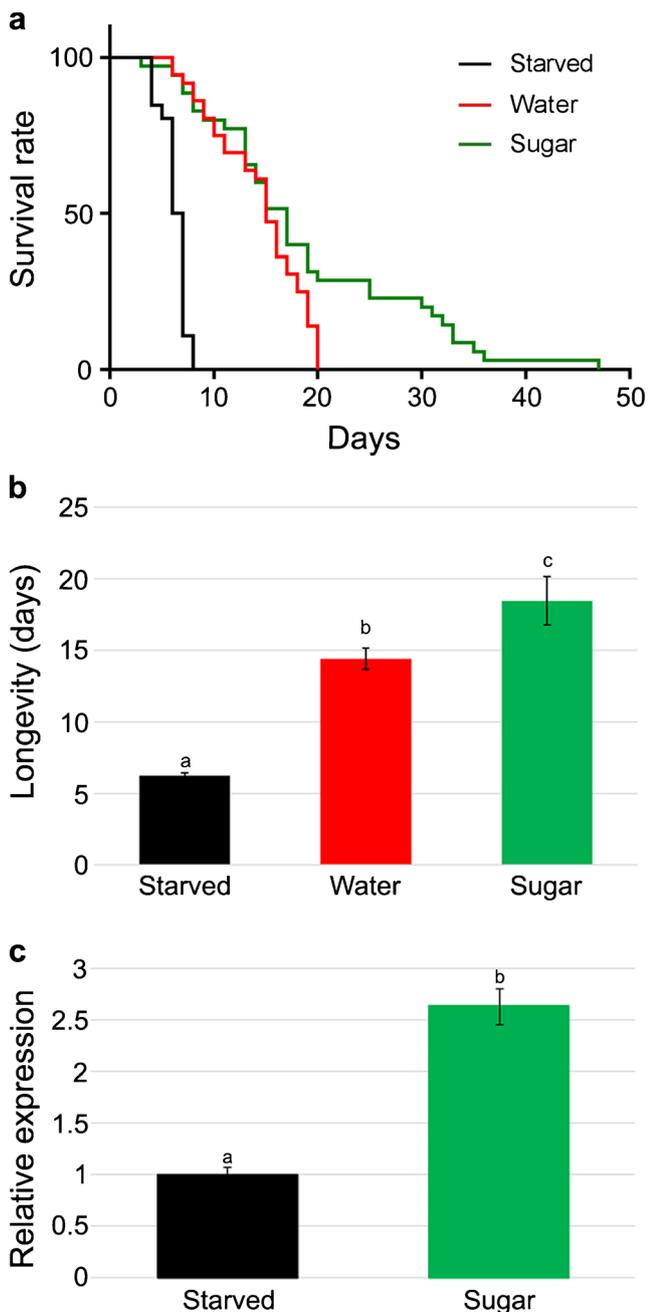


Fig. 9 Longevity of adult *H. illucens* and effects of the diet on the expression levels of digestive enzymes. **a** Survival curves of flies under different dietary conditions. **b** Fly longevity under different nutrient conditions. The values are reported as mean \pm s.e.m. of at least 40 individuals. Different letters denote significant differences (ANOVA test followed by Tukey's test. ANOVA p value < 0.001 , Tukey's test p values: Water vs. Starved $p < 0.001$, Sugar vs. Starved $p < 0.001$, and Sugar vs. Water $p < 0.05$). **c** qRT-PCR analysis of *Hic- α -glucosidase* in the midgut of starved (Starved) and fed flies (Sugar). The values are reported as relative expression mean \pm s.e.m. of three experiments. Different letters denote significant differences (unpaired t test followed by Tukey's test. p value < 0.05)

metamorphosis: this could explain why an early and complete degeneration of the yellow body in the pupa is not needed in

H. illucens and the timing of the degeneration of the larval midgut is different from that observed in those holometabolous species whose adult insect does not feed (Franzetti et al. 2015; Romanelli et al. 2016).

The midgut epithelium of the adult BSF is mainly formed by columnar cells that are responsible for the digestive processes. The activity of this organ is supported by endocrine and stem cells. Only closed-type endocrine cells, which remain close to the basal lamina and do not extend through the whole epithelium thickness (Fujita and Kobayashi 1977), with the presence of a large number of electron-dense granules in the cytoplasm (Endo and Nishiitsutsuju-Uwo 1981; Nishiitsutsuju-Uwo and Endo 1981), were observed. The number of stem cells, identified on the basis of their morphology (Franzetti et al. 2015), was limited, which is in accordance with the lack of expression of H3P in adult samples. The absence of intestinal damage or pathogenic bacteria in our rearing conditions, two strong inducers of intestinal stem cell proliferation in adult *D. melanogaster* (Amcheslavsky et al. 2009; Buchon et al. 2013), could maintain these cells in a quiescent state. Morphological analysis highlighted two peculiar aspects that characterize the midgut epithelium of the adult insect: the lack of copper cells and the absence of peritrophic matrix (PM). Copper cells are a peculiar cell type that in Diptera are able to acidify the middle midgut lumen thanks to the secretion of protons (Shanbhag and Tripathi 2009). In *D. melanogaster* larvae, the administration of copper in the diet causes copper cells to acquire an orange fluorescence signal due to the formation of a complex between copper and metallothioneins (McNulty et al. 2001). The absence of a fluorescent signal in our feeding experiments with cupric chloride, as well as the absence of a midgut region with a very acidic pH in the lumen, confirm the morphological evidence. In contrast, copper cells in the midgut epithelium of BSF larvae contribute to the establishment of a strongly acidic pH (about pH 2) in the middle midgut (Bonelli et al. 2019). This feature, differing between the two developmental stages of BSF, could be due to their feeding habits: at variance with the adults, the larvae grow on substrates that can be highly contaminated by microorganisms and the very acidic pH of the middle midgut lumen helps kill them (Bruno et al. 2019; Padilha et al. 2009). Our morphological investigation was not able to detect the presence of PM in all the midgut districts analyzed: this result did not appear to be an artifact, nor was the acellular sheath lost during dissection of the organ, as the lumen content was clearly visible and in close contact with the microvilli. The absence of PM could be explained by considering different aspects related to the feeding habits of the insect: (i) it has been suggested that fluid-feeding species might not need PM for the mechanical protection of the midgut epithelium (Lehane 1997); (ii) according to Billingsley and Lehane (Billingsley and Lehane 1996), the degree of microbial contamination of the liquid diet may be a more important

factor determining presence or absence of the PM, so that insects feeding on a less infected liquid diet tend to lack PM; and (iii) Villanon and collaborators (Villalon et al. 2003) demonstrated that the absence of PM increases the rate of protein hydrolysis; thus, despite the protective action, this acellular layer could partially restrict hydrolytic enzyme movement from the midgut cells to the lumen.

The midgut of the adult BSF is endowed with features typical of a functional epithelium. In addition to the presence of well-developed microvilli, abundant mitochondria under the brush border, rough endoplasmic reticulum and secretory vesicles, all features that indicate a secretory and absorbing activity of these cells, the evaluation of enzymatic activity revealed that this organ is able to digest macromolecules. A significant enzymatic activity involved in both protein and sugar digestion was measured in the midgut homogenate. Total proteolytic activity and APN activity are responsible for digesting proteins, from the initial to the final phase of this process (Terra and Ferreira 1994). Surprisingly, differently from other brachyceran flies such as *D. melanogaster* (Chng et al. 2014) and *M. domestica* (Pimentel et al. 2018; Shina 1975; Terra et al. 1988a) and from *H. illucens* larvae (Bonelli et al. 2019), we did not record α -amylase activity in the midgut of adult BSF. However, it was demonstrated that the expression of amylase is repressed in *D. melanogaster* flies fed on high sugar diets (Benkel and Hickey 1986; Chng et al. 2014; Hickey and Benkel 1982). Since we fed flies with fully ripe banana pulp, which contains a high percentage of reducing sugars (33.6–33.8%) and sucrose (52.0–53.2%) and a very low percentage of starch (2.6–3.3%) (Lii et al. 1982), monosaccharides are already available to be absorbed and they can also be produced by the hydrolysis of sucrose thanks to sucrase activity. Thus, it is possible that the expression of amylase was repressed in our experimental conditions. Moreover, when we provided sugar to the flies, we observed an upregulation of α -glucosidase expression levels compared to starved flies, confirming that the diet can modulate the expression of digestive enzymes.

The results on midgut morphology and physiology, together with the presence of a typical sponging mouthpart, led us to investigate the function of the alimentary canal in terms of food ingestion and transit of the bolus. All our evidence supports data indicating that *H. illucens* flies possess a fully functional alimentary canal: feeding experiments with food coloring and gold-conjugated protein A, video recording and evaluation of fecal spots clearly demonstrate that the alimentary canal of the fly is endowed with motility and that bolus transit in the lumen occurs. As shown by the movie, fecal spots are produced by fed flies. However, we cannot exclude that some of the spots observed on filter paper derive from regurgitation, i.e., the expulsion of material from any location within the foregut out of the oral cavity (Rivers and Geiman 2017). This process, which can be associated with different functions

in flies, such as food processing or elimination of excess water, involves the crop, a foregut organ present in Diptera (Stoffolano et al. 2008; Stoffolano and Haselton 2013). Although spots deriving from secretion and excretion processes that occur in the alimentary canal of flies are not easy to identify on the basis of their morphology (Rivers and Geiman 2017), the presence of a large crop associated to the gut of adult *H. illucens*, as well as the spots observed both in fed and unfed insects, suggest that BSF might regurgitate. It must be highlighted that fecal spots visible in unfed flies are produced in smaller numbers than in fed flies. Moreover, it cannot be excluded that these spots may be due not only to regurgitation but also to the elimination of meconium after eclosion (Rivers and Geiman 2017).

There is general agreement that *H. illucens* adults emerge relatively free of pathogens and, due to their relatively short lifespan compared to other flies, they do not eat. This common belief not only is in contradiction with the few previous studies that used different substrates to rear BSF (Bertinetti et al. 2019; Nakamura et al. 2016) but is definitely disproved by our evidence that the adult BSF is able to ingest and process food, produce frass and may even regurgitate. In addition, our feeding experiments clearly show that food administration affects the longevity of the fly, confirming previous results (Bertinetti et al. 2019; Nakamura et al. 2016). This evidence suggests that the energy requirements of the fly do not depend exclusively on reserves accumulated during the larval stage as reported in the literature (Sheppard et al. 2002; Tomberlin and Sheppard 2002; Tomberlin et al. 2002). In contrast, the ability of the fly to ingest and process food can be exploited to increase its performance in terms of lifespan and oviposition (Bertinetti et al. 2019). In this scenario, our study contributes to pave the way for a deeper understanding of the nutrient requirements of the adult and a better exploitation of this insect in mass rearing processes. Moreover, the feeding behavior of adult *H. illucens* should be carefully considered in view of the recommendations reported in the EFSA opinion (EFSA Scientific Committee 2015) concerning the safety of insects used for feed production and the lack of pathogenic effects, as also required by European Commission Regulation No 2017/893 (European Commission 2017). In fact, regurgitation, defecation and transstadial transmission have been described as potential routes of pathogen transmission in nonbiting flies (Graczyk et al. 2001, 2005).

Conclusions

Thanks to a multidisciplinary approach, our study clearly demonstrates for the first time that *H. illucens* adults can feed and provides an in-depth description of the morphofunctional features of the fly midgut. This information not only directs attention to the safety of this species as feedstuff but could also

represent a useful platform of knowledge to improve the performance of BSF in mass rearing procedures. The use of large-scale screenings will be useful to evaluate the expression profile of genes coding for key proteins/peptides involved in BSF midgut functions (i.e., digestive enzymes, transport proteins, antimicrobial peptides and signaling molecules) and will also allow to better analyze the effects of different diets on the performances of the fly.

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Compliance with ethical standards

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

Ethical approval All applicable international, national and/or institutional guidelines for the care and use of animals were followed.

References

- Amcheslavsky A, Jiang J, Ip YT (2009) Tissue damage-induced intestinal stem cell division in *Drosophila*. *Cell Stem Cell* 4:49–61
- Benkel BF, Hickey DA (1986) Glucose repression of amylase gene expression in *Drosophila melanogaster*. *Genetics* 114:137–144
- Bernfeld P (1955) Amylases, α and β . *Methods Enzymol* 1:149–158
- Bertinetti C, Samayoa AC, Hwang SY (2019) Effects of feeding adults of *Hermetia illucens* (Diptera: Stratiomyidae) on longevity, oviposition, and egg hatchability: insights into optimizing egg production. *J Insect Sci* 19:19
- Billingsley PF, Lehane MJ (1996) Structure and ultrastructure of the insect midgut. In: Lehane MJ, Billingsley PF (eds) *Biology of the insect midgut*. Springer, Dordrecht, pp 3–30
- Bonelli M, Bruno D, Caccia S, Sgambettera G, Cappelozza S, Jucker C, Tettamanti G, Casartelli M (2019) Structural and functional characterization of *Hermetia illucens* larval midgut. *Front Physiol* 10:204. <https://doi.org/10.3389/fphys.2019.00204>
- Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72:248–254
- Bruno D, Bonelli M, De Filippis F, Di Lelio I, Tettamanti G, Casartelli M, Ercolini D, Caccia S (2019) The intestinal microbiota of *Hermetia illucens* larvae is affected by diet and shows a diverse composition in the different midgut regions. *Appl Environ Microbiol* 85:e01864–e01818
- Buchon N, Osman D, David FP, Fang HY, Boquete JP, Deplancke B, Lemaitre B (2013) Morphological and molecular characterization of adult midgut compartmentalization in *Drosophila*. *Cell Rep* 3:1725–1738
- Caccia S, Chakroun M, Vinokurov K, Ferre J (2014) Proteolytic processing of *Bacillus thuringiensis* Vip3A proteins by two *Spodoptera* species. *J Insect Physiol* 67:76–84
- Charney J, Tomarelli RM (1947) A colorimetric method for the determination of the proteolytic activity of duodenal juice. *J Biol Chem* 171:501–505
- Chng WA, Sleiman MSB, Schupfer F, Lemaitre B (2014) Transforming growth factor beta/activin signaling functions as a sugar-sensing feedback loop to regulate digestive enzyme expression. *Cell Rep* 9:336–348
- Cickova H, Newton GL, Lacy RC, Kozanek M (2015) The use of fly larvae for organic waste treatment. *Waste Manag* 35:68–80
- Coch Frugoni JA (1957) Tampone universale di Britton e Robinson a forza ionica costante. *Gazz Chim Ital* 84:403–407
- Dow JAT (1986) Insect midgut function. *Adv Insect Physiol* 19:187–328
- Dubreuil RR (2004) Copper cells and stomach acid secretion in the *Drosophila* midgut. *Int J Biochem Cell Biol* 36:745–752
- EFSA Scientific Committee (2015) Scientific opinion on a risk profile related to production and consumption of insects as food and feed. *EFSA J* 13:4257–4317
- Endo Y, Nishiitsutsuju-Uwo J (1981) Gut endocrine cells in insects: the ultrastructure of the gut endocrine cells of the lepidopterous species. *Biomed Res* 2:270–280
- European Commission (2017) Commission regulation (EU) 2017/893 of 24 May 2017 amending Annexes I and IV to regulation (EC) no 999/2001 of the European Parliament and of the Council and Annexes X, XIV and XV to commission regulation (EU) no 142/2011 as regards the provisions on processed animal protein. *Off J Eur Union L* 138:92–116
- Franzetti E, Huang ZJ, Shi YX, Xie K, Deng XJ, Li JP, Li QR, Yang WY, Zeng WN, Casartelli M, Deng HM, Cappelozza S, Grimaldi A, Xia Q, Feng Q, Cao Y, Tettamanti G (2012) Autophagy precedes apoptosis during the remodeling of silkworm larval midgut. *Apoptosis* 17:305–324
- Franzetti E, Romanelli D, Caccia S, Cappelozza S, Congiu T, Rajagopalan M, Grimaldi A, de Eguileor M, Casartelli M, Tettamanti G (2015) The midgut of the silkworm *Bombyx mori* is able to recycle molecules derived from degeneration of the larval midgut epithelium. *Cell Tissue Res* 361:509–528
- Fujita T, Kobayashi S (1977) Structure and function of gut endocrine cells. *Int Rev Cytol Suppl* 187–233
- Giangaspero A, Broce AB (1993) Micromorphology of the prestomal teeth and feeding-behavior of *Musca autumnalis*, *M. larvipara* and *M. osiris* (Diptera, Muscidae). *Med Vet Entomol* 7:398–400
- Gobbi P, Martinez-Sanchez A, Rojo S (2013) The effects of larval diet on adult life-history traits of the black soldier fly, *Hermetia illucens* (Diptera: Stratiomyidae). *Eur J Entomol* 110:461–468
- Graczyk TK, Knight R, Gilman RH, Cranfield MR (2001) The role of non-biting flies in the epidemiology of human infectious diseases. *Microbes Infect* 3:231–235
- Graczyk TK, Knight R, Tamang L (2005) Mechanical transmission of human protozoan parasites by insects. *Clin Microbiol Rev* 18:128–132
- Gullan PJ, Cranston PS (2014) *The insects: an outline of entomology*. Wiley-Blackwell, Chichester, pp 156–189
- Hakim RS, Baldwin K, Smagghe G (2010) Regulation of midgut growth, development, and metamorphosis. *Annu Rev Entomol* 55:593–608
- Hickey DA, Benkel B (1982) Regulation of amylase activity in *Drosophila melanogaster*: effects of dietary carbohydrate. *Biochem Genet* 20:1117–1129
- Hogsette JA (1992) New diets for production of house flies and stable flies (Diptera, Muscidae) in the laboratory. *J Econ Entomol* 85:2291–2294
- Holmes LA, Vanlaerhoven SL, Tomberlin JK (2012) Relative humidity effects on the life history of *Hermetia illucens* (Diptera: Stratiomyidae). *Environ Entomol* 41:971–978
- Holmes LA, Vanlaerhoven SL, Tomberlin JK (2013) Substrate effects on pupation and adult emergence of *Hermetia illucens* (Diptera: Stratiomyidae). *Environ Entomol* 42:370–374

- Kovacs F, Medveczky I, Papp L, Gondar E (1990) Role of prestomal teeth in feeding of the house fly, *Musca domestica* (Diptera, Muscidae). *Med Vet Entomol* 4:331–335
- Lee CY, Cooksey BA, Baehrecke EH (2002) Steroid regulation of midgut cell death during *Drosophila* development. *Dev Biol* 250:101–111
- Lehane MJ (1997) Peritrophic matrix structure and function. *Annu Rev Entomol* 42:525–550
- Lemaitre B, Miguel-Aliaga I (2013) The digestive tract of *Drosophila melanogaster*. *Annu Rev Genet* 47:377–404
- Lemos FJA, Terra WR (1991) Digestion of bacteria and the role of midgut lysozyme in some insect larvae. *Comp Biochem Physiol* 100:265–268
- Li YB, Yang T, Wang JX, Zhao XF (2018) The steroid hormone 20-hydroxyecdysone regulates the conjugation of autophagy-related proteins 12 and 5 in a concentration and time-dependent manner to promote insect midgut programmed cell death. *Front Endocrinol (Lausanne)* 9:28
- Lii CY, Chang SM, Young YL (1982) Investigation of the physical and chemical properties of banana starches. *J Food Sci* 47:1493–1497
- Makkar HPS, Tran G, Henze V, Ankers P (2014) State-of-the-art on use of insects as animal feed. *Anim Feed Sci Technol* 197:1–33
- Marianes A, Spradling AC (2013) Physiological and stem cell compartmentalization within the *Drosophila* midgut. *Elife* 2:e00886
- McNulty M, Puljung M, Jefford G, Dubreuil RR (2001) Evidence that a copper-metallothionein complex is responsible for fluorescence in acid-secreting cells of the *Drosophila* stomach. *Cell Tissue Res* 304:383–389
- Meneguz M, Schiavone A, Gai F, Dama A, Lussiana C, Renna M, Gasco L (2018) Effect of rearing substrate on growth performance, waste reduction efficiency and chemical composition of black soldier fly (*Hermetia illucens*) larvae. *J Sci Food Agric*
- Micchelli CA, Perrimon N (2006) Evidence that stem cells reside in the adult *Drosophila* midgut epithelium. *Nature* 439:475–479
- Montali A, Romanelli D, Cappelozza S, Grimaldi A, de Eguileor M, Tettamanti G (2017) Timing of autophagy and apoptosis during posterior silk gland degeneration in *Bombyx mori*. *Arthropod Struct Dev* 46:518–528
- Muller A, Wolf D, Gutzeit HO (2017) The black soldier fly, *Hermetia illucens*—a promising source for sustainable production of proteins, lipids and bioactive substances. *Z Naturforsch C* 72:351–363
- Nakamura S, Ichiki RT, Shimoda M, Morioka S (2016) Small-scale rearing of the black soldier fly, *Hermetia illucens* (Diptera: Stratiomyidae), in the laboratory: low-cost and year-round rearing. *Appl Entomol Zool* 51:161–166
- Nguyen TTX, Tomberlin JK, Vanlaerhoven S (2013) Influence of resources on *Hermetia illucens* (Diptera: Stratiomyidae) larval development. *J Med Entomol* 50:898–906
- Nguyen TTX, Tomberlin JK, Vanlaerhoven S (2015) Ability of black soldier fly (Diptera: Stratiomyidae) larvae to recycle food waste. *Environ Entomol* 44:406–410
- Nishiitsutsuju-Uwo J, Endo Y (1981) Gut endocrine cells in insects: the ultrastructure of the gut endocrine cells of the cockroach midgut. *Biomed Res* 2:30–44
- Ohlstein B, Spradling A (2006) The adult *Drosophila* posterior midgut is maintained by pluripotent stem cells. *Nature* 439:470–474
- Ohlstein B, Spradling A (2007) Multipotent *Drosophila* intestinal stem cells specify daughter cell fates by differential notch signaling. *Science* 315:988–992
- Padilha MH, Pimentel AC, Ribeiro AF, Terra WR (2009) Sequence and function of lysosomal and digestive cathepsin D-like proteinases of *Musca domestica* midgut. *Insect Biochem Mol Biol* 39:782–791
- Parthasarathy R, Palli SR (2008) Proliferation and differentiation of intestinal stem cells during metamorphosis of the red flour beetle, *Tribolium castaneum*. *Dev Dyn* 237:893–908
- Pimentel AC, Montali A, Bruno D, Tettamanti G (2017) Metabolic adjustment of the larval fat body in *Hermetia illucens* to dietary conditions. *J Asia Pac Entomol* 20:1307–1313
- Pimentel AC, Barroso IG, Ferreira JMJ, Dias RO, Ferreira C, Terra WR (2018) Molecular machinery of starch digestion and glucose absorption along the midgut of *Musca domestica*. *J Insect Physiol* 109:11–20
- Rivers D, Geiman T (2017) Insect artifacts are more than just altered bloodstains. *Insects* 8
- Romanelli D, Casartelli M, Cappelozza S, de Eguileor M, Tettamanti G (2016) Roles and regulation of autophagy and apoptosis in the remodelling of the lepidopteran midgut epithelium during metamorphosis. *Sci Rep* 6:32939
- Sehnal F, Zitnan D (1996) Midgut endocrine cells. In: Lehane MJ, Billingsley PF (eds) *Biology of the insect midgut*. Springer, Dordrecht, pp 55–85
- Shanbhag S, Tripathi S (2009) Epithelial ultrastructure and cellular mechanisms of acid and base transport in the *Drosophila* midgut. *J Exp Biol* 212:1731–1744
- Sheppard DC, Newton GL, Thompson SA, Savage S (1994) A value-added manure management-system using the black soldier fly. *Bioresour Technol* 50:275–279
- Sheppard DC, Tomberlin JK, Joyce JA, Kiser BC, Sumner SM (2002) Rearing methods for the black soldier fly (Diptera: Stratiomyidae). *J Med Entomol* 39:695–698
- Shina M (1975) Amylase activity in the midgut of *Sarcophaga ruficornis* and *Musca domestica*. *Entomol Exp Appl* 18:290–296
- Stoffolano JG Jr, Haselton AT (2013) The adult Dipteran crop: a unique and overlooked organ. *Annu Rev Entomol* 58:205–225
- Stoffolano JG, Acaron A, Conway M (2008) “Bubbling” or droplet regurgitation in both sexes of adult *Phormia regina* (Diptera: Calliphoridae) fed various concentrations of sugar and protein solutions. *Ann Entomol Soc Am* 101:964–970
- Takashima S, Younossi-Hartenstein A, Ortiz PA, Hartenstein V (2011) A novel tissue in an established model system: the *Drosophila* pupal midgut. *Dev Genes Evol* 221:69–81
- Terra WR (1990) Evolution of digestive systems of insects. *Annu Rev Entomol* 35:181–200
- Terra WR, Ferreira C (1994) Insect digestive enzymes: properties, compartmentalization and function. *Comp Biochem Physiol B* 109:1–62
- Terra WR, Ferreira C (2005) Biochemistry of digestion. In: Gilbert LI, Iatrou K, Gill SS (eds) *Comprehensive molecular insect science*, vol 4. Elsevier Pergamon Press, Oxford, pp 171–224
- Terra WR, Espinoza-Fuentes FP, Ferreira C (1988a) Midgut amylase, lysozyme, aminopeptidase, and trehalase from larvae and adults of *Musca domestica*. *Arch Insect Biochem* 9:283–297
- Terra WR, Espinoza-Fuentes FP, Ribeiro BM, Ferreira C (1988b) The larval midgut of the housefly (*Musca domestica*): ultrastructure, fluid fluxes and ion secretion in relation to the organization of digestion. *J Insect Physiol* 34:463–472
- Terra WR, Ferreira C, Baker JE (1996a) Compartmentalization of digestion. In: Lehane MJ, Billingsley PF (eds) *Biology of the insect midgut*. Springer, Dordrecht, pp 206–235
- Terra WR, Ferreira C, Jordão BP, Dillon RJ (1996b) Digestive enzymes. In: Lehane MJ, Billingsley PF (eds) *Biology of the insect midgut*. Springer, Dordrecht, pp 153–194
- Tettamanti G, Grimaldi A, Casartelli M, Ambrosetti E, Ponti B, Congiu T, Ferrarese R, Rivas-Pena ML, Pennacchio F, Eguileor M (2007) Programmed cell death and stem cell differentiation are responsible for midgut replacement in *Heliothis virescens* during prepupal instar. *Cell Tissue Res* 330:345–359
- Tettamanti G, Salo E, Gonzalez-Estevéz C, Felix DA, Grimaldi A, de Eguileor M (2008) Autophagy in invertebrates: insights into development, regeneration and body remodeling. *Curr Pharm Des* 14:116–125

- Tettamanti G, Cao Y, Feng Q, Grimaldi A, de Eguileor M (2011) Autophagy in Lepidoptera: more than old wine in new bottle. *Invertebr Surviv J* 8:5–14
- Tomberlin JK, Sheppard DC (2002) Factors influencing mating and oviposition of black soldier flies (Diptera: Stratiomyidae) in a colony. *J Entomol Sci* 37:345–352
- Tomberlin JK, Sheppard DC, Joyce JA (2002) Selected life-history traits of black soldier flies (Diptera: Stratiomyidae) reared on three artificial diets. *Ann Entomol Soc Am* 95:379–386
- Tomberlin JK, Adler PH, Myers HM (2009) Development of the black soldier fly (Diptera: Stratiomyidae) in relation to temperature. *Environ Entomol* 38:930–934
- Villalon JM, Ghosh A, Jacobs-Lorena M (2003) The peritrophic matrix limits the rate of digestion in adult *Anopheles stephensi* and *Aedes aegypti* mosquitoes. *J Insect Physiol* 49:891–895
- Vinokurov KS, Elpidina EN, Oppert B, Prabhakar S, Zhuzhikov DP, Dunaevsky YE, Belozersky MA (2006) Diversity of digestive proteinases in *Tenebrio molitor* (Coleoptera: Tenebrionidae) larvae. *Comp Biochem Physiol B Biochem Mol Biol* 145:126–137
- Vogel H, Muller A, Heckel DG, Gutzeit H, Vilcinskas A (2018) Nutritional immunology: diversification and diet-dependent expression of antimicrobial peptides in the black soldier fly *Hermetia illucens*. *Dev Comp Immunol* 78:141–148
- Wang YS, Shelomi M (2017) Review of black soldier fly (*Hermetia illucens*) as animal feed and human food. *Foods* 6

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