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JOURNAL OF INSECTS AS FOOD AND FEED (2024) DOI:10.1163/23524588-00001422

Journal of Insects as Food and Feed
brill.com/jiff

RESEARCH ARTICLE

Strike while the iron is hot: unravelling the impact of dietary iron on two edible insects

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Received 18 October 2024 | Accepted 25 November 2024 | Published online 17 December 2024

Abstract

Edible insects are key players in the alternative protein market. Alongside their high protein content, edible insects contain abundant trace minerals, including iron. Given the lack of sustainable and bioavailable iron sources, edible insects are a promising iron source in both human and livestock diets. However, little is known about how the iron concentration in edible insects can be manipulated. This study aims to investigate the impact of dietary iron at different concentrations on the growth, survival, and nutrient composition of two edible insect species larvae: black soldier fly (BSF, *Hermetia illucens*) and yellow mealworm (YM, *Tenebrio molitor*). Substrate iron content was increased using ferric ammonium citrate (FAC), ranging from 267-563 mg/kg (0 to 10 mM FAC) for YM and 323-6637 mg/kg (0 to 100 mM FAC) for BSF. Larval weight of YM was unaffected by dietary iron content, but survival rates significantly decreased by 4% at the 10 mM diet. In BSF larvae, no differences in body weight or survival were noted between dietary iron treatments, but cuticular lesions potentially indicative of iron overload appeared at 100 mM diet. When exposed to excess dietary iron, both YM and BSF showed significant increases in iron content in a dose-dependent manner, with BSF larvae exhibiting a threefold increase from 372 mg/kg to 999 mg/kg. Both species exhibited significant reductions in zinc on iron-enriched diets. Calcium levels in BSF strongly correlated with dietary iron concentration, rising by over 20% at the 100 mM diet, a phenomenon not previously reported. Finally, across all dietary iron concentrations, no significant effects on protein and fat content or soluble protein composition were observed in either species. All together data suggest that BSF larvae can act as a precious source of both iron and calcium for food and feed formulations through substrate manipulation.

Keywords

black soldier fly – dietary iron – diet manipulation – novel iron sources – yellow mealworm

1 Introduction

Iron deficiency is the most prevalent hidden nutrient deficiency worldwide, with more than 24% of the world population suffering from iron deficiency anaemia (GBD, 2023). Similarly, iron deficiency anaemia is also widespread among livestock, particularly pigs, with over 60% of piglets suffering from this condition (Kim *et al.*, 2018).

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For human consumption, heme-based iron is currently the most bioavailable form of iron available, which is found mostly in animal-based sources (Schönfeldt and Hall, 2011). However, the production of these animal-based products requires extensive use of water, energy, and land, posing significant global economic and sustainability burdens (Parlasca and Qaim, 2022). Iron supplementation is a common practice for both humans and livestock to combat iron deficiency. Nevertheless, this approach is associated with health risks and low bioavailability. In humans, iron supplements can lead to various side effects, such as gastrointestinal issues, fatigue and dizziness, and poor iron absorption rates (Tolkien *et al.*, 2015). In piglets, iron supplementation can result in antioxidant deficiency and the induction of hepcidin expression, which reduces iron absorption (Szudzik *et al.*, 2018). These observations underscore the need for novel, sustainable and bioavailable iron sources, for humans and livestock alike.

Edible insects are a viable alternative protein source for both feed and food, owing to their lower requirements for water, land and energy in rearing (van Huis *et al.*, 2021). Many edible insects have an iron content similar to beef, and a Caco-2 iron bioavailability assay suggested similar absorption between insects and beef (Launder-Dada *et al.*, 2016; Mwangi *et al.*, 2018). There is a knowledge gap regarding the effective utilization of iron from edible insects, given the high levels of antinutritional factors that can inhibit absorption (Mwangi *et al.*, 2022). However, the use of fractionated insect products may offer a promising approach to harness this iron as a viable nutritional source. It is hypothesised that the iron availability potential in insects is rooted in entoferritin, the predominant iron transporter protein in insects (First *et al.*, 2023). Although it is evident that substrate iron content influences iron concentrations in insects (Seyedalmoosavi *et al.*, 2023), little is known about how this affects their development and nutritional profiles. For instance, *Hermetia illucens* larvae reared on different waste streams with varying iron contents have shown increased iron concentrations when reared on a high-iron waste stream (Seyedalmoosavi *et al.*, 2023).

This study investigates how the targeted addition of iron affects the overall development and nutritional content of edible insects. The effects of dietary iron on the growth, survival, macro- and micronutrients content of two key insect species in the food and feed industry: black soldier fly (BSF, *Hermetia illucens*) and yellow mealworm (YM, *Tenebrio molitor*) larvae were measured under a diet intervention trial.

2 Methods

Yellow mealworm rearing

Ferric ammonium citrate (FAC, CAS number 1185-57-5) was obtained from Merck (Darmstadt, Germany). The iron salt was dissolved in water (FAC solution) in a concentration 0, 1,2.5, 5 and 10 mM (Table 1). Mealworm feed provided by InsectenKwekerij van de Ven V.O.F (Deurne, the Netherlands) was mixed using a magnetic stirrer with the different FAC solutions at a 1:1, FAC solution feed ratio (v/w). The wet feed was then dried overnight at 50 °C with ventilation set on 100% overnight in an incubator (Binder GmbH, Tuttlingen, Germany). After drying, the substrate was ground with a blender (Waring, Stamford, CT, USA) and returned to the incubator at 50 °C with ventilation set on 100% for another night. Finally, the feed was ground again with a blender into a fine powder with a dry matter (DM) content of approximately 90% to finalize the YM substrate. Final iron content in the substrates varied from 267 to 563 mg/kg DM, including the non-spiked iron content of the feed (Table 1). Each YM substrate was sampled for freeze-drying and homogenization prior to mineral analysis as described below.

The YM larvae (InsectenKwekerij van de Ven V.O.F, Deurne, the Netherlands) were reared in a controlled environment at 25 °C, dark environment and 50% relative humidity in a rearing container (15.5 × 10.5 × 6 cm). For each diet (Table 1), 500 larvae, approximately 1 cm in length and weighing approximately 26 mg each, were placed on 20 grams of YM substrate and 15 grams of carrots. Every other day, the YM were provided with 10 grams of fresh YM substrate and 15 grams of fresh carrots. Dead YM were removed at each feeding event to prevent cannibalism. Upon the appearance of the first pupae, 20 days after exposing the larvae to the experimental substrate, the YM were transferred to a control YM substrate diet for two days to clear excess iron-spiked diet from their gut (Figure 1). On day 22, the YM larvae were separated from the feed using a sieve (pore size 1 mm), weighed (three sets of 30 random larvae), and individually counted.

For each replicate, 5 g of larvae were sacrificed with CO_2 for further preparation of soluble fractions. The remaining larvae were sacrificed by flash-freezing with liquid nitrogen, followed by freeze-drying and stored at −80 °C until proximate and mineral analyses. Each diet was done in triplicate.

TABLE 1 Iron concentrations (mg/kg dry substrate) of substrates used for rearing the yellow mealworm larvae and black soldier fly larvae

Yellow mealworm larvae substrate	Diet				
	Control*	1 mM	2.5 mM	5 mM	10 mM
Fe	267	293	350	407	563
Black soldier fly larvae substrate	Diet				
	Control*	10 mM	25 mM	50 mM	100 mM
Fe	323	943	2300	2740	6637

* Control substrates were not mixed with ferric ammonium citrate; the iron content is based on the starting iron concentrations of the feeds.

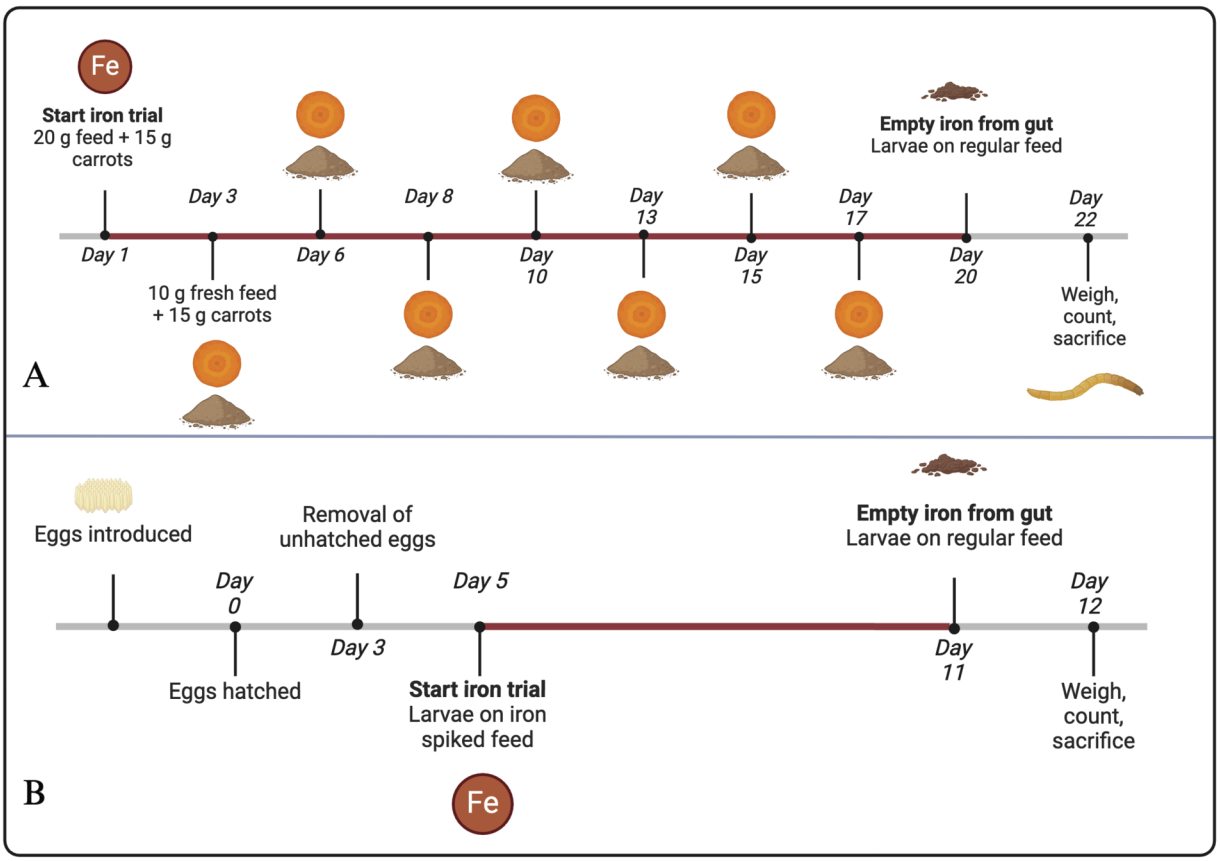

FIGURE 1 Timeline of the rearing period of the yellow mealworm (A) and black soldier fly (B) larvae. Created with BioRender.com.

Black soldier fly rearing

Before mixing with FAC solution, chicken feed (Kuikenopfokmeel no. 1, Kasper FaunaFood, the Netherlands) was sieved (pore size 1.0 mm). FAC solutions were prepared by dissolving FAC in water at concentrations of 0, 10, 25, 50 and a 100 mM (Table 1). The FAC concentrations incorporated in the BSF diet were tenfold higher than those tested in the YM substrate, as preliminary trials indicated the lower FAC concentrations did not affect larval weight or survival of BSF larvae, whereas at 5 mM FAC YM performance was negatively affected (Figure 2). Sieved chicken feed was combined with the FAC solution in a 1:2 ratio (w/v) and mixed with a magnetic stirrer for 30 min at room temperature to prepare the BSF substrate. Final iron content in the substrates varied from 323 to 6637 mg/kg, including the non-spiked iron content of the feed (Table 1). Each BSF substrate was sampled for freeze-drying and homogenization prior to mineral analysis as described below.

Eggs from BSF were obtained from Protix (Bergen op Zoom, the Netherlands). Approximately 10,000 eggs were distributed into 10 rearing containers, each containing 600 g of wet BSF substrate, with a feed to water ratio of 1:2 (w/v). The lids of the rearing containers had a window (8.0 × 5.5 cm) covered with nylon mesh to allow ventilation. The day of the first larvae hatching

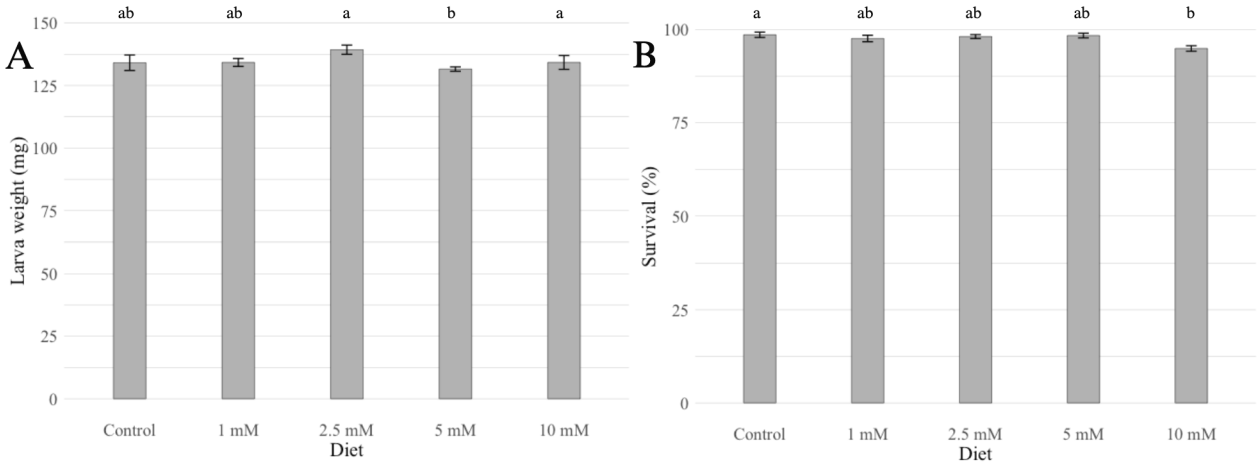

FIGURE 2 (A) Average weight (mg) of YM larvae; (B) Survival (%) of YM larvae. Both measurements were taken 22 days after larvae were introduced to substrates with varying concentrations of ferric ammonium citrate: control, 1 mM, 2.5 mM, 5 mM, and 10 mM. Bars having no letter in common differ significantly (one-way ANOVA followed by Tukey's HSD test; $P < 0.05$).

was designated as day zero of the trial. Insect rearing was carried out at 27 °C and a relative humidity of 70% in the dark.

On day 3, any unhatched eggs were removed to ensure uniformity among the larvae. On day 5, a homogeneous group of larvae that were manually chosen and collected, weighing approximately 10 mg each, were introduced to the FAC-spiked BSF substrate (Table 1, Figure 1A). For each dietary group, 250 larvae were placed on 105 g of wet BSF substrate per 100 larvae with a feed to water ratio of 1:2 (w/v). Each rearing container held a sample size of 250 larvae. After six days on the iron-spiked diet, the larvae were transferred to a control BSF substrate to clear the iron-spiked diet from their guts. On day 12, the larvae were separated from the substrate using a sieve, washed with room temperature tap water, individually counted and weighed (three sets of 30 random larvae).

For each replica, 5 g of larvae were sacrificed with CO_2 for further preparation of soluble fractions. The remaining larvae were sacrificed by flash-freezing with liquid nitrogen, followed by freeze-drying and stored at –80 °C until proximate and mineral analyses. Each diet was done in triplicate.

Insect parameters

Survival

Survival was calculated as described by Oonincx *et al.* (2019). Survival is defined as the percentage of larvae remaining at the end of the rearing period relative to the number of larvae at the beginning of the rearing period.

Larval weight

Average weight of the larvae was determined by randomly collecting 30 larvae from each rearing container at the end of the rearing period weighing them collectively and dividing the value by 30. This process was repeated three times for each replicate.

Lesion documentation

Freeze-dried BSF reared on 100 mM FAC diet were visually inspected using an Olympus SZX12 microscope equipped with a Euromex sCMEX-20 camera, using ImageFocus software. Images of specific lesions on the larvae were captured.

Proximate composition

Sample preparation

Firstly, flash-frozen insects were placed in pre-weighed boxes, covered with plastic lids. A hole was cut in each lid and covered with tissue to allow water sublimation. For the substrate samples, approximately 20 mL of substrate was placed in 50 mL Greiner tubes, covered with tissue, and secured with rubber bands. The insect and substrate samples were freeze-dried with the Super Modulyo freeze dryer (Edwards, Burgess Hill, UK). After freeze-drying, the containers with samples were weighed and immediately transferred to new airtight containers. These containers were then stored in vacuum bags in the freezer at –80 °C until sample homogenization.

Insect samples were homogenized using the Freezer/CryoMill® 6775 (SPEX SamplePrep, NJ, USA). The CryoMill operated with dual samples under automatic liquid nitrogen filling, with a pre-cool time of 3 min, a run time of 3.5 min, and a cool time of 1 min. Milling was

performed in three cycles at a rate of 15 cps. Substrate samples were homogenized by placing 8-10 g of the sample in the Retsch Ball Mill MM400 (Retsch, Haan, Germany) for 45 s at a frequency of 25/s.

Soluble fraction was prepared by grinding CO_2-sacrificed larvae with Milli-Q water at a 1:4 ratio. The resulting slurry was centrifuged at 4,700 g for 45 minutes at 4 °C followed by supernatant (soluble fraction) collection. This soluble fraction was then stored at –80 °C until further use.

Dry matter content

The dry matter (DM) content of the freeze-dried larvae was measured using a DAB 200-2 Moisture analyzer (KERN and Sohn GmbH, Balingen, Germany). To determine the DM, one gram of the sample was heated at 120 °C until complete dryness. Dry matter content represents the fraction of dried material relative to the fresh weight of the larvae.

Fat content

Fat extraction was based on the Official AOAC method 920.39 (AOAC, 2023), with minor modifications. The fat content of the larvae was determined using a SOXTHERM® Extraction unit (Gerhardt GmbH and Königswinter, Germany). In this procedure, four grams of freeze-dried insect powder were placed into extraction thimbles and covered with grease-free cotton wool. The fat was extracted using petroleum ether (boiling range 40-60 °C) at an extraction temperature of 150 °C for a 2 h. Following extraction, the canisters were left in a fume hood overnight to allow any residual solvent to evaporate. The remaining fat content in the canisters was weighed the next day to and calculated as a percentage of the DM.

Protein content and molecular weight distribution

The nitrogen content of the larvae was quantified using a DUMAS Flash EA 1112 Protein analyser (ThermoFisher Scientific, MA, USA). Cellulose (Merck, Darmstadt, Germany) was used as a blank, and D-methionine (Thermo Scientific Chemicals, Waltham, MA, USA) was utilized to construct the calibration curve. The protein content of the samples was calculated assuming a nitrogen-to-protein conversion factor of 4.75 for the yellow mealworm larvae and 4.67 for the black soldier fly larvae as established for these two species respectively (Janssen *et al.*, 2017). The Protein samples were expressed as a percentage of the DM.

Protein composition was assessed using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions. The soluble fraction samples were run on 12-well NuPAGE™ Novex™ 12% Bis-Tris gels. The electrophoresis was carried out using NuPAGE™ MOPS SDS Running Buffer and NuPAGE™ Antioxidant, followed by staining with Coomassie Brilliant Blue R-250 Staining Solution. Thermo Scientific™ Spectra™ Multicolor Broad Range Protein Ladder (10-225 kDa) was used as a molecular weight marker. The gels were scanned using a GS-900 Calibrated Densitometer (Bio-Rad, USA), and ImageLab (version 6.1, Bio-Rad, USA) was employed for documenting the gels.

Ash content

The total content of non-combustible inorganic elements, called ash, was measured following the AOAC ash determination protocol (Thiex *et al.*, 2012), with minor modifications. In brief, one gram of freeze-dried insect powder was incinerated at 550 °C for 5 hours using a Gallenkamp Muffle Furnace (Gemini B.V., Apeldoorn, the Netherlands). Post-incineration, the samples were left to cool overnight in a desiccator before weighting. After weighing, concentrations were calculated and expressed as a percentage of the dry matter.

Trace mineral analysis

Larvae and substrate mineral quantification was carried out by the Soil Chemistry Laboratory (CBLB, Wageningen University and Research, the Netherlands). Briefly, 300 mg of the insect sample was treated with concentrated nitric acid and hydrochloric acid. The mixture was then heated in a microwave. Following this, hydrogen peroxide was added, and the mixture was reheated in the microwave. After allowing the undissolved silica particles to settle, the supernatant was collected for analysis using Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-AES, Thermo iCAP-6500 DV; Thermo Fisher Scientific, USA). The mineral content was expressed as g/kg DM (Ca, K, Mn, P, Zn) or mg/kg (Fe). Bioaccumulation factor (BAF) is defined by Daş *et al.* (2023) as the larval iron concentration divided by the substrate iron content.

Data analysis

Statistical analyses were conducted using RStudio (version 1.3.1073). A one-way ANOVA was performed to evaluate differences among groups, followed by Tukey's Honestly Significant Difference (HSD) post-hoc test for pairwise comparisons with alpha set at 0.05. Pearson correlation analysis was employed to assess the relationship between substrate iron concentration and the

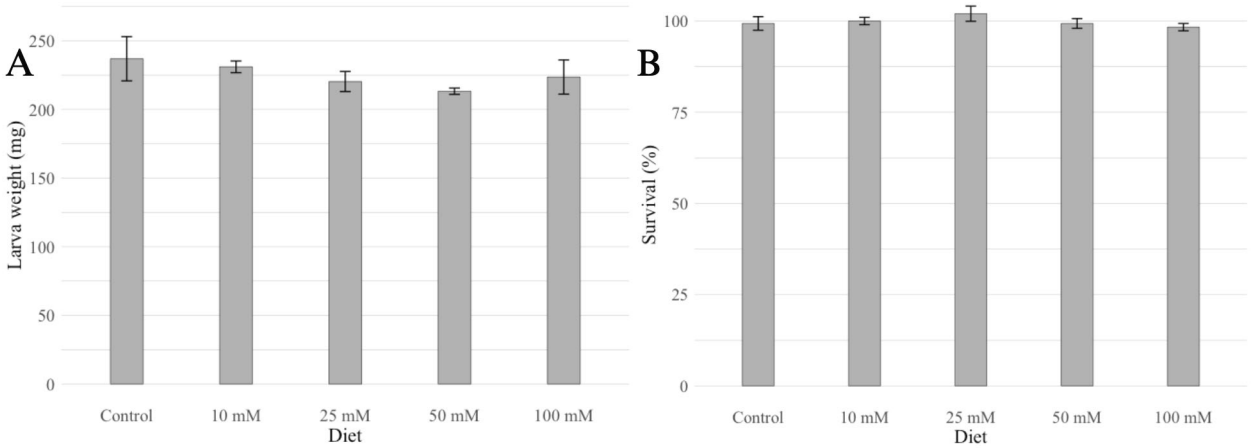

FIGURE 3 (A) Average weight (mg) of BSF larvae; (B) Survival rates (%) of BSF larvae. Both measurements were taken 6 days after 5 days old homogenous larvae were introduced to substrates with varying concentrations of ferric ammonium citrate: control, 10 mM, 25 mM, 50 mM, and 100 mM. No significant differences between diets were found for either the average weight or survival rate of the BSF larvae.

mineral contents. Correlations were classified as moderate for Pearson correlation coefficients (r) ranging from 0.75 to 0.9, or from –0.75 to –0.9. Correlations were considered strong when r was ≥ 0.9 or ≤ –0.9.

3 Results and discussion

Insect parameters

Yellow mealworm (YM)

Larvae reared on control diet weighed 134.1 ± 5.3 mg (Figure 2A). The observed range of larval weight at this rearing time aligns with values reported in the literature (Kim *et al.*, 2016; Rumbos *et al.*, 2022). The YM reared on substrate with 2.5 mM of FAC reached a similar weight (139.3 ± 3.2 mg) as the larvae on control and 1mM FAC diets. Further increase of FAC concentration to 5 mM significantly decreased larval weight to 131.5 ± 1.5 mg compared to the 2.5 mM Diet. At the highest tested concentration of 10 mM FAC, larval weight (134.2 ± 4.7 mg) was significantly higher than the 5 mM FAC diet but was similar to that on control, 1 and 2.5 mM FAC diets. Thus, there was no clear trend in the effect of iron concentration on larval weight of YM.

Survival of YM larvae on control diet was the highest (98.6%) compared to all FAC diet reared larvae. Increase in FAC content up to 5 mM resulted in survival of 97.6%. On 10 mM FAC diet, survival dropped to 94.9%, being significantly lower than on control diet (Figure 2B). The survival of YM larvae in this study is high compared to other studies. The larvae in this study exhibited survival ranging from 94% to 99%, while previous studies reported survival of 70% to 90% in various feeding experiments (Liu *et al.*, 2020; Rumbos *et al.*, 2022; Zim *et al.*, 2021). The high survival rate observed in our study suggests that the larvae were reared under suitable conditions. Prior to this study, the effect of iron excess on YM survival has not been documented. However, exposure to similar excess dietary iron can reduce growth rates, larval size, oviposition, and locomotive functionality in insects such as *Drosophila melanogaster* and *Lymantria dispar* (dos Santos *et al.*, 2024; Keena, 2022). Although those studies did not measure survival, they did demonstrate that excess dietary iron negatively affects larval development and health. This negative impact aligns with the findings reported here on the YM larvae when exposed to excess dietary iron. Given the significant impact of iron supplementation on YM larvae, they were not reared on the tenfold iron diet used for BSF larvae (Table 1).

Black soldier fly (BSF)

The weight and survival of BSF larvae did not significantly differ across the FAC concentrations tested (0-100 mM; Figure 3). The weight range of BSF larvae at a similar age, between 200-250 mg per larvae, is in line with literature (Kießling *et al.*, 2023; Seyedalmoosavi *et al.*, 2022). Larval weight change in response to increased iron concentrations has not previously been reported for BSF, but the effect of heavy metals is well-documented. In a study conducted by Wu *et al.* (2020), larvae were reared on diets spiked with copper concentrations ranging from 100 to 800 mg/kg DM and cadmium concentrations from 10 to 80 mg/kg DM. The study demonstrated that BSF larvae do not exhibit significant changes in larval weight when reared on diets

TABLE 2 Protein, fat, and ash content (% dry matter) of YM and BSF larvae reared on diets with different ferric ammonium citrate concentrations. Each nutritional value is compared between the FAC diets within the two insect species

Yellow mealworm					
	Control	1.0 mM	2.5 mM	5.0 mM	10.0 mM
Protein (%/DM)	38.5 ± 0.5	38.1 ± 0.0	38.0 ± 0.1	38.7 ± 0.3	38.3 ± 0.2
Fat (%/DM)	34.8 ± 3.0	35.7 ± 2.3	35.9 ± 1.4	35.7 ± 1.2	35.5 ± 2.0
Ash (%/DM)	3.6 ± 0.1	3.4 ± 0.5	3.7 ± 0.1	3.8 ± 0.2	3.5 ± 0.1
Black soldier fly					
	Control	10.0 mM	25.0 mM	50.0 mM	100.0 mM
Protein (%/DM)	32.9 ± 0.8	33.3 ± 0.8	33.6 ± 0.8	33.8 ± 0.1	33.3 ± 0.1
Fat (%/DM)	28.5 ± 1.6	30.8 ± 0.5	30.6 ± 0.6	30.4 ± 1.5	31.1 ± 1.3
Ash* (%/DM)	10.3^{ab} ± 0.1	10.2^{a} ± 0.0	10.3^{ab} ± 0.3	10.8^{b} ± 0.2	11.5^{c} ± 0.2

* Different letters indicate significant differences ($P < 0.05$). Mean groups lacking common letters are not significantly different ($P > 0.05$).

with varying levels of cadmium or copper, that is similar to the effect of the excess of dietary iron observed in the current study. However, it should be noted, that biological effects and known lethal or toxic concentrations of different metals vary, making it challenging to directly compare effects of iron concentrations with cadmium or copper (Gupta and Gupta, 1998).

Similar to the larval weight, BSF larval survival was unaffected by dietary iron concentrations (ranging from 99-102%). The survival rates slightly exceeding 100% are likely due to human error, as the larvae were counted manually. Survival of nearly 100% during an 11-14 days rearing period is a common in BSF reared in suitable conditions (Cheng *et al.*, 2017). However, survival can vary depending on the feed source. For example, BSF reared on fish-based diets and digested sewage sludge, which are both contaminated with various heavy metals, exhibited survival rates of 47% and 39%, respectively (Lalander *et al.*, 2019; Nguyen *et al.*, 2013). The low survivability of larvae reared on sewage sludge (39%) was associated with a lower protein conversion rate compared to those reared on undigested sewage sludge, which had 79% survival (Lalander *et al.*, 2019). Cai *et al.* (2018) reared larvae on artificial diets spiked with 11 different heavy metals, mimicking the content found in such waste streams, to investigate the specific effect of heavy metals on BSF larvae survival in contaminated waste streams Although iron was not included in the study, none of the tested heavy metals affected larval survival when exposed to these artificial diets. These findings align with the current study, indicating that metal content, including iron, has a lesser impact on BSF larval survival compared to factors like substrate processing, moisture content and macronutrient bioaccessibility.

Several BSF larvae reared on the 100 mM diet (6637 mg/kg DM of iron) displayed a dark, three-layered lesions with an approximate diameter of 400 to 800 μm (Figure 3). Although this phenomenon has not been previously documented in insects, forensic signs of iron toxicity have been characterized in other animals. For instance, bronze skin hyperpigmentation and the presence of a black-greyish, rigid pancreas have been observed in humans (de Carvalho Machado and Dinis-Oliveira, 2023). Iron is not known to be directly involved in cuticle formation in BSF larvae, but calcium is. As elaborated below, calcium levels of BSF larvae levels are correlated with dietary iron concentrations in the substrate (Table 3). Amorphous calcium carbonate and calcite are abundant in the larval cuticle and their presence increases with larval growth (Rebora *et al.*, 2023). This could explain the appearance of these lesions. This finding could be used as an indication of extreme iron concentrations in waste streams fed to BSF larvae.

Proximate composition

Fat content, protein content and soluble protein composition of the two larvae species were not significantly altered by the incorporation of FAC in the substrate. For the BSF larvae, protein content ranged from 32.9% to 33.8% DM, and fat content ranged from 28.5% to 31.1% DM. For the YM larvae, protein content ranged from 38.0% to 38.7% DM, and fat content ranged from 34.8% to 35.9% DM (Table 2, Figure 4). Protein content, bioaccessibility and fat content have been shown to be the main drivers for protein and fat alteration of both BSF and YM larvae (Fischer and Romano, 2021; Jankauskienė *et al.*, 2024; Wu *et al.*, 2006). These findings further support the conclusion that substrate processing, macronutrient composition and form are the primary determi-

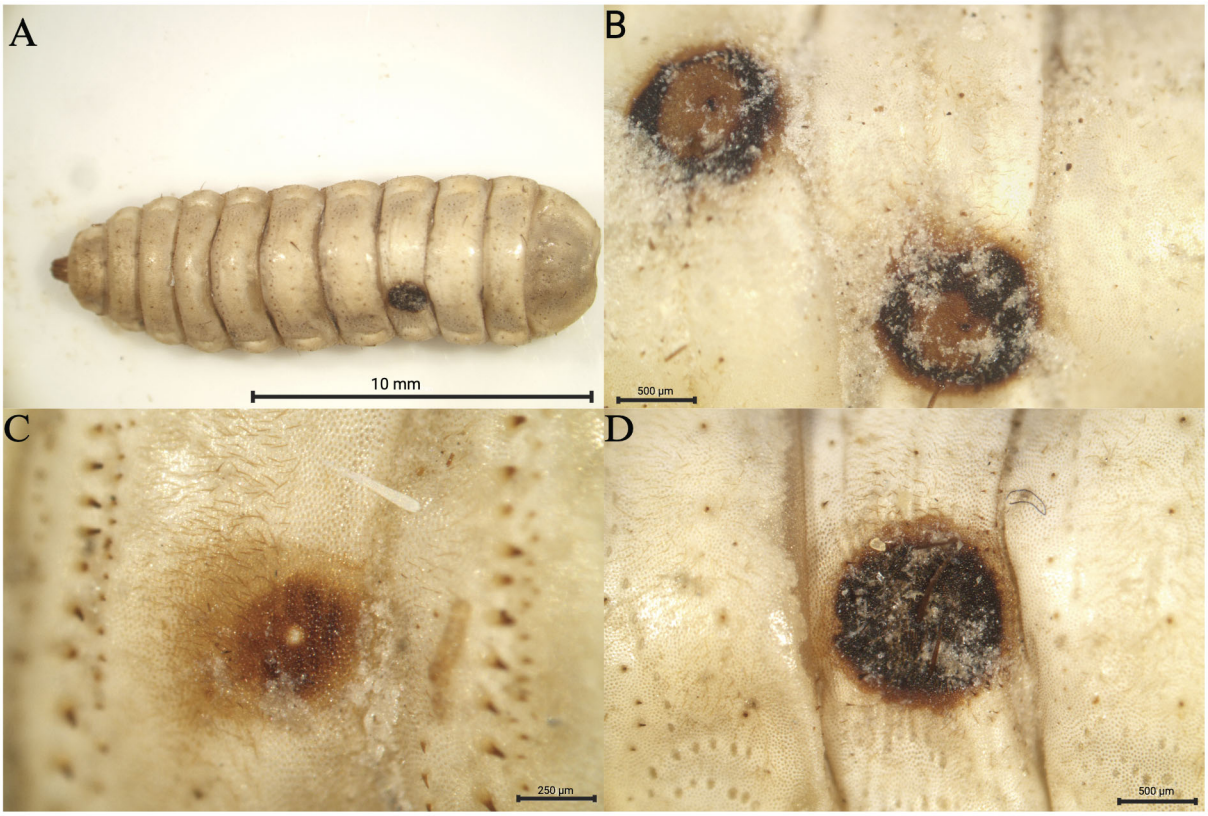

FIGURE 4 BSF reared in the 100 mM FAC diet. Image captured using an Olympus SZX12 microscope equipped with a Euromex sCMEX-20 camera. The taken pictures focus on the lesions found on the larvae reared on the 100 mM diet. The scale bars in the pictures are 10 mm (A), 500 µm (B), 250 µm (C), and 500 µm (D).

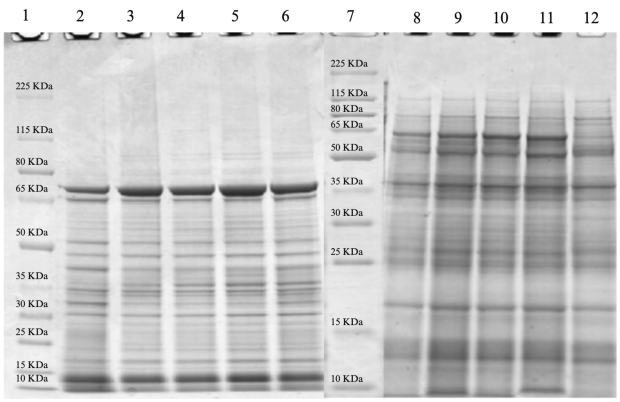

FIGURE 5 Molecular weight distribution of BSF and YM larvae soluble fraction by SDS page under reducing conditions. Lane 1: Marker (10-225 kDa); Lane 2: BSF larvae control; Lane 3: BSF larvae 10 mM; Lane 4: BSF larvae 25 mM; Lane 5: BSF larvae 50 mM; Lane 6: BSF larvae 100 mM; Lane 7: Marker (10-225 kDa); Lane 8: YM larvae control; Lane 9: YM larvae 1 mM; Lane 10: YM larvae 2.5 mM; Lane 11: YM larvae 5 mM; Lane 12: YM larvae 10 mM.

nants of larval macronutrient composition, with iron playing a negligible role.

Ash content of YM larvae did not differ significantly between the different FAC diets, with 3.4% DM on the 1 mM diet being the lowest and 3.8% DM on the 5 mM FAC diet being the highest. These ash concentrations are aligned with previously reported YM larvae inorganic content ranging from 1% DM to 5% DM. An increase of dietary iron concentration from FAC in this study did not significantly alter the ash content of YM larvae. It is suggested that the relatively low proportion of iron, comprising less than 0.5% DM of the total larval mineral composition (Costa *et al.*, 2020), is insufficient to significantly affect the total ash content.

The ash content of the BSF larvae was highest on the 100 mM FAC diet, at 11.5% DM, was significantly higher compared to all other diets. The ash content reported in this study falls within the previously reported range for BSF larvae, which varies widely from 3% DM to 17% DM depending on factors such as strain, diet and rearing conditions (Boafo *et al.*, 2023; Weko *et al.*, 2023). Unlike protein and fat content, ash is not reported to be dose-dependent on any specific macronutrient (Boafo *et al.*, 2023). Similar to YM larvae, the total iron content in BSF larvae ranks it among microminerals, comprising less than 0.5% of the total mineral content (Chia *et al.*, 2020). Despite this, an increase in ash content by 1.3% DM was observed. Given the small proportion of iron in

the total mineral composition of the larvae, it is unlikely that increased iron alone could account for the rise in ash content. Instead, the data suggest that the elevated dietary iron in the BSF substrate may have facilitated the accumulation of other minerals, notably calcium, which contributed to the observed increase in ash content, as mentioned below.

Trace minerals

Iron

A significant dose-dependent positive correlation between iron concentration in the substrate and YM larval iron content was found (Table 3). The YM larvae reared on diets containing up to 5 mM FAC have shown a significant increase of just under 20% in iron content (50.33 to 58.66 mg/kg) without compromising larval growth or survival. Oppositely, a negative dose-dependent trend was observed between the LM substrate iron and the iron BAF of the larvae. Iron bioaccumulation was significantly higher in the control group (0.18) compared to all other treatments, while the 10 mM diet (0.11) showed significantly lower bioaccumulation than all other treatments (Table 3, Figure 2). These results suggest that although iron levels in YM larvae can be increased through dietary manipulation, the extent of accumulation is limited due to the low conversion rate.

A significant dose-dependent increase in iron content was also observed in BSF larvae with increasing concentrations of FAC in their substrate (Table 3). At the highest dosage (100 mM), the iron content in the larvae nearly tripled, from 372 mg/kg DM to 999 mg/kg DM, while larval size and survival remained similar (Table 3, Figure 3). These results are consistent with the study by Seyedalmoosavi *et al.* (2023), who explored rearing BSF larvae on substrates mixed with various fertilizers to simulate sewage waste and resulting in iron content of the substrates between 337 and 5,852 mg/kg DM. However, a decrease in iron BAF was observed, with the highest value in the control diet (1.15). Bioaccumulation then plateaued at 25 mM (0.27), showing similar values in the 50 mM and 100 mM treatments. The lack of significant differences among these three highest iron treatments highlights the BSF larvae's capacity to accumulate iron even at very high substrate iron concentrations. The consistent BAF values across diets with more than 25 mM FAC and above may indicate that iron absorption at high concentrations occurs via a passive paracellular pathway, driven by a density gradient. Unlike protein-regulated gut absorption, paracellular transport involves the passive movement of water and charged molecules through tight junctions between epithelial cells (Hollander and Kaunitz, 2020). This phenomenon has not been investigated in edible insects regarding iron and warrants further study. In the work of In Seyedalmoosavi *et al.* (2023), larvae reared on substrates with varying iron content showed lower larval iron levels (150-557 mg/kg DM), compared to the current findings (372-999 mg/kg DM). Notably, larvae reared on a substrate mimicking sewage containing 337 mg iron/kg DM, accumulated less than half the iron (150 mg/kg DM) compared to larvae reared on a sewage mimic with 351 mg iron/kg DM, which accumulated 339 mg/kg DM. The latter treatment is comparable to the iron BAF found in the control diet (1.15). Additionally, larvae reared on a sewage mimic substrate with 5,852 mg iron/kg DM resulted in a larval iron content of 557 mg/kg DM, leading to a lower iron BAF when compared to the 100 mM treatment (0.15). Thus, the strong correlation observed in the current study between substrate iron and larval iron content and the higher iron BAF (Table 3), as opposed to the findings of Seyedalmoosavi *et al.* (2023), may be attributed to differences in iron accessibility in the substrates. Missirlis *et al.* (2006) demonstrated that FAC inclusion in the substrate significantly increases iron levels in *Drosophila melanogaster*, another fly species, suggesting that FAC can act as a bioavailable iron source for BSF larvae as well. Thus, it is suggested that the FAC iron is more bioavailable compared to the iron in fertilizer mimics, which may be less soluble and more prone to interactions with anti-nutritional factors, making it less accessible to the larvae. Pre-processing methods might improve the bioavailability of iron in waste materials for BSF larvae, as suggested by this comparison.

Zinc

Increasing the dietary iron in both the BSF and YM substrate decreased the zinc concentration of the larvae, from 128 (Control) to 117 (10 mM) mg/kg in the YM larvae and from 183 (Control) to 125 mg/kg for the BSF larvae (100 mM; Table 3). The dose-depended relationship between iron intake to zinc levels is not studied in insects but is well known amongst other organisms. Iron is a known antinutritional factor of zinc in mammals (Meadows *et al.*, 1983). For instance, piglets exposed to excessive dietary iron have a dose-dependent zinc decrease (Middleton *et al.*, 2021). This might be as iron and zinc share similar transporters and receptors, such as the ZIP protein family and DMT1 (Hansen *et al.*, 2009; Cousins *et al.*, 2006). Although this relationship in insects has not been investigated, ZIP proteins were identified in insects to deliver both iron

TABLE 3 Trace mineral composition (W/W dry matter) of YM and BSF reared on diets with different iron (FAC) concentrations. Each value is compared between the iron treatments within each of the two insect species

Yellow mealworm						
Diet	Control	1.0 mM	2.5 mM	5.0 mM	10.0 mM	Pearson correlation coefficiency (r)
Substrate iron content (mg/kg)	267	293	350	407	563	
Bioaccumulation factor (BAF) of iron	0.18^{a}	0.18^{a}	0.15^{b}	0.14^{b}	0.11^{c}	
Iron* (mg/kg DM)	$50.3^{a} \pm 2.0$	$55.3^{bc} \pm 0.5$	$54.0^{ab} \pm 0.0$	$58.6^{c} \pm 2.3$	$64.0^{d} \pm 1.0$	0.91^{xx}
Zinc* (mg/kg DM)	$128.4^{a} \pm 1.5$	$126.6^{ab} \pm 3.3$	$117.5^{c} \pm 2.6$	$120.3^{bc} \pm 3.2$	$117.3^{c} \pm 2.6$	-0.70^{x}
Calcium (g/kg DM)	1.1 ± 0.0	1.1 ± 0.0	1.1 ± 0.1	1.2 ± 0.2	1.1 ± 0.1	−0.10
Potassium (g/kg DM)	8.6 ± 0.3	8.6 ± 0.1	8.3 ± 0.2	8.7 ± 0.1	8.4 ± 0.2	−0.22
Magnesium (g/kg DM)	2.0 ± 0.1	2.0 ± 0.0	1.9 ± 0.1	2.0 ± 0.0	1.9 ± 0.0	−0.45
Phosphorus (g/kg DM)	7.0 ± 0.3	7.1 ± 0.1	6.8 ± 0.2	7.2 ± 0.2	6.8 ± 0.2	−0.28
Black soldier fly						
Diet	Control	10.0 mM	25.0 mM	50.0 mM	100.0 mM	Pearson correlation coefficiency (r)
Substrate iron content (mg/kg)	323	943	2,300	2,740	6,637	
Bioaccumulation factor (BAF) of iron	1.15^{a}	0.53^{b}	0.27^{c}	0.21^{c}	0.15^{c}	
Iron* (mg/kg DM)	$372.6^{a} \pm 31.2$	$505.3^{ab} \pm 39.1$	$626.6^{bc} \pm 74.8$	$791.3^{c} \pm 12.5$	$999.3^{d} \pm 125.1$	0.95^{xx}
Zinc* (mg/kg DM)	$183.8^{a} \pm 15.0$	$154.5^{b} \pm 12.1$	$136.3^{bc} \pm 10.1$	$125.8^{c} \pm 2.9$	$125.6^{c} \pm 9.0$	-0.75^{x}
Calcium* (g/kg DM)	$26.5^{a} \pm 1.5$	$27.0^{a} \pm 0.53$	$28.1^{ab} \pm 0.5$	$30.1^{bc} \pm 1.3$	$31.9^{c} \pm 0.5$	0.91^{xx}
Potassium (g/kg DM)	11.5 ± 0.2	11.5 ± 0.1	11.2 ± 0.4	10.9 ± 0.3	10.9 ± 0.4	−0.67
Magnesium (g/kg DM)	2.5 ± 0.1	2.5 ± 0.0	2.5 ± 0.03	2.5 ± 0.0	2.5 ± 0.1	0.15
Phosphorus (g/kg DM)	7.6 ± 0.5	7.7 ± 0.2	7.6 ± 0.2	7.8 ± 0.2	7.8 ± 0.5	0.18

* Different letters indicate significant differences ($P < 0.05$). Mean groups lacking common letters are not significantly different ($P > 0.05$). The Pearson correlation coefficient (r) between the mineral content and the iron in the substrate is defined as follows: ^{x} indicates a moderate correlation ($0.7 \leq r < 0.9$ for moderate positive correlation or $-0.9 < r \leq -0.7$ for moderate negative correlation), and ^{xx} indicates a strong correlation ($r \geq 0.9$ for strong positive correlation or $r \leq -0.9$ for strong negative correlation).

and zinc, and the transporter DMT1 is known to interact with both metals in insects (Bettedi *et al.*, 2011; Missirlis, 2021). Therefore, it is possible that excessive dietary iron inhibits zinc absorption due to competition for both DMT1 and ZIP proteins capacity in BSF and YM.

Calcium

Calcium levels were similar across all diets for the YM (from 1.1 to 1.2 g/kg). In mammals calcium is known to decrease iron absorption due to noncompetitive inhibition of the DMT1 transporter (Thompson *et al.*, 2010). The calcium absorption pathway in insects begins primarily in the midgut, where calcium uptake is driven by a calcium concentration gradient (Taylor, 1985). Although the specific mechanisms of calcium absorption in the insect midgut remain underexplored, evidence suggests that this process may involve passive and active transport pathways. A secondary reabsorption of calcium occurs in the Malpighian tubules, facilitated by calcium-specific channels such as Dmca1A and Dmca1D (Browne and O'Donnell, 2018; Dube *et al.*, 2000). This reliance on specific transporters and calcium concentration might explain the minimal effect on calcium absorption observed in the diets provided, despite the competition on the DMT1 pathway.

Interestingly, a strong correlation was found between substrate iron content to BSF larvae calcium content (from 26.5 to 31.9 g/kg; Table 3) when dietary iron was increased. Notably, this approximate 5.4 g/kg increase in calcium partially explains the 1% significant increase in the total insect ash content (Tables 2 and 3). There is a gap in literature regarding how increased iron intake affects calcium levels in arthropods. However, calcium has been shown to inhibit iron absorption in humans (Lönnerdal, 2010). Understanding this phenomenon requires further research.

Phosphorus

No significant differences in phosphorus content were observed within the larvae of the two insect species across the iron treatments. The phosphorus content ranged from 6.8 to 7.2 g/kg in the YM larvae and from 7.6 to 7.8 g/kg in the BSF larvae (Table 3). Ferric citrate hydrate is a medicine used for patients suffering from chronic kidney disease to lower phosphorus serum levels (*Iida et al.*, 2013). Ferric iron binds to phosphorus ions, making it insoluble, lowering absorption. Similarly, the free iron in FAC could interact with phosphorus. This potential interaction did not affect YM and BSF larvae phosphorus levels.

Potassium

The most abundant mineral in the larval body, potassium, was not affected by dietary iron concentrations. The potassium content ranged from 8.3 to 8.7 g/kg in the YM larvae and from 10.9 to 11.5 g/kg in the BSF larvae (Table 3). The absorption pathways of potassium occur primarily via a concentration gradient through Na+/K+-ATPase pumps, a conserved mechanism common in all animals. This pathway has minimal connection to the DMT1 and ZIP transporters involved in iron absorption found in insects (Gorman, 2023; Terra and Ferreira, 2020; Thabet *et al.*, 2016).

Magnesium

The FAC diets did not affect magnesium content in the larvae of either insect species. The magnesium content ranged from 1.9 to 2 g/kg in the YM larvae and 2.5 g/kg in the BSF larvae (Table 3). Iron has not been documented to affect magnesium absorption. Moreover, magnesium increases nonheme iron absorption in humans (Neda *et al.*, 2022).

4 Conclusion

This study investigated the effects of dietary iron on the growth, survival, and nutritional composition of larvae of the yellow mealworm and the black soldier fly.

Survival at high iron concentrations (10 mM) decreased YM survival, indicating sensitivity to iron overload. In contrast, BSF larvae demonstrated resilience across the tested iron levels. Nutritional analysis revealed a dose-dependent increase in iron content in both species, with BSF larvae showing a significant rise from 372 to 999 mg/kg. This increase correlated with a decrease in zinc content in both species of larvae, suggesting competition for absorption. An unexpected rise in calcium levels was observed in BSF larvae across the diets. At the highest iron concentration (100 mM), stress-induced black lesions appeared, suggesting structural modifications of the exoskeleton related to the observed increase in calcium, a crucial mineral for exoskeletal development. Characterization of these lesions and their source could enable the use of BSF larvae as a detection tool for extreme iron conditions in waste streams, warranting further research.

Ferric ammonium citrate (FAC), a commercial iron supplement, was used in this study due to its previous success in enhancing iron content and increasing entoferritin detection in insects. Iron offered as ferric ammonium citrate is likely more bioavailable than naturally occurring iron in substrates. Future research should identify substrate processing methods to enhance the bioavailability of naturally occurring iron in substrates, to further improve the larvae's nutritional profile. The switch to an FAC-free diet in the final days of rearing further supports the effective assimilation of iron by the larvae, as iron was likely retained within the larvae. This suggests an enhancement in the larvae's iron content and potentially improved bioavailability, if bound to iron binding proteins. This still requires further identification of protein expression and concentration.

These findings underscore the potential of dietary manipulation to enhance the nutritional profile of BSF larvae, positioning them as a promising sustainable source of iron and calcium for food and feed applications.

Acknowledgements

The authors would like to thank Protix (Bergen op Zoom, the Netherlands) and InsectenKwekerij van de Ven V.O.F (Deurne, the Netherlands) for providing the insect materials essential for this experiment. Special thanks are extended to Machiel Stam, Senna Smit, Jesper Tros, and Stephan Ruiters for their assistance with the larvae counting. The authors would also like to express their gratitude to Dr. Hans Smid for assisting in documenting the BSF lesions and to MFA Rona David for helping with the layout and figure design.

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