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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., 2011a, 2011b; 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).

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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 × 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 × 16 × 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.

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.

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.

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