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Upgrading ammonia-nitrogen from manure into body proteins in black soldier fly larvae

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A B S T R A C T

Nitrogen (N) losses via ammonia (NH_3) emissions from manure is one of the main environmental burdens resulting from livestock production. Feeding manure to black soldier fly larvae (BSFL) is envisioned as a new circular strategy to recover manure-N, reduce its environmental impact, and upgrade it into insect proteins to be used in animal feed. However, so far, it remained unknown if BSFL could incorporate N from NH_3 in manure into the larval body mass. Here, using the stable isotope ^{15}N in NH_3, we demonstrate that at least 13% of pig manure NH_3-N can be incorporated into BSFL body mass. Within the larval body, the tracer was found in both insoluble and soluble nitrogen fractions, including proteins. We discuss interventions that could increase the incorporation of NH_3-N into larval proteins and with that reduce NH_3 emissions from manure. Our results provide the first reliable quantification of NH_3-N assimilation in manure-fed larvae and contributes to quantifying the potential of BSFL for manure management, and as a circular protein source.

1. Introduction

Surplus manure in livestock-dense regions is a source of environmental pollution, with ammonia (NH_3) emissions being one of the main environmental burdens (Gerber et al., 2013; Leip et al., 2015). Ammonia emissions contribute to acidification and eutrophication, and are an indirect source of the potent greenhouse gas (GHG) nitrous oxide (Cameron et al., 2013; Chadwick et al., 2015; Strokal et al., 2016; Wang et al., 2017). Ammonia can be released from manure through ammonification via two pathways. In the first pathway, the organic nitrogen (N) present in the solid feces (e.g., as undigested dietary or fecal-endogenous proteins) is degraded to NH_3 or its ionic form ammonium (NH_4^+) by microbial fermentation (Chen et al., 2020; Maeda et al., 2011). In the second and most important pathway, NH_3 is released from manure due to microbially produced ureases present in faces, which hydrolyzes urinary urea into NH_3 and CO_2 (Aarnink and Elzing, 1998; Dai and Karring, 2014a). Some groups of microbes assimilate this NH_3 as a source of N for *de novo* amino acid synthesis and other metabolic processes (Kenealy et al., 1982; Kuypers et al., 2018). However, by hydrolyzing urea with microbial ureases, NH_3 molecules are volatilized, thus causing environmental pollution.

A key requirement to improve the environmental sustainability of the livestock sector is to reduce manure-related NH_3 and GHG emissions (Gerber et al., 2013). Even though the biological and physicochemical mechanisms of manure-related emissions are well known (Dai and Karring, 2014b; Sigurdarson et al., 2018), and different manure management methods and mitigation strategies for emissions are available (Flotats et al., 2011), manure-related emissions are a persistent environmental problem in livestock-dense regions. While manure NH_3 emissions due to urea hydrolysis could be largely avoided by collecting livestocks solid feces separately from urine (Vries et al., 2013), in most large-scale livestock housing systems solid feces and urine are mixed and stored together. In such systems, the mitigation of NH_3 and greenhouse gas (GHG) emissions mainly depend on costly strategies such as the use of urease inhibitors and acidifiers, the export of manure outside manure-surplus areas, and the use of manure valorization practices such as composting and anaerobic digestion (Kuhn et al., 2018; Sigurdarson et al., 2018). Many of these practices are known to reduce manure-related emissions (Hou et al., 2017; Wang et al., 2017), but most are costly and not widely adopted by farmers unless incentives for their adoption are available. In this context, novel manure management methods which are sustainable, circular and accessible are being

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explored.

Manure bioconversion with black soldier fly larvae (BSFL) is envisioned as a new potentially sustainable, and circular management method to treat surplus manure (van Huis, 2019). If safety standards for metals, antibiotics, pathogens and parasites are met (Sanchez Matos et al., 2020) the larval biomass obtained after bioconversion could be used as protein-rich ingredient. Thereby, it can replace feed ingredients having a high environmental impact, such as soybean and fish meal, and contribute to the circular economy (Heuel et al., 2021; Van Zanten et al., 2015).

In addition, the residual material of the bioconversion process (i.e., mix of uneaten manure, and insect frass and exuviae) is considered an attractive fertilizer because of improved humification properties relative to fresh manures (Wang et al., 2021). In the last years, various studies reported the bioconversion efficiency of BSFL on different manure types (see Sanchez Matos *et al.* 2020 for an overview), and recent studies quantified NH_3 and GHG emissions during this process (Chen et al., 2019; Parodi et al., 2021). Although more quantitative environmental data is becoming available for broader sustainability assessments, the role that BSFL bioconversion can have in reducing NH_3 emissions through its direct use has so far received limited attention.

After pig manure bioconversion with BSFL, one quarter of the N originally contained in the manure can be accumulated in the larval body mass (Parodi et al., 2021). However, it remains unknown how much of this N is derived from NH_3. Pathways for glutamine synthesis from NH_3-N has been found in moths (Drews et al., 2000; Hirayama et al., 1997), and in cockroaches by the endosymbiont *Blattabacterium* (Sabree et al., 2009). In addition, the microbiota present in the rumen of ruminants, and to some extent also in the digestive tract of monogastric animals, provides their hosts with microbial amino acids derived from NH_3-N (Columbus et al., 2014; Pengpeng and Tan, 2013; Torrallardona et al., 2003; van Erp et al., 2020). Given the high content of intestinal microbes in manure and the influence that the diet has on BSFL microbiota (Bruno et al., 2019), it is hypothesized that some NH_3-N is incorporated in the larval body mass via exogenous (i.e., in the substrate) or endogenous (i.e., in the larval digestive system) microbial assimilation, followed by the absorption of microbial amino acids by the larvae. Thus, the objectives of this research are to quantify the incorporation of NH_3-N into the larval body mass and larval proteins after pig manure bioconversion with BSFL using the stable isotope ^{15}N ammonium in $^{15}NH_4Cl$, and to construct a NH_3-N mass balance of the bioconversion process among the larvae and residues. The outcomes of this study are relevant to elucidate the potential of manure bioconversion with BSFL for reducing manure NH_3 emissions and to upgrading NH_3 as a circular protein source for animal feed.

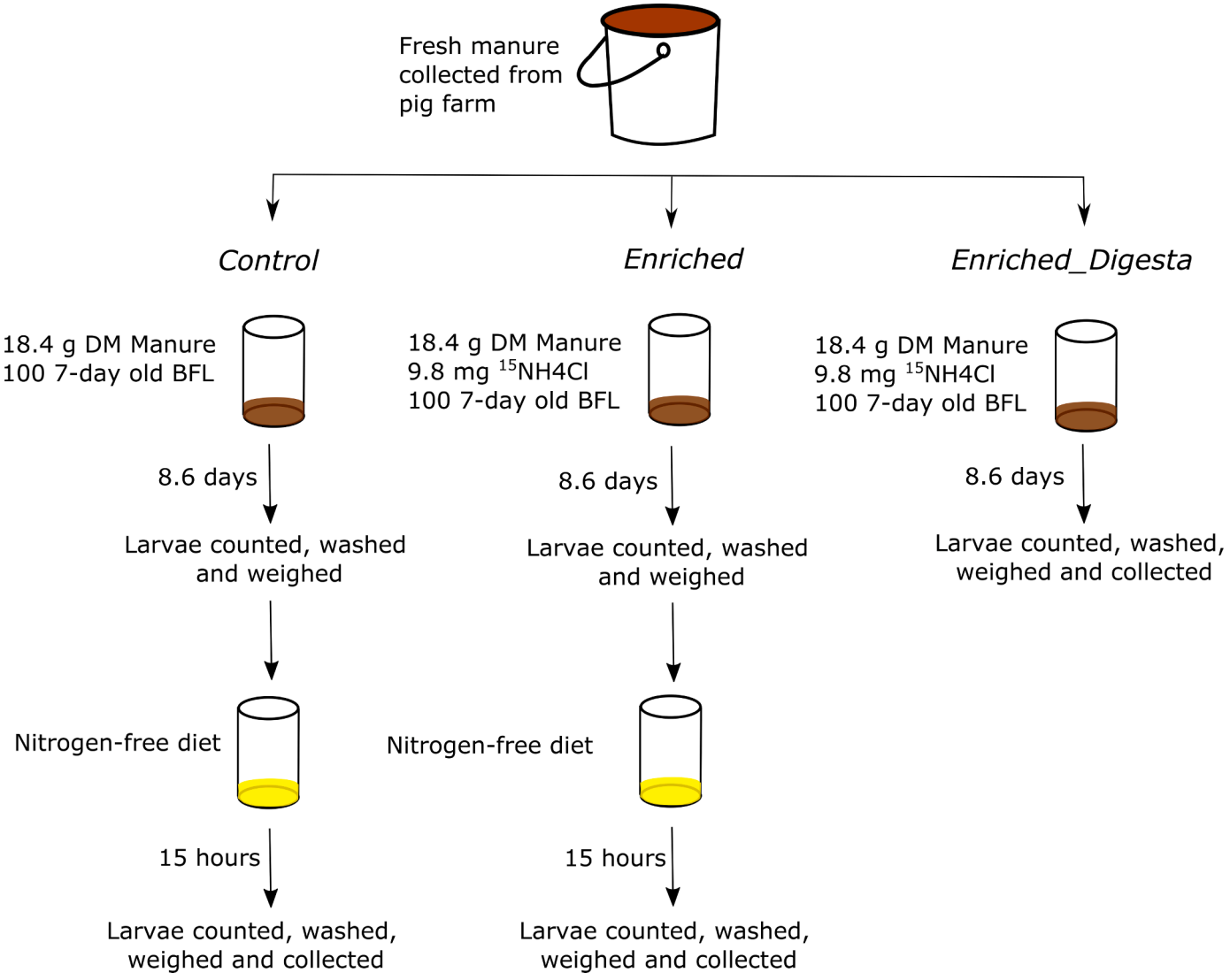

Fig. 1. Flow diagram of the experimental design. Each treatment had five replicates.

2. Materials and methods

2.1. Experimental design

A flow diagram of the experimental design is presented in Fig. 1. BSFL were reared under three different treatments named *Control, Enriched*, and *Enriched_Digesta* (Fig. 1). The larvae reared under the treatment *Control* were placed in a container that contained fresh pig manure. After 208 h (i.e., 8.6 days), the average time on which the first prepupae were observed in previous experiments, larvae were removed from the containers, counted, washed with water, dried with paper towel, and weighed. The residual material was collected. Subsequently, the washed larvae were placed overnight in a substrate made of a nitrogen-free diet. This was done to replace the content of the larval intestines with a nitrogen-free material to maintain the same $^{15}N/^{14}N$ ratio, and thus ensure that during the isotopic quantification only absorbed ^{15}N in the larval body mass was measured. Then, larvae were removed, counted, washed, weighed, and stored at -20 °C. The larvae reared under the treatment *Enriched* passed through the same steps described for the *Control* treatment, except that the larvae were initially reared in fresh pig manure mixed with $^{15}NH_4Cl$ (Fig. 1). The *Enriched_Digesta* larvae were reared as in the *Enriched* treatment, but without the subsequent nitrogen free diet; thus, once harvested from the ^{15}N-enriched residual material, they were directly stored at -20 °C. The *Enriched_Digesta* treatment was included to compare larval total nitrogen and ^{15}N values when guts were filled with enriched digesta, and hence have a benchmark value for larval samples which also contained unabsorbed ^{15}N. No residual material was collected for this treatment as it was expected to have the same composition as the residual material from the *Enriched* treatment. Each treatment had five replicates. Rearing details are presented in section 2.2.

2.2. Larvae rearing

Pig feces excreted within the last 24 h, that were in contact with urine, were collected in a commercial pig fattening farm in Rhenen, the Netherlands, and transported to the experimental facilities of Wageningen University & Research. A preliminary dry matter (DM) analysis was performed to rapidly determine the amount of manure that needed to be added to each container. This was done by drying a homogeneous 2 g sample of fresh manure in a moisture analyzer (Ohaus MB-90, Parsipany, United States). Once the DM was known (i.e., 24.5 %), 15 cylindric plastic containers (diameter 8 cm, height 11 cm) were filled with 75 g of pig manure, equivalent to 18.4 g of dry manure. The height of the manure layer inside each container at the start was 1.3 cm. Subsequently, 9.8 mg of ^{15}N ammonium-chloride ($^{15}NH_4Cl$, Sigma-Aldrich, see Supplementary Information SI.1 for details), diluted in 400 μL of distilled water were pipetted in the *Enriched* and *Enriched_Digesta* treatments and mixed with the manure using a small spoon. The containers of the *Control* treatment received 400 μL of distilled water. Subsequently, 100 seven-day old BSFL, also referred to as starter larvae, were added to each container. The larval density in manure was 1 larvae/cm^3, the individual weight of each starter larva was 3.4 ± 0.2 mg (mean ± standard deviation) and the feed rate was equivalent to 23 mg DM manure/larva/day, as suggested by Parodi *et al.* (2021), although all manure was provided at once. The starter larvae were reared on a substrate containing 30% wheat bran and wheat flour, and 70% water by Bestico B.V. (Berkel and Rodenrijs, the Netherlands). All containers were placed in a custom-made climate chamber at 28 °C and 70 % relative humidity under a 12:12 light:dark regime for 208 hours (8.6 days). The residual material from the *Control* and *Enriched* treatments and the larvae from the *Enriched_Digesta* treatment were collected, weighed, and stored at -20 °C. The larvae from the *Enriched* and *Control* treatments were moved for 15 h to new plastic containers containing 50 g of a nitrogen-free diet mixed with 40 ml of deionized water (see SI.2 for details on the ingredient composition of the diet) prior to sample collection and storage (see section 2.1 and Fig. 1). Larval survival ranged between 99 and 100%, and the average fresh larval weight was determined by weighting all larvae obtained per container and dividing the weight by the number of surviving larvae.

2.3. Sample processing and nutrient analyses

Samples of the *Enriched* and *Control* residual material were freeze-dried for 96 h starting at -80 °C until stable weight was reached, grounded by mortar and pestle, and stored at –20°C for DM, total N and ^{15}N analysis. Samples of larvae from all three treatments were subjected to the same processing steps as for the residual material, but after grinding, passed through a defatting process and subsequently a protein extraction process. This defatting was a precautionary measure to protect the equipment used to measure ^{15}N (see section 2.4) and was not suitable to quantify true fat content. Protein extraction was done to quantify ^{15}N in larval insoluble and soluble N fractions.

2.3.1. Defatting

The ground, freeze-dried larvae were defatted by mixing and centrifuging 2-3 g twice with n-hexane at a ratio of 1:4 g:ml. Centrifugation was done for 5 min at 1000 g and 23 °C. To remove n-hexane, the solid centrifuged material was placed in an incubator for 4 h at 30 °C, and subsequently placed overnight in a fume hood for drying. Homogenous samples of defatted larvae were freeze-dried and stored at –20°C for analysis of DM, total nitrogen and ^{15}N content, and used for protein extraction.

2.3.2. Protein extraction

Protein was extracted by immersing 1.5 to 2.5 g of defatted larvae in 0.2 M Na_2HPO_4 / 0.2 M $NaH_2PO_4 \bullet 2H_2O$ buffer in a ratio of 1:4 (g:ml), at pH 8. The solution was vortexed, shaken (Multi Reax, Germany) for 60 min, and centrifuged for 5 min, at 4500 g and 23 °C. After centrifugation, the supernatant was collected, and the remaining pellet passed again through the same protein extraction process. The second supernatant was then mixed with the first one, and the pellet was freeze-dried and stored at -20 °C for DM, total N and ^{15}N analysis. The supernatant, which was the soluble larval fraction, was expected to be mainly composed of soluble protein. The pellet, the insoluble larval fraction, was mainly assumed to contain insoluble protein and other N non-protein compounds (Janssen et al., 2017). To corroborate if ^{15}N was found in soluble larval proteins and not in other N-containing compounds, the supernatant was centrifuged (5 min, 1000 g, 23 °C) and dialysed. Dialysis was performed at 4 °C with a cut-off of 3 kDa (SnakeSkin™ Dialysis Tubing, 3.5K MWCO, 22 mm) using 2000 ml ultrapure water in two rounds of 3 h each. The dried and dialysed supernatant (i.e., here referred as dialysed soluble larval fraction) were freeze-dried and stored at -20 °C for DM, total N and ^{15}N analysis. Overall, the protein extraction process yielded an insoluble larval fraction and a dialysed soluble larval fraction.

2.4. DM, total N and ^{15}N analysis

The DM content of freeze-dried samples was estimated by oven drying samples at 70 °C overnight. Sample quantity varied; the quantities of freeze-dried samples were 0.5 g for manure, 0.2 g for ground larvae, 0.2 g for defatted larvae, 0.5 g for pellet of insoluble larval fraction, 0.1 g for dialysed soluble larval fraction and 0.5 g for residual material, on average. As the experimental unit was one container, we had five biological replicates per sample. Dry matter and total N were determined *in simplo*, except for fresh manure for which DM was done in duplicate. For N-NH_3 and ^{15}N determination, analyses were performed in duplicate. Total N was quantified with the Dumas method using Flash EA 1112 N/Protein analyzer (Thermo Fisher Scientific, USA). Sample quantities used for Dumas averaged 15 mg for manure, 9 mg for defatted larvae, 14 mg for insoluble larval fraction, 10 mg for dialysed soluble

larval fraction and 14 mg for residual material. Ammonia-nitrogen was determined via colorimetric determination in fresh manure samples of 5 g. ^{15}N enrichment was measured after combustion in an elemental analyzer (Flash 2000 organic elemental analyzer HT O/H- N/C, Thermo Scientific) with the use of a continuous flow isotope ratio mass spectrometer (Conflo IV, Thermo Scientific) in samples of 1 to 1.5 mg. Given that ^{15}N was not measured in the starter larvae, it was assumed starter larvae had the same ^{15}N content as the larvae at the end of the *Control* treatment.

2.5. ^{15}N and nutrient balance calculations

The total amount of ^{15}N stored in the larvae ($15NL_{Total}$, in mg) was calculated using equation (1):

$$15NL_{Total} = FW*DM*TN*15Nat \quad (1)$$

Where *FW* is the fresh weight (mg) of the larvae harvested from each replicate at the end of the experiment, *DM* is the dry matter (%) of fresh larvae, *TN* is the total nitrogen content (%) in DM larvae, $15Nat$ is the percentage of ^{15}N atoms (at %) relative to the total N atoms ($^{14}N + ^{15}N$) in larvae.

While the $15NL_{Total}$ of the control treatment shows the background content of ^{15}N in the larvae, for the enriched treatments the total $15NL_{Total}$ shows the sum of the background^{15}N in the larvae and the tracer ^{15}N that was incorporated in the larvae. Equation (2) was used to calculate the total amount of ^{15}N (mg) from tracer origin in the larvae of the *Enriched* and *Enriched_Digesta* treatments ($15NL_{tracer}$):

$$15NL_{tracer} = 15NL_{Total} - 15NL_{Background} \quad (2)$$

Where, $15NL_{background}$ (mg) is the estimated background ^{15}N content in the enriched larvae. $15NL_{background}$ was calculated using equation (1), using the average $15Nat$ for the larvae of the control group for all replicates.

Equation 3 was used to calculate the percentage (%) of tracer ^{15}N found in the larvae (*tL*):

$$tL = \frac{15NL_{tracer}}{15Ntracer}*100 \quad (3)$$

Where, $15Ntracer$ is the amount (mg) of ^{15}N contained in the tracer added at the start of the experiment. Considering that 9.8 mg of $^{15}NH_4Cl$ were added to each replicate of the enriched treatments, and that N constitutes 27% of the molecular weight of NH_4Cl and the purity of ^{15}N atoms in the tracer was 98%, $15Ntracer$ was 2.61 mg.

The total amount of ^{15}N in the residual material, the amount of ^{15}N from tracer origin in the residual material and the percentage of tracer ^{15}N found in the residual material (*tR*) were calculated similarly to equations (1), (2), and (3) used for the larvae. Because gaseous ^{15}N and other aerial losses were not measured, for the ^{15}N balance the percentage of ^{15}N losses (%) (*tLo*) was estimated using equation (4):

$$tLo = 100 - (tL + tR) \quad (4)$$

The total amount of ^{15}N in the larval insoluble and dialysed soluble fractions was calculated using equation (5).

$$15N_f = FW*DM*Y_f* TN_f*15Nat_f \quad (5)$$

Where, $15N_f$ is the amount of ^{15}N (mg) in fraction *f* (i.e., insoluble larval fraction and dialysed soluble larval fraction), *FW* is the fresh weight (mg) of the larvae harvested from each replicate at the end of the experiment, *DM* is the dry matter (%) of fresh larvae, *Y* is the percentage of the dry mass recovered (%) after extraction of each nitrogen fraction *f* (see section 2.3.2), *TNf* is the total nitrogen content of fraction *f* and $15Nat_f$ is the ^{15}N content (at %) of fraction *f*. The amount of ^{15}N from tracer origin and the percentage of tracer ^{15}N in fraction *f* were calculated similarly to equations (2) and (3).

2.6. Statistical analyses

Data was analyzed in R version 4.0.3 (R Core Team, 2019). All analyses and visualizations are reproducible and accessible (see statement on Supporting Information). Results were expressed as mean ± standard error (n = 5). Student's t-test and two-way ANOVA with post-hoc Tukey tests were used to test whether there were differences among treatments, after verifying normality (Shapiro test) and homogeneity of variances.

3. Results and Discussion

3.1. Larval performance

The larval weight once harvested from manure were similar between the *Enriched, Control* and *Enriched_Digesta* treatments (Table 1). Considering this, and that larval weights ranging from 79 to 82 mg were almost the same to the 84 ± 9 mg as reported by Parodi *et al.* (2021), who used the same diet (i.e., pig manure), feeding rate and incubation time, it can be presumed that the tracer ^{15}N nor experimental conditions limited larval growth. Furthermore, the larvae from the *Enriched* and *Control* treatments that passed through the nitrogen free diet had equal final weights, which were similar to those prior to the exposure to the nitrogen free diet (Table 1). These results show that the larvae of the *Enriched* and *Control* treatments did not lose weight after being exposed to the nitrogen free diet and suggest that the nitrogen free diet did not influence larval final weights.

3.2. Total nitrogen and ^{15}N content in larval samples

Larval total N concentration differed between treatments. The larvae and the dialysed soluble larval fraction from the *Enriched_Digesta* treatment had higher nitrogen levels than those from the *Enriched* and *Control* treatments (Fig. 2). On average, the insoluble larval fraction from the *Enriched_Digesta* treatment contained more nitrogen than the other two treatments, however, statistically the difference was marginally non-significant (P = 0.069 – 0.093, see Table S2 for details). There were no differences in the larval N content between the *Enriched* and *Control* treatments (Fig. 2, P = 0.806), confirming that the tracer did not alter the N deposition in the larvae.

The higher nitrogen content in larvae from the *Enriched_Digesta* treatment was likely due to nitrogen contained in their gut, whereas this was replaced by nitrogen free diet in the larvae from the *Enriched* and *Control* treatments. This is supported by the fact that larval total N in the *Enriched_Digesta* (Fig. 2) was equal to 6.9 ± 0.2 g/100 g DM as reported by Parodi *et al.* (2021) for manure-fed larvae, while the larval total N of the *Enriched* and *Control* treatments were slightly lower (Fig. 2). The midgut is the most important digestive organ in insects (Caccia et al., 2019), with feed residence times ranging from 154 to 195 minutes in BSFL (Gold et al., 2020). It is therefore expected that during the 15 h that the larvae were exposed to the nitrogen free diet their gut content was replaced several times effectively removing N contained in their digestive tract.

The higher ^{15}N enrichment of almost a factor 2 in the larval samples of both *Enriched* and *Enriched_Digesta* compared to the *Control* (Fig. 2), confirmed that the tracer-derived ^{15}N was incorporated in the larval

Table 1
Fresh weight (mg) of individual larvae (mean ± std error, n = 5) before and after exposure to the nitrogen free diet for each treatment. The larvae from treatment *Enriched_Digesta* were not exposed to the nitrogen free diet. Values within columns and rows without letters in common are significantly different (P < 0.05). See Table S1 for statistical parameters.

Treatment	After manure	After N free diet
Enriched	82 ± 1.76 ^{ab}	81 ± 1.07 ^{ab}
Control	82 ± 1.06 ^{ab}	85 ± 1.26 ^{a}
Enriched_Digesta	79 ± 1.16 ^{b}	-

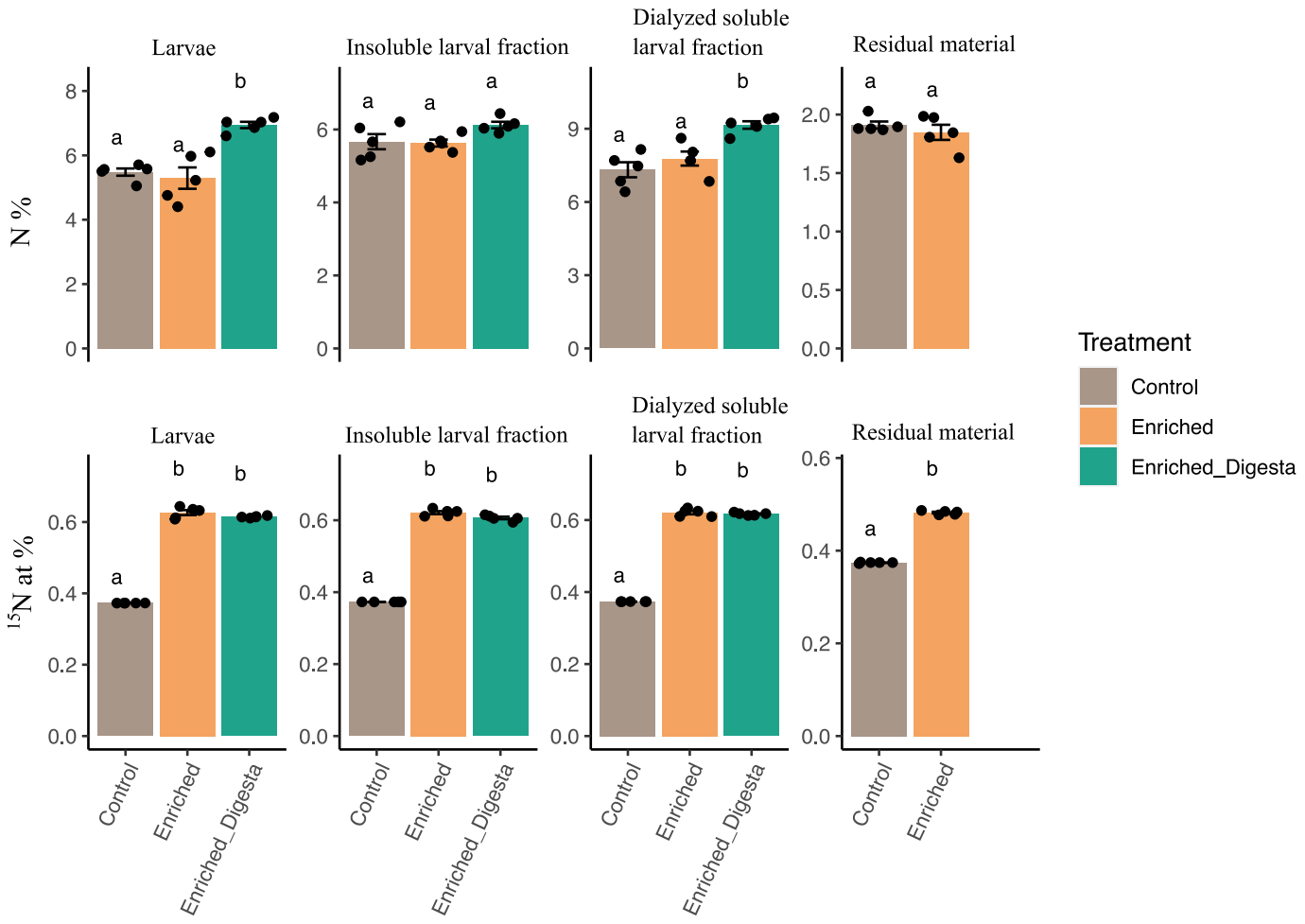

Fig. 2. Total nitrogen (N) and ^{15}N enrichment (^{15}N) of larvae, insoluble larval fraction, dialysed soluble larval fraction and residual material (mean ± std error, n = 5). N values expressed as % are reported on a DM basis. ^{15}N expressed as at % (atom percentage of ^{15}N atoms relative to total N (^{14}N + ^{15}N)). Different letters indicate that values are significantly different ($P < 0.05$). See Table S2 for exact N and ^{15}N values and statistical parameters, and Table S3 for background data.

body mass. Unlike total N, ^{15}N enrichment was equal for most larval samples (i.e., larvae, insoluble N and soluble N fractions) of both *Enriched* and *Enriched_Digesta* treatments (Fig. 2), suggesting that the ^{15}N concentration in the frass-digesta was too low to change the relative atom abundance of ^{15}N in the larval samples. This is supported by the fact that the ^{15}N abundance in the residual material was lower (Fig. 2) than in the larval samples, and that most of the tracer ^{15}N was lost (see section 3.3, Fig. 3B). The background ^{15}N enrichment found in the larvae of the *Control* treatment was within the 0.355 – 0.377 at % range, present in most naturally-occurring nitrogen materials (Robinson, 2001).

3.3. *Total N, ^{15}N tracer balance and ^{15}N allocation in larvae*

The nitrogen balance shows that between 25 to 27 % of the nitrogen contained in the manure was deposited in the larvae, 52 to 54 % was found in the residues and 19 to 23 % was likely lost to the air as NH_3 emissions (Fig. 3A). In addition, the nitrogen balance reported here was similar to Parodi *et al.* (2021), who also quantified emissions in a complete mass balance approach and found that 25% of the nitrogen provided in pig manure was found in the larvae, 53% in the residues and 20% was lost as NH_3 emissions.

The balance of the tracer ^{15}N showed that 13% of the tracer ^{15}N was found in the larvae, 11% in the residues, and 76% was lost (Fig. 3B). ^{15}N proportional losses were much higher than proportional total nitrogen losses. Considering that 80 to 90 % of the nitrogen contained in the manure was NH_3-N (see Table S5 for total nitrogen and NH_3-N in fresh manure, and Table S6 for N content in supernatant from manure), it was expected that total nitrogen losses were going to be similar to the ^{15}N losses, but this was not the case. It is therefore likely that while some ^{15}N was lost via NH_3 emissions, a substantial amount of ^{15}N was lost from the residues pool during the cleaning of larvae once extracted from the enriched residual material, or during gut digesta cleaning with the nitrogen free diet.

Within the larvae, 17% of the ^{15}N was found in dialysed soluble fraction and 78% in the insoluble fraction. The remaining 10% was likely lost during dialysis either as soluble protein or other N-containing compounds. The main pool of ^{15}N was in the insoluble larval fraction. Likely, at pH 8 this primarily consisted of proteins, and chitin (Azzollini et al., 2020). A smaller proportion could have been present in other non-protein nitrogen compounds such as melanin (Caligiani et al., 2018), nucleic acids and phospholipids (Janssen et al., 2017). A previous study that quantified the composition of nitrogen-containing compounds in whole BSFL, found 84% of the nitrogen in proteins, 10% in chitin, and 6% in other N-compounds such as melanin (Caligiani et al., 2018). If all the chitin and other N-compounds were in the insoluble larval fraction (i.e., assuming 13 % of insoluble larval fraction was chitin and 8% in melanin), at least 79% of the ^{15}N in the insoluble larval fraction could have been present in insoluble proteins. Thus, when considering soluble and insoluble proteins, at least 10% of the NH_3-N contained in manure could be incorporated into larval proteins and 3% into other N-compounds such as chitin and melanin. Future studies

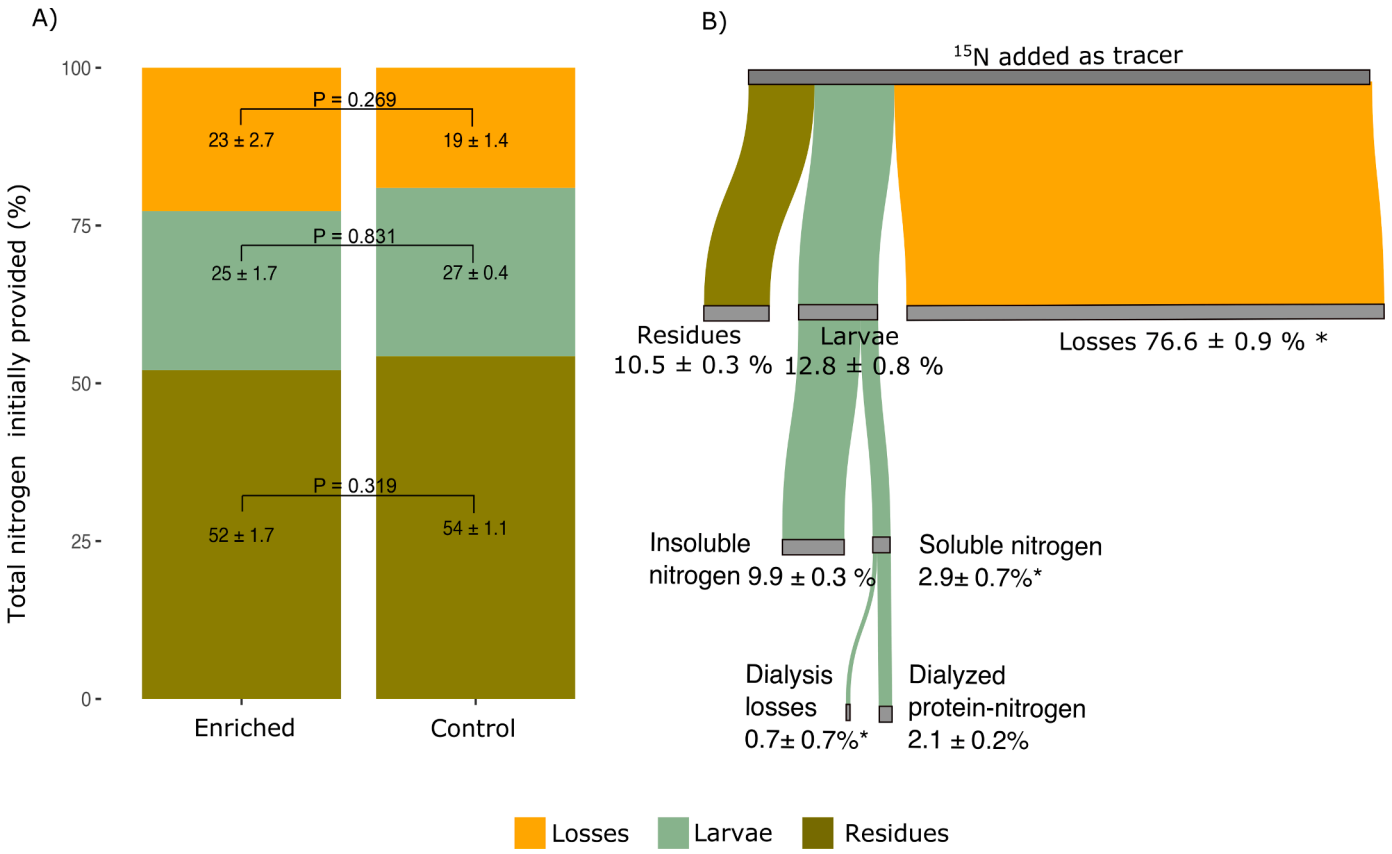

Fig. 3. A) Total nitrogen balance of the *Enriched* and *Control* treatment. B) ^{15}N tracer balance for the *Enriched* treatment. Percentages refer to the initial amount of total N or ^{15}N tracer. Values with an asterisk * were calculated by difference. See Table S3-S4 for background data for both nutrient balances.

could quantify ^{15}N enrichment in amino acids, chitin and melanin to confirm this claim.

3.4. Opportunities and outlook

In this study we show that 13% of manure NH_3-N can be stored in the larval body mass and incorporated into proteins. Elucidating the mechanisms by which NH_3-N is assimilated into BSFL is key to explore interventions aimed to maximize the 13% reported here. Maximizing manure NH_3-N assimilation in BSFL reduces manure NH_3 emissions and increases the amount of circular protein that BSFL supply. Two simultaneous pathways could be involved in the assimilation of NH_3-N in BSFL larval proteins. One of the pathways is via transamination inside the larvae, where NH_3 is used as a N-donor during amino acid synthesis (Chen and Bachmann-Diem, 1964; Seshachalam et al., 1992). If the tracer ^{15}N was the N donor, amino acids and proteins could be enriched with tracer ^{15}N. As with higher larval densities more larvae compete for the same substrate, the chances of larvae incorporating NH_3-N as an N donor for the synthesis of non-essential amino acids via transamination might increase. The second pathway is via microbial biomass acting equivalent to an "external rumen", where NH_3-N is used for the synthesis of microbial amino acids, subsequently used by larvae feeding on these microbes. The assimilation of NH_3 by microbes is enzymatically driven by glutamate deshydrogenase (GDH) and glutamine synthetase (Reitzer, 2014). The gene that encodes for GDH is present in animals, plants and microorganisms (Hudson and Daniel, 1993). Unlike the larval transamination pathway, the microbial biomass pathway could reduce manure NH_3 emissions if NH_3-N assimilation in larvae is maximized via rearing conditions. For instance, substrates inoculated with microbes can increase both bioconversion and larval weights (Mazza et al., 2020; Wong et al., 2021; Xiao et al., 2018), although this is not always the case (Gold et al., 2021; Hasnol et al., 2020). Future assessments should elucidate if increased bioconversion due to specific microbial inocula is correlated with larger NH_3-N assimilation in manure-based diets. Inocula from *Enterococcus faecium* strain C2 and *Bacillus coagulans* strain B1 can reduce NH_3 emissions from manure by 53% and 31%, respectively (Xiao et al., 2021), and could be good candidates to improve NH_3-N assimilation in BSFL. Another potential intervention to increase NH_3-N microbial and larval assimilation is to increase the C:N ratio of manure to foster microbial growth and promote higher microbial nitrogen utilization. Such carbon sources could be supplied from low value fiber-rich leftovers or high-carbohydrate leftovers which are non-edible to livestock. However, negative effects on larval performance parameters such as final weight, survival and development time, and GHG emissions should be evaluated to determine the net benefits of this intervention (Barragan-Fonseca et al., 2019).

If the limit of microbial NH_3-N assimilation is reached, as can occur in the rumen (Pengpeng and Tan, 2013), interventions should prevent NH_3 volatilization from the residual material. Keeping the substrate slightly acidic to favor the NH_4: NH_3 equilibrium towards NH_4, without hampering the growth of beneficial bacteria that assimilate NH_3-N, would contribute to this. Instead of the expensive acidifiers used for manure management, the substrate pH could be kept slightly acidic by mixing manure with an acidic feed ingredient that might improve the nutrient quality of manure and increase larval yields. Such acidic ingredient could come from food waste pre-treated with lactic acid bacteria (Sabater et al., 2020), or acidic fruit wastes such as citrus and tomatoes. After BSFL harvest, however, it is key to implement management practices of the residual material to avoid NH_3 volatilization during storage and application. The residual material is the main pool of nitrogen after BSFL bioconversion (Parodi et al., 2021) and its emissions are one of the main sources of GHG in BSFL bioconversion systems

(Mertenat et al., 2019). Thus, without proper post-harvest management of the residual material, the NH_3 reduction gains obtained via larval NH_3-N assimilation could be offset.

BSFL have the potential to become an important element in the circular economy. Future holistic assessments covering not only the net environmental benefits and disadvantages of BSFL at a supply-chain and food systems level, but also the economic, social and safety dimensions associated to its use, are needed to elucidate the role of manure-fed BSFL in future food systems

3.5. Conclusions

In this study we show that during manure bioconversion with BSFL 13% of the NH_3-N contained in manure was incorporated into the larval biomass. Within the larval body, 77% of the NH_3-N was found in the insoluble larval fraction, and 17 % in the dialysed soluble fraction. Our results suggest that manure-fed black soldier fly larvae can reduce ammonia pollution from manure and can thereby contribute to a circular and sustainable protein supply.

Notes

The authors declare no competing financial interest.

Author contribution

Alejandro Parodi: Conceptualization, Investigation, Formal analysis, Visualization, Writing - original draft. **Qifa Yao**: Investigation, Data curation. **Walter J.J. Gerrits**: Conceptualization, Validation, Writing - review & editing, Supervision. **Maryia Mishyna**: Conceptualization, Validation, Writing - review & editing. **Catriona M.M. Lakemond**: Conceptualization, Validation, Writing - review & editing. **Dennis Oonincx**: Validation, Writing - review & editing .**Joop J.A. Van Loon**: Conceptualization, Validation, Writing - review & editing, Supervision.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that cdocould have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.resconrec.2022.106343. Raw data and custom R scripts developed for the analyses and visualizations are available at doi.org/10.4121/19376129.

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