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Comparative proteome profiling of egg, larva and adult *Hermetia illucens* (Diptera: Stratiomyidae)

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The black soldier fly (BSF), *Hermetia illucens*, is the insect with the greatest potential to contribute to sustainable human development because of its nutritional characteristics, low environmental impact, and bioremediation ability. In this study, we characterized and quantified the proteome of three BSF stages: egg, larva, and adult, using bottom-up proteomics. A total of 6,116 proteins were identified across all BSF developmental stages. Processes related to information processing, such as chromatin structure, replication, transcription, translation, and the cell cycle, were notably more abundant in eggs than in larvae and adults. Nevertheless, metabolic processes, such as amino acid, carbohydrate, lipid, and nucleotide metabolism, were more abundant in larvae and adults than in eggs. The quantitative analysis revealed four expression clusters of proteins involved in amino acid transport and metabolism, carbohydrate metabolism, coenzyme transport and metabolism, energy production, inorganic ion transport and metabolism, lipid metabolism, posttranscriptional modification and translation. Furthermore, the detection of xenobiotic detoxification proteins, cytochromes, coesterases, and GSTs, suggests that BSF can metabolize xenobiotics. This overview of the BSF proteome provided information for further investigations as biotechnological or practical applications based on newly identified detoxification enzymes, such as enhancing rearing practices with new diet formulations or bioremediation wastes.

Keywords Shotgun proteomics, Developmental stage, Detoxification, Bioremediation, Metabolic processes, Insect rearing

The sustainable exploitation of natural resources can be further enhanced by integrating nature-based solutions to address the problems of overproduction, solid organic waste disposal, and the growing demand for natural raw materials[1]. Natural bioactive components are found in various natural sources, such as plants, animals, and microorganisms[2]. The exploitation of insect mass culture as a promising alternative source of proteins and lipids has increased in recent years. Insects are highly efficient bioconverters, generating a low environmental impact since they emit fewer greenhouse gases, consume less water and reduce the risk of zoonoses[3,4]. The black soldier fly (BSF), *Hermetia illucens* (L., 1758) (Diptera: Stratiomyidae), can contribute to sustainable human development because of its nutritional characteristics, low environmental impact, and bioremediation ability, among other advantages. Many studies have described promising farmed insects that provide multiple services and uses for humans[5,6]. Saprophagous larvae are highly polyphagous and consume vegetal and animal substrates such as food waste, manure, animal corpses, and other organic materials[7,8]. They represent one of the most efficient and quick fly species that consumes raw waste[9]. Consequently, BSF has a potential role in human waste management and could participate in a sustainable circular economy through waste bioconversion[10–12]. Moreover, the activity of BSF larvae reduces the number of noxious bacteria and insect pests, such as the common fly or *Drosophila* flies, in waste. The biomass generated by BSF larvae is rich in proteins and lipids and is successfully used to feed fish such as rainbow trout[13,14] or Atlantic salmon[15]. Nutritional value of BSF larvae could be enhanced by specific diets as shown by the over-accumulation of metabolites in BSF larvae after rosemary[16] or fungi[17,18]

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supplementation. The high fat content of larvae could also be a source for biodiesel production[19,20]. In addition, various products of high biological value can be extracted from BSF larvae as chymotrypsin-like proteases, serine endopeptidases[21] or antimicrobial peptides with activity against bacteria, fungi, and viruses[22–24]. Finally, insects have a high protein content and are rich in essential amino acids. Consequently, BSF, which is currently used in animal feed, represents a potential source of nutrients for humans[25].

The polyphagy of BSF larvae is a central feature of interest in this species. First, BSF can modulate nutrient digestion and absorption, which affect diet quality[26]. Then, digestive enzymes, which are found in the gut as amylases, lipases, and proteases, ensure that the first stage of food digestion[9] is associated with the gut microbiota[27,28]. Finally, different biochemical mechanisms transform the digested elements into valuable ones, such as proteins or lipids. Nevertheless, their accumulation can be influenced by diet quality[29] and larval growth performance[11]. The food conversion rate can also be affected by the diet composition and the presence of secondary metabolites or toxins (pesticides, fungal agents), negatively affecting BSF production. Metabolic enzymes such as cytochrome P450 monooxygenases (cytochromes), carboxylesterases (coesterases), and glutathione-S-transferases (GSTs) can support the detoxification process[30,31]. The mechanisms involved in these processes in BSF larvae have rarely been studied.

Previous biological, genomic, and proteomic studies on BSF have focused on the larval instars used for biotechnology applications. Nevertheless, studies examining other developmental stages are controversial. Until recently, adults were considered unable to eat[5]. However, a recent morphological and functional study formally demonstrated that BSF adults possess an operative digestive system[32]. To date, no studies have characterized the BSF egg proteome. In other insect species, this characterization has been fundamental in developmental biology studies and for identifying enzymes with high biotechnological value[33–36]. Proteomic studies of BSF eggs and adults could lead to the characterization of new cellular processes and future applications.

Here, we characterized and quantified the proteomes of three developmental stages of BSF, egg, larva, and adult, via a bottom-up proteomics strategy. The analyses focused on metabolic processes related to carbohydrates, lipids and amino acids and xenobiotic detoxification processes involving the metabolic enzymes cytochromes, coesterases, and GSTs. As a result, we provide valuable information about the whole life cycle of BSF, revealing potential functions related to regulatory mechanisms of development, evolution, and interaction with the surrounding environment (bacteria, diet, etc.). This knowledge provides relevant information for optimizing the breeding conditions of BSF, developing new biotechnology based on three BSF developmental stages, and producing quality proteins as required by industry.

Results

Protein identification Overview and Functional annotation by KOG

A total of 6,116 proteins were identified across the three BSF developmental stages. By stage, 4344 proteins were identified in the egg, 3543 in the larva, and 4031 in the adult (Table 1). The proteomes shared by the three individuals at each stage were 2261, 2149, and 2360 proteins for eggs, larva, and adults, respectively (Table 1). A comparison of the proteomes between the BSF stages revealed that 1768 proteins were shared by all three stages: 1718 unique to the egg, 911 unique to the larva, and 949 unique to the adult (Fig. 1A). Notably, the highest number of proteins was detected in the egg stage. Quality control analyses confirmed the consistency of sample distributions across biological replicates (Supplementary Fig. 1).

On the basis of the complete dataset, the PCA clustered each stage separately, with low variation among the three individuals within each stage (Fig. 1B). Each protein dataset was annotated, achieving an annotation yield of 90–92% using eggNOG-mapper, 89–91% using eukaryotic orthologous groups (KOG), and 88–99% using protein families (PFAM) (Table 2). To analyze the processes in which the proteins participate and to assess the distribution of unique proteins at each developmental stage, the percentage of proteins associated with different pathways was determined through KOG analysis (Fig. 1C).

Compared with the larval and adult stages, the egg stage presented the highest percentage of assigned proteins in each category. The most abundant processes in the egg were replication, recombination, and repair (100%); cell cycle control, cell division, and chromosome partitioning (88%); transcription (83%); chromatin structure and dynamics (75%); translation and ribosomal structure (72%); RNA biogenesis, processing, and modification (67%); nuclear structure (60%); and intracellular trafficking, secretion, and vesicular transport (59%). In the larva, the most abundant processes were secondary metabolite biosynthesis, transport, and catabolism (64%); cell wall/membrane/envelope biogenesis (58%); carbohydrate transport and metabolism (52%); defense mechanisms (50%); posttranslational modification, protein turnover, and chaperones (41%); and amino acid transport and metabolism (39%). In adults, the most abundant processes were extracellular structures (52%),

Biological replicates	Egg	Larva	Adult
R1	3181	2837	2986
R2	3521	2853	3095
R3	2961	2721	3369
Panproteome	**4344**	**3543**	**4031**
Shared Proteins	2261	2149	2360

Table 1. Number of proteins identified in each individual per stage, total number of unique proteins per stage (panproteome), and number of proteins shared among the three individuals at each stage. Significant values are in bold.

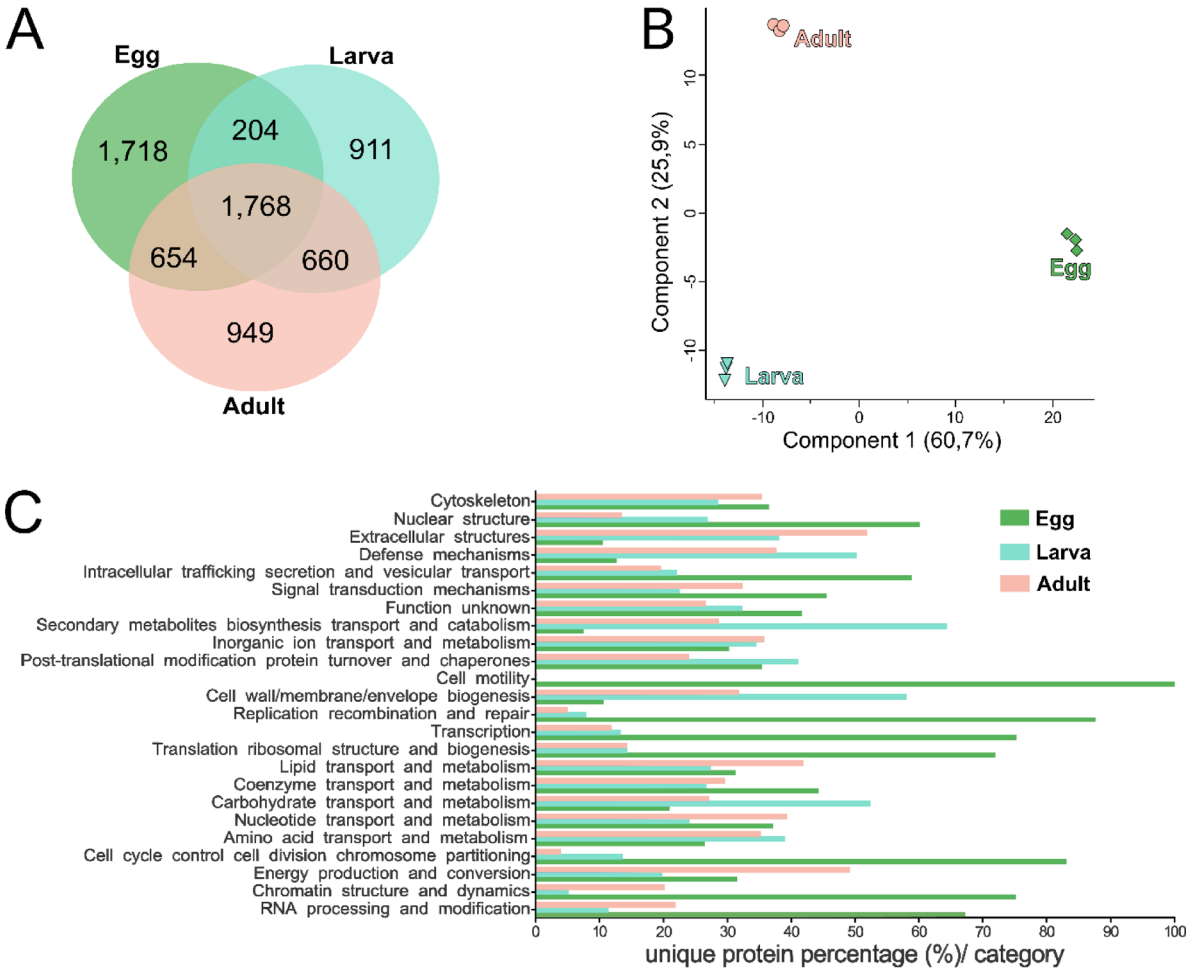

Fig. 1. Comparative proteomic overview across BSF developmental stages. (**A**) Venn diagram showing shared and unique proteins identified in eggs, larva, and adults. (**B**) Principal component analysis (PCA) of protein profiles from three biological replicates per stage. (**C**) Functional classification of identified proteins according to KOG categories, showing the number of proteins assigned to each function per stage.

Stage	Number of identified proteins	eggNOG	KOG	PFAM
Egg	4344	4006 (92.2%)	3947 (90.9%)	3887 (89.5%)
Larva	3543	3198 (90.3%)	3157 (89.1%)	3119 (88.0%)
Adult	4031	3689 (91.5%)	3604 (89.4%)	3557 (88.2%)

Table 2. Functional annotation of each stage's panproteome using eggNOG (Evolutionary Genealogy of Genes: Nonsupervised Orthologous Groups), KOG (Clusters of Orthologous Groups of proteins), and PFAM (protein families) databases. Annotation yields are shown in parentheses.

energy production and conversion (49%), lipid transport and metabolism (42%), and nucleotide transport and metabolism (39%).

In summary, processes related to information processing were significantly more common in the egg stage than in the larva and adult stages, whereas metabolic processes were more common in the larva and adult stages than in the egg stage.

Analysis of protein clustering at different BSF stages

To determine which proteins significantly varied in expression (i.e. DEPs), we compared the three developmental stages of BSF. First, we identified 1688, 2042 and 2269 quantifiable proteins (Fig. 2B) and 802, 839 and 1136 DEPs for larva vs. egg, adult vs. egg and adult vs. larva, respectively (Fig. 2A).

A greater number of quantifiable proteins and DEPs were found between adults and larva.

To further investigate the functional organization of differentially expressed proteins, we performed a complementary analysis constructing protein–protein interaction (PPI) networks using STRING v12.5 for

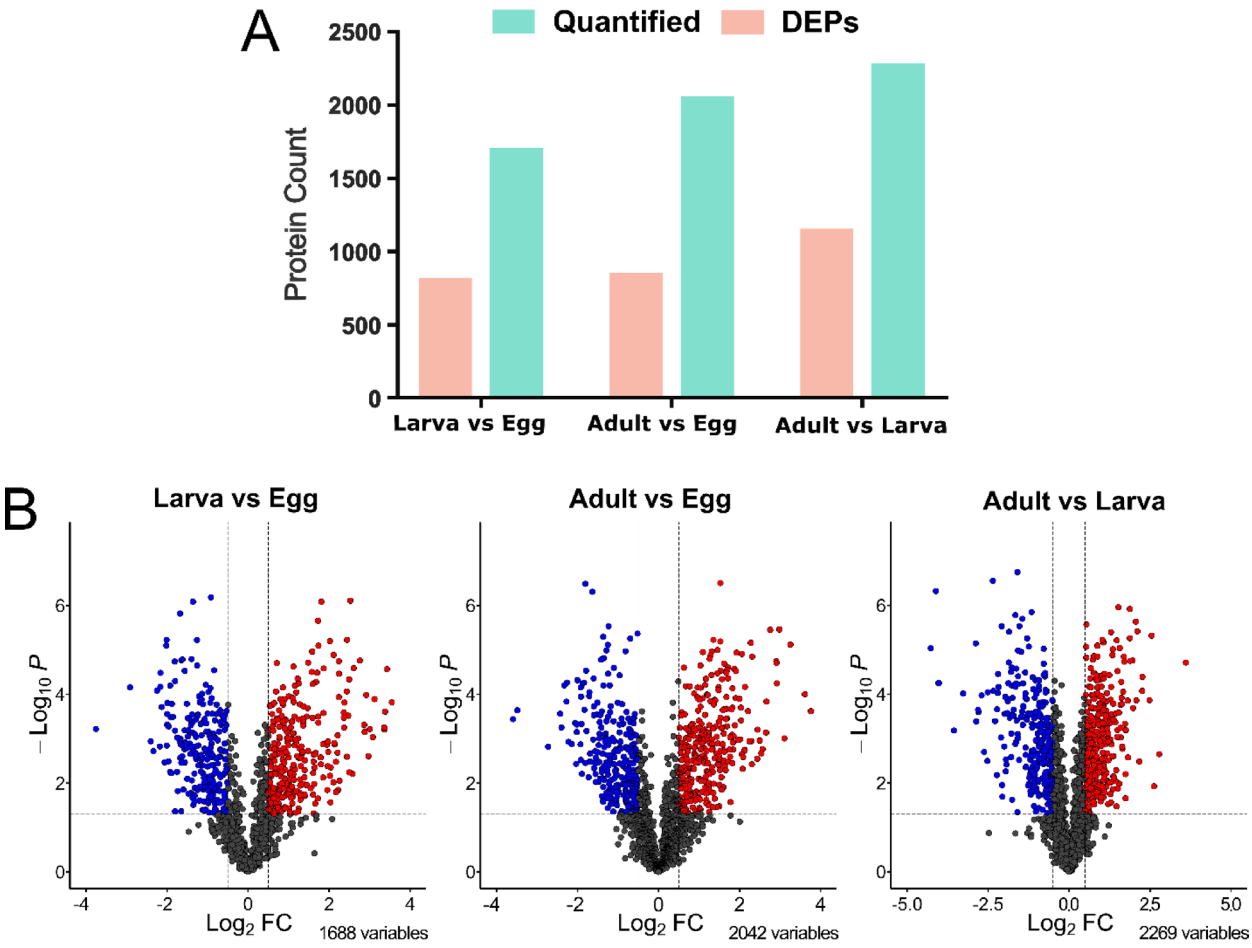

Fig. 2. Differential proteomic analysis across BSF developmental stages. Bar plot showing the number of quantified proteins (aqua teal) and differentially expressed proteins (DEPs) (soft pink) for each pairwise comparison: Larva vs Egg, Adult vs Egg, and Adult vs Larva. Volcano plots display quantified proteins for each comparison. Vertical dashed lines indicate the 0.5-fold change threshold; the horizontal line marks the *p*-value = 0.05 cutoff. Red and blue dots represent significantly upregulated and downregulated proteins, respectively.

each developmental comparison: adult vs. egg, adult vs. larva, and larva vs. egg. High-confidence interactions (score ≥ 0.7) were used, and sequence mapping was carried out against the recently published *H. illucens* proteome in UniProt. A total of 893, 1136 and 802 proteins were input for the adult vs. egg, adult vs. larva, and larva vs. egg comparisons, respectively, resulting in 591 (66.2%), 798 (70.2%) and 572 (71.3%) nodes mapped per network. All networks were significantly more connected than expected by chance ($p < 1.0e-16$), with high edge counts (9,877–15,088), average node degrees of 33.4–37.8, and clustering coefficients between 0.336 and 0.346 (Supplementary Figs. 4–6). Functional enrichment analysis based on KEGG pathways revealed consistent overrepresentation of central metabolic routes, including "Metabolic pathways", "Carbon metabolism", "Oxidative phosphorylation", "Citrate cycle (TCA cycle)", "Fatty acid degradation", "Ribosome" and "Protein processing in endoplasmic reticulum" (Supplementary Figs. 7A–C). These results highlight a progressive activation of biosynthetic and catabolic processes during BSF development, and support the robustness of the differential proteome analysis by confirming a high number of proteins mapped into coherent functional networks.

To explore the functional landscape of the DEPs beyond canonical enrichment terms, we additionally analyzed the frequency of Gene Ontology Biological Process (GO:BP) annotations across the three comparisons. Although classical enrichment analyses did not yield statistically significant terms directly related to reproduction, this approach revealed a consistent representation of development- and reproduction-related categories throughout the life cycle of *H. illucens*. The most frequent GO:BP terms included "Developmental process", "Anatomical structure development", and "Multicellular organism development", particularly in the adult vs. larva comparison (178, 175, and 150 proteins, respectively), followed by adult vs. egg (122, 120, 105) and larva vs. egg (120, 117, 104). Additionally, proteins annotated with "Reproduction" and "Reproductive process" were present across all comparisons, indicating progressive involvement during development (Supplementary Fig. 3). Notably, several vitellogenin-related proteins (e.g., XP_037926969.1, XP_037923239.1, and XP_037923786.1) were associated with these biological processes, supporting their role in insect reproduction. Together, these findings highlight functionally relevant developmental transitions that may not be captured by standard enrichment analyses.

Next, 1091 proteins were significantly differentially expressed across the three BSF stages and were divided into eleven clusters (Supplementary Fig. 2). Clusters 02, 04, 05, and 06 were composed of 198, 43, 11 and 15 proteins, respectively, and presented consistent expression between developmental stages (Fig. 3A). The proteins in clusters 02, 04, and 05 were highly expressed in eggs but weakly expressed in larva and adults. In cluster 06, the expression of these proteins was higher in larva but lower in eggs and adults. Additionally, out of 21 pathways, we highlighted six KOG pathways: carbohydrate metabolism, coenzyme transport and metabolism, energy production, inorganic ion transport and metabolism, lipid transport and metabolism and amino acid transport and metabolism. These genes were relevant within the three developmental stages (Fig. 3B).

To synthesize and analyze the protein interactions of these four clusters, the mean relative abundance scaled as the Z score of the proteins was plotted (Fig. 4). The heatmap revealed that the expression profile across the clusters was consistent with the trajectories shown in the profile plots. Figure 4 shows the protein distribution in the KOG pathways of amino acid transport and metabolism, carbohydrate metabolism, coenzyme transport and metabolism, energy production, inorganic ion transport and metabolism, lipid metabolism, posttranscriptional modification and translation, and protein–protein interactions in the STRING v12.0 database mapping against *Drosophila melanogaster*. As shown in the profile plots (Fig. 3A), the analysis of these clusters revealed that the

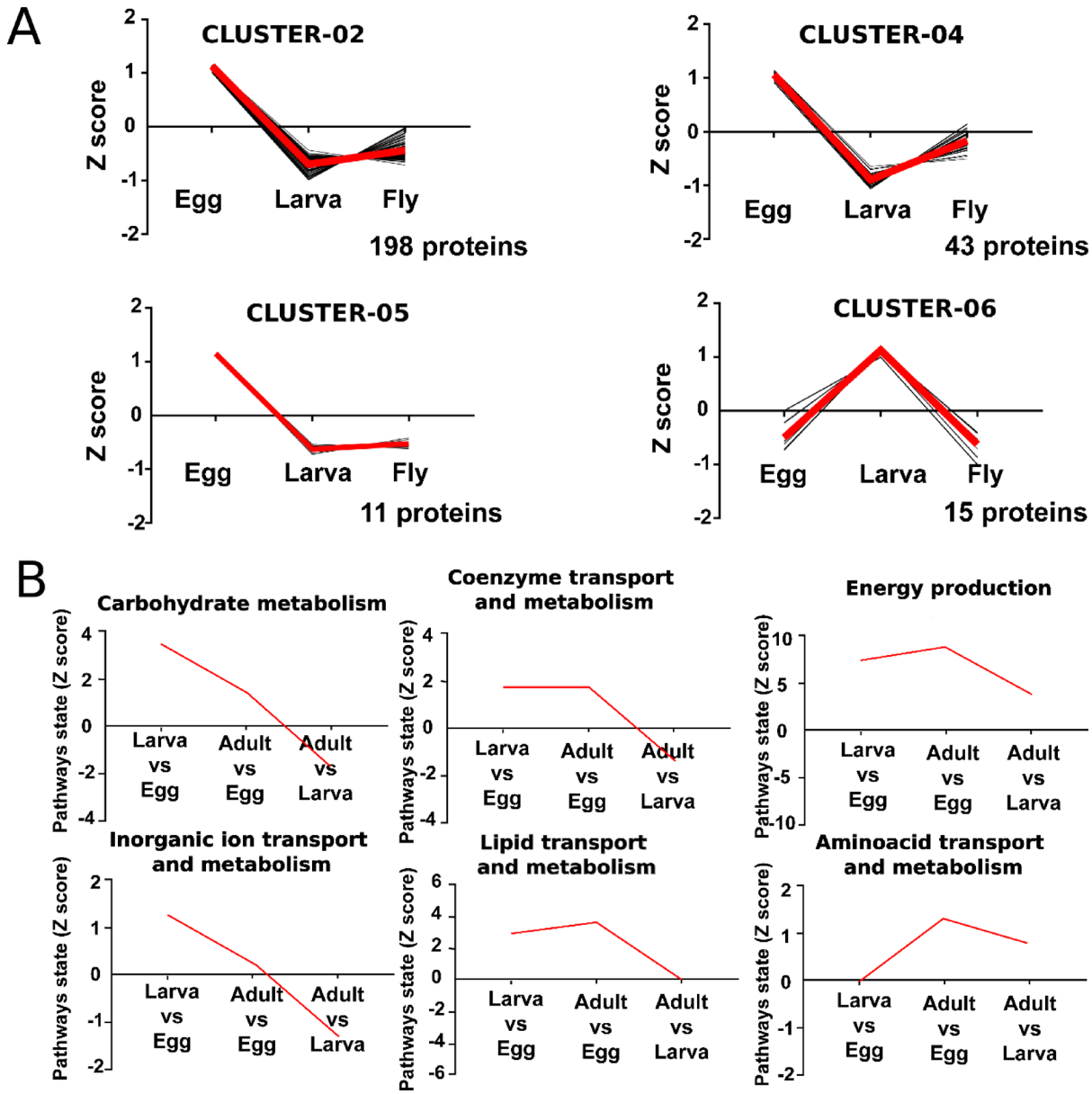

Fig. 3. Protein expression clusters and relative activity of key KOG categories across BSF developmental stages. (**A**) Scaled expression trajectories (Z-score) of protein clusters showing consistent patterns across egg, larva, and adult stages. Each plot indicates the cluster name and number of proteins included. (**B**) Line plots of the relative activity (Z-score) of selected KOG categories across pairwise comparisons: larva vs egg, adult vs egg, and adult vs larva. Categories with noticeable activity shifts are highlighted.

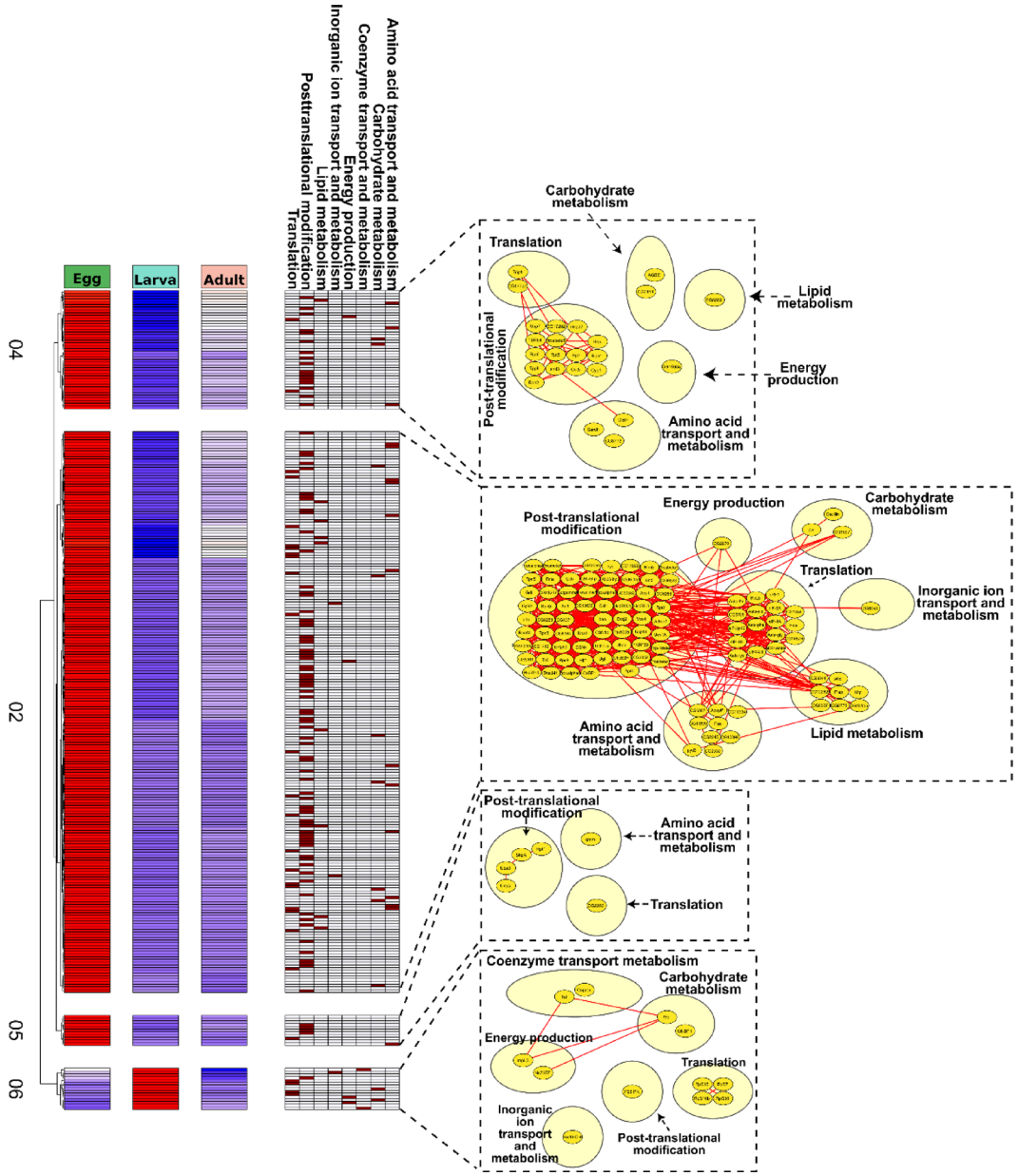

Fig. 4. Relevant biological processes represented in the most consistent protein expression clusters across BSF developmental stages. From left to right: heatmap showing the scaled protein abundance (Z-score) for each developmental stage (Egg, Larva, and Adult); KOG annotation indicating the functional classification of proteins within each cluster; and STRING protein–protein interaction network highlighting the main biological processes enriched in each expression pattern. Clusters were selected based on their consistency across stages and biological relevance.

proteins in clusters 02, 04, and 05 presented a higher mean abundance in egg and a lower mean abundance in larva and adults. In contrast, the proteins in cluster 06 had a higher abundance in larva and a lower abundance in eggs and adults. In cluster 02, the largest cluster, proteins belonged predominantly to posttranscriptional modification, with numerous interactions within the proteins involved in this process, and with proteins belonging to the processes of translation and lipid metabolism. For cluster 04, the most relevant biological process was post modification with 15 of 24 proteins, but with only one and four interactions with proteins from Translation and Amino acid transport and metabolism, respectively. In cluster 05, three processes were represented Post Transcriptional modification, Amino acid transport, metabolism, and Translation. The proteins of the first process presented successive interactions. Finally, the cluster 06 was composed of proteins that mainly participated in Translation, and in interacting processes such as Energy Production, Carbohydrate metabolism and Coenzyme Transport and Metabolism.

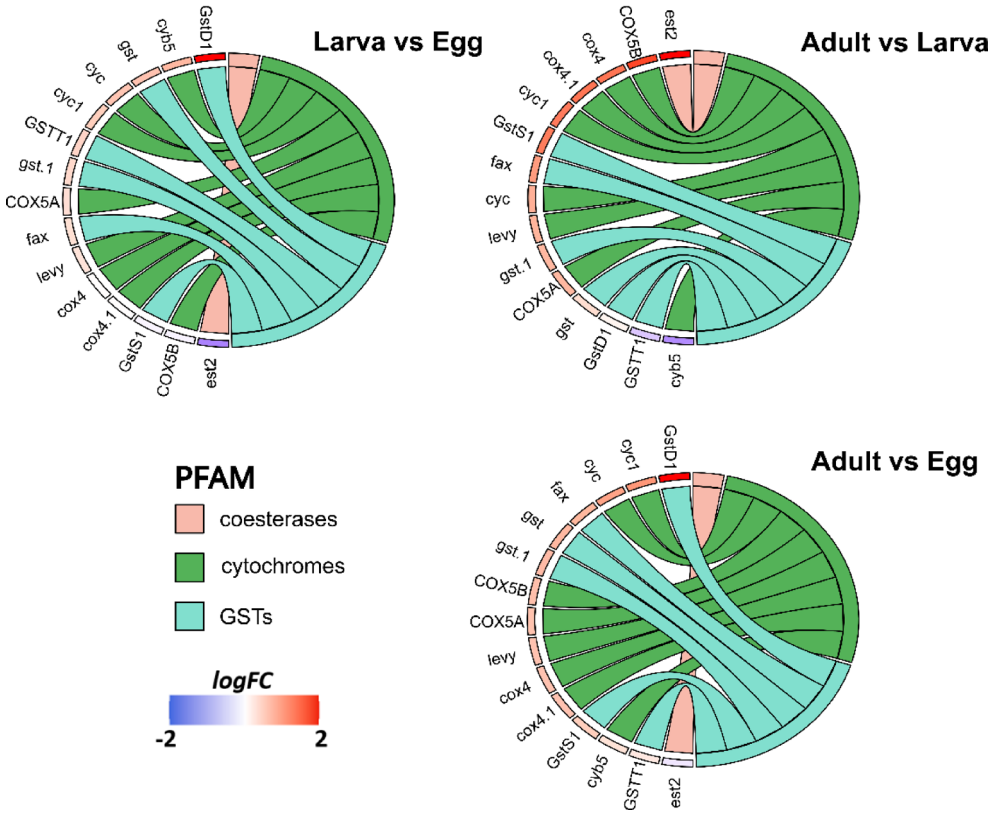

Fig. 5. Proteins involved in bioremediation-associated PFAM terms across BSF developmental stages. Chord plots showing bipartite networks between proteins (left semicircle) and their associated PFAM domains (right semicircle), including GSTs (aqua teal), cytochromes (leaf green), and coesterases (soft pink). Each panel represents one pairwise comparison: Larva vs Egg, Adult vs Egg, and Adult vs Larva. The outer colored bars next to each protein node indicate differential expression status: red for upregulated and blue for downregulated proteins.

Bioremediation- and detoxification-associated protein families (PFAMs)

The protein annotation performed using the PFAM database identified 182 proteins belonging to three major detoxification-related families: 32 coesterases, 118 cytochromes, and 32 glutathione S-transferases. From these, we highlighted 15 proteins quantified across the three developmental comparisons, which were plotted on a chord plot showing their relative abundances for the larva vs. egg, adult vs. egg, and adult vs. larva comparisons (Fig. 5). In general, cytochrome proteins (cyc) such as cytochrome c (cyc-c), cytochrome c-1 (cyc1), Levy, isoform A (levy), and cytochrome c oxidase subunits 5A (COX5A), IV (cox4 and cox4.1), and 5B (COX5B) were consistently upregulated in both larva and adult stages when compared to egg, and also in the adult vs. larva comparison. Similarly, glutathione S-transferases including isoform D (GstD1), S1 (GstS1), T1 (GSTT1), and 0.1 (gst.1), as well as failed axon connections, isoform D (fax), showed the same trend. The only coesterase family member, carboxylic ester hydrolase (est2), was downregulated in egg vs. larva and egg vs. adult comparisons, but upregulated in adult vs. larva.

In addition to the classic detoxification enzymes described above, we identified a diverse set of enzymes with potential biological and biotechnological relevance in *H. illucens* (Supplementary Table 1). Among these, antimicrobial peptides (AMPs) were the most represented, with ten proteins detected, including defensin-A-like (XP_037915075.1), cecropin-like peptide 1 (XP_037918984.1), and attacin-A-like (XP_037916366.1), predominantly present in egg and adult stages. One AMP (XP_037924140.1) displayed significant differential abundance between larva and eggs, highlighting potential stage-dependent antimicrobial functions. In contrast, a single laccase (multicopper oxidase) was identified, exclusively in larval samples, suggesting a specialized role in this developmental phase. The MBL-fold/β-lactamase-like family comprised three proteins, with beta-lactamase-like protein 2 homolog (XP_037921214.1) consistently detected across all stages and showing significant differences between adult flies and larva. These stage-specific patterns, together with PFAM-based functional annotations, support a broad enzymatic repertoire encompassing antimicrobial defense, oxidative catalysis, and β-lactamase-related activities.

Discussion

Using a bottom-up approach, we performed the first proteomic description, which included three BSF developmental stages: Egg, L5 Larva and Adult. A total of 6,116 proteins were identified, of which 28.9% were

shared by the three stages, 28.1% were unique to egg, 14.8% were unique to larva, and 15.5% were unique to adults. More than 88% of these proteins were annotated in the databases eggNOG, KOG, and PFAM.

This comprehensive proteomic dataset not only delineates stage-specific and shared components of the BSF proteome but also provides a solid foundation for downstream functional analyses. Our functional network analyses confirm the robustness of the differential proteome dataset, with PPI networks showing significantly higher connectivity than expected by chance and most DEPs forming coherent functional modules. Enrichment patterns across stages likely reflect shifts in energy allocation for growth, metamorphosis, and reproductive preparation, consistent with evidence that diet influences metabolic investment and reproduction in *H. illucens*[37]. The recurrent detection of developmental and reproductive GO terms, including vitellogenin-related proteins, suggests progressive activation of reproductive machinery, supported by seasonal effects on oviposition and reproductive costs[38]. These results provide a comprehensive molecular framework for BSF development and a reference for identifying stage-specific biomarkers, with potential relevance to artificial reproduction strategies.

A total of 1091 differentially expressed proteins were identified and divided into 11 clusters. Hierarchical clustering separated eggs from the other two stages, but the variation in protein expression between stages was dependent on each cluster. The proteins involved in bioremediation belonging to the Cytochrome and GST families were generally more highly expressed in larva and adults than in eggs. This valuable information from egg to adult BSFs revealed potential functions related to regulatory mechanisms of development, evolution, and interactions with the surrounding environment. This knowledge will be helpful for enhancing rearing methods and providing potential new biotechnology uses for the less studied stages: egg and adult.

Previous investigations have focused on the BSF larva[39] because this is the main stage used as the final product, e.g., for feeding breeding animals and in the cosmetics industry. In our study, we obtained 6,116 identifications among the three stages and 3543 for the sole L5 larva. Previous larval studies in which instars were not precisely assigned identified approximately 500 proteins, whereas others characterized around 5,000 proteins across the five larval instars.[40,41]. Previously, these authors annotated between 26 and 66% of the larva proteins[41]. In our study, each dataset of proteins was annotated to obtain an annotation yield of 90 to 92% using eggNOG-mapper, 89 to 91% using EuKaryotic Orthologous Groups (KOG) and 88 to 99% using protein families (PFAM). This enhancement mainly resulted from the increasing amount of information available in proteomics databases. This knowledge will provide a better understanding of the pathways and functions involved in BSF development.

One of the insights acquired in the present study is the description of the egg proteome. During this critical stage, the embryo fully develops into a larva. Approximately 1.8-fold more unique proteins were found in the egg stage than in the other two stages. Specifically, the number of egg proteins was greater in 15 of 24 pathways noted in the KOG, corresponding to 62.5% of the total number of proteins, highlighting pathways related to metabolic processes and storage information and processing. These results confirmed that active embryogenesis required specific biological processes for embryo development. Cluster 2 included numerous proteins and interactions involved in these processes (Fig. 4). The same relevant changes in development processes from egg to larva were observed in *Bactrocera latifrons* (Hendel) (Diptera: Tephritidae)[42] and *Apis mellifera* L. (Hymenoptera: Apidae). Gala and collaborators (2012) reported that proteins related to energy production, metabolism development and amino acid metabolism were strongly expressed in honeybee embryos[43]. In the case of BSF egg, we discovered a high percentage of unique proteins that participate in processes related to transport together with storage information and processing. Interestingly, when the DEPs in the KOG category were analyzed, we observed that the relative activity of the abovementioned processes was increased mainly in larva and that processes related to amino acid metabolism and transport were favored in egg. Moreover, analyses of unique proteins related to the transport of amino acids and nucleotides revealed an increase in the larval stage. However, when comparing DEPs, the relative activity of this pathway, such as carbohydrate metabolism and organic ion transport, was increased mainly in egg. This phenomenon may be related to the fact that, in L5, the larva has a higher food intake; therefore, pathways related to metabolic processes are increased regardless of the number of proteins[44,45].

The second insight of this study is the description of the adult proteome. Previous studies on BSF adults have focused mainly on reproductive aspects via biological[46–48], ecological[49] and morphological[32] experiments. However, the potential of BSF adults as providers of the final product has rarely been considered. Indeed, metamorphosis highly modifies an individual's composition and weight, reducing interest in adult-based products. In the adult stage, a significant number of unique proteins related to energy production and conversion were identified according to the KOG annotation. However, for DEPs, most of the processes evaluated increased with respect to egg but not larva, which may be related to the above phenomenon. Our results could favor new investigations for applications in industrial processes, such as those performed for larva, e.g., antibacterial substances[24] or digestive enzymes[21,27]. These findings are consistent with proteomic studies in different larval stages[50].

Understanding the digestive system is essential for improving applications and BSF mass rearing[39]. By exhibiting more cytochromes, coesterases, and glutathione S-transferases than adults, larva can efficiently digest food and organic waste[9,51]. Indeed, cytochromes play essential roles in developmental processes, xenobiotic metabolism, phytochemical detoxification, and insecticide resistance[52,53]. In *Drosophila melanogaster* (Diptera: Drosophilidae), CYP303a1, identified here in egg (XP_037916470.1), is essential for wing extension and likely plays a role in the metabolism of an ecdysteroid-like molecule[53], whereas CYP6a9, 40 variants identified here as XP_037904182.1, is related to DDT resistance[54]. The characterization of these enzymes can be used to understand the growth performance of larva and to formulate an optimized diet that suppresses noxious byproducts or organic waste used to feed the larva. For this last objective, the role of the gut microbiota also needs to be considered[55,56]. From these three families, adults exhibited 94 proteins, 25 unique to this stage, showing the capacities of adults to detoxify exogenous molecules. These results will be useful to develop adult diets. Some proteins as XP_037920550.1 belonging to SGNH hydrolase-type esterase should be evaluated to

their capacity of ester bond hydrolysis which is central in the decomposition of pyrethroids[57]. Other coesterases showed their role in promoting PET depolymerization[58].

In this way, nutriproteomics (how diet affects protein synthesis) has great potential for optimizing artificial diets for mass rearing, as is the case for *Arma chinensis* (Fallou) (Hemiptera: Pentatomidae), a biocontrol agent[59]. Overall, OMICS approaches will be helpful for improving selection practices for identifying specific lineages with the desired traits[60], taking advantage of phenotypic plasticity in the development and waste conversion of BSF[61]. Another result could be the enhancement of the BSF larva meal based on metabolic processes. Interdisciplinary approaches are needed to understand the interactions between BSF and its controlled environment and to optimize the final BSF product (meal composition, lipid extraction).

Within this framework, we also identified proteins of biotechnological relevance, with distinct abundance patterns across developmental stages. In particular, the identification of stage-specific antimicrobial peptides (AMPs) and oxidoreductases expands current views of *H. illucens* developmental physiology. The predominance of AMPs in eggs and adults is consistent with innate immune provisioning in early development and pathogen defense in reproductively active stages, a pattern echoed across Diptera and specifically reported in BSF under immune challenge[62,63]. The larva-restricted detection of a laccase aligns with the high oxidative demand of detritivorous feeding and substrate turnover, matching reports that insect laccases (multicopper oxidases) catalyze phenolic and lignin transformations with broad biocatalytic potential[64–66]. The widespread occurrence of β-lactamase-like (MBL-fold) proteins—including forms present in all stages—fits the multifunctionality of the MBL scaffold beyond classical antibiotic resistance and points to roles in xenobiotic handling in microbe-rich substrates[67]. Collectively, these patterns support biochemical plasticity across the BSF life cycle and motivate downstream tests of AMPs, laccases, and MBL-fold enzymes for antimicrobial, biocatalytic, and biodegradation applications[66,68].

This study provides the most comprehensive proteomic overview of *H. illucens* to date, spanning all developmental stages—egg, larva, and adult—and identifying more than 6,000 proteins. The dataset offers a solid foundation for future research aimed at understanding and functionally characterizing proteins relevant to both basic biology and technological innovation. We acknowledge certain limitations: the small amount of biological material remaining after proteomic analysis prevented further validation by qPCR or Western blot, and the limited availability of *H. illucens*-specific antibodies, together with known discrepancies between transcript and protein abundance, constrains transcript-based validation. Even so, these results substantially advance our molecular knowledge of BSF and open promising opportunities for harnessing newly identified enzymes and optimizing rearing practices, including tailored dietary formulations.

Methods

Insect rearing and sampling

Each stage of BSF was provided by F4F (Food 4 Future, Talca—Chile). From the third instar, the larvae were reared on vegetable and fruit wastes in a chamber with 80% humidity and 18 °C. Individuals were collected from the main rearing population without distinguishing between male and female: 12-h-old eggs, fifth-instar larvae, and two-day-old adults were collected and immediately processed.

Protein extraction and digestion and sample preparation for nLC-MS/MS

Protein extraction was carried out as previously described[69]. Three individuals at each developmental stage (egg, larva, and adult) were selected and individually processed. The BSF samples were lyophilized overnight, ground in a mortar with a pestle in liquid nitrogen until obtaining a fine powder and resuspended in lysis buffer composed of 10 mM Tris–HCl (pH 8.0), 140 mM NaCl, 1% SDS, 25 mM DTT and 1 mM EDTA. Proteins were subjected to precipitation using 5:1 (v/v) cold 100% acetone and incubated overnight at -80 °C. Then, the mixture was centrifuged at 15,000 × g for 10 min, the supernatant was discarded, and the pellet was washed three times with acetone at 90%, dried in a rotary concentrator at 4 °C, and finally resuspended in 8 M urea with 25 mM ammonium bicarbonate (pH 8.0). The proteins were quantified with a Qubit protein assay, where 100 µg was reduced with 20 mM DTT for one hour, alkylated with 20 mM iodoacetamide in the dark for one hour, diluted ten times with 25 mM ammonium bicarbonate (pH 8.0) and digested with trypsin/LyC (Promega) at a 1:50 ratio overnight at 37 °C. Peptides were cleaned using Pierce C-18 Spin Columns (Thermo Scientific, USA) according to the protocol suggested by the manufacturer. The eluted peptides were dried via a rotary concentrator at 4 °C, resuspended in 2% acetonitrile (MERCK Germany) with 0.1% v/v formic acid (MERCK Germany) and quantified via direct detection (MERCK Millipore).

Mass spectrometry analysis

A NanoElute (Bruker Daltonics) liquid chromatography system was used. Two hundred nanograms of tryptic peptides were separated within 90 min at a flow rate of 400 nL/min on an Aurora Series CSI reversed-phase column (25 cm × 75 µm i.d. C18 1.6 µm) (IonOpticks, Australia) at 50 °C. Mobile phases A and B were water and acetonitrile with 0.1 vol% formic acid, respectively. The B percentage linearly increased from 2 to 17% within 57 min, followed by an increase to 25% B within 21 min and further to 35% within 13 min, followed by a washing step at 85% B and re-equilibration. All samples were analyzed on a timsTOF Pro (Bruker Daltonics) hybrid trapped ion mobility quadrupole time-of-flight mass spectrometer via a CaptiveSpray nanoelectrospray ion source. The mass spectrometer was operated in data-dependent acquisition (DDA) mode for ion mobility-enhanced spectral library generation. The accumulation and ramp times were 100 ms each, and mass spectra were recorded in the range from m/z 100–1700 in positive electrospray mode. The ion mobility was scanned from 0.6 to 1.6 Vs/cm^2. The overall acquisition cycle of 1.16 s comprised one full TIMS-MS scan and 10 parallel accumulation-serial fragmentation (PASEF) MS/MS scans.

Database searching

Tandem mass spectra were extracted using Tims Control version 2.0 software. All MS/MS samples were analyzed using PEAKS Studio X + (Bioinformatics Solutions, Waterloo, ON Canada; version 10.5[2019-11-20]), which was set up to search the *Hermetia illucens* protein database, available in NCBI (24,794 entries, [2022-01-02]), with trypsin assumed to be a digestion enzyme. PEAKS studio X + was configured with fragment ion mass tolerance of 0.05 Da. and parent ion tolerance of 50 ppm. The carbamidomethyl of cysteine was specified as a fixed modification. Deamination of asparagine and glutamine, oxidation of methionine and acetylation of the N-terminus were specified as variable modifications. FDR estimation was included using a decoy database. FDR < 0.01 and 1 minimal unique peptide per protein were used for identification.

Bioinformatics analysis

Perseus (v1.6.15.0) was used for bioinformatics and statistical analysis using the intensity matrix from PEAKS Studio X + protein identification[70]. The label-free quantification (LFQ) intensity data were normalized to the Z score. Only proteins with 100% valid values were considered for analysis. To determine the variability of each biological replicate and developmental stage, a principal component analysis (PCA) was performed. To compare multiple groups, one-way ANOVA, controlled by a permutation-based FDR threshold of 0.05, was used to identify significant differences in protein abundance during the BSF developmental stages. Proteins with p values less than 0.05 were considered significant. Protein clusters were determined via hierarchical clustering analysis through Euclidean distances. Panproteomes were annotated through eggNOG-mapper[71,72] using the following databases: Clusters of Orthologous Groups of proteins[73] and the protein families PFAM database[74]. Protein–protein interaction (PPI) networks and pathway enrichment analyses were performed by mapping protein sequences to the *Drosophila melanogaster* dataset in the STRING v12.0 database[75]. For *H. illucens*, the proteome was manually uploaded to STRING v12.5[76], as this feature was available at the time of analysis (July 2, 2025), and the resulting interaction networks are provided in the Supplementary Material. The resulting network for *Drosophila melanogaste* was imported and processed in Cytoscape v.3.91[77]. The charts were created via R v.3.6.0 with ComplexHeatmap v.2.0.0[78], GOplot[79] and base packages.

Data availability

The mass spectrometry proteomics data have been deposited in the ProteomeXchange Consortium via the PRIDE partner repository under the dataset identifier PXD042131.

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Author contributions

Conceptualization: P.A., C.V.; Methodology: M.H., G.N., P.S.; Software: G.N., P.S.; Validation: P.S., P.A.; Formal analysis: G.N., P.S.; Investigation: P.A.; Resources: C.E.; Data curation: P.S., P.A., G.N.; Writing – original draft: P.S., P.A.; Writing – review & editing: P.A., C.V.; Visualization: P.S.; Supervision: C.V., M.H.; Project administration: C.V., M.C.A., R.D.; Funding acquisition: C.V. All authors have read and approved the final version of the manuscript.

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Declarations

Competing interests

The authors declare no competing interests.

Additional information

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