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RESEARCH ARTICLE

Make it a standard? The creation and variability assessment of a consensus standard protocol for Tenebrio molitor larvae feeding trials

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Abstract

Interest in the nutrition of the yellow mealworm (*Tenebrio molitor* L.) larvae is on the rise, leading to an increase in publications on this topic. The absence of a standard protocol and resulting differences in experimental designs reduces comparability among studies and impedes research on mealworm nutrition. To address this, a consensus standardised protocol was developed specifically for the evaluation of mealworm larval growth and performance in feeding trials. The efficacy of this protocol was evaluated through an international ring test involving seven partners using two wheat brans as dry feed (a standard bran and a local bran) at 27 °C and 60% relative humidity. As experimental units, plastic crates filled with 2.1 kg of bran and 10,000 4-week-old larvae were used with six replicates. Agar gel was provided as wet feed *ad libitum*. The mean individual larval weight and the number of larvae per crate were determined weekly until either three or more replicates ran out of feed or pupation exceeded 10%. At harvest, the total larval fresh biomass and amount of frass was determined. Larval samples were taken for chemical analysis. To assess the protocol, the within (repeatability) and between (reproducibility) laboratory variability was calculated for each parameter. The repeatability was good (limit at 12% (standard) and 14% (local)). The reproducibility was poorer with a limit 2.7 times higher for the standard feed (36%) and 3.8 times higher for the local feed (55%). For both feeds, the total larval fresh harvest, amount of frass and the larval protein concentration were the most consistent both within and among laboratories. The highest variability was observed at the early life stages and for the larvae ash content. The detailed consensus standard protocol and repeatability/reproducibility estimates can be used as basis for future mealworm feeding trials, comparing results and future improvements.

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Keywords

feeding substrate – insect nutrition – ring test – standard protocol – yellow mealworm

1 Introduction

The yellow mealworm, *Tenebrio molitor* L. (Coleoptera: Tenebrionidae), is one of the most studied edible insect species for food and feed applications (Rumbos and Athanassiou, 2023). It's nutritional requirements have attracted scientific interest as early as the 1950's (Fraenkel, 1950), when scientists were seeking for a method to eradicate pests from stored grains. More recent scientific interest is justified, as *T. molitor* larvae have a high nutritional value (Finke, 2015; Stull *et al.*, 2019), are efficient in converting feed into insect biomass (Oonincx *et al.*, 2015), can thrive on a variety of organic side-streams and wastes (Van Peer *et al.*, 2021). Furthermore, their production has low land and water requirements (Oonincx and De Boer, 2012), especially when produced in a circular economy by upcycling food waste that would otherwise be landfilled, composted or used for biomethane production (Paris *et al.*, 2024). Scientific interest in the yellow mealworm has increased over the last decade in parallel with the rising interest in its commercial exploitation. This has propelled mealworms to occupy a significant portion of the edible insect market today (Pippinato *et al.*, 2020). Important milestones towards the commercialisation of the yellow mealworm and the industrialisation of its production were the approvals at EU level, allowing *T. molitor* larvae to be used as ingredient in aquafeeds (EU, 2017) and poultry and swine feeds (EU, 2021), as well as for human consumption (EFSA, 2021).

Much research on *T. molitor* by both public institutions and industry has been directed towards larval nutrition and the evaluation of substrates as potential feedstocks. Several research groups have used various experimental protocols in mealworm feeding trials (Van Broekhoven *et al.*, 2015; Rumbos *et al.*, 2021; Fondevila and Fondevila, 2022; Montalbán *et al.*, 2023). However, the lack of a standard protocol for mealworm feeding trials complicates the comparison of the results across studies, posing challenges and risks in drawing conclusions. For instance, the growth and performance of *T. molitor* larvae can greatly vary depending on several parameters, such as larval density (Deruytter and Coudron, 2021), feed particle size (Naser El Deen *et al.*, 2022), wet feed provision and distribution (Deruytter *et al.*, 2021), temperature and relative humidity (Ribeiro *et al.*, 2018) or strain (Adamaki-Sotiraki *et al.*, 2021; Rumbos *et al.*, 2021). Even apparently identical control diets, such as wheat bran, from different geographic origin have been shown to yield different biomass in mealworm larvae (Paris *et al.*, 2022). Given the growing interest and the increasing number of publications on mealworm rearing, it becomes evident that the establishment and use of a standard protocol for mealworm feeding trials could render the results from different research facilities comparable and thereby more relevant. The recently published standardised protocol for feeding experiments with larvae of the black soldier fly (BSF), *Hermetia illucens* L. (Diptera: Stratiomyidae), paves the way toward this direction (Deruytter *et al.*, 2023).

Based on the above, the aim of the present study was to develop a consensus protocol, evaluate this protocol and propose it as standardized protocol for *T. molitor* larvae feeding trials. This was achieved through the formation of an international consortium of research groups and companies working with yellow mealworms. The group met regularly to discuss and thoroughly analyse all the factors that should be standardised, in order to design a protocol that is both aligned with industrial production methods, is scientifically robust, and can easily be adopted and performed by other researchers and institutes. Subsequently, a ring test among the participating research groups was performed to evaluate the consensus protocol and define the within partner variability (repeatability) and between partner variability (reproducibility).

2 Materials and methods

The protocol described below summarises the procedure developed and performed by the consortium during the ring test. The complete version of the consensus and proposed standard protocol is available as Supplementary Annex S1. It is important to note that the partners were free to choose when the experiment was performed, hence they were not executed simultaneously.

Tenebrio molitor *larvae populations*

In total five *T. molitor* populations were used for the ring test (identical population for partners A, C and D). In

TABLE 1 Proximate composition (%DM) of the standard wheat bran used in the ring test, as well as the local wheat brans used by partners (a-g). Partner b provided the standard feed and therefore has no local diet. A nitrogen to protein conversion factor of 6.25 was used

Parameter		b (standard bran)	a	c	d	e	f	g
Dry matter	(% as fed)	86.8	87.2	93.3	n.d.	88.8	85.8	86.0
Crude protein	(% DM)	18.2	15.9	18.1	25.4	17.6	17.4	17.2
Crude Fat	(% DM)	5.5	4.7	3.5	4.5	3.0	n.d.	4.3
Starch	(% DM)	24.3	9.5	n.d.	n.d.	n.d.	n.d.	14.9
Ash	(% DM)	5.8	6.7	4.1	5.0	6.1	4.6	6.1

n.d. = not determined; DM = dry matter.

Supplementary Table S1, a detailed description of the origins of the different populations, as well as the rearing conditions of the stock colony for each partner, are provided.

Dry and wet feed

Two types of wheat bran (particle size <2 mm) were used as a sole dry feed, i.e. a bran purchased by each partner from a local retailer and a standard bran provided to all partners from a single batch by partner B (Table 1). An amount of 2.1 kg of wheat bran was introduced initially in each crate, resulting in a feed layer height around 5 cm. Samples of both wheat brans were kept frozen at –20 °C for further analysis. Agar gel was used as moisture source and was prepared by dissolving 40 g of agar powder in 2 L of boiling water and pouring into a 60 × 40 cm crate. The resulting agar layer of approximately 1 cm thickness was cut into 1 cm^3 cubes after solidifying.

Nursing newly-emerged larvae

To obtain newly-emerged larvae for the trial, wheat bran (particle size <2 mm) was used as oviposition substrate for the adult beetles. An amount of 250 g of sexually mature beetles were put in 60 × 40 cm oviposition crates together with wheat bran. A mesh was placed by most partners in between the wheat bran, to avoid egg cannibalism by the adults. To acquire enough eggs and subsequent newly-emerged larvae, at least 6 oviposition crates were prepared. Adult beetles were provided with agar cubes and kept at 27 °C and 60% relative humidity (RH). They were kept in the dark, with the exception of partner G (8h light). The beetles were allowed to oviposit for 4 days (d) to minimize the age and size variability of the offspring. After this 4 day interval, beetles were removed (End of oviposition = Day 1; Week 1) and the oviposition crates were left undisturbed under the aforementioned climatic conditions. Newly-emerged larvae were left to grow undisturbed for 2 weeks after the end of the oviposition period, after which agar was provided three times per week *at libitum*. The agar cubes were well distributed over the crate and were spaced no more than 10 cm in between (Deruytter *et al*., 2021).

Experimental design

At the start of W5 (D29 after oviposition), the contents of all oviposition crate replicates were combined and sieved with a 0.5 mm sieve to remove frass and the mixture was weighed and gently mixed to have a homogeneous distribution of the larvae and accurate subsampling (see also Supplementary Figure S1). Subsamples were taken to determine the number of larvae and their corresponding mean individual weight. Special attention was paid for the determination of the initial number of larvae. Briefly, at least 3 subsamples of approximately 5 ml or 2 g (depending on adult fertility), containing at least 100 larvae per sample, were used to estimate the number and weight of the larvae. All larvae in each subsample were counted. A Coefficient of variance (CoV = standard deviation/average × 100) <10% was considered acceptable. If the CoV was higher than 10%, one or more additional samples were counted. The mean individual larval weight was calculated by dividing the total larval weight with the number of larvae. The mixture was then again gently, but thoroughly, mixed. A certain amount of the mixture (larvae + feed) containing an estimated 10,000 larvae (based on the calculations) was introduced in each experimental crate. An additional amount of bran was added in each crate to reach a total of 2.1 kg wheat bran per crate. As experimental units, plastic crates (outer dimensions 60 × 40 cm = 2,400 cm^2 of which approximately 2,000 cm^2 inner surface) were used. There were six replicates for each treatment. Experimental crates were maintained in climate rooms under constant conditions at 27 °C,

60% relative humidity (RH) and total darkness. The crates were kept at a distance from the walls and the floor of the climate room to avoid the influence of different microclimates (Deruytter *et al.*, 2019).

After setting up the experiment, no additional dry feed was added in each crate, but agar gel was provided *ad libitum* and at least three times per week. Once a week a subsample was taken from each crate to determine the mean individual larval weight, following the same subsampling process described above. Both larvae and substrate were placed back into the rearing crates after subsampling and weighing. The experiment was terminated when three or more replicates ran out of feed (<10% of the initial feed was present, here defined as the fraction between 0.5 and 2 mm) or when more than 10% of the larvae reached the pupal stage. At harvest, the total crate content was weighed. Afterwards, the content was carefully sieved to harvest all larvae and pupae. A 2 mm sieve was used to separate the larvae and pupae from the remaining content and a 0.5 mm sieve was used to separate the frass from the leftover feed (feed, dried agar, exuviae, etc.) in each crate. Larvae and pupae were separated and samples were taken as previously described to determine their mean individual weight. From each experimental crate two samples of larvae and frass were taken and kept frozen at –20 °C for further analysis. The feed conversion ratio (FCR) was determined by dividing the feed provided by the live biomass increase.t

Sample preparation and chemical analyses

Samples of local feeds and frass were dried and ground using various methods prior to analysis for the determination of their nutritional content, e.g. protein, fat, ash, and dry matter. Samples were afterwards analysed internally by each partner. The laboratory analytical methods performed by each partner followed internationally accepted standards. Methods and equipment used to prepare the samples, as well as the laboratory analytical methods are presented in Supplementary Table S2. In the case of larval composition analysis, partners sent dried larval samples to partner D for analysis using a near-infrared (NIR) analyser (FOSS NIRS™ DS2500 SR). The NIR was independently calibrated based on a 150 mealworm samples assessed by a certified laboratory.

Statistical analyses

The statistical analysis on repeatability (within partner variability) and reproducibility (between partner variability) were done identical as for black soldier flies, described in Deruytter *et al.* (2023). The calculations were done in accordance to the 'Standard practice for conducting an interlaboratory study to determine the precision of a test method' of the ASTM (E691-20) with the required minimum of 6 participants familiar with *T. molitor*. The test was performed blind with the partners not aware of each other's results. Briefly, to determine the repeatability and reproducibility the following steps were taken. Potential inconsistent results were marked 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). Potential outliers were assessed in depth assessing possible errors in the set-up, typos, calculation errors, or other causes. When a partner was removed from a dataset for a variable, these data were not replaced. Individual outliers, 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 repeatability and reproducibility standard deviation which is the same as the within and total variance of a one-way analysis of variance if no outliers were removed. This can then be used to $(1.96 \times \sqrt{2})$ to determine the 95% repeatability and reproducibility limits. Finally, a normalization was performed by dividing this limit with the average value of the parameter in order to compare the outcome. These are called the normalised (r% and R%) and are defined as: 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%. Hence, a lower value indicates a lower inter and/or intra laboratory variability.

3 Results

In general, the larvae grew to an average weight of 111 mg at harvest with an estimated survival of 89% and an FCR of 2.1 on the standard feed. As shown in Figure 1 and Table 2, both the average of a parameter and the – within laboratory – variation (size of the boxplot) can be very different between partners and feeds. In general, the larvae reared on the local wheat bran gained less weight (98.6 mg at harvest) with a higher FCR (2.4).

The repeatability (within partner variability) and reproducibility (between partner variability) of the biological and chemical parameters are listed in Table 3. The estimated repeatability limits are very similar for both the standard and local wheat bran for most individual parameters and on average (on average 12% for

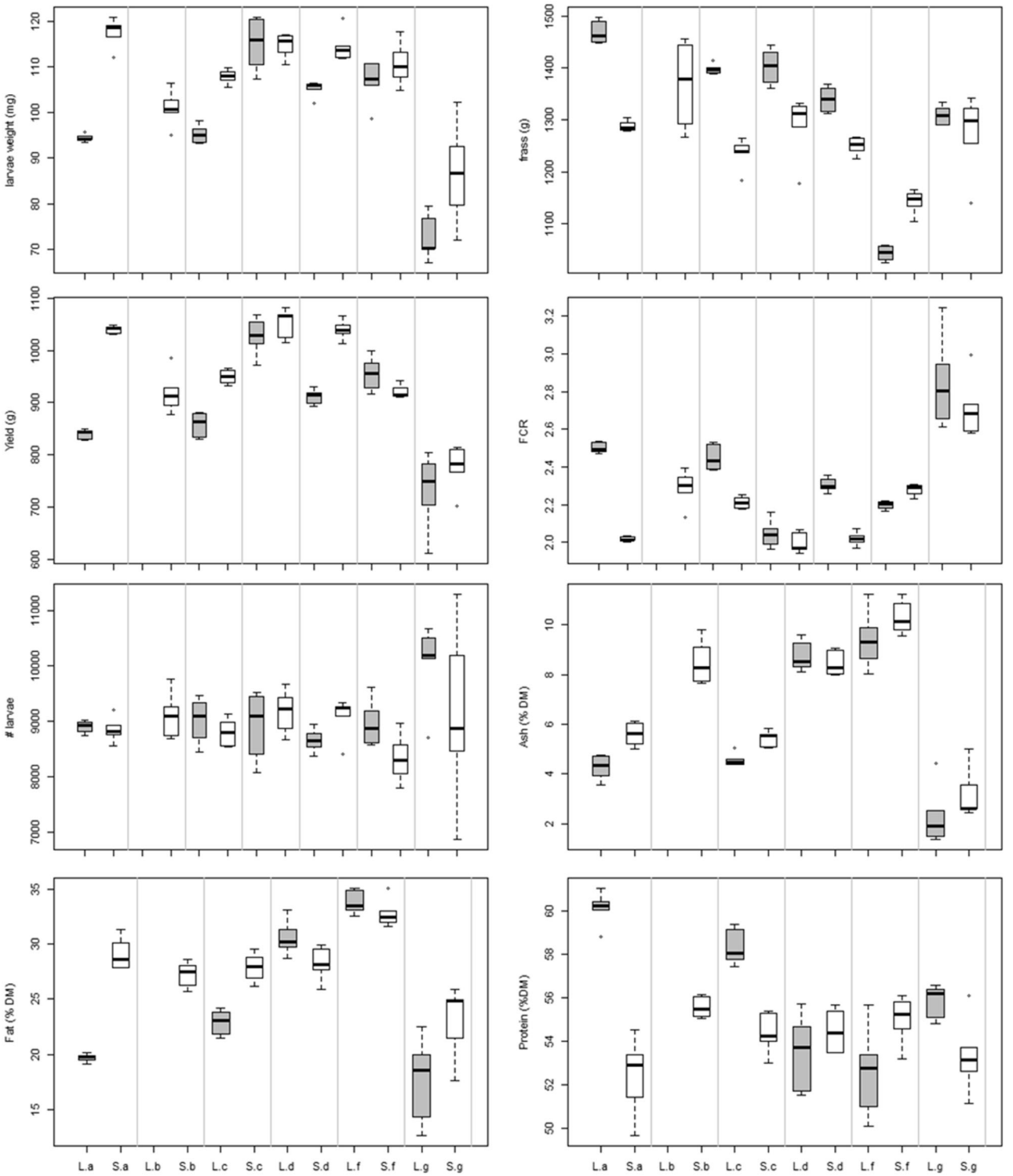

FIGURE 1 Boxplots with the mean, interquartile distance and whiskers as min and max value of the harvest parameters for the different feeds (L = local feed, Grey; S = standard feed = white) and partners (a-g). Partner b provided the standard feed and therefore has no local diet, hence no L.b. For partner E, no chemical data was available. DM = dry matter, # = estimated number (of larvae), yield: total live harvest per crate (g), Fat = crude fat content of the larvae, Protein = nitrogen content of the larvae * 6.25.

standard and 14% for local). The reproducibility limit values are, as expected, higher than the repeatability limits, for the standard feed they are 2.7 times higher (averaging 36%) and for the local feed 3.8 times higher (averaging 55%). For both feeds, the parameters determined at the end of the experiment and the larval protein concentration were the least variable both within and among laboratories. Among the evaluated parame-

TABLE 2 Details on the growth, harvest and chemical composition parameters for all partners for the standard feed (a-g). A correction factor of 6.25 was used for the protein calculation. WW = wet weight; DM = dry matter; SD = standard deviation, NA = Not applicable, # = estimated number (of larvae), FCR = feed conversion ratio

		a	b	c	d	e	f	g
5 weeks	mg/larvae WW	3.9	6.7	5.5	NA	4.6	4.9	2.9
	SD	NA	NA	NA	NA	0.3	0.2	NA
6 weeks	mg/larvae WW	9.9	15.0	17.8	10.3	12.2	12.1	12.9
	SD	0.5	0.9	1.2	1.1	0.7	0.8	1.4
7 weeks	mg/larvae WW	22.1	30.5	44.9	25.7	33.6	30.1	24.9
	SD	0.9	1.4	1.6	1.9	2.0	1.5	3.2
8 weeks	mg/larvae WW	51.1	57.0	85.9	53.1	69.4	57.2	42.9
	SD	1.8	3.5	2.2	5.7	4.6	3.0	10.4
9 weeks	mg/larvae WW	90.0	88.9	107.9	114.9	102.7	87.4	78.5
	SD	2.5	5.0	1.4	2.5	5.4	6.1	8.5
10 weeks	mg/larvae WW	117.7	100.9	NA	NA	NA	110.7	86.6
	SD	3.1	3.7				4.6	11.6
End weight	mg/larvae WW	117.7	100.9	107.9	114.9	114.4	110.7	83.8
	SD	3.1	3.7	1.4	2.5	3.2	4.6	12.4
#larvae		8848	9105	8802	9178	9101	8332	9063
	SD	216	400	234	369	348	417	1525
Yield	g	1039	928	950	1054	1040	920	762
	SD	9	41	13	27	17	9	72
FCR		2.02	2.29	2.21	1.99	2.02	2.28	2.80
	SD	0.01	0.09	0.03	0.05	0.03	0.03	0.26
Frass	g	1288	1369	1236	1291	1250	1142	1281
	SD	10	78	27	58	16	22	76
Larvae DM	% WW	32.8	33.0	34.6	41.7	31.5	NA	NA
	SD	0.5	1.3	0.5	2.7	1.1	0.0	0.0
Larvae Ash	% DM	5.6	8.5	5.5	8.4	NA	10.3	3.0
	SD	0.5	0.8	0.3	0.5	0.0	0.7	1.1
Larvae Fat	% DM	29.1	27.3	27.9	28.2	NA	32.8	22.2
	SD	1.4	1.2	1.3	1.5	0.0	1.2	3.6
Larvae Protein	% DM	52.5	55.5	54.3	54.4	NA	55.0	53.6
	SD	1.7	0.5	0.9	1.0	0.0	1.1	1.7

ters, the highest variability was observed for the larval weight at the early life stages, as well as for the ash content of the larvae harvested at the end of the trial.

4 Discussion

Over the last few years, there has been a notable increase in the volume of published data related to feeding experiments with *T. molitor* larvae. For instance, much research on this field has been directed towards the rearing of *T. molitor* larvae on agri-food byproducts, an approach fully aligned with circular economy principles that can be promoted through insect farming (Oonincx *et al.*, 2015; Mancini *et al.*, 2019; Harsányi *et al.*, 2020; Morales-Ramos *et al.*, 2020; Rumbos *et al.*, 2022). Although of high significance, the results of these studies are usually not fully comparable, as they have been attained using various experimental protocols differing in many parameters (e.g. environmental conditions, larval density, scale of experimentation or experimental units, presence and type of wet feed supplements, etc.). Based on the above, the generation of reliable and comparable data is mandatory to lay a robust foundation for future advancements of the sector.

Repeatability or within partner variability and reproducibility or between partner variability are key indicators of credibility of findings in scientific research, especially when biological and ecological parameters are in question (Cassey and Blackburn, 2006). Stan-

TABLE 3 Repeatability and reproducibility of the biological and chemical parameters for the standard and local feed

		Standard diet					Local diet					BSF	
		Average	SD	r%	R%	n	Average	SD	r%	R%	n	r %	R%
6 weeks LW	mg	12.9	2.7	**21**	**63**	7	11.1	3.1	**18**	**80**	7		
7 weeks LW	mg	30.1	7.7	**15**	**73**	7	23.8	6.4	**18**	**77**	7		
8 weeks LW	Mg	62.3	13.2	**17**	**61**	6	46.9	11.6	**19**	**72**	7		
9 weeks LW	mg	98.6	11.5	**12**	**34**	6	80.0	21.8	**15**	**78**	7		
10 weeks LW	mg	108.0	7.7	**11**	**22**	4	89.7	15.6	**11**	**50**	5		
Final LW	mg	111.1	6.0	**8**	**17**	6	98.6	13.5	**11**	**40**	7	32	63
#larvae		8,909	334	**11**	**14**	6	9,102	444	**11**	**20**	7	20	69
Yield	g	986.2	64.8	**5**	**19**	6	892.6	91.2	**10**	**30**	7	21	39
FCR		2.1	0.1	**6**	**19**	6	2.4	0.2	**9**	**28**	7		
frass	g	1,263	75	**9**	**19**	6	1,333	137	**7**	**30**	7	10	56
Larval DM	% WW	33.0	1.3	**8**	**13**	4	NA						
Larval ash	% DM	6.9	2.6	**28**	**108**	6	6.3	2.9	**37**	**135**	6	8	18
Larval fat	% DM	28.3	2.8	**14**	**30**	6	26.8	5.7	**12**	**60**	5	20	53
Larval protein	% DM	54.2	1.1	**7**	**8**	6	56.0	2.8	**6**	**15**	6	5	18

LW = larvae weight, FCR = feed conversion rate, WW = wet weight, DM = Dry matter, SD = standard deviation, r% = standardized repeatability limit, R% = standardized reproducibility limit, n = number of partners for the estimation, # = estimated number (of larvae), FCR = feed conversion ratio, BSF = black soldier fly data (Deruytter *et al.*, 2023) as comparison.

dardised test protocols validated through ring tests have been commonly proposed as a means to mitigate variability in trials with insects. For instance, Klein *et al.* (2022) proposed standardized semi-field tests to assess the impacts of plant protection products on the bumblebee, *Bombus terrestris* (L.) (Hymenoptera: Apidae). Similarly, a standardised bioassay was developed to evaluate the toxicity of residues of veterinary pharmaceuticals against the face fly, *Musca autumnalis* L. (Diptera: Muscidae) (Römbke *et al.*, 2010). Efforts to standardise experimental procedures and subsequently enhance the repeatability and reproducibility of the applied methods used in edible insects research are scarce. In a recent study, Deruytter *et al.* (2023) proposed an experimental protocol for feeding trials with *H. illucens* larvae, and reported considerable variation among the results obtained by the partners participating in a ring test performed to validate a protocol. Along these lines, the current study is, to our knowledge, the first attempt to suggest a standardised feeding experiment protocol for *T. molitor* larvae and to estimate the within and between laboratory variability of that protocol.

In general, the protocol resulted in larvae that are in terms of growth, survival and composition similar or better when compared to other studies (Deruytter *et al.*, 2019; 2021; Deruytter and Coudron, 2021; Harsani *et al.*, 2020; Mancini *et al.*, 2019; Rumbos *et al.*, 2021; 2022). This indicates the robustness to generate reliable data for the assessment of mealworm larvae feeding substrates. Additionally, the overall normalised repeatability limit was low, indicating low intra-laboratory variability, with few parameters above 20% and the most important parameters (e.g. FCR) at or below 10%. This means that 95% of the replicate values of a single experiment should fall within 10% of the estimated average value. The good repeatability is also evident when comparing the mealworm protocol with the BSF protocol (Deruytter *et al.*, 2023: table 3). More specifically, with the exclusion of the chemical measurements, the normalized repeatability limit of the mealworm protocol was up to 4 times lower compared to the BSF protocol. Indicatively, for the total yield, one of the most important industrial parameters, an r% of 5% was reported for the mealworm protocol compared to an r% of 20.5% for BSF. This is hardly a surprise, as BSF larvae live and feed in a moist substrate resulting in additional factors, such as the pH of the feeding substrate, and the dynamic of the substrate temperature, potentially adding to the overall variability of outcomes (Ma *et al.*, 2018; Meneguz *et al.*, 2018; Schreven *et al.*, 2022; Yakti *et al.*, 2022). These factors are not, or much less, of relevance to mealworm experiments. Especially because agar gel was used further reducing the potential variability of the wet feed. Finally, the repeatability is very similar for both the standard and the local feed for all evaluated parame-

ters, something that, although expected, verifies that the repeatability of the protocol is feed source independent.

Although a good repeatability is very important during experimentation, reproducibility is often neglected and to our knowledge never determined for mealworm trials. To ensure that results are reproducible and comparable among studies, the normalised reproducibility limit or among laboratory variability should be as low as possible when implementing an experimental protocol. In the present study, for the comparable 'end of experiment' parameters, i.e. the final larval weight, the estimated number of larvae (survival) and the biomass yield, the variability was on average only one third of the estimated variability with the BSF standard protocol (Deruytter *et al.*, 2023). The larval protein content was the least variable between partners, both for the standard and the local feed, which is in accordance with the results of previous studies that reveal a consistent pattern for *T. molitor* larval protein content due to their ability to regulate their body protein content regardless of the dietary protein content (Ramos-Elorduy *et al.*, 2002; Van Broekhoven *et al.*, 2015; Rumbos *et al.*, 2021).

The variability between partners did increase in all parameters when using the local wheat bran as diet. This was expected as an additional variable (diet) is introduced. However, in practice, it is impossible to always use the same wheat bran over time or among partners. Wheat bran has no fixed composition and depending on the wheat and milling process the feed parameters may differ among suppliers, especially the starch, sugar and lignin content (Stevenson *et al.*, 2012; Heuzé *et al.*, 2015). Therefore, it is recommended to include in future studies the chemical composition of the wheat bran, and by expansion the composition of any feed used. Furthermore, wheat bran can potentially be contaminated with pesticides that, although below the maximum residue level for conventional livestock, may still impede the larval growth (Meyer *et al.*, 2021, 2022 and 2023) and therefore could potentially be a source of variation between partners.

Additionally, it has been documented that the provision of wet feed as moisture source is critical for the performance and growth of *T. molitor* larvae (Urs and Hopkins, 1973), and the results of more recent studies have further supported this point of view (Oonincx *et al.*, 2015; Adamaki-Sotiraki *et al.*, 2021; Deruytter *et al.*, 2021; Rumbos *et al.*, 2021). Therefore, special care should be taken with mealworm feeding trials to ensure that a wet feed is adequately provided to the *T. molitor* larvae. In most feeding trials with mealworms usually vegetables (e.g., carrot, potato, apple, etc.) are used as a wet feed. However, since the nutrient composition and moisture of the vegetables or related materials provided as a moisture source is not stable and may vary among batches, their use as moisture source may result in variations in insect development. Moreover, as vegetables dry out quickly and are prone to microbial deterioration, they have to be replaced frequently, which is often time consuming and labour intensive. Agar however is a stable, non-toxic and easily available gelling agent with a near absence of nutrients. Therefore, it is recommended to use agar as moisture source in mealworm feeding trials. This can ensure that the observed variations in feeding trials are only due to the dry feeds tested.

Finally, although for most evaluated parameters the repeatability and reproducibility of the proposed protocol was low compared to the BSF protocol, this was not always the case, indicating that there is still room for future improvement. For instance, surprisingly, the larval ash content was very variable within and among partners, especially when compared to the results of the BSF standard protocol. Currently, it is unknown if this effect could be attributed to and whether this is a biological or a laboratory/technical variability, however this should be further assessed. Potential gains could also be made in reducing the variability of the size of the young larvae (first weeks), by reducing the variation in the initial average weight. Currently this was based on age, and although reared in identical ways, the initial estimated weight was still variable (2.9-6.7 mg). Initializing the experiment within a specific weight range (e.g. 3-5 mg) could be beneficial. Reducing the variability by sieving the larvae through – a set of – sieves could also be investigated to start the experiment with a fixed size and lower initial variability of the individual weights.

5 Conclusion

To conclude, the low r% and R% of the proposed protocol in the present study indicate its suitability to be used for mealworm larvae feed experiments to further investigate the nutritional requirements of *T. molitor* larvae as they are not as thoroughly studied as those of most livestock animals and farmed fish. These, first, repeatability and reproducibility values can also be used as guidelines to check, compare and or perform a quality control on the variability of other protocols and experiments. Although, the protocol can be implemented *as is* in future studies the authors invite other researchers to use it as a basis to build more advanced experimental protocols improving the reproducibility and scope

of this initial protocol. Finally, from an applied point of view, this study highlights the significance of collaborative efforts within the edible insect sector, involving various stakeholders to enhance the knowledge on repeatability and reproducibility of experimentation with edible insects.

Supplementary material

Supplementary material is available online at: https://doi.org/10.6084/m9.figshare.27276537

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