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Traceability of insects as feed: stable isotope ratio analysis of *Hermetia illucens* larvae and pre-pupae reared on different protein sources

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RESEARCH ARTICLE

It is with profound sadness that family, friends, students, colleagues and co-authors of Prof. Andrea Amici announce his peaceful passing on July 14th 2022, at the age of sixty-four. Thank you Andrea for your passion, your teachings, your kindness, your subtle and always smart irony, and your helpful support in every occasion. You will always be with us.

Abstract

Insects attract the interest of the agri-food sector because of their advantages as feed for livestock. The reason for this lies in the role of insects as alternative protein source to soybean and fish meal, common but expensive materials, and in their ability to proliferate on waste substrates, thus allowing a sustainable livestock management. Insects for feed purposes can however be problematic, in particular with regard to the safety of the substrates the insects would be reared on. Therefore, effective tracking methods are needed. This study focused on the investigation, by means of isotope ratio mass spectrometry, of larvae and pre-pupae of *Hermetia illucens* L. (black soldier fly (BSF)) reared on diets including protein sources of vegetable origin (wheat gluten), animal origin (bovine blood meal), or a combination of the two, in order to detect differences in their isotope ratios for $\delta^{13}C$ and $\delta^{15}N$. Nitrogen isotopic ratios did not follow an incremental pattern between diets, thus not allowing the discrimination of the BSF larvae and pre-pupae, presumably due to differences in the metabolism of insects that could have influenced the fractionation of nitrogen. In the case of the carbon isotopic ratio an increasing trend was observed, directly correlating the carbon isotopic signature of BSF larvae and pre-pupae to the level of replacement of the vegetable protein source with the animal one. According to our observations, it is therefore foreseeable that the study of carbon isotope ratios could be a promising method for the traceability of insects intended for animal feeding. Given the paucity of studies in this regard, further research effort is needed to fully explain some observation reported in the present study and to ascertain the reliability of the ratio analysis of insects as a tool for tracing their feeding history.

Keywords: insects rearing, stable isotope ratio analysis, bovine blood meal, feed safety, larvae and pre-pupae

1. Introduction

Insects are considered promising substitutes for fish meal (FM), due to the ability of different species of fishes to tolerate partial or total substitution of that main component of their diets with insects or insect meals (Gasco *et al.*, 2020; Makkar *et al.*, 2014). A critical point of the conventional animal husbandry concerns the economic implications of feeding stuffs purchasing: for poultry and fish farming, these costs contribute to 60-80% of the total economic costs of production, in particular for protein components, constituting 70% of such costs (Ssepuuya *et al.*, 2017). In 2014, about 10% of the fish caught or reared was reduced to meal and oil; FM is generally derived from fish particularly

rich in fishbones because they are considered unfit for human consumption, and is regarded as high-level feedstuff for pigs, poultry and aquafeed formulation. However, the extensive use that has been made of FM in recent years has contributed to raise its market prices (FAO, 2018). In fact, since January 2000 the price of FM has increased dramatically, from $416 to $1,681 per metric ton in just 10 years. As of August 2021, the price stands at $1,507.97 per metric ton (Index Mundi, 2021). Several options are regarded as possible solutions to solve the so called 'fish meal trap'. On one hand, since 2016 a growing percentage (25-35%) of FM has been produced from fish waste, which could however lead to a reduction in FM nutritional value as feed (Index Mundi, 2021). On the other hand, the use of protein feedstuffs of plant origins in salmonid aquaculture, such as soybean meal, is hampered by high concentrations of carbohydrates and anti-nutritional factors (Hart *et al.*, 2007). To meet the needs of the feed industry it is plausible that insect meal could become a notable substitute for other feeds. In particular the larval stages of *Hermetia illucens* (black soldier fly (BSF)) are well-known for their interesting nutrient profile and the possibility of mass-rearing on a wide range of organic substrates with reduced environmental impact; these characteristics allow them to be considered potentially interesting resources for animal feed purposes (Cammack and Tomberlin, 2017; Danieli *et al.*, 2019; Gasco *et al.*, 2020; Hart *et al.*, 2007; Schiavone *et al.*, 2017; Van Huis, 2013). Indeed, several studies have explored the possible applicability of BSF larvae in aquaculture. For example, Newton *et al.* (2005) reported good results on substitutability of FM with BSF meal included at levels between 7.5 and 15% in the diet of American catfish (*Ictalurus punctatus*), and Stadtlander *et al.* (2017), observed promising results with BSFL meal in the diet of the rainbow trout (*Oncorhynchus mykiss*). The use of insect meal is also suitable for poultry feeding: birds of the Ardennese breed seem to tolerate very well substitutions with BFS up to 8% (Moula *et al.*, 2018), while Ruhnke *et al.* (2018) reported that a 16% inclusion level of BSF in the diet of ISA Brown chicken does not result in any differences with respect to the controls for parameters such as feed-conversion ratio, feed intake, body weight and egg production.

Along with the increasing interest in using insects for farm animal feeding purposes, the concerns regarding safety and traceability of such type of feedstuff is growing. As a matter of fact, the European Food Safety Authority (EFSA, 2015) stated that meanwhile the use of some insect species could be allowed, some relevant topics linked to their safe use as feed and, in particular, the traceability of their production chain as well should be ensured. The concept of traceability is originally interpreted as 'the ability to follow, in real time, or rebuild (off line) the logistic path of single or composite products' (Vernède *et al.*, 2003), and therefore indicates the ability to trace any food, feed, animal-producing food or food-for-food substance through any stage of production, processing and distribution (Regulation (EC) No 178/2002 (EC, 2002)). The objective of traceability is hence to ensure it is possible to implement counter-measures such as product recall and damage control (Vernède *et al.*, 2003) in such a way as to contribute to the quality of products. The traceability is necessary to address the potential risks that may arise from food and feed consumption, and so to ensure that foodstuff, and feed as well, may reach sufficient safety levels. It is therefore compulsory that, in the event of the identification of actual or presumed risks, the national authorities and operators are able to trace the products to the source in order to quickly isolate the risk factors and prevent contaminated products from reaching the consumer (Chhikara *et al.*, 2018; EC, 2007; Vernède *et al.*, 2003). As an example, the reported higher microbial loads of insects, such as crickets (*Acheta domesticus L.*), raises concern for their use in the feed and food sectors, thus leading to the need for adequate labeling regulations in order to ensure the protection of consumers' health (Fernandez-Cassi *et al.*, 2018). There is also concern about the possibility that insect reared on animal origin substrates, as well as on animal litter, could represent a risk of transmission of pathogens to the animals that feed on them or on insects-derived products (Eilenberg *et al.*, 2015; Fernandez-Cassi *et al.*, 2018). Due to the paucity of scientific evidences about the full safety of insects intended for feed purposes and reared on wastes of animal origin and animal by-products (Gałęcki and Sokół, 2019; Ruhnke *et al.*, 2018), the use of such substrates is limited or downright prohibited for insect mass rearing. As a matter of fact, in the Regulation (EU) 2017/893 (EC, 2017) laying down rules for the use of insects in farmed fish feeding, a limited number of feedstocks of animal origin has been included in the list of admissible growing substrates for insects (dairy and eggs), while ruminant proteins, catering waste, meat-and-bone meal and manure are not allowed. Methods for distinguishing insects grown on feedstock of animal or plant origin are thus welcomed in the light of tracing the life history of mass-reared insects intended for animal feed.

Isotope ratio mass spectrometry (IRMS) applications for relative determinations of stable isotopes of carbon ($^{13}C/^{12}C$), nitrogen ($^{15}N/^{14}N$), oxygen ($^{18}O/^{16}O$) and hydrogen (D/H) has been exhaustively used for traceability purposes of fish and poultry products (Bell *et al.*, 2007; Van Raamsdonk *et al.*, 2017). Notwithstanding the great diffusion in the scientific literature of the use of IRMS in the life science and food sector as well, there is still a lack of studies specifically focused on dietary effects on isotope ratios of insects, with special regard to the related traceability issues. Some studies regarding the isotopic abundances in insects do exist; however, some concern the insects' position within the trophic chains, as for Scrimgeour *et al.* (1995) and Chikaraishi *et al.* (2011), but also on the discrimination of captivity reared vs wild

moths (Lepidoptera) (Hood-Nowotny *et al.*, 2016). Matos *et al.* (2018) explored how the isotopic signature of the blowflies *Calliphora* sp. (a holometabolous insect) was influenced by diet (carrion). Belghit *et al.* (2021), instead, bred *H. illucens* on diets with increasing replacement of bovine haemoglobin powder, carrying out various types of analyses, including elemental analyser-isotope ratio mass spectrometry (EA-IRMS) on bulk BSF larvae (but not in the pre-pupae) and compound specific isotope ratio analysis (CSIA) on amino acids.

Taking all that into consideration, the labelling in arthropods to study their feeding behaviour appears feasible (de D'Aubeterre *et al.*, 1999) thus making IRMS as a potential tool for the traceability of the rearing history of insects intended for feed purposes, given that the use of animal derived feedstock for insects' rearing may be in conflict with the current legislation. In line with these premises, the main objectives of this study were: (1) to investigate whether it is possible to deduce, by applying carbon and/or nitrogen isotope ratio mass spectrometry techniques, the type of diet with which larvae of *H. illucens* are grown, with particular reference to animal derived protein feedstock such as bovine blood meal; (2) to study the BSF growth performance and efficiency of conversion of different dietary blends of plant and animal protein sources, and the chemical composition, as protein and fat contents, of both BSF larvae and pre-pupae, mainly with the aim of supporting the IRMS data interpretation.

2. Materials and methods

Experimental design and diets formulation

For the present study, three growing trials lasting 21 days each were carried out. In each experimental trial, four different iso-nitrogenous experimental diets (ED1-ED4) were formulated and tested. These diets shared the presence of corn starch, winter wheat straw and corn oil, and were differentiated instead by the more specifically proteinaceous ingredients, i.e. wheat gluten purchased in a wholesale food store and blood meal (Linfa Bovisang, Italy), added to the other diets' components in inversely proportional amounts (Table 1).

Proximate composition and gross energy of the experimental diets

For the four experimental diets, the composition was analysed on the dry matter (DM) basis. The dry matter and ash (ASH) content were analysed by drying 10 grams of diets in forced air oven at 105 °C until constant weight, which were subsequently incinerated in a muffle furnace at 550 °C (AOAC method No. 942.05) (AOAC, 2000). The crude fat content (EE) was assessed in 3 g diet aliquots by means of a Soxhlet apparatus (AOAC method No. 920.39) (AOAC, 2000). For the measure of the neutral detergent fibre ($aNDF_{OM}$) the Van Soest method was used (Goering and Van Soest, 1970) by using a fibre analyser (ANKOM Technology, Macedon, NY, USA). For the analysis of crude proteins (CP), the Kjeldahl method was used (AOAC method No. 978.04) (AOAC, 2000). Combining the values thus obtained for CP, ASH, EE and ($aNDF_{OM}$), it was subsequently possible to obtain the values for non-fibrous carbohydrates (NFC), as described by Sniffen *et al.* (1992). The gross energy content (GE) (MJ/kg DM) of diet samples was obtained through a Parr 6200 Isoperibol Calorimeter (Parr Instrument Company, Moline, IL, USA) according to the manufacturer's instructions.

Black soldier fly rearing and production

The rearing of *H. illucens* for the purposes of the present work took place at the Minilivestock Facility of the Didactic-Experimental Agricultural Farm 'Nello Lupori' of the University of Tuscia (Viterbo, Italy). All of the larvae, pupae and adults used in this study belonged to a unique BSF stock of approximately 5,000 BSF larvae (BSFL) (Progetto Hermetia, Gioia Tauro, Italy). The insects were reared in a climatic chamber under controlled temperature and relative humidity, as previously described by Danieli *et al.* (2019). Briefly, 100 pre-pupae (BSFP) per batch were placed in round plastic containers in order to perform pupation on pet litter and emergence, and the resulting adult flies were maintained in an 80×35×85 cm flight cage made of a semi-rigid, self-supporting steel net (3×3 mm mesh) covered by a 3 mm thick methacrylate top. Through a system of spotlights placed 90 centimetres above the floor of the flight cage, it was possible to provide the necessary amount of light, equal to an intensity of 109.7 μmol/m^2/s (Danieli *et al.*, 2019), for a period of 10 hours/day. Some branches of plastic climbing plants 70 cm long were available inside the flight cage to allow for the free expression of the flies' lekking behaviour (Tomberlin and Sheppard, 2001). Mated females laid clutches of eggs within an oviposition box, in which packs of single-face fluted cardboard strips (3 flutes/cm) were suspended approximately 3 cm above a thick layer of winter wheat middlings in distilled water (weight ratio of approximately 1:2) as an attractant. Every day at 9:00 a.m., the oviposition packs were opened, and the cardboard

Table 1. Ingredients of the experimental diets and their percentage contribution (% w/w) to the diet composition.

Ingredients	ED1	ED2	ED3	ED4
Corn starch	55	55	56	56
Wheat straw	27	27	27	27
Wheat gluten	15.5	11	5	0
Blood meal	0	4.5	9.5	14.5
Corn oil	2.5	2.5	2.5	2.5

strips were checked for clutches; if present, the clutches were transferred to thin plastic strips (six per strip) with the aid of a small steel rounded-end spatula. Plastic strips were then suspended at approximately 1 cm above a layer of 15 g winter wheat middlings saturated with water (approximate ratio of 1:1) into small circular starter *larvaria* (12 cm ID, 8 cm high). Additional amounts of saturated middlings were added three days after hatching. At six days post-hatching, a part of the young BSFL was used for the experimental purposes and the rest was reared at a density of 10,000 larvae/m^2 on a corn meal/alfalfa/wheat bran diet (50:30:20) at an individual feeding rate of 12.2 mg DM/day to obtain a successive cohort of breeders. For the experimental trials, the six-days old BSFL were initially separated by stacking sieves of the ASTM series with progressively narrower meshes (Danieli *et al.*, 2019). The young larvae were placed on the highest sieve and allowed to go through the lower sieve. A 'cold' light source was used to encourage the descent of the larvae that, because of their photophobic tendencies, tried to escape from the light by crawling downwards. The purpose of such screening was to isolate the larvae between 1 and 2 mm in diameter, in order to ensure the best possible dimensional uniformity of the specimens to be used for the following experiment.

Black soldier fly experiments

To set up the experimental trials, a total of 195 six-days old BSFL were manually counted and randomly assigned to each replicate of the four experimental diets. Each group of BSFL was placed in 12×12 cm plastic containers equipped with holed slits covered with metal grids, in order to ensure proper gas exchange, while preventing larvae from escaping. The density of the larvae in such containers was 1.35 larvae/cm^2. After supplying the animals with enough water-saturated nourishment (2.35 g of dry diets per container a day) for 3 to 4 days, pouring the necessary amount of feed previously weighed directly into the containers and sprinkling with deionised water using a variable volume P5000 pipette (Eppendorf, Germany), to saturate the fresh substrates, the containers were transferred to the climatic chamber. Adequate nourishment was provided every 2 or 3 days until the end of each trial. As emerged by saturation test performed beforehand, the amount of water needed to saturate each experimental diets amounted to 13 ml of water per 9.75 g of diet: the lack of differences in saturation levels between experimental diets is attributable to wheat straw (saturable with 13 ml per 2.7 g), occurring in the same proportions in all diets.

Black soldier fly samples preparation

At the end of each trial, the BSFL and BSFP were separated manually, and after washing to remove the dried substrate remnant, their overall weight was measured according to Danieli *et al.* (2019). The insects were then placed in an air-forced stove at 36-37 °C until constant weight. Subsequently, the dry weight of larvae and pre-pupae was measured with a bench analytic scale (Mettler-Toledo, USA) and recorded. The insects were then grounded with a kitchen dry homogeniser to obtain an insect meal sufficiently fine to allow the subsequent analytical procedures. Samples of BSFP and BSFL meal were separately subjected to extraction of the fat component by the Soxhlet method, as described by Danieli *et al.* (2019). The defatted meals thus obtained were then deprived of the residual ether used for the extraction process by means of a vacuum stove set at a temperature of 60 °C for 30 min. The recovered fat matter through the Soxhlet extractor device (PBI, Milan, Italy) was weighed and transferred with a stainless-steel spatula into a 2 ml centrifuge test tube for the subsequent IRMS determinations. Defatted meal samples were quantitatively recovered from the Soxhlet apparatus and transferred to falcon tubes, ready for IRMS analysis. The total nitrogen content of BSFL and BSFP whole and defatted was carried out along with the determination of isotope ratios (Section 2), given its substitutability of the Dumas method with respect to the Kjeldahl one (Simonne *et al.*, 1997). The percentage of nitrogen obtained from the isotope analysis was multiplied by the conversion factor 6.25, thus obtaining estimated values for the CP content of the insect meals. In addition, the CP to ethereal extract ratio was calculated.

Stable isotope determinations on the experimental diets, black soldier fly fat and defatted meal samples

Samples of the experimental diets, BSFL and BSFP, fat and defatted meals were subjected to IRMS at the laboratory of the Research Institute on Terrestrial Ecosystems (IRET), National Research Council (NRC) of Porano (Italy). On diets and whole and defatted meal samples, both carbon ($^{13}C/^{12}C$) and nitrogen ($^{15}N/^{14}N$) isotope ratios were measured. In the case of BSF/P fat samples, only the analysis of the $^{13}C/^{12}C$ ratio was carried out as the expected virtual absence of nitrogen in the ethereal fraction made the analysis of $^{15}N/^{14}N$ superfluous. In details, all the prepared samples were quantitatively processed in a Dumas combustion elemental analyser (NA1500, CARLO ERBA, Milan, Italy) and admitted to a continuous flow, triple collector isotope ratio mass spectrometer (CF-IRMS; ISOPRIME, Cheadle Hulme, UK). The electron impact source and relative optical parameters of the mass spectrometer were properly tuned to analyse stable isotope ratios of carbon ($^{13}C/^{12}C$) and nitrogen ($^{15}N/^{14}N$) on gaseous CO_2 and N_2, respectively. The measurements were anchored on the Vienna Pee-Dee Belemnite (VPDB) and atmospheric N_2 scales by means of international standards provided by International Atomic Energy Agency (IAEA) and United States Geologic Survey. The ranges of standards used for calibration were from -30.0 to -10.4‰ for $\delta^{13}C$ and from -30.4 to +375.3‰ for $\delta^{15}N$: NBS-22 fuel oil, IAEA-CH6, USGS25 ammonium sulphate, USGS40 L-glutamic, IAEA-600 caffeine, USGS42

human hair (Tibetan), USGS43 human hair (Indian), IAEA-305 A labelled ammonium sulphate, USGS26 ammonium sulphate, IAEA 305 B labelled ammonium sulphate. We further processed one internal standard every 10 samples, within each analytical run, in order to correct for fine scale fluctuations of the analyser performances. Isotope composition values were then calculated as the deviation from the unit of the ratio of the isotope ratio of a sample to that of the international standard:

$$\delta_A = \frac{R_A}{R_{std}} - 1 \quad (1)$$

where R_A and R_{std} are respectively the isotope ratio of the sample ($^{13}C/^{12}C$ or $^{15}N/^{14}N$) and of the international standard, i.e. VPDB for $\delta^{13}C$ and atmospheric nitrogen for $\delta^{15}N$ (IAEA, 1993). The measurement precision, expressed as standard deviation of 10 repeated measurements, was 0.1‰ for $\delta^{13}C$ and 0.2‰ for $\delta^{15}N$.

Statistical analysis

All the data were analysed by ANOVA through Statistica 10 (StatSoft Inc., Tulsa, OK, USA). To test significance between experimental diets it was resorted to the contrasts analysis. The relationship of the isotopic signature for C and N in different sample types (insects and diets) was also investigated using Pearson's product moment correlation. The T-test for dependent samples was used for the comparisons between BSFL and BSFP. Significance was always declared at $P \leq 0.05$. In order to ensure that the parameters which values were percentages or between 0 and 1 could have a normal distribution for the statistical analysis, the arcsine transformation as described by Zar (2014) was applied. Tabulated data are in the original form.

3. Results

Characterisation of the experimental diets

The CP content measured in aliquots of the diets used in the growing trials resulted slightly augmented in relation to the incremental substitution of corn gluten with blood meal (Table 2). However, the CP content of the blood meal including diets (ED2-ED4) were within a +5% deviation from the ED1. The other compositional traits of the diets resulted fairly variable in response to the corn gluten to blood meal substitution.

The GE of and diets ED1 and ED2 showed a higher caloric content than those richer in blood meal (ED3 and ED4) that, on average (17.24 MJ/Kg DM), had -6% GE content than the average value for ED1 and ED2 (18.34 MJ/Kg DM). Both the C and N isotopic composition increased according to the level of inclusion of blood meal in the formulation (Table 2) and the ED4 resulted in an increase of +1.3‰ and +0.37‰ in ^{13}C and ^{15}N respectively than the all-plant diet (ED1).

Table 2. Proximate composition (g/kg DM; unless otherwise stated), gross energy content (MJ/kg DM), and C and N isotopic composition (‰) of the experimental diets.

Parameter[1]	ED1	ED2	ED3	ED4
DM (g/kg)	898.0	902.4	900.7	899.1
CP	142.6	150.9	152.3	158.4
EE	7.7	4.0	4.7	3.3
ASH	40.4	47.7	46.4	48.3
$aNDF_{OM}$	231.1	256.1	289.1	334.0
NFC	578.2	541.3	507.5	456.0
GE	18.20	18.48	17.20	17.28
$\delta^{13}C$	-18.81	-18.46	-17.90	-17.51
$\delta^{15}N$	0.42	0.54	0.65	0.79

[1] $aNDF_{OM}$ = neutral detergent fibre, organic matter basis; ASH = ashes; CP = crude protein; DM = dry matter; EE = ether extract; GE = gross energy; NFC = non-fibrous carbohydrates.

Growth parameters, yields and composition of black soldier fly larvae and pre-pupae

No statistical differences were observed for the total number of BSF specimens recovered (TR) at the end of the trials, as well as the overall survivorship (SR) (Table 3). Regarding the larval development, the contrast of ED4 against the other treatments was significant ($P<0.05$) only for the BSFL recovered at the end of the trials, with no significant effects of the diet for other contrasts investigated. However, for the BSFP/BSFL ratio a marginal significance ($P=0.052$) was highlighted in the case of contrast between ED4 against the other experimental diets (Table 3).

As far as the growing performances and the feedstock conversion ratio (Table 4), contrasts were significant ($P<0.01$) for both the wet (WWG) and dry weight gain (DWG) in the case BSFP only, with the ED2 ones showing higher values in comparison with the others reared on ED1, ED3 or ED4. In addition, the DWG of the ED4 BSFP resulted lower ($P<0.05$) if contrasted against the other diets.

Regarding the insects' chemical composition, the diet did not play a role in the final content of CPs and ether extract of the BSFL nor in their CP/EE ratios (Table 5), with the exception showed by the contrast between ED4 BSFL and the larvae fed on the other experimental diets ($P<0.05$), even though the significance of the ANOVA model was not recorded (Table 5). The diet affected the CP, but not the EE content of the BSFP (Table 5) with the ED1 pre-

Table 3. Effect of the diets on survival of black soldier fly larvae (BSFL) and larval development to the pre-pupal (BSFP) stage.

Parameter[1]	ED1	ED2	ED3	ED4	SEM[2]	*P*-value	Contrasts (*P*-value)		
							ED1 vs ED2+ED3+ED4	ED1+ED2 vs ED3+ED4	ED4 vs ED1+ED2+ED3
TR (n)	167.5	160.8	166.3	176.8	9.9	0.276	0.973	0.497	0.346
SR (%)	85.9	82.4	85.3	90.6	5.1	0.910	0.920	0.636	0.496
TR_{BSFL} (n)	58.3	30.5	35.5	69.0	9.3	0.044	0.253	0.426	0.028
TR_{BSFP} (n)	109.3	130.3	130.8	107.8	13.7	0.509	0.417	0.972	0.354
BSFP/BSFL	2.2	5.3	4.2	1.7	0.8	0.935	0.195	0.445	0.052

[1] BSFP/BSFL = recovered pre-pupae/larvae ratio; SR = survival rate (TR/seed larvae); TR = total recovered specimens at the end of the trials; TR_{BSFL} = black soldier fly larvae recovered at the end of the trials; TR_{BSFP} = black soldier fly pre-pupae recovered at the end of the trials.
[2] SEM = standard error of the mean (pooled).

Table 4. Effect of the diet on the wet and dry weight gains (g/*larvarium*) for the black soldier fly feeding larvae (BSFL), pre-pupae (BSFP), all specimens (ALL) and the overall feed conversion ratio (FCR).[1]

		Diets				SEM	*P*-value	Contrasts (*P*-value)			
		ED1	ED2	ED3	ED4			ED1 vs ED2+ED3+ED4	ED1+ED2 vs ED3+ED4	ED2 vs ED1+ED3+ED4	ED4 vs ED1+ED2+ED3
BSFL	WWG	7.1	5.1	5.7	8.6	1.2	0.247	0.661	0.420	0.183	0.092
	DWG	2.4	1.7	1.9	2.8	0.5	0.384	0.666	0.516	0.248	0.160
BSFP	WWG	8.1	12.3	9.8	8.0	0.8	0.014	0.785	0.150	0.003	0.059
	DWG	3.0	4.5	3.6	2.9	0.3	0.010	0.089	0.112	0.002	0.033
ALL	WWG	15.2	17.4	15.4	16.6	0.5	0.084	0.613	0.896	0.512	0.816
	DWG	5.4	6.2	5.5	5.7	0.2	0.150	0.674	0.810	0.486	0.982
	FCR*	9.3	8.1	9.1	8.8	0.4	0.181	0.350	0.430	0.071	0.237

[1] ALL = black soldier fly pre-pupae and larvae; DWG = dry weight gain; SEM = standard error of the mean (pooled); WWG = wet weight gain.

Table 5. Effect of the diet on content of crude protein (CP) and ether extract (EE) (g/kg DM) and their ratio (CP/EE) of black soldier fly larvae and pre-pupae.[1]

		Diets				SEM	*P*-value	Contrasts (*P*-value)		
		ED1	ED2	ED3	ED4			ED1 vs ED2+ED3+ED4	ED1+ED2 vs ED3+ED4	ED4 vs ED1+ED2+ED3
BSFL	CP	421.6	413.4	442.0	380.8	17.8	0.152	0.653	0.724	0.044
	EE	312.8	294.6	242.9	269.5	39.7	0.717	0.409	0.312	0.824
	CP/EE	1.78	1.91	2.14	1.61	0.48	0.822	0.750	0.754	0.595
BSFP	CP	403.3	439.4	424.8	420.8	8.0	0.036	0.013	0.860	0.861
	EE	310.8	325.2	326.5	315.8	15.0	0.876	0.530	0.849	0.781
	CP/EE	1.31	1.38	1.33	1.35	0.08	0.919	0.614	0.950	0.899

[1] BSFL = larvae; BSFP = pre-pupae; SEM = standard error of the mean (pooled).

pupae showing 5.8% less CP content that the other ones (on average 428.3 g/kg DM).

Isotopic analysis of black soldier fly larvae and pre-pupae

In whole (W) and defatted larvae (D) as well as in their fat matter (F), the carbon isotopic abundance was affected by the diet (Table 6) and all the contrast tested showed high significance (Table 6), substantiating the increasing numerical pattern observed. The diet played a role also for the nitrogen isotopic ratios, but the ED4 BSFL showed $\delta^{15}N$ values that did not differ from the others, falling in the range of value spanned by the ED1-ED3 BSFL (Table 6).

As seen for the BSFL, the inclusion of blood meal in the diet resulted in an increased carbon isotopic composition of the whole pre-pupae, the defatted matter and the residual fat, and all the contrasts explored were significant (Table 7). Also, the nitrogen isotopic composition of the pre-pupae (whole or defatted) was affected by the diet but a clear relationship with the level of inclusion of the blood meal was not observed (Table 7). As a matter of fact, the contrasts for the insects reared on ED1 (total plant protein source) against all those reared on ED2-ED4 were highly significant (Table 7), but the ED4 counterparts had similar values (Table 7).

Correlation analysis of isotope composition

Very strong positive correlations were observed between the $\delta^{13}C$ values of both whole and defatted BSFL or BSFP and the C and N isotopic abundances of the diets (Table 8). As expected, the $\delta^{13}C$ of the fat of BSFL and BSFP was positively correlated with the C isotope abundances of the experimental diets (Table 8). Negative but significant correlations were observed for the $\delta^{13}C$ and the $\delta^{15}N$ values in the diets and in BSFL, but the $\delta^{15}N$ of the BSFP resulted uncorrelated to the C and N isotopic abundances of the diets (Table 8).

As expected, the $\delta^{13}C$ values of both BSFL and BSFP were positively correlated though the correlation coefficient (r) for the fat matter was the highest (Table 9). Whole and defatted BSFL had $\delta^{13}C$ values inversely correlated with the

Table 6. Effect of the diet on the nitrogen and carbon isotopic signature (δ ‰) of the black soldier fly larvae (BSFL), their defatted matter, and fat.[1]

		Diets				SEM	*P*-value	Contrasts (*P*-value)		
		ED1	ED2	ED3	ED4			ED1 vs ED2+ED3+ED4	ED1+ED2 vs ED3+ED4	ED4 vs ED1+ED2+ED3
$\delta^{13}C$	W	-16.8	-16.0	-15.6	-15.0	0.1	<0.001	<0.001	<0.001	<0.001
	D	-17.5	-16.5	-16.1	-15.5	0.1	<0.001	<0.001	<0.001	<0.001
	F	-16.0	-16.2	-15.7	-14.9	0.1	<0.001	<0.001	<0.001	<0.001
$\delta^{15}N$	W	10.2	10.3	8.3	9.2	0.2	<0.001	0.004	<0.001	0.110
	D	10.5	10.4	8.9	9.3	0.3	<0.001	0.004	<0.001	0.063

[1] D = defatted BSFL; F = fat matter; SEM = standard error of the mean (pooled); W = whole BSFL.

Table 7. Effect of the diet on the nitrogen and carbon isotopic signature (δ ‰) of the black soldier fly pre-pupae (BSFP), their defatted matter, and fat.[1]

		Diets				SEM	*P*-value	Contrasts (*P*-value)		
		ED1	ED2	ED3	ED4			ED1 vs ED2+ED3+ED4	ED1+ED2 vs ED3+ED4	ED4 vs ED1+ED2+ED3
$\delta^{13}C$	W	-17.6	-17.4	-16.1	-15.3	0.2	<0.001	<0.001	<0.001	<0.001
	D	-18.8	-18.2	-16.8	-16.1	0.2	<0.001	<0.001	<0.001	<0.001
	F	-16.3	-16.5	-15.8	-15.0	0.1	<0.001	<0.001	<0.001	<0.001
$\delta^{15}N$	W	9.8	8.9	7.8	9.8	0.2	<0.001	<0.001	0.016	0.001
	D	9.8	9.2	7.8	9.7	0.2	<0.001	0.001	0.001	0.005

[1] D = defatted BSFP; F = fat matter; SEM = standard error of the mean (pooled); W = whole BSFP.

Table 8. Correlation analysis of carbon and nitrogen isotope ratios (‰) of the diets and the values recorded in the black soldier fly larvae (BSFL) and pre-pupae (BSFP).[1,2]

	BSFL $\delta^{13}C$			BSFP $\delta^{13}C$			BSFL $\delta^{15}N$		BSFP $\delta^{15}N$	
	W	D	F	W	D	F	W	D	W	D
Diet $\delta^{13}C$	0.93***	0.86***	0.82***	0.86***	0.88***	0.79***	-0.57**	-0.51**	-0.14	-0.17
Diet $\delta^{15}N$	0.94***	0.88***	-	0.84***	0.87***	-	-0.51**	-0.49**	-0.10	-0.11

[1] D = defatted BSF; F = fat matter; W = whole BSF.
[2] **P<0.01, ***P<0.001.

Table 9. Correlation analysis of carbon and nitrogen isotope ratios (‰) between black soldier fly larvae (BSFL) and pre-pupae (BSFP).[1,2]

	BSFP $\delta^{13}C$			BSFP $\delta^{15}N$	
	W	D	F	W	D
BSFL $\delta^{13}C$	0.67***	0.85***	0.90***	-0.18	-0.29
BSFL $\delta^{15}N$	-0.52*	-0.44*	-	0.66***	0.74***

[1] D = defatted BSFP; F = fat matter; W = whole BSFP.
[2] *P<0.05, ***P<0.001.

corresponding values in BSFP, though de degree of such relationship was quite low. The carbon isotopic abundances of BSFL did non correlate with the $\delta^{15}N$ values of the BSFP, but their $\delta^{15}N$ values were positively correlated with the corresponding values in whole and defatted BSFP (Table 9).

4. Discussion

Effect of the diet on the growing performance and composition of black soldier fly pre-pupae and larvae

Despite the different GE content of the experimental diets, increasing the level of substitution of plant protein from wheat gluten with blood meal had little effect on the growing performance of BSF with the exception of the final number of BSFL, that was higher for the all-animal protein diet (ED4) in comparison with ED1-ED3 (Table 3). That result was conditioned by the numerical outcomes concerning the final number of BSF (TR_{BSFL}) and the number of BSFP recovered on ED4 which were the highest and lowest, respectively. Similar results were observed by Danieli *et al.* (2019), but in that case the main cause of delay was imputable to a very high NFC content of the diet (689 g/kg DM). In contrast, in this study the NFC content of the ED4 was the lowest (Table 2), hence a different issue should be the cause of the observed delay in the larvae-to-pre-pupae passage. Probably because of combined effects that will need further study effort, it seemed reasonable that blood meal as the only protein source extended the feeding larvae stage, thus reducing the number of specimens passing to the pre-pupal phase during the 21 days of the trials. The yield was marginally affected by the type of protein source used in the formulation of BSF diets. Wet and dry weight gain of ED2 BSFP resulted higher than the ED1, ED3 and ED4 counterparts, but the ED4 BSFP showed the lowest DWG and thus it can be affirmed that inclusion of blood meal as the only protein source in the diet of BSF can give low dry matter yield, but a blend of wheat and bovine blood proteins (i.e. the ED2 diet) can exert positive effects on the weight gain of BSF in the last larval stage. As far the insects' composition, the protein content was the lowest if the BSFL were reared on the all-animal protein diet (ED4), but, on the contrary, the pre-pupae of BSF reared on all-plant diet (ED1) had lower CP content than the others. Considering all these data it can be hypothesised that the total substitution of gluten wheat with blood meal as a source of proteins in BSF diets had some impact on the development of the feeding larvae (BSFL), reducing their nitrogen retention and leading to lighter pre-pupae. Overall, these results confirmed previous reports (Cammack and Tomberlin, 2017; Danieli *et al.*, 2019; Gligorescu *et al.*, 2018) regarding that, though being a polyphagous and scavenger species (Cammack and Tomberlin, 2017), *H. illucens* shows variable growing performances and rearing yields according to different growing substrates.

Isotope analysis

The average $\delta^{13}C$ values recorded for BSF in this study ranged between -18.8‰ (defatted BSFP reared on ED1) and -14.9‰ (fat matter of the BSFL fed on ED4) (Tables 6-7). These results lay in the upper part of the range described by Pianezze *et al.* (2021) (from -31.2 up to -12.2‰, on defatted matter) studying the isotopic ratios of 35 different edible insect species. For the diets' formulation, it was not possible to find corn gluten (C4 plant) in the retail market, in place of which was used wheat gluten (C3 plant). Since C3 and C4 plants fix carbon differently, their $\delta^{13}C$ values

are also expected to differ (approximately -27 and -10‰ for C3 and C4 plants, respectively) (Hyodo, 2015), and this has certainly had a role on the average $\delta^{13}C$ of the diets and of the BSF as well. As a reference, the observed enrichment in ^{13}C of whole BSFP and BSFL in comparison with the isotopic abundance of their diets ($\Delta^{13}C$) (Spence and Rosenheim, 2005) ranged from +0.96‰ (BSFP reared on ED2) up to +2.46‰ (BSFL fed on ED2) (data not shown), and fell in the range (-2.6 to +6.1‰) reported in a survey of the literature concerning isotopic studies carried out on 27 different insect species (Spence and Rosenheim, 2005). The only research found concerning the carbon isotopic abundance of BSF larvae (Belghit *et al.*, 2021) was carried out on the aminoacidic fraction on larvae reared on a standard poultry feed, of which the $\delta^{13}C$ was not reported, spiked with bovine haemoglobin. Belghit *et al.* (2021) showed that, except for the essential amino-acids methionine and leucine, the others had $\delta^{13}C$ values higher than -30‰ and nine out of eighteen amino-acids had isotopic ratios from to -25 to -15‰, with minor shifts depending on the amount of bovine haemoglobin included. Thus, the data gathered in the present study on defatted BSF larvae and pre-pupae would be positioned in the upper part of the $\delta^{13}C$ values reported by Belghit *et al.* (2021) for the amino-acidic component of *H. illucens* larvae. However, any further comparisons would be amiss due to differences of sample processing and lack of details given by Belghit *et al.* (2021) on the composition and carbon isotopic ratios of the diets used.

Notably, the $\delta^{13}C$ values of larvae and pre-pupae reported in the present study (Tables 6-7) clearly reflected the C isotopic abundance of the diets (Table 2), as also outlined by the strong and highly significant correlations (Table 8), since $\delta^{13}C$ values, though generally high due to the presence of corn starch ($\delta^{13}C$ of -11.96‰, data not shown), followed incremental trends according to the progressive substitution in the diets of vegetable proteins (from wheat gluten) with the animal ones (from blood meal). Also, the $\delta^{13}C$ values of BSFL and BSFP correlates with the $\delta^{15}N$ of the diets (Tables 8) but this outcome is easily explainable because of the strong correlation existing between the C and N isotopic abundances of the experimental diets (r=0.993; $P<0.01$) (data not shown).

A noticeable difference between the BSF larvae and pre-pupae is that the former, for all diets and in the case of whole and defatted samples, and even for the lipid fraction, showed values of $\delta^{13}C$ consistently higher than those of the pre-pupae (average values of -16.01 and -16.63‰, respectively; T-test for dependent samples, $P<0.01$). These differences could be due to the phase transition undertaken by the larvae which entails, among other things, the cessation of feeding (pre-pupae do not feed), but, unfortunately, there are not similar studies to which these observations can be directly compared with.

Importantly, in both BSFL and BSFP, the all-plant diet gave $\delta^{13}C$ values that can allow the discrimination of the whole insects (W) as well as their defatted (D) and fat (F) matter from the BSF reared on diets including more than 4.5% of blood meal as substitute of the plant protein source (wheat gluten). In the blowfly (*Calliphora vicina*) larvae, pre-pupae and adults raised on three different types of carrion (beef, pork, chicken), Matos *et al.* (2018) observed variations of $\delta^{13}C$ up to 8% in non-essential amino acids depending on different types of carrion the insects fed on, suggesting the possibility of identifying the origin of their diet. However, it should be taken in consideration that the discriminating power of $\delta^{13}C$ could not be directly applied to BSFL/P fed on a diet with relevant increases of the C4-to-C3 plant feedstock ratio that could result in a 13C enrichment in the BSFL/P overlapping the ones observed for the ED2-ED4 BSF samples. It is worth pointing out that in the experimental conditions of the present study, the isotopic ratios of the pre-pupae could be considered more reliable than those of the larvae as it cannot be excluded the possibility that, because the BSFL were still able to feed (unlike the pre-pupae) at the end of the trials, the undigested substrate within their gastrointestinal tract at the time of killing may have potentially affected the isotopic ratios of the meal obtained from the BSFL. A countermeasure that could be adopted in future studies could consist in preventing the larvae from feeding for at least 24 hours before killing, so that digestion and/or expulsion of residual material would take place. However, the differences highlighted for the $\delta^{13}C$ depending on the experimental diets cannot be due to the presence of undigested feed within the gastrointestinal tract of the BSFL, because if that was the case, their isotopic ratios would have been closer to those of the diets. On the contrary, the $\delta^{13}C$ values were observed to diverge from the diets' isotopic abundances to a higher degree in comparison to the (non-feeding) pre-pupae. These findings allow us to infer that no relevant interference can be expected from undigested matter should the IRMS be used as a tool for certifying the rearing history of unpurged BSF larvae intended for protein meal production.

Looking at the data in Tables 6 and 7, the $\delta^{13}C$ ranking shows that the larvae's fat exhibited lower ^{13}C depletion with respect to the defatted counterpart and, reasonably, whole insects showed intermediate values. At a first sight, that order sounds surprisingly opposite to the well-known tendency of the organism fat matter to show relatively higher ^{13}C depletion than other compounds. The biochemical bases of such isotope fractionation have been masterfully formulated by De Niro and Epstein (1978). However, in our case the situation was quite different from other studies found in literature (Pianezze *et al.*, 2021), for this study describes variable carbon isotope fractionation between the animals' compartments with respect to the isotopic abundance of the diets. In this study, the main (if not the sole) source of lipids in the BSF diet was the corn oil, whose

$\delta^{13}C$ value was very stable at -16.3‰ and highly enriched with respect to C3 metabolism plant oils (e.g. soybean, sunflower, cottonseed *ecc.*; range: -32.4/-25.4‰) (Jahren and Kraft, 2008; Kelly *et al.*, 1997). The fatty acid (FA) profile of corn oil is dominated by the poly-unsaturated fraction (PUFA) that sum up to about 60% of the total fatty acids, followed by the mono-unsaturated fraction that account for about one fourth (Dupont *et al.*, 1990). In connection to the corn oil C isotopic abundance and acidic profile, a very relevant point is that BSF lipid metabolism (anabolism) mainly concerns the saturated short chain FAs, only partially the longer chain monounsaturated fatty acids (MUFA), but not the PUFA. Hence, the main corn oil components (PUFA and part of the MUFA), characterised by very high $\delta^{13}C$ values, show a tendency to be accumulated in the larvae rather than being processed. Convincing evidence on this issue has been reported by Hoc *et al.* (2020), who studied the D/H ratio in different FAs extracted from BSF larvae provided or not with deuterated water. In addition, also the BSF larvae saturated fatty acids (SFA) pool was expected to have a relatively enriched $\delta^{13}C$ value, considering that the anabolic pathway of lipids is based on the glycolysis of the carbohydrate source (Hoc *et al.*, 2020) that, in our case, was the corn starch. Our IRMS measurements of this ingredient ranged from -11.9 down to -12.0‰. From these values, rather than from the diet $\delta^{13}C$ mean values (-18.8/-17.5‰, Table 2), the lipid-related depletion due to SFA *de-novo* synthesis should be accounted for. In comparison to the C isotopic abundance of the proteins of BSF larvae and prepupae, all the aforementioned reasons, *per se*, could not be sufficient to justify the anomalous behaviour recorded in Tables 6-7, if a further point is not duly considered. Most of the DW of the defatted BSFL/P is represented by proteins (see for example the data reported in Danieli *et al.* (2019)). In our experiment, the main sources of proteins were the wheat gluten (-27.9/-27.8‰, our data) and/or the blood meal (-20.0/-19.9‰, our data) (the diets formulation is shown in Table 1). A ^{13}C enrichment of 3.7‰ was reported by Webb *et al.* (1998) in muscular tissue of wheat-fed growing locusts. Hence, considering the ED1 BSFL or BSFP, it is reasonable that the BSFL/P proteins would have had a carbon isotope composition much more depleted than the fat (e.g. about -24‰). It is thus likely that the nitrogen metabolism of the insects (concerning the proteins and the chitin as well) played a role on the final defatted matter $\delta^{13}C$ values listed in Tables 6-7. In support to these reasonings, in Gratton and Forbes (2006) the $\delta^{13}C$ of fat tissue (including the reproductive system) appeared to be enriched when compared to that of the muscular tissue in insects. In particular, it was shown that after a diet shifting (preys reared on soybean vs corn), the carbon isotope composition in the young multicoloured Asian ladybeetle and seven-spotted ladybeetle was higher in fat tissues (passing from about -26.5 up to about -17.8‰ in 14 days) than in muscles (approximately -25.8/-22.5‰). The explanation given by Gratton and Forbes (2006) is rooted on a mixed growth-metabolic turnover model that, at least partially, can apply also to our study case. Finally, the unusual ranking of $\delta^{13}C$ among fat, defatted and whole BSFL/P presented in Tables 6-7 seems to have a true subtle and complex biological basis that merit further insights.

Another surprisingly outcome of the study was the absence of a well-defined trend for the $\delta^{15}N$ of the insects depending on the protein source substitution level. For both BSFL and BSFP, in fact, the insects grown on the all-plant diet (ED1) were not unequivocally discriminable from the counterparts grown on diets including only animal proteins (Tables 6-7). However, moderate, though significant, relationships were observed among the $\delta^{15}N$ values of BSFL and their diets (Table 8), but the same relationship was not observed for the BSFP (both whole and defatted). Though the contrasts tested were significant, on average the $\delta^{15}N$ values of the ED1 BSFP (wheat gluten as a protein source, 15.5% of the diet DM) and the ED4 BSFP (bovine blood meal as a protein source, 14.5% of the diet DM) were the same (9.8‰), therefore indicating that the total replacement of the protein sources is not detectable through the study of the nitrogen isotopic ratios. These findings seemed unexpected looking at the results obtained on the carbon isotopic abundance and at the increased $\delta^{15}N$ values of the experimental diets ED1-ED4 (Table 2). Chikaraishi *et al.* (2011) studied the isotopic changes of adult bees, wasps, and hornets or their amino acids in relation to the trophic position (herbivorous, omnivorous and predators, respectively), and found $\delta^{15}N$ values between 1.6 and 5.1‰ for bees, between 1.1 and 7.2‰ for wasps and between 4.5 and 5.6‰ for hornets. The data produced by Chikaraishi *et al.* (2011), therefore, suggest that the amount of animal protein in the diet can have no-univocal effects on bulk $\delta^{15}N$ of insects. A totally different situation was found looking at the amino acidic $\delta^{15}N$ values through which it was possible to assign more precisely the right trophic level to bees (herbivorous), wasps (omnivorous) and hornets (predators) (Chikaraishi *et al.*, 2011). Unfortunately, in the present study it was not performed the isotopic analysis of BSF amino acids and further studies on the topic should be undertaken as it may be very relevant within the frame of feed/food security (Belghit *et al.*, 2021).

However, the unexpected trend of $\delta^{15}N$ in relation to the different composition of the experimental diets observed for larvae and pre-pupae prompted us to think about the possible causes. Assuming that there were no mistakes in the administration of diets to insects or in the sampling of insect meals for isotopic analysis, as it would have affected the results of the carbon isotopic abundance, at first it was hypothesised that the anomalous behaviour of the $\delta^{15}N$ may be due to effects of the nitrogen metabolism of the larvae related to the amino acid composition of the diets. As no amino-acidic analysis were performed, it has been resorted to data from literature to carry out a correlation

analysis between the amino acid profiles of the larvae of *H. illucens* (Heuzé and Tran, 2016) and those of the two protein sources used for the experimental diets: bovine blood meal (Heuzé and Tran, 2016), and wheat gluten (Woychik *et al.*, 1961). Initially, comparisons were made for all amino acids, and then only for those considered essential (namely histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, valine). Considering all the amino acids, an increasing trend for the correlation coefficients was observed between the amino acid profile of *H. illucens* meals and diets ED1 (r=0.50; $P<0.05$), ED2 (r=0.60; $P<0.01$) and to ED3, with the latter showing the highest correlation value (r=0.78; $P<0.001$) (data not shown). Also noteworthy is the value observed for ED4 (r=0.77; $P<0.001$) (data not shown), slightly lower than the value observed for ED3. Hence, the complete amino-acid profiles of the experimental diets tended to match increasingly that of the feeding BSF larvae as the substitution of wheat gluten with blood meal increased overcoming the 50% threshold. On the other hand, looking at the essential amino acids only, the highest correlation values were obtained for ED2 and ED3 (r=0.83; $P<0.01$) with the value for ED4 slightly lower (r=0.80; $P<0.05$) (data not shown). Again, the lowest correlation value compared to the amino acid profile of the larvae was observed for ED1 (r=0.68; $P<0.05$) (data not shown). The lowest values for $\delta^{15}N$ were observed in the case of the ED3 diet for whole larvae and pre-pupae (Tables 6-7). Furthermore, as just discussed, the ED3 diet showed the second highest correlation value for all amino acids, as well as the highest for essential amino acids compared to the amino acid profile of *H. illucens* larvae (Heuzé and Tran, 2016).

Considering all these data, it could therefore be inferred that ED2 and ED3 had, though potentially, an amino acid profile more suitable to meet the nutritional requirements of growing BSFL. As mentioned, this could have influenced the amino acid metabolism of the insects with possible effects on the isotopic fractionation, which would therefore have led to the peculiar trend described for the $\delta^{15}N$. Supporting this hypothesis is also the fact that the increase on the dry weight of the pre-pupae resulted the highest in the case of ED2 (Table 4). On the other hand, it can be assumed that higher quality protein sources (i.e. having an aminoacidic profile very similar to that of the consumer) should not necessarily lead to a ^{15}N-depletion, as showed by Webb *et al.* (1998) for the locust (*Locusta migratoria*). It was also possible that the ED2, and possibly the ED3, represented fair better diets for BSLF than ED1 and ED4 while reducing nitrogen recycling, a biochemical pathway that has been shown to induce an anomalous ^{15}N-erichement in undernourished or fastened insects with respect to the nitrogen isotopic abundance of the diet (Scrimgeour *et al.*, 1995). In the light of this latter evidence, and in the lack of reference data for BSFL coming from different but comparable studies, the high nitrogen isotopic abundances, especially for the ED1 and ED4 BSFL (Table 6) or BSFP (Table 7) compared with those of the respective diets (Table 2), seems to find a reasonable explanation. However, no solid basis for any of these hypotheses were found in literature. For this reason, in future studies it may be appropriate to investigate in more depth the effects of the different amino acid combinations of the diets on the isotopic fractionation of nitrogen in *H. illucens*.

It is also particularly interesting to observe the variation in the $\delta^{15}N$ between larvae and pre-pupae. Similar to what has been discussed for the carbon isotopic abundances, in whole as well as defatted larvae higher values for $\delta^{15}N$ were observed (9.7 and 9.6‰, respectively) compared to the whole or defatted pre-pupae (9.1 and 9.0‰, respectively) (T-test for dependent samples; $P<0.001$ and $P<0.05$, for whole and defatted specimen respectively) (data not shown). There are evidences that in some insects the phase transition leads to a ^{15}N-enrichment, as seen in *Chironomus acerbiphilus* (Diptera) (Doi *et al.*, 2007) and also in *Baetis tricaudatus* (Ephemeroptera) (Wesner *et al.*, 2017), but Chikaraishi *et al.* (2011) did not observe any variations of the $\delta^{15}N$ values during the complete metamorphosis from the larva to the adult insect in three species of wasps (*Parapolybia indica, Polistes japonicus japonicus,* and *Polistes rothney iwatai*). However, the results shown in the present study are like those reported by Matos *et al.* (2018) who observed a surprising ^{15}N-depletion (-1.0‰, on average) passing from the blowfly larvae to the pupae. The authors mention that this depletion may be due to the metamorphic change from larvae to adult involving complex metabolic processes wherein the body tissues become more enriched in ^{15}N in each life stage due to the release of depleted nitrogenous excreta, and suggest separate analysis of puparium and meconium could provide a better understanding of nitrogen fractionation of these early stages (Matos *et al.*, 2018).

5. Conclusions

This paper explored the effect of the substitution of plant proteins from gluten wheat with animal proteins from blood meal looking at the growth of BSF larvae and the main composition of feeding larvae and pre-pupae, while exploring the use of isotopic ratios to trace the feeding history of *H. illucens* larval stages intended of animal feeding. Overall, shifting from the plant to the animal protein-based diets for BSF gave poor, if any, result suggesting that, from a merely scientific standpoint, there may be low interest in using, for example, bovine blood meal as a protein source for rearing BSF in comparison with fully allowed plant-derived counterparts. However, as this issue cannot be neglected in realistic productive scenarios, it is out most relevant to lay on fast and easy methods to trace the feeding history of *H. illucens*.

From this standpoint, the nitrogen isotopic ratio did not prove decisive for any traceability purposes. The reasons for these results are still to be clarified. On the contrary, the analysis of the carbon isotopic abundance showed promising discriminant power to distinguish unprocessed larvae and pre-pupae of BSF reared on all-plant diet from the animal protein fed counterparts. However further studies are needed to test the reliability of the $\delta^{13}C$ as (process) traceability marker for BSF larvae and pupae intended for animal feeding, with particular regard to the definition of thresholds that can be adapted to variable rearing condition. The existing interest in the possible use of insects as animal feedstuff should encourage further studies in the perspective of desirable regulatory openings. In turn, the development of traceability systems capable of determining the use of forbidden substrates in insect rearing could help demonstrate that, despite concerns about the healthiness of insect and their products, they can be profitably exploited in order to counteract the critical issues of finding alternative animal protein sources for livestock feeding.

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Conflict of interest

We declare that the authors have no conflict of interest, financial or otherwise.

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