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Standardisation of quantitative resource conversion studies with black soldier fly larvae

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REVIEW ARTICLE

Abstract

Using larvae of the black soldier fly (*Hermetia illucens*; BSF) to convert low-value residual organic resources into high-value products like protein-rich animal feed ingredients and biofuel while managing organic waste has developed into a global industry. Considering the associated exponential increase in publications dealing with diet conversion efficiency by BSF larvae, it is timely to suggest procedures to arrive at an improved harmonization and reproducibility among studies. This means establishing protocols for describing the basic experiment design, fly colony origin, rearing procedures, reference and experimental feeding substrates, and sampling preparations including microbiota and chemical analyses. Such standardised protocols are instrumental to allow conversion efficiencies to be calculated. Some of these parameters are relatively easy to describe such as giving the origin and rearing conditions, while others are more challenging (e.g. description of microbe community). In this article we discuss and propose such procedures with the aim to arrive at standardisation of how future resource conversion studies with BSF larvae are conducted and how results are communicated.

Keywords: genetic status, *Hermetia illucens*, microbiota, waste management, standard operating procedure

1. Introduction

Historically, the black soldier fly (BSF, *Hermetia illucens* (L.); Diptera: Stratiomyidae) was viewed as a medical-veterinary pest due to its potential to produce accidental myiasis (Bonnet, 1948; Meleney and Harwood, 1935), or contamination of poultry wastes (Axtell and Arends, 1990; Axtell and Edwards, 1970); however, it was considered valuable to the forensic sciences as the presence of its larvae on human remains could be used to estimate a time of colonization (Lord *et al.*, 1994; Tomberlin *et al.*, 2005). The first publication on the BSF larvae as feed probably dates back to 1973 (Hale, 1973). An earlier publication mentioned the natural control of house fly, *Musca domestica* L. (Diptera: Muscidae) by BSF larvae in manure (Furman *et al.*, 1959). Another application has been the use of BSF larvae as a tool to manage waste, in particular manure (Sheppard *et al.*, 1994). The interest to study BSF larvae during the last year has shown an exponential increase: In 2017 and 2018, more publications (173) appeared than during the preceding 15 years (2002 to 2016) (124) (Web of Science, consulted 19 December 2018). This growing interest has to do with the capacity of BSF larvae to convert low-value residual organic streams into high-value protein products as feed for fish and production animals (Henry *et al.*, 2015; Makkar *et al.*, 2014), and for biofuel or biofertiliser (Rehman *et al.*, 2018; Wang *et al.*, 2017b).

Many governments now pursue a circular economic policy. A study of seven European nations determined a shift to a circular economy would reduce each nation's greenhouse-gas emissions by up to 70% (Wijkman and Skånberg, 2017). The reprocessing of goods and materials generates jobs and saves energy while reducing resource consumption and waste (Stahel, 2016). How to optimally use the capacity of

BSF larvae to contribute to a more circular economy of food production will be an important area for future studies.

The larval diet is crucial for the economics of BSF production due to the trade-offs between the costs of the feed substrate (resource) (Onsongo *et al.*, 2018), its nutritional quality, and the effects on larval development and body composition (e.g. protein, fat) (Barragan-Fonseca *et al.*, 2017), environmental footprint (Smetana *et al.*, 2016), and safety of the insect product (Purschke *et al.*, 2017). Moreover, larvae can be used for the management of various sorts of waste (Van Huis and Tomberlin, 2017). To quantify the efficiency of conversion of the feed given to BSF larvae, numerous methodologies have been applied (Bosch *et al.*, 2019), which hamper comparison among studies. Rather than reviewing variations in methodologies applied as described in the scientific literature, this discussion paper proposes standardisation of methods to quantify diet conversion efficiency by BSF larvae and aims at harmonisation among studies and reproducibility of results to improve the overall scientific rigour in this developing field of research. As the larvae have an intimate relationship with microbiota (De Smet *et al.*, 2018), which affects the conversion efficiency, sampling procedures for microbial analyses are also included. Furthermore, we present background information to increase awareness of factors impacting results and suggest avenues for future research. The structure of the sections below follow that for potential sections in the 'materials and methods' of a typical manuscript describing diet conversion efficiency by BSF larvae. Directions and checklists in boxes at the end of specific sections (Box 1-7) are provided to facilitate design, execution and communication of future resource conversion studies.

2. Experimental design

Developing an appropriate experiment design is predicated on the explicit question being asked by the research. Development of a concise question allows the researcher to then proceed to stating the hypothesis being tested that is potentially refuted by data generated. Common practice should be to complete these first two steps prior to tackling the experiment design. Furthermore, the question, and/or hypothesis, should be clearly stated at the conclusion of the introduction of the resulting paper. Doing so allows for the reviewer, and future reader, to understand specifically what is being tested. From the author perspective, clearly stating this information up front could also decrease the variability of reviews received from the journal as the individuals asked to comment will know clearly what is being tested.

The purpose of the experiment design is to allow the researcher to ask specific questions *a priori*. Doing so reduces potential bias as related to navigating the experiment once it is up and running as well as after data have been collected. Once a design is put into place, researchers will need to understand what to expect from the experiment and potential impact of data generated. Specifically, researchers should take care not to extrapolate their data beyond the experiment conducted; however, commentary on potential impact beyond the experiment is encouraged as long as the concepts are presented as hypothetical.

A well-developed experiment design has clearly defined treatments. In most cases, a control is used comparatively. Therefore, it is important to concisely state these treatments in the materials and methods. From the perspective of a diet study, the control potentially would be the standard diet (Section 3). Using a control will allow for two items to be accomplished: (1) most importantly, determining if the treatment tested impacted the variables being measured; and (2) providing data for all future studies to utilize globally in terms of comparison. The first aspect is far more important for the study being conducted; however, the second allows for posterity to continue to use the data generated. This aspect provides relevance to data into the future. One thing to note, not all studies will possess a control. Lacking such a facet in an experiment does not decrease the value of the study. But, broad applications (e.g. relevance to other studies) of the data could be limited. We therefore favour and encourage study designs in which a reference diet as a positive control treatment is included.

Defining a replicate as related to the treatments tested is critical. This factor is often overlooked or misapplied in terms of being defined. One should differentiate between pseudoreplicates and experimental replicates. According to Hurlbert (1984) 'Pseudoreplication most commonly results from use of inferential statistics to test for treatment effects with data from experiments where either treatments are not replicated (though samples may be) or replicates are not statistically independent.' It follows that individual flies in a cage or larvae in a container are not true experimental replicates but are considered to be pseudoreplicates. For conversion studies, we consider containers as experimental units (Section 7). As such, the replicates are based on a single generation or population, which is restrictive in terms of data applicability to all BSF larvae around the world (i.e. limited scale relevant to the population tested). Future studies may be performed on a larger scale and include multiple generations and populations providing the replicates for a study. In a nutshell, bench-top studies do not necessarily translate into industrial scale results. Therefore, researchers should be cautious with describing the global relevance of the data generated. It is in general advised to have at least 4 replicates for each diet being evaluated.

3. Origin, rearing history and genetic status

The population from which the larvae used in feed conversion studies are performed needs to be specified as precisely as possible since it can impact experiment results and is therefore essential information to allow comparisons among studies. The origin should be specified as: (1) the geographic location (defined as degrees latitude and longitude) on which specimens have been collected from a natural field population; and (2) the history of the colony, defined as a genetically isolated population that has been domesticated and has undergone selection for increased adaptation to the specific circumstances (e.g. diet, temperature, light spectrum and intensity, day/night cycle, humidity, etc.) under which this population was kept since its domestication. This adaptation could consequently affect how efficiently the larvae convert test diets during experiments.

In scenarios where researchers obtain larvae from an insect producer, the company should be mentioned and, if the producer is willing to share it, the information outlined previously should be provided. If larvae originate from a producer that does not want to disclose this information, such should be explicitly stated in the manuscript. Furthermore, voucher specimens need to be curated in a museum and such information stated in resulting publications. Doing so will allow future researchers access to these materials to assess additional information about the biology (e.g. genetics) and life-history of the population used.

Colony genetic status

Differences in conversion efficiency of the same feed or feeds with similar nutrient composition reported in the literature may be explained by genetic differences between colonies maintained in the respective laboratories. Such differences in performance between genetic lines are well known for livestock species like chickens and pigs. Although there are very few published data on comparisons of BSF strains, indications have been found that the origin of BSF can also affect experimental results (Zhou *et al.*, 2013). Genetic differences can also be caused by: (1) genetic drift; (2) mutations; or (3) inevitable selection occurring after initial colony establishment. Differences between insect colonies of a given species are among the most likely causes of contrasting experimental results in entomology; however, few studies have explicitly tested this contention (Berthier *et al.*, 2010). Captive populations of insects maintained for many generations in genetically isolated conditions are commonly inbred (Francuski *et al.*, 2014; Sørensen *et al.*, 2012). It can be of interest to estimate the degree of inbreeding in a colony. For this it is necessary to know the number of generations the colony has gone through since its establishment. Quantitative information on the degree of homozygosity of genes that are selectively neutral is highly desirable (e.g. obtained by analysis of molecular genetic markers such as microsatellites; Liu *et al.*, 2015). If known, the founder population size (i.e. the number of individuals sampled from a natural population of which the progeny was used to start the colony) is important to report. In the process of establishing a colony from a natural population it is likely to pass through genetic bottlenecks that can drastically reduce the effective population size (i.e. the number of individuals producing the next generation). For example, population genetic calculation predicts that alleles can be lost after 50 generations of inbreeding due to genetic drift if the effective population size is 200 individuals whereas allele frequencies are relatively stable when the effective population is 2,000 adults, fluctuating between 40 and 60% when initial frequency is 50% (Johnson, 2007). And as previously stated, if voucher specimens of the insects studied are deposited in a public collection and their registration number is stated, the specimens can be used for comparative purposes, e.g. genome sequencing. Finally, if individuals from other origins have been introduced into the population in order to allow outbreeding, it should be stated how this was done (i.e. through controlled mating or only adding feral specimens into a cage with adults), how often, and which numbers were introduced.

Standard rearing diet

As larvae may originate from a population that is adapted to a specific standard-rearing diet (Wang *et al.*, 2017a), it is of value to provide details of this diet. The main crude ingredients of the standard diet on which the colony is maintained (e.g. chicken feed diet containing wheat and soybean meal as main ingredients), their proportions in the rearing diet (e.g. respectively 60.4 and 22.0%) and the (commercial) supplier need to be provided. Reporting the proximate nutrient contents of the standard diet is desirable. In case a documented defined diet was used the original reference should be included (e.g. Gainesville house fly diet; Hogsette, 1992) and any modifications should be specified. The duration of the rearing cycle (i.e. egg to egg) together with mean pupal biomass provide comparative information on performance of the colony on their standard diet.

Describing the procedures and conditions is necessary to facilitate pupation and eclosion as well as the housing of adults and the collection of egg masses. For an example of such description we refer to Sheppard *et al.* (2002). The handling of the neonates that are used for diet testing is described in Section 6.

Box 1. Origin, genetic status and rearing conditions – crucial information to report.

Origin, genetic status and rearing conditions:
- Origin of the insects used to set up the colony.
- Founder population size.
- Minimum effective population size.
- Number of generations in the lab.
- Introduction of individuals from outside the colony if applicable.
- Current rearing conditions:
  - Diet composition; ingredients, suppliers and product names.
  - Abiotic conditions: temperature, light intensity and spectral composition, photoperiod, air relative humidity.
- Duration of rearing cycle.
- Substrate for pupation and eclosion.
- Conditions for reproduction: adult nutrition and oviposition substrate.

4. Reference diet

We propose using a reference diet to rear the neonates for the initial 5 days (Section 6). When switching the larvae to the experimental diets, parallel groups of larvae should be reared on this reference diet until the set harvest moment (see below). The assessment of their growth performance facilitates comparisons among studies. At present, few studies used a reference diet and those that had one differed. To facilitate harmonisation among studies it is ideal to create a reference diet using well-defined ingredients that are available around the globe and for a low cost. The ingredients for the Gainesville diet (50% wheat bran, 30% alfalfa meal, and 20% corn meal; Hogsette, 1992) might not be available around the globe, and is likely to vary in composition among producers and regions in the world. When obtaining such ingredients, be aware of the potential presence of (residues of) insecticides that can impact study results.

A more defined alternative is to compose a meridic diet out of well-defined ingredients at specified inclusion levels. It is necessary to include the name of the (commercial) supplier and the specific product name. For example, a protein often incorporated in meridic diets for insects is the major milk protein casein. As there are different casein proteins (α, β, γ, κ) and different ways to make and tailor casein to different applications, it is essential to include the specific product name used in the diet. In line with studies in other insects (Burton, 1970; Singh, 1983), we stimulate researchers to develop such a diet and evaluate it for basic parameters of larval performance (e.g. growth, development rate, conversion efficiency).

As for preparation of such a reference diet, the dry ingredient mixture should be thoroughly mixed with tap water, either by hand in case of sub-kg amounts or by motor-driven mixers in case of higher amounts. The composition of the diet as offered to the larvae should be reported in terms of its contents of dry matter, nitrogen and ash. Chemical analyses to be performed are indicated in Section 9.

5. Experimental diets

As discussed above, designing an experiment partially hinges on proper definition of treatments to be implemented. As with any feeding trial, or diet experiment, defining the diets being tested is critical. Generalized descriptions including qualitative terminology are difficult for others to implement in their own designs and hinder comparison of results. For anyone attempting to conduct such studies, two steps need to be followed when preparing experiment design.

The initial step is to define the diet. This step includes quantified measures of the materials used to produce the diet. Data on where the materials originated, in particular the company and product name and storage method (e.g. used within 24 h of production, frozen, stored on a shelf at 27 °C during a stated period of time) are critical for future replication. Basic characteristics (e.g. moisture, protein, fat, ash) of the diet are essential information for feeding trials. If possible additional data on the nutrient composition (e.g. amino acids, vitamins, and minerals) may contribute to explain differences in larval performance between studies.

The second step is to clearly define the preparation of the diets used in the study. Such information includes specifics on cooking (e.g. 100 °C for 20 min), cleaning (e.g. materials were sorted manually and all plastics removed, or material was washed in 27 °C tap water with 10% bleach for 10 min prior to used), handling (e.g. materials were placed in a grinder (give mesh size, make and model of grinder, company name and location) operating at 'X' rpm for X min), and mixing (e.g. 10% of item A was mixed with 90% of item B). In all cases, such data could be included in the publication produced.

Box 2. Reference and experimental diets – crucial information to report.

Diet composition and preparation:
- Diet ingredients: supplier and product name.
- Storage method and duration if applicable.
- Preparation methods.
- Analysis of contents: dry matter, ash, N and total fat.

6. Rearing neonates

The pre-trial rearing of neonate larvae used in dietary studies may impact the primary parameters indicating substrate suitability. Although published data are lacking, it seems plausible that development time, larval and prepupal weight, and possibly larval protein and fat content are influenced by larval pre-treatment. Hence, standardisation is important to allow direct comparisons between dietary studies. If not using neonates directly with the treatments, but rather neonates aged on a 'starter' diet, they should be provided with an excess of the reference diet (Section 3) from the moment of hatching. This diet should be mixed with water as described in Section 3 and placed in the rearing container. As larval aggregation facilitates early development, it is advised to allow several thousands of eggs to hatch together. One hundred larvae consume approximately 18 g of dry chicken feed during their development (Oonincx *et al.*, 2015). If the larvae are allowed to develop up till one third of their final weight on the starter diet, they require approximately 6 g (dry matter; DM) per 100 larvae. Per clutch of e.g. 500 eggs a max. of 500 neonate larvae hatch, which require 30 g (DM) of the reference diet (Pastor *et al.*, 2015). The size of the rearing container should be such that the layer is around 5 cm thick and not more than 10 cm thick (Brits, 2017). The material of the rearing container needs to be accurately described with special attention for the cover of the container (if present), as this influences air flow and potential moisture loss. The rearing container with the reference diet and the egg clutches on top should then be placed in a climate controlled location with a temperature of 30 °C (Tomberlin *et al.*, 2009) and a relative humidity of 70% (Holmes *et al.*, 2012). If the egg clutches are positioned on top of moistened filter paper the hatch rate can be assessed. A photoperiod of 12 h per day is suitable (Oonincx *et al.*, 2015), but not required for larval development (Biancarosa *et al.*, 2018; Liland *et al.*, 2017). It is strongly advised to measure substrate temperatures during rearing as these can deviate from ambient temperatures. If the substrate temperature is elevated more than 5 °C compared to the ambient temperature layer thickness should be decreased and/or ventilation should be increased to reduce substrate temperatures.

When reared for five days (i.e. 120 h) post-hatching at 30 °C, the larvae are ready to be transferred into the experimental container. This duration is in line with common practice and with Sheppard *et al.* (2002). Moist substrate tends to adhere to the larval integument. Therefore, the larvae need to be cleaned prior to being subjected to experimental treatments. A subsample of the substrate-larvae mixture can be taken from the rearing container with a spoon, placed in a kitchen sieve and gently rinsed with lukewarm water (~30 °C). Subsequently larvae should be carefully dried on a kitchen towel. As a fixed duration of the rearing period can result in different development stages due to colony differences and possibly due to batch-to-batch variations in nutritional quality of the substrate, the development stage should be determined when the treatments are started. This can be done based on the number of spiracle openings and the size of the head capsule as shown by Gobbi (2012). If a small number of larvae, for instance 100, is to be used per replicate these should be counted and weighed as a group. If the number of larvae to be used in one replicate is far larger, for instance 10,000, multiple (>3) subsamples of about 100 cleaned larvae can be taken to determine the average larval weight and then the appropriate total mass of larvae can be calculated. It is advisable to create a representative sample of young larvae at the start of the experiment and chemically analyse these. Results can be used to quantify the resource bioconversion efficiency (Section 11).

7. Rearing larvae

Diet conversion studies with 5-d old larvae should be executed similarly to the rearing of neonates, as described in the previous section, including the abiotic conditions. A known amount of the experimental diet (Section 4) is placed in a container. The water and the diet are then homogenized by carefully stirring. Water is added so that the diet has an optimal consistency. This optimum depends on the physico-chemical properties of the dry material but often approximates a final DM content of about 30% (Cammack and Tomberlin, 2017). Two types of feeding regimes are possible; either the full amount of feed can be offered at the start of the experiment, or larvae can be offered fresh diet at intervals. The latter regime is more labour intensive, but can prevent over- or underfeeding if the amount of available feed is estimated accurately. This will result in a more accurate quantification of the diet conversion efficiency. The container used during the experiment should be adequately described with special attention to the ventilation surface. A known number of larvae is transferred from the neonate rearing container as described in the previous paragraph and carefully placed on top of the substrate in the middle of the tray without spreading them out. Density affects parameters such as development time, survival, and weight (Barragan-Fonseca *et al.*, 2018; Diener *et al.*, 2009). Therefore, the feeding ration (i.e. the amount of feed available per larva) needs to be considered, especially if a one-time feeding regime is used. The bioavailable nutrients and energy provided *via* the diet and *via* microbiota living in the diet determine larval performance. Feeding ration can be based on mg of e.g. fresh matter, DM, organic matter, N, or based on gross energy. Fixing one nutritional parameter will affect other parameters and thereby larval performance. Researchers are stimulated to carefully consider such effects in conversion efficiency studies and discuss such factors when interpreting results.

As temperature and air flow can influence larval development, it is important to correct for potential differences due to the location within the climate-controlled space. Hence, all containers should be placed in a random order within the climate-controlled space and subsequently rotated randomly every other day. Each container can then be considered and used as a replicate (i.e. an experimental unit). If the setup allows for this, e.g. experiments are conducted in climate respiration chambers (Gerrits and Labussière, 2015; Oonincx *et al.*, 2010), it would be of interest to quantify N volatilisation (ammonia), greenhouse gas emissions, heat production, oxygen consumption and carbon dioxide emissions to gain further insight in larval and microbial metabolism, substrate utilisation, and environmental impact of insect production.

Box 3. Rearing of neonates and larvae – crucial information to report.

Rearing neonates until offering experimental diet:
- Egg collection, handling, hatching of neonates.
- Amount of diet provided and description rearing boxes.
- Environmental conditions (temperature, relative humidity, photoperiod).
- Days of pre-trial rearing.
- Larval handling procedures.
- Larval weight and developmental stage.

Rearing larvae on experimental diet:
- Number of larvae per experimental container.
- Feeding frequency and ration.
- Amount of diet provided and water content.

8. Harvesting larvae

Timing the larval harvest is crucial and requires consideration. Different strategies have been applied, ranging from a fixed day for all treatments (e.g. Liland *et al.*, 2017) to harvesting at appearance of first prepupa (e.g. Oonincx *et al.*, 2015) to 100% prepupal formation (e.g. Diener *et al.*, 2011). To our knowledge, in practice, the insects are in general harvested in the larval stage and in some cases self-harvesting is applied. This might be an argument to time the harvest after a fixed number of days or when the first larva has become a prepupa (i.e. when most larvae are still actively feeding and growing). The downside of the latter is that the performance of one single animal determines the timing of harvest of the whole batch. Harvesting when 50% of the larvae have become prepupae would provide a more general assessment of development time. In the last phase, however, the animals stop feeding and lose weight. For the main aim, determining conversion efficiency, having 100% of prepupae will underestimate the potential larval biomass that can be produced per unit of diet. We propose to time the harvest at 5% of the starting number of larvae being in the prepupal stage or to harvest after a fixed number of days of rearing.

The procedure for harvesting larvae and prepupae will depend on the scale (e.g. 100 larvae versus 10,000) and whether the amount of residue is to be quantified and chemically analysed. In the latter case, larvae and prepupae need to be picked out from the residue using a forceps with minimal amounts of adhering residue. Larvae and prepupae are counted and weighed separately. Larvae and prepupae are gently washed in a sieve with running lukewarm water, dried using paper tissues, and weighed again in order to determine the live weight of the larvae and the dry weight of the residue that was washed off. The total residual material is calculated as the sum of the weight of the residue in the rearing container (pre-weigh empty containers before the experiment) plus the weight of residue adhering to the larvae. After these procedures, larvae and prepupae can be combined again.

The larvae and prepupae are often frozen, or killed in hot water and then frozen. Note that in some countries such as the Netherlands, larval welfare is under debate and it is questioned whether larvae can feel pain (i.e. are they 'sentient beings'?). The Council on Animal Affairs in the Netherlands advised to treat insects in production systems as sentient animals (RDA, 2018). This would mean that we need to take care of proper killing methods that minimise distress. For research purposes, we propose to freeze the larvae after harvesting at e.g. -20 °C for >16 h, blanching or shredding which would result in death with minimal distress and no interference with subsequent analyses for conversion studies.

Growth curves of larvae fed a specific feeding substrate provide valuable insights including the evaluation of optimal timing for harvest and we stimulate the inclusion of these curves in studies. Harvesting larvae (e.g. subsampling, cleaning, weighing, returning or not returning) throughout the rearing period to determine the growth curves is in principle not advisable. On the one hand, larvae which are not returned, change diet to larvae ratios and this affects the conversion efficiency. On the other hand, larvae which are returned, might have a lower survival due to handling which consequently affects conversion efficiency (Tinder *et al.*, 2017). It is, therefore, advised to use separate replicates per time point when assessment of growth curves is performed. At large scale (i.e. 10,000 larvae or more), subsampling a small number of larvae (0.5%) is deemed to not greatly disturb the larvae or affect the conversion efficiency. Although unstudied to our knowledge, experiments on growth of individual larvae are unrepresentative for practice.

Box 4. Harvesting larvae – crucial information to report.

Harvesting:
- Timing of harvest.
- Procedures for collecting, counting, cleaning, weighing of larvae and prepupae.
- If applicable, procedure to quantify amount of residue.
- Handling of larvae and prepupae after counting and weighing.

9. Sample preparation and chemical analyses

How materials such as diets, larvae, and residues (mixture of diet left-over and frass) are (sub)sampled, processed, stored, and finally analysed in the laboratory will impact study outcomes. These aspects require careful consideration during the research and authors should provide details of each aspect in their articles. It is evident that (sub)sampled materials should be representative of the original material. International standards for general sample preparation are available (e.g. ISO, 2012) and additional requirements are described in standards for specific analyses. Processing operations such as drying and grinding are instrumental for creating homogeneous sample material suitable for the various analyses. Suitability of drying and grinding procedures depend on the type of sample (diet, insect, residue). Samples containing more than 15% moisture should be dried. Freeze-drying is preferable to oven-drying at 70 °C when loss of volatile compounds such as short-chain fatty acids and ammonia is likely or when damage to the structure of the sample and heat-sensitive components such as amino acids and vitamins should be minimised (De Jonge and Jackson, 2013). Grinding is generally performed using a centrifugal mill with a 1 mm mesh sieve. Materials with a high fat content such as insects may need a different type of grinder (not forced through a mesh), ground frozen or in liquid N, or need extraction of the fat prior to grinding. Dried and ground samples are stored in airtight containers away from heat and light.

The chemical characterisation of diets, larvae and substrate residues allows calculations of the conversion efficiencies for the DM, organic matter (OM; 100% – crude ash on % DM basis), and nitrogen (N). For the required laboratory analyses there are different internationally accepted procedures (e.g. from the International Organization for Standardization or from the Association of Official Analytical Chemists) which authors can use and refer to. If applicable, modifications to these procedures should be clearly described. For example, as sample material can be limited, the weigh-in per analysis might deviate from the standard procedures, the number of technical replicates for the analysis might be reduced or an alternative procedure described in the literature might be chosen. The nature of the material can require additional sample preparations. Larval fat content can be high (15.0-34.8% of DM; Makkar *et al.*, 2014), which can hamper proper sampling or interfere with specific analyses for instance acid detergent fibre (ADF; Fahey *et al.*, 2019). We therefore advise authors to describe the details of sample preparation, the amount of sample weighed in, and the number of replicates per performed analysis. For understanding the larval performance and optimising diets, various additional analyses of the diets can be undertaken and we highly recommend researchers to perform these analyses next to the DM, crude ash (for OM) and N analyses. Also the fat and carbohydrate fractions (sugars, starch, and fibres) impact the nutritional value of the diet. In line with other scientific journals (e.g. Animal Feed Science and Technology), we consider the analysis of crude fibre not acceptable to quantify fibre content or to estimate the non-fibrous carbohydrate content (i.e. N-free extract) of dietary ingredients, diets or larvae. The neutral detergent fibre and ADF assays are the preferred procedures to estimate dietary insoluble fibre content and acid detergent lignin assay is suitable to estimate the dietary lignin content. Analyses of total dietary fibre, insoluble dietary fibre and soluble fibre can also be of value. In addition, we stimulate the analyses of starch and, if deemed of relevance, free sugars which allow estimation of non-starch polysaccharides, and estimation of soluble and insoluble fibre fractions (De Leeuw *et al.*, 2008). It should be noted that the N content can be analysed using the Kjeldahl (ISO, 2005) or Dumas (ISO, 2008) methods. The Kjeldahl method might give lower N values than the Dumas method because inorganic forms of N, such as nitrates and nitrites, may be incompletely reduced during digestion (Etheridge *et al.*, 1998). For the larval biomass, the protein content can be estimated by quantifying the amino acid contents or multiplying the analysed N content with 4.67 (Janssen *et al.*, 2017). When performing amino acid analyses, some amino acids might be difficult to accurately quantify due to interference of likely glucosamine or galactosamine (e.g. Bosch *et al.*, 2016) and an alternative approach is required. Part of the insect N content originates from chitin. Though there is no international standardised and accepted procedure to quantify the chitin content, several studies have estimated chitin using different approaches (e.g. Finke, 2007; Janssen *et al.*, 2017; Liu *et al.*, 2012). Depending on the nature of the residual resources under study, various other laboratory analyses are relevant to perform and provide valuable insights in nutritional value and safety of insect biomass. It goes beyond the scope of this article to provide directions for these analyses and we stimulate colleagues to explore recent publications in this and other journals (e.g. Charlton *et al.*, 2015; Diener *et al.*, 2015; Van der Fels-Klerx *et al.*, 2016). Though chemical analyses of the residue are not required to quantify conversion efficiency (see below), it can provide valuable insights in the flow of chemical compounds from the diet to the insects, the residue or into the air. Furthermore, the concentrations of N, phosphorous (P) and potassium (K) in the feed residue are of interest when it is considered as fertiliser.

The nutritional value of a diet is determined by the chemical composition, and the physical properties such as hydration and particle size distribution can impact conversion efficiency. For the hydration properties, diets can be characterised by determining their capacity to swell, hold or bind water (Guillon and Champ, 2000). Different methods are available to characterise particle size such as sieving (dry or wet), laser diffraction, image analysis, and light scattering. As diets are fed in moist form, particle size distribution is better measured via wet sieving than dry sieving (e.g. Poppi *et al.*, 1980). Finally, the pH is of interest to monitor as dietary pH can impact microbial growth and the pH will change during larval rearing due to microbial products and compounds excreted by the insects e.g. short-chain fatty acids, ammonia, uric acid.

Box 5. Sample preparation and chemical analyses – crucial information to report.

Sample preparation:
- Procedures for homogenisation, sampling, drying, grinding, and storage.

Chemical analyses:
- Procedures for each type of analysis; in any case for dry matter, ash, and nitrogen.

10. Sample preparation and microbial analyses

When considering the utility of BSF larvae as feed and fuel, one must also consider the contribution of symbiotic microbes to BSF conversion of a wide variety of organic wastes, and resulting larval biomass. Many insects meet the bulk of their nutritional needs from both substrate and gut-associated microbes or microbial metabolites (Dillon and Dillon, 2004; Yun *et al.*, 2014). Excess nutrients and fat generated in this process are stored in the insect fat body. Gut-associated microbes rapidly adapt to changes in the insect diet through changes in population and the sensing of signalling compounds and degradation enzymes that facilitate digestion through absorption and metabolism of complex molecules (Dillon and Dillon, 2004; Engel and Moran, 2013a,b; Yun *et al.*, 2014). Microbes contribute largely to the fatty acids used as energy reserves in insect cells serving to aid in vitamin absorption. Studies have shown that some bacterial signalling pathways are known to influence BSF larval health, host development, metabolic homeostasis, and/or behaviour (Engel and Moran, 2013a; Jordan and Tomberlin, 2017; Zheng *et al.*, 2013a). Additionally, non-pathogenic commensal bacteria isolated from BSF larvae have been shown to enhance (i.e. probiotics) manure reduction and larval development, and to mediate oviposition (De Smet *et al.*, 2018; Yu *et al.*, 2011; Zheng *et al.*, 2013a). Despite this, little is known about BSF microbiology, particularly the microbial structure residing within the BSF gut or animal or human pathogens associated with BSF as a feed. Therefore, characterization of microbes is important for understanding the comprehensive physiology of the gastrointestinal tract microbiota. Recent work, however, suggests that these bacterial communities are surprisingly dynamic during BSF successive life stages (Varotto Boccazzi *et al.*, 2017; Wynants *et al.*, 2018; Zheng *et al.*, 2013b).

When designing an experiment involving microbial analyses, quality sample collection, handling, and preservation should also take into account downstream analyses, such as whether analysis of live microbes (culture) or nucleic acid isolation will be conducted, with care for experimenter safety with respect to the environmental background of the sample, and regard for potential pathogenicity. Samples for culture should be cryostored, and/or placed into appropriate storage medium that preserves microbial viability (Camacho-Sanchez *et al.*, 2013; Laulier *et al.*, 1995). Depending upon intent of analyses, samples may either be dissected and/or surface sterilized by submersing in 10% bleach for 1 min, followed by two successive submersions in sterile water, prior to preservation (Linville and Wells, 2002). Ethanol or appropriate nucleic acid stabilization solution are suitable preservatives for DNA or RNA applications (Camacho-Sanchez *et al.*, 2013; Menke *et al.*, 2017; Wong *et al.*, 2012). In all instances, aseptic technique and personal protective equipment such as gloves and lab coat at minimum, should be employed to prevent exogenous microbe or genomic contamination, and should be performed, as appropriate in a clean bench or biosafety cabinet.

Despite the limitations of culture dependent methodologies, many regulatory agencies require these for monitoring product quality (Stewart, 2012; Theron and Cloete, 2000). Implementation of these practices may therefore be necessary for BSF larvae as feed. Furthermore, microbial isolation in pure culture with classical microbiological methodology allows for phenotypical or genotypical analysis and distinct microbial identification. For this, samples must be placed in or onto a nutrient medium sufficient for growth of the species present. Media chosen varies from provision of nutrients to a broad spectrum of microbes, to selective and differential media for the isolation of targeted genera or species. Medium chosen, additives for enrichment, and temperature and incubation time should be specific to the desired microorganisms to be isolated. Once isolated, microbial identity can be confirmed by phenotype, microscopy, or a variety of molecular methods.

While microbial culture remains largely the gold standard for physiological characterization, most microbes are not culturable; therefore, molecular biology and bioinformatics provide the tools for characterization of microbial communities previously inaccessible by culture methods. These technologies can also be utilized to understand effects on larval feeding rates and overall processes of animal health important to agricultural science. However, for both culture-dependent and culture-independent methodologies, data must be collected that are specific to the experiment, such as (but not limited to) diet composition, larval age, rearing conditions, and time point of sample collection. Samples should be collected of both diet and larvae prior to experimental treatment, and at the end of the experiment. This is at minimum, as samples collected during designated experimental time points also provide valuable data regarding temporal microbial shifts that could be correlated to larval physiological states and waste conversion. Both technical and biological replicates should be included, with attention to sample size calculated for appropriate statistical rigor, as discussed below. Furthermore, additional samples should be collected for controls if nucleic acid isolation is to take place, as described in subsequent paragraphs.

Nucleic acids can be isolated using commercially available kits or in-house methods. A great deal of consideration for isolation methodology should be considered depending on sample matrix type. For instance, high lipid content and polysaccharides, substances such as humic acids, proteases, as well as other substances in larvae and waste could potentially inhibit downstream molecular analyses (Lienhard and Schäffer, 2019; Ridgeway and Timm, 2014). Methods should include steps to mitigate these potential inhibitors. But with every isolation, isolation controls should be included. Positive and negative controls are vital to interpret subsequent molecular results. Additionally, a positive control matrix spike should be included where killed or live microbes are added to a tube along with an additional larval or waste sample. We recommend the number of negative controls be equal to 10% of sample number. Following isolation, resulting nucleic acids should be validated for quantity and integrity (determination of degradation) determined through 230:260:280 ratios, gel electrophoresis and/or by using a bioanalyser.

Once nucleic acids are isolated, molecular methods can be used to determine specific microbial presence and abundance, gene expression, viability, or microbial community profiling, depending upon whether DNA or RNA is used. Polymerase chain reaction (PCR) is a widely used technique for detection of targeted organisms or products. The method relies upon the selection of primers that are complementary to a specific segment of genomic DNA, as well as inclusion of other reagents, to exponentially amplify the targeted DNA (National Human Genome Research Institute, 2015). As with isolation, PCR controls should be included with every PCR run and are also necessary for monitoring aspects of the PCR reaction such as reagent activity, cycling parameters, potential contamination, and for data interpretation (Kralik and Ricchi, 2017). Controls should include a PCR positive control that includes a tube containing target DNA as a template, and a negative control tube that does not include a DNA template. These controls should be included with every PCR run, and alongside isolated DNA samples and isolation controls.

Microbial community profiling using genetic-based methods and a taxonomically informative marker can also be used to provide a robust analysis of larval or waste associated microbial communities, since genes or organisms present can be used for identification without biases often encountered through live culture. Common sequencing techniques include the use of universal primers targeting 16S or 18S ribosomal RNA (rRNA) for prokaryotes or eukaryotes, respectively (Malacrinò, 2018; Wang *et al.*, 2014). Large parts of these regions are highly conserved, however, variable regions with interspecific polymorphisms are also present and are useful for determining differences in microbes with higher taxonomic resolution. Resulting sequences can be compared to sequencing databases for taxa-referenced matches to 16S or 18S. Implementation of methods described above regarding sample collection and isolation are especially important for sequencing methodologies as these are very sensitive to exogenous DNA contamination that result in false positive results for microbial communities. This is particularly important when considering the presence of rare taxa in sequencing data. For all sequencing data, sequencing depth, fragment size, sequencing platform, and whether pair-end should be noted. These data will be important in comparing data across studies and for determining validity of data. Additionally, post-sequencing bioinformatics pipelines and statistics should be carefully chosen based on robustness of database, parameters, and error rates.

Box 6. Sample preparation and microbial analyses – crucial information to report.

Sample preparation:
- Data with respect to timepoint and rearing conditions at time of sampling.
- Preservation methods.

Microbial analyses:
- Nucleic acid isolation methodology.
- For PCR: Molecular target, primers used, and cycling parameters.
- For Sequencing: Sequence platform, depth, and fragment size.
- Bioinformatics pipeline.
- Statistics used.

11. Calculations of larval performance parameters

Basic parameters to evaluate the performance of the larvae on the experimental diet(s) include survival and development time. Survival is the percentage of larvae that survived the duration of the experiment. The development time is the duration in days from the start of the experiment until e.g. when 5% of the larvae are in the prepupal stage (Section 7). Determination of the total larval biomass and the number of larvae at the end of the experiment allows estimation of the average larval body weight (in mg). The weights of diet provided, the larvae and residue (Section 6 and 7), in combination with the chemical analyses (Section 9), allow estimations of how efficiently larvae converted the experimental diets into larval biomass or how efficiently larvae reduced it. The most straightforward formula to express conversion efficiency is based on larval biomass (L) per amount of diet provided (D): (L/D)×100% (e.g. Rehman *et al.*, 2017). This formula relates to the feed conversion ratio also reported in the literature, which is the amount of diet needed per unit of larval biomass increase (D/L). This is, however, only informative if all diet offered has been consumed, which cannot be established for BSF larvae studies. Furthermore, conversion efficiencies on fresh matter basis can be obscure as considerable variation is present in the DM levels of the diets (12.3% in Lardé (1990) to 31.7% in Oonincx *et al.* (2015)) and the larvae (17.9% in Tschirner and Simon (2015) to 38.8% in Finke (2013)). For example, if a diet would be converted for 20% on fresh matter basis, using the previous DM values for diets and larvae, on a DM basis the conversion efficiency can vary from 11 to 63%. We therefore recommend to express conversion efficiencies on a DM basis (see also Waldbauer, 1968).

When the experiment starts when larvae are 5-d-old (Section 6), the larval biomass gained is determined by correcting the larval biomass determined at the end of the experiment for the larval biomass at the start (in g DM). The bioconversion efficiency (BE) can then be estimated with the following formula: $((L_{end} - L_{start}) / D) \times 100\%$. BSF larvae mix the diet with excreta and leave exuvia behind in the feed substrate; this mixture is consumed by the larvae, likely resulting in coprophagy. This behaviour limits true quantification of the amounts of diet consumed and faeces produced. Some studies extend the formula for BE and reported the so-called efficiency of conversion of ingested food (ECI) formula (Waldbauer, 1968). For ECI, the amount of food ingested is estimated by the amount of diet provided during the experiment corrected for the amount of diet left at the end of the experiment (in g DM): $((L_{end} - L_{start}) / (D_{start} - D_{end})) \times 100\%$. The formula for efficiency of conversion of digested food (ECD) (Waldbauer, 1968) has also been used and includes correction for the amount of faeces (F) at the end of the experiment to estimate the amount of digested diet (in g DM): $((L_{end} - L_{start}) / (D_{start} - D_{end} - F_{end})) \times 100\%$. Based on the considerations given above, in our opinion the derived parameters ECI (referring to 'ingested food') and ECD (referring to 'digested food') cannot be estimated correctly and to prevent misinterpretations these parameters should be avoided. Procedures using indigestible markers such as silicon (Clissold *et al.*, 2018) are currently under development and will provide new opportunities to assess actual conversion efficiencies. In the literature, the residue (R) at the end of the experiment consisting of diet, exuvia and excreta is quantified (in g DM) and used to correct the amount of diet provided: $((L_{end} - L_{start}) / (D_{start} - R_{end})) \times 100\%$. The outcome of this formula can be of value and we propose to label this formula bioconversion efficiency corrected for residue (BER). Note that fractional efficiency measures such as ECI, ECD and BER have inherent statistical pitfalls, see e.g. Raubenheimer and Simpson (1994) and Raubenheimer (1995). Next to using the BE or BER formula to estimate the conversion of dietary DM into larval DM, the formulae also allow the estimation of the conversion of dietary N into larval N.

For waste management studies, the overall degradation (Diener *et al.*, 2009) or reduction rate (Rehman *et al.*, 2017) are estimated based on the amount of diet provided during the experiment and residue obtained at the end of the experiment (in g DM): $((D - R) / D) \times 100\%$. The waste reduction index can then be obtained by dividing the reduction rate by the number of days that the larvae were fed the experimental diet.

Box 7. Larval performance and conversion efficiency – crucial information to report.

Larval performance:
- Survival (%).
- Growth rate, preferably reported as growth curves.
- Fresh weight of larvae and of prepupae at harvest.
- Total dry matter, organic matter, and N in insect biomass (larvae + prepupae) at harvest.

Conversion efficiency:
- Bioconversion efficiency and/or bioconversion efficiency corrected for residue.
- If applicable, overall degradation, reduction rate and/or waste reduction index.

12. Concluding remarks

Considering the exponential increase in publications dealing with diet conversion efficiency by BSF larvae, it is timely to suggest procedures to arrive at a much needed harmonization and reproducibility among studies. We provide directions and checklists for researchers when conducting and documenting experiments in which resource conversion by BSF larvae is studied and quantified. However, it is not yet possible to establish unambiguous standardised procedures due to the lack of basic knowledge and reference values. For example, although we favour using a meridic diet as a reference, it still needs further development as more data are needed on performance of the larvae when fed this diet in terms of development rate, growth curves and conversion efficiency. We limited the procedures to those that are essential for quantifying conversion efficiency and its replicability. In the different sections we include examples of methodologies and analytical techniques which are available or under development. We would like to stimulate colleagues to invest in studies that improve and extend our methodologies for this rapidly growing field of research, which will also allow us to further enhance our fundamental understanding of biological principles of resource utilisation by BSF larvae and the commensal and symbiotic microbiota.

The type of experimental design is the first to consider in which the main concerns are to establish validity, reliability, and replicability. The design of the experiment determines the validity, which refers whether the study will answer the research questions without bias and whether the study findings can be generalized to other contexts (Andrade, 2018). Reliability is the degree of consistency of a measure, giving the same result when repeated under the same conditions. Replicability means that when another team uses the same measurement procedure, the same measuring system, under the same operating conditions, in the same or a different location on multiple trials, they would arrive at the same results (Plesser, 2018).

The origin and genetic status of the colony as well as the rearing diet often determine the outcomes. For the diet a standard rearing diet is proposed to facilitate comparisons among studies. When experimental diets are considered they need to be defined. The rearing of neonates may not be used directly for the experiments and therefore we propose to make a distinction between them and the older (5-d) larvae and describe for both the (a)biotic and handling conditions.

The time of harvesting differs often between experiments and varies between number of larval days and percentage of pre-pupae present. For a number of reasons we propose to time the harvest of the larvae either when 5% of them are in the prepupal stage or to use a certain number of days but then their development stage should be reported. Appropriate measures need to be made of how to collect and handle the larvae and the residue at harvest. If measuring during development is required we propose to use replicates as handling larvae repeatedly during the study may affect survival and thereby influence experimental results.

It is becoming increasingly clear that microbes associated with plants and insects can influence plant-insect interactions (Shikano *et al.*, 2017) and likewise the substrate-insect interactions. BSF has a high diversity of bacterial species (Zheng *et al.*, 2013b) among locations (Wynants *et al.*, 2018). The larvae change their microbial community depending on the nutrient source (Jeon *et al.*, 2011). BSF larvae may also change the chemical composition of the substrate (Myers *et al.*, 2008) and modify the microbial composition, such as harmful bacteria (Erickson *et al.*, 2004; Lalander *et al.*, 2013). Microbes play a role in stimulation of oviposition (Zheng *et al.*, 2013a) and very likely in larval development (Liu *et al.*, 2008). However, they can also be useful in bioengineering biowaste management systems associated with BSF such as co-treatment of biowaste with beneficial microbes (Gold *et al.*, 2018). BSF larvae survive under harsh conditions and are remarkably resistant to bacterial infections. The potent antibacterial peptides in BSF hold promise in view of the highly problematic emergence of bacterial strains resistant to common antibiotics that are used to fight human and animal infections (Müller *et al.*, 2017; Park *et al.*, 2015; Vogel *et al.*, 2018). Because bacteria can be retained through successive life stages (Zheng *et al.*, 2013b) it is recommended to scrutinize the initial bacterial load and diversity on these flies before introduction into waste or feed to mitigate any inadvertent disease transmission, having consequences for any experiment dealing with food or feed safety. However, although characterization of microbes of the substrate and the gut is important, one has also to realize that the bacterial communities are diverse and dynamic during BSF's successive life stages. Several methodologies are proposed to isolate and identify the microbes.

To calculate survival and development time of the larvae is rather straightforward , though procedures for accurately determining survival rate on the larger scale need to be developed. To estimate how efficiently larvae can convert the substrate into larval biomass needs measuring the weight of the substrate provided, the larvae and the residue. In order to use a standard methodology we suggest to calculate BER on a DM and N basis. New methods currently under development will provide new opportunities to assess actual conversion efficiencies. In waste management, one wants to know how much waste is reduced and a reduction rate (index) can be calculated.

It is challenging to standardise biological experiments due to the inherent genetic and phenotypic variability of organisms and the effects of abiotic conditions. Control over the independent variables is required to identify causes of changes in the dependent variable. Often the procedures of data generation are insufficiently documented and data processing is arbitrary; for that reason standardization at multiple levels is essential (Schilling *et al.*, 2008). In this article we have tried to provide coherent ideas of how to arrive at standardization of future BSF studies with the aim of rigorous assessment of its efficiency to convert decaying organic matter into high-quality nutrients.

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