Science of the Total Environment 831 (2022) 154840

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Short Communication

Is there any biological insight (or respite) for insects exposed to plastics? Measuring the impact on an insects central carbon metabolism when exposed to a plastic feed substrate

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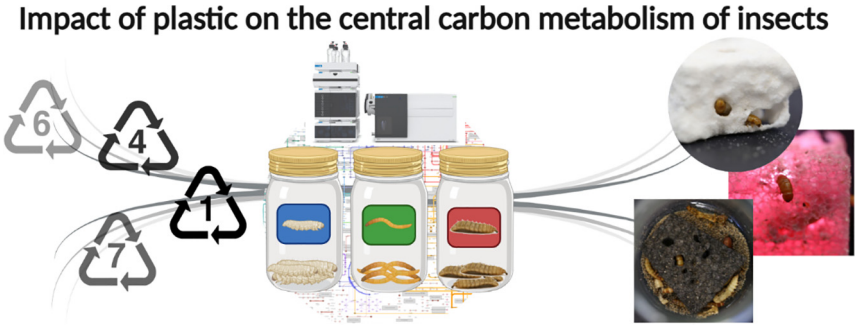
HIGHLIGHTS

- Black soldier fly (BSF) larva had a positive pyrimidine metabolism interaction with PET.
- Alternative purine metabolism pathway was expressed in BSF for non-PET plastics.
- BSF exhibited a generalised gut symbiont breakdown when reared on plastics.
- Mealworm (MW) and Wax moth (WM) were metabolically active on PLA and expanded foams.
- WM exhibited an increased gut symbiont Vitamin B6 metabolism on supplemented plastic.

GRAPHICAL ABSTRACT



ARTICLE INFO

Editor: Rafael Mateo Soria

Keywords:
Metabolomics
Biotransformation
Plastic
Biological impacts
Plastic metabolism

ABSTRACT

Insects used to treat organic waste streams and produce valuable protein products are increasingly exposed to plastic contaminated source material assimilating plastic carbon into organic biomass, which is pervasive and hazardous to organisms. Our understanding of this increased insect-plastic interaction remains limited and needs urgent scientific attention if plastic biodegradation and production rates of quality protein are to be improved. Herein, we investigated the biochemical impact of various plastics using three insect models. Black Soldier Fly (BSF), Mealworm (MW), and Wax Moth (WM) larva were each exposed to a plastic substrate (PET, PE, PS, Expanded PE, PP, and PLA) as the primary carbon source for five days to explore any positive metabolic benefits in terms of insect performance and plastic degradation potential. Central carbon metabolism (CCM) metabolites were analyzed via a targeted tMRM liquid chromatography triple quadrupole mass spectrometry (LC-QqQ-MS) method. Unique expressed pathways were observed for each insect model. When reared on PET, BSF larvae were found to have an elevated pyrimidine metabolism, while the purine metabolism pathway was strongly expressed on other plastics. BSF also exhibited a downregulated Vitamin B6 metabolism across all plastics, indicating a likely gut-symbiont breakdown. The MW and WM model insects were metabolically more active on PLA and expanded foam plastics. Further, WM exhibited an elevation in Vitamin B6 metabolism. This data suggests a positive insect-specific interaction towards certain plastic types that warrants further

Abbreviations: BSF, Black Soldier Fly; MW, Mealworm; WM, Wax Moth; PET, Polyethylene terephthalate; PE, Polyethylene; PS, Polystyrene; PP, Polypropylene; PLA, Polylactic Acid.

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http://dx.doi.org/10.1016/j.scitotenv.2022.154840
Received 14 January 2022; Received in revised form 17 March 2022; Accepted 22 March 2022
Available online 31 March 2022

investigation. It is anticipated that through deeper insight into the metabolic impact and benefits afforded from certain plastics, an insect biotransformation pipeline can be established that links fit-for-purpose insect models to individual plastic types that address our growing plastic waste issue.

1. Introduction

Global plastic production and its waste accumulation are exceeding recycling and biodegradation rates (Lebreton and Andrady, 2019). Characteristic plastics have various degrees of biodegradability; however, all plastic is considered a source of carbon (fossil fuel derived) and is continuously degraded and comminuted into smaller particles over time (Zhu, 2021). This accumulation of plastic carbon particles in the environment is pervasive and poses a hazard to organisms (Allen et al., 2020; Lim, 2021). Further, plastic particles cause numerous downstream organic waste processing problems, where the end product is often intended as compost (Kawecki et al., 2021), feedstock (Tonini et al., 2021), or producing insects used for food and animal feed (Braun et al., 2021). It is here that the assimilation of plastic carbon into insect biomass can occur, among other transportation pathways (Lusher et al., 2017; Taipale et al., 2019).

The ability of some insects to consume different waste substrates (including plastics) and effectively convert plastic carbon into viable and valuable protein is a specific focus being explored by researchers globally, not only as a means of addressing growing global plastic waste reserves but also as a source of nutrition (Ites et al., 2020; Mertenat et al., 2019; Salomone et al., 2017; Scala et al., 2020; van Huis, 2020; Wynants et al., 2019). Typically, plastic waste occurs as a physical and chemical contaminant, which pervades all environments, making it inevitable that invertebrates will come into physical contact with plastic carbon and most likely ingest it (Oliveira et al., 2019). Waste management systems that apply insects to process organic waste (i.e., supermarket waste etc.) require paper and plastic packaging to be removed during the de-packaging phase of treatment (Ites et al., 2020). Their introduction into the organic waste stream is viewed to negatively impact nutrient availability, which plays a significant role in insect phenology, development, and behaviour (Chapman, 1998). Moreover, such contaminants can negatively impact organic waste conversion efficiencies and process retention times (e.g., process throughput and quality of end products). Therefore, confirming how insects respond to different plastic sources and their fate if consumed will be important knowledge to obtain if insects are to be considered a legitimate source of protein to supplement or replace traditional protein sources (van Huis, 2020; van Huis et al., 2021). Furthermore, identifying which insects are equipped and capable of biotransforming specific plastic types will help to develop fit-for-purpose insect biotransformers matched to beneficial plastic carbon substrates. One avenue to explore this interaction is via metabolomics, specifically through the analysis of central carbon metabolism (CCM) metabolites.

The CCM metabolites comprise a suite of compounds within the most basic cellular pathways present in all living organisms (Bar-Even et al., 2012). CCM facilitates numerous biosynthetic and metabolic pathways in response to nutrient availability and helps to direct available nutrients for various physiological requirements (Matsuda et al., 2017). The profile of CCM metabolites can therefore provide important information on how metabolic processes are being activated, and the deviation from normal processes during health, disease, and environmental perturbation (Beale et al., 2021; Beale et al., 2022; Shah et al., 2019; Shah et al., 2021; Siegel et al., 2014). In addition, previous studies have shown that CCM is involved in a myriad of pathways connected to nutrient availability and show how organisms, such as insects, can divert or partition functions to compensate or maintain essential metabolic services in response to nutrient-limited environments or changes to nutrient availability (Ankrah et al., 2020). This is particularly pertinent when exploring the biochemical perturbations caused by plastics and affords the analysis of CCM as an effective tool to explore the interplay between plastic as a resource and insect metabolic processes. Here we used targeted CCM metabolomics to measure the larvae response of three different insect species when exposed to different classes of plastic during larval development. The three insects under study were: Black Soldier Fly (BSF), Mealworm (MW) and Greater Wax Moth (WM).

The three insect candidates, one from each of three orders *Diptera*, *Lepidoptera* and *Coleoptera*, are well studied; and MW and WM have been shown to degrade different plastics (Billen et al., 2020; Yang et al., 2021a; Yang et al., 2021b). Depending on the species, insects are more effective and efficient at food conversion to protein, which is suitable for livestock feed and food, requiring less space, water, and energy. As such, insects are increasingly recognised as sustainable mini livestock (van Huis, 2020). Further, the candidates here have relatively short lifecycles, fast growth rates and are easy to rear on a range of organic food stuffs that make them suitable for large scale production. BSF larvae are cosmopolitan and applied to upcycle many organic substrates. Compared to WM and MW, BSF lack well developed mandibles and are less likely to chew plastic and make an interesting contrast that focuses degradation potential on enzymes (Bruno et al., 2020). WM consumes large amounts of bee wax and may give them an advantage to metabolize substances such as plastics (Kong et al., 2019). Bee wax contains a range of chemical compounds including alkanes and alkenes (Hepburn et al., 2014). Mealworm (MW) is the most investigated larval model in plastic biodegradation (Pivato et al., 2022), and it has been proposed as a model in micro- and nanoplastic toxicology (Sanchez-Hernandez, 2021). Furthermore, the frass of MW and WM has a high potential in sustainable agriculture as an organic fertilizer (Houben et al., 2020; Watson et al., 2021), thus increasing the interest of mealworms in multiple environmental services.

Further, the CCM data will provide insight to which insect have a positive metabolic response to various plastics and direct future research to how insects can be used as a plastic waste biotransformer.

2. Material and methods

2.1. Model insects

Hermetia illucens, BSF, were sourced from Goterra® (Canberra, ACT, Australia). BSF was reared for four generations at the CSIRO Insect Laboratory (Ecosciences Precinct, Dutton Park, Brisbane, Australia) in a constant environment room (CER) set at a 14:10 h Light-Dark (LD) cycle, with a 60% relative humidity (RH) at 26 °C. BSF eggs hatched, and neonates reared on a mix of 50% Barastoc golden yolk® poultry pellets (Melbourne, VIC, Australia) and wheat bran (supermarket sourced) that was mixed with deionised distilled (DI) water. Four-day old larvae were then transferred to a diet of commercially sourced mixed vegetables and milk powder. *Galleria mellonella*, WM was procured from the Queensland Department of Agriculture and Fisheries (DAF) colony which had been in culture for >30 plus generations and then reared for three generations at the CSIRO Insect Laboratory in a CER set at 14:10 LD 50% RH 26 °C. WM was reared in one-litre glass jars and fed a mix of commercially sourced yeast, honey, glycerol and Farex® baby cereal, the same diet used by DAF. *Tenebrio molitor*, MW, were purchased from Pisces® commercial pet food supplier (Brisbane, Australia) then reared for two generations at the CSIRO Insect Laboratory in a CER set at 14:10 LD 50% RH 26 °C. MW was reared in open stainless-steel dishes and fed on wheat bran, fresh carrot, and sweet potato.

2.2. Artificial feeding of plastic

The impact of different common plastics on insect metabolism was evaluated in a controlled plastic-insect exposure study (Fig. 1). Larvae for each insect was exposed to plastic alone or plastic supplemented (coated) with a supplement, namely molasses. Supplemented plastic was dipped in molasses:water (50:50, v/v) and air-dried overnight which left a film over the

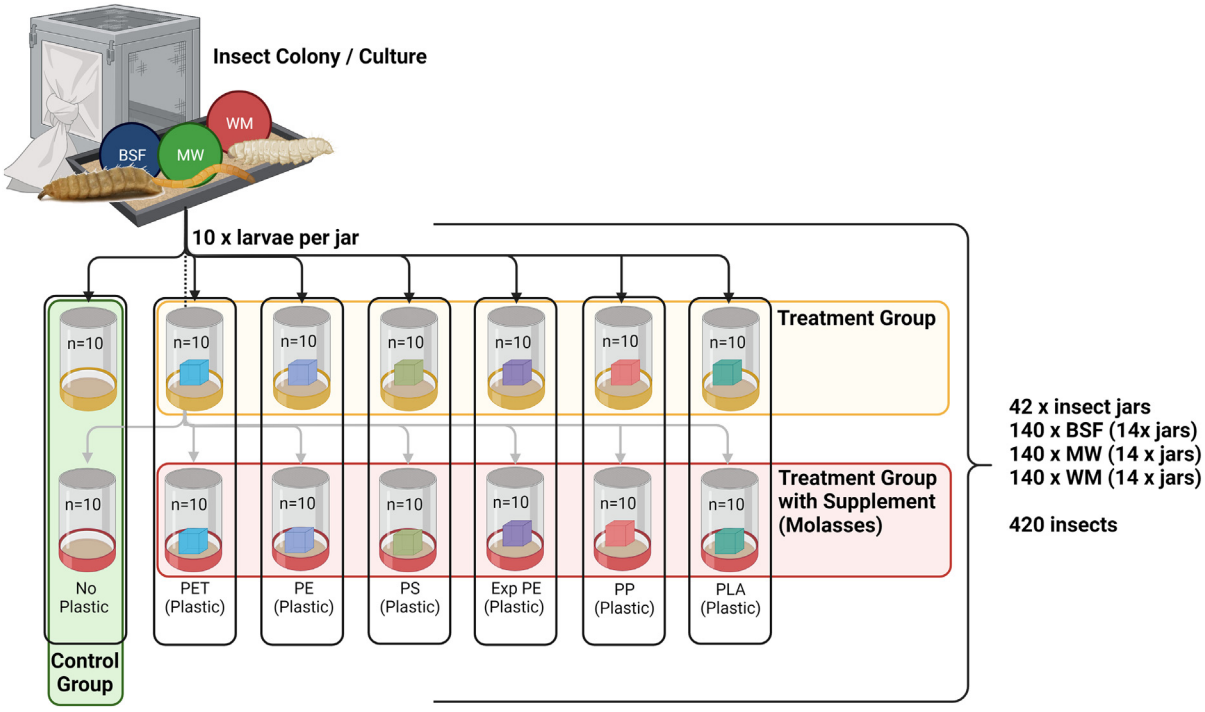

Fig. 1. Graphical overview of the experimental design. Note, BSF, MW and WM are defined as Black Soldier Fly, Mealworm and Wax Moth, respectively. PET, PE, PS, PP and PLA are defined as polyethylene terephthalate, polyethylene, polystyrene, polypropylene and polylactic acid, respectively. Exp PE is defined as expanded foam polyethylene.

plastic surface which was not thick or viscous and did not impede insect behaviour or movement.

Briefly, a series of 14 glass jars (150 mL) were allocated per insect, of which seven glass jars were set aside for supplemented treatments. For each series of glass jars, one contained control diet and no plastic, and six were seeded with small blocks of the various plastics under assessment and contained no diet. The control (plastic-free) groups were fed 5 mL of diet as described above. This was replicated for each insect model (see Fig. 1).

Ten larvae of each species were then placed into individual glass jars that were barcoded for sample tracking. A single piece of weighed plastic was added to each treatment glass jar. Table 1 gives the plastic used for the treatment groups as defined by the Plastics Identification Code of Practice from Plastics and Chemicals Industries Association Australia (PACIA) (Generation and Weickhardt, 2006). To distinguish the two different forms of PE assessed, the PE used in glass jar 5 was denoted as expanded (Exp) PE. For the supplemented treatments, the weighed plastic pieces were submerged in a solution of 50% molasses in deionised (DI) water. The plastic was air-dried overnight before placing it in the jars and adding the larvae. The supplemented control comprised 2.5 mL of molasses solution added to 5 mL of diet.

The introduction of molasses was to promote interaction with the plastic blocks seeded in the glass jars since it presents a ready source of metabolizable energy. Molasses is composed of 40–60% sugars (sucrose, glucose and fructose), 2–5% small organic acids, in up to 25% water (Clarke, 2003).

The age of the larvae for each of the three species exposed to plastics was six days, four and seven weeks for BSF, WM and MW, respectively. The larvae were secured in the jar using a metal screw top lid. A 40 mm diameter hole was cut from the centre of each lid and aluminium insect mesh was placed inside of the lid to cover the hole. Fine nylon 1 mm^2 gauze was placed between the metal lid and metal mesh to stop larvae from escaping. Every second day, each jar received a fine mist of DI water and dead larvae were removed. All insects were exposed to plastic in each jar for approximately 116 ± 6 h (5 days). The age of the larvae and the duration of exposure to plastic were based on when each insect was most actively feeding. For example, 5 days represent ~30% of larvae development time for BSF.

After exposure, individual larvae were removed, tripled washed in MilliQ water, blotted on a paper towel, and placed individually in a 5 mL barcoded pre-weighted cryotube. Each tube with larvae was then reweighed, placed into dry ice before storing at −80 °C for CCM metabolomics analysis.

Table 1
Summary of plastic treatments.

Jar	PACIA*	Description
1, 8	No Plastic	5 mL of diet media specific to each insect.
2, 9	PET	Fine PET fiber polyester upholstery fill.
3, 10	PE	Pink-coloured expanded low-density polyethylene foam block.
4, 11	PS	Poly-foam Australia® fish box.
5, 12	(Exp) PE	Expanded polyethylene foam sheet.
6, 13	PP	Polypropylene Tera cell.
7, 14	PLA	3D printed porous polylactic acid blocks (Qty 2; 8 × 8 × 8 mm).

Note, the asterisks (*) denotes plastics as defined by the Plastics Identification Code of Practice from Plastics and Chemicals Industries Association Australia (PACIA). All plastics were characterised by CSIRO (Clayton, VIC).

2.3. Plastic loss measurement

At the end of the exposure period, jar remnant material (i.e., any remaining plastic, broken off plastics, frass, and silk) were processed and analyzed for plastic loss. The jar remnant was immersed in 1 M HCl:ethanol (2:1, v/v) solution, mixed, and incubated at room temperature for 3 days, with periodic manual stirring. The solution was then filtered through a vacuum manifold fitted with a 0.45 μm PVDF filter membrane (47 mm diameter). The filtered liquid was then subjected to thermal gravimetric analysis (TGA).

Unfiltered plastic pieces were rinsed with DI water and dried at 60 °C before being weighed. Small jar materials and insect frass were subjected to Fenton digestion (Koppenol, 1993) to remove biological organic material. The undigested solid was filtered onto PDVF filter through abovementioned method and applied for TGA-based measurement. The filter membrane containing post-filtration solid material was loaded to alumina crucibles. The samples were then analyzed on a Mettler Toledo TGA2

instrument (Mettler-Toledo Ltd., Port Melbourne, VIC, Australia) under a 50 mL min^{-1} nitrogen flow. The heating program comprised of three stages: 10 °C min^{-1} from 25 to 150 °C to dry out all residual moisture and low molecular weight organic compounds, 5 °C min^{-1} from 150 to 550 °C to detect any plastic decomposition, and finally 25 °C min^{-1} from 550 to 800 °C to clean up any organic materials. Data were analyzed with Mettler Toledo STARe Evaluation software. The derivative thermogravimetric (DTG) curve was calculated from the mass evolution data. The peak at the same decomposition temperature as the reference plastic was integrated and corrected to obtain the mass loss amount.

2.4. Central carbon metabolism (CCM)

CCM metabolites were extracted from freeze-dried whole insect larvae using a modified extraction method (Gyawali et al., 2021). Briefly, 20 mg of freeze-dried biomass was reconstituted with 100 μL MilliQ Water and combined with 450 μL of ice-cold (−20 °C) methanol:ethanol (50% v/v; LiChrosolv®, Merck, Darmstadt, Germany) spiked with 1 ppm Succinic Acid $^{13}C_2$ (Cambridge Isotope Laboratories, Inc., Tewksbury, MA, USA) and vortexed for 2 min. The samples were centrifuged (Centrifuge 5430R, Eppendorf, Hamburg, Germany) at 14,000 *g* at 4 °C for 5 min. The supernatant was transferred and filtered using a positive pressure manifold (Agilent PPM48 Processor, Agilent Technologies, Santa Clara, CA) with Captiva EMR cartridges (40 mg, 1 mL; Agilent Technologies, Australia) to remove the lipid fraction. The cartridges were then washed with two 200 μL aliquots of MilliQ water:methanol:ethanol (2:1:1, v/v/v). The combined filtered supernatant and cartridge washes, representing the insect polar CCM metabolite fraction, were combined in a 1.5 mL high recovery vial (30 μL reservoir, silanized glass vials, Agilent Technologies, Australia) and dried in a SpeedVAC (10 mBar). The metabolite fraction was reconstituted with 100 μL MilliQ water:methanol (4:1, v/v) spiked with 100 ppb of L-Phenylalanine (1-^{13}C). CCM metabolites were measured on an Agilent Infinity Flex II UHPLC coupled to an Agilent 6470 Triple Quadrupole Mass Spectrometer (QqQ-MS) as previously reported Gyawali et al. (2021) and Sartain (2016). The residual relative standard deviation (RSD) of the internal standards were 3.16% (Succinic Acid, 1,4-$^{13}C_2$) and 1.98% (L-Phenylalanine, 1-^{13}C). Procedural blanks (n = 6) and pooled biological samples (n = 6) were randomly dispersed throughout the sequence.

2.5. Statistical analysis

CCM data were first '*blank*' subtracted, normalised to the spiked internal standard (Succinic Acid $^{13}C_2$) and sample biomass (20 mg). Features with >50% missing values per treatment group were removed, and any remaining missing values were replaced with 1/5 of the minimum positive value for each variable. In total, 171 central carbon metabolism metabolites were measured across all insect samples (Supplementary Figs. S1 and S2 provide as an overview of the model insect control groups). The acquired data were then normalised by the sample median, log_{10}-transformed, and scaled using the mean-centered value and divided by the standard deviation of each variable. Data were then subjected to univariate and multivariate statistical analysis using MetaboAnalyst 5.0 (Pang et al., 2021) and SIMCA 17.01 (Sartorius AG, Goettingen, Germany). Pathway impact analysis and enrichment were performed in MetaboAnalyst 5.0 using the model insect *Drosophila melanogaster* (fruit fly) KEGG database. Significant metabolites were identified using a fold change threshold ≥ 1.5, and a p-value ≤ 0.05; pathway impact is determined from a pathway topology analysis and its the cumulative percentage from the matched metabolite nodes (Pang et al., 2021).

3. Results and discussion

3.1. Effect of exposure to plastic on insect larva weight and survival

There were subtle effects of treatment on larval weights across treatments and insects (Table 2). The mean larval weights (±SD) of surviving larvae across all treatments were 97.0 ± 20.9 mg, 102.4 ± 20.6 mg, and 37.2 ± 13.0 g for BSF, MW, and WM respectively. Mean larval weights were not significantly different between control treatments and plastic treatments regardless of supplementation, apart from BSF on PLA with supplement. This treatment returned a 28.7% reduction in mean larval weight compared to that of the supplemented control treatment (p-value = 0.0231). There was a significant effect of supplementation on WM mean larval weights with supplemented larva exhibiting a 13.2% reduction in weight (p-value = 0.014 unpaired *t*-test; p-value = 0.0075 paired *t*-test).

Treatment did not affect the percentage survival of BSF or MW (Table 2). BSF and MW larva recorded 100 and 97.1% survival respectively. While there was no effect of the plastic treatment on WM survival, there was a significant effect of supplementation (p-value = 0.0022); supplemented MW larva displayed 90.0% survival whereas the rate for unsupplemented WM was 61.4%. While the focus of the work herein was to determine the metabolic effect of exposure of insects to plastic, more work is needed to assess the impact of exposure for longer periods in terms of fitness variables (i.e., ability to complete larvae development, time to complete development, adult size, mating, etc.).

3.2. Effect of exposure to plastic on the central carbon (CC) metabolome on insect larva

3.2.1. Black Soldier Fly

The supervised orthogonal partial least squares-data analysis (OPLS-DA) statistics revealed that BSF fed on different plastic shared a high degree

Table 2
Summary of larval weights (mean ± SD) and the number of surviving larva (n of 10) for each insect and treatment group.

Jar	PACIA*	Supplement	BSF	MW	WM
			Mean larval weight (±SD, mg), n	Mean larval weight (±SD, mg), n	Mean larval weight (±SD, mg), n
1	No Plastic	No	97.63 ± 15.70, (n = 9)	111.00 ± 18.69 (n = 9)	39.15 ± 5.76 (n = 8)
2	PET	No	94.58 ± 25.11, (n = 10)	107.10 ± 22.97 (n = 10)	41.81 ± 15.63 (n = 8)
3	PE	No	93.16 ± 23.01, (n = 10)	102.40 ± 25.47 (n = 10)	35.10 ± 7.15(n = 4)
4	PS	No	84.99 ± 19.28, (n = 10)	101.90 ± 21.62 (n = 10)	39.48 ± 9.88 (n = 5)
5	Exp PE	No	101.70 ± 22.48, (n = 10)	103.10 ± 23.34 (n = 10)	39.94 ± 7.13 (n = 5)
6	PP	No	94.69 ± 12.83, (n = 10)	96.10 ± 19.28 (n = 10)	46.03 ± 10.48 (n = 6)
7	PLA	No	91.05 ± 19.44, (n = 10)	86.88 ± 16.82 (n = 10)	42.01 ± 16.92 (n = 7)
8	No Plastic	Yes	114.00 ± 17.30, (n = 10)	110.30 ± 14.34 (n = 8)	37.04 ± 13.72 (n = 8)
9	PET	Yes	111.60 ± 16.92, (n = 10)	96.05 ± 17.08 (n = 10)	38.34 ± 10.31 (n = 9)
10	PE	Yes	96.73 ± 27.92, (n = 10)	114.10 ± 18.93 (n = 10)	34.40 ± 12.10 (n = 10)
11	PS	Yes	95.35 ± 19.40, (n = 10)	92.78 ± 21.12 (n = 9)	31.00 ± 9.93 (n = 10)
12	Exp PE	Yes	106.90 ± 18.66, (n = 10)	104.50 ± 20.92 (n = 10)	34.58 ± 15.57 (n = 9)
13	PP	Yes	95.10 ± 15.80, (n = 10)	100.70 ± 18.60 (n = 10)	39.60 ± 23.24 (n = 7)
14	PLA	Yes	81.27 ± 19.28, (n = 10)	107.80 ± 20.30 (n = 10)	31.22 ± 12.39 (n = 10)

Note, BSF is defined as Black Soldier Fly; MW is defined as Mealworm; WM is defined as Wax Moth.

of similarity in terms of expressed metabolites after 5 days of exposure (Supplementary Fig. S3A), clustering into 4 groups. Groups consisted: Group 1 (PET); Group 2 (Exp PE); Group 3 (PE, PS, PP, and PLA); and the control cohort (Group 4). The loadings plot is indicative of the metabolites contributing to the separation of the differing CCM towards these groups (Supplementary Fig. S3B); 21 metabolites were differentially expressed (elevated or depleted, FC > 1.5), while 15 were statistically significant (p-value < 0.05; Supplementary Fig. S4).

Pathway enrichment and impact analysis of the 15 significantly differential metabolites (Fig. 2) indicated altered expression in several metabolic pathways involving nucleic acids, amino acids such as alanine, aspartate, glutamate, arginine and proline, and butanoate (via 4-aminobutanoate) and vitamin B6 (via pyridoxine) (Fig. 2). While most of these pathways were found to be upregulated in the PET treatment, vitamin B6 metabolism was observed to be downregulated in all plastic treatments with respect to the control group.

Vitamin B6 is a known co-factor in several other biochemical reactions of which 140 downstream pathways are involved (Parra et al., 2018), including fatty acid synthesis. Depletion of key intermediates such as pyridoxine and 4-pyrridoxate, combined with elevated γ-aminobutyrate (GABA) indicate potential Vitamin B6 and Ala-Asp-Glu metabolic pathways pooling via GABA intermediates and increased fatty acid synthesis (Huang et al., 2016). *N*-acetyl galactosamine (NAcGal) and uridine 5-diphosphate (UDP) are known to be the precursors of nucleotide sugars. These precursors are metabolised by microbial UDP-galactopyranose mutase to form glycophospholipids which are required for microbial adhesion (Lamarre et al., 2009), and antigenic signalling in insects (Ichimiya et al., 2015). Combined with the observations of Gravelat et al. (2013) and elevated GABA, NAcGal and UDP levels, our study suggests that an increased trans-trans-muconic acid, potentially originating from plastic polymers (Seow et al., 2012), which may have aided towards elevated synthesis of UDP-galactopyranose related glycophospholipids by gut microbes. Interestingly, elevated trans-trans-muconic acid and 4-aminobenzoic acid suggested plastic degradation activity (Myhrstad et al., 2020) in the insect gut, as was reported from earthworms (Huerta Lwanga et al., 2018). However, more research on the insect microbiome contribution is needed to ascertain this phenomenon for the insects under study here.

Further, the presence of plastic appeared to cause an oxidative stress in BSF. Elevated homocysteine (glutathione metabolism intermediate) and 4-aminobenzoic acid, combined with a downregulated pyrimidine and upregulated purine metabolism was observed. This suggests a switch from purine metabolism, which is aimed at generating the necessary substrates for basic cellular processes (Pedley and Benkovic, 2017), towards pyrimidine metabolism. The effects of oxidative stress caused by plastics, resulting in upregulated stress response pathways such as glutathione metabolism, combined with perturbed nucleotide metabolism has been shown recently in marine microbiota (Ye et al., 2021a) and Zebrafish microbiota (Dimitriadi et al., 2021). Further, oxidative stress, inflammation, and metabolic alterations due to plastics have been observed in other aquatic and terrestrial invertebrates (Sanchez-Hernandez, 2021), impacting a wide variety of cellular responses and linked to immune response. However, further insect microbiota-based studies are needed to shed more light on this mechanism for the insect models herein with a specific focus on biomarkers of oxidative stress and plastic toxicity.

3.2.2. Mealworm

Unlike BSF, the supplement had a significant influence on the sample clustering among MW. The effect of the supplement was to enhance clustering within treatments and improve discrimination between treatments (data not shown). Thus, further analyses were applied to the metabolomic data from the supplement treatments only.

Three distinct clusters between the various treatments were observed (Supplementary Fig. S5), with discrimination between a grouping formed by the insect metabolomes of the PET, PS, and Exp PE (combined), and the PLA and Control treatment clusters. Of the 33 differentially expressed metabolites across the clusters (FC > 1.5), 24 were observed to be statistically significant. While guanine and L-dihydroorotic acid were downregulated, the remaining 22 metabolites were upregulated (Supplementary Fig. S6).

MW showed upregulated starch and sucrose metabolism, pentose phosphate pathway (PPP), and amino sugar and nucleotide sugar pathway, as demonstrated by the elevated sugar phosphates, nucleotide sugars in addition to *N*-acetyl glucosamine (NAcGlu) and glucosamine 6-phosphate. Furthermore, synthesis of cofactors such as β-nicotinamide adenine nucleotide (β-NAD) and flavin adenine nucleotide (FAD) elevated via elevated purine metabolism intermediates (Fig. 3).

The cofactor accumulation in combination with upregulated PPP and purine metabolism and elevated 3-dehydroshikimate indicate an altered

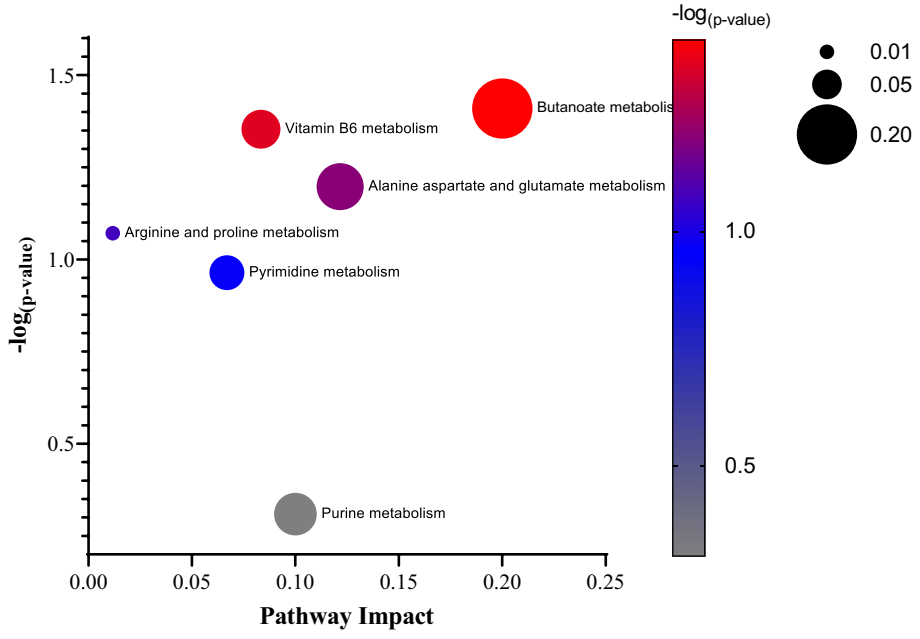

Fig. 2. Pathway impact analysis using significantly differential metabolites from the acquired Black Soldier Fly (BSF) CCM analysis. Bubble size represents the pathway impact score (0–1.0) that accounts for the metabolite centrality and criticality in terms of pathway expression. Bubble colour is according to the -Log_2 of the p-value.

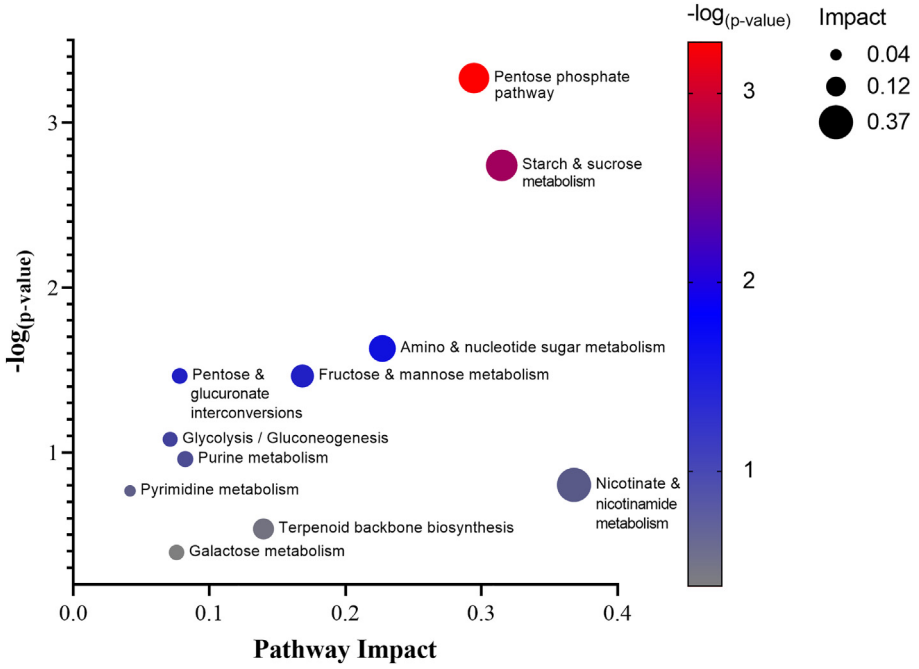

Fig. 3. Pathway impact analysis using significantly differential metabolites from the acquired Mealworm (MW) CCM analysis. Bubble size represents the pathway impact score (0–1.0) that accounts for the metabolite centrality and criticality in terms of pathway expression. Bubble colour is according to the -Log_2 of the p-value.

metabolism due to oxidative stress in the MW gut potentially caused by the plastic. However, in this study, compared to BSF, the oxidative stress appeared to be less severe, as the stress response pathways such as glutathione metabolism in MW were not significantly changed. Additionally, elevated trehalose 6-phosphate, a key intermediate regulatory product and a primary energy source in insects (Iordachescu and Imai, 2008) pointed toward an increased carbohydrate-related metabolism that could be linked to energy expenditure towards plastic degradation. The elevated trehalose 6-phosphate along with other intermediates such as nucleotide sugars and NAcGlu intermediates also indicated antigenic signalling and plastic degradation at a considerably greater magnitude when compared to BSF.

3.2.3. Wax Moth

As with the MW insect model, the supplement was also found to have a significant and similar influence on the WM treatment clustering, i.e., an addition of the supplement improved resolution within and between clusters (data not shown). The metabolomic data of the supplemented treatment show separation between the Control cluster and two groups of

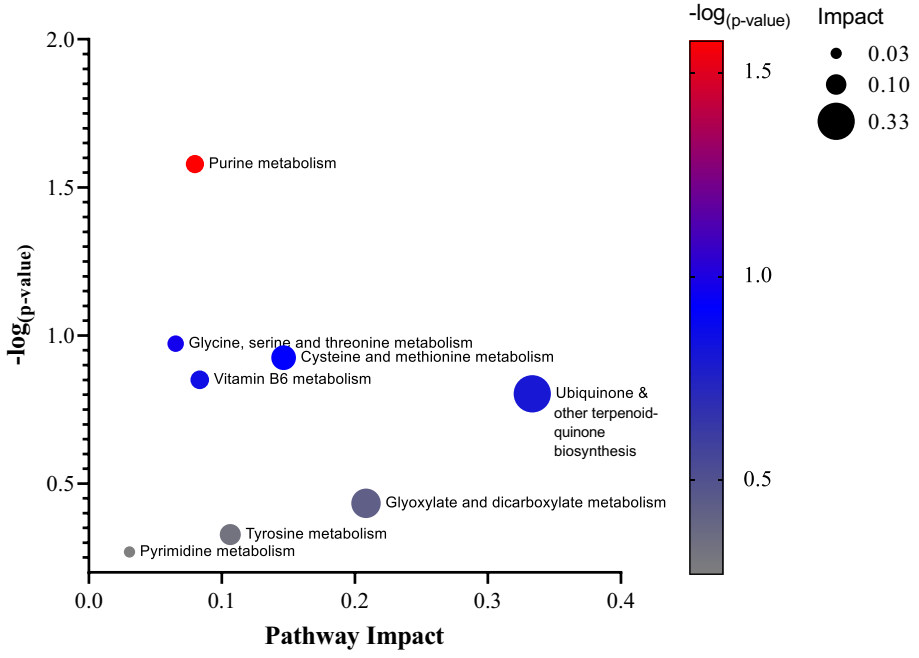

Fig. 4. Pathway impact analysis using significantly differential metabolites from the acquired Wax Moth (WM) CCM analysis. Bubble size represents the pathway impact score (0–1.0) that accounts for the metabolite centrality and criticality in terms of pathway expression. Bubble colour is according to the -Log_2 of the p-value.

clusters from the plastic treatments (Supplementary Fig. S7). Group 1 was comprised of the metabolites strongly expressed because of foam plastic treatments (Exp PE and PET), and Group 2 by the hard plastic treatments (PE, PP, and PLA) (Supplementary Fig. S7). The clusters produced by the PS treatment overlapped groupings 1 and 2 (data not shown), and for simplicity were not included in further analysis. Across the control and the two plastic groupings, 27 metabolites were differential upregulated or downregulated; of which, 19 were statistically significant (Supplementary Fig. S8).

Although the WM larvae expressed several pathways (Fig. 4), tryptophan metabolism and glyoxylate and dicarboxylate metabolism leading to purine metabolism, and oxidative stress response pathways such as cysteine and methionine metabolism, were upregulated. Tyrosine metabolism, which has been implicated in phenotypes associated with reproduction, development, and nymph survival, demonstrating a highly pleiotropic role was also downregulated. More importantly, within the tryptophan metabolism, the elevation of kynurenine intermediates indicates a possible motoric dysfunction due to the neurotoxic and apoptotic effects of these metabolites, as observed in adult flesh flies (Cerstiaens et al., 2003) and ladybird beetles (*Henosepilachna vigintioctopunctata*) (Ze et al., 2021). As such, more research is needed to explore the multigenerational impact of plastic on WM, if supplementation can account for any developmental deficiencies.

3.3. Plastic weight losses post exposure to insect larva

Every attempt was made to measure the plastic material post exposure to insect larva using a common plastic accounting measurement method used for the measurement of microplastics in waste effluents (Ye et al., 2021b). This was done to measure the magnitude of plastic lost during the experiment (post 5 days), if any, per surviving insect larva. As illustrated in Fig. 5, PET, PE, PS, Exp PE and PLA when un-supplemented (in addition to PE supplemented) exposed to BSF larva exhibited varying degrees of plastic loss. However, with the exception to the PE plastics, the reliability of measurement was outside the acceptable thresholds for reporting (i.e., the error was more than 15%). This could be an artefact of the sample preparation protocol reducing the plastic mass and causing consequential losses. In any case, these data are annotated with an asterisk and the magnitude of loss is maintained at −0.5 mg per surviving insect larva to illustrate a loss was observed, although the reporting error indicates this loss is much higher. More work is needed to ensure reliable plastic weights are consistently obtained, prior to and post-exposure. Similarly, unsupplemented WM on PE and (supplemented) PS demonstrated significant plastic loss post-exposure. PET, PLA and supplemented PE and Exp PE were lower in mass but not reliably measured and annotated with an asterisk. PET, PS, and Exp PE exposed to MW for 5 days were measured at a significantly lower mass. These observations anecdotally correlate with the CCM data described above. Yet, it is acknowledged that a more reliable plastic measurement is needed to establish these links in CCM measurements with key physical insect performance metrics (weight and survival rates) coupled with observed plastic measurements.

4. Summary of findings

These data suggest the ability of each insect to perform important functions via alternate pathways when reared on a plastic substrate. The addition of a food supplement, here molasses, ultimately had a net positive impact in terms of energy utilisation for the MW and WM insect models, as demonstrated by an increased survival rate for WM and better sample groupings in the supervised multivariate analysis when supplemented compared to the unsupplemented equivalent. However, the addition of the supplement had no significant difference to the BSF model insects on plastic.

The BSF larvae had a positive metabolic interaction with the PET, a negative/neutral interaction with the expanded Exp PE plastic. Similarly, the MW and WM model insects, particularly WM, performed well in terms of CCM outputs when on PLA and foam-based plastics. This may be a result of the insects' ability to degrade the plastic into small particles and exhibit a positive gut microbiome interaction as a result. The positive CCM correlation with specific plastics types is summarised in Fig. 6 and is indicative of plastic substrates that warrant further investigation using further analytical approaches (such as lipidomics, microbiome compositional analysis, and detailed plastic degradation assessments). These approaches will provide information related to the assimilation and potential conversion of plastics into insect biomass.

4.1. Study limitations and benefits

The current study provides valuable biochemical insight into the impacts plastic has on insect CCM metabolites. However, it is acknowledged that the study is not without limitations. For example, the insect biomass variance within treatments and across treatment groups (both with and without supplement) is a significant limitation of this study. While 10 larvae were used per jar, the weight distribution variance at the end of the experiment varied as much as 25% between individual larvae. It is noted that the Metabolomics Standard Initiative guideline documents for minimum reporting standards for biological samples set a minimum number of biological replicates to three (n = 3) per treatment group (Sumner et al., 2007). While we meet this requirement for the insects harvested from all treatment jars, even when accounting for removed larva, the biochemical changes across the total biomass distribution may bias these results due to the weight variation observed. To overcome this limitation and facilitate a qualitative omics assessment of measurable CCM biomolecules, all

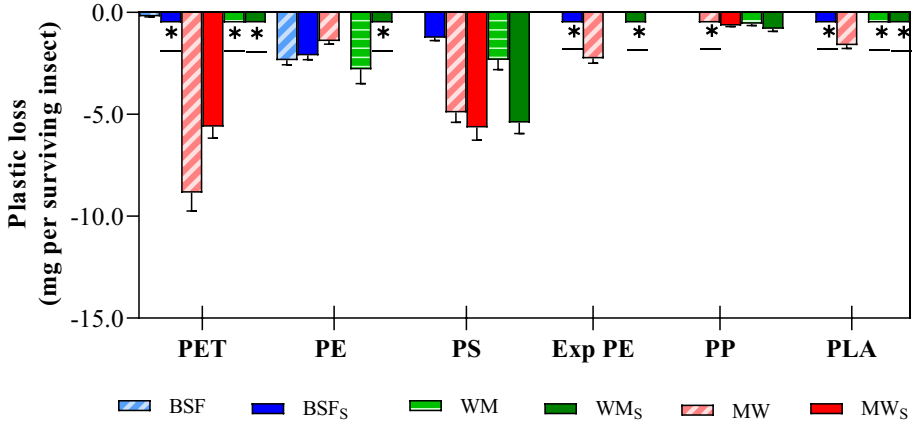

Fig. 5. Summary of plastic losses post-exposure to insect larva for 5 days. BSF, MW, and WM are defined as Black Soldier Fly, Mealworm, and Wax Moth, respectively. The 'S' subscript indicates the addition of a molasses supplement to the plastic substrates. PET, PE, PS, PP, and PLA are defined as polyethylene terephthalate, polyethylene, polystyrene, polypropylene, and polylactic acid, respectively. Exp PE is defined as expanded foam PE.

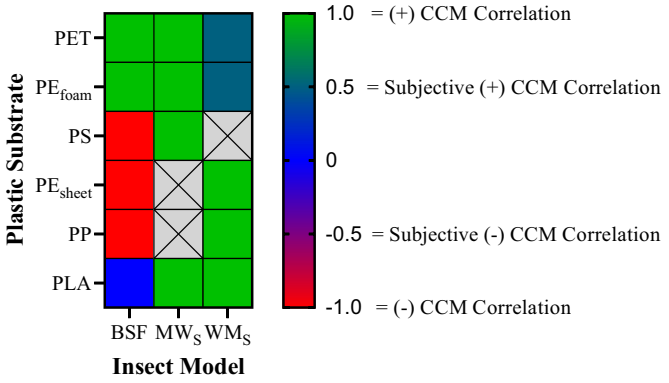

Fig. 6. Global overview summary of positive and negative CCM correlations with various plastic substrates. Note, the 'S' subscript indicates the addition of a molasses supplement to plastic substrates.

sampled larvae were normalised to a standard freeze-dried biomass unit and via the utility of labelled standards that were added at different stages of the extraction procedure.

Another limitation is the assessment of micro- and nano-plastics within the expelled insect frass and excreta. While we observed some positive correlation with measured CCM metabolites on certain plastic substrates, visual inspection of the jars post-experiment indicated that the MW and WM had burrowed into some of the expanded foam plastic substrates and visual residues of plastic were noted in the jars. To increase the amount of plastic degradation observed, the ratio of insect biomass to plastic could be increased, to try and enhance any physical effects that may occur. In addition, increasing our understanding of the additives commonly used in commercial plastic products and their impact on insects and the insect microbiome warrants more research. This can be done by analysing plastic chemical residues (plasticizers, anti-oxidizers, lubricants, etc.) bioaccumulating in insect larvae, and being expelled into insect frass in parallel to additional omics-based measurements (such as metabolomics, lipidomics, and proteomics).

Future studies should extend the analyses herein to screen for organic plastic residues within dissected insect biomass, gut, and expelled frass. In addition to screening for known chemical plastic by-products, a detailed multi-omics analysis of the insect biomass, gut microbiome, and expelled frass that is investigated over multiple insect generations reared on plastic will enable the impacts of plastics on these insects to be obtained and help create a biotransformation workflow that utilises insects. Such an approach will also assist distinguish the metabolites produced by larval tissues from those coming from gut symbionts (gut microbiome). This is graphically depicted in Fig. 7.

CRediT authorship contribution statement

David J. Beale: Methodology, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization. **Rohan M. Shah**: Data curation, Writing – original draft, Writing – review & editing; **Anna Marcora**: Methodology, Investigation, and Writing - Review & Editing; **Andrew Hulthen**: Methodology, Investigation, and Writing - Review & Editing; **Avinash Karpe**: Data curation, Writing – original draft, Writing – review & editing; **Khoa Pham**: Methodology, Investigation, Data Curation and Writing - Review & Editing; **Gene Wijffels**: Conceptualization, Methodology, Investigation, Writing - Original Draft, Writing - Review & Editing, Supervision, Project administration, and Funding acquisition; **Cate Paull**: Conceptualization, Methodology, Investigation, Writing - Original Draft, Writing - Review & Editing, Supervision, Project administration, and Funding acquisition.

Declaration of competing interest

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

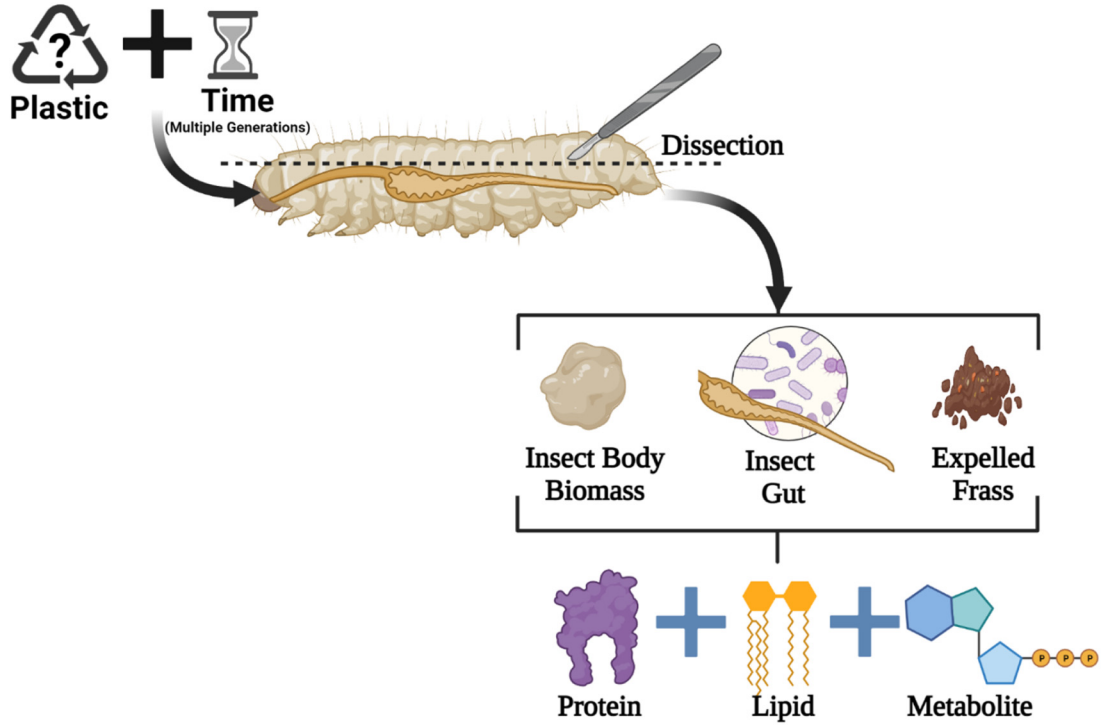

Fig. 7. Proposed multiple generational insect plastic exposure study using multi-omics approaches coupled with traditional plastic degradation data. Note, this includes the protein, lipid, and metabolite profile from dissected insect body biomass, insect gut, and expelled frass fractions and analyzed from multiple generations of insects reared on a plastic substrate.

Acknowledgements

The authors would like to acknowledge Goterra Pty Ltd. for supplying BSF and Katie Hillyer for assisting with the analysis of preliminary insect larva samples. Appreciation is extended to Professor Michelle Colgrave, Dr. Owain Edwards, the CSIRO Protein Mission and the broader Insect Biotransformation Team for their intellectual contributions and comments over the course of this study. The authors would like to thank the internal CSIRO reviewers who provided input and comments on earlier manuscript drafts. Lastly, the 1290 Infinity II Flex pump coupled to a 6470 LC-QqQ-MS instrument used in this study were provided by Agilent Technologies to the CSIRO for the determination of central carbon metabolism metabolites. All images and figures were created or modified using BioRender.com.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2022.154840.

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