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SPECIAL ISSUE ARTICLE

Standardising black soldier fly larvae feeding experiments: an initial protocol and variability estimates

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Abstract

There is a growing interest in the ability of black soldier fly (*Hermetia illucens*) larvae (BSFL) to convert low-value organic residues into high-value products. This leads to more publications with conversion data for various organic resources. However, these results are rarely comparable between laboratories due to differences in study protocols. This hinders comparisons among studies, the use of results in practice, and overall advancement in BSFL conversion research. Therefore, a standardised research protocol was developed for nursing, rearing and harvesting of BSFL for feed assessment. The utility of this protocol, was assessed via an international ring test with 9 partners. One batch of Gainesville diet (wheat bran (50%), alfa-alfa (30%) and maize (20%)) was produced and distributed among the partners to avoid dietary variations. Five-day-old BSFL larvae were used for the growth trial with six replicates per partner. Average larval weight was assessed after 3 days, 7 days, and harvest (>10% prepupae). Total yield and frass were recorded, and samples were chemically analysed to allow the quantification of the conversion efficiency. The results were used to calculate the within and between partner variability of the protocol. The results indicate that for the biological parameters (average weight, yield and density) the within partner variability was 24% and the between partner variability was 60%. For the assessed chemical parameters (N, fat, ash, P, K, pH), both the

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within and between variability was lower (respectively 9 and 28%). The results of this study give a first indication of the variability that can be expected within and between BSFL feeding experiments for different parameters and can therefore serve as guideline when developing a new experimental designs, assess standard operating procedures and other applications. The protocol can be used as first basis for future feed experiments, improving the comparability of results.

Keywords

BSF – ring test – standard protocol – feed – nutrition

Introduction

The black soldier fly (BSF – *Hermetia illucens*, Linnaeus, 1758) is considered as one of the most promising species for converting low-value residual organic substrates to high-value products (Fowles and Nansen, 2020; Gasco *et al.*, 2020). The bioconversion via their larvae fits within the circular economy concept, where waste from one system is utilised in another process reducing disposal efforts and producing value along the production chain. For example, a recent review by Surendra *et al.* (2020) indicated the plethora of substrates that BSF larvae (BSFL) can consume and convert, including fruit and vegetable wastes, slaughterhouse wastes, sludge, and manure.

Both public research institutes and private companies are keen on investigating and optimising rearing substrates to improve the performance (e.g. growth and feed conversion), quality, and quantity of the larvae. However, there are currently no guidelines or protocols available on how to perform such feeding experiments, thus resulting in a mismatch of experimental designs and variable protocols (Bosch *et al.*, 2020). Variation in BSFL performance can be caused by many factors, such as batch size (Yakti *et al.*, 2022; Yang and Tomberlin, 2020), larvae density and feed availability (Padmanabha *et al.*, 2020; Parra Paz *et al.*, 2015), feeding regimes (e.g. one-time feeding *vs* daily feeding; Barragan-Fonseca *et al.*, 2018), temperature (Gligorescu *et al.*, 2018; Shumo *et al.*, 2019; Yakti *et al.*, 2022) or genetic differences (Kaya *et al.*, 2021). Differences in experimental protocols make it difficult, or impossible, to compare the conclusions even though each experiment is, on its own, performed well and scientifically sound. With a rapidly increasing interest in BSFL farming and the increasing number of publications, standardization is urgently needed to improve the intra- and inter-institutional exchange, utility, and comparability of results. This comparability of results will further advance our understanding of BSFL feeding, conversion efficiency and production, as well as enable a more efficient time and resource use for both the public and the private sectors.

The standardization of BSFL feeding experiments received an initial push from the work of Bosch *et al.* (2020).The conclusions of this paper were discussed at the 2019 EAAP (European Federation of Animal Science) conference in Ghent (Belgium), and this discussion made clear that a standard protocol for feeding experiments needed to be developed. An international coalition of BSF researchers was then established, consisting of members of the EAAP working group on standardisation, the EU horizon project SUSINCHAIN (SUStainable Insect CHAIN) working group on standardisation, and several independent researchers and companies. Online discussions were held on the experimental design and rearing conditions, and which factors should be standardised. The common goal was to ensure that the protocol was straightforward and feasible to be performed by most institutions regardless of their location or financial realities. In addition to be scientifically sound, the protocol had to be applicable on a large scale as well, in order to allow the industry to better benefit from the obtained results. This consensus protocol was assessed via a ring test, determining its repeatability and reproducibility. The aim of the present study is to share the consensus protocol and guidelines for BSF feeding experiments, as well as the variability regarding repeatability and reproducibility of the generated results in the ring test. Furthermore, the experienced challenges in executing the protocol and suggested improvements of the protocol are addressed.

Materials and methods

The approach below is a summary of the consensus protocol followed by the coalition partners in the ring test. The extended and detailed protocol is provided in

the supporting information: Annex 1. In that protocol, points of attention and possible improvements based on the results of this study are already highlighted to improve the execution of future feeding experiments.

BSF larvae populations

The origin of the different populations used in this study is outlined in detail in Supplementary Table S1. In total 8 different populations were used (identical population for partner A and B). The true origin of most populations is unknown but all were reared in laboratory or industrial conditions for an estimated 40 generations or more. The minimum effective population size was estimated at around 1,000 flies for the academic partners up to 10 million for the industrial partners. The climatic conditions were similar between the partners but the feed used to maintain the colony differed considerably.

Experimental diet

A single batch of 350 kg of Gainesville diet (Hogsette, 1992; Table 1) was produced at the Experimental Facility of the Department of Agricultural, Forest and Food Sciences (University of Turin – UNITO, Italy). Corn, alfalfa, and wheat bran were purchased from Mangimi Monge Snc (Torre San Giorgio, CN, Italy). Corn and alfalfa pellets were hammer-milled (Ceccato Olindo, M4; Italy) to pass a 2-mm sieve, whereas wheat bran was already in ground form (3-mm or smaller). Feedstuffs were mixed for 10 min after which the feed was divided into 5 kg bags and stored at room temperature in a dry place until shipment to the partners. Due to customs restrictions, individual unmixed feedstuffs were sent to the Canadian partner, where the feed was prepared on site. Each partner stored a sample of the dry feed in a dry place pending chemical analyses. The chemical composition of the diet is shown in Table 1 (for methods see below). Gross energy was analysed with an adiabatic calorimetric bomb (C7000; IKA, Staufen, Germany), and determined to be 16.5 MJ/kg.

TABLE 1 Ingredient and analysed chemical composition (% as is) of the Gainesville diet[1]

Composition	Content
Ingredient	
Wheat bran	50.0
Alfalfa	30.0
Corn	20.0
Chemical	
Dry matter	88.9
Crude ash	5.8
Crude protein[2]	13.7
Crude fat	2.9
Starch	26.0
Sugar	3.7
NDF	33.4
ADF	15.5
ADL	3.7
P	0.53
K	1.18

1 NDF = neutral detergent fibre; ADF = acid detergent fibre; ADL = acid detergent lignin.
2 Crude protein as N × 6.25.

Nursing the neonates

The procedure for nursing BSF neonates (0 to 5-days-old-larvae) to determine the amount of feed, larvae density, and growth time was assessed in a pilot study prior to the ring test (see Supporting information: Annex 2). In brief, eggs (24 to 48 h old) were collected from fly cages and placed on a mosquito mesh inside of an empty crate, covered with a lid. The lid of the crate was perforated and sealed with a fine fabric (mesh size of approximately 200 μm). This allowed air exchange and avoided the escape of hatched BSFL. The crate was placed at 27 °C and 60 to 80% relative humidity (RH). The crates were checked daily and 0 to 24 h old larvae (0-DOL) were collected to start the experiment (approx. 3 days after egg harvesting). This method was preferred over letting eggs hatch directly above a crate containing feed as the feed-less method eliminates differences in hatch rate and hatching time (up to 24 h).

One gram of newly hatched neonates (<24 h of age) were placed in a 20 × 30 cm crate (no lid, with the height being not standardised) on top of a mixture of 0.3 kg of a mix of standard Gainesville diet and 0.7 l tap water (25-30 °C). The crates were placed at 27 °C, 60% RH in the dark. This method resulted in 45,000 to 50,000 larvae / g neonates after 5 days with an average weight of 3.8 mg / larvae. A minimum of two crates were set up to ensure that enough BSFL were available. When the larvae were 5 days old (5-DOL), the crate contents were weighed, combined, and gently homogenised and three subsamples were randomly taken. The number of larvae (at least 100) and total weight of all larvae in the subsample was determined to calculate the survival and average weight. Six portions of the 5-DOL/frass mixture were then taken with an estimated number of 15,000 larvae per portion.

Rearing the larvae

Six plastic crates of 60 × 40 cm (n = 6) were pre-weighed and either stacked or placed on a shelf; crate height was not standardised. The diet consisted of a mix of 3.33 kg of dry feed and 6.67 kg of tap water (26-28 °C). Hence, a total of 10 kg wet diet was added to each crate. The diet was gently homogenised to ensure an equal distribution of the moisture. A 25 g sample was taken from each crate for chemical analyses and for pH determination. Finally, 15,000 5-DOL were added and the crates were randomly placed at 27 °C and 60% RH in the dark. If the crates were stacked, ventilation between the crates was ensured and an empty crate was placed on top and also at the bottom to minimise possible edge effects (e.g. cold floor). The experiment was performed at this scale, to mimic industrial rearing including the dynamics that come with a large larvae number (e.g. heat production (Meneguz *et al.*, 2018a; Yakti *et al.*, 2022; Yang and Tomberlin, 2020)), while still being manageable by hand.

Larval growth was monitored per crate by determining the average weight of the larvae at day 3 and 7 of the experiment. Subsamples were taken from five locations per crate; from each corner, at 10 cm from each edge and one from the centre (Supplementary Figure S1). This method aimed to attain representative samples, without disturbing the crate contents. The five subsamples were pooled to one sample with at least 100 larvae. All the larvae in the sample were counted, washed, dried with a paper towel, and weighed to determine their average wet weight. Thereafter, they were dried at 60 °C for 72 h to determine their DM content.

The experiment was terminated when at least 10% of the BSF attained the prepupal stage. This was assessed through a daily visual check after the first prepupae were observed. All the replicas were harvested on the same day. At harvest, the total weight of the crate was determined, and the content was harvested by sieving (openings of 2 to 4 mm depending on larval size and availability). Both the harvested larvae and the frass were weighted, and a sample was taken from each crate. The frass sample was stored at –20 °C for chemical analyses, while the larval sample was rinsed with lukewarm, demineralised water, and then dried with a paper towel. After that, the larvae and prepupae were split and a part was used to determine the average larval weight (at least 100 larvae). The larvae were stored at –20 °C for further chemical analyses.

Sample preparation and chemical analyses

Samples were analysed by internal or external laboratories. In line with current practices, analytical procedures were allowed to differ, but they had to adhere to internationally accepted standards. Therefore, sample preparation and analytical procedures differed among the institutes and contributed to between-institute variation. Samples of the feed, larvae, and frass were prepared by drying and/or grinding using different procedures. For the details of equipment and procedures used for sample preparation, as well as the references to the laboratory analytical procedures, see Supplementary Table S2. For the feed, larvae, and frass, each institute organised the analysis of dry matter, ash, and nitrogen. Larvae were also analysed for crude fat content and the frass for phosphorus and potassium contents. Feeds were further chemically characterised by one institute through the analyses of starch (NEN, 1974), sugars (EC1971), NDF (Van Soest *et al.*, 1991), ADF (Van Soest *et al.*, 1991), and ADL (Van Soest *et al.*, 1991). Due to differences in equipment availability and financial possibilities among the institutions, some chemical parameters could not be measured by all the partners.

Calculations and statistical analyses

The main goal of the ring test was to assess the within and between partner variability, which are the repeatability and reproducibility of the protocol. For all the variables, repeatability and reproducibility were determined in accordance with protocol E691-20 of the ASTM (Standard practice for conducting an interlaboratory study to determine the precision of a test method). The ring test did meet the minimum requirements of the ASTM protocol on the number of participants (six) and familiarity (i.e. previous experience with insect feeding experiments). The partners were blind to the results of the other partners to avoid any bias. Table 2 lists the definitions for repeatability and reproducibility used in this study.

The analyses entailed different stages. In the first stage, possible inconsistent results were flagged using the Mandel's h and k consistency statistics at the 0.5% significance level (cut-off k: 1.73, cut-off h: 2.23). Results that were flagged as potential outliers were investigated in depth assessing possible errors in the set-up, typos, calculation errors, or other causes to assess whether to keep them in the further analysis or were removed. Some inconsistent results proved to be typos or the use of different units (e.g. mg vs g) and could easily be resolved, while for others the reason remained unknown. When a partner was removed from a dataset

TABLE 2 List of definitions used in this study, based on the definitions in the ASTM 691-20 protocol

Parameter	Definition
Repeatability (r)	Precision of results from tests conducted within the shortest practical time period on identical material by the same test method in a single laboratory.
Reproducibility (R)	Precision of results from tests conducted on identical material by the same test method in different laboratories.
Repeatability and reproducibility standard deviation (S_r and S_R)	Standard deviation of test results obtained under repeatability and reproducibility conditions.
Repeatability and reproducibility limit (r and R)	The value below which the difference between two individual test results obtained under repeatability and reproducibility conditions may be expected to occur with a probability of approximately 95%.
Normalised repeatability and reproducibility limits (r% and R%)	The value below which the normalised difference between two individual test results obtained under repeatability and reproducibility conditions may be expected to occur with a probability of approximately 95%.

for a variable, these data were not replaced. However, when individual values were flagged as an outlier, they were replaced by the average of that partner according to the recommendations of evaluating laboratory consistency with missing data of the ASTM protocol. The resulting dataset was then used to calculate the S_r and S_R which is the same as the within and total variance of a one-way analysis of variance if no outliers were removed. S_r and S_R were multiplied by 2.8 ($1.96 \times \sqrt{2}$) to determine the 95% repeatability and reproducibility limits. A normalisation was performed by dividing this limit with the average value of the parameter in order to compare the outcome (r% and R%). Bioconversion efficiency of dry matter (BE_{DM}) was calculated as g dry weight gained / g dry feed provided × 100%. The bioconversion efficiency of nitrogen (BE_N) was calculated as the g nitrogen larval biomass / g nitrogen provided via the diet × 100%.

Due to variations in the estimated number of larvae at the end of the experiment (due to differences in survival and/or subsampling errors), a linear regression was performed to assess the relation between the estimated number of larvae in a crate and the yield or average larval weight of that crate. The obtained linear model was thereafter used to standardise the yield or average larval weight to 15,000 larvae for each partner to account for the differences in final larvae density.

Results and discussion

The number of papers assessing feeding substrates for BSFL has increased over the last few years and the published results are considered essential for both the academia and the rapidly growing BSF industry. Considering that BSF farming is an approach for waste management and feed production, much of published research focuses on the utilisation of different waste streams and by-products of the food and biofuel industries, which could vary in composition based on the source and pre-processing of the used by-products. It proves difficult to compare results obtained from different studies, due to many factors that could differ among them and, in turn, influence larval growth and bioconversion (e.g. larvae density, feeding regime, or temperature). The standardisation and harmonisation of experimental protocols aims to improve the comparability among trials by minimising differences in BSFL growth conditions and provide better insights into larval performance on tested substrates. Similar efforts to explore variation among institutes and to harmonise procedures have been undertaken for other animals such as rainbow trout and broiler chicken (e.g. Nichols *et al.*, 2018; Ravindran *et al.*, 2017). To our knowledge, this study is the first to develop and evaluate a standardised protocol, and to estimate the repeatability (within laboratory variability) and reproducibility (between laboratory variability) for BSFL feeding experiments.

Overall, the standardised experimental protocol with the Gainesville diet resulted in a final average weight

of 92 mg (range 63-156 mg) with a total yield of 1.18 kg (range 0.63-1.46 kg) and a dry matter content of 27% (range 26-32%). The latter underlines the need to express yield and bioconversion on DM basis rather than fresh weight basis – or at least include the DM% in any report (Bosch *et al.*, 2020). Compared to the other parameters in this study, larvae were relatively consistent in ash content (avg. 116; range 107 to 126 g/kg DM) and N content (avg. 82; range 75 to 90 g/kg DM; or 35 to 42% CP of the DM using a conversion factor of N to CP of 4.67 (Janssen *et al.*, 2017)). Crude fat varied more among partners, (avg. 172; range 132 to 231 g/kg DM. The in BE_{DM} and BE_N were on average 12 and 42% (range 7.7-15.1% and 35.4-47.6%). These ranges are similar to previously published values (Arabzadeh *et al.*, 2022; Barragan-Fonseca *et al.*, 2018; Chia *et al.*, 2020; Meneguz *et al.*, 2018a; Tinder *et al.*, 2017; Van Looveren *et al.*, 2023).

The data of each partner is presented in Figure 1 and Table 3 (detailed information in Supplemantary Table S3). It is evident that, despite the efforts to standardise procedures, differences among partners were still observed and changed depending on the time or parameter in question. For example, the BSFL dry weight after 3 days was 2.8 times higher for partner I compared to the average of the other partners, but similar at harvest (25 mg vs 25.8 mg on average). A second example is the BSFL weight at harvest: from 63 to 156 mg fresh weight and 17 to 51 mg on a dry weigh basis, indicating a factor 2.5 to 3 for this parameter. Plausible explanations for this variation could be differences in laboratories environment, variation in plasticity responses to new diet, survival, as well as differences in the genetic background (Kaya *et al.*, 2021; Khamis *et al.*, 2020; Sandrock *et al.*, 2022).

An estimation of the within (repeatability limit, r%) and between laboratory variability (reproducibility limit, R%) can indicate robustness of a protocol. The summary of the normalised repeatability and reproducibility limit estimates for the variables related to the larvae (both biological and chemical parameters) and the frass is presented in Table 4. For biological variables (average weight, yield, number of larvae in crate (density)), the normalised repeatability limit was on average 24% (range: 17.6-33.7%). The normalised reproducibility limit was about 2.5 times higher with an average of 60% (range: 32.2-95.1%). This implies that the difference between the lowest and highest measured value within an experiment should (on average) not exceed 24%, or 60% between experiments (depending on the parameter and with an identical set-up).

Although the repeatability limit of the average fresh and dry weights at day 3 was near average to the other parameters (22-28.4 vs 24%), the reproducibility limit

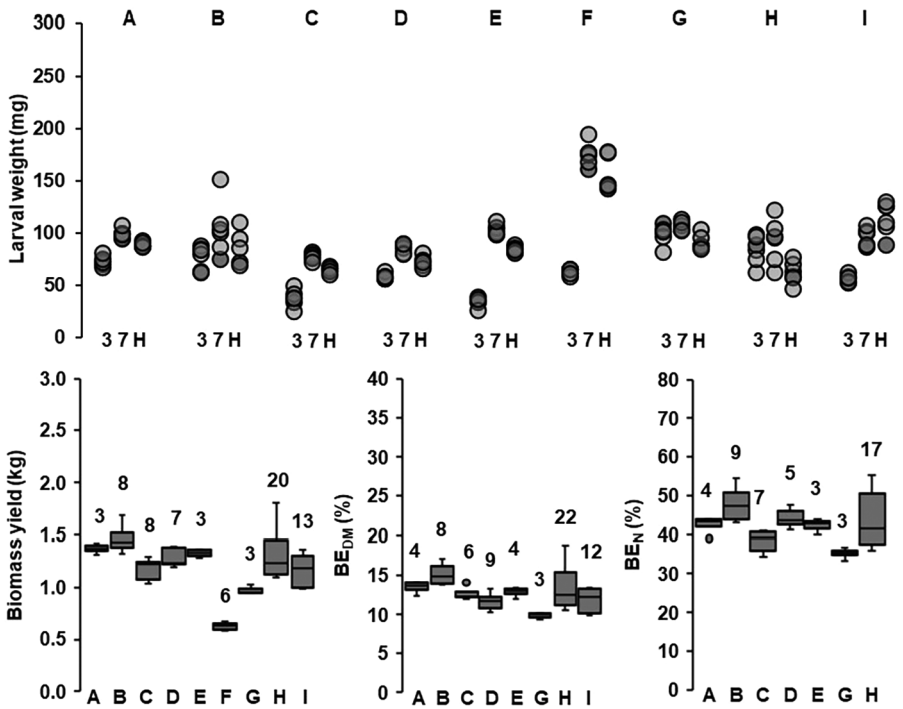

FIGURE 1 Top: The average larval fresh weight (mg) at day 3, 7 and at harvest (H) for the different partners (A-I) for each replicate. Bottom: the biomass yield at harvest (kg FW), bioconversion efficiency (BE) and nitrogen bioconversion efficiency (BE_N) with the coefficient of variation above each boxplot. Note that the BE calculations could not be made for all partners.

TABLE 3 Larval and frass variables for the different partners (A-I) (average ± SD, n = 6)[1]

	Partner								
Parameter	A	B	C	D	E	F	G	H	I
Larvae at 3 days									
BW mg wet	74 ± 5	78 ± 11	60 ± 3	40 ± 8	58 ± 4	85 ± 14	64 ± 3	37 ± 4	101 ± 9
BW mg dry	17 ± 1	19 ± 3	13 ± 1	9 ± 2	12 ± 1	NA	15 ± 1	8 ± 1	37 ± 9
Larvae at 7 days									
BW mg wet	100 ± 5	105 ± 26	87 ± 4	79 ± 3	99 ± 8	94 ± 21	176 ± 11	105 ± 5	108 ± 4
BW mg dry	28 ± 1	30 ± 9	23 ± 1	22 ± 1	21 ± 2	NA	57 ± 5	28 ± 2	32 ± 1
Larvae at harvest									
BW mg wet	89 ± 2	87 ± 17	74 ± 5	66 ± 3	115 ± 16	63 ± 11	156 ± 17	86 ± 3	92 ± 7
BW mg dry	24 ± 0	24 ± 5	20 ± 1	17 ± 1	31 ± 5	17 ± 3	51 ± 8	22 ± 1	25 ± 2
Yield kg wet	1.37 ± 0.04	1.46 ± 0.12	1.28 ± 0.08	1.18 ± 0.1	1.17 ± 0.15	1.20 ± 0.11	0.63 ± 0.04	1.33 ± 0.03	0.97 ± 0.03
Yield kg dry	0.36 ± 0.02	0.40 ± 0.03	0.35 ± 0.02	0.30 ± 0.02	0.32 ± 0.04	0.36 ± 0.08	0.20 ± 0.01	0.34 ± 0.01	0.26 ± 0.01
Number × 1000	15.5 ± 0.7	15.7 ± 3.6	18.2 ± 1.0	18.1 ± 1.9	10.6 ± 1.2	19.0 ± 0.8	4.1 ± 0.6	15.5 ± 0.9	10.1 ± 0.8
N g/kg DM	78 ± 1	75 ± 1	87 ± 2	80 ± 1	85 ± 2	80 ± 4	NA	78 ± 1	90 ± 2
Fat g/kg DM	159 ± 4	161 ± 10	132 ± 16	201 ± 9	160 ± 18	147 ± 29	NA	231 ± 8	188 ± 32
Ash g/kg DM	111 ± 3	115 ± 3	112 ± 3	111 ± 3	126 ± 5	126 ± 6	NA	107 ± 3	119 ± 3
BE_{DM} %	13.5 ± 0.6	15.1 ± 1.2	12.7 ± 0.8	11.4 ± 0.7	11.9 ± 1.5	13.3 ± 2.9	7.7 ± 0.3	12.9 ± 0.5	9.9 ± 0.3

TABLE 3 (Continued)

	Partner								
Parameter	A	B	C	D	E	F	G	H	I
BE_N	42.8	47.6	44.1	38.4	41.3	43.6	NA	42.5	35.4
%	± 1.9	± 4.1	± 2.1	± 2.6	± 5.1	± 7.3		± 1.4	± 1.1
Frass									
Weight	2.79	3.12	1.95	1.92	1.85	2.12	2.24	2.26	1.88
kg wet	± 0.2	± 0.24	± 0.07	± 0.04	± 0.07	± 0.16	± 0.11	± 0.10	± 0.06
Weight	1.18	1.26	1.20	1.24	1.00	1.13	NA	1.23	1.33
kg dry	± 0.02	± 0.04	± 0.04	± 0.02	± 0.04	± 0.09		± 0.03	± 0.07
N	29	20	24	23	30	29	NA	24	32
g/kg DM	± 1	± 1	± 1	± 1	± 1	± 2		± 1	± 1
Ash	NA	122	128	120	134	137	NA	128	126
g/kg DM		± 3	± 3	± 6	± 3	± 3		± 2	± 1
K	27	NA	28	25	31	31	NA	24	NA
g/kg DM	± 0		± 1	± 0	± 1	± 1		± 1	
P	15	NA	12	18	16	16	NA	13	NA
g/kg DM	± 0		± 1	± 1	± 1	± 1		± 0	
pH	8.81	8.75	8.62	9.08	8.87	NA	8.47	8.74	8.81
	± 0.05	± 0.05	± 0.03	± 0.08	± 0.1		± 0.14	± 0.12	± 0.09

1 BW = body weight; N = Nitrogen; DM = dry matter; BE_{DM} = dry matter bioconversion efficiency; BE_N = nitrogen bioconversion efficiency; K = potassium; P = phosphorus; NA = not available.

was the highest of the study (R% > 90%). This might be due to exponential growth in this period. Differences of a few hours (the protocol stated 3 days and not 72 h), initial weight of the larvae, or (a)biotic conditions may have had a large impact. The average weight after 7 days had the lowest variation (low r% and R%). This reduced variability combined with the more industrial perspective may advocate the use of a fixed day endpoint when compared to a biological endpoint (X% of (pre)pupae) for future BSF feed experiments. Nevertheless, when substrates with different nutritional values/energy are tested, a fixed day endpoint harvest may cause a bias in the assessment when one treatment (low nutrients) enters a starvation mode, while others still have adequate available nutrients.

At the time of harvest, the variability on the yield (and calculated BE) was near average compared to the other parameters (21 vs 24%), but the variability (both r% and R%) of the average larval weight (wet and dry) was near record high and notably higher than at day 7. This may, in part, be due to a difference in percentage of prepupae (32%, SD 12%) and other biological/experimental influences. Nonetheless, the main reason may be the variability in larvae density (estimated number of larvae per crate; R%: 68.9), even though the standard protocol stated 15,000 larvae per crate. This variability could be due to sampling/estimation errors at the start of the experiment or differences in mortality during the experiment. Differentiating between these two options at harvest is impossible based on the employed protocol. Previous studies reported a positive correlation between the amount of feed provided and the final larval weight (Diener *et al.*, 2009), and improved yield at higher densities when the larvae are harvested simultaneously (Yakti *et al.*, 2022). Similarly, in this study, a significant positive correlation was detected between the estimated num-

TABLE 4 Descriptive statistics and normalised repeatability and reproducibility limits for parameters related to larval and frass parameters[1]

Parameter	Average	SD	r%	R%	n
Larvae at 3 days					
BW, mg wet	66.6	21.2	28.4	92.8	9
BW, mg dry	13.6	4.5	22.0	95.1	7
At 7 days					
BW, mg wet	96.5	9.62	17.6	32.2	8
BW, mg dry	26.1	4.14	20.6	48.2	7
Larvae at harvest					
BW, mg wet	84.2	16.6	32.1	62.5	8
BW, mg dry	22.6	4.6	33.7	65.3	8
Yield, kg wet	1.24	0.15	20.5	38.7	8
Yield, kg dry	0.33	0.04	21.1	40.0	8
Number, ×1000	15.3	3.6	20.0	68.9	7
N, g/kg DM	82	5	4.5	17.8	8
Fat, g/kg DM	172	31	19.7	53.2	8
Ash, g/kg DM	115	7	7.9	18.0	8
BE_{DM}, %	11.9	2.2	21.4	39.4	8
BE_N, %	41.7	4.5	21.5	31.3	8
Frass					
Weight, kg wet	2.21	0.43	10.4	55.7	8
Weight, kg dry	1.19	0.10	14.5	26.6	8
N, g/kg DM	26	4	10.9	46.8	8
Ash, g/kg DM	128	6	7.2	14.7	7
K, g/kg DM	28	3	6.7	29.7	6
P, g/kg DM	15	2	12.6	39.6	6
pH	8.81	0.14	2.5	5.1	7

1 SD = standard deviation; r% = normalised repeatability limit; R% = normalised reproducibility limit; n = the number of partners included in the analysis; BW = body weight; DM = dry matter; BE_{DM} = dry matter bioconversion efficiency; BE_N = nitrogen bioconversion efficiency.

ber of larvae per crate at the time of harvest and the yield (yield (g) = 600.5 + 0.0402 × # larvae; $R^2 = 0.65$) and a strong negative correlation with the average weight (average larval wet weight (mg) = 169.5 – 0.0055 × # larvae; $R^2 = 0.84$; Supplementary Figure S6). Therefore, it is fair to assume that density differences resulted in poor reproducibility of the biological harvest parameters.

Data were normalised based on the linear models for yield and average larval wet weight to assess the theoretical potential repeatability and reproducibility of the standard protocol if all partners had 15,000 larvae per crate. This reduced the repeatability of the fresh weight from 32.1 to 26.3% and for the yield from 20.5 to 13.3%. The reproducibility reduced from 62.5 to 28.6% for the average larval weight and for the yield from 38.7 to 35.7%. This indicates that it is of vital importance that similar number of larvae per crate should be considered when comparing feeding experiments across different labs and production sites. Furthermore, the number of larvae at the end of the experiment should be

determined in order to estimate and include any effect related to survival and/or escaped larvae. It is suggested to: 1) use at least 3 subsamples with at least 100 larvae in each sample, 2) avoid any unintentional bias (mainly towards larger larvae) by counting all the larvae in a sample, and 3) strive for a coefficient of variance lower than 10% between the 3 subsamples (SD / mean × 100%). If not, more subsamples should be taken or the subsample protocol should be re-evaluated. The findings of this study indicate the importance of reporting these variables in publications to improve the comparability: estimated number at the start and estimated number at harvest and, by extension, crate size, feed quantity and timing, and sampling methods.

In general, the chemical parameters (N, fat, ash, P, K, pH) could be determined much more precise with a repeatability and reproducibility limit about half of the biological parameters: average r% of 9% (range: 2.5-19.7) and average R% 28% (range: 5.1-53.2; Table 4). Importantly, the variation in nitrogen (or protein) within (4.5%) or between partners (17.8%) was among the lowest values measured in this study. The highest r% and R% values for a chemical parameter were found for the crude fat content, which could be due to a larger inherent variability, differences in methods and extraction efficiency among labs (Ramos-Bueno *et al.*, 2016; Smets *et al.*, 2021), to differences in larvae density leading to a different nutrient availability for BSFL (Yakti *et al.*, 2022) or due to genetic differences among colonies.

Frass seems to be very constant within an experiment (low r%), especially the final pH that displays the lowest r% of the whole study (2.5%). The reproducibility, on the other hand, was in the same range as the other variables with the notable exception of the pH. This is in concordance with a previous study that reported a convergence of the pH near the end of a feeding experiment (Meneguz *et al.*, 2018b).

In practice, the r% and R% values can be used as a quality control guidance. A higher than expected within laboratory variability (r%) may, for example, warrant a check if the homogeneity of the experimental climate room/set-up is adequate. Heterogeneous ventilation may affect the microclimate and lead to variability in larval growth. On the other hand, a large deviation from the expected value range (outside reproducibility limit) may be, for example, due to differences in overall ventilation, miss-calibrated/drifted temperature sensor or genetic differences. The standardised protocol presented in this study enables quality control over experiments by signalling deviations in performance within control treatments. Considering the between-laboratory variability of identical BSF feeding experiments in this ring test, it is clear that comparing small differences in results among studies should be done with caution especially when different protocols are used. Due to the absence of similar studies, it is unclear whether the values obtained in the current study are normal for insect feeding experiments.

Conclusion

The current study aimed to provide an initial protocol for BSFL feeding experiments and provide the first estimates on the reproducibility and repeatability for a broad range of biological and chemical parameters. The protocol (SI annex 1) can be used in its present form, as a basis, for future experiments. Yet, the results indicate that even with the protocol, variation within and between laboratories is far from fully resolved, especially for the younger larvae (day 3). Despite this, using a standard protocol would improve the comparability of scientific results across studies and facilitate future meta-analyses. To further improve this initial protocol, additional research is needed to understand the impact of currently unstandardised variables, such as ventilation and genetic differences, on repeatability and reproducibility. Furthermore, the data clearly indicates that special care should be taken when estimating the number of larvae (at start and harvest) and report them appropriately. These findings cannot only be used in concordance with the protocol but can also be used as guidance and control for other (non-feed) experiments providing a first insight into the expected results and variability.

Supplementary material

Supplementary material is available online at:
https://doi.org/10.6084/m9.figshare.24139395

Conflict of interest and funding statement

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