Insects' superpower: waste bioconversion by *Hermetia illucens* larvae

Il superpotere degli insetti: bioconversione di materiale di scarto mediata da larve di *Hermetia illucens*

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SUMMARY

Over the coming decades it will be mandatory to face the problem of feeding the increasing world population, limiting the depletion of food resources, the unsustainable exploitation of agricultural land and water, and environmental pollution. The higher demand for human food requires an increasing production of feeds for livestock, poultry, and aquaculture. Another serious concern is represented by food waste since one third of all food produced globally is wasted. These critical challenges require innovative solutions and approaches for the recognition of new biological resources, innovative production and consumption cycles, and alternative waste processing, recycling and disposal procedures. Insects must be considered as a high potential source of animal protein for feed production, as well as a primary agent for organic waste reduction and bioconversion into a usable protein product. In particular, the larvae of the black soldier fly (BSF), *Hermetia illucens* (Diptera: Stratiomyidae), are good candidates for this bioconversion process, because they grow on decomposing organic materials including animal waste, fish offal, decayed fruits and vegetables, and the dry-matter of larvae-prepupae grown on these substrates contain a very high percentage of protein and fat that can be used as feedstuff. While in recent years a lot of efforts have been devoted to address important issues that could support the growth of the emerging industrial sector of edible insects and their exploitation in the feed market, information on the biology of *H. illucens* is still scarce.

The aim of this PhD thesis was to analyse BSF midgut and fat body, two organs that are involved in the digestion, absorption, and storage of nutrients in the larva. Moreover, the feeding habits of the fly were investigated.

In the first chapter an in depth description of the morphological, ultrastructural, and functional properties of the midgut of *H. illucens* larvae is reported. The data collected shed light on the unexpected complexity of this organ and allowed us to propose a functional model of the larval midgut of this insect that clearly shows how each region of the midgut possesses peculiar features and functions.

The second chapter reports an in depth study of the microbiota of *H. illucens* larval midgut taking into account pivotal aspects such as the midgut spatial and functional regionalization, as well as microbiota and nutrient composition of the feeding substrate. The analyses performed clearly demonstrate that the presence of different midgut regions associates to differences in microbial density and composition.

In the third chapter it is shown how the diet provided to the larvae can affect different morphological and functional features of the midgut. Larvae grown on a vegetable mix diet show differences in the morphology of the midgut epithelium, as well in the activity of digestive enzymes and accumulation of long-term storage molecules. A study performed in the larval fat body, described in chapter four, demonstrated similar morphofunctional changes also in this organ.

The last chapter reports information about the feeding habits of the adult insect, an aspect that has been neglected so far. Our results demonstrate that, the larval midgut of *H. illucens* is completely removed during metamorphosis and a new adult epithelium is formed. In addition, functional experiments indicate that the newly formed midgut epithelium of the fly is endowed with digestive activity.

In conclusion, our study not only provides insights into the biology of BSF, but also represents a useful platform of knowledge for the best exploitation of this insect in bioconversion processes and, hopefully, take advantage of the huge potential offered by the growing edible insect industry.

RIASSUNTO

Un'impegnativa sfida per il futuro sarà quella di soddisfare le richieste alimentari di una popolazione mondiale in costante crescita, cercando di sfruttare al meglio e in modo sostenibile le risorse, i terreni coltivabili e l'acqua, limitando nel contempo l'inquinamento ambientale. Per soddisfare la richiesta crescente di cibo sarà necessario incrementare la produzione di mangimi per l'allevamento animale e far fronte al grave problema dello spreco alimentare e dello smaltimento dei rifiuti. Queste sfide cruciali richiedono soluzioni innovative e strategie volte alla ricerca di nuove risorse alimentari, alla realizzazione di nuovi cicli di produzione e di consumo, e alla progettazione di nuovi sistemi per il trattamento, il riciclo e lo smaltimento dei rifiuti. Gli insetti rappresentano una soluzione per la riduzione di rifiuti organici che possono, nello stesso tempo, essere bioconvertiti in proteine ad elevato contenuto nutrizionale. In particolare, le larve del dittero *Hermetia illucens* (black soldier fly, BSF), sono ottimi candidati per processi di bioconversione, perché in grado di crescere su materiale organico in decomposizione di diverso tipo, tra cui rifiuti di origine animale, frattaglie di pesce, frutta e verdura. Inoltre, le larve cresciute su questi substrati sono caratterizzate dal possedere una percentuale molto elevata di proteine e grassi e possono essere utilizzate per la formulazione di mangimi animali. Sebbene negli ultimi anni siano stati condotti diversi studi al fine di sostenere la crescita dell'emergente settore industriale degli insetti commestibili e la valorizzazione degli stessi nel mercato della mangimistica, le informazioni riguardanti la biologia di *H. illucens* rimangono scarse.
Lo scopo di questo lavoro di tesi è stato quello di analizzare l'intestino medio ed il corpo grasso di *H. illucens*, due organi

responsabili dei processi di digestione, assorbimento ed accumulo dei nutrienti nello stadio larvale di questo insetto. Inoltre, sono state studiate le abitudini alimentari dello stadio adulto di questo dittero.
Nel primo capitolo viene presentata un'approfondita analisi morfofunzionale dell'intestino medio larvale di *H. illucens*. I risultati ottenuti hanno dimostrato l'elevata complessità di quest'organo e hanno consentito di costruire un modello funzionale dell'intestino medio larvale di questo insetto, che evidenzia peculiari caratteristiche e funzioni delle diverse regioni di quest'organo.
Il secondo capitolo descrive uno studio del microbiota dell'intestino medio larvale di *H. illucens*, focalizzandosi su specifici aspetti quali la regionalizzazione spaziale e funzionale di questo organo, il microbiota e la composizione nutrizionale del substrato di crescita. Le analisi eseguite dimostrano chiaramente che ad ogni regione dell'intestino medio è associata una specifica densità e composizione batterica.
Nel terzo capitolo viene descritto come il substrato di crescita fornito alle larve influisca su diverse caratteristiche morfologiche e funzionali dell'intestino medio. In particolare, larve cresciute su una dieta di origine vegetale mostrano differenze morfologiche, nell'attività degli enzimi digestivi e nell'accumulo di molecole energetiche a livello dell'intestino medio. Uno studio simile è stato condotto sul corpo grasso delle larve di questo insetto, secondo quanto riportato nel capitolo quattro in cui viene mostrato come la dieta vegetale determini cambiamenti morfofunzionali anche in questo organo.
L'ultimo capitolo presenta uno studio riguardante le capacità alimentari dell'adulto di *H. illucens*, un aspetto finora poco studiato. I nostri risultati dimostrano che l'intestino medio larvale viene completamente rimosso durante la metamorfosi, e viene sostituito

da un nuovo epitelio intestinale che permarrà nell'adulto. Inoltre, esperimenti di tipo funzionale indicano che l'intestino neoformato è funzionale e dotato di attività digestiva.

In conclusione, la nostra ricerca non solo fornisce conoscenze riguardanti la biologia di *H. illucens*, ma rappresenta anche una piattaforma di conoscenze utilizzabile per consentire un migliore sfruttamento di questo insetto nei processi della bioconversione, ed un significativo incremento delle potenzialità degli insetti nel settore mangimistico.

INTRODUCTION

The world nutritional requirement is foreseen to increase in the next decades. In fact, the world population will reach more than 9.5 billion people within the next thirty years (United Nations, 2017) and this will lead to an increasing demand for human food: in particular, Food and Agriculture Organisation (FAO) estimates that food production will need to double (Van Huis et al., 2013).

Nowadays the food production systems, however, use about 70% of agricultural land and are responsible for the generation of a high quantity of anthropogenic greenhouse gases (GHGs), that amount at 31% of the total emissions (Foley et al. 2011; Tukker and Jansen, 2006). In particular, livestock contributes to exacerbate this scenario: in fact, a large part of GHGs produced by food production system can be ascribed to this sector (Steinfeld et al., 2006), and the emission of GHGs for meat production will increase by 40% within 2050 (Pelletier and Tyedmers, 2010). Carbon dioxide (CO_2), methane (CH_4), and nitrous oxide (N_2O) are the most important GHGs and CH_4 and N_2O have a considerable high global warming potential (Oonincx et al., 2010). Moreover, although ammonia (NH_3) is not considered a GHG, it is associated with environmental pollution because its emission from urine and manure can indirectly form N_2O due to the conversion by denitrifying bacteria (Wrage et al., 2001). According to recent estimates, more than 60% of all anthropogenic NH_3 emission is caused by livestock (Steinfeld et al., 2006). These GHGs are considered one of the major problems since they are responsible for climate change (Oonincx et al., 2010). A high level of methane production is caused by ruminants, which are characterised by a low feed conversion rate and long reproduction intervals. These two characteristics determine high dietary

requirements, costs, and resources for the maintenance of these animals (Steinfeld and Gerber, 2010).

Another relevant issue in this setting is represented by the global crop production. In fact, the rising demand for food will cause an increase of 100-110% in the global crop demand by 2050 (Tilman et al., 2011). As a consequence, a higher land cleaning and intensive use of existing croplands will be necessary to produce more crop and satisfy the growing demand of food. However, the resulting environmental impact is uncertain (Godfray et al., 2010). What is known it is that at present agriculture is the major cause of anthropogenic-induced climate change. In fact, about one-third of all GHGs emission, including CO_2 produced by deforestation, methane produced by rice paddies, and nitrogen oxide released by nitrogen fertilizers, is caused by agriculture (Sachs, 2010).

Livestock currently uses around one third of the earth surface, including permanent pasture and arable lands used to produce livestock feed (FAO, 2006; FAO, 2015; McMichael et al., 2007). Also the demand for grain and protein-rich nutrients as feed for livestock will increase: it is estimated that for one kilogram of high quality animal protein produced, about 6 kilograms of plant protein are necessary (Pimentel and Pimentel, 2003; Trostle, 2008). In 2012, more than 40% of the world's crops was destined to produce animal feed and the situation will progressively worsen (Alexandratos and Bruinsma, 2012; Foley et al., 2011). As a results of this trend, the prices of the most important agricultural crops, along with those of beef, pork and poultry, will rise of more than the 30% in the forthcoming years (Nelson et al., 2009).

Finally, a further severe concern is represented by food loss and waste. Food loss refers to the edible food mass, destined to the production of human food, that is lost throughout the supply chain

production. On the other hand, food waste is the waste at the end of the food chain produced by retailers and consumers (Parfitt et al., 2010). Today, the total quantity of food that is lost or wasted by the food supply chain is about one-third of the food globally produced, amounting at around 1.3 billion tons per year (Gustavsson et al., 2011). These estimates include food loss due to mechanical damage, spillage and degradation during handling, storage, transportation, processing, distribution, and consumption at the household level (Gustavsson, 2011). Moreover, a large volume of food is degraded or consumed by pests (FAO, 1981).

For all these reasons, it is fundamental to implement strategies for improving the global production systems to obtain more food with fewer inputs, less environmental impact, and reducing GHGs emission. It is also necessary to find alternative sources of animal protein reducing, at the same time, the waste produced by the food supply chain. Insect proteins could represent part of the solution to these problems (Van Huis and Tomberlin, 2017).

ALTERNATIVE PROTEIN SOURCES

Because of all the issues described above, one of the major challenges for the next future will be the development of new technologies for the production of alternative and sustainable sources of protein.

Among the potential candidates there are seaweeds. In 2015, the global aquaculture production amounted at about 29 million tonnes of aquatic plants (FAO, 2017a). Depending on the species, the protein content of seaweeds vary in a range from 3% to 50%. For example, the red seaweed *Porphyra tenera* (commonly known as

"nori") presents a 47% protein dry mass, a value even higher than soybean (Fleurence, 1999).

Another alternative protein source is the cultured meat from stem cells (Post, 2012). By exploiting the ability of stem cells to divide and differentiate into a specific cell type, it is possible to make them growing and differentiate *in vitro* into muscle cells. Specific cells of muscle tissue (myosatellite cells) are used to start the *in vitro* culture (Allen et al., 1997). Once isolated, these cells fuse and form myofibers (Wolpert et al., 1998). Then, by adding a mixture of collagen, myofibers can be harvested and used as meat (Fayaz and Fayaz, 2011).

Minilivestock have been proposed in the last decades as a valid alternative source of protein. Many small animals including rodents, frogs, snails, and arthropods (i.e., insects) reared as food and feed, belong to this group (Paoletti, 2005). Farming and eating rodents, for example, could be helpful to counteract scarcity of food and malnutrition (Gruber, 2016). Moreover, insects are a natural renewable resource since they represent the most abundant animals in the world in terms of individuals and number of species, and are characterized by high nutritional values (Barroso et al., 2014).

THE GREAT POTENTIAL OF INSECTS AS FOOD AND FEED

The possibility to use insects to counteract the rising demand for meat and fish production could represent an interesting opportunity for a number of reasons.

First, insects show a higher capability to convert feed mass into body mass than other animals. This high feed conversion ratio allows insects to better transform feed into weight gain (Oonincx et al.,

2015). Moreover, some insects are able to grow on a wide variety of organic substrates, including side streams and byproducts, thus reducing environmental contamination (Newton et al., 2005). In addition, insect rearing require less water than other animals, and is characterized by a lower environmental impact and low GHGs emission (Oonincx et al., 2010). Finally, the use of insects present few animal welfare issues and low risk for zoonotic infection transmission (Van Huis et al., 2013). Some of these issues are detailed below.

1-Feed conversion ratio

In farming activities, the feed conversion ratio (FCR) is a parameter useful to evaluate the efficiency of the conversion of feed by an animal into the output (for example meat, milk, and eggs). FCR depends on different factors as the type of animal, the quality of feed and the conditions in which the animals is kept, as well as the processes used to obtain the meat. For example, the calculated FCR for chicken is 2.5, for pork is 5, and for beef is around 10 (Smil, 2002). The situation is completely different for insects which require less feed for their growth. Studies on *Acheta domesticus* showed that to obtain 1 kilogram of insects only 1.7 kg of feed are necessary (Collavo et al., 2005), making crickets 2 times more efficient in converting feed into meat than chickens, 4 times than pigs, and 12 times than cattle (Van Huis, 2013). The ability of insects to convert feed more efficiently is due to the fact that they are poikilothermic organisms. For this reason, they do not use metabolic energy to maintain constant body temperature over the ambient value (Van Huis, 2013). Moreover, it is worthy to note that the percentage of digestibility of crickets is about 80% (Nakagaki & DeFoliart, 1991) compared to chicken and pork, where the percentage of the edible

weight is about 55% of fresh weight (Flachowsky, 2002). Another relevant aspect is the amount of protein content: chicken, pork, and beef contain 200, 150, and 190 g of protein per kilogram of edible weight, respectively (Flachowsky, 2002), while insects contain higher level of proteins (Rumpold and Schlüter, 2013).

2-Rearing substrate

Different insect species are able to grow on substrates that are not used by humans and other animals, such as organic side streams like manure, pig slurry, and compost. Moreover, some of these species received a great attention because of their ability to bioconvert organic waste, which today amounts at 1.3 billion of tons per year (Gustavsson et al., 2011; Veldkamp et al, 2012). Among them there are the black soldier fly (BSF) (*Hermetia illucens*), the common housefly (*Musca domestica*), and the yellow mealworm (*Tenebrio molitor*). BSF is a saprophage able to convert organic refuse such as dairy, poultry, and swine manure in body mass, while reducing dry matter mass by up to 58% (Diener et al., 2009; Sheppard et al., 1994). Thanks to their ability to grow on these substrates, BSF larvae are able to make the waste safe, reducing microelements that are dangerous for the environment (Myers et al., 2008) and pathogens (Erickson et al., 2004; Liu et al., 2008). The larvae of other insect species, as *Musca domestica* and *Tenebrio molitor*, are very efficient in the bioconversion of organic waste, from municipal organic waste to dried and cooked waste materials derived from fruit, vegetables, and cereals. Then the larvae can be used as a protein source for poultry diets (Ocio et al., 1979; Ramos-Elorduy et al., 2002).
The use of organic side streams as feed for these insect species could be useful to reduce biowaste, although the current legislation on food and feed does not allow the use of insects reared on these

substrates as feedstuffs, given the unknown risks about pathogens, heavy metals, and organic pollutants (see “Legislation and food security”).

3-Water use

It is estimated that by 2025 about 1.8 billion people will be living in regions with scarcity of water (FAO, 2012).

The global demand for water is significant. Agriculture, for example, consumes about 70% of fresh water (Pimentel et al., 2004). Moreover, livestock is one of the major water user: in fact, the quantity of water needed to produce 1 kg of animal protein is 100 times greater than that needed to produce 1 kg of grain protein. This value considers the virtual water, i.e., the total amount of water necessary for the entire process of protein production, including place and time of production and the efficiency in using water (Chapagain and Hoekstra, 2003). So, for the production of meet from livestock, it is necessary not only water for animal grow, but also that for the production of the final item. Considering this parameter, for the production of 1 kg of chicken, pork, and beef about 2300, 3500, and 22000 litres of virtual water, respectively, are necessary (Chapagain and Hoekstra, 2003). For the latter, estimates reach up to 43000 litres if indirect water inputs, such as forage and grain feed crops, are considered (Pimentel et al., 2004).

At present, estimates about the amount of virtual water needed for insect rearing procedures are not available. It is expected that this would be much lower than livestock, considering that some insect species do not need water in their diet, while others are able to live entirely on substrates that already contain water, as vegetable waste and organic side streams.

4-Greenhouse gas emissions

As described above, a large part of the total GHGs emission is caused by livestock rearing, amounting approximately at 18% (Steinfeld et al., 2006). Among insects, only few orders, such as Blattodea, Isoptera, and Coleoptera, are able to produce methane (Hackstein and Stumm, 1994). On the other hand, insects reared as food and feed sources, such as yellow mealworm, crickets, and locusts, show reduced GHGs emission compared to pigs and beef cattle (2-122 g/kg mass gain for insects than 80-1130 g/kg and 2850 g/kg mass gain for pigs and cattle, respectively). Moreover, these insect species are friendlier in NH_3 production than pigs (Oonincx et al., 2010). Since these are lab-scale evaluations, it is mandatory to understand the real difference on a larger scale compared to pork and beef production.

5-Risk of zoonoses

The incidence of livestock disease increases when animals are bred in high-density conditions. This situation is exacerbated by the emergence of antimicrobial resistance (Furtula et al., 2010; Smith et al., 2007).

One of the biggest problems about this scenario is the cost caused by livestock disease: epidemics such as avian influenza (H5N1), foot-and-mouth disease (FMD), bovine spongiform encephalopathy (BSE), and swine fever, just to cite the most famous, cost billions of euros every year (King et al., 2006). For example, in the United Kingdom BSE in 1996-1997 and FMD in 2001 caused a loss of around 2.5 and 8.6 billion pounds, respectively (Atkinson, 1999; Beef Checkoff Program, 2017).

These diseases are zoonotic, causing infections in humans and in wild or domestic animals (Cleaveland et al., 2001). Moreover, the intensification of animal production, in addition to climate change, is one of the causes of the increase of zoonotic diseases. Multiple human health problems are caused by zoonoses, since livestock animals are taxonomically close to humans. Insects, on the other hand, are taxonomically very distant from human. For this reason, it is expected that they present a lower risk of zoonoses than livestock animals, though research in this area is needed, especially if waste products are used to rear them.

European Food Safety Authority (EFSA) published a document about the risk profile caused by the use of non-transformed insects as food and feed (2015). EFSA declared that the risk profile in using insects is very low or lesser than the existing protein sources, as long as insects are not reared on substrates derived from ruminants or from humans (for example manure). Since the transformation process further reduce the biological hazards, the EFSA document is also valid for the use of processed animal proteins (PAPs) obtained from insects.

6-Food security and legislation

Organic waste and other substrates suitable to rear insects could contain pathogens, mycotoxins, pesticides or heavy metals. These compounds may accumulate in larvae and prepupae, thus causing problems to livestock animals, such as monogastrics (e.g., pig and poultry), where an over-exposure to mycotoxins induce mutagenic, carcinogenic or teratogenic effects (Tola et al., 2016).

Different studies evaluated the accumulation of these compounds in BSF larvae reared on contaminated substrates (Bosch et al., 2017; Diener et al., 2015; Lalander et al., 2013; Purschke et al., 2017).

Although they did not find any accumulation of mycotoxins and pesticides in the larvae, the results were different for heavy metals. In fact, a considerable accumulation of cadmium and lead was observed in larvae and prepupae reared on contaminated substrates, though the concentration of these metals was less than the initial amount in the food (Dinier et al., 2015; Purschke et al., 2017). For these reasons, it is very important to monitor the heavy metal concentration in the feed provided to insects and also in feedstuffs produced with insects, focusing the attention on cadmium and lead.

Today, in Europe, there is a strict regulation for the use of insects as feed. In fact, although it is allowed to feed all animal species, except ruminants, with whole live insects and the derived fat, PAPs derived from insects are allowed only for pets, fur animals, and aquaculture species (Commission Reg. (EU) 2017/893 amending TSE Reg. (EC) 999/2001 and ABP Enforcement Reg. (EU) 142/2011). Moreover, only few insect species are admitted to this purpose: black soldier fly (*Hermetia illucens*), common housefly (*Musca domestica*), yellow mealworm (*Tenebrio molitor*), lesser mealworm (*Alphitobius diaperinus*), house cricket (*Ancheta domesticus*), banded cricket (*Gryllodes sigillatus*), field cricket (*Gryllus assimilis*).

INSECTS AS FEED

Aquaculture represents one of the fastest growing food producing sector over the last three decades and has shown an increasing demand for protein for fish feed and competition for feed inputs, especially protein related, with other forms of livestock. The world aquaculture production continues to increase at around 4-5% per year. In 2016 the global fish production amounted at 170 million

tons, divided in 90 million tons as capture fisheries and 80 million tons as aquaculture, where finfish farming is the most important type of product in all continents. However, about 14.3 million tonnes of the total production are used as fish meal and fish oil to feed fish (FAO, 2017b). Due to its high nutritional value in terms of energy and protein content, fish meal is considered a premium feed ingredient in aquaculture though, for these reasons, it is very expensive. Soybean meal is used as alternative protein feed ingredient in fish meal, thanks to its high content of protein and low cost. Although the soybean price is lower than fish meal, the rising demand for aquaculture products caused an increase in the price. Moreover, the use of vegetable proteins can affect fish health, thus altering several parameters of the immune system in different fish species (Bell et al., 2006; Lin and Shiau, 2007; Montero et al., 2010; Mourente et al., 2007).

If we consider the increasing global population, to maintain at least the current level of per-capita consumption of aquatic foods derived from aquaculture (estimated 20.2 kg in 2016) (FAO, 2017b), additional 23 million tonnes thereof by 2020 will be required (FAO, 2017a). Aquaculture could represent a solution, though this sector largely depends on the availability of quality feeds and relative prices. FAO states that: “the sustainability of the aquaculture sector is more likely to be closely linked with the sustained supply of terrestrial animal” (FAO, 2017a).

As described above, insects can represent a valid alternative protein source. In particular, the most promising species for industrial production are the black soldier fly, the housefly, the yellow mealworm, the lesser mealworm, silkworm, and some grasshopper species (Van Huis, 2013).

Larvae of flies are able to grow and convert different waste materials that are not moreover exploitable. For example, housefly larvae can convert poultry manure that cannot be used as feedstuff for poultry because of the low energy and the high content of uric acid. On the other hand, housefly larvae are able to grow on this waste substrate, producing pupae with 60% proteins, which have a good content of the most important amino acids, such as arginine, lysine and methionine (El Boushy, 1991). Furthermore, dehydrated fly larvae present a better protein supplement for turkey than soybean (Zuidhof et al., 2003). The yellow mealworm is a good protein source for catfish (Ng et al., 2001) and bloiler chickens (Ramos-Elorduy, 2002). In addition, also silkworm pupae represent good feed for poultry (Wijayasinghe & Rajaguru, 1977). Crickets contain a considerable amount of digestible amino acids, which are advantageous for poultry because of the presence of a good source of dietary nitrogen, thanks to the high level of lysine, methionine, and cysteine (Wang et al., 2005).

Hermetia illucens

The black soldier fly (BSF) (Diptera: Stratiomyidae) is a non-pest cosmopolitan insect native to the Neotropics (Marshall et al., 2015).

Figure 1. The black soldier fly. Photo by Daniele Bruno

Adults of *H. illucens* are large, about 20 mm long, with a black-colored body (Figure 1). Under laboratory conditions, mating takes place two days after eclosion and oviposition is accomplished four days later (Tomberlin and Speppard, 2002). The female lays down from 500 to 900 eggs in cracks, in proximity of the substrate, to promote larval growth. The eggs show an oval shape with a pale yellow colour and hatch approximately in four days. The larval stage is composed by five instars, lasting from 13 to 18 days, and during this period the larvae are insatiable feeders (De Smet et al., 2018). BSF kept under laboratory conditions, at 27 °C, need 22-24 days to develop from the egg to the prepupa and 40-43 days from the egg to the adult (Tomberlin et al., 2002). However, under unfavourable conditions, about 4 months may be necessary for BSF to reach the adult phase (Figure 2) (Furman et al., 1959).

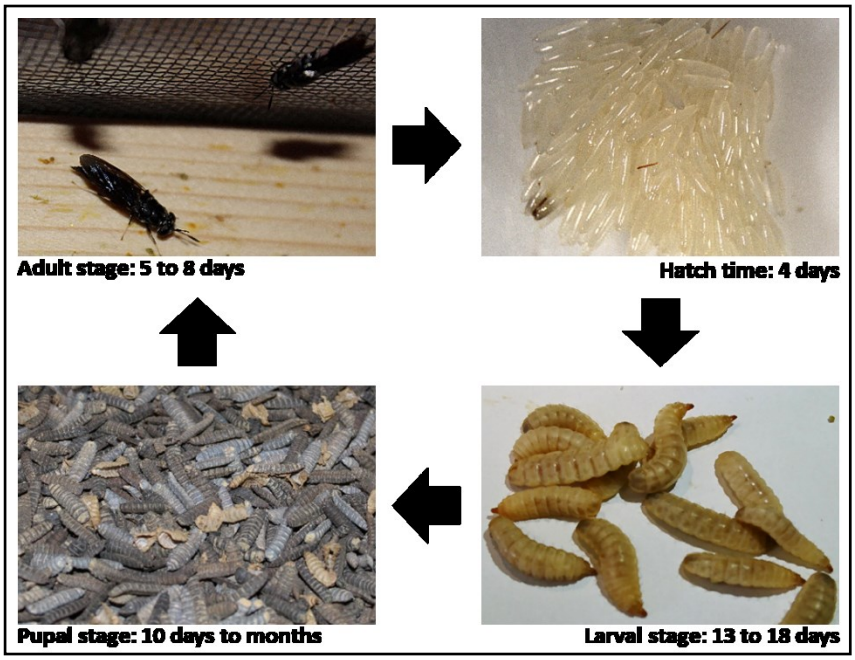

Figure 2. *Hermetia illucens* life cycle. Photos by Daniele Bruno

This saprophagous insect is able to grow on very different substrates, i.e., manure, organic waste, vegetable and fruits in advanced decomposition phase, waste from distilleries, and human feces (Banks et al., 2014; Cheng & Lo, 2015; Čičková et al., 2015; Yurina and Karagodin, 2018; Nguyen et al., 2015; Paz et al., 2015; Xiao et al., 2018; Zhou et al., 2013). Moreover, *H. illucens* larvae are used in forensic entomology to estimate post-mortem intervals, since their ability to grow on cadavers (Pujol-Luz et al., 2008).

BSF larvae have the ability to reduce and bioconvert organic waste, obtaining insects that are characterized by good nutritional values in terms of proteins and fat, which can be used as animal feed for fish, chicken, and pigs. In fact, these larvae are able to convert manure to body mass containing 42% of protein and 35% of fat (Sheppard et al., 1994), making this insect a good source of feed for livestock and aquaculture (Bondari & Sheppard, 1987; Newton et al.,

2005). Moreover, since this insect lacks omega-3, when BSF larvae are reared on fish offal, this fatty acid and other lipids reveal to be present in the larval body, making BSF a good alternative for fish meal and fish oil (St. Hilaire et al., 2007). This approach allows the manipulation of the larval diet to improve the nutrient composition of the fly biomass. Furthermore, BSF larvae are able to reduce the pollution potential of manure substrate, by reducing the total amount of different microelements, as nitrogen and phosphorous, and heavy metals as cadmium, copper, iron, lead, chromium, nickel, zinc and others (Sheppard et al., 2008). In addition, larvae reared on manure, especially chicken manure, are able to reduce potentially harmful bacteria, such as *Escherichia coli* and *Salmonella enterica* (Erickson et al., 2004; Liu et al., 2008).

Another important issue is the production of biodiesel from this insect (Leong et al., 2016; Li et al., 2011) and antimicrobial peptides (Müller et al., 2017; Vogel et al., 2018) from *H. illucens* larvae.

The processes of food digestion, nutrient absorption and accumulation of long-term storage molecules in insect larvae are mediated by two specific organs: the alimentary canal and the fat body. A deep characterization of these two organs is therefore fundamental to better understand how BSF larvae are able to grow on waste materials, transforming them in proteins useful for the production of feedstuff.

INSECT MIDGUT AND FAT BODY

The insect alimentary canal is composed by three specific regions called foregut, midgut, and hindgut. The foregut is the first part of the gut and is involved in the ingestion and transport of the food to the midgut. The midgut (Figure 3) is responsible for the production

of digestive enzymes and the absorption of nutrients derived from the digestion. This region is the central part of the alimentary canal, followed by the hindgut, where the absorption of water and salts takes place, before the elimination of frass (Lehane and Billingsley, 1996).

The insect midgut is a monolayered epithelium formed by three specific cell types: columnar cells, endocrine cells, and stem cells.

- **Columnar cells**

  Columnar cells represent the most common cell type present in the midgut of all insect species. These cells are involved in different functions as the synthesis and secretion of enzymes, the production of peritrophic matrix, nutrient absorption, and storage of organic and inorganic products (Martoja & Ballan-Dufrançais, 1984). They show a big nucleus and a cytoplasm rich in rough endoplasmic reticulum and Golgi. There is also a high amount of mitochondria in the cytoplasm, suggesting the active transport role of these cells. In addition, the apical plasma membrane is lined by well-developed microvilli, which allow the absorption of nutrients. The digestion of the food in the midgut lumen is allowed by the release of secretory vesicles that contain digestive enzymes (Billingsley & Lehane, 1996). Furthermore, this cell type is also able to store glycogen, lipids, and minerals from the ingested diet (Martoja & Ballan-Dufrançais, 1984).

- **Endocrine cells**

  Endocrine cells have been described in the midgut epithelium of a large number of insect species at different developmental stages (Hecker et al., 1971; Hecker, 1977; Kobayashi, 1971). Their main feature is the presence of a high number of electron-dense granules in the cytoplasm. The granules can show very

different size, from small (200 nm) up to very high dimension, occasionally reaching 800 nm (Nishiitsutsuji-Uwo and Endo, 1981). These cells can be classified in open- and closed-type: the former depart from the basal lamina and extend the cytoplasm for the entire epithelium thickness, contacting the midgut lumen with a surface covered by microvilli (Fujita & Kobayashi, 1977), thus suggesting their receptor-secretory role (Endo & Nishiitsutsuji-Uwo, 1981). Conversely, closed-type endocrine cells do not extend throughout the midgut epithelium but are limited to the basal membrane (Fujita & Kobayashi, 1977).

- **Stem cells**

  Stem cells are characterized by an undifferentiated morphology and are dispersed throughout the midgut epithelium. These cells show a high nucleus-to-cytoplasm ratio, a dense cytoplasm, and few differentiated organelles. In different insect species, they can be observed as single cells, paired or in groups at the base of the epithelium (Böhringer-Schweizer, 1977; Ohlstein and Spradling, 2006).

  Stem cells play a role into the replacement of midgut epithelium during larva-larva and larva-pupa moult (Baldwin & Hakim, 1991), allowing the growth of the midgut epithelium, and they are involved in cell replacement following injury (Spies & Spence, 1985), thus restoring the integrity of the epithelium.

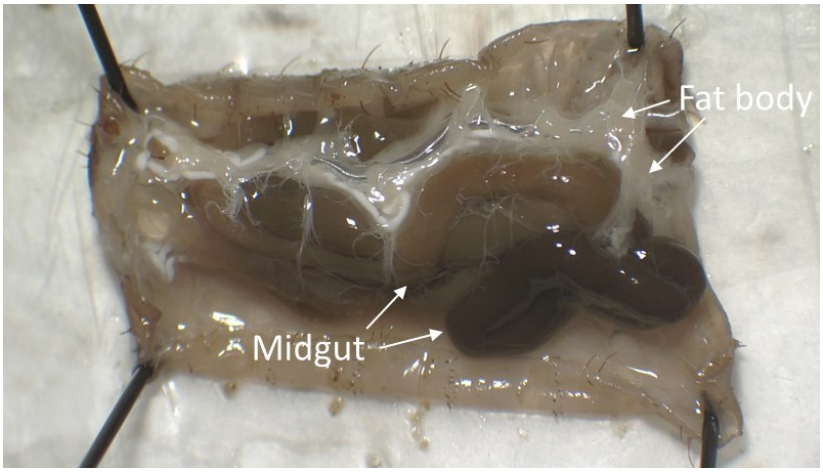

Figure 3. Dissected *H. illucens* larva in which the midgut and the fat body are visible. Photo by Daniele Bruno

The fat body (Figure 3), an unique organ among insects, plays an important role during the entire length of the insect life, since it stores and releases energy, in terms of glycogen and triglycerides, depending on the insect needs (Arrese et al., 2010; Law & Wells, 1989). It is a large organ distributed in the insect body and is composed by adipocytes that contain lipid droplets. The level of nutrient reserves accumulated during the larval stage affects different processes of the insect life, as growth, metamorphosis, and egg development (Mirth & Riddiford, 2007). It is generally believed that the adult of BSF does not eat, so the energy necessary to complete development and promote egg laying is derived from the storage molecules accumulated in the fat body during the larval stage (Tomberlin et al., 2002).

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AIM OF THE STUDY

The aims of the present study were:

- the morphological, physiological, and molecular characterization of *H. illucens* larval midgut;
- the evaluation of intestinal microbiota in *H. illucens* larvae fed on different diets;
- the analysis of the morphofunctional changes of the larval midgut and fat body in relation to the diet provided to the larvae;
- the morphological and functional characterization of the midgut of adult BSF and the evaluation of the feeding habits of the fly.

CHAPTER 1

Structural and functional characterization of *Hermetia illucens* larval midgut

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ABSTRACT

The larvae of *Hermetia illucens* are among the most promising agents for the bioconversion of low quality biomass, such as organic waste, into sustainable and nutritionally valuable proteins for the production of animal feed. Despite the great interest toward this insect, the current literature provides information limited to the optimization of rearing methods for *H. illucens* larvae, with particular focus on their efficiency in transforming different types of waste and their nutritional composition in terms of suitability for feed production. Surprisingly, *H. illucens* biology is a neglected aspect and a deep understanding of the morphofunctional properties of the larval midgut, the key organ that determines the extraordinary dietary plasticity this insect, is completely overlooked. The present study aims at filling this gap of knowledge.

Our results demonstrate that the larval midgut is composed of distinct anatomical regions with different luminal pH and specific morphofunctional features. The midgut epithelium is formed by different cell types that accomplish nutrient digestion and absorption, acidification of the lumen of the middle region, endocrine regulation, and growth of the epithelium. A detailed characterization of the activity of enzymes involved in nutrient digestion and their mRNA expression levels reveals that protein and carbohydrate digestion is associated to specific regions of this organ. Moreover, a significant lysozyme activity in the lumen of the anterior and middle regions of the midgut was detected. This enzyme, together with the strong acidic luminal pH of middle tract, may play an important role in killing pathogenic microorganisms ingested with the feeding substrate.

The collected evidence led us to propose a detailed functional model of the larval midgut of *H. illucens* in which each region is characterized by peculiar features to accomplish specific functions. This platform of knowledge sets the stage for the development of rearing protocols to optimize the bioconversion ability of this insect and its biotechnological applications.

INTRODUCTION

Hermetia illucens (Linnaeus, 1758) (Diptera: Stratiomyidae), the black soldier fly (BSF), is a common and widespread fly in tropical and temperate regions. The insect is traditionally supposed to be of American origin (Rozkošný, 1983; Wang and Shelomi, 2017) although this hypothesis was recently questioned, advancing a possible Palearctic origin (Benelli et al., 2014). Adults do not bite or sting, and are not described as vector of any specific diseases (Wang and Shelomi, 2017). In contrast with adults, which do not need to feed according to several reports (Sheppard et al., 1994; Sheppard et al., 2002; Tomberlin and Sheppard, 2002; Tomberlin et al., 2002; Tomberlin et al., 2009), the larvae of this holometabolous insect are voracious and grow on a wide variety of organic matters (Nguyen et al., 2015; Wang and Shelomi, 2017) thanks to a well-developed mandibular-maxillary complex (Kim et al., 2010).

From the pioneering study dated back to the end of the seventies of the last century (Newton et al., 1977), a growing body of evidence indicates that *H. illucens* larvae are among the most promising agents for the bioconversion of low quality biomass (e.g., organic waste and byproducts of the agri-food transformation chain) into sustainable and nutritionally valuable proteins and lipids for the production of animal feed (Van Huis, 2013; Van Huis et al., 2013;

Barragan-Fonseca et al., 2017; Wang and Shelomi, 2017). Moreover, *H. illucens* larvae are considered a potential source of bioactive substances, such as antimicrobial peptides (AMPs), a wide group of small cationic molecules that are currently studied as a possible alternative to conventional antibiotics and food or feed preservatives (Buchon et al., 2014). The production of a wide array of AMPs by *H. illucens* could be related to the alimentary habits of the larvae that feed on a variety of decomposing organic substrates, typically rich in microorganisms (Müller et al., 2017; Vogel et al., 2018). Furthermore, the ability of the larvae to grow on almost all organic matter makes this insect a potential source of enzymes able to degrade complex substrates that can have important industrial applications. For example, a cellulase has been characterized from *H. illucens* gut microbiota (Lee et al., 2014) and a recent review reported that BSF larvae represent a source of cellulose-, chitin-, and lignin-degrading enzymes (Müller et al., 2017). The interest in *H. illucens* as bioconverter and ingredient for animal feed or source of bioactive molecules is also demonstrated by the increasing number of newly founded companies that deal with BSF mass rearing (https://ilkkataponen.com/entomology-company-database/).

In the last decade, despite the increasing number of papers that demonstrate the broad range of applications that can derive from the exploitation of BSF larvae, little information on the biology of this insect has been obtained. This lack of knowledge may strongly hamper the exploitation of *H. illucens* as a source of proteins, lipids, and bioactive molecules as well as any other possible future biotechnological development. Among the subjects that need deep consideration there are the characterization of the immune system, the description of the gut microbiota and its relationship with the rearing substrates, the fine definition of the critical requirements for

insect development, and the characterization of the morphology and physiology of the larval midgut, which has a primary role in food digestion and nutrient absorption. The latter topic, which is essential to better comprehend the extraordinary dietary plasticity of the larva and optimize the exploitation of its bioconversion capability, is the object of this study.

Although the general properties of the larval midgut of non-hematophagous Diptera belonging to the taxon of Brachycera have been already defined (Terra et al., 1996; Nation, 2008), an exhaustive and comprehensive morphofunctional characterization of this organ, with particular attention to the role and the properties of its different districts, has never been performed. Basically, the information regarding the larval midgut of Brachycera species is limited to *Musca domestica* Linnaeus, 1758 and *Drosophila* spp. In the larval midgut of *Drosophila* spp. at least three regions are distinguishable from a morphological and physiological point of view (Dimltriadis and Kastritsis, 1984; McNulty et al., 2001; Dubreuil, 2004; Shanbhag and Tripathi, 2009). Ultrastructural features of midgut cells and the mechanisms of acid and base transport across the midgut epithelium responsible for the pH values recorded in the lumen have been studied (Shanbhag and Tripathi, 2009). The anterior region is characterized by a neutral-slightly alkaline luminal pH, as well as the first part of the posterior region, while the second part of the posterior region is strongly alkaline and the middle midgut is highly acidic (Shanbhag and Tripathi, 2009). A body of evidence indicates that copper cells, a peculiar cell type that is present in the first part of the middle midgut, are involved in the acidification of the lumen of this region (Dubreuil et al., 1998; McNulty et al., 2001; Dubreuil, 2004; Shanbhag and Tripathi, 2009). These cells, described for the first

time by Strasburger (1932), are also known as cuprophilic or oxyntic cells.

In the larval midgut of *M. domestica* three regions can be also recognised, with a slightly acidic pH in the lumen of the anterior and posterior midgut and a strongly acidic pH in the middle region (Terra et al., 1988: Lemos and Terra, 1991a). At variance with *Drosophila* spp., in *M. domestica* a functional characterization of the larval midgut, especially regarding the digestive properties associated to the midgut regions, has been accomplished (Espinoza-Fuentes and Terra, 1987; Espinoza-Fuentes et al., 1987; Terra et al., 1988; Lemos and Terra, 1991b; Lemos et al., 1993; Jordão et al., 1996; Pimentel et al., 2018).

Regarding *H. illucens* larvae, a morphofunctional study of the midgut has never been performed. Only few data about the biochemical properties of digestive enzymes are present in the literature. Kim and coworkers (Kim et al., 2011a) investigated the enzymes released by salivary glands and gut. The authors evidenced that clarified gut homogenates have high amylase, lipase, and protease activities, but they did not explore if these activities are associated to a specific region of the organ. Moreover, a qualitative and quantitative comparison of the digestive enzymatic activity from *H. illucens* and *M. domestica* evidenced that BSF larvae possess more digestive enzymes with higher levels of activity compared to the housefly (Kim et al., 2011a), a finding that supports the extraordinary digestive capability of *H. illucens*. Finally, two serine proteases were cloned and characterized (Kim et al., 2011b; Park et al., 2012).

The present work aims to provide an in depth description of the morphological, ultrastructural, and functional properties of the midgut epithelium of *H. illucens* larvae, focusing the attention on the peculiar characteristics of each region in which this tract of the

alimentary canal can be subdivided. In particular we defined: (i) the morphology and ultrastructural features of the cells that form the midgut epithelium; (ii) the value of the luminal pH in the different midgut regions with particular attention to the district where a strongly acidic pH is present; iii) the enzymes that are involved in carbohydrate and protein digestion and the midgut region where they hydrolyze the substrates; and (iv) the expression levels of genes encoding for digestive enzymes in each midgut region. These data allowed us to propose a functional model of the larval midgut of *H. illucens* that clearly shows how each region of the midgut possesses peculiar features and functions.

MATERIALS AND METHODS

Insect rearing

H. illucens larvae 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, Milano, Italy).

BSF adults were kept at 27 ± 0.5 °C, under a 12:12 h light:dark photoperiod, 70 ± 5% relative humidity with water supply and egg traps to promote oviposition. Eggs were collected in a Petri dish (9 × 1.5 cm), maintained at 27 ± 0.5 °C until hatching, and then subjected to a weaning procedure as described in Pimentel et al. (2017). Lots of 300 larvae were grown on Standard diet for Diptera (Hogsette, 1992), composed by 50% wheat bran, 30% corn meal, and 20% alfalfa meal mixed in the ratio 1:1 dry matter:water. The larvae were maintained at 27.0 ± 0.5 °C, 70 ± 5% relative humidity, in the dark. The feeding substrate was added with fresh diet every two days. All experiments were performed on actively feeding last

instar larvae weighing between 180 and 230 mg. For the expression analysis of phospho-Histone 3, midgut samples collected from larvae molting from third to fourth instar were used.

Isolation of midgut epithelium and midgut juice

Larvae were anesthetized on ice with CO_2. The gut was isolated in Phosphate Buffered Saline (PBS, 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.76 mM KH_2PO_4, pH 7.4) at 4 °C. The midgut, with the enclosed intestinal content, was isolated and subdivided into five regions: anterior midgut, middle midgut 1 and 2, posterior midgut 1 and 2 (see Figure 1 and section "General organization of the larval midgut and pH of the midgut lumen" in Results).

After isolation, the midgut regions used for morphological analyses were fixed as described below, whereas for immunohistochemical analyses the midgut regions were immediately frozen in liquid nitrogen and kept in liquid nitrogen until use.

For the rest of the analyses, the midgut epithelium was separated from the gut content as follows. The peritrophic matrix of each midgut region, with the enclosed intestinal content, was isolated, lightly blotted on filter paper and placed into microcentrifuge tubes. Samples were then centrifuged at 15000 × g for 10 min at 4 °C. Supernatant, i.e., the midgut juice, was collected and used fresh for pH determination, or stored at -80 °C for the enzymatic assays. The midgut epithelium was lightly blotted on filter paper, placed into cryovials and weighed. Tissues were stored in liquid nitrogen for aminopeptidase N assay, Western blot, and molecular analyses. For the determination of digestive enzyme activity and measurement of the luminal pH only anterior midgut, middle midgut 2 (for simplicity named middle midgut), and posterior midgut 2 (for simplicity named

posterior midgut) were used, to avoid contaminations of midgut content between tracts.

Light and transmission electron microscopy

After isolation, midgut samples were 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 2 h at room temperature, specimens were dehydrated in ascending ethanol series and embedded in Epon/Araldite 812 mixture resin. Sections were obtained with a Leica Reichert Ultracut S (Leica, Nussolch, Germany). Semi-thin sections were stained with crystal violet and basic fuchsin and then observed with an Eclipse Ni-U microscope (Nikon, Tokyo, Japan) equipped with a TrueChrome II S digital camera (Tucsen Photonics, Fazhou, China). Thin sections were stained with lead citrate and uranyl acetate and then observed with a JEM-1010 transmission electron microscope (Jeol, Tokyo, Japan) equipped with a Morada digital camera (Olympus, Münster, Germany).

Immunohistochemistry

After isolation, midguts were fixed in 4% paraformaldehyde in PBS overnight at 4 °C. Specimens were then dehydrated in ascending ethanol series and embedded in paraffin. Sections (7-µm-thick), obtained with microtome Jung Multicut 2045, were deparaffinized, rehydrated, and pre-incubated for 30 min with PBS containing 2% bovine serum albumin (BSA) before incubation with anti-H^+ V-ATPase, subunit V1, antibody (Ab 353-2, dilution 1:10000 in 2% PBS/BSA), for 1 h at room temperature. After washing with PBS, sections were incubated for 1 h at room temperature with an anti-guinea pig Cy2-conjugated secondary antibody (dilution 1:200 in 2% PBS/BSA, Jackson ImmunoResearch, Pennsylvania, USA). After

washes with PBS, sections were incubated with DAPI (100 ng/ml in PBS) for nuclear staining and then washed. Slides were mounted in Citifluor (Citifluor Ltd, London, UK) with coverslips and analyzed with a Nikon Eclipse Ni microscope equipped with TrueChrome II S digital camera. The primary antibody was omitted in controls.

Western blot analysis

Tissues were homogenized with T10 basic ULTRA-TURRAX (IKA, Staufen, Germany) in RIPA buffer (150 mM NaCl, 2% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), and 50 mM Tris, pH 8.0) (1 ml/0.14 g of tissue), added with 1× protease inhibitor cocktail (1 mM AEBSF, 800 nm Aprotinin, 50 µM Bestatin, 15 µM E64, 20 µM Leupeptin, 10 µM Pepstatin A) and phosphatase inhibitors (1 mM sodium orthovanadate and 5 mM sodium fluoride) (Thermo Fisher Scientific, Massachusetts, USA). The homogenate was centrifugated at 15000 × g for 15 min at 4 °C and the pellet discarded. Bradford assay (Bradford, 1976) was used to determinate the protein concentration of the supernatant. Clarified homogenates were denaturated at 98 °C in 4X gel loading buffer for 5 min and loaded on 8% or 12% Tris-glycine acrylamide gel for SDS-PAGE analysis (60 µg protein/lane). Proteins were then transferred to a 0.45 µm nitrocellulose membrane (GE Healthcare Life Sciences, Little Chalfont, UK). The membranes were blocked with 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 the following antibodies: anti-phospho-Histone 3 (Merck-Millipore, Massachusetts, USA), diluted 1:1000 in TBS/2% milk, anti-H^+ V-ATPase, diluted 1:10000 in TBS/5% milk, and anti-GAPDH (Proteintech, Illinois, USA), diluted 1:2500 in TBS/5% milk. After three washes with TBS/0.1% Tween 20, antigens of H3P

and GAPDH were detected with an anti-rabbit (dilution 1:7500 in TBS/5% milk; Jackson ImmunoResearch Laboratories), and H^+ V-ATPase with an anti-guinea pig (dilution 1:10000 in TBS/5% milk, Jackson ImmunoResearch Laboratories) HRP-conjugated secondary antibody. After three washes with TBS/0.1% Tween 20, immunoreactivity was detected by SuperSignal chemiluminescence substrate (Thermo Fisher Scientific).

RNA extraction and qRT-PCR

Total RNA was extracted from 15-40 mg of frozen tissue by using TRIzol reagent (Thermo Fisher Scientific), according to manufacturer's instructions. DNA contamination was removed by using TURBO DNA-free Kit (Thermo Fisher Scientific), then the purity of RNA was assessed by quantification and the integrity of RNA was tested by electrophoresis on 1% agarose gel. RNA was retrotranscribed with M-MLV reverse transcriptase (Thermo Fisher Scientific). qRT-PCR was performed with iTaq Universal SYBR Green Supermix (Biorad, California, USA) using a 96-well CFX Connect Real-Time PCR Detection System (Biorad). The primers used for qRT-PCR are reported in Table 1. To calculate the relative expression of genes of interest, the $2^{-\Delta\Delta Ct}$ method was used, with *HiRPL5* (*Hermetia illucens* Ribosomal Protein L5) as a housekeeping gene. The primers used for *Hi RPL5* were designed on conserved regions of *RPL5* in other insect species and the sequence was checked by sequencing the PCR product.

Table 1. Sequence of primers used in this study.

Gene name	Accession number	Primer sequences
HiTrypsin	HQ424575	F: ATCAAGGTCTCCCAGGTC
		R: GGCAAGAGCAATAAGTTGGAT
HiChymotrypsin	HQ424574	F: AGAATGGAGGAAAGTTGGAGA
		R: CAATCGGTGTAAGCAGAGACA
HiRPL5		F: AGTCAGTCTTTCCCTCACGA
		R: GCGTCAACTCGGATGCTA

Determination of midgut juice pH

The pH of the midgut juice isolated from midgut samples as described above was measured by universal indicator strips with a resolution of 0.5 pH unit (Hydrion Brilliant pH Dip Sticks, Sigma-Aldrich, Milano, Italy). Samples of midgut juice of the different regions were extracted from at least 15 larvae and the experiment was repeated on 14 independent batches of larvae.

Functional characterization of copper cells

Last instar larvae were transferred to Standard diet prepared with a water solution containing 4 mM $CuCl_2$ and allowed to feed for 6, 12, 24, and 48 h. Control larvae were fed on diet prepared with water without $CuCl_2$. At the indicated time points, 15 larvae were collected, anesthetized on ice with CO_2 and dissected. The whole gut was removed and placed in ice-cold PBS. The midgut was divided in five regions as described above and fixed overnight at 4 °C in 3.7% formaldehyde in PBS. Tissue samples were then rinsed in PBS and mounted on microscope slides with mounting medium (2:1 glycerol:PBS). Copper-dependent fluorescence was observed with Olympus BX50 fluorescence microscope at 365 nm excitation wavelength, analyzing the emitted fluorescence between 585 and 620 nm.

At the same time points (6, 12, 24 and 48 h), midgut juice from 15 larvae was collected from both control and copper-feed larvae, and the luminal pH of anterior, middle, and posterior midgut region was measured as indicated above.

Enzymatic assays on midgut juice

Frozen samples of midgut juice were thawed at 4 °C and protein concentration was determined by the method of Bradford (1976), using BSA as standard. Assays were performed under conditions in which product formation was linearly associated with enzyme concentration.

Total proteolytic activity.

The total proteolytic activity in midgut juice samples was assayed with azocasein (Sigma-Aldrich), measuring its degradation by release of azo chromophore (Charney and Tomarelli, 1947; Vinokurov et al., 2006; Caccia et al., 2014). Different volumes of midgut juice were diluted to 100 µl with Universal Buffer (UB), which has a constant ionic strength at different pH values (Coch Frugoni, 1957); the pH used for the assays is indicated in the captions to figures and in the Results. Then, diluted samples were incubated for 30 min at 45 °C with 200 µl of 1% (w/v) azocasein solution dissolved in UB. The reaction was terminated by adding 300 µl of 12% (w/v) trichloroacetic acid (TCA) at 4°C, then the mixture was maintained for 30 min on ice to favor undigested substrate precipitation, and it was clarified by centrifugation at 15000 × g for 10 min at 4 °C. An equal volume of 500 mM NaOH was added to the supernatant and absorbance was measured at 440 nm with a spectrophotometer (Pharmacia Biotech Ultrospec 3000 UV-Visible, Biochrom Ltd. Cambridge, England). One unit (U) of total proteolytic activity was defined as the amount of enzyme that causes an increase in

absorbance by 0.1 unit per min per mg of proteins. Controls were run on midgut juice samples heated to 100 °C for 5 minutes to denature proteolytic enzymes (three replicates for each region of the midgut). The mean absorbance of controls (2.6 ± 0.5 U) was identical for the different midgut regions (unpaired t-test) and was subtracted from the total proteolytic activity that was measured in each experiment. The total proteolytic activity in midgut juice samples from the posterior midgut was assayed at different temperatures ranging from 10 °C to 70 °C.

For total proteolytic activity inhibition assays, midgut juice from posterior region of the midgut was diluted 1:10 in UB at pH 8.5 and preincubated with 5 mM phenylmethanesulfonyl fluoride (PMSF, Sigma-Aldrich) or 0.1 mM E-64 (Sigma-Aldrich) for 15 min at 25°C. Inhibitor was omitted in controls. Total proteolytic activity in the absence and in the presence of inhibitors was measured as described above. Preliminary experiments were performed to verify the dose-dependent effect of the inhibitors on proteolytic activity, and the concentrations that guaranteed maximum inhibition were used.

Chymotrypsin- and trypsin-like proteolytic activity

Chymotrypsin- and trypsin-like proteolytic activity in midgut juice samples was assayed with N-succinyl-ALA-ALA-PRO-PHE-p-nitroanilide (SAAPPpNA, Sigma-Aldrich) and Na-Benzoyl-D,L-arginine 4-nitroanilide hydrochloride (BApNA, Sigma-Aldrich), respectively, measuring their degradation by release of p-nitroaniline (pNA) (Hosseininaveh et al., 2007). Different volumes of midgut juice were diluted to 300 µl with UB at pH 8.5. The diluted samples were then incubated with 300 µl of 10 mM SAAPPpNA solution dissolved in UB or with the same volume of 10 mM BApNA

solution in UB obtained from a stock solution of 100 mM BApNA in dimethyl sulfoxide for 10 min at 45 °C. The reaction was terminated by addition of 600 µl of ice-cold 12% TCA and after 5 min at 25 °C the absorbance was measured at 405 nm. One unit (U) of proteolytic activity was defined as the amount of enzyme that causes an increase in absorbance by 0.1 unit per min per mg of proteins.

Controls were run on midgut juice samples heated to 100 °C for 5 minutes to denature proteolytic enzymes (three replicates for each region of the midgut). The mean absorbance of controls (8.3 ± 1.4 U and 3.0 ± 0.4 U for chymotrypsin- and trypsin-like proteolytic activity, respectively) was identical for the different midgut regions (unpaired t-test) and was subtracted from the activity measured in each experiment.

Trypsin-like activity in midgut juice samples from the posterior region was assayed at different temperatures, ranging from 10 °C to 70 °C, at pH 8.5 to identify the optimum temperature, and at different pH values, ranging from 3.0 to 10.5, at 45 °C to identify the optimum pH.

α-amylase activity

α-amylase activity in midgut juice samples was assayed using starch as substrate, measuring the amount of maltose released (Bernfeld, 1955). A standard curve was determined through linear regression of the maltose absorbance at 540 nm. Different volumes of midgut juice were diluted to 595 µl in Amylase buffer (AB) (20 mM NaH_2PO_4, 6.7 mM NaCl, pH 6.9). Then, the diluted samples were incubated with 90 µl of 1% (w/v) soluble starch solution in AB. Controls without midgut juice sample and controls without substrate were performed for each experiment. All samples were incubated for 30 min at 45 °C, and, after the addition of 115 µl of Color Reagent Solution (CRS)

(1 M sodium potassium tartrate, 48 mM 3,5-dinitrosalicylic acid, 0.4 M NaOH), heated at 100 °C for 15 min and then cooled in ice to 25 °C, mixed by inversion, and their absorbance was measured at 540 nm. Considering the standard curve and the absorbance in control samples, the production of maltose from enzymatic hydrolysis of starch was calculated. 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. Midgut juice samples heated to 100 °C for 5 minutes to denature amylolytic enzymes showed no α-amylase activity. α-amylase activity in midgut juice samples from the anterior region was assayed at different pH values ranging from 3.0 to 10.5 to identify the optimum pH.

Lysozyme activity

Lysozyme activity in midgut juice samples was assayed with *Micrococcus lysodeikticus* lyophilized cells (Sigma-Aldrich), measuring the rate of lysis of bacterial cells. Different volumes of midgut juice were diluted to 20 µl with 66 mM potassium phosphate buffer, pH 6.2. Then, the diluted samples were incubated with 980 µl of a suspension of 0.02 % (w/v) *Micrococcus lysodeikticus* lyophilized cells in the same buffer. The mixture was subjected to continuous absorbance reading at 450 nm at 45 °C. One unit/ml (U/ml) of lysozyme activity was defined as the amount of enzyme that causes a decrease in absorbance by 0.001 unit per min per ml of midgut juice sample.

Aminopeptidase N activity in midgut homogenates

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). After thawing, midgut samples were homogenized with a microtube

pestle in 50 mM Tris-HCl, pH 7.5 (1 ml/100 mg tissue). Different volumes of homogenate were diluted to 800 µl with the same buffer and then 200 µl of 20 mM L-leucine p-nitroanilide were added. The diluted samples were subjected to continuous absorbance reading at 410 nm at 45 °C. One unit/mg (U/mg) of APN activity was defined as the amount of enzyme that releases 1 µmol of pNA per min per mg of proteins.

Statistical analyses

Statistical analyses were performed with R-statistical software (ver. 3.3.2). The following analyses were performed: one-way analysis of variance (ANOVA) followed by Tukey's test, paired and unpaired t-tests. Statistical differences between groups were considered significant at p-value ≤ 0.05. The statistical analysis performed for each experiment and the p-values are reported in the captions to figures.

RESULTS

General organization of the larval midgut and pH of the midgut lumen

Three regions were clearly recognizable in the alimentary canal isolated from *H. illucens* larvae, i.e., the foregut, the midgut, and the hindgut (Figure 1A). This organization of the gut into three regions of different embryonic origin characterizes all insects (Nation, 2008) and allows the sequential function of the gut, i.e., food ingestion, digestion and absorption of nutrients, and elimination of frass. At variance with other Brachycera (Terra et al., 1988; Dubreuil, 2004), the larval gut of *H. illucens* did not present gastric caeca (Figure 1A). The midgut, involved in the production and secretion of digestive enzymes, and the absorption of nutrients represented the

intermediate and the longest part of the digestive system (Figure 1B). In non-hematophagous brachycerous larvae examined so far, the midgut is at least subdivided into three distinct regions: anterior, middle and posterior, with the middle segment characterized by a strongly acidic pH of the lumen content (Terra et al., 1988; Lemos and Terra, 1991a; Shanbhag and Tripathi, 2009). Gross morphology and pH values of midgut juice isolated from different midgut districts revealed distinct regions also in the midgut of *H. illucens* larvae. The anterior midgut (AMG) (Figure 1C), with an acidic lumen content (Table 2) was formed by a deeply infolded layer, the middle region was characterized by a narrow and short tract (middle midgut 1, MMG1) that continued in a segment characterized by a larger diameter (middle midgut 2, MMG2) and absence of infolding (Figure 1D). The middle region, as reported for other non-hematophagous brachycerous larvae, was strongly acidic (Table 2). The posterior midgut, downstream of a constriction at the end of the middle tract and characterized by an alkaline luminal pH (Table 2), was the longest region and showed a short first tract (posterior midgut 1, PMG1) followed by a second one darker in color (posterior midgut 2, PMG2) (Figure 1E).

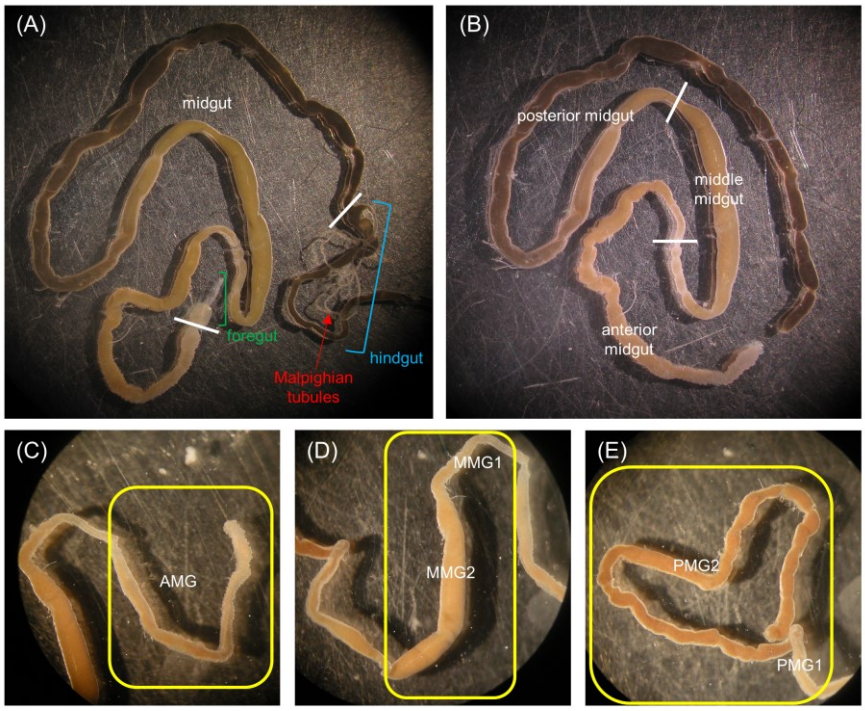


Figure 1. Anatomy of the alimentary canal of *H. illucens* larvae and definition of the midgut regions. The short foregut is followed by a very long midgut; the beginning of the hindgut is identifiable by the insertion of Malpighian tubules (A). The midgut can be subdivided into three main regions: anterior, middle, and posterior (B). In (C), (D), and (E) details of each midgut region are reported. Anterior midgut (C); middle midgut (D) in which a first narrow and short tract (MMG1) is followed by a segment with a larger diameter (MMG2); posterior midgut (E) in which two tracts are recognizable, a first short part (PMG1) and a second tract darker in color (PMG2).

Morphological characterization of the midgut epithelium and regional differentiation

A detailed morphological characterization of the midgut epithelium was performed for all the five regions described above (see "General organization of the larval midgut and pH of the midgut lumen" and Figure 1). Three main cell types were found along the whole length of the midgut, i.e., columnar, endocrine, and stem cells. Columnar cells were the most representative cell type of the epithelium and

were characterized by the presence of microvilli on the apical membrane and basal infolding (Figure 2A). The endocrine cells were localized in the basal region of the epithelium and presented electron-dense granules in the cytoplasm (Figure 2B). Finally, stem cells were randomly distributed at the base of the epithelium (Figure 2C). These cells were able to proliferate during larva-larva molt (Figure 2D), as confirmed by Western blot analysis of phospho-Histone 3: a 17-kDa band (weight of the protein) revealed a significant presence of this mitotic marker during the molting stage, which was absent in intermolt period (last instar larvae actively feeding) (Figures 2E, S1).

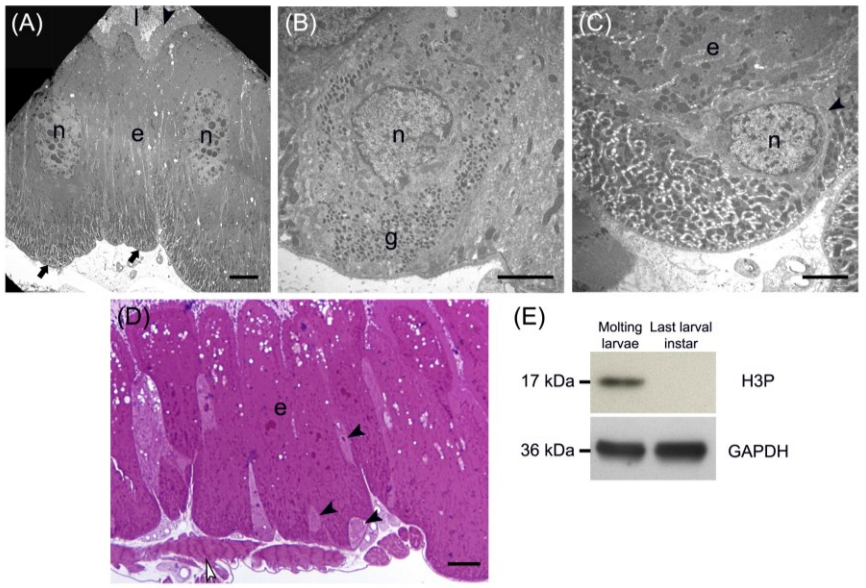


Figure 2. **Main cell types present in the larval midgut epithelium.** (A): columnar cells display a developed basal infolding (arrows) and long microvilli (arrowhead). (B): endocrine cells with electron-dense granules (g) inside the cytoplasm. (C): stem cell (arrowhead) located at the base of the midgut epithelium. (D): the amount of stem cells (arrowheads) increases during larva-larva molt. (E): Western blot analysis of phospho-Histone 3 (H3P). e: epithelium; l: lumen; open arrowheads: muscle cells; n: nucleus. Bars: 5 µm (A), 2 µm (B, C), 10 µm (D).

Besides the three cell types described above, every midgut district presented typical features, indicating a marked regional differentiation of this organ. The AMG showed a thick epithelium (Figure 3A) formed by columnar cells with a developed basal infolding (Figure 3B). These cells were characterized by abundant rough endoplasmic reticulum (RER) (Figure 3C), and numerous electron-dense granules and mitochondria were present in the apical region under the microvilli (Figure 3D).

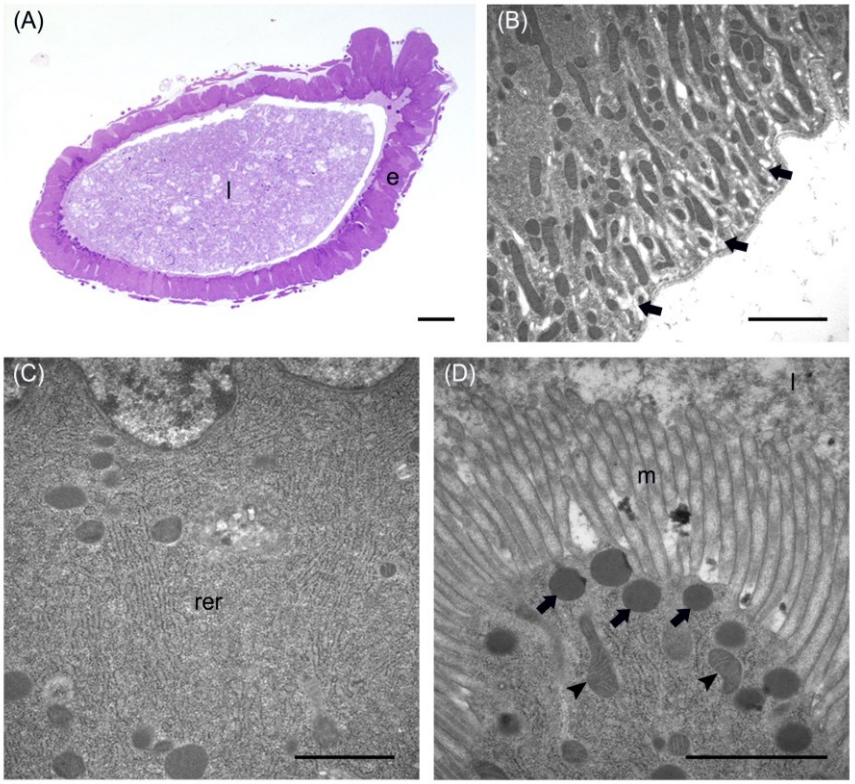


Figure 3. **Morphological organization of the anterior midgut.** (A): cross-section of anterior midgut. (B): wide basal infolding (arrows) in columnar cells. (C): rough endoplasmic reticulum (rer) in the cytoplasm of columnar cells. (D): electron-dense vesicles (arrows) in the apical part of columnar cells, under the microvilli (m). e: epithelium; l: lumen; arrowheads: mitochondria. Bars: 100 µm (A), 2 µm (B), 1 µm (C, D).

The epithelium of MMG1 contained specialized cells, i.e., copper cells, characterized by a cup shape (Figure 4A), a big central nucleus (Figure 4B) and long microvilli (Figure 4B). A peculiar trait of these cells was the presence of several and elongated mitochondria inside each microvillus (Figures 4C, D). On the apical membrane of the cells portasome-like particles could be observed (Figure 4E). The presence of H^+ V-ATPase, previously shown to be associated to portasomes (Shanbhag and Tripathi, 2009; Zhuang et al., 1999), was investigated by immunohistochemistry and Western blot analysis. Western blot revealed seven bands with molecular mass of 13-kDa, 14-kDa, 27-kDa, 34-kDa, 55-kDa, 56-kDa, and 67-kDa, corresponding to most of the subunits of the cytoplasmic V_1 complex of H^+ V-ATPase (Figure 4F). Immunostaining confirmed the presence of H^+ V-ATPase only in MMG1, specifically associated to the apical membrane of copper cells (Figures 4G-K).

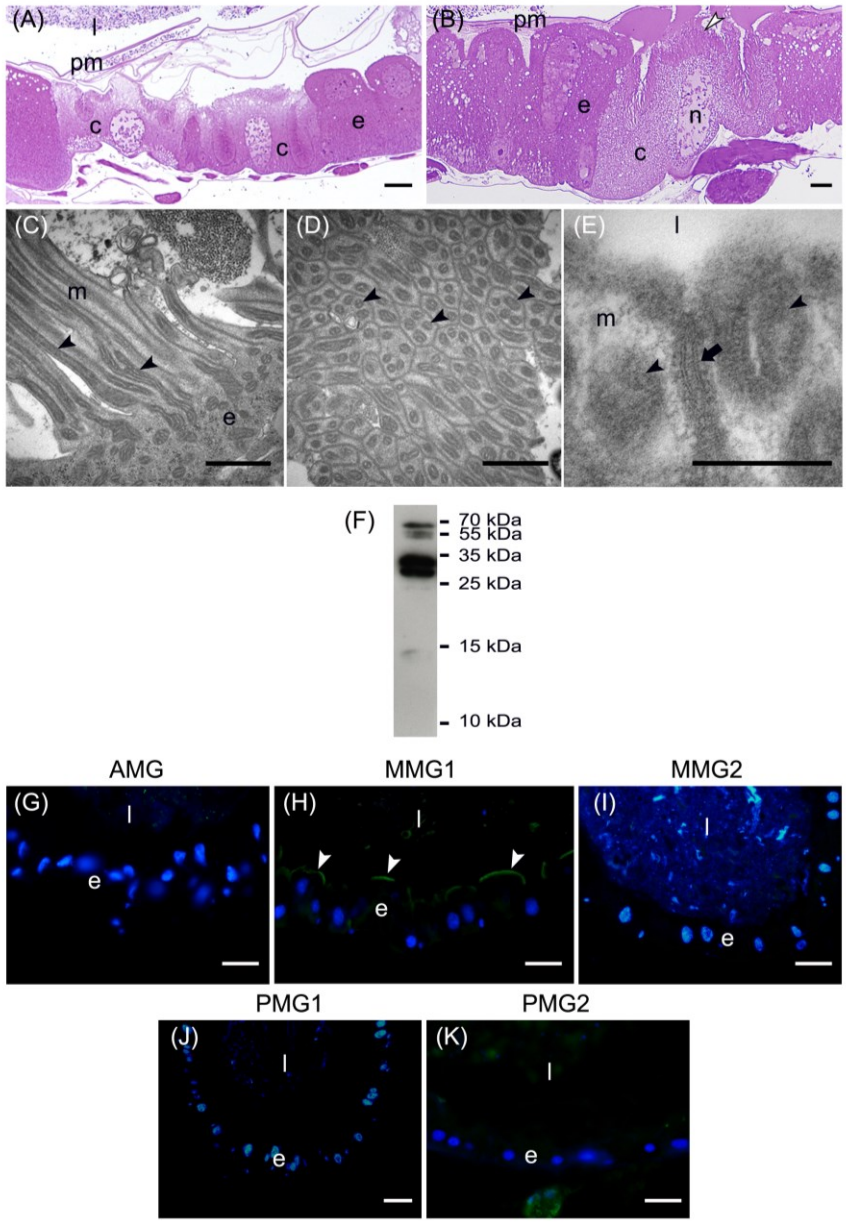


Figure 4. Morphological organization of MMG1. (A, B): cuprophilic cells (c) characterized by the long microvilli (open arrowheads). (C, D): Elongated mitochondria (arrowheads) are visible in longitudinal (C) and cross (D) section of microvilli (m). (E): Portasome-like structure (arrow) in the apical surface of microvilli. Mitochondria (arrowheads) are visible. (F): Western blot analysis of H^+ V-ATPase. (G-K): H^+ V-ATPase immunolocalization (white arrowheads) in the different midgut regions. e:

epithelium; l: lumen; n: nucleus; pm: peritrophic matrix. Bars: 20 µm (A, G-K), 10 µm (B), 1 µm (C, D), 200 nm (E).

The ability of these cells to acidify the middle midgut lumen, thanks to the secretion of proton into the lumen *via* H^+ V-ATPase, was evaluated. In copper-fed larvae of *Drosophila melanogaster* Meigen, 1830, copper cells have been shown to acquire an orange fluorescence signal due to the formation of a copper ions-metallothionein complex and, in turn, the acid-secreting activity of these cells is reduced (McNulty et al., 2001). To verify whether copper cells in *H. illucens* midgut showed similar features, larvae fed on diet containing cupric chloride for different time points (6, 14, 24 and 48 h) were examined. MMG1, the region containing copper cells, from control larvae reared without $CuCl_2$ in the diet did not exhibit orange fluorescence when examined under UV excitation for all the time points considered (Figure 5A and data not shown). Conversely, the same midgut tract of larvae fed on copper-containing diet showed a fluorescence signal starting from 14 h that increased significantly over time (Figures 5B-E). Midgut cells of the other regions showed an orange fluorescence only in larvae fed on copper-containing diet for 48 h (data not shown). We also examined the relationship between copper-dependent fluorescence and midgut lumen pH. Midgut juice samples from anterior, middle, and posterior region of the midgut were extracted from larvae fed for 24 h on copper-containing diet and control diet, and the pH was measured using universal indicator strips. As indicated in Figure 5F, copper feeding reduced larval midgut acidification in the middle midgut, whereas no variation was recorded in the anterior and posterior midgut.

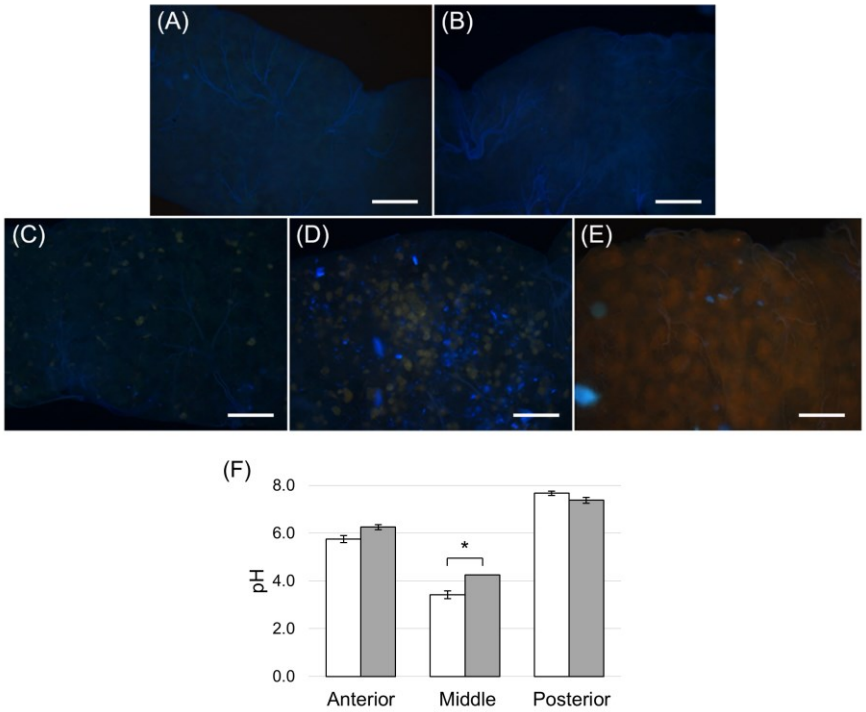


Figure 5. Copper feeding experiments. (A): dissected midgut from control larvae reared on standard diet without $CuCl_2$ for 24 h. (B, C, D, E): dissected midgut from larvae reared on standard diet added with $CuCl_2$ for 6, 14, 24 and 48 h, respectively. (F): pH measurement of midgut juice samples from anterior, middle and posterior region of midgut isolated from larvae fed for 24 h on control (white bar chart) and copper-containing diet (grey bar chart). The values are reported as mean ± SEM of at least 3 experiments. Only in the middle midgut a significant difference between groups was observed (unpaired *t*-test: *p<0.01). Bars: 100 µm (A-E).

MMG2 showed a wide lumen surrounded by a thin epithelium (Figure 6A) formed by large flat cells with a big elongated nucleus (Figure 6B) and a very short brush border (Figure 6C).

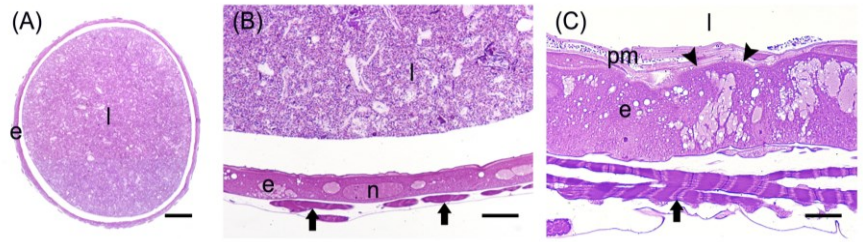


Figure 6. Morphological organization of MMG2. (A): cross-section of MMG2. (B, C): Details of the large flat cells that forms the thin epithelium in this midgut region. The short brush border (arrowheads) is visible in (C). e: epithelium; l: lumen; arrows: muscle cells; n: nucleus; pm: peritrophic matrix. Bars: 200 µm (A), 20 µm (B), 10 nm (C).

The posterior midgut displayed peculiar morphological features. In this region, the epithelium was thick (Figure 7A) with a well-developed brush border (Figure 7B). In particular, columnar cells in PMG1 differed from those of PMG2 for the numerous electron-dense granules localized under the microvilli (Figure 7B). Similarly to the anterior midgut, abundant RER (Figure 7C) was observed. Columnar cells of PMG2 showed very long microvilli (Figure 7D).

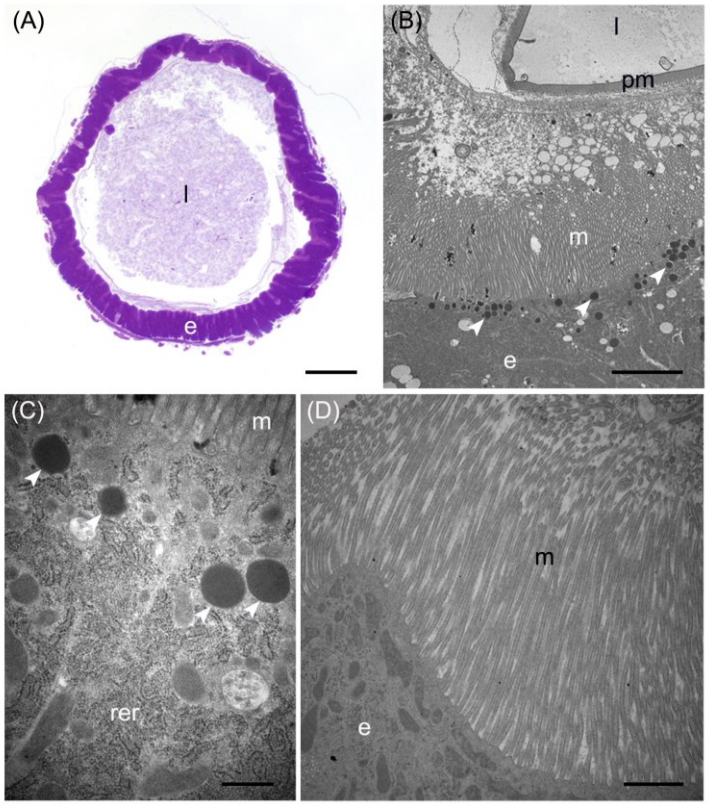


Figure 7. Morphological organization of the posterior midgut (PMG1 and PMG2). (A): cross-section of PMG1. (B, C): rough endoplasmic reticulum (rer), microvilli (m), and electron-dense vesicles (arrowheads) in the apical part of columnar cells in PMG1. (D): brush border of the epithelial cells in PMG2. e: epithelium; l: lumen; pm: peritrophic matrix. Bars: 100 µm (A), 5 µm (B), 500 nm (C), 1 µm (D).

Distribution of digestive enzymes in the midgut

A previous study indicated that salivary glands of *H. illucens* larvae are a minor source of digestive enzymes and that digestion is mainly accomplished by the midgut (Kim et al., 2011a). To evaluate whether the morphological differences observed in the different midgut regions, as well as the deep variation in the luminal pH along this organ, corresponded to a functional regionalization, we examined

the activity of enzymes involved in protein and carbohydrate digestion in each midgut region.

We first measured the total proteolytic activity in the midgut juice isolated from the anterior, middle and posterior region using azocasein as substrate at pH values as close as possible to those present in the lumen of each region. As reported in Figure 8A, the posterior tract showed the highest activity that was more than forty- and twenty-fold higher than in the anterior and middle region, respectively. Since the posterior midgut may have a major role in protein digestion, a detailed characterization of the total proteolytic activity in this region was performed. First, we evaluated the dependence of the total proteolytic activity on temperature (Figure 8B). The highest activity was recorded at 45 °C, while beyond this temperature the residual activity declined, reaching approximately 10% at 10 and 70 °C. Since the lumen of the posterior midgut had an alkaline pH (Table 2), we evaluated if the total proteolytic activity measured in this region could be ascribed to serine proteases, the major family of endopeptidases that shows a significant activity at alkaline pH values (Terra and Ferreira, 1994; 2005).

Table 2. pH values in the lumen of the midgut regions of *H. illucens* larvae. Different letters indicate statistically significant differences between groups (mean ± SEM, number of replicates in parenthesis. ANOVA test followed by Tukey's test. ANOVA p-value<0.001, Tukey's test p-values: Middle *vs* Anterior p<0.001, Posterior *vs* Anterior p<0.001, Posterior *vs* Middle p<0.001).

Midgut region	Mean ± SEM (n)
Anterior	5.9 ± 0.1 (14)[a]
Middle	2.1 ± 0.1 (14)[b]
Posterior	8.3 ± 0.2 (14)[c]

When the proteolytic activity was measured at acidic pH (pH 5.0), a highly significant decrease of the enzymatic activity (approximately 13-fold reduction) was observed compared to that recorded at pH 8.5 (Figure 8C). Moreover, PMSF, a rather specific, competitive, and irreversible inhibitor of serine proteases, caused a significant reduction of the total proteolytic activity compared to controls, with a percent of inhibition of 56.5 ± 1.4% (Figure 8D). On the contrary, no inhibition was observed with E-64, an irreversible inhibitor of a wide range of cysteine proteases (Figure 8D).

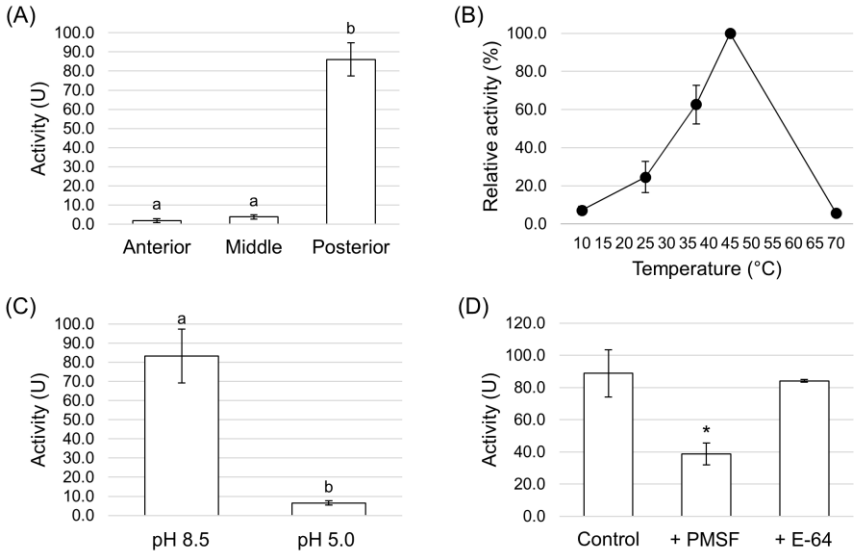


Figure 8. Total proteolytic activity in the different midgut regions. (A): total proteolytic activity in midgut juice extracted from anterior, middle and posterior midgut. For each tract the enzymatic assay was performed at pH as close as possible to that of the lumen (pH 6.0 for the anterior midgut, pH 5.0 for the middle midgut and pH 8.5 for posterior). The values are reported as mean ± SEM of at least 3 experiments. Different letters denote significant differences (ANOVA test followed by Tukey's test. ANOVA *p*-value<0.001, Tukey's test *p*-values: Middle *vs* Anterior *p*=0.975, Posterior *vs* Anterior *p*<0.001, Posterior *vs* Middle *p*<0.001). (B): dependence of total proteolytic activity on temperature in midgut juice from the posterior region of the midgut performed at pH 8.5. Relative activity values (%) are reported as mean ± SEM of at least 3 experiments and are expressed as percentage of the highest activity over the

temperature range examined. (C): total proteolytic activity in midgut juice from the posterior midgut measured at pH 8.5 and 5.0. The values are reported as mean ± SEM of 3 experiments. Different letters denote significant differences (paired t-test: *p*-value<0.05). (D): total proteolytic activity in midgut juice from the posterior midgut measured at pH 8.5 in the absence (control) and in the presence of serine- (PMSF) and cysteine- (E-64) protease inhibitors. The values are reported as mean ± SEM of 5 experiments. A significant difference between groups was observed between the activity measure in the presence of PMSF *vs* control (paired t-test: **p*-value<0.01).

Trypsin- and chymotrypsin-like enzymes are the major serine proteases involved in protein digestion in most insects (Terra and Ferreira 1994, 2005). To verify the involvement of these enzymes in digestion processes, we first measured mRNA levels of two isoforms of these enzymes (Kim et al., 2011b) in the different midgut districts by qRT-PCR. Both genes were highly expressed in the posterior midgut, with mRNA levels significantly higher in PMG1 than PMG2 for *HiTrypsin* (Figures 9A, B).

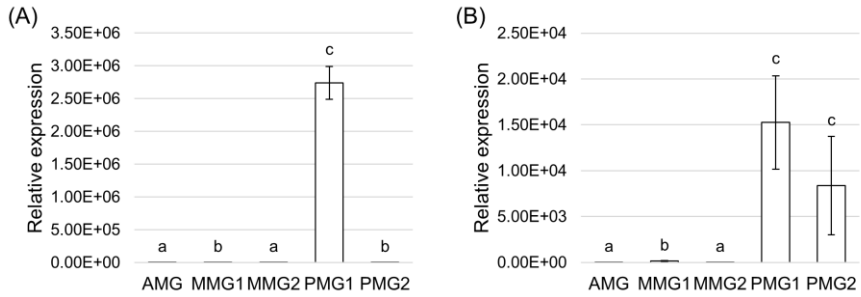


Figure 9. Expression profile of serine proteases. (A, B): qRT-PCR analysis of *HiTrypsin* (A) and *HiChymotrypsin* (B) in the different midgut regions. Values represent mean ± SEM (different letters denote significant differences compared to anterior midgut using ANOVA followed by Tukey's test.).

By using specific substrates, we measured the tryptic and chymotryptic activity in the different midgut regions. Figure 10A reports trypsin-like activity in the midgut juice from anterior, middle

and posterior region of the midgut using BApNA as substrate. No activity was recorded in the anterior and middle region, while a significant trypsin-like activity was present in the posterior region. To better characterize the tryptic activity, we evaluated its dependence on temperature and pH. The optimal temperature, as for the total proteolytic activity (Figure 8B), was 45 °C (Figure 10B). The evaluation of pH effect on trypsin activity using buffers at pH ranging from 3.0 to 10.5, at temperature of 45 °C, evidenced that the highest BApNA hydrolysis occurred at pH values from 7.0 to 10.5, with an optimum at pH 8.5, and decreased significantly at acidic pH (Figure 10C). We also measured the activity of chymotrypsin-like proteases in the midgut juice extracted from the three regions of the midgut using SAAPPpNA as substrate (Figure 10D). The highest activity was recorded in the lumen content of the posterior midgut, confirming that this district is the main site where protein digestion by endopeptidases belonging to serine proteases family occurs.

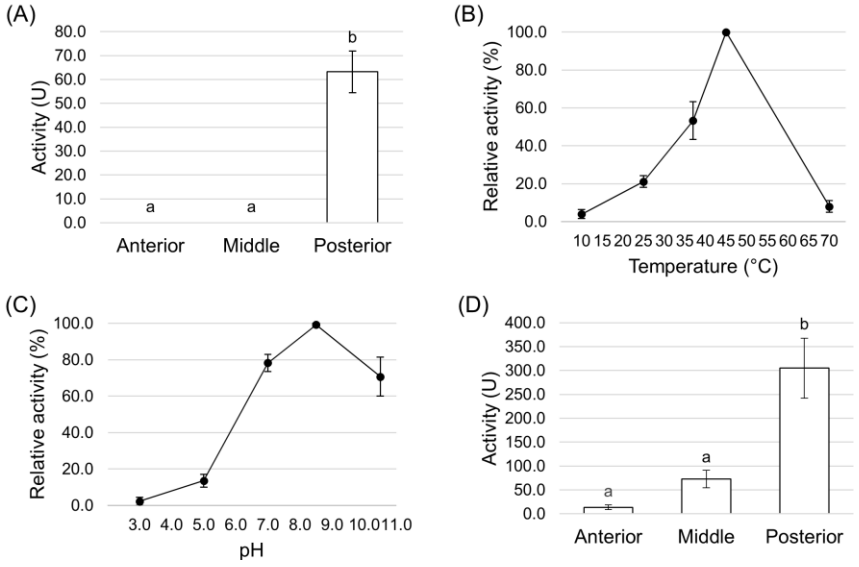


Figure 10. Trypsin and chymotrypsin activity in the different midgut regions. (A): trypsin activity in midgut juice extracted from anterior, middle and posterior midgut. For each tract the enzymatic assay was performed at pH 8.5. The values are reported as mean ± SEM of 3 experiments. Different letters denote significant differences (ANOVA test followed by Tukey's test. ANOVA *p*-value<0.001, Tukey's test *p*-values: Middle *vs* Anterior *p*=1.000, Posterior *vs* Anterior *p*<0.001, Posterior *vs* Middle *p*<0.001). (B): dependence of trypsin activity on temperature in midgut juice from the posterior region of the midgut performed at pH 8.5. Relative activity values (%) are reported as mean ± SEM of at least 3 experiments and are expressed as percentage of the highest activity over the temperature range examined. (C): dependence of trypsin activity on pH in midgut juice from the posterior region of the midgut performed at 45 °C. Relative activity values (%) are reported as mean ± SEM of at least 3 experiments and are expressed as percentage of the highest activity over the pH range examined. (D): chymotrypsin activity in midgut juice extracted from anterior, middle and posterior midgut. For each tract the enzymatic assay was performed at pH 8.5. The values are reported as mean ± SEM of at least 3 experiments. Different letters denote significant differences (ANOVA test followed by Tukey's test. ANOVA *p*-value<0.05, Tukey's test *p*-values: Middle *vs* Anterior *p*=0.845, Posterior *vs* Anterior *p*<0.05, Posterior *vs* Middle *p*=0.061).

To define whether the posterior midgut was also responsible for the final phase of protein digestion in which single amino acids are produced, we measured the activity of aminopeptidase N (APN), one of the abundant exopeptidase families present in the midgut brush border of insect larvae (Terra and Ferreira 1994). APN activity was recorded only in tissue homogenates prepared from the posterior midgut (Figure 11).

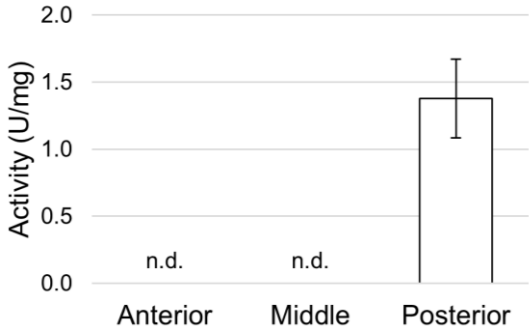


Figure 11. **Aminopeptidase N activity in the different midgut regions using L-leucine p-nitroanilide as substrate.** The values are reported as mean ± SEM of at least 3 experiments. In the anterior and middle midgut no activity was detectable (n.d.).

α-amylase activity in midgut juice samples from anterior, middle and posterior regions of the midgut was also determined. The highest activity was recorded in the anterior midgut, although a significant digestion of starch occurred also in the posterior region; conversely, no activity was present in the middle midgut (Figure 12A). Since in *H. illucens* larvae the pH of the lumen in the three midgut region was rather different (Table 2), to assess how this chemical parameter influenced α-amylase activity, a detailed evaluation of pH dependence in the anterior midgut was performed. A relative activity higher than 80% was recorded at pH values ranging from 5.0 to 8.0, with the highest substrate hydrolysis between pH 6.0 and 7.0. At pH values lower than 5.0 and higher than 8.0 α-amylase activity significantly decreased and dropped to zero at pH 3.0 and 10.0 (Figure 12B). Therefore, the luminal pH value of the anterior and posterior midgut (Table 2), the regions where hydrolysis of internal α-1,4-glycosidic bonds of polysaccharides (i.e., starch and glycogen) occurs (Figure 12A), fits with the optimum pH range of α-amylase activity (Figure 12B).

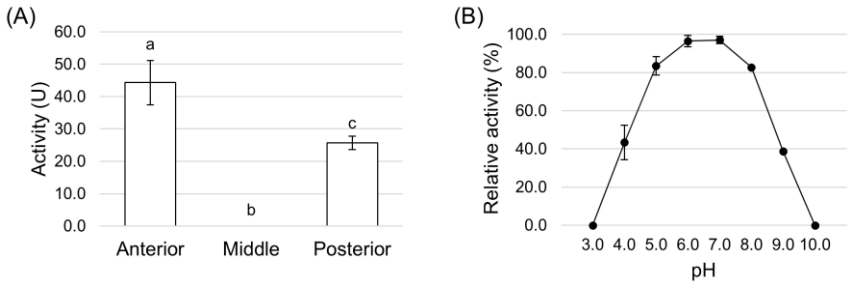


Figure 12. α-amylase activity in the different midgut regions. (A): α-amylases activity in midgut juice extracted from anterior, middle and posterior midgut. The values are reported as mean ± SEM of at least 3 experiments. Different letters denote significant differences (ANOVA test followed by Tukey's test. ANOVA *p*-value<0.001, Tukey's test *p*-values: Middle *vs* Anterior *p*<0.001, Posterior *vs* Anterior *p*<0.05, Posterior *vs* Middle *p*<0.05). (B): dependence of α-amylases activity on pH in midgut juice from the anterior region of the midgut. Relative activity values (%) are reported as mean ± SEM of at least 3 experiments and are expressed as percentage of the highest activity over the pH range examined.

Finally, since the midgut can be involved in the enzymatic clearance of ingested microorganisms (Lemos and Terra, 1991a; Lemos et al., 1993; Terra and Ferreira, 1994; Padilha et al., 2009), we measured the activity of lysozyme, which catalyzes the hydrolysis of 1,4-β-glyosidic bonds between N-acetylmuramic acid and N-acetyl-D-glucosamine residues in the peptidoglycan of the cell wall of many bacteria, thus compromising the integrity of the structure and causing the lysis of the cell. Lysozyme activity was measured in midgut juice samples extracted from anterior, middle, and posterior region of the midgut. The highest activity was present in the middle midgut, whereas the other two regions showed significantly lower activities, especially the posterior (Figure 13).

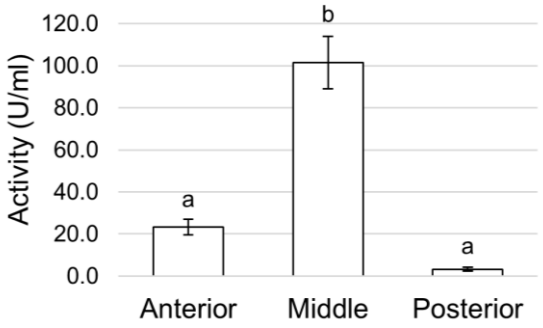


Figure 13. **Lysozyme activity assayed in the different midgut regions measuring the rate of lysis of *Micrococcus lysodeikticus*.** The values are reported as mean ± SEM of at least 3 experiments. Different letters denote significant differences (ANOVA test followed by Tukey's test. ANOVA *p*-value<0.001, Tukey's test *p*-values: Middle *vs* Anterior *p*<0.001, Posterior *vs* Anterior *p*=0.394, Posterior *vs* Middle *p*<0.001).

DISCUSSION

The great interest towards *H. illucens* larvae for their ability to bioconvert low quality substrates, such as organic waste materials, into valuable biomass (Barragan-Fonseca et al., 2017; Müller et al., 2017; Wang and Shelomi, 2017) is not supported by a knowledge of the physiology of this insect, in particular of the functional properties of the midgut, which is involved in nutrient digestion and absorption. This lack of information can negatively affect the exploitation of the bioconversion ability of *H. illucens* since the dietary plasticity of the larvae and the efficiency of bioconversion strictly depend on the physiological properties of this organ. In this work we fill this gap of knowledge performing an in depth morphofunctional characterization of the larval midgut.

Our results indicate that the midgut of *H. illucens* larvae shows a marked regionalization and each region possesses peculiar morphological and functional features (Figure 14).

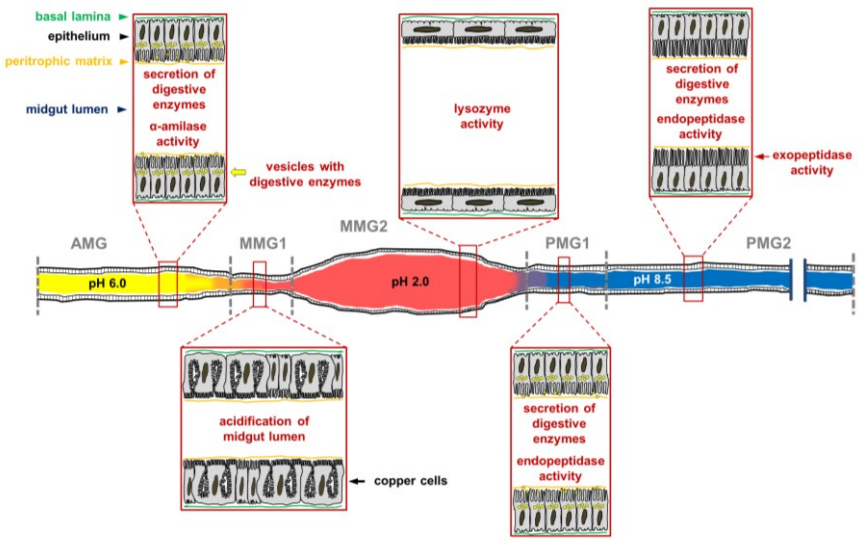


Figure 14. Schematic representation of *H. illucens* larval midgut in which the main morphofunctional features of each region of the organ are reported. AMG: anterior midgut, MMG1: first tract of the middle midgut, MMG2: second tract of the middle midgut, PMG1: first tract of the posterior midgut, PMG2: second tract of the posterior midgut.

A key parameter that confirms midgut regionalization is the pH of the lumen. In fact, the three main regions of the midgut present very different pH values, being acidic, strongly acidic and alkaline in the anterior, middle and posterior midgut, respectively. Although the pH values of the two first regions are similar to those measured in other Brachycera larvae (Terra et al., 1988; Shanbhag and Tripathi, 2009), the alkaline pH in the posterior midgut is not a common feature: in fact, although in *D. melanogaster* the pH of the lumen in this region is higher than 10 (Shanbhag and Tripathi, 2009), in *M. domestica* a slightly acidic pH (pH 6.8) was measured (Terra et al., 1988). It has to be highlighted that luminal pH plays a crucial role in the functional properties of the midgut since it influences digestive enzyme activity, solubility of nutrients, neutralization of toxic

ingested compounds, and gut microbiota (Appel, 1994; Nation, 2008), and therefore has a great impact on midgut physiology.

Our data show that the epithelium of the anterior midgut of *H. illucens* larvae is characterized by the presence of columnar cells that possess structural and ultrastructural properties typical of secretory cells and are likely responsible for the production and secretion of amylases and lysozyme, whose activity has been recorded in the lumen of this region. Therefore, ingested polysaccharides begin to be degraded in the anterior midgut by soluble amylases that show the highest activity in this tract. In other brachycerous larvae, such as *M. domestica*, amylase expression (Pimentel et al., 2018) and activity (Espinosa-Fuentes and Terra, 1987) have been recorded especially in the posterior midgut, as well as the majority of digestive enzymes (Espinosa-Fuentes and Terra, 1987). It is difficult to explain the different localization of carbohydrate digestion in the two species on the base of their alimentary habits because both insect larvae are saprophagous and feed on very similar substrates, thus a possible explanation may reside in the phylogenetic distance between the two species (Wiegmann et al., 2011).

The first part of the middle midgut (MMG1) is characterized by the presence of copper cells. ATP produced by the elongated mitochondria inside microvilli is readily available to be used by H^+ V-ATPase localized in the apical membrane of these cells. By Western blot analysis multiple bands that correspond to the different subunits of the V1 complex were revealed. In particular, the 67- and 56-kDa bands correspond to the A and the B subunit, respectively, that are responsible for the binding of ATP (Novak et al., 1992; Wieczorek et al., 1999). The remaining bands correspond to other V1 subunits, as E (27-kDa) (Gräf et al., 1994a), F (14-kDa), G (13-kDa)

(Gräf et al., 1994b; Gräf et al., 1996; Lepier et al., 1996), H (55-kDa), and D (32-kDa) (Merzendorfer et al., 2000). Our data and evidence reported in the literature for other brachycerous larvae (Terra and Ferreira, 1994; Dubreuil et al., 1998; Dubreuil, 2004) indicate that copper cells, thanks to their ability to secrete H^+, are responsible for the acidification of middle midgut lumen. In fact, as demonstrated in *D. melanogaster* larvae (McNulty et al., 2001), when this ability is impaired by copper ingestion and the metal selectively accumulates in these cells, the pH value in the middle midgut increases. As previously suggested, the orange fluorescence signal is probably due to the formation of a complex between copper ions and a protein belonging to the metallothionein family (McNulty et al., 2001). It has been demonstrated that two metallothionein genes are constitutively expressed in the middle midgut of *D. melanogaster* larvae and adult, whereas are inducible in other regions of the gut (Durliat et al., 1995). A similar pattern can also occur in *H. illucens* larvae, since the fluorescence signal appears more rapidly in copper cells (starting from 14 h of feeding on copper-containing diet) than in cells of other midgut regions, where the signal is visible only after 48 h. The physiological meaning and the relationship between florescence due the formation of the complex copper-metallothionein and acid secretion are not clear. Different hypotheses have been made (McNulty et al., 2001), but we can exclude that the reduction of middle midgut acidification by copper is due to nonspecific and detrimental effect of the metal on midgut cells, since the anterior and the posterior regions maintained their ability to regulate their own lumen pH. Another key factor essential for the acidification of the lumen of the middle midgut may be carbonic anhydrase, since this enzyme generates H^+ ions that are transported into the lumen (Shanbhag and Tripathi, 2008; 2009).

The epithelium present in the second part of the middle midgut (MMG2) shows a peculiar morphology since it is formed by large, flat cells. This region does not appear to be involved in digestive processes. Neither carbohydrate nor significant protein digestion takes place in this region, but only lysozyme activity has been recorded. The high activity of this enzyme in the middle midgut, together with the strong acidic luminal pH, suggests an important role of this region in killing pathogens ingested with the feeding substrate, as proposed for other brachycerous larvae (Lemos and Terra, 1991a; Lemos et al., 1993; Padilha et al., 2009). Recently, lysozyme and extreme pH values in the middle midgut of *H. illucens* larvae were indicated among the possible agents responsible for shaping the microbiota composition of the midgut and the microbial density along the different regions of this organ (Bruno et al., submitted).

Our data indicate that the posterior midgut plays a fundamental role in protein digestion. In this region, endo- and exo-peptidases accomplish the hydrolysis of peptide bonds that leads to the production of free amino acids. Columnar cells are the main cell type present in the posterior region, but they show different morphological features in the first and in the second part of this district that confer different functional properties to these two tracts. Columnar cells present in the first part of the posterior midgut (PMG1) are likely characterized by secretory activity. This evidence is supported by qRT-PCR data that demonstrate a primary involvement of PMG1 in the production of serine proteases, i.e., trypsin and chymotrypsin. These two enzymes are responsible for the initial phase of protein digestion that occurs in the posterior midgut. Moreover, since the assay conditions for the measurement of trypsin and chymotrypsin activity are the same and both specific

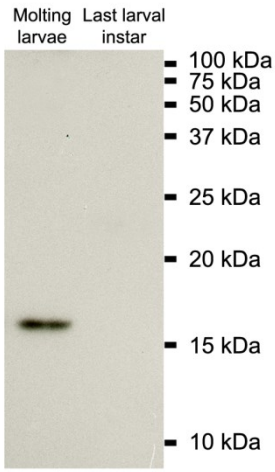
chromogenic substrates release p-nitroaniline after the hydrolysis, it is possible to compare the relative activity of the two enzymes. Considering their activity in the posterior region of the midgut, chymotrypsin-like enzymes appear to be the major serine proteases responsible for the initial phase of protein digestion. In the second part of the posterior midgut (PMG2), columnar cells have microvilli that are longer than in other regions while morphological traits ascribable to a secretory activity are less evident, supporting a main role of this tract in nutrient absorption. Moreover, APN activity is recorded only in the posterior region, confirming that this district plays a fundamental role in protein digestion from the initial to the final phases of the process, the latter producing single amino acids that, in turn, can be absorbed. *H. illucens* larvae are able to grow on a great variety of organic matter (Nguyen et al., 2015; Barragan-Fonseca et al., 2017; Wang and Shelomi, 2017), including vegetal materials (Jucker et al., 2017), a substrate rich in tannins and other secondary metabolites which bind to proteins at low pH values affecting the efficiency of digestion (Felton et al., 1989; Felton et al., 1991; Apple, 1994). Considering this evidence, the digestion of protein in the posterior midgut, a region with an alkaline pH of lumen, could ensure the best exploitation of these important nutrients. Moreover, the involvement of endopeptidases belonging to serine proteases, which have an optimum pH value in the alkaline range, fits with the pH values recorded in this region of the midgut. Apparently, endopeptidases able to work at acidic or very acidic pH, such as cysteine and aspartic proteases, whose involvement in protein digestion has been demonstrated in other insects including brachycerous larvae (Espinosa-Fuentes and Terra, 1987; Lemos and Terra, 1991a; Lemos and Terra, 1991b; Terra and Ferreira, 1994; Padilha et al., 2009), play a marginal role in *H. illucens* larvae, since

the total proteolytic activity measured in the anterior and middle regions of the midgut is negligible compared to posterior midgut. In this region serine proteases are active and therefore represent the major enzymes responsible for the initial phase of protein digestion. We have also unraveled the influence of temperature on proteolytic activity in the midgut of BSF larvae. In accordance with previous findings (Kim et al., 2011a) we observed an optimum temperature around 45 °C. This result is not surprising if we consider that BSF larvae exhibit a very peculiar tendency to aggregate into the feeding substrate forming larval clusters in which the temperature increases due to the larval overcrowding and the heat generated by their movement (Parra Paz et al., 2015). In our rearing conditions (environmental temperature of 27.0 ± 0.5 °C), a temperature higher than 40 °C was recorded within the clusters, therefore proteolytic enzymes can work at their optimum temperature. This feature can strongly contribute to the efficiency of BSF larvae in the bioconversion of feeding substrates.

In conclusion, our work represents the first comprehensive description of the morphofunctional features of *H. illucens* larval midgut. It not only sheds light on the unexpected complexity of this organ, but also represents a useful platform of knowledge for the best exploitation of this insect in bioconversion processes.

SUPPLEMENTARY MATERIALS

Figure S1. Western blot analysis: full lane of phospho-Histone 3.



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CHAPTER 2

The intestinal microbiota of *Hermetia illucens* larvae is affected by diet and shows a diverse composition in the different midgut regions

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ABSTRACT

The larva of the black soldier fly (*Hermetia illucens*) has emerged as an efficient system for the bioconversion of organic waste. Although many research efforts are devoted to the optimization of rearing conditions to increase the yield of the bioconversion process, microbiological aspects related to this insect are still neglected. Here we describe the microbiota of the midgut of *H. illucens* larvae showing the effect of different diets and midgut regions in shaping microbial load and diversity. The bacterial communities residing in the three parts of the midgut, characterized by remarkable changes in luminal pH values, differed in terms of bacterial numbers and microbiota composition. The microbiota of the anterior part of the midgut showed the highest diversity that gradually decreased along the midgut, whereas bacterial load had an opposite trend, being maximal in the posterior region. The results also showed that the influence of the microbial content of ingested food was limited to the anterior part of the midgut and that the feeding activity of *H. illucens* larvae did not affect significantly the microbiota of the substrate. Moreover, a high protein content compared to other macronutrients in the feeding substrate seems to favor midgut dysbiosis. The overall data indicate the importance of taking into account the presence of different midgut structural and functional domains, as well as substrate microbiota, in any further study that aims at clarifying microbiological aspects concerning *H. illucens* larval midgut.

IMPORTANCE

The demand for food of animal origin is expected to increase by 2050. Since traditional protein sources for monogastric diets are

failing to meet the increasing demand for additional feed production, there is an urgent need to find alternative protein sources. The larvae of *Hermetia illucens* emerge as efficient converters of low quality biomass into nutritionally valuable proteins. Many studies have been performed to optimize *H. illucens* mass rearing on a number of organic substrates and to maximize quantitatively and qualitatively the biomass yield. On the contrary, although insect microbiota can be fundamental for bioconversion processes and its characterization is mandatory also for safety aspects, this topic is largely overlooked. Here we provide an in depth study of the microbiota of *H. illucens* larval midgut taking into account pivotal aspects such as the midgut spatial and functional regionalization as well as microbiota and nutrient composition of the feeding substrate.

INTRODUCTION

The black soldier fly (BSF), *Hermetia illucens* (Diptera: Stratiomyidae), is a true fly that occurs worldwide in tropical and temperate regions. Adults of this insect have never attracted interest because they do not approach humans, do not bite and are not known to vector pathogens. On the contrary, BSF larvae have been object of intense research efforts because of their remarkable utility for humans that take advantage of their feeding regime of generalist detritivore (1). In particular, BSF larvae are largely used in forensic entomology to estimate human postmortem interval (2, 3) but the major promising potential of the voracious BSF larvae is their use as efficient bioconverters (4-6). Indeed, BSF larvae can be reared in mass cultures on a very wide variety of organic waste (e.g. crop and food processing residues, food waste, manure and feces)

leading to the conversion of low quality material into valuable biomass. The latter is exploitable for the isolation of bioactive compounds (e.g. antimicrobial peptides, chitosan and degrading enzymes), biodiesel production, and as feed or feed ingredients (mainly for their content of high-quality proteins and lipids) for poultry, aquaculture, and livestock (1, 6). The production of BSF larvae is technically simple, cost-effective and environmentally sustainable (1, 6). However, the rate of waste recycling and the final value of the biomass obtained depend on the rearing strategy, in terms of feeding substrate composition, feed consumption rate, and environmental parameters (i.e. temperature, humidity and photoperiod) (1). For this reason, many research efforts now focus on the characterization of nutrient and micronutrient content of BSF larvae in response to different rearing conditions and substrates, in order to optimize biomass yield and quality (1, 6-10).

Safety aspects concerning the microbiological load of intermediate and final products of bioconversion processes are also crucial, especially when BSF is exploited for feed applications. In principle, this issue can be approached by classical food microbiology methods to establish whether a product meets the recommendations imposed by current hygiene criteria. On the other hand, an in-depth characterization of BSF larvae microbiota and the factors that influence its composition is particularly important. Microbiota composition is known to impact insect health and performance, and has to be considered in the effort to optimize biomass yield (11). In addition, the analysis of the microbiota could allow the identification of bacterial species with peculiar and unique characteristics, such as the capacity to degrade complex substrates, as cellulose, hemicellulose and lignin (12), or xenobiotics. These microorganisms, or even the enzymes responsible for the

degradation, could be isolated and exploited at industrial level for waste recycling and bioremediation. Populations of gut bacteria able to compete with pathogens or to act as probiotics could be boosted for the improvement of BSF larvae performances and bioconversion efficiency or may be used in other animal hosts with similar purposes. Moreover, the study of BSF microbiota has a strong potential in contributing to the global problem of the identification of new antimicrobials. Indeed, BSF larvae feeding activity is able to reduce the bacterial load of substrates and, importantly, this capacity is not accompanied by the accumulation of pathogens in their gut (13-16). Such evidence implies the presence of potent antimicrobial effectors produced by BSF larvae and their intestinal microbiota. It should be pointed out that the latter is implicated in turn in the maintenance of gut homeostasis and supports gut immune functions (11, 17, 18).

A few studies on microbiota of BSF larvae have already been performed (19, 20). Rearing substrate and insect development stage have a significant impact on the overall composition of the microbial community (19, 20). A very critical issue that these preliminary microbiological surveys have not taken into account is the high complexity of the gut of fly larvae. In fact, this organ, and in particular the midgut, shows peculiar regional structural and functional features associated with changes of luminal pH (21-23). The differences in gut morphology and epithelial architecture along different intestinal tracts of some insects are in fact accompanied by remarkable differences in physiological, metabolic and immune features that impact on microbiota composition (24-26). These complex relationships have been exhaustively described in the model insect, the fly *Drosophila melanogaster* (Diptera: Drosophilidae) (17, 27-30).

In all insect, the digestive tract is divided into three regions with different embryonic origin and peculiar morphological and functional features: a short initial tract, the foregut, a long midgut where digestion and absorption occur and a final hindgut where water, salts, and other molecules are absorbed prior to elimination of the feces. Even though a detailed morphofunctional description of *H. illucens* larval midgut is lacking, it is expected that, as in other non-hematophagous brachycerous Diptera, discrete regions with peculiar pH values can be recognized along the midgut and that each distinct midgut region possesses its own features, at both structural and functional levels, and a peculiar resident microbiota (17, 24-30). In the present work we analyzed the effects of different diets and their microbial community on the midgut microbiota of BSF larvae, and the impact of the insect feeding activity on the diet microbiota. Most importantly, we analyzed the different tracts of BSF larval midgut separately, and highlighted the need of having future research on BSF larval midgut considering each midgut domain independently.

MATERIAL AND METHODS

Insect rearing

BSF eggs were collected from a colony established in 2015 at the University of Insubria (Varese, Italy), and maintained in a humid chamber at 27°C until hatching. The eggs were laid on a Petri dish (9×1.5 cm) with the experimental diet. Three diets were used in the current study: standard diet for Diptera (Standard), a diet containing fruits and vegetables (Veg Mix), and a diet based on fish feed (Fish). Standard diet (31), was composed by wheat bran (50%), corn meal (30%) and alfalfa meal (20%) mixed in the ratio 1:1 dry matter/water

(approximately 13% protein, protein/carbohydrate ratio 1:1). Veg Mix diet was composed by seven fruits and vegetables (apple, banana, pear, broccoli, zucchini, potato and carrot) in equal quantity and appropriately minced (approximately 1% protein, protein/carbohydrate ratio 1:9). Fish diet was composed by fish meal (FF type, Mazzoleni SpA, Bergamo, Italy), mixed in the ratio 1:1 dry matter/water (approximately 35% protein, no carbohydrates). Percentages are calculated on diet weight, including water. The values in parenthesis concerning protein and carbohydrate content were estimated on data available on the web for Standard and Veg Mix diet, whereas for Fish diet they were reported in the product data sheet. Nipagin (Methyl 4-hydroxybenzoate) was added to the diet administered to larvae the first 4 days after hatching to avoid mold growth (a 18% (w/v) stock solution in absolute ethanol was prepared; each gram of Veg Mix diet was added with 20 µl of this stock solution, whereas each gram of Standard and Fish diet was added with 1 ml of a 1.7% (v/v) dilution in water of the stock solution). Four days after hatching, 300 larvae were placed in a plastic container (16×16×9 cm), and fed *ad libitum* with the three experimental diets described above without nipagin. The larvae were maintained at 27.0 ± 0.5 °C, 70 ± 5% relative umidity, in the dark. Fresh diet was added every two days, until larvae reached the last larval instar. Five independent rearing groups were set up for each diet. Random samples of 30 individuals were weighed every two days. For each experimental diet, the sampling and the annotation of the larval weight were made in triplicate. Before weighing, the larvae were washed in tap water to remove diet matter from their body and then wipe dried. The weights were recorded until 25% of insects reached the pupal stage. Last instar,

actively feeding larvae were used for the measurement of midgut lumen pH and microbiota analyses.

Determination of pH in the midgut lumen with colorimetric indicators

The presence of different pH in the midgut lumen of *H. illucens* larvae was assessed using phenol red and bromophenol blue, two pH indicators that assume different coloration at different pH values. Bromophenol blue is yellow at pH values lower than or equal to 3.0, blue at pH higher than or equal to 4.6; phenol red is yellow at pH lower than or equal to 6.8, fuchsia at pH higher than or equal to 8.2, with a gradual color transition for intermediate values. *H. illucens* larvae were fed *ad libitum* with Standard diet until they reached the last instar as described above. Larvae with a weight ranging between 180 and 200 mg were selected and transferred to plastic containers on Standard diet added with 0.2% (w/w) bromophenol blue or phenol red. After 24 h the larvae were removed from the diet, placed in a plastic tube, and anesthetized on ice with CO_2. The guts were isolated and the coloration of the midgut content was evaluated by means of a stereomicroscope.

Collection of midgut and diet samples and RNA extraction

Last instar larvae were washed with 70% ethanol in autoclaved distilled water and then dissected with the help of a stereomicroscope, under a horizontal-flow hood, by using sterile tweezers and scissors, to avoid cross-contaminations of the samples. Each midgut was isolated in autoclaved 1× PBS (Phosphate Buffered Saline: 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.4) in a sterile Petri dish (5.5×1.3 cm). Once collected, the midgut was divided into three districts: anterior, middle, and posterior region (see Results and Fig.1). For the

dissection of each larva a new Petri dish was used, and tweezers and scissors were washed with 70% ethanol in water. For each diet, pools of five midgut regions samples for each of the five replicates of insect rearing were collected in a cryovial, immediately put into TRIzol reagent (Life Technologies, Carlsbad, CA, USA), and kept at -80°C until total RNA extraction that was performed according to the manufacturer's instructions. Briefly, after homogenization with eppendorf-fitting pestles to lyse samples in TRIzol reagent, total RNA was precipitated with isopropanol, washed with ethanol, and suspended in RNase-free water. Samples of fresh (before administration to larvae) and conditioned diets (on which larvae have fed) were also immediately put into TRIzol reagent and kept at -80°C until total RNA extraction. Ten samples of both fresh and conditioned diets were collected for each of the 5 experimental replicates on the 3 different feeding substrates.

RNA concentration was assessed by measuring the absorbance at 280 nm, with a Varioskan™ Flash Multimode Reader (Thermo Scientific, Waltham, MA, USA), and sample purity was evaluated by assessing 260/280 nm absorbance ratio. Total RNA preparations were then treated with TURBO DNase I (Life Technologies), according to the manufacturer's instructions and RNA quality was checked by electrophoresis on 1% agarose gel.

qRT-PCR for relative bacterial load determination

Total RNA was isolated as described above. The relative bacterial load in the three midgut regions (n=5 for each sampling point containing pools of 5 midgut portions each), was quantified by normalization of the relative expression of the *16S rRNA* gene (SRP accession number SRP064613; *16S rRNA* forward primer: ACTCCTACGGGAGGCAGC, *16S rRNA* reverse primer:

ATTACCGCGGCTGCTGGC) to that of the ribosomal protein L5 gene of *H. illucens* (*Hi RPL5*). The primers used for *Hi RPL5* (*Hi RPL5* forward primer: AGTCAGTCTTTCCCTCACGA, *Hi RPL5* reverse primer: GCGTCAACTCGGATGCTA) were designed on conserved regions of *RPL5* in other insect species and their sequence checked by sequencing the PCR product. Changes in relative bacterial loads were measured by one-step qRT-PCR (58-60), using the SYBR Green PCR Kit (Applied Biosystems, Carlsbad, CA, USA), according to the manufacturer's instructions, using the primers reported above. Relative gene expression data were analyzed using the $2^{-\Delta\Delta CT}$ method (61-63). Expression data were normalized taking into account the differences in the area of the cross-section of the different intestinal tracts (81,000 ± 7,300 μm^2, 250,000 ± 17,200 μm^2 and 46,000 ± 1,700 μm^2 for the anterior, middle and posterior midgut, respectively, n=10 for each tract) by dividing the C_T values (for both *16S rRNA* and *Hi RPL5* transcripts) by the area of the cross-section of the corresponding midgut tract. The areas were calculated using the diameter of the lumen of each midgut tract obtained by direct measurement on the micrographs of different cross-sections acquired from semithin cross- sections of BSF larval midguts stained with crystal violet and basic fuchsin, prepared for light microscopy analysis (64). For validation of the $\Delta\Delta C_T$ method the difference between the C_T value of *16S rRNA* and the C_T value of *Hi RPL5* transcripts [ΔC_T = C_T (16S rRNA)-C_T (*Hi RPL5*)] was plotted versus the log of two-fold serial dilutions (200, 100, 50, 25 and 12.5 ng) of the purified RNA samples. The plot of log total RNA input versus ΔC_T displayed a slope lower than 0.1 (y=1.3895 - 0.0137x, R^2=0.0566), indicating that the efficiencies of the two amplicons were approximately equal.

Analysis of the microbiota and bioinformatics of the *16S rRNA* gene sequencing data

After extraction, 400 ng of RNA were reverse-transcribed into cDNA with random primers using RETROscript (Life Technologies), according to the manufacturer's instructions. The midgut microbiota was assessed by sequencing of the amplified V3-V4 region of the *16S rRNA* gene as recently described (65). Demultiplexed, forward and reverse reads were joined by using FLASH (66). Joined reads were quality trimmed (Phred score < 20) and short reads (< 250 bp) were discarded by using Prinseq (67). High quality reads were then imported in QIIME1 (68). Operational Taxonomic Units (OTUs) were picked through *de novo* approach and uclust method and taxonomic assignment was obtained by using the RDP classifier and the Greengenes database (69), following a pipeline previously reported (65). To avoid biases due to different sequencing depth, OTU tables were rarefied to the lowest number of sequences per sample. Statistical analyses and visualization were carried out in R environment (https://www.r-project.org). Alpha-diversity analysis was carried out in QIIME on rarefied OTU tables. Kruskal-Wallis and pairwise Wilcoxon tests were used to determine significant differences in alpha diversity parameters, weighted Unifrac distance or in OTU abundance. Permutational Multivariate Analysis of Variance (non-parametric MANOVA) based on Bray Curtis distance matrix was carried out to detect significant differences in the overall microbial community composition among the different parts of the midgut or as affected by the type of diet, by using the *adonis* function in R *vegan* package.

Statistical analysis

Data were analyzed using Prism (GraphPad Software Inc. version 6.0b, San Diego, CA, USA) software using One-Way ANOVA with Tukey's multiple comparison test to compare bacterial load and parameters of larval performances within any single diet treatment. Two-Way ANOVA analysis followed by Bonferroni's post-hoc tests, when significant effects were observed (*P* value<0.05), was carried out on bacterial load as affected by different diet treatment and different midgut trait. When necessary transformation of data was carried out, to meet assumptions of normality. Levene's test was carried out to test the homogeneity of variance.

Accession number(s)

The 16S rRNA gene sequences produced in this study are available at the Sequence Read Archive (SRA) of the National Centre for Biotechnology Information (NCBI), under accession number SRP064613.

RESULTS

Determination of the pH values of the midgut lumen content

Since luminal pH is a good marker for midgut regionalization in flies (21-23), we evaluated how the pH of the lumen content of BSF larvae changed along the midgut in order to have a clear identification of the regions in which this organ could be subdivided. For this purpose, last instar *H. illucens* larvae were fed with diet containing two pH indicators, bromophenol blue and phenol red. The color of the luminal content of larvae fed with diet containing bromophenol blue was clearly visible through the isolated epithelium (Fig. 1A). The anterior region of the midgut presented a blue color, indicating that its luminal content has a pH ≥ 4.6. Then, a marked change was observed, since the middle region turned

yellow, revealing that its lumen has a pH ≤ 3. Moving towards the posterior midgut, the color gradually turned blue. Bromophenol blue turns at pH values between 3.0 and 4.6, thus differences in the pH values of the anterior and posterior midgut contents could not be evidenced. Figure 1B shows the gut isolated from a larva fed with diet containing phenol red, a dye that turns yellow at pH ≤ 6.8 and fuchsia at pH ≥ 8.2. Since the anterior and the middle regions of the midgut presented a golden yellow color, whereas the posterior midgut content appeared fuchsia, it is possible to state that the luminal content of the anterior and middle regions have an acidic pH and the posterior has an alkaline pH. The evidence obtained with phenol red supported and completed results obtained with bromophenol blue. In conclusion, the luminal content of the midgut of *H. illucens* larvae presents different pH values: the anterior region has an acid luminal content, the middle region presents a strongly acid pH (pH ≤ 3) and the posterior region has an alkaline luminal content. These three regions are separated by transition zones, in which the pH values gradually change (Fig. 1A and B). Taking into account this evidence, we could easily distinguish three main regions of the larval midgut of *H. illucens*, a fundamental aspect to isolate midgut samples for the analyses reported below (Fig. 1C).

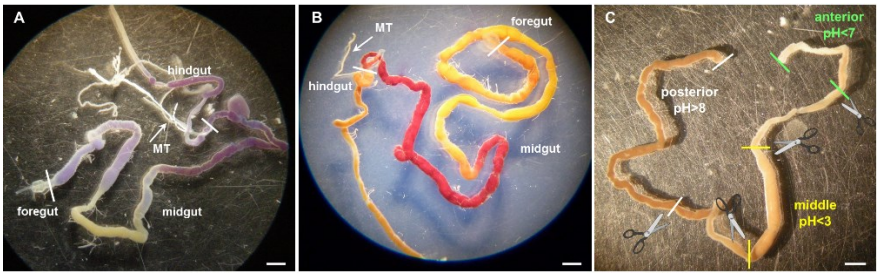

Figure 1. Determination of pH values in BSF larvae midgut lumen (A and B) and definition of the midgut portions for the microbiota analysis (C). In (A) and (B) the anatomy of the larval BSF gut is visible. The short foregut

is followed by a very long midgut. The beginning of the hindgut (which extends out of the field of view) is easily recognizable by the insertion of Malpighian tubules (MT), structures involved in excretion in insects and that deliver the primary urine into hindgut lumen. The whole guts isolated from *H. illucens* larvae fed with diet containing bromophenol blue (A) or phenol red (B) pH indicators show the presence of different pH values along the midgut lumen. (C) Image of the midgut, that is subdivided in a relatively short and thick anterior midgut, a middle midgut characterized by an enlarged highly acidic portion (stomach) and the posterior midgut. The bars of different color highlight the position of the cuts for the isolation of the portions used for microbiota analyses. Bars: 2 mm.

Insect performances on different diets

The microbiota analyses were performed on larvae reared on three different feeding substrates: Standard diet, an optimal diet for fly larvae rearing (31), Veg Mix diet, containing a mixture of fruits and vegetables, and Fish diet, based on fish meal (see Material and Methods for detailed composition). We thus evaluated the performances of the BSF larvae on these substrates. The maximum weight reached before pupation by BSF larvae reared on Standard diet was significantly higher than that of larvae reared on the other two two diets (Table 1) ($F_{(2,12)} = 15.50$, P=0.0005, df = 14; one-way ANOVA). There was also a trend in the increase of larval period duration ($F_{(2,12)} = 12.00$, P=0.0014, df = 14). This was particularly evident for the larvae reared on Fish diet, that showed doubled developmental time and almost halved maximum weight compared to larvae grown on Standard diet (Table 1).

Table 1. Length of BSF larval cycle and maximum weight at pupation for the different diets used in this study[a].

Diet	Larval period (days)[b]	Maximum wt (mg)[b]	Day of sample collection for microbiota analysis
Standard	18 ± 1 (5) A	218 ± 8 (5) A	16
Veg Mix	24 ± 2 (5) A	195 ± 5 (5) B	22
Fish	36 ± 3 (5) B	173 ± 3 (5) C	30

[a]Data are expressed as mean ± standard error, with number of experiments in parenthesis. For each experiment, at least 20 larvae were monitored for development time and weight.
[b]Different letters denote statistical differences (One-Way ANOVA).

Evaluation of relative bacterial counts in the different regions of BSF larval midgut

The bacterial loads in different midgut regions of *H. illucens* larvae (Fig.1C) were determined by quantitative reverse transcription PCR (qRT-PCR) on RNA samples in order to narrow in the analysis on live bacteria. The results demonstrate that the profile of the relative bacterial counts in the different midgut regions was similar for the three diets. In particular, while anterior and middle midgut had comparable bacterial loads, they were higher in the posterior portion (Fig. 2) (Standard $F_{(2,12)} = 8.869$, n=5, $P=0.0043$, df = 14; Veg Mix $F_{(2,12)} = 295.51$, n=5, $P<0.0001$, df = 14; Fish $F_{(2,12)} = 33.882$, n=5, $P<0.0001$, df = 14; one-way ANOVA). We observed a statistically significant interaction between the effects of diet and midgut region on bacterial load ($F_{(4,36)} = 17.601$, $P<0.0001$) which was significantly affected from both the considered independent variables (diet $F_{(2,36)} = 23.339$, $P<0.0001$; midgut region $F_{(2,36)} = 137.170$, $P<0.0001$).

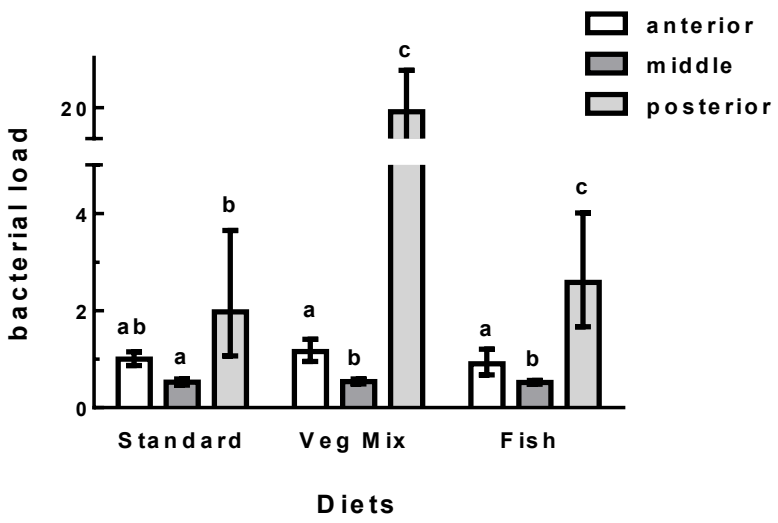

Figure 2. Relative quantification of bacterial load by qRT-PCR in the different tracts of the midgut of BSF larvae reared on different diets. The values reported are the mean ± standard error (n=5 for each sampling point containing pools of 5 midgut portions each) of the relative expression of the *16S rRNA* gene normalized to that of the *Hi RPL5* gene (see "qRT-PCR for relative bacterial load determination" in Materials and Methods). Different letters denote significant differences for each diet (One-Way ANOVA).

Microbiota composition in the different regions of BSF larval midgut and diet substrates

We analyzed the microbiota by *16S rRNA* gene sequencing starting from cDNA obtained from RNA samples in order to consider communities of live bacteria. A total of 2,175,325 high quality reads were analyzed, with an average of 29,000 reads/sample. Our study included also the analysis of the microbiota of the feeding substrates prior to BSF larvae administration (fresh diet) and after BSF larvae feeding (conditioned diet). This is particularly important because BSF larvae feed and develop inside the food substrate, which is not renewed but periodically added with fresh one. The

anterior part of the midgut was always characterized by a high microbial diversity (P<0.05), that progressively decreased going from the anterior to the posterior part (Fig. 3), and this trend happened regardless of the diet. The microbiota of the feeding substrate showed a strong impact in shaping the midgut microbiota in larvae fed with Standard or Fish diet, at least in the first regions of the midgut (Fig. 4); by contrast, the microbiota of Veg Mix diet was not found in the midgut (Fig. 4).

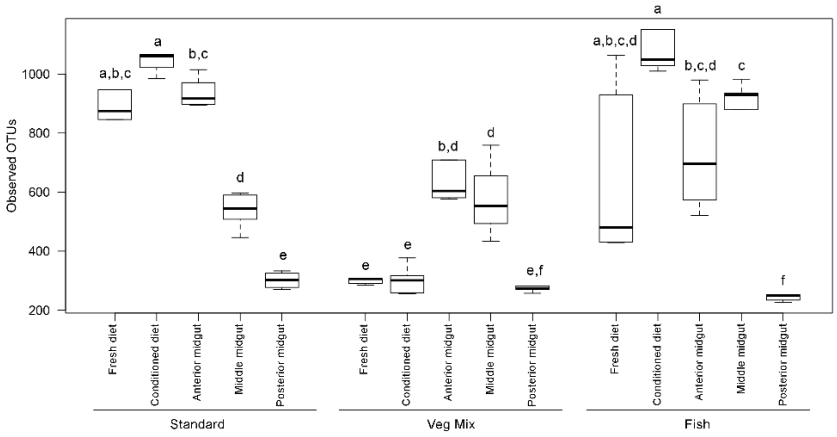

Figure 3. Microbial diversity. Box plot showing the number of Observed OTUs in the different samples, as detected by high-throughput sequencing of the *16S rRNA* gene. Boxes represent the interquartile range (IQR) between the first and third quartiles, and the line inside represents the median (second quartile). Whiskers denote the lowest and the highest values within 1.5×IQR from the first and third quartiles, respectively. Different letters indicate a significant difference (P<0.05) as obtained by pairwise Wilcoxon's tests. "Fresh diet" and "conditioned diet" refer to the analysis of the microbiota of the feeding substrates just after preparation and after larval feeding, respectively.

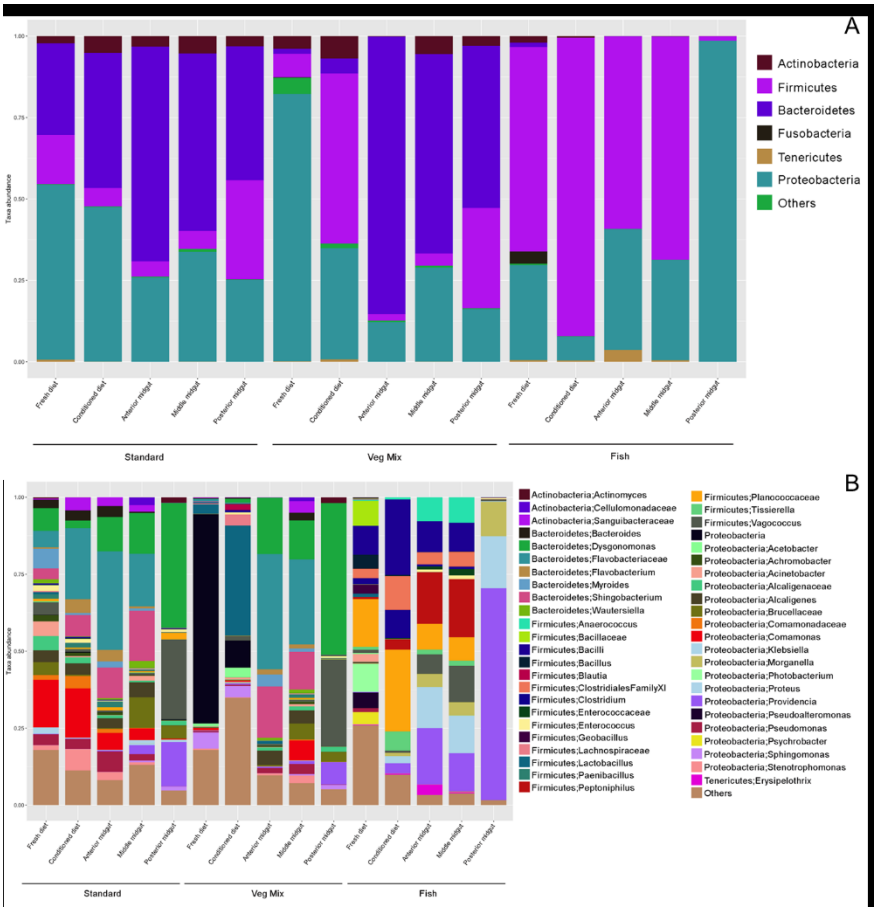

Figure 4. Incidence of the major bacterial taxonomic groups. The stacked bar chart shows the relative abundance of bacterial phyla (A) and genera (B) identified in midgut and diet samples analyzed. The order of the taxa in each bar is the same provided in the legend. Values are the average of 5 replicates. Genera and phyla with abundance < 2% in at least 5 samples are summed up and showed as "others".

The posterior part always showed a significantly different microbiota when compared with middle and anterior part of the midgut, as determined by MANOVA based on Bray Curtis distance (Standard $F_{(2,12)} = 24.945$, $P<0.001$; Veg Mix $F_{(2,12)} = 46.287$, $P<0.001$; Fish $F_{(2,12)} = 16.968$, $P<0.001$) and the composition of the microbiota in this region reflected a strong selection of the species that were

present in the food substrate, an aspect of particular extent for Fish diet (Fig. 4 and 5). The composition of the microbiota determined a clear differentiation of the samples according to both midgut portion and diet (Fig. 5). Indeed, a significant effect of both diet type and midgut region was found by MANOVA, for both the independent variables (diet $F_{(2,36)} = 57.047$, $P<0.001$; midgut region $F_{(2,36)} = 39.256$, $P<0.001$) and for the interaction between them ($F_{(4,36)} = 19.540$, $P<0.001$). Fish diet microbiota seemed to have the strongest effect on the gut microbiota, leading to a higher abundance of Proteobacteria taxa in the posterior tract of the midgut, while Firmicutes prevailed in the anterior and middle tract (Fig. 4A). On the contrary, the midgut of BSF larvae fed with Standard and Veg Mix diets were more similar and characterized by higher levels of Bacteroidetes (Fig. 4).

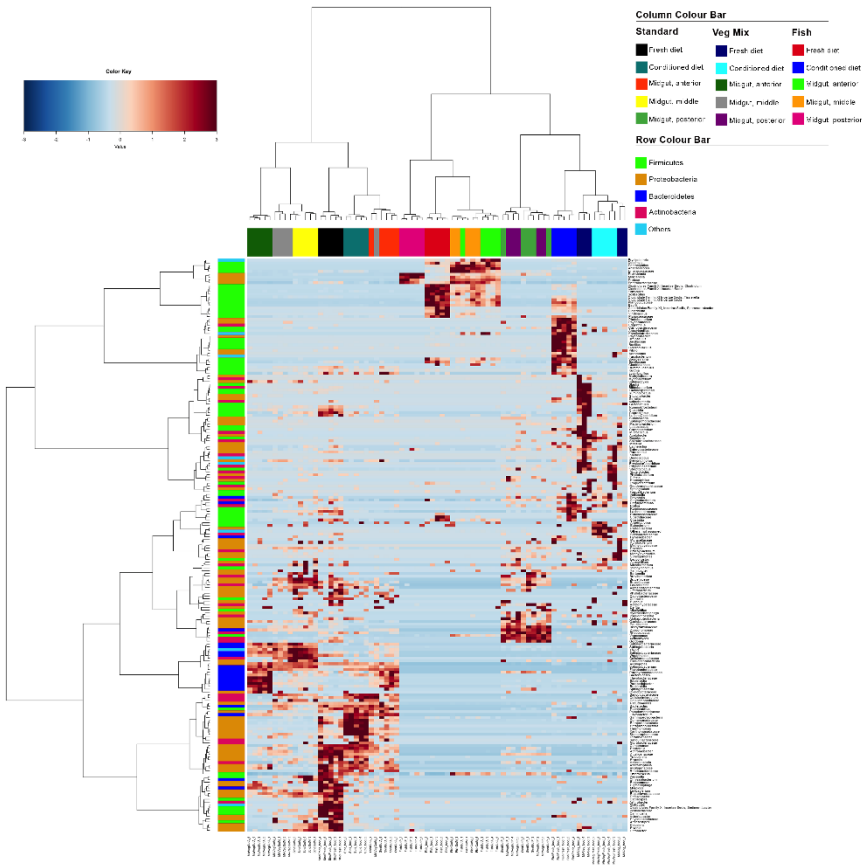

Figure 5. Heatplot based on microbiota composition at genus level. Hierarchical Ward-linkage clustering based on the Spearman's correlation coefficient of the microbial taxa abundance. Column bar is color-coded according to the type of diet and the midgut region. Row bar is colored according to the taxa assignment at phylum level. The color scale represents the scaled abundance of each variable, denoted as Z-score, with red indicating high abundance and blue indicating low abundance.

Indeed, the midgut of larvae fed with Fish diet showed significantly higher weighted Unifrac distance from Standard and Veg Mix diets, compared to the distance of between Standard and Veg Mix, in all the three portions (Fig. S1). Although the larvae feed and develop into the diet, the data show that BSF larvae do not significantly alter microbiota composition of the substrate, except for an increase in

Lactobacillus population in Veg Mix diet (Fig. 4B). A complete list of the taxa identified is reported in Supplementary Tables S1, S2, S3, S4.

DISCUSSION

Despite the great and exponentially increasing interest in BSF larvae for bioconversion (4-6) and bioremediation (32), several aspects concerning the biology of this insect are still neglected. Surprisingly, there is still paucity of information on its intestinal microbiota (11), an issue that should be instead considered a priority for an organism that can be used for such purposes. A recent review on the microbial community associated to BSF (11) highlights knowledge gaps and provides suggestions on aspects that are still to be unraveled, rather than presenting a summary of the available data.

Firstly, none of the few studies on BSF intestinal microbiota has taken into account the correlation between the different regions of the midgut of this insect and the microbiota. In this paper we provide evidence that discrete regions can be recognized along the midgut of BSF larvae as clearly demonstrated by the differences in the luminal pH (Fig. 1). Anterior region is characterized by an acid luminal content, followed by a strongly acidic middle region and an alkaline posterior tract. These data are partially in accordance with previous reports on non-hematophagous brachycerous Diptera. Indeed, in the larvae of *Musca domestica* (Diptera: Muscidae) three main segments can be identified: the anterior and the posterior midgut are characterized by a slightly acidic luminal pH, while the middle midgut presents a very low pH in the lumen (21) that is generated by the so called “copper cells”, a distinctive cell type present in the acidic segment of the midgut of flies (23, 33-35). The

midgut of *D. melanogaster* larvae presents distinct regions as well (23, 35) with different pH of the luminal content: the anterior segment and the anterior part of the posterior segment is between neutral to mild alkalinity, while the middle segment is highly acidic and the posterior part of the posterior segment is highly alkaline (23). The differences of the pH in fly midgut regions are associated to peculiar physiological, immune and microbiological features (22, 26-28, 30).

Here we demonstrate that in BSF larvae the presence of different midgut regions associates to differences in microbial density and composition. We have observed that each tract is characterized by a different bacterial load, which is higher in the posterior compared to the anterior midgut. Interestingly, microbial diversity has an opposite trend, since it gradually decreases along the midgut, suggesting that a selection of fewer taxa takes place. A simple explanation may be a reduced flow rate of luminal content to the posterior region due to the possible presence of sphincters or epithelium folding. In alternative or in addition, most bacteria are killed in the anterior and middle region and only a selection of the initial microbiota proliferate in the posterior midgut using the available nutrients, thus leading to higher numbers. This process of selection may result by the balanced combination of extreme pH values in the middle region of the midgut and the activity of antimicrobial peptides, lysozyme and digestive enzymes produced and secreted by midgut cells into the lumen of anterior and middle midgut (17, 21, 27, 36, 37).

To understand whether and how food affects the microbial communities that colonize the digestive tract of BSF larvae, we have examined dietary substrates that strongly differ in terms of nutrient composition. In particular, the three diets were characterized by a

different protein/carbohydrate ratio, a parameter that has been demonstrated to impact on the gut microbiota (38-40) and insect performances (41-43). Indeed, we detected differences in BSF larvae development on the different diets. A major novelty introduced by our study is the characterization of the microbiota of the dietary substrates, an aspect that was previously overlooked (11) and that could strongly affect the composition of the bacterial community of the midgut. In addition, we studied the influence of feeding activity of BSF larvae on dietary substrates. A comparative analysis of the results shows that diet composition plays a major role in shaping the diversity of the midgut microbiota. Similarly, the microbiota present in the diet influences the composition of the microbiota resident in the anterior/middle tracts of the midgut and less the one occurring in the posterior that presented a very narrow selection of the species in the food substrate. Interestingly, BSF larvae do not have detrimental effects on the microbiota of the substrates on which they feed and develop. They are not able to significantly change the bacterial community of the Standard and Fish diet substrates, and, although an increase of a specific population (i.e. *Lactobacillus*) occurs in Veg Mix substrate, these bacteria are known as non-pathogenic for their potential probiotic properties for humans (44-46) and some species are involved in detoxification of pesticides and xenobiotics in humans and insects (47-50). This evidence is in contrast with previous claims about the capacity of BSF larvae to change the microbiota of substrates and, in particular, to reduce pathogenic bacteria of substrates (1, 11), but is a valuable trait for an organism that has to be mass-reared for bioconversion and bioremediation on a variety of substrates.

The differences found in the microbiota of larvae fed on different diets could reflect their physiological performances and

bioconversion efficiency, and the posterior midgut, where the resident microbiota results from a selection of microbes present in previous midgut tracts, may have a relevant contribution in nutrient conversion and thus in energy harvest and overall fitness. Standard and Veg Mix diets were associated to an overall similar microbiota composition, both leading to increased levels of *Bacteroidetes* in the midgut, bacteria known as glycan degraders because of the presence of polysaccharide utilization loci in their genome (51). Genera of *Sphingobacterium* and *Dysgonomonas* were particularly abundant, likely reflecting a remarkable potential for complex polysaccharide degradation, and worthy to be isolated and explored for biotechnological purposes. *Bacteroidetes* have been identified as core members of the gut microbiome in many *Drosophila* species across the globe and also in other insects, including termites and honeybees (52), and several have xylanases directly involved in hemicellulose digestion (53, 54). On the other hand, Fish diet apparently induces a more putrefactive environment, with a microbiota severely dominated by *Proteobacteria* (Fig. 4A), mainly *Providencia* (Fig. 4B), which are highly transmitted vertically throughout insect life cycle (11) but can also be pathogens of many organisms including humans and insects (55). On the basis of the above consideration, Fish diet may induce a gut dysbiosis which may contribute to the reduced performance that we detected for BSF larvae reared on Fish diet compared to the other two feeding substrates. These data, along with a previous study performed on the same insect (7), suggest that unbalanced diets with a high protein/carbohydrate ratio content are not optimal for BSF larvae rearing.

Despite the great potential of *H. illucens* larvae (see Introduction for details), information on its microbiota is surprisingly very limited.

Apart from a recent study on mycobiota (56), only two studies have previously examined the microbiota of *H. illucens* larvae. In the first study (19, Table 2) the microbiota of the entire gut from larvae reared on three different feeding substrates were investigated. In the second one (20, Table 2) the microbiota analysis was performed on whole larvae. The differences in the experimental samples analyzed make it difficult to compare the results from those studies and, for the same reason, results from previous studies and the present. Moreover, both studies completely overlooked the bacteria communities present in the feeding substrates, that we demonstrated can affect midgut microbiota composition. Nevertheless, as summarized in Table 2, a limited comparison can be done. In Zheng et al. (20), larvae were reared on a diet with a composition very similar to Standard diet used in this study and the major Phyla that characterize the microbiota quite match (both considering each midgut tract separately or the average value of the different tracts).

Table 2. Short summary of the data on microbiota composition of *H. illucens* larvae from present work and published studies[a].

Authors and yr (reference) or source	Sample	Feeding substrate	Major phyla	%[b]
Jeol et al., 2011 (19)	larval gut	Food waste	*Bacteroidetes*	67.4
			Proteobacteria	18.9
			Firmicutes	9.4
			Fusobacteria	2.0
			Actinobacteria	1.9
		Cooked rice	*Proteobacteria*	54.0
			Firmicutes	47.3
			Unclassified	3.5

		Calf forage	*Proteobacteria* *Actinobacteria* *Firmicutes* *Bacteroidetes*	31.1 24.6 23.5 20.5
Zheng et al., 2013 (20)	whole larvae	Gainesville diet[c]	*Bacteroidetes* *Firmicutes* *Proteobacteria* *Actinobacteria*	54.4 20.0 16.0 9.0
Present study	larval midgut	Standard diet	*Bacteroidetes* *Proteobacteria* *Firmicutes* *Actinobacteria*	41.5 (A:65.9, M:54.4, P:41.1) 28.2 (A:25.9, M:33.7, P:25.2) 13.6 (A:4.7, M:5.6, P:30.4) 3.9 (A:3.2, M:5.3, P:3.1)
		Veg Mix	*Bacteroidetes* *Proteobacteria* *Firmicutes* *Actinobacteria*	65.4 (A:85.2, M:61.2, P:49.8) 19.1 (A:12.2, M:28.9, P:16.2) 15.7 (A:2.0, M:28.9, P:16.2) 11.6 (A:0.1, M:3.8, P:30.8)
		Fish diet	*Proteobacteria* *Firmicutes*	55.5 (A:37.1, M:30.8, P:98.6) 43.0 (A:59.1, M:68.6, P:1.4)

[a]In all the studies the microbiota composition was obtained by *16S rRNA* gene sequencing. Estimated on the basis of the histogram presented in references 19 and 20.

[b]Only percentages >1% are reported. For the present study, the % reported is the average of the percentages in the three different midgut portions (A: anterior, M: middle, P: posterior) that are specified in parenthesis.

[c]Gainesville diet is composed by 20% corn meal, 30% alfalfa meal, and 50% wheat bran, saturated with water.

This evidence, along with the differences associated to the microbiota of larvae reared on different substrates, suggests that diet composition had a role in shaping bacterial communities. In particular, when diets were very unbalanced (i.e. cooked rice and Fish diet) the diversity of microbial communities decreased compared to nutritionally more balanced diets. In those unbalanced diets *Proteobacteria* were the major group identified, whereas in all other cases *Bacteroidetes* was one of the dominant phyla (Table 2). Interestingly, our data (Table 2) demonstrate that the overall gut microbiota does not mirror the microbiota composition of each tract, confirming the relevance of working with each tract separately.

Our study focused on the effect of midgut morphofunctional regionalization in shaping the residing microbiota. Future work on microbiota in the hindgut of *H. illucens* larvae is also needed, although the establishment of a stable bacterial community in the hindgut of insect larvae is problematic (due to the molts during the larval period that involve the removal of the cuticle lining the hindgut epithelium) and often requires the presence of special structures that provides a stable environment for bacterial colonization (57), structures that have never been reported for *H. illucens* larvae.

In conclusion, the presence of different midgut domains, diet composition and diet microbiota have a non-negligible effect on BSF larvae microbial ecology. These factors and their interdependence are going to play a major role for a proper exploitation of the biotechnological uses of insects.

SUPPLEMENTARY MATERIAL

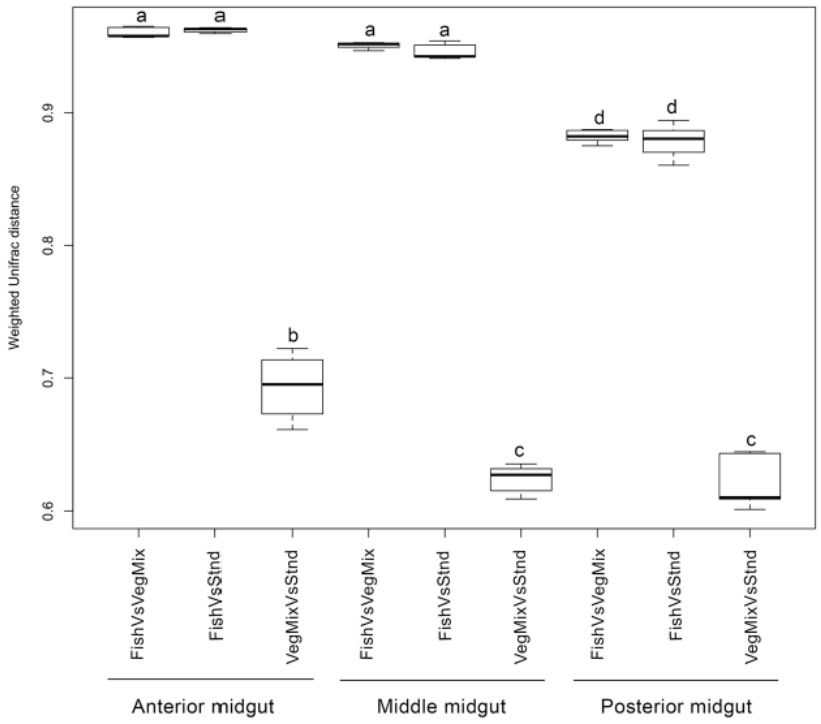

Figure S1. Boxplots showing Weighted Unifrac distance between anterior, middle and posterior midgut samples of BSF larvae fed with different diets. Each point represents the average distance between one sample and all the samples from the other group.

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S.C., M.C., D.E. and G.T. designed the research; M.B., D.B., F.D.F. and I.D.L. performed the experiments; F.D.F. and I.D.L. analyzed data and contributed figures and tables; S.C., M.C., D.E. and G.T. wrote the article.

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CHAPTER 3

The feeding substrate affects morphological and functional features of the *Hermetia illucens* larval midgut

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In preparation

ABSTRACT

The use of insects as a primary agent for organic waste reduction and bioconversion into usable protein products, as insect protein for animal feed to complement traditional plant sources, could contribute to solve problems related to the management of organic waste and, at the same time, free up land to grow crops for direct consumption by the human population. In this setting, the larvae of black soldier fly (BSF), *Hermetia illucens* (Diptera, Stratiomyidae), emerge as a relevant issue since they can convert low quality biomass as food waste, organic residues, and byproducts of the agri-food transformation chain into nutritionally valuable proteins.

Among the most promising substrates for rearing *H. illucens* larvae, fruit and vegetable waste provided in large amounts by large-scale retail trade and wholesale markets, poses limited chemical and microbiological risks compared to other waste substrates, and fulfils the requirements of the European Commission Regulation N°2017/893 on the use of insects for aquaculture.

In this study we investigated the impact of two feeding substrates with different nutritional composition (i.e. a diet composed of vegetables and fruits and a standard diet for Diptera), on the morphology and physiology of the *H. illucens* larval midgut. Our data show a diet-dependent adaptation process of this organ: in particular, differences in the morphology, activity of digestive enzymes, and accumulation of long-term storage molecules were observed. The diet-dependent plasticity of the midgut, the organ involved in the digestion and absorption of nutrients, may be responsible for the ability of BSF larvae to growth and develop on such different substrates.

INTRODUCTION

With an increasing world population estimated to reach nearly 10 billion in 2050 (United Nations, 2017), it is mandatory to face the crucial problem of feeding the planet, limiting food waste and the unsustainable exploitation and exhaustion of natural resources, such as land and water. The population growth, together with rising per capita demand for meat in developing countries (FAO, 2017), will lead to an increase in the global demand for food of animal origin in the next decades, in particular for pigs, poultry and fish (Alexandratos and Bruinsma, 2012; Waite et al., 2014). Moreover, the consequent need to increase the production of traditional feedstuff (e.g., soy, fishmeal) raises serious concerns in terms of land and water use, overfishing, diversion of resources from direct human food production (Steinfield et al., 2006; Alexandratos and Bruinsma, 2012; Mekonnen and Hoeksta, 2012; Cashion et al., 2017), and economic costs (Verbeke et al., 2015; Chaalala et al., 2018).

Another serious global problem is food loss and waste, since one third of the food produced globally is lost or wasted throughout the entire supply chain (Gustavsson et al., 2011). In addition to policies to reduce the consumption of food of animal origin and the production of food waste, a promising perspective is to consider insects for the bioconversion of organic waste into proteins for sustainable and large-scale feed production: insect-based feeds are comparable in terms of nutritional value with fishmeal and soy-based feed formulae and can represent a more environmentally friendly alternative to conventional feedstuff (St-Hilaire et al., 2007; Diener et al., 2009; Oonincx et al., 2010; Kroeckel et al., 2012; Van Huis et al., 2013; Van Huis, 2013; Barroso et al., 2014; Makkar et al.,

2014; Oonincx et al., 2015; Magalhães et al., 2017; Van Huis and Oonincx, 2017; Halloran et al., 2018).

In this context, the black soldier fly (BSF), *Hermetia illucens* (Linnaeus, 1758) (Diptera: Stratiomydidae) emerges as a relevant issue since the larvae of this insect are able to grow on a wide variety of organic matter (Nguyen et al., 2015; Barragan-Fonseca et al., 2017; Wang and Shelomi, 2017), and the meal prepared from BSF larvae is suitable for partially replace traditional feedstuff for livestock and aquaculture (Barragan-Fonseca et al., 2017). In fact, *H. illucens* larvae contain high protein levels with a good quality amino acidic profile, and other macro- and micro-nutrients useful for animal feed (Spranghers et al., 2017). However, it must be taken into consideration that the feeding substrate strongly affects *H. illucens* larval growth, and protein, fat and fatty acid content in larvae and prepupae (Barragan-Fonseca et al., 2017; Wang and Shelomi, 2017). Even considering only organic matters of vegetal origin, nutrient composition of larvae and prepupae and performance indicators, such as duration of the larval development, and larval and pupal weights, vary significantly (Jucker et al., 2017). Nonetheless, *H. illucens* larvae show high survival rate on very different substrates, unlike other insects used for feedstuff production (Oonincx et al., 2015; Van der Fels-Klerx et al., 2016). However, the choice of the insect feeding substrates must consider not only their impact on larval composition and insect development, but also criteria regarding food security and economic and environmental sustainability. In recent years, many low-cost substrates such as manure, kitchen waste, municipal organic waste, and fruit and vegetable waste have been proposed (Nguyen et al., 2015; Barragan-Fonseca et al., 2017; Wang and Shelomi, 2017). However, in the European Union, it is not allowed to rear insects destined for

animal feed on feces, manure, catering waste, and other organic matter containing products of animal origin (Regulation (EC) 767/2009; Regulation (EC) 1069/2009; Regulation (EU) 142/2011). Former foodstuffs not containing meat and fish, such as production surplus or misshapen products, are instead among the main substrates currently used in the European insect production (EFSA Scientific Committee, 2015). The share of non-animal origin matter of the global food waste is about 86% and three quarters of this percentage are represented by roots, tubers, fruit, and vegetables waste (World Resource Institute, 2011). For this reason, fruit and vegetable waste, provided in large amounts by large-scale retail trade and wholesale markets, could represent a suitable feeding substrate to rear *H. illucens* larvae. However, although information on performances and nutritional composition of larvae and prepupae of *H. illucens* reared on fruit and vegetable waste is available (Jucker et al., 2017; Spranghers et al., 2017), it is essential to establish if and how a feeding substrate with a particularly low protein carbohydrate ratio, such as fruit and vegetable, determines morphofunctional adaptations in the midgut, the organ involved in digestion and nutrient absorption. These adaptations could be necessary to better exploit an unbalanced supply of nutrients, but have a metabolic cost for the insect and negatively affect development and nutrient composition of the larvae.

A deep morphofunctional characterization of *H. illucens* midgut has been already performed by Bonelli et al., (submitted), on larvae reared on Standard Diet (SD) for Diptera (Hogsette, 1992), and it has been demonstrated that this organ presents a marked regionalization, and the different midgut regions shows peculiar features and functions. In the present work, we investigated with a multidisciplinary approach a broad spectrum of morphofunctional

properties of the BSF larval midgut reared on two different feeding substrates: Standard Diet (SD) for Diptera, used as control, and a Vegetable Mix Diet (VMD), composed of different vegetables and fruits, mimicking fruit and vegetable waste. Differences in larval growth rate, activity of digestive enzymes, morphological features of midgut cells, and long-term storage molecules in the midgut tissue were evaluated in relation to the provided diet. Our results indicate that *H. illucens* larval development, as well as morphofunctional features of the midgut, are strongly influenced by the nutritional composition of the diet.

MATERIALS AND METHODS

Insect rearing

Hermetia illucens larvae used in this work derived from a colony established in 2015 at University of Insubria (Varese, Italy).

Two specific substrates, with different nutrient composition, were used to rear the larvae: i) Standard Diet (SD) for Diptera (Hogsette, 1992), composed by 50% of wheat bran, 30% of corn meal and 20% of alfalfa meal, mixed in the ratio 1:1 dry matter/water ; ii) Vegetable Mix Diet (VMD), composed by seven vegetables (apple, banana, pear, broccoli, zucchini, potato and carrot) in equal quantity and appropriately minced. The VMD was realized miming the fruit and vegetable waste, but choosing ingredients available all year round, in order to standardize the experimental conditions. The rearing methods of the larvae on both diets were described in Pimentel et al. (2017). The larvae were maintained at 27.0 ± 0.5 °C, 70 ± 5% relative humidity, in the dark. For all the experiments larvae at last instar were used.

Determination of nutrient content of the diets

Three samples of fresh VMD were lyophilized with freeze-dryer (Martin Christ GmbH, Alpha 2-4 LD plus) under 12-15 mbar at -80 °C and then analyzed for the determination of nutrient content. Samples of SD powder were analyzed as they were. Samples were analyzed at the Department of Agronomy, Food, Natural Resources, Animals and Environment, University of Padua, Agripolis, Legnaro PD, Italy.

Measurement of larval growth rate

Batches of 300 larvae were placed in plastic containers and random samples of 30 individuals were weighed every two days starting from the fourth day after hatching. For each experimental diet, the sampling and the annotation of the larval weight were made in triplicate. Before weighing, the larvae were washed in tap water to remove diet matter from the body and then wiped dry. The weight was recorded until 25% of insects reached pupal stage.

Midgut juice extraction and midgut epithelium isolation

Larvae were anesthetized on ice with CO_2. The gut was isolated in Phosphate Buffer Solution (PBS) (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.76 mM KH_2PO_4, pH 7.4) at 4 °C. The regions of the midgut were collected depending on the analysis performed, according to Bonelli et al. (submitted). For all the experiments, except for the detection of ferric iron, the midgut was subdivided into three regions, i.e. anterior, middle, and posterior.

The peritrophic matrix from each midgut region, with the enclosed intestinal content, was isolated from the epithelium, centrifuged at 15000 × g for 10 min at 4 °C to remove the insoluble material, and supernatant (midgut juice) was collected. The midgut juice from 15 larvae was used as fresh sample for the luminal pH measurement, or stored at -80 °C for the enzymatic assays. The epithelium of the

posterior region of the midgut, deprived of the peritrophic matrix, was placed into cryovials, each containing the midgut epithelium collected from 15 larvae, and stored in liquid nitrogen for aminopeptidase N activity assay.

Luminal pH measurement

The pH of the midgut juice extracted from anterior, middle and posterior regions of the midgut was measured using universal indicator strips with a resolution of 0.5 pH unit (Hydrion Brilliant pH Dip Sticks, Sigma-Aldrich, Italy). The experiment was repeated on six independent samples for each diet.

Morphological analysis of the larval midgut

The larval midgut was processed for morphological analysis as described in Bonelli et al. (submitted). Briefly, after fixation in glutaraldehyde 4% in 0.1 M Na-cacodylate buffer, the specimens were dehydrated in ethanol ascending series and then embedded in epoxy resin (Epon/Araldite 812 mixture). 0.6-µm-thick sections were obtained with a Leica Reichert Ultracut S (Leica, Germany), stained with crystal violet and basic fuchsin, and then observed under Eclipse Ni-U microscope (Nikon, Japan) equipped with the digital camera TrueChrome II S (Tucsen photonics Co. Ltd, China).

Histochemical analysis of the larval midgut

For the detection of glycogen, the isolated midgut, with the enclosed intestinal content, was subdivided in the three regions as described above and immediately fixed in 4% paraformaldehyde in distilled water for 2 h at room temperature and then overnight at 4 °C. After dehydration in ascending ethanol series, the specimens were embedded in paraffin (Franzetti et al., 2015), and 7-µm-thick sections were obtained using microtome Jung Multicut 2045 (Leica). After deparaffinization, the sections were stained with periodic

acid-Schiff kit (PAS) (Bio-Optica, Italy) according to the manufacturer's instructions to detect the glycogen deposits in the midgut tissues, and then analyzed under Eclipse Ni-U microscope equipped with digital camera.

For the detection of ferric iron, whole mount staining of the midgut was performed. After isolation, the tissue was fixed in 4% paraformaldehyde in distilled water for 20 minutes, and then stained with Perls' stain kit (Bio-optica) according to the manufacturer's instructions. Each region of the midgut was analyzed under NSZ-606 Zoom Stereo Microscope (Xiamen Phio Scientific Instruments Co. Ltd, China) equipped with TrueChrome II S digital camera.

Total proteolytic activity assay

Total proteolytic activity in midgut juice samples was assayed with azocasein (Sigma-Aldrich), measuring its degradation by release of azo chromophore (Charney and Tomarelli, 1947), as reported by Bonelli et al. (submitted). Assays were performed with Universal Buffer (UB), a buffer with constant ionic strength at different pH values (Coch Frugoni, 1957). The pH used for the assays is indicated in the captions to figures and in the Results section. 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.

Chymotrypsin- and trypsin-like proteolytic activity assay

Chymotrypsin- and trypsin-like proteolytic activity in midgut juice samples were assayed with N-succinyl-ALA-ALA-PRO-PHE-p-nitroanilide (SAAPPpNA, Sigma-Aldrich) and Na-Benzoyl-D,L-arginine 4-nitroanilide hydrochloride (BApNA, Sigma-Aldrich), respectively, measuring their degradation by release of p-

nitroaniline (pNA) (Bonelli et al., submitted). These assays were performed on midgut juice samples obtained from the posterior region, using UB at pH 8.5. One unit (U) of chymotrypsin- and trypsin-like proteolytic activity was defined as the amount of enzyme that causes an increase in absorbance by 0.1 unit per min per mg of proteins.

Aminopeptidase N activity assay

The activity of aminopeptidase N (APN) was assayed using L-leucine p-nitroanilide as substrate, measuring its degradation by release of p-nitroaniline (pNA), as reported by Bonelli et al. (submitted). Assays were performed on the homogenate of posterior midgut epithelium in50 mM Tris-HCl, pH 7.5. One unit/mg (U/mg) of APN activity was defined as the amount of enzyme that releases 1 µmol of pNA per min per mg of proteins.

α-amylase activity assay

α-amylase activity in midgut juice samples was assayed with starch as substrate, measuring its hydrolysis by the amount of maltose released (Bernfeld, 1955) as reported by Bonelli et al. (submitted). These assays were performed in amylase buffer (20 mM NaH_2PO_4, 6.7 mM NaCl, pH 6.9). 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.

Lysozyme activity assay

Lysozyme activity in midgut juice samples was assayed with *Micrococcus lysodeikticus* lyophilized cells, measuring the rate of lysis of these cells as reported by Bonelli et al. (submitted). These assays were performed on midgut juice samples extracted from the middle region of the midgut, in 66 mM potassium phosphate buffer, pH 6.2. Even though the luminal pH of the middle region is very

acidic (Bonelli et al., submitted), it was not possible to measure lysozyme activity at pH values lower than 6.2 since in those conditions the enzyme is unable to lyse *Micrococcus lysodeikticus*, the substrate used in the classic method for lysozyme activity assay (Gagliardi et al., 1986; Pitotti et al., 1991). One unit/mL (U/mL) of lysozyme activity was defined as the amount of enzyme that causes a decrease in absorbance by 0.001 unit per min per mL of midgut juice sample.

Statistical analyses

Statistical analyses were performed with R-statistical software (ver. 3.3.2). The following analyses were performed: paired and unpaired t-tests. Statistical differences between groups were considered significant at p-value ≤ 0.05. The statistical analysis performed for each experiment and the p-values are reported in the captions to figures.

RESULTS

Larval growth rate

The larvae reared on both experimental diets (SD and VMD) were weighted every two days to evaluate differences in the length of the larval period and the maximum weight reached. The curves reported in Figure 1 show that the larvae reared on SD reached the pupal stage more rapidly than those grown on VMD (18 days vs 25 days, respectively). The diet affected not only the length of the larval period, but also the maximum weight reached before pupation. In fact, larvae reared on VMD reached a maximum mean weight 10.5 ± 0.6% (mean ± SEM) less than larvae reared on SD.

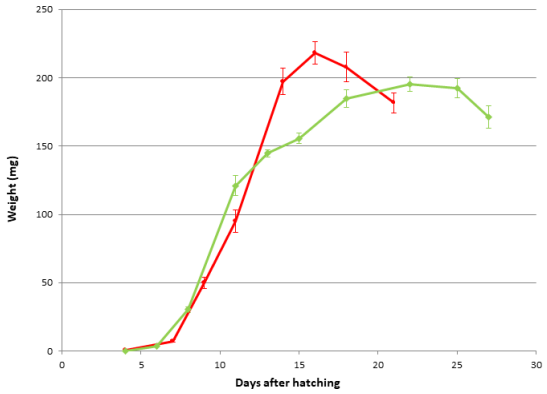

Figure 1. Larval grow rate. Larvae reared on SD are indicated in red while larvae grown on VMD are in green.

Nutrient content of the diets

For the evaluation of the nutritional content, VMD samples were weighted before and after the lyophilization process to calculate the water loss. Moreover, the amount of water lost during the analyses was considered. The nutrient content of the two diets is reported in Table 1.

Table 1. Nutritional composition of the two experimental diets. The values of the fresh diets are reported.

	Standard diet[a] (%)	Vegetable mix diet[b] (%)
Ash	2.3	0.5
Protein	6.4	1.2
Lipid	1.2	0.1
Fiber	6.7	0.5
Hemicellulose	9.9	0.4
Cellulose	4.4	0.5
Lignin	1.7	0.1
Starch	8.6	1.3
Glucose and fructose	1.5	1.5

	Standard diet[a] (mg/kg)	Vegetable mix diet[b] (mg/kg)
Iron	119	3.1
Copper	4.2	0.8
Nickel	0.8	0.1
Zinc	22.4	2.1

[a] 55% water
[b] 88% water

Measurement of the midgut lumen pH

To evaluate if the feeding substrate could affect the pH of the midgut lumen, this parameter was measured in the three midgut regions of larvae reared on SD and VMD. As reported in Table 2, no significant differences were found between the pH values recorded in each midgut region of larvae reared on the two diets. The pH values are in agreement with those already reported (Bonelli et al., submitted), being acidic in the anterior midgut, strongly acidic in the middle, and alkaline in the posterior.

Table 2. **pH values in the lumen of *H. illucens* midgut regions.** Mean ± SEM, number of replicates in parenthesis. No statistically significant differences were recorded among diet groups for each midgut region (unpaired t-test).

	SD	VMD
Anterior midgut	5.8 ± 0.1 (6)	6.0 ± 0.1 (6)
Middle midgut	2.4 ± 0.2 (6)	1.8 ± 0.2 (7)
Posterior midgut	8.3 ± 0.4 (6)	8.8 ± 0.1 (7)

Morphological analysis of the larval midgut

The midgut of larvae reared on both diets was analyzed by optical and electron microscopy to evaluate if the feeding substrate could affect the morphology of the cells. The diet administered to the larva did not seem to cause substantial differences in the anterior midgut (Fig. 2A, B): in both cases columnar cells showed a big central

nucleus, basal infoldings, and a well-developed brush border. Moreover, a large amount of dark vesicles, probably containing digestive enzymes, were clearly visible under the microvilli.

The middle midgut of *H. illucens* larvae is characterized by a first district with an epithelium in which copper cells are present and a second one characterized by a thin epithelium formed by large flat cells (Bonelli et al., submitted). All these cells showed the same characteristics, regardless of the feeding substrate: copper cells displayed the typical cup shape whit a big central nucleus and developed microvilli (Fig. 2C, D), while large flat cells presented an elongated nucleus and very short microvilli (Fig. 2E, F).

The most consistent change in the morphology of the epithelium was observed in the posterior midgut (Fig. 2G, H). In fact, the brush border of columnar cells showed a different length depending on the diet. In particular, columnar cells of larvae reared on VMD were characterized by microvilli that were longer than those of larvae reared on SD.

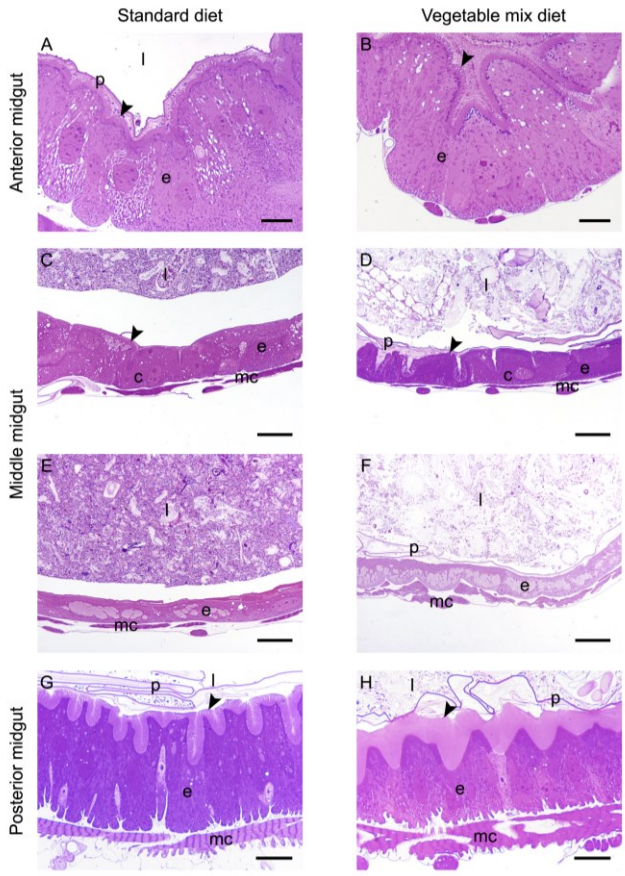

Figure 2. Morphological comparison of midgut from larvae reared on SD and VMD. (A, B): cross-section of the anterior midgut. (C-F): copper (C, D) and large flat cells (E, F) in the middle midgut of larvae. (G, H): cross-section of the posterior midgut. Larvae grown on VMD (H) show microvilli (arrow) that are longer than those of larvae reared on SD (G). c: copper cells; e: epithelium; l: lumen; mc: muscle cells; p: peritrophic matrix. Bars: 10 µm (A, B), 20 µm (C-H).

Histochemical characterization of the larval midgut

Detection of glycogen – PAS staining

The comparison of glycogen deposits in the midgut of larvae reared on the two diets evidenced a major difference in the anterior midgut. In fact, glycogen accumulation was more consistent in larvae reared on SD than on VMD (Fig. 3A, B). Moreover, the

deposits in this region of the midgut were not localized in a specific area of the cell, but were sparse into the cytoplasm. In the middle and posterior midgut, the glycogen deposits showed almost the same presence in larvae grown on both diets (Fig. 3C-F), although their distribution differed in the two midgut regions. In fact, glycogen deposits were sparse in the cytoplasm in the middle midgut (Fig. 3C, D), while in the posterior midgut they were present in the apical region of columnar cells (Fig. 3E, F).

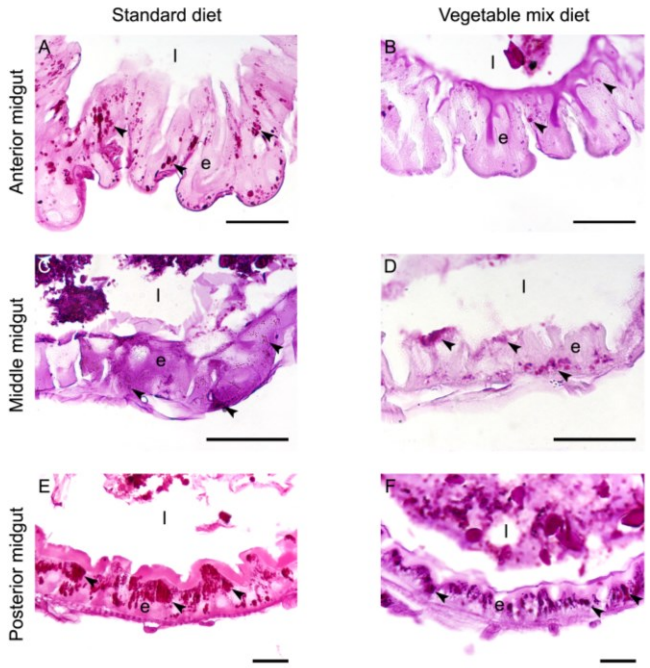

Figure 3. Comparison of glycogen accumulation in the three midgut regions of larvae reared on SD and VMD - PAS staining. (A, B): anterior midgut of larvae reared on SD (A) shows a higher accumulation of glycogen (arrowheads) than VMD (B). (C-F): middle (C, D) and posterior (E, F) midgut of larvae reared on the two diets do not show significant differences in glycogen accumulation. e: epithelium; l: lumen. Bars 50 µm (A, B, E, F), 20 µm (C, D).

Detection of ferric iron - Perls' method

Ferric iron showed a broader distribution in the whole midgut of larvae grown on SD compared to VMD (Fig. 4). The presence of this

compound in the first part of the posterior midgut (Fig. 4C, D) in larvae reared on the two diets could be attributed to iron cells, a peculiar cell type that has been identified in the midgut epithelium of other insects (Metha et al., 2009). Apart from this characteristic localization of ferric iron, in the anterior (Fig. 4A, B) and in the second part of the posterior region (Fig. 4E, F), the presence of ferric iron in larvae reared on SD was higher than in VMD (Fig. 4A, E). Regardless of the feeding substrate, the middle midgut did not show any staining (Fig. 4C, D), as previously observed in other Diptera (Poulson and Bowen, 1952; Mandilaras et al., 2013).

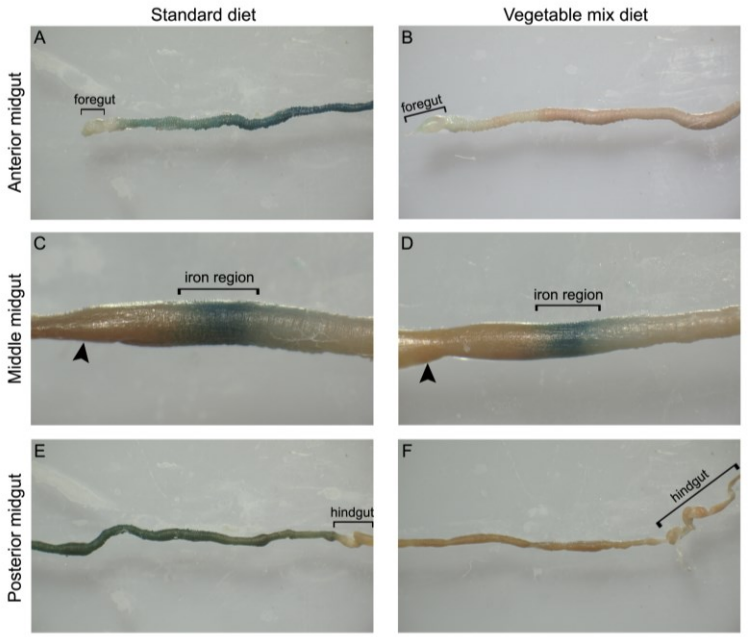

Figure 4. Comparison of iron accumulation between the three midgut regions of larvae reared on SD and VMD - Perls' method. (A, B): a higher iron accumulation in the anterior midgut of larvae reared on SD (A) compared to VMD (B) can be observed. (C, D): iron region of the first part of the posterior midgut of larvae reared on the two diets. The middle midgut (arrowheads) not shows accumulation of iron in both larvae. (E, F): a higher accumulation of iron in the posterior midgut of larvae grown on SD than VMD is visible.

Enzymatic assays

To assess if *H. illucens* larvae can modulate the digestive efficiency in response to the nutrient composition of the feeding substrate, the activity of enzymes involved in protein and carbohydrate digestion was measured in larvae reared on both diets.

The total proteolytic activity was assayed on midgut juice samples using azocasein as substrate, at a pH as close as possible to the luminal pH of the investigated region (pH 6.0 for the anterior midgut, pH 5.0 for the middle midgut and pH 8.5 for posterior). As reported in figure 5, for all midgut regions we observed a significantly higher proteolytic activity in larvae reared on VMD, a feeding substrate with a lower protein content compared to SD (Table 1). For both diets, the highest proteolytic activity was measured in the posterior region.

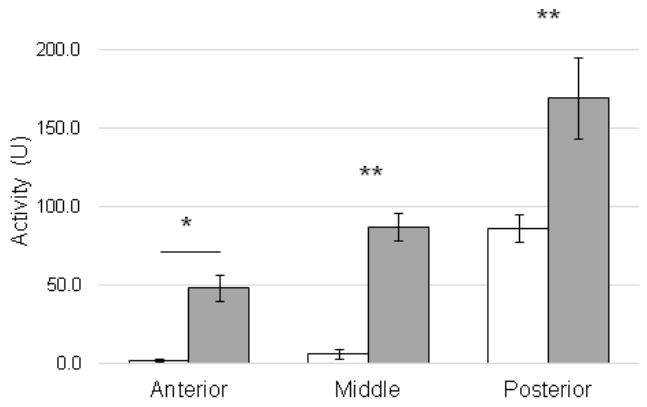

Figure 5. Total proteolytic activity in midgut juice extracted from anterior, middle, and posterior midgut of larvae reared on SD and VMD. For each tract the enzymatic assay was performed at pH as close as possible to that of the lumen (pH 6.0 for the anterior midgut, pH 5.0 for the middle midgut and pH 8.5 for posterior). The values are reported as mean ± SEM of at least 3 experiments. Asterisks indicate statistically significant differences between diet groups (unpaired t-test: *p-value < 0.05, **p-value < 0.01).

Previous data obtained on larvae reared on SD indicated that protein digestion essentially occurred in this tract and the enzymes involved in the initial phase of digestion of these macromolecules were mostly serine proteases (Bonelli et al., submitted). To establish if the increase in proteolytic activity observed in this region in larvae reared on VMD was due to a major activity of serine proteases, we evaluated the activity at acidic pH. When the proteolytic activity was measured at acidic pH (pH 5.0), a significant decrease of the enzymatic activity (five-fold reduction) was observed compared to that recorded at pH 8.5 (total proteolytic activity at pH 8.5: 169.7 ± 25.7 U, at pH 5.0: 34.1 ± 5.3 U, mean ± SEM of 4 experiments, paired *t*-test: *p*-value < 0.01). This result suggests that the increase in total proteolytic activity in the posterior region in larvae reared on VMD could be mainly due to serine proteases.

The two best studied serine proteases involved in protein digestion in insects are trypsin- and chymotrypsin-like proteases (Terra and Ferreira, 1994; Terra *et al.*, 1996). Their activity was measured in midgut juice samples from the posterior midgut using the specific substrates BApNA and SAAPPpNA, respectively. As shown in figure 6, there was no significant difference in trypsin-like activity among larvae reared on the two diets (Fig. 6A), while chymotrypsin-like activity was significantly higher in larvae reared on VMD (Fig. 6B), more than double of that measured in larvae reared on SD.

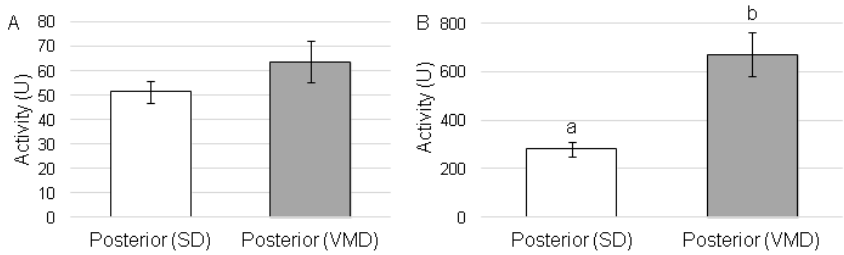

Figure 6. Trypsin- and chymotrypsin-like activity in midgut juice extracted from posterior midgut of larvae reared on SD and VMD. The values are reported as mean ± SEM of at least 3 experiments. For trypsin-like activity (A) no statistically significant difference among diet groups was recorded (unpaired t-test). For chymotrypsin-like activity (B) different letters indicate statistically significant difference among diet groups (unpaired t-test: p-value < 0.01).

In addition to the evaluation of the activity of enzymes present in the midgut lumen and involved in the initial phase of protein digestion, the activity of enzymes responsible for the final phase of digestion able to the release of single amino acids from small peptides was measured. In particular, we assayed the activity of APN, an exopeptidase that is anchored to the midgut brush border membrane (Terra and Ferreira, 1994). These assays were performed on the homogenate of posterior midgut epithelium. As shown in figure 7, APN activity was significantly higher in larvae reared on VMD, reaching values that were six-fold higher than those measured in larvae reared on SD.

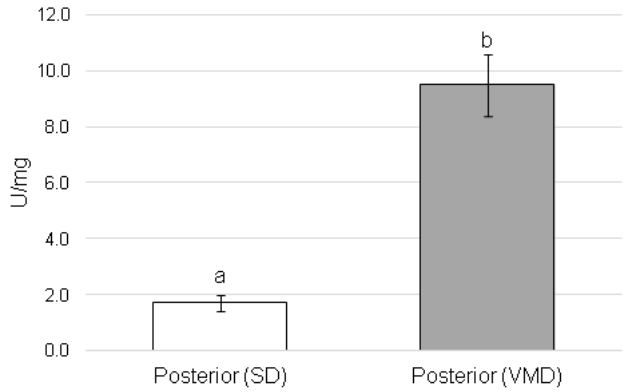

Figure 7. Aminopeptidase N activity in the posterior midgut of larvae reared on SD and VMD. The values are reported as mean ± SEM of at least 3 experiments. Different letters indicate statistically significant difference among diet groups (unpaired t-test: p-value < 0.01).

To examine carbohydrate digestion, we compared the activity of α-amylase (Terra and Ferreira, 1994; Terra *et al.*, 1996) in larvae reared on the two diets. As reported in figure 8 no significant difference in α-amylase activity was observed in the anterior and middle regions, while in the posterior region the activity was significantly lower in larvae reared on VMD, with a sixty-fold reduction compared to than that measured in larvae reared on SD.

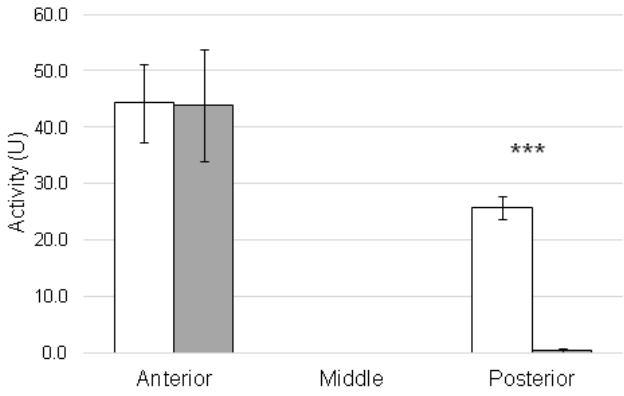

Figure 8. α-amylase activity in midgut juice extracted from anterior, middle, and posterior midgut of larvae reared on SD and VMD. The values are reported as mean ± SEM of at least 3 experiments. Asterisks indicate statistically significant differences between diet groups (unpaired t-test: ***p-value < 0.001).

Finally, the activity of lysozyme, an enzyme responsible for the degradation of peptidoglycan present in the cell wall of many bacteria, was measured in the middle midgut (Fig. 9).

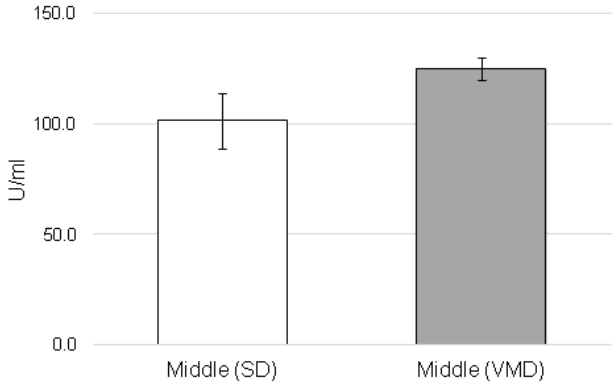

Figure 9. Lysozyme activity in midgut juice extracted from middle midgut of larvae reared on SD and VMD. The values are reported as mean ± SEM of at least 3 experiments. No statistically significant difference among diet groups was recorded (unpaired t-test).

We focused the attention on this tract since it has been suggested that lysozyme, together with the low pH value of the lumen of this region, is responsible for killing pathogens ingested with the diet in brachycerous larvae (Espinoza-Fuentes and Terra, 1987; Terra and Ferreira, 1994; Bonelli et al., submitted; Bruno et al., submitted). No significant difference was observed between larvae reared on the two diets.

DISCUSSION

The larvae of the black soldier fly are suitable for the bioconversion process (Rehman et al., 2017a; Wang et al., 2017; Xiao et al., 2018), since they are able to grow and convert a wide variety of different waste material, such as agro-industrial by-products, kitchen waste, fruit and vegetable waste, and manure into nutritionally valuable protein (Meneguz et al., 2018; Nguyen et al., 2013; Rehman et al., 2017b; Salomone et al., 2017). In particular, the bioconversion of fruit and vegetable waste into protein useful for production of

feedstuffs (Magalhães et al., 2017; Newton et al., 1977; Schiavone et al., 2017) is a promising approach since in the EU insects destined for animal feed cannot be reared on organic matter containing products of animal origin (European Commission Regulations N°767/2009, 1069/2009 and 142/2011) and recently the European Commission Regulation N°2017/893 has partially uplifted the feed ban rules regarding the use of processed animal proteins from BSF and other six insect species for aquaculture. Regarding *H. illucens*, no information is available in literature about the morphofunctional modification of the midgut when the larvae are reared on substrates with different nutrient content. In the present work, we evaluated growth performances, activity of digestive enzymes, morphological features of midgut epithelium and long-term storage molecules changes in this tissue in larvae reared on vegetable mix diet (VMD) and standard diet (SD) for Diptera (Hogsette, 1992) to understand if the extraordinary feeding plasticity of BSF larvae corresponds to adaptations of the larval midgut, the organ directly involved in the bioconversion process.

It is known that several factors, such as temperature, humidity, density of insects in the rearing substrates influence the development of BSF larvae (Barragan-Fonseca et al., 2018; Harnden and Tomberlin, 2016; Holmes et al., 2012). For this reason, the larvae used in this study were kept at the same temperature, humidity and density conditions, to avoid bias due to these parameters and evaluate the impact only of the rearing substrates. Diet composition seems to be the major factor that influences larval performances, and the time required to complete the larval development decrease with the increase of diet quality. In particular, larvae show a high development rate on substrates with appropriate nutritional values such as chicken feed, on which after

12 days the first prepupae were observed (Spranghers et al., 2017), while poor performances were described on substrates such as the rice straw waste, on which 38 days are necessary to have 50% of larvae to reach pupal stage (Manurung et al., 2016). Our data demonstrated that VMD is suitable for rearing *H. illucens* larvae although a lower developmental rate was observed compared to control diet. The nutritional composition of the two diets is very different in terms of protein, starch, and lipids, and larvae reared on VMD reached the pupal stage approximately one week later than larvae reared on control diet. Moreover, maximum weight reached before pupation by larvae reared on fruit and vegetable is about 10% lower than that those reared on SD. These differences are probably caused by the lower nutritional content of a diet only composed by fruits and vegetables (Table 1).

Conversely, different growth pattern of BSF larvae reared on the two diets corresponds to different enzymatic expression and activity in the larval midgut. No information is available about the regulation of digestive enzymes in response to different nutritional composition of the diet, in particular in relation to different protein and carbohydrate content and their ratio, probably because of the very recent interest in insect farming. In fact, these issues have been deeply investigated in other arthropod species with consolidate economic interest such as shrimps (Brito et al., 2000; Muhlia-Almazán et al., 2003; Tantikitti et al., 2016; Shao et al., 2017). Our work represents the first attempt to shed light on relation between digestive enzyme activity and diet nutritional content in insects. Larvae of *H. illucens* reared on VMD show an increased activity of enzymes involved in both first and final phase of protein digestion, with higher total luminal proteolytic activity in all three midgut regions and higher APN activity in the homogenate of posterior

midgut epithelium, respectively. Regarding carbohydrate digestion, α-amylase activity does not differ between diets in the anterior midgut, while it is strongly increased in the posterior midgut of larvae reared on SD. We hypothesize that these changes are an adaptation to better exploit a diet with a non-optimal nutritional content: in the presence of a low-protein diet, as for VMD, an increase in proteolytic activity is observed, while when larvae are reared on SD, which has a protein content 6-fold higher than VMD, the protein demand is satisfied without any need to increase protease activity, and the larvae invest on amylolytic enzymes to better exploit the carbohydrate content in the feeding substrate.

We also analyzed lysozyme activity, an enzyme involved in enzymatic killing of bacteria and fungi ingested with the diet. No difference in lysozyme activity was observed among larvae reared on the two substrates. The expression of these enzymes was recently analyzed in *H. illucens* larval gut by Vogel et al. (2018). The authors reported that only some lysozyme coding genes are upregulated in diets with a high bacterial load. Moreover, an overall similar microbiota composition in the midgut of *H. illucens* larvae reared on SD and VMD has been described and the two diets do not induce gut dysbiosis (Bruno et al., 2018, submitted). Therefore, it is reasonable that no significant variation in lysozyme activity was recorded in larvae fed on the two diets.

We demonstrated that the nutrient composition of the feeding substrate not only influences the length of larval development and activity of digestive enzymes, but also induces morphological modifications in midgut cells. Many studies demonstrated that diets induce ultrastructural changes in midgut cells of many insects. The modifications regard fluctuations in the number and structure of lysosomes (Sutherland et al., 2002), proliferation of both smooth

and rough endoplasmic reticulum (Houk and Vardy, 1982), and the increase of the basal labyrinth surface (Rudin and Hecker, 1979). Moreover, in other insects changes in structures associated to the midgut take place, for example the strong rearrangement of the perimicrovillar membrane forming a sort of peritrophic matrix after blood feeding in Hemiptera (Billingsley and Downe, 1983). The diet can also alter the structure of microvilli: in *Drosophila melanogaster* Meigen, 1830 (Diptera: Drosophilidae) variations in the shape and length of microvilli were observed, an effect similar to that caused by starvation (Li et al., 2009). Also in *H. illucens* larvae we observed changes in the morphology of midgut cells, and in particular in the length of microvilli. This modification is restricted to the posterior midgut, the region mainly involved in nutrient absorption (Bonelli et al., submitted). The higher length of microvilli in larvae reared on VMD probably responds to the need of a higher absorbing surface and represents an adaptation to the low nutritional content of VMD in terms of proteins.

Midgut cells of *H. illucens* larvae also showed differences in glycogen accumulation. Glycogen reserves are useful to sustain the insect during metamorphosis (Franzetti et al., 2015), a process that in *H. illucens* needs about twelve days to occur. During this period, indeed, glycogen deposits are mobilised and progressively reduced (Bruno et al., in preparation). Our data demonstrate that larvae reared on VMD show lower accumulation of this long-term storage molecule than larvae reared on control diet, especially in the anterior midgut. The higher presence of glycogen reserves in larvae reared on the control diet could be related to the higher presence of carbohydrate level in SD than VMD (Table 1). Regardless of the feeding substrate, we observed that glycogen accumulation occurs differentially in the three midgut regions. In particular, the anterior

and the posterior midgut are mainly involved in glycogen storage, indicating that the cells in the midgut epithelium accomplish different metabolic functions (Turunen and Crailsheim, 1996).

We also evaluated the accumulation of microelements in midgut cells of larvae grown on the two diets. In particular, our attention focused on iron. Several studies demonstrated that this element is fundamental for the development of Diptera (Law, 2002; Missirlis et al., 2007) due to its role as a cofactor for different enzymes involved in crucial physiological functions. Among these there are those responsible for respiration (Warburg, 1925) and synthesis of DNA (Clark, 1994), ecdysone (Chavez et al., 2000; Warren et al., 2002), and lipids (Navarro et al., 2010). The accumulation of iron in insect midgut is attributed a peculiar cell type, called iron cells: in D. melanogaster these cells are present in the so called iron region, located in the middle midgut, after the so called copper region (Poulson and Bowen, 1952; Metha et al., 2009). Ferritin is the major protein responsible for iron storage in insects (Pham and Winzerling, 2010), and is constitutively expressed in iron cells (Mandilaras et al., 2013). The expression of this protein is inducible in other midgut cells. In fact, it has been demonstrated that when the iron content in the diet is high, ferritin encoding genes are also expressed in the anterior and posterior midgut cells, allowing iron accumulation also in these regions (Poulson and Bowen, 1952; Mandilaras et al., 2013). Our analyses demonstrate that an iron region located after the copper region is present also in *H. illucens* larval midgut, and that, depending on the diet, different iron accumulation occurs. In particular, the higher iron content in the SD (Table 1) determines an accumulation of this microelement not only in the iron region, but also in the anterior and in the posterior midgut. By contrast, the very

low quantity of iron in VMD limits the accumulation to the iron region.

In conclusion, our work represents the first attempt to clarify how different feeding substrates with different nutrient composition affect the morphology and some physiological parameters of *H. illucens* larval midgut. Our results demonstrate that the extraordinary feeding plasticity of this insect is correlated with the plasticity of this organ, which is responsible for digestion and nutrient absorption. Although vegetal materials are suitable to rear *H. illucens* larvae (Jucker et al., 2017), more in depth studies are necessary to optimize their bioconversion, opening up interesting application perspectives.

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CHAPTER 4

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Metabolic adjustment of the larval fat body in *Hermetia illucens* to dietary conditions

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ABSTRACT

The black soldier fly (BSF), *Hermetia illucens*, has great economic importance because of its ability to degrade a wide variety of organic products, including vegetable waste. The conversion of organic waste into valuable nutrients, that can be isolated from larvae and prepupae, is a widely exploited strategy to produce protein for animal feed and, consequently, comprehending the mechanisms that regulate nutrient accumulation in this insect could improve the production of insect-derived meal quantitatively and qualitatively.

Since the fat body participates in metabolizing proteins, fat, and sugars in insects, detailed knowledge of this organ and of its modifications in relation to insect food intake could provide interesting clues about the nutritional value of the larvae, a fundamental aspect from an applied perspective. To this end, we performed a morphofunctional and molecular characterization of the fat body of sixth instar *H. illucens* larvae reared on different food substrates, focusing on markers related to nutrient accumulation.

We demonstrate that a protein-poor diet affects both lipid and protein accumulation in fat body cells as well as the expression of key genes involved in these metabolic processes. Our study not only represents the first characterization of the larval fat body in this insect, but also confirms the central role of this organ in nutrient accumulation and substantiates the hypothesis of producing larvae with higher nutritional value by manipulating the diet.

INTRODUCTION

Hermetia illucens (Diptera: Stratiomyidae), also known as black soldier fly (BSF), is a widely distributed insect species (Martínez-

Sánchez et al., 2011) with great economic importance because of its ability to degrade a wide variety of organic products. In particular, BSF larvae can grow on different organic substrates, consuming daily a quantity of food equal to twice their weight (Sheppard et al., 1994; Čičková et al., 2015, Nguyen et al., 2015). Moreover, dried prepupae contain a high percentage of protein (37-63%) and fat (7-39%) (Barragan-Fonseca et al., 2017). Therefore, this versatility can be exploited to transform organic waste into larvae that can be used as a source of protein for animal feed (Makkar et al., 2014). In addition, the oil extracted from dried larvae can be utilized for biodiesel production (Li et al., 2011 a, b; Leong et al., 2016). According to Rozkosný (1983), the adults cannot eat because they do not have functional mouthparts and therefore they depend exclusively on the reserves accumulated during the larval stage (Gobbi et al., 2013). For these reasons, the quality and quantity of food administered to the insect as well as the nutrient storage processes at larval stage affect the development of the larva, impacting on the protein content in the insects and thus their suitability for producing animal feed.

The fat body is distributed along the whole length of the insect body and is mainly localized under the integument and around the organs (Dean et al., 1985). It represents the main tissue responsible for accumulating nutrients, i.e., fat, carbohydrates, and proteins (Ad et al., 1985; Arrese and Soulages, 2010). A high level of these nutrient reserves guarantees a fine modulation of larval growth rate, metamorphosis and egg production in the adult insect (Mirth and Riddiford, 2007; Hahn et al., 2008). Lipid metabolism plays a crucial role in insect growth and reproduction and supplies energy during starvation. Lipids are mainly stored in droplets within fat body cells, namely, in trophocytes (Martin and Parton, 2006), which play a

central role in fat and energy metabolism owing to the action of perilipins, e.g., lipid storage droplet 1 (Lsd1) and 2 (Lsd2) (Olofsson et al., 2009; Arrese and Soulages, 2010). In particular, Lsd2 expression promotes lipid accumulation, while Lsd1 is responsible for the lipolytic activity triggered by the adipokinetic hormone (Teixeira et al., 2003; Patel et al., 2005). Glucose is stored in the fat body as glycogen and provides trehalose to other organs. Trehalose can be allocated to supply energy or synthesize chitin (Steele, 1982; Lockey, 1988). It is also a source for the synthesis of saccharides, which are required for adaptation to cold (Storey, 1997) or drought (Watanabe et al., 2002). In insects, hexamerins are called larval serum proteins (LSP) and play a major role in protein accumulation (Telfer and Kunkel, 1991). First isolated from *Calliphora erythrocephala*, the fly hexamerin (calliphorin or LSP-1) is produced by the fat body and constitutes the main soluble protein in the hemolymph (Munn et al., 1969). Later on, LSP-1 was described in other Diptera species (Munn and Greville, 1969). A second hexamerin, LSP-2, is present in this insect order as well. It has a lower methionine and aromatic amino acid content than LSP-1 and is mobilized mainly in gonadotrophic cycles, differently from LSP-1 which is uniformly consumed throughout development (Capurro et al., 2000; Hahn et al., 2008).

The main aim of this study was to perform a morphofunctional analysis of the larval fat body in *H. illucens*. In particular, we focused our attention on protein accumulation, testing how differential availability of proteins in the insect diet affects the expression of genes related to nutrient accumulation. *H. illucens* is a good model for studying nutrient reserves for two reasons: 1) since the fly does not feed, all the nutrients used in the adult phase rely on the reserves accumulated during the larval stage; 2) BSF larvae are

increasingly used as an ingredient for animal feed. Thus, knowledge of aspects related to energy metabolism is of primary importance from an applied perspective since they could impact on the final quality of the insect-derived meal.

MATERIALS AND METHODS

Experimental animals

H. illucens larvae used in this study were obtained from a colony kept in the laboratory for 24 months, that was established starting from larvae purchased from a local dealer (Redbug, Milano, Italy).

The eggs were collected after deposition and subjected to a hatching procedure as follows. The eggs were laid on a Petri dish (9 x 1.5 cm) with a standard diet for Diptera [wheat bran (50%), alfalfa meal (20%), and corn meal (30%) mixed in the ratio 1:1 dry matter/water (Hogsette, 1992)] until hatching. Nipagin (Methyl 4-hydroxybenzoate) (0.15% v/v) was added to the diet to avoid mold growth. Newly hatched larvae were maintained under these conditions for four days. After the weaning phase, 200 larvae were transferred to a plastic container (16 x 16 x 9 cm) and fed *ad libitum* with the experimental diet, which was renewed every three days. Two diets were used in this study: 1) a standard diet for Diptera prepared as described above; 2) a vegetable mix composed of seven vegetables (equal weight): carrots, broccoli, zucchini, potatoes, apples, pears, and bananas. Vegetables were shredded in a blender before administering them to the larvae. Nutritional information on the diets is reported in Table 1. The experiment on dietary protein manipulation was made by adding 5% (w/w) casein to the vegetable mix. The larvae were maintained at 27 ± 0.5 °C, 70% relative humidity, in the dark.

Table 1. Protein, fat, and carbohydrate content of the experimental diets. The values of the fresh diet were calculated based on data from United States Department of Agriculture (https://ndb.nal.usda.gov/ndb/) as follows: standard diet (NDB numbers: 20077, 45037554, 20014) and vegetable mix (NDB numbers: 09040, 09003, 09252, 11090, 11352, 11477, 11124). The values are shown as percentage (w/w).

	Standard diet[a] (%)	Vegetable mix[b] (%)
Protein	11.3	1.2
Fat	3.5	0.2
Carbohydrate	55.2	12.6
Starch	30.5	4.3
Sugar	0.4	6.0

[a] 225 kcal/100 g; 27% water.
[b] 52.4 kcal/100 g; 85% water.

Actively feeding sixth instar larvae (230-340 mg) were anesthetized on ice prior to dissection. In each experiment, fat body isolated from at least three larvae was examined, unless otherwise specified.

Light microscopy and transmission electron microscopy (TEM)

Fat body samples were 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 at room temperature, the samples were dehydrated in an ethanol series and embedded in resin (Epon/Araldite 812 mixture). The sections were obtained with a Leica Reichert Ultracut S (Leica, Nussloch, Germany). Semi-thin sections (0.60 µm) were stained with crystal violet and basic fuchsin and then observed with an Eclipse Ni-U microscope (Nikon, Tokyo, Japan) equipped with a digital camera (Nikon DS-5M-L1). Thin sections (70 nm) were stained with lead citrate and uranyl acetate

and observed with a JEM-1010 transmission electron microscope (Jeol, Tokyo, Japan) equipped with a Morada digital camera (Olympus).

Histochemistry

The fat body was isolated from the larva, immediately embedded in PolyFreeze cryostat embedding medium, and stored at - 80 °C. Cryosections (8 µm) were obtained with a Leica CM 1850 cryostat and stored at - 20 °C until use. To evidence lipid droplets, cryosections were firstly incubated with Baker's fixative (5% formaldehyde, 0.32 M sucrose, 0.15 M sodium cacodylate, 90 mM $CaCl_2$) for 10 min and then treated with Oil Red O (O.R.O.) solution (Bio-Optica) for 15 min. The samples were then incubated with 100 ng/mL DAPI (4',6-diamidino-2-phenylindole) for 5 min. For Periodic Acid-Schiff (PAS) reaction, sections were processed with the Bio-Optica Histopathological PAS kit according to the manufacturer's instructions. Control samples for the PAS reaction were pretreated with diastase which breaks down glycogen. Samples were analyzed with a Nikon Ni-U microscope.

qRT-PCR

The fat body was isolated from the larva, immediately frozen in liquid nitrogen, and stored at – 80 °C. RNA was extracted from the tissue using Trizol Reagent (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA was treated with a TURBO DNA-free Kit (Life Technologies) to remove genomic contamination. Retrotranscription was performed with M-MLV reverse transcriptase (Life Technologies), according to the manufacturer's instructions.

cDNA was used as template for qRT-PCR with the efficiency ranging from 94% to 100%. Primers are listed in Supplementary Table 1.

Reactions were carried out at a final primer concentration of 0.4 µM, 2 µL of cDNA, and iTaq Universal SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) in a total volume of 15 µL. Amplification was performed in CFX Connect Real-Time System (Bio Rad) as follows: 95 °C for 10 min, 95 °C for 15 s (45 cycles), and 60 °C for 45 s. The PCR product was submitted to melting curve analysis to confirm the specific amplification of the target.

RNA and protein quantification

Fat body was dissected and stored as described in the "qRT-PCR" section. 60-100 mg were used for RNA and protein extraction using Trizol Reagent (Life Technologies), according to the manufacturer's instructions. RNA was quantified by measuring the absorbance at 260 nm with a spectrophotometer. Protein concentration was determined according to Bradford (1976), using bovine serum albumin as standard.

RESULTS AND DISCUSSION

***H. illucens* fat body organization**

To obtain information about the general organization of the BSF fat body, we first performed a morphofunctional characterization of this tissue collected from larvae reared on the standard diet.

The fat body was formed by a series of thin sheets distributed in the hemocoel around the gut (perivisceral fat body) and bound to the body wall (peripheral fat body). Notwithstanding local, minimal variations, the tissue was composed of trophocytes arranged in layers of few cells (Fig. 1a). The cytoplasm of trophocytes was mainly occupied by two types of roundish structures, associated to nutrient accumulation. They showed a different morphology (Fig. 1b) and were characterized by differing affinities for selected histochemical

stainings: 1) abundant lipid droplets, freely distributed in the cytoplasm; they were white colored after crystal violet-fuchsin staining (Fig. 1a, b) and reacted with O.R.O. (Fig. 1c); 2) small round granules, characterized by a purplish color following violet-fuchsin staining, were less abundant, and concentrated around the nuclei (Fig. 1a, b). TEM analysis showed a crystal-like organization of the granules (Fig. 1d, e), which clearly indicates they are protein (Tojo et al., 1980). These protein granules appeared fluorescent under ultraviolet light (Fig. 1f), as expected for proteins with aromatic amino acid residues (Locke and Collins, 1965), evidence that substantiates the massive presence of hexamerins in the granules (Haunerland, 1996). Glycogen granules could be observed among the lipid droplets (Fig. 1d).

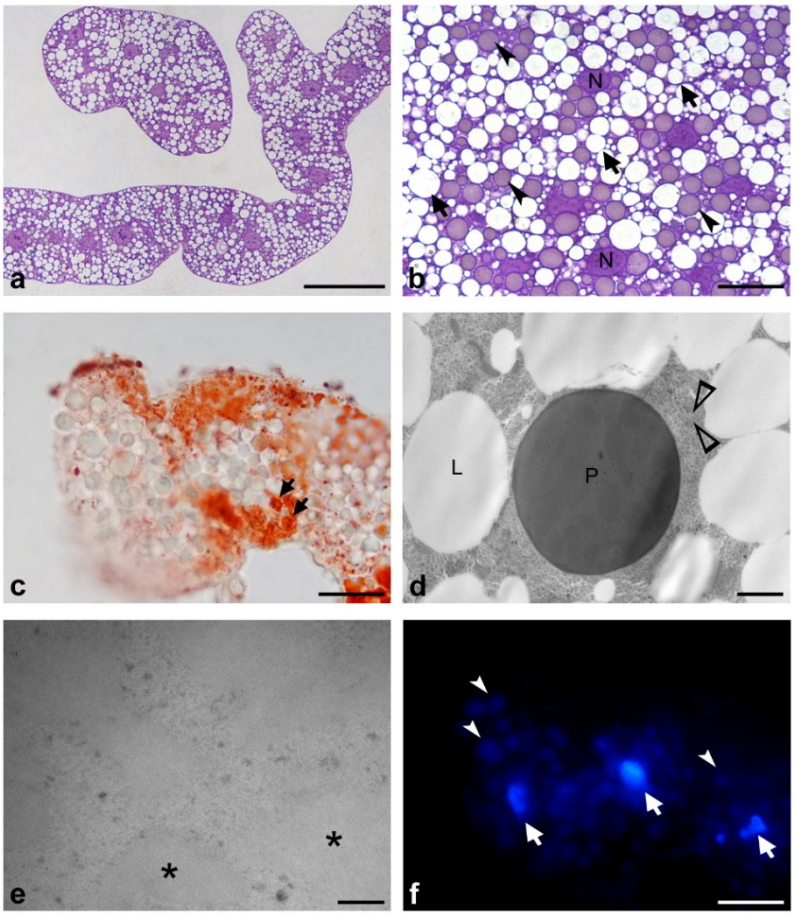

Figure 1. General morphology of *H. illucens* larval fat body. (a) The fat body is formed by thin sheets with few cells. (b) Trophocytes contain a large number of lipid droplets (arrows) and nuclei (N) surrounded by protein granules (arrowheads). (c) O.R.O. specifically stains lipid droplets (arrows). (d, e) TEM analysis reveals the presence of glycogen granules (open arrowheads) among lipid droplets (L) and confirms the crystal-like organization of the protein granules (P), which are better visible at higher magnification (asterisks). (f) The protein granules (arrowheads) can be easily seen due to their intrinsic fluorescence around the DAPI-stained nuclei (arrows). a, b: crystal violet-basic fuchsin staining; c: O.R.O.; d, e: TEM; f: DAPI.
Bars: 50 µm (a), 20 µm (b, c, f), 1 µm (d), 200 nm (e). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Morphological modifications of the fat body in relation to the diet

To analyze the potential modifications of the fat body in relation to nutrient availability, we compared the fat body from larvae reared on the standard diet with that of larvae grown on the vegetable mix. Variation in diet notably modified the tissue morphology (for a comparison see Fig. 2a and b). In particular, both protein granules and lipid droplets were affected (Fig. 2d, e). In fact, trophocytes of larvae fed on the standard diet (Fig. 2a, d, 3c) presented a higher number of protein granules around the nuclei than larvae grown on the vegetable mix (Fig. 2b, e, 3d). Moreover, the lipid droplets from the fat body of larvae fed on the vegetable mix (Fig. 2e) were larger than those from larvae grown on the standard diet (Fig. 2d).

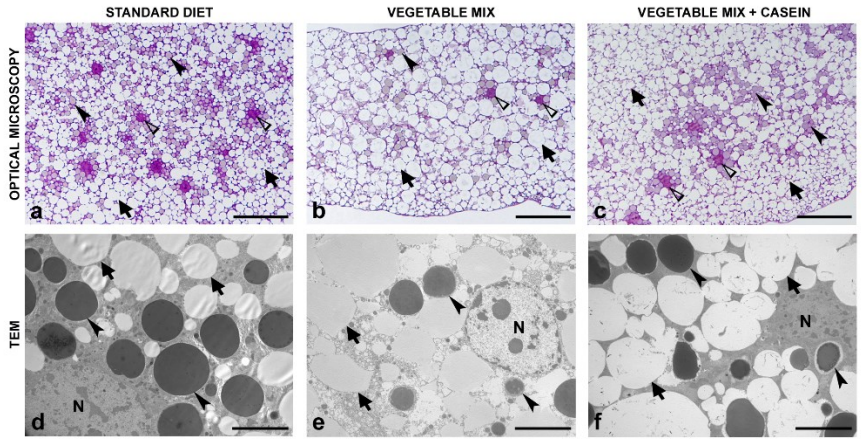

Figure 2. Morphology of the fat body in larvae reared on different diets. The fat body of larvae fed on the standard diet has more protein granules (arrowheads) (a) than the fat body of larvae reared on the vegetable mix (b). The protein granule (arrowheads) density is recovered in the larvae fed on the vegetable mix supplemented with casein (c). The lipid droplets (arrows) in the fat body are smaller when larvae are fed on the standard diet (d) than when grown on the vegetable mix (e). The size of lipid droplets (arrows) is reduced when casein is added to the vegetable mix (f). N and open arrowhead: nucleus. a-c: crystal violet-basic fuchsin staining; d-f: TEM.

Bars: 50 µm (a, b, c), 5 µm (d, e, f). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

The increased intensity of O.R.O. staining in fat body samples of larvae reared on the vegetable mix confirmed the variation in size of the lipid droplets (Fig. 3a, b). PAS staining demonstrated a higher glycogen content in the fat body of larvae reared on the standard diet (see Fig. 3e, f for experimental samples, and Fig. 3g, h for diastase-treated samples). These results demonstrate that the fat body of larvae fed on the vegetable mix has a lower content of protein granules and glycogen, but a higher quantity and density of lipid droplets than larvae reared on the standard diet.

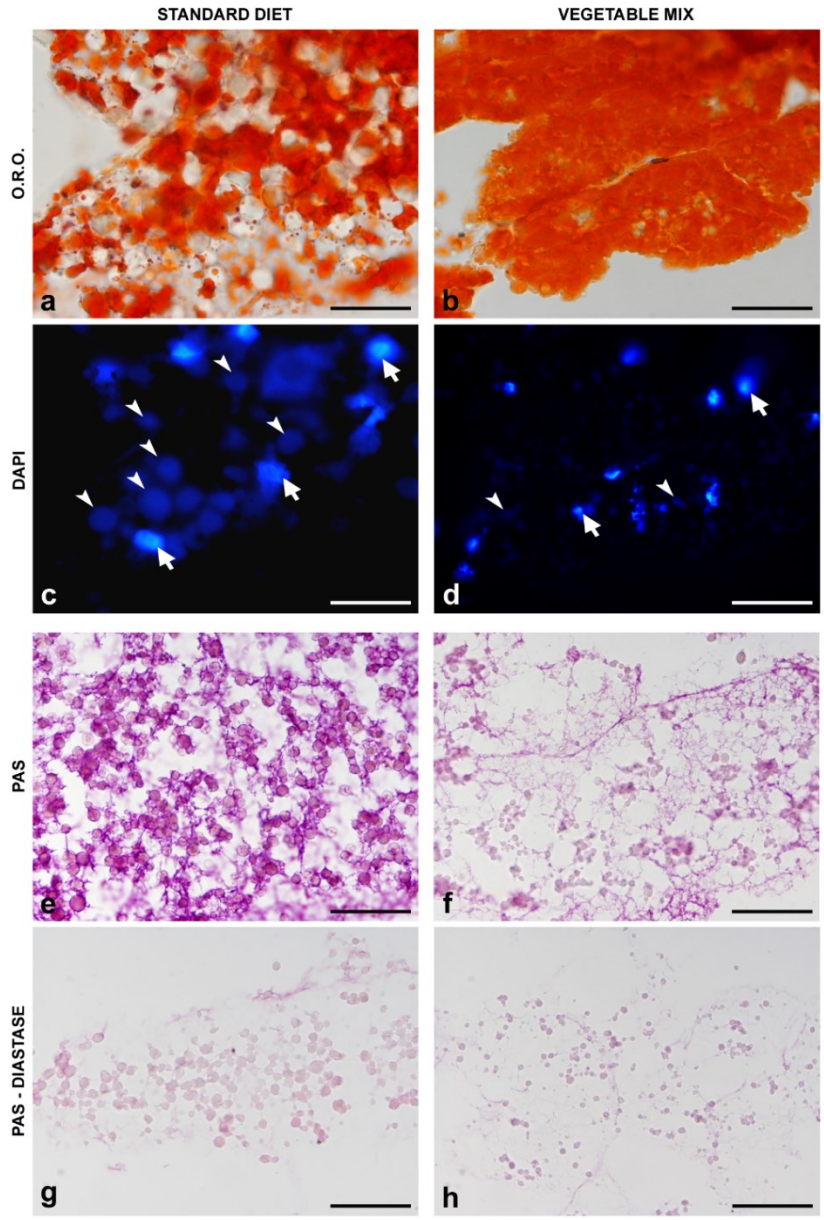

Figure 3. Histochemical analysis of the fat body from larvae grown on different diets. The lower lipid content and the higher number of protein granules of larvae grown on the standard diet (a, c) compared to larvae reared on the vegetable mix (b, d) are confirmed by histochemistry (red staining for lipids and blue fluorescence for protein granules). Larvae fed on the standard diet show more glycogen (magenta staining) in their fat body cells (e) than larvae grown on the vegetable mix (f). Glycogen

deposits disappear after the treatment with diastase (g, h). a-d: O.R.O.-DAPI staining; e, f: PAS; g, h: PAS-diastase.
Bars: 50 µm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Since the growth of larvae on a protein-poor diet (i.e., vegetable mix) induced consistent modifications of the fat body and the storage reserves were distributed differently inside the tissue, we tested whether adding protein to the vegetable mix could restore the morphological organization of the fat body. To this aim we chose casein, a protein source with a good balance of essential amino acids, which is broadly used to formulate insect diets (Cohen, 2015). According to our hypothesis, the density of protein granules could be fully recovered and the size of the lipid droplets was partially restored by adding 5% casein to the vegetable mix (Fig. 2c, f). As predicted, the protein supply increased the RNA and protein content of the larval fat body (Table 2). It is worth noting that nutrient withdrawal is known to reduce transcriptional rates of RNA polymerase (Jona et al., 2000; Moir and Willis, 2013) and consequently affects RNA production. Here, RNA levels were experimentally increased by supplying casein. This result not only confirms the recovery of morphological features in the fat body, but also demonstrates that protein accumulation within the round granules depends on the larval protein intake.

Table 2. Effect of different diets on the RNA and protein content in the larval fat body. VEG, vegetable mix; VEG+CASEIN, vegetable mix supplemented with 5% (w/w) casein. Mean ± SEM of four biological replicates. Data are presented as µg of RNA or protein per g of fresh tissue. Data were compared using Student's *t*-test and were considered significantly different for $p<0.05$.

	RNA (µg/g tissue)	Protein (µg/g tissue)
VEG	0.97 (±0.07)	4412 (±407)
VEG + CASEIN	2.39 (±0.17)	6558 (±516)
	t-test $p = 0.0001$	*t*-test $p = 0.009$

In summary, these data demonstrate that morphofunctional modifications occur in the larval fat body in response to differing nutrient availability.

Nutrient supply and gene expression

The modifications observed in the fat body in relation to the diet administered to the larva prompted us to investigate the potential pathways that are involved in this response. Thus, we analyzed some key genes that play a pivotal role in protein and lipid accumulation/mobilization in the fat body of larvae reared on vegetable mix with or without protein supplementation.

Supplementing casein to the protein-poor diet (i.e., vegetable mix) increased the expression of the hexamerin genes, i.e., *HiLSP-1* and *HiLSP-2*, which are responsible for protein accumulation in the fat body (Fig. 4a, b). Adding protein also affected the expression of *HiLsd1*, which regulates the lipolytic activity, but did not modify *HiLsd2* expression, a gene responsible for lipid accumulation (Fig. 4c, d).

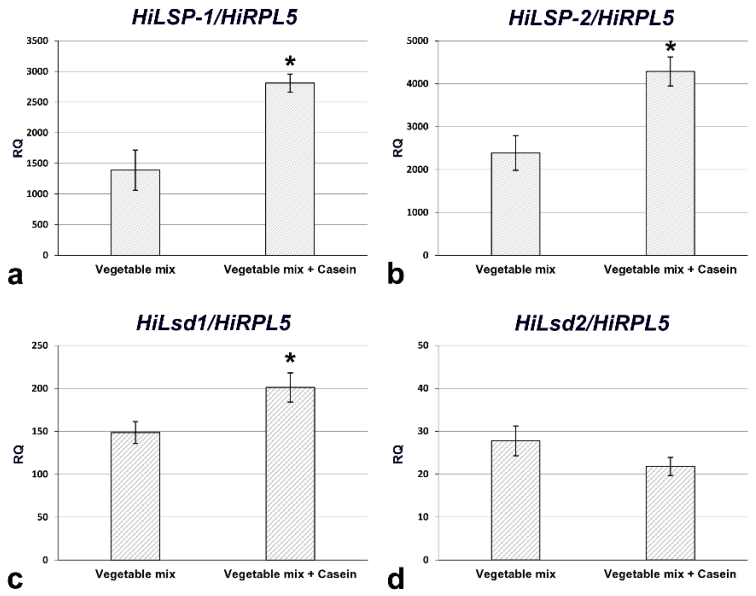

Figure 4. Effect of different diets on gene expression. Relative quantification (RQ) of the expression of genes related to nutrient accumulation in *H. illucens* fat body using RPL5 gene as internal control. a: *HiLSP-1*; b: *HiLSP-2*; c: *HiLsd1*; d: *HiLsd2*. The figures are mean ± SEM of four biological replicates. Data were compared with Student's *t*-test and are indicated with an asterisk when significantly different ($p<0.05$).

Hexamerins are known as the major reserve proteins in insects (Telfer and Kunkel, 1991). The increased gene expression of both hexamerins (Fig. 4a, b) produced a higher protein content in the fat body, as demonstrated by fat body morphology and protein quantification assay (Table 2). It is expected that protein content is also concomitantly higher in the hemolymph since, in feeding larvae, hexamerins are mainly secreted into the hemocoel, while they are only partially retained in the fat body (Munn et al., 1969). As a protein-poor food substrate, hexamerin expression was lower with the vegetable mix. Interestingly, the same expression pattern was previously observed in *Drosophila melanogaster* larvae undergoing starvation (Handke et al., 2013). Hexamerin LSP-2 is

accumulated in females of *Sarcophaga crassipalpis* that feed on protein (liver), resulting in positive effects on reproduction (Hahn et al., 2008). The importance of this gene at later developmental stages could be envisaged in *H. illucens* as well since the reserves accumulated in the larvae are used during the adult phase (Gobbi et al., 2013). Considering the conserved function of *LSP-2*, modulating its expression in BSF substantiates a link between protein accumulation in the larva and oviposition at the adult phase.

HiLsd1 was upregulated in larvae fed on the vegetable mix supplemented with casein (Fig. 4c). This perilipin is known to activate lipolysis in lipid droplets when phosphorylated by protein kinase A in response to adipokinetic hormone (Patel et al., 2005). In addition to regulating the function of Lsd1 by phosphorylation, the transcription of its gene is downregulated during starvation (Grönke et al., 2005). Probably, the low protein content of the vegetable mix triggers the same regulatory response of starvation and, when casein is added to the vegetable mix, *HiLsd1* gene expression is subjected to a transcriptional increase. The modulation of *HiLsd1* gene expression is thus a clear indication that food composition affects lipolysis, causing different lipid/protein rates in the larvae depending on the diet. Conversely to *D. melanogaster*, where the modulation of *Lsd2* gene expression directly affects lipid storage (Grönke et al., 2003), no variation in transcription of this gene was observed in *H. illucens* (Fig. 4d), excluding its involvement in differential triacylglycerol storage under these experimental conditions. These data show that a low protein content in the diet not only affects protein accumulation in the larvae, but also increases lipid accumulation as triacylglycerol. Similarly, in sugar-fed *D. melanogaster* larvae the expression of genes involved in fat biosynthesis is upregulated compared to yeast-fed (protein-rich

substrate) larvae (Zinke et al., 2002). Thus, lipid accumulation appears to be related to the activation of fat biosynthesis. Free sugar, which represents the most abundant carbon source in vegetable mix, is used to produce triacylglycerol by the larvae, accumulating it in the fat body (Carvalho et al., 2012).

In summary, our results demonstrate that metabolic adjustment to a protein-rich diet determines the accumulation of protein in the larval fat body, due to higher expression of hexamerin genes, a marker that can thus be used to monitor the nutritional status of the larvae. The use of protein-poor diets can thus lead to the production of poorly nourished larvae with a lower protein and a higher lipid content.

CONCLUSIONS

The fat body of BSF larva is mainly composed of trophocytes, which are rich in lipid droplets and protein granules. The low protein content in the diet affects both protein and lipid accumulation in these cells, leading to a lower abundance of protein granules and larger, more abundant, lipid droplets. Moreover, a protein-poor diet leads to low expression levels of the two hexamerin genes (*HiLSP-1* and *HiLSP-2*) and the fat-related gene *HiLsd1*. Gene expression increases and morphological features are recovered after adding protein to the diet. Taken together, our results underscore the central role of the fat body in producing nutrient-rich larvae by manipulating the insect diet.

SUPPLEMENTARY MATERIALS

Supplementary Table 1. Primer sequences used in this study. LSP, Larval serum protein; Lsd, Lipid storage droplet; RPL5, Ribosomal protein L5.

Primer name	Sequence
*HiLSP-1*_FW	ACATCAACGACAACCGTGAA
*HiLSP-1*_RV	GAGTACATGGTGCGGTCCTT
*HiLSP-2*_FW	CCAGGACTTCGCTACTACGG
*HiLSP-2*_RV	GTCAATGACGTCCATGATGC
*HiLsd1*_FW	GAAATTTGGGATCGGAACCT
*HiLsd1*_RV	GGCTTCCTTTCCTTGCTTCT
*HiLsd2*_FW	CCGATGATCCTGTCCTTCAT
*HiLsd2*_RV	TTTAATTTGGCGGGAAACAG
*HiRPL5*_FW	AGTCAGTCTTTCCCTCACGA
*HiRPL5*_RV	GCGTCAACTCGGATGCTA

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CHAPTER 5

The adult *Hermetia illucens* (Diptera: Stratiomyidae) is endowed with a functitional digestive system

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Keywords: black soldier fly, feeding habits, insect midgut, metamorphosis, tissue remodeling, stem cells

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ABSTRACT

The increasing demand for food of animal origin and, consequently, additional feed production has generated an urgent need for alternative and more sustainable protein sources. In this setting, the larvae of black soldier fly (BSF), *Hermetia illucens*, emerge as a relevant resource since they can convert low-quality biomass into nutritionally valuable proteins. While in recent years considerable effort has been invested to address important issues that could support the growth of the emerging industrial sector of edible insects and exploit BSF larvae in the feed market, including their use as a source of bioactive molecules, information on the biology of *H. illucens* 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 need to eat. In the present work, we therefore investigated the remodeling process of BSF larval midgut during metamorphosis, analyzed the morphology and function of the adult midgut, and evaluated the feeding habits of the fly.

Our results demonstrate that, as in other holometabolous insects, the larval midgut of *H. illucens* is removed during metamorphosis and a new pupal-adult epithelium is formed by proliferation and differentiation of intestinal 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. This new scenario not only opens up the possibility to manipulate the

feeding substrate of the fly to improve its performances in mass rearing procedures, but could also provide insights into the safety of using this insect for feed purposes.

INTRODUCTION

The larvae of the black soldier fly (BSF), *Hermetia illucens* (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 (Cickova et al., 2015; Meneguz et al., 2018; Nguyen et al., 2015). 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, the European Commission Regulation N°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 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 and Sheppard, 2002; Tomberlin et al., 2002; Tomberlin et al., 2009). 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 one study has evaluated the longevity of adult BSF fed on sugar, but the functional properties of the digestive system were not considered (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 involved in food digestion and nutrient absorption (Dow, 1986). Notwithstanding its apparent simplicity (the insect midgut consists of only a single-layered epithelium surrounded by a basal lamina and striated muscle fibers), it is characterized by a marked regionalization and cellular diversity. This organization is necessary to optimize digestion by enabling sequential functions ranging from 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 non-hematophagous dipteran larvae examined so far, the midgut presents three regions with differing luminal content pH (Bonelli et al., submitted; Dubreuil, 2004; Lemos and Terra, 1991; Shanbhag and Tripathi, 2009; Terra et al., 1988b). As in other holometabolous insects, the adult midgut of the common fruit fly, *Drosophila melanogaster*, a model species among Diptera, is generated de novo during metamorphosis. In fact, the larval midgut degenerates completely during larva-adult transition 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 characterized 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 analyzed 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 monitored the feeding habits of the fly, investigating the morphology and function of the midgut of the adult insect and its ability to exploit the ingested nutrients by means of vital stains and measuring digestive enzyme activity.

Our results demonstrate that, similarly to other holometabolous insects, the larval midgut of *H. illucens* is completely removed during metamorphosis and a new pupal-adult epithelium 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 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).

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

The larvae were reared on standard diet for Diptera (Hogsette, 1992) as previously reported (Bonelli et al., submitted; Pimentel et al., 2017). After eclosion, the flies were maintained at 27.0 ± 0.5 °C and 70 ± 5% relative humidity. A 36W/765 FLUO lamp (Osram, 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 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 (SEM)

For three-dimensional 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, The Netherlands).

Light microscopy and transmission electron microscopy (TEM)

The midgut was isolated from last instar larvae, pupae (day 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 a Nikon Eclipse Ni-U microscope (Nikon, Japan) equipped with a TrueChrome II S digital camera system (Tucsen Photonics, China). Thin sections were stained with uranyl acetate and lead citrate and observed by using a Jeol JEM-1010 electron microscope (Jeol, Japan) equipped with an Olympus Morada digital camera (Olympus, Germany).

Analysis of stem cell proliferation

Midguts were isolated from pupae (day 4, 8, and 10) and flies (day 1) and homogenized with a T10 basic ULTRA-TURRAX (IKA, 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, 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, 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 1:1,000 in 2% milk in TBS; Merck-Millipore) and anti-GAPDH antibody (dilution 1:2,500 in 5% milk in TBS; Proteintech, USA) to ensure equal gel loading. Antigens were revealed with an anti-rabbit HRP-conjugated secondary antibody (diluted 1:7500; Jackson ImmunoResearch Laboratories, USA) and immunoreactivity was detected with SuperSignal chemiluminescence substrates (Thermo Fisher Scientific, USA).

Histochemistry

Midgut samples were isolated from last instar larvae and pupae (day 4 and 8), embedded in polyfreeze cryostat embedding medium after dissection, and stored in liquid nitrogen until use. Subsequently, 7-m-thick cryosections were obtained with a Leica CM 1850 cryostat and slides were immediately used or stored at -20 °C. Sections were processed with Bio-Optica Histopatological kit (Bio-Optica, 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 (day 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, 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, 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) 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 °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 × g for 10 min at 4 °C and supernatant was collected. Protein concentration was determined by 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 °C. The reaction was stopped by adding 300 µl of 12% (w/v) trichloroacetic acid at 4 °C. The samples were maintained for 30 min on ice and then centrifugated at 15,000 × g for 10 min at 4 °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 substrate (Bernfeld, 1955). A standard curve was determined through linear regression of the maltose absorbance at 540 nm. Frozen samples of midgut were thawed at 4 °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 × g for 10 min at 4 °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 were 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 °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 °C for 15 min, then cooled in ice to 25 °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 midgut were thawed at 4 °C and homogenized in 1 ml/100 mg tissue of Tris-HCl 50 mM, pH 7.5. Samples were then centrifuged at 15,000 × g for 10 min at 4 °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 continuous absorbance reading at 410 nm at 45 °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, USA). Frozen samples of midgut were thawed at 4 °C and homogenized in the Invertase Hydrolysis buffer provided with the kit (40 µl buffer/1 mg of midgut sample). The homogenate was centrifuged at 15,000 × g for 10 min at 4 °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 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 Polimerase (Promega, USA) (95 °C for 30 s, Tm - 2 °C for 30 s, 72 °C for 30 s, 35 cycles) and a MyCycler Thermal Cycler System (Bio-Rad, USA). The primers used for PCR are listed in Table 2. The primers for *Hiα-glucosidase* were designed on conserved regions of this gene in other insect species and the sequence was checked by sequencing the PCR product.

Table 2. Sequence of primers used in this study.

Gene name	Accession number	Primer sequences	Melting temperature (Tm)	Length of amplificate
HiTrypsin	HQ424575	F: ATCAAGGTCTCCCAGGTC R: GGCAAGAGCAATAAGTTGGAT	56 °C	126 bp
HiChymotrypsin	HQ424574	F: AGAATGGAGGAAAGTTGGAGA R: CAATCGGTGTAAGCAGAGACA	57 °C	109 bp
Hiα-glucosidase	-	F: GGCTTTCAGTTGCTCCGTTA R: AGGCTCGTTATTGATGTCGC	58 °C	127 bp

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 x 30 mm Petri dish. A filter paper (214 cm^2) was put on 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., 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, USA). For all treatments, flies were dissected every 24 hours from 1 to 6 days after beginning of the experiment. For the first and second treatments the alimentary canal was mounted with Citifluor (Citifluor Ltd, UK) 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 section "Light microscopy and transmission electron microscopy".

Fly longevity

Forty newly emerged flies (<15 h after eclosion) were placed in 30 x 30 x 30-cm cages and maintained under the environmental conditions reported in the section "Experimental animals". 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 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 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 mantained open by cuticular ribs (Fig. 1C), giving them a superficial similarity to 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* (Broce and Elzinga, 1984; Giangaspero and Broce, 1993; Kovacs et al., 1990). The mouthparts presented many sensilla (Fig. 1B) protruding from the cuticle, which are probably involved in chemo- and mechano-reception.

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

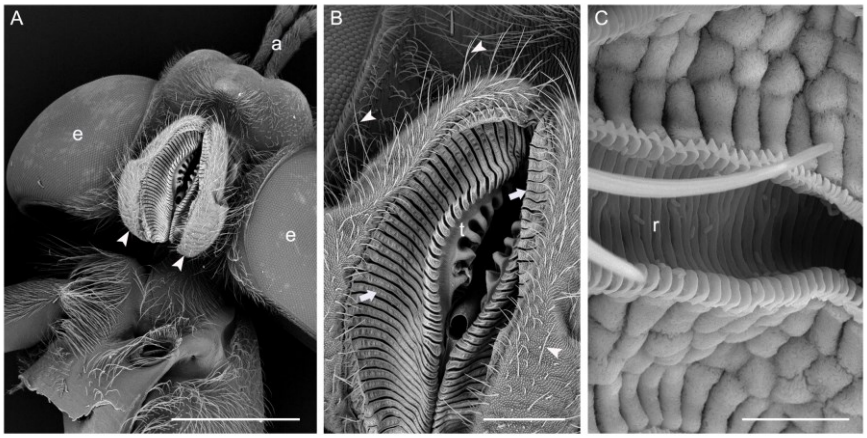

Figure 1. SEM analysis of BSF adult mouthparts. (**A**) Ventral view of BSF head in which everted labella (arrowheads) are visible. (**B**) View of the sponging mouthparts where tooth-like structures (t), pseudotracheae (arrows), and sensilla on the labella (arrowheads) can be observed. (**C**) Detail of pseudotracheae characterized by cuticular ribs (r). a: antennae; e: compound eye. Bars: 1 mm (**A**), 200 µm (**B**), 10 µm (**C**).

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 (Hakim et al., 2010); 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., submitted), 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 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 (Figs. 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 (Figs. 2C, F). At late pupal stage (day 10), a newly forming epithelial layer was visible, while the larval cells were pushed in the lumen (Figs. 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).

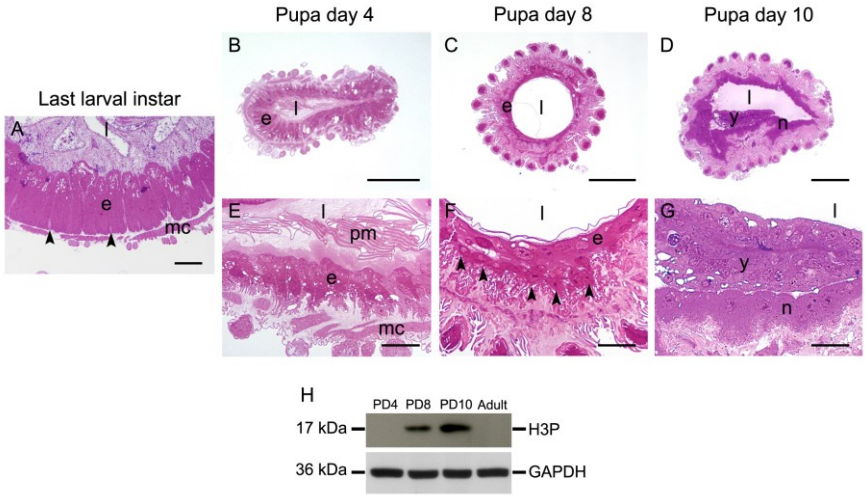

Figure 2. Modification of the larval midgut during metamorphosis. (**A**) Cross-section of the midgut epithelium of last instar larvae. Stem cells (arrowheads) are visible at the base of the epithelium. (**B, E**) Cross-sections

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 H3 (H3P). (**E, F, G**) are details at higher magnification of (**B, C, D**), respectively. e: epithelium; l: lumen; pm: peritrophic matrix; mc: muscle cells. Bars: 25 μm (**A, E-G**), 100 μm (**B-D**).

During the pupal stage (day 8-10) the newly forming midgut was characterized by features that are typical of a secretory/absorptive epithelium (Figs 3A-D). In fact, the apical membrane formed microvilli (Fig. 3A), abundant rough endoplasmic reticulum and mitochondria could be observed in the cytoplasm (Fig. 3B), and the cells were linked by septate junctions (Fig. 3C). Moreover, glycogen granules and lipid droplets were present in the cytoplasm (Figs. 3A, D). 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 (Figs. 2D, G, 3E). 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 (Figs. 3E, F) and unusual organization of the nuclear chromatin (Fig. 3G), the large part of the cells showed an intact morphology (Fig. 3G) 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. 3H).

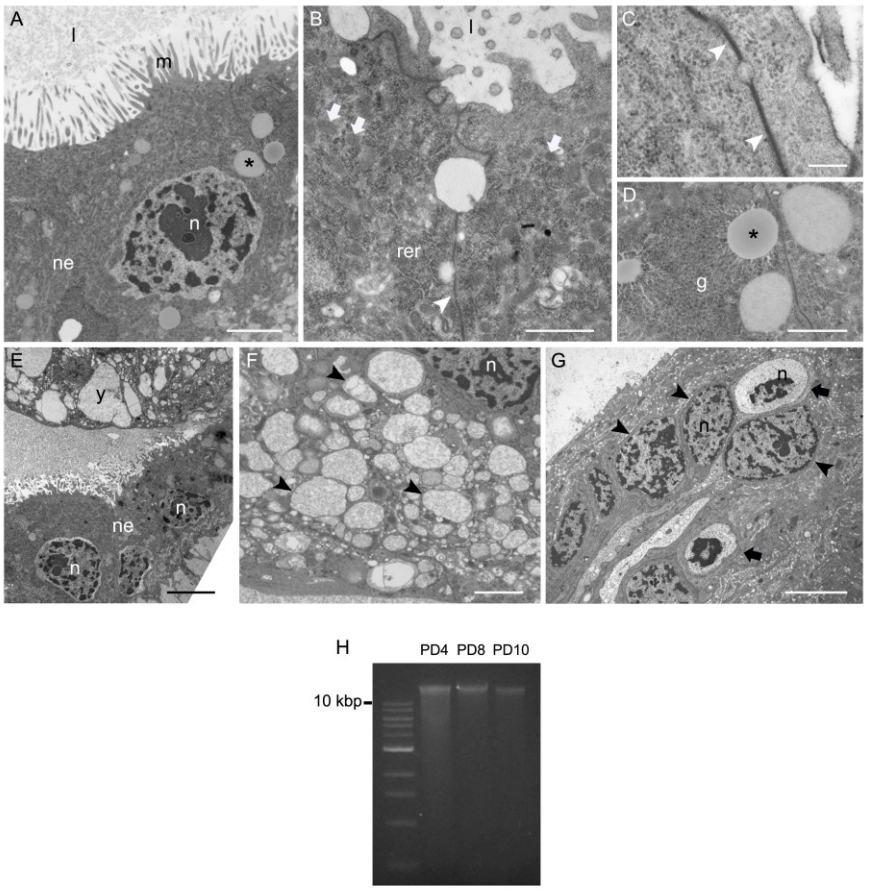

Figure 3. Ultrastructural analysis of pupal midgut. (**A-D**) The newly forming midgut epithelium (ne) displays microvilli (m) in the apical membrane (**A**), abundant rough endoplasmic reticulum (rer), and mitochondria (arrows) in the cytoplasm (**B**). Septate junctions (arrowheads) are present among columnar cells (**B, C**). Glycogen (g) and lipid droplets (asterisks) can be observed in the cytoplasm (**A, D**). (**E**) The newly forming midgut epithelium (ne) progressively detaches from the larval midgut, which forms the yellow body (y). (**F**) Vacuoles (arrowheads) in the cytoplasm of yellow body cells. (**G**) Yellow body that contains a high number of cells with an intact nucleus (arrowheads) and some cells with pyknotic nuclei (arrows). (**H**) Ladder analysis of genomic DNA from midgut cells at pupa day 4, 8, and 10. l: lumen; n: nucleus. Bars: 2 µm (**A, F**), 1 µm (**B, D**), 500 nm (**C**), 5 µm (**E, G**).

The histochemical analysis gave evidence that long-term storage molecules were mobilized during larva-pupa transition: in particular, a reduction of glycogen (Figs. 4A-C) and lipid (Figs. 4G-I) reservoirs was detected in the larval midgut epithelium at early pupal stage (Figs. 4A, B, G, H) and no reactivity was present inside the yellow body (Figs. 4C, I). Specificity of the PAS reaction towards glycogen was confirmed by treatment with diastase (Figs. 4D-F).

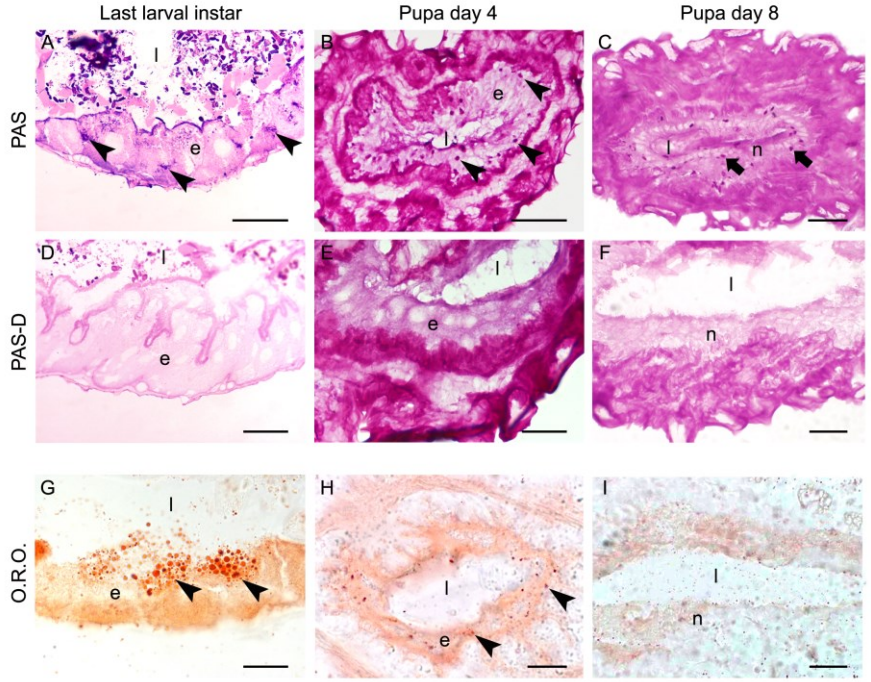

Figure 4. Histochemical analysis of the midgut epithelium. **(A-C**) PAS. Staining for PAS reveals that glycogen deposits (purple, arrowheads) disappear in the larval midgut epithelium at pupal stage. A signal is visible only in the newly forming midgut epithelium (arrows). (**D-F**) Glycogen deposits 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 pupal stage. e: larval epithelium, l: lumen; n: newly forming epithelium. Bars: 50 µm (**A, B**), 20 µm (**C-E, G, H**), 10 µm (**F, I**).

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. 5A). A large crop was associated to the foregut (Fig. 5B). 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 (Figs. 5C-H). While the anterior (Figs. 5C, F) and posterior (Figs. 5E, 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 (Figs. 5D, G).

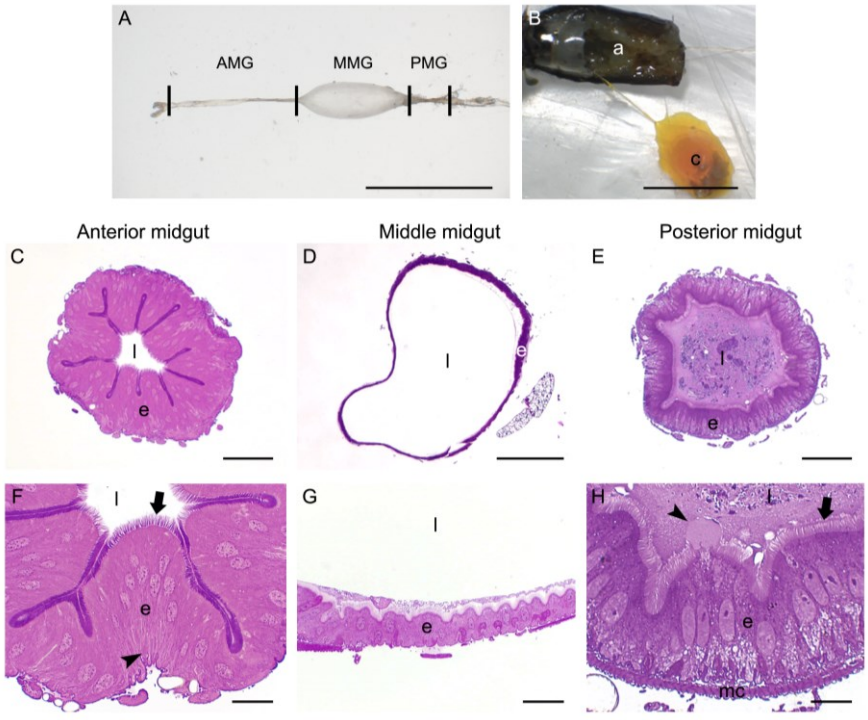

Figure 5. Morphological analysis of the adult midgut. (**A**) General view of the adult midgut subdivided in anterior (AMG), middle (MMG), and posterior (PMG) midgut. (**B**) General view of the crop (c) associated to the gut. (**C, F**) Cross-section of the anterior midgut characterized by a thick epithelium 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**) are details at higher magnification of (**C, D, E**), respectively. a: abdomen of the fly; e: epithelium; l: lumen; mc: muscle cells. Bars: 5 mm (**A**), 3 mm (**B**), 50 μm (**C, E**), 200 μm (**D**), 10 μm (**F-H**).

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. 6A) and apical brush border (Fig. 6B), and were linked by septate junctions (Fig. 6C). A great number of mitochondria was present in the apical region of the cells (Figs. 6B, D). Glycogen granules and abundant rough endoplasmic reticulum were visible in the cytoplasm (Fig. 6D). This evidence correlated well with a consistent secretory activity. In fact, we observed microvilli with an enlarged tip (Fig. 6E), 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. 6F). Interestingly, the peritrophic matrix was not present and the lumen content was in direct contact with the brush border (Figs. 6G, H). The midgut epithelium was supported by a thick basal lamina (Fig. 6A, I) and muscle (Fig. 6A). In all three midgut regions, endocrine cells localized in the basal region of the epithelium (Fig. 6J), containing electron-dense granules in the cytoplasm (encircled by a

membrane) (Fig. 6K), were visible. Sparse, small round cells were localized in the basal region of the epithelium (Fig. 6L): 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 pupal stage (Fig. 3G), these cells showed a vacuolated cytoplasm, but the nucleus appeared intact (Fig. 6M).

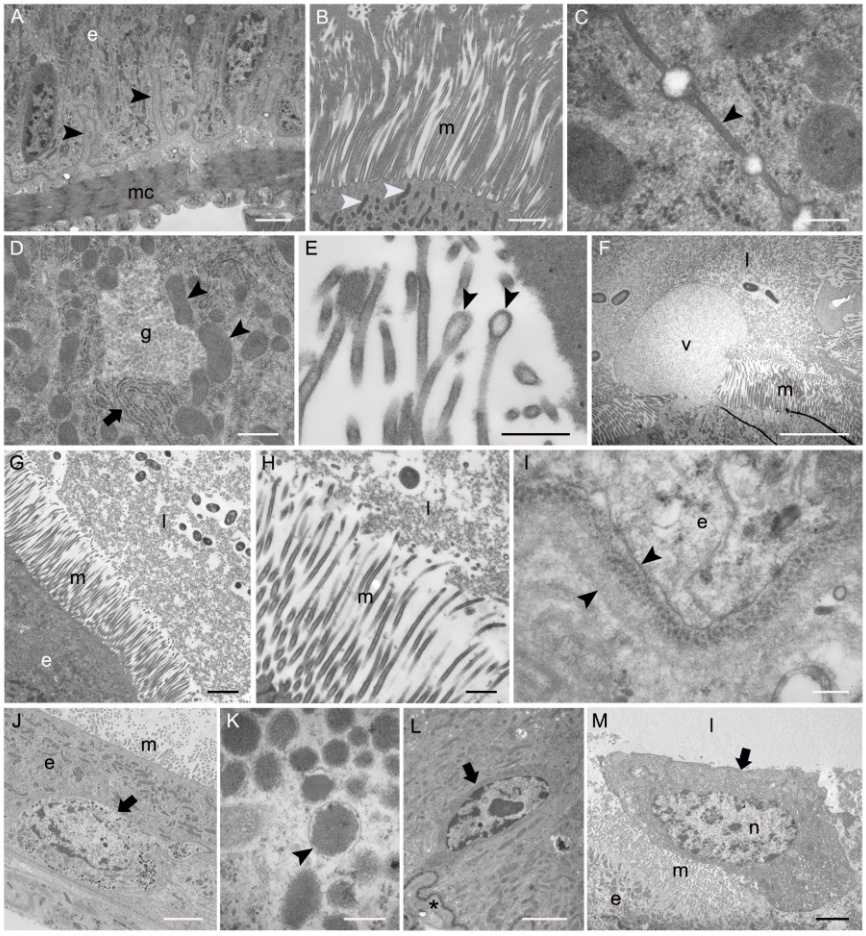

Figure 6. Ultrastructural analysis of the adult midgut - TEM. (**A**) A thick muscle layer (mc) surrounds the epithelium, characterized by developed basal infolding (arrowheads). (**B**) A high number of mitochondria (arrowheads) are present in the apical region of the cells, under the brush border (m). (**C**) Detail of septate junctions (arrowhead) 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 (arrowheads). (**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 brush border (m) due to the absence of a peritrophic matrix. (**I**) A thick basal lamina (arrowheads) 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 (arrowhead) (**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). e: epithelium; l: lumen; m: microvilli. Bars: 2 μm (**A, G, H, J, L, M**), 1 μm (**B**), 500 nm (**D, E**), 200 nm (**C, I, K**), 5 μm (**F**).

Analysis of the 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) (Figs. 7A, A'). In addition, the analysis of the labella (Figs. 7B, B'), of the dorsal and ventral translucent windows on the abdomen (Figs. 7C-D'), and of the fecal spots (Figs. 7E, 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.

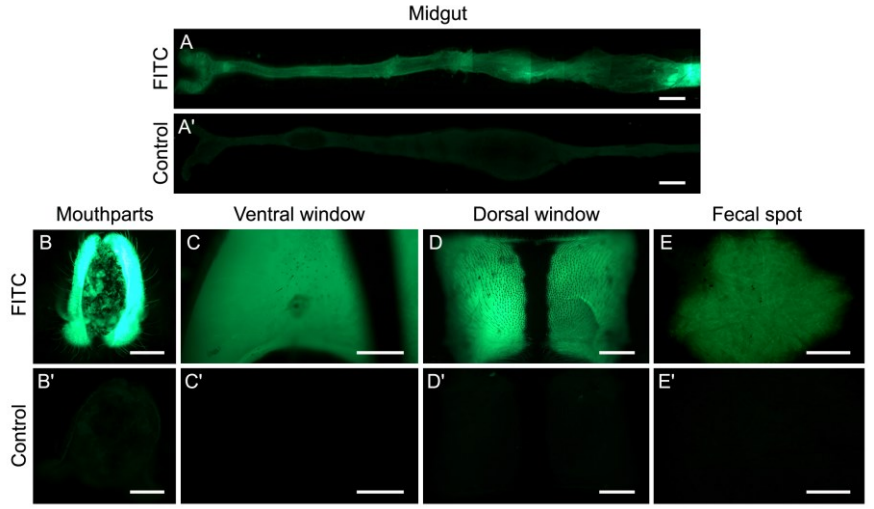

Figure 7. Analysis of the transit of ingested food - FITC. (**A-D**) Midgut, mouthparts, and ventral and dorsal translucent windows of flies fed with banana and FITC. (**E**) Fecal spot produced by flies fed with banana and FITC. (**A'-E'**) Midgut, mouthparts, and ventral and dorsal translucent windows, and fecal spot of unfed flies or fed with banana (control). Bars: 500 µm (**A, A', E, E'**), 250 µm (**B-D, B'-D'**).

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 Figures 8A-C, while in control insects (no food substrate administered) some spots were observed from day 1 to day 6, the number of fecal spots rapidly increased from day 1 up to day 12 in animal fed with food coloring. It is noteworthy that, while in control flies the color of the spot was light brown, the spots were dark purple in fed animals (Figs. 8D, E), thus demonstrating the transit of the stain throughout the alimentary canal. Production of fecal spots could be clearly observed in the video (Figs. 8F-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 six days after administering the food substrate and analyzed at the ultrastructural level. TEM analysis demonstrated the presence of gold particles in the midgut lumen (Figs. 8I, J).

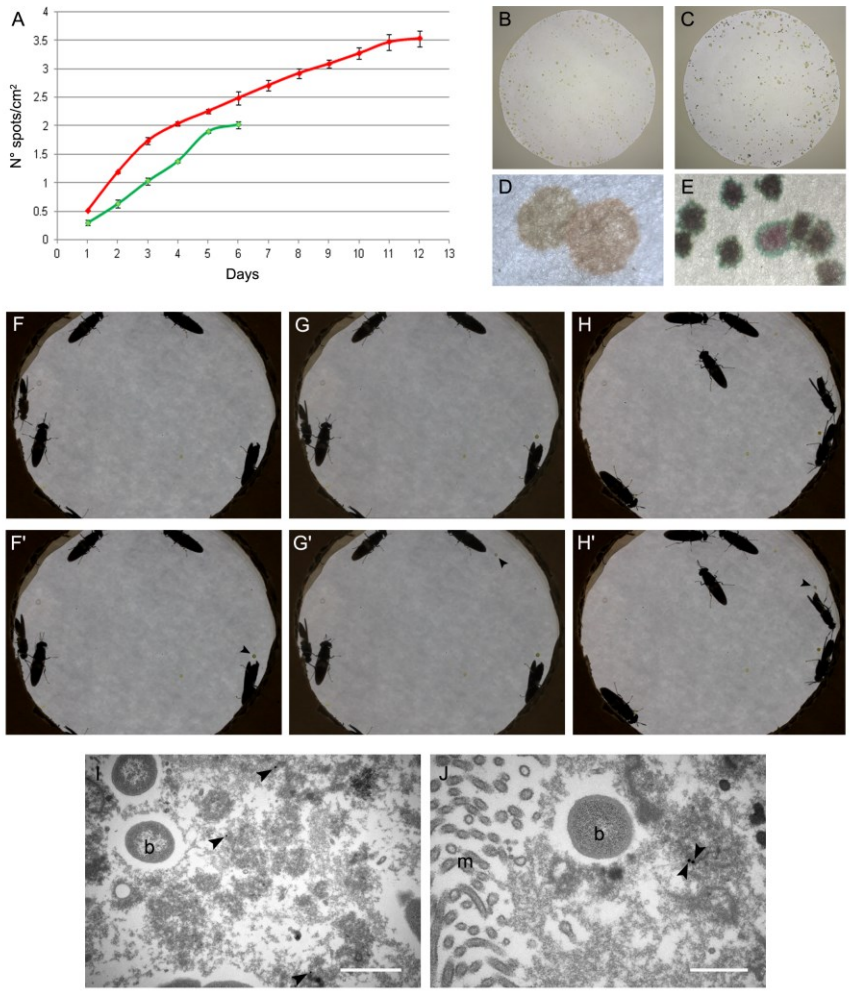

Figure 8. Evaluation of the transit of ingested food - Food coloring and gold-conjugated protein A. (**A**) Spot counting. Flies fed with banana to which purple food coloring was added are indicated in red; starved flies

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**) are details at higher magnification of (**B, C**), respectively. b: bacteria; m: microvilli. Bars: 1 µm (**I**), 500 nm (**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 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 evaluated the activity of enzymes involved in the final phase of sugar and protein digestion, i.e., sucrase and aminopeptidase N, two hydrolytic enzymes present in the apical membranes of insect absorptive epithelia (Terra and Ferreira, 1994). The activity of both enzymes could be measured in the homogenate of the adult midgut (Table 3).

Table 3. Activity of digestive enzymes. Mean ± SEM, with number of replicates in parentheses, expressed in units as described in Materials and methods.

Total proteolytic activity (U)	α-amylase activity (U)	APN activity (U/mg)	Sucrase activity (U/ml)
1.64±0.46 (4)	Non-detectable	0.27±0.05 (4)	30.45±1.33 (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→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. 9).

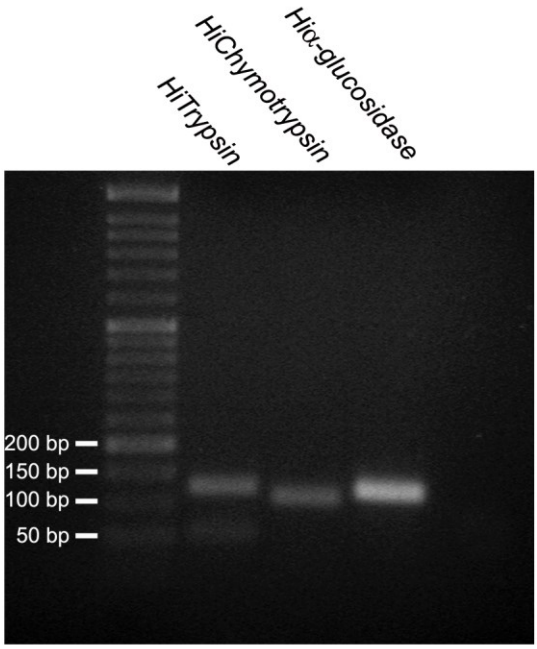

Figure 9. PCR analysis of the expression of *HiTrypsin*, *HiChymotrypsin*, and *Hiα-glucosidase* in the midgut of adult BSF.

Fly longevity under different nutrient conditions

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 five days after beginning the experiments, and all the insects died about one week after eclosion. On the contrary, a higher survival rate was observed when water or water and sugar

cube were provided to flies (Fig. 10A). A significant difference in fly longevity was observed under the three different conditions, with the best performance obtained when water and suger cube were provided to insects (Fig. 10B).

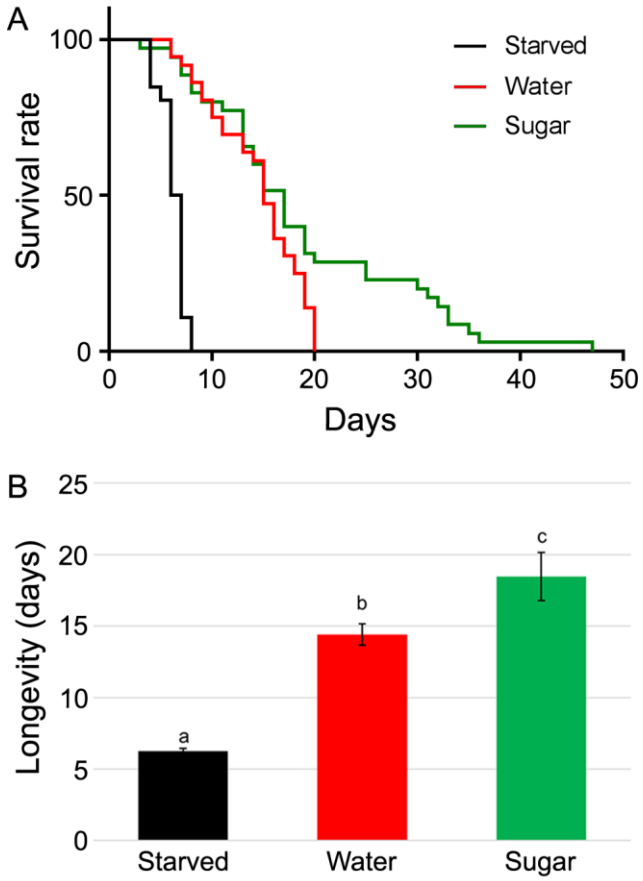

Figure 10. (**A**) Survival curves of flies under different dietary conditions. (**B**) Fly longevity under different nutrient conditions. The values are reported as mean ± SEM of at least 40 individuals. Different letters denote significant differences (ANOVA test followed by Tukey's test. ANOVA *p*-value<0.001, Tukey's test *p*-values: Water *vs* Starved *p*<0.001, Sugar *vs* Starved *p*<0.001, Sugar *vs* Water *p*<0.05).

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 takes 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 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 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 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; Tettamanti et al., 2008; Tettamanti et al., 2011). In *Bombyx mori* it has been previously demonstrated that the complete digestion of larval midgut cells by autophagy, followed by their death, and the release of their content in the lumen of the newly forming epithelium can contribute to trophically substain 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 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 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 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., submitted). 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., in press; 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 (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 (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 brachicerous flies such as *M. domestica* (Shina, 1975; Terra et al., 1988a) and *H. illucens* larvae (Bonelli et al., submitted; Pimentel et al., 2018), α-amylase activity was not recorded, evidence indicating the inability of the fly to digest complex sugars. However, monosaccharides ready to be absorbed can be obtained by the hydrolysis of sucrose thanks to sucrase activity.

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 food processing or elimination of excess water, involves the crop, a foregut organ present in Diptera (Stoffolano and Haselton, 2013; Stoffolano et al., 2008). 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 contradicts previous studies that used sugar solutions to rear BSF flies (Furman et al., 1959; Nakamura et al., 2016), but is definitely contradicted 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 Nakamura's results (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 could be exploited to increase its performance in terms of lifespan and oviposition. In this scenario, our study paves 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 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 the European Commission Regulation N°2017/893 (European Commission, 2017). In fact, regurgitation, defecation, and transtadial transmission have been described as potential routes of pathogen transmission in nonbiting flies (Graczyk et al., 2001; Graczyk et al., 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.

SUPPLEMENTARY MATERIALS

Supplementary 1 - Evaluation of the presence of copper cells in the midgut of adult *H. illucens*

The presence of copper cells in the midgut of adult BSF was investigated through two different approaches.

Materials and methods

A) Evaluation of midgut juice pH

H. illucens flies were fed *ad libitum* with banana, renewing the food substrate every day. Flies were dissected at day four: the lumen content was recovered from the midgut and spotted on pH indicator strips.

B) Copper feeding trials

H. illucens flies were fed *ad libitum* on standard diet prepared with a water solution containing 4 mM $CuCl_2$, renewing the diet every day. Flies were dissected at day four: the midgut was isolated and

analyzed under fluorescence microscope at 365 nm excitation wavelength.

Results

Copper cells are able to acidify the lumen of BSF larval midgut through the extrusion of H^+, leading to a very acidic pH (pH = 2.1 ± 0.1) in the middle midgut region (Bonelli et al., submitted). In addition, in copper-fed fly larvae, the accumulation of this metal in copper cells determines the emission of an orange fluorescence (Bonelli et al., submitted; McNulty et al., 2001), probably due to the formation of a metallothionein-copper complex (McNulty et al., 2001). In *H. illucens* flies, the anterior-middle region of the midgut showed a slightly acidic pH (Fig. S1A), while the posterior midgut presented a basic pH (Fig. S1B). Therefore, the absence of a midgut region with a very acidic pH in the lumen strongly suggests that the epithelium does not contain copper cells. The analysis of the midgut from flies subjected to copper feeding trials did not reveal any orange fluorescence due to the metal intake (Fig. S1C), thus confirming the lack of copper cells in the midgut of adult BSF.

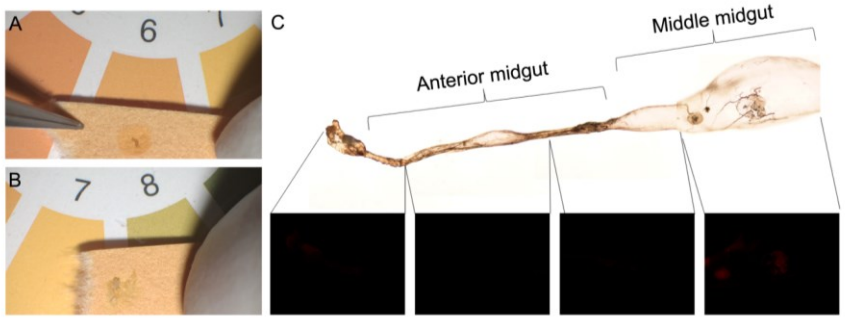

Figure S1. (**A-B**) pH value in the anterior-middle (**A**) and posterior (**B**) midgut lumen as shown by pH indicator strips. (**C**) Evaluation of fluorescence generated by the metallothionein-copper complex in fed-copper flies.

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Supplementary 2 - Analysis of the transit of ingested food by video recording.

The supplementary video is available at the following link: https://www.dropbox.com/s/9y2ms6aywpfjgp5/Hermetia%20video1.mp4?dl=0

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CONCLUDING REMARKS

In this thesis an in-depth study on the larval midgut and fat body of the black soldier fly, *Hermetia illucens*, was reported. The larvae of this insect are able to grow and develop on different waste substrates and, thanks to their high content of protein and lipids, they represent an interesting resource for the production of aquaculture feedstuff. Although in the literature different studies on the rearing methods for BSF larvae are available, little is known about the biology of this insect. In particular, the investigation of the midgut and the fat body, two organs that are involved in nutrient digestion, absorption, and storage, has been completely overlooked. The present study aimed at filling this gap of knowledge. The major achievements of our research are:

1) A morphological and physiological characterization of the larval midgut was obtained. This led to the identification of five distinct regions, characterized by specific cell types and functional activities. In detail, the anterior midgut is involved in the initial phase of digestion. The first part of the middle midgut is responsible for the acidification of the central region of the alimentary canal through the action of copper cells, while in the second part the production of lysozyme is needed to kill bacteria ingested with the food. In the posterior midgut, proteases are secreted in the lumen and nutrient absorption occurs.
2) A microbiota analysis of the larval midgut was performed. We investigated in detail how different diets affect the microbial ecology of the three major midgut regions and analysed, qualitatively and quantitatively, the bacterial distribution in each midgut region. An increase in the

number of bacteria from the anterior to the posterior midgut was observed, along with a progressively reduction of microbial diversity. Moreover, we demonstrated that the composition of the diet affects the microbial ecology of BSF midgut.

3) The influence of a low-protein diet (VMD), composed by fruit and vegetables, on the larval growth rate and the morphofunctional features of the larval midgut and fat body was assessed. A marked variation in the length of the larval cycle and a different activity of digestive enzymes were observed in larvae fed with the VMD. Moreover, morphological changes of midgut cells and different accumulation of micro- and macro-nutrients were identified. Similar changes were observed in the fat body. In particular, we showed that a diet with low protein content affects the accumulation of proteins and lipids in this organ.
4) It is a common belief that the adult stage of *H. illucens* does not need to eat and depends exclusively on reserves accumulated during the larval stage. We demonstrated that the midgut epithelium of the adult is formed during metamorphosis and functional experiments indicate that it is endowed with digestive activity. This scenario opens up the possibility to manipulate the diet of the adult insect to improve its performances in mass rearing procedures, and leads to focus attention on the safety of this species as feedstuff.