A multifactorial proteomics approach to sex-specific effects of diet composition and social environment in an omnivorous insect

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Abstract
Rearing conditions may elicit noticeable plastic responses in life-history traits of living organisms. Diet composition and the social environment have proven to influence prominent traits such as survival, body size, fecundity, and life span. Nevertheless, the physiological mechanisms underlying such responses are largely unknown. In this study, we investigated changes in the proteome of the house cricket Acheta domesticus subjected to diets of different nutritional composition (i.e., protein to carbohydrates ratio) and two distinct social environments (i.e., solitary or in groups). We measured the relative abundances of 685 proteins identified in whole-body cricket samples using high-performance liquid chromatography coupled to tandem mass spectrometry. Differential expression of proteins induced by diet composition and social environment in female and male A. domesticus was assessed in a data-independent proteomics approach. Additionally, we performed a functional analysis of the differentially expressed proteins using comprehensive databases (KEGG and GO). We found that sex alone explained a significant portion (40.87%) of the relative protein abundance variation. Males had a higher representation of proteins involved in metabolic pathways and locomotion. In contrast, females exhibited a higher abundance of proteins related to genetic processes regulation and nutrient catabolism. Moreover, diet composition and social environment induced sex-specific changes in a smaller set of proteins with particular roles. Females had their protein profile affected by diet composition and social environment. The involved proteins were mainly related to several protein synthesis stages, carbohydrate metabolism, and muscle development. In contrast, males were only affected by diet composition, overexpressing proteins related to hormone production, carbohydrate metabolism, and apparently depositing excess protein in the cuticle when fed with a protein-rich diet. Evidently, diet had a more substantial influence on the proteome of the cricket.
Proteins are the primary functional molecules in many different physiological processes (Müller et al., 2020). Although the proteome (i.e., the identity and abundance of the proteins in an organism) is moderately conserved across organisms (Müller et al., 2020), the variation in protein abundance and interactions due to age (i.e., life-stage) or environmental differences results in disparate phenotypes (Heck & Neely, 2020). Previous studies have demonstrated that the proteome is responsive to environmental factors (Baer & Millar, 2016; Diz & Calvete, 2016; Diz et al., 2012) and social interactions (Valcu & Kempenaers, 2015). For instance, the protein expression profile can be directly linked to dietary changes (Afshar et al., 2013), effects of thermal stress (Manjunatha, 2020), and division of labor in social animals (Li et al., 2010; Quque et al., 2019).

Historically, transcriptomic-based approaches (i.e., gene expression analysis) have been the preferred tool to infer physiological mechanisms underlying plastic responses toward environmental changes (Alvarez et al., 2015; Zuk & Balenger, 2014). However, recent studies claim that the large scale study of protein expression profiles by proteomics presents a closer proxy to the actual phenotype (Baer & Millar, 2016; Diz & Calvete, 2016; Diz et al., 2012; Feder & Walser, 2005; Valcu & Kempenaers, 2015). By focusing on proteins, the post-transcriptional and post-translational regulation processes are also taken into account (Cox & Mann, 2011). Therefore, the proteome would inform more accurately about biological functions than other “omics” (Baer & Millar, 2016; Diz et al., 2012). Furthermore, recent advances in the techniques to identify and quantify proteins (Collins et al., 2017; Gillet et al., 2012) and novel bioinformatics tools (e.g., Čuklina et al., 2018; Lambert et al., 2020; Robinson et al., 2010) allow to construct highly replicable data analysis pipelines for complex datasets and detect differential expression of proteins in multifactorial experiments. For instance, by using the data-independent acquisition (DIA) scheme, previous issues on proteomics reproducibility and under-sampling have been overcome (Zhang et al., 2020).

Proteomics represents a valuable tool to address ecological and physiological hypotheses (Baer & Millar, 2016; Diz & Calvete, 2016; Diz et al., 2012). However, there still only scarce examples of ecological studies making use of this technique (Diz & Calvete, 2016). Most studies in this regard have focused on model organisms such as Saccharomyces (yeast), Arabidopsis (rockcress), Drosophila (fruit fly), and Danio (zebrafish), as their reference proteome is already well studied (Heck & Neely, 2020; Müller & Grossniklaus, 2010). By taking advantage of the fast development of mass spectrometry-based proteomics techniques, investigations using nonmodel species of particular ecological or economic interest can be performed to unravel the physiological mechanisms underlying the phenotypic plasticity in response to changes in environmental factors.

As an example, changes in rearing conditions such as temperature (Clissold & Simpson, 2015), diet (Afshar et al., 2013; Zou et al., 2013), and organism density (Imre et al., 2004; Swift et al., 1996; Van Buskirk, 1989) may elicit critical phenotypic changes in many taxa. Considered independently, nutrition has shown to directly affect determinant traits of animals such as development time, reproduction, and longevity (Gutiérrez, Fresch, et al., 2020; Gutiérrez, Phung, et al., 2020; Lushchak et al., 2017; Templeman & Murphy, 2018). Previous studies indicate that broad dietary changes (e.g., natural versus artificial diet) have a noticeable impact on gene expression profiles, which may induce changes in physiological pathways in a range of insect species (Afshar et al., 2013; Zou et al., 2013). Yet, the physiological mechanisms of the effects derived from diets differing in their macronutrient composition (i.e., using the geometric nutrition approach, Raubenheimer et al., 2009) have been rarely studied. In like manner, the social environment can be of significant influence in determining an organism phenotype (Crocker & Hunter, 2018; Peters & Barbosa, 1977; Tammaru et al., 2000). However, few studies have shown its potential physiological effects through changing gene expression patterns only (Swift et al., 1996; Zhang, He, et al., 2020).

In the present study, we use the house cricket Acheta domesticus (L., 1758) as a model organism. This cosmopolitan insect species is widely used for behavioral and physiological studies (e.g., Crocker & Hunter, 2018; Gray, 1997; Nosil, 2002), and mass-produced as food (Oppert et al., 2020; Udomsil et al., 2019). In the cricket A. domesticus, protein makes up the bulk of its body composition in terms of macromolecules. The protein content ranges from 18% to 42% in fresh weight (Gutiérrez, Fresch, et al., 2020) and from 60% to 70% in dry weight (Udomsil et al., 2019). To the extent of our knowledge, no study to date has addressed changes in the proteome of this species in response to experimental rearing conditions. In fact, proteomics has rarely been used to study the effects of breeding conditions on experimental animals (e.g., differences in diet constitution and social environment, Sundekilde et al., 2020). Even though recent studies have used transcriptomics in a comparative fashion (e.g., Drinnenberg et al., 2014; Oppert et al., 2020), no study to date had estimated the complete protein-coding genes or proteome of the house cricket A. domesticus. However, genomic studies in related cricket species have identified 17,871 coding genes for Gryllus bimaculatus (Gryllidae) and 12,767 for Laupala kohalensis (Trigonididae) (Ylla et al., 2020).
In a recent study (Gutiérrez, Fresch, et al., 2020), we subjected the house cricket *A. domesticus* to a factorial experiment where diet composition and social environment were manipulated. Such experimental factors are considered largely influential on the survival, development, and fitness of animals (Clark et al., 2015; Crocker & Hunter, 2018; Dávila & Aron, 2017; Han & Dingemanse, 2017; Swift et al., 1996). We showed that *A. domesticus* exhibited considerable sex-specific differences to disparate rearing conditions. Overall, diet composition affected development time, life span, and fecundity. The social environment affected the survival of immature crickets, food consumption, and lipid content of the house crickets. Although the previously described outcomes demonstrate the remarkable plasticity in many traits of this insect species, the underlying physiological mechanisms for such responses have remained largely unknown. Here, we studied the proteome of crickets subjected to the previously described experiment utilizing high-performance liquid chromatography coupled to tandem mass spectrometry (HPLC-MS/MS) (Aebensold & Mann, 2016). We applied a bottom-up approach (i.e., protein digestion is carried out prior mass spectrometric analysis) for the quantitation and characterization of proteins of the cricket *A. domesticus* using the method of sequential window acquisition of all theoretical mass spectra (SWATH-MS), a highly reproducible data-independent acquisition (DIA) technique for comparative proteomics (Collins et al., 2017; Gillet et al., 2012).

We aimed to unravel physiological changes by detecting differentially expressed proteins in both female and male crickets feeding on either of two diets differing in nutritional composition and housed either solitarily or in groups. The selection of experimental conditions (as explained in Gutiérrez, Fresch, et al., 2020) was based on previous studies that demonstrated that the social environment (Jonsson, 2017; Prokopy & Roitberg, 2001), and the proportion of macronutrients (Harrison et al., 2014) have a striking effect on cricket survival and development. Even though our experiment may appear as merely exploratory and descriptive, we were motivated by the following hypotheses: (a) natural differences in the proteome and enriched pathways of male and female cricket would be evident due to sex-specific traits and disparate resource investment strategy between the two sexes (i.e., energy storage, mate finding, reproduction); (b) crickets fed with the protein-rich diet will exhibit overexpression of storage proteins (Haunerland, 1996); (c) females living in groups will evidence enrichment of metabolic pathways related to lipid deposition as suggested by our previous study (Gutiérrez, Fresch, et al., 2020); and (d) females will show physiological responses to diet composition related to reproduction and longevity, as previous studies have shown that a protein-rich diet increases fecundity and shortens insect life span (Gutiérrez, Fresch, et al., 2020; Harrison et al., 2014). Specifically, we expected to find underexpression of proteins involved in the nutrient-sensing TOR (target of rapamycin) pathway in females fed with a balanced diet. Previous studies have shown that in many eukaryote species, inhibition of the TOR pathway is related to increased life span (reviewed by Lushchak et al., 2017).

## 2 MATERIALS AND METHODS

### 2.1 Experimental design and sample collection

In this study, we used samples collected from a recent experiment (Gutiérrez, Fresch, et al., 2020) in which we subjected the house cricket *A. domesticus* (L. 1758) to distinct experimental conditions. In that experiment, we manipulated the diet composition (diets contained either 1:1 or 3:1 protein to carbohydrate ratio), and the social environment (insects reared either in solitude during their whole life cycle or at a density of 800 inds./m²—i.e., six individuals in every container). For further details on experiment setup and insect husbandry, see Gutiérrez, Fresch, et al. (2020). Here, we studied the proteome of the house cricket *A. domesticus* following a three-factor experimental design (i.e., Diet × Social Environment × Sex) with eight resulting experimental treatments. We collected five biological replicates (i.e., whole insects) for every experimental treatment (N = 40). All insects were collected 15 days after the adult molt to avoid introducing confounding factors caused by age-related physiological differences. All insects were alive at the moment of collection and immediately frozen at −20°C.

### 2.2 Sample preparation

Five biological replicates (i.e., whole crickets) from every one of the eight experimental treatments (N = 40) were subjected to proteome analysis using a bottom-up approach (Kettman et al., 2001). Additionally, three samples of each diet (i.e., 3:1 and 1:1) were also processed to identify the dietary protein profile accurately and exclude such diet-derived proteins from the final cricket proteome dataset. All reagents were obtained from Merck KGaA (Germany) unless otherwise stated.

Samples were individually homogenized in extraction buffer in a 1:20 w:v ratio (6 M urea, 1 M thiourea, 0.05 M Tris-HCl, pH = 8.0) using an Ultra-Turrax (T18, IKA®-Werke GmbH & Co., Germany) at 1,030 g for 2 min. Subsequently, the samples were centrifuged at 10,410 g for 1 h at 4°C (Z326K, Hermle, Germany). The supernatant containing the extracted proteins was stored at 4°C and used for posterior procedures. The protein concentration for each sample was determined by using the Bradford protein quantification assay (Bradford, 1976). In brief, the protein concentration of every sample is calculated through a linear regression model of a known reference sample (bovine serum albumin) using absorption spectroscopy (Spectrostar nano, BMG Labtech, Ortenberg, Germany).

Two technical replicates were prepared by precipitating 500 μg of protein per sample with acetone and left overnight at −20°C. Posteriorly, samples were transferred to digestion buffer (6 M urea, 0.1 M Tris-HCl) and treated with solutions of dithiothreitol (for reduction) and then iodoacetamide (for alkylation) to prevent reformation of disulfide bonds. Later, all samples were digested to peptides with 10 μg of trypsin overnight at 37°C. The digested peptides were submitted to solid-phase extraction (Strata™-X 33 μm, Phenomenex,
Germany) to remove impurities, and the eluent was evaporated in a vacuum concentrator at 30°C and 150 g (RVC 2–18 CDplus, Martin Christ Gefriertrocknungsanlagen GmbH, Germany). The peptides were dissolved in 50 µl of a solution of 3% acetonitrile and 0.1% formic acid. An additional 1:50 (v/v) dilution with the same solution was performed to allow for injection in the microscale liquid chromatography system. Samples were finally stored at −20°C until measurement took place.

2.3 | Sample analysis (HPLC-MS/MS)

The subsequent chromatographic and mass spectrometric parameters were identical in both acquisition modes: information-dependent acquisition (IDA) and sequential window acquisition of all theoretical mass spectra (SWATH). An aliquot of 5 µl of every digested sample (about 1 µg of peptides) was injected on an analytical column preceded by a guard column (YMC-Triart C18, pore size 120 Å, length 150 mm for analytical column and 5 mm for guard column, inner diameter 0.3 mm, particle size 3 µm) using an M3 MicroLC system (Sciex, Darmstadt, Germany). The mobile phase was composed of 0.1% formic acid in water (Solvent A) combined with 0.1% formic acid in acetonitrile (Solvent B). A 3%-40% B three-step gradient of 73 min was used for peptide separation at 7 µl/min and 40°C prior column wash at 80% B for 3 min and equilibration at 3% B for 8 min. The mass spectrometric analysis was conducted in a positive ion mode and high sensitivity mode on a TripleTOF® 6,600 mass spectrometer (Sciex, Darmstadt, Germany) with a total acquisition time of 85 min. HPLC gradient and ion source parameters are available in Table S1 in the supplementary material.

IDA was performed on eight representative cricket samples (one from every experimental treatment) and two digested diet samples (one of each diet type). In IDA, a predetermined number of precursor ions are selected and analyzed, this step was necessary for the generation of SWATH variable windows setting and spectral ion library. The instrument cycle lasted 2.25 s and consisted of an MS survey scan with an accumulation time of 250 ms, followed by MS/MS scans for the top 30 precursor ions above the intensity threshold of 150 cpsi each with an accumulation time of 65 ms. Additional IDA parameters were as follows: MS/MS resolution, LOW; precursor charge state, from 2 to 5; mass tolerance, 100 ppm; exclude former target ions for 12 s. Following IDA analysis, SWATH was conducted on all digested cricket and diet samples. Fifty precursor isolation windows were defined using the SWATH Variable Window Calculator (Sciex) based on the density of precursors across the m/z range in a corresponding IDA run (cricket or diet), with a minimum window width of 3 m/z and a window overlap of 1.0 Da. The instrument cycle lasted 1.6 s and included one MS survey scan with an accumulation time of 50 ms, and SWATH-MS/MS scans with each an accumulation time of 30 ms. The MS/MS resolution was defined as UNIT. All 80 cricket samples (40 biological replicates * two technical replicates) were measured consecutively in the same MS machine over three days to avoid batch effects, yet, the order of the samples was not randomized. A clean run with the injection of methanol lasting 20 min was performed every five samples to clean the separation column.

2.4 | In silico construction of the reference proteome

In order to construct the reference proteome, several RNA-Seq datasets of *A. domesticus* were downloaded from the Sequence Read Archive (https://www.ncbi.nlm.nih.gov/sra, the datasets can be accessed with the codes SRR1552491, SRR2230498, SRR6001368, SRR6250553, SRR6250554). Sequencing adapters were removed using BBduk from BBTools v37.93 (Bushnell & Rood, 2018) by matching a 23-mer with a single mismatch to an adapter reference. Low-quality regions at the 3´-end were removed applying a PHRED 20 quality threshold, and sequence reads with an average quality below PHRED 20 were entirely discarded. Preprocessing success was manually inspected by FastQC v0.11.5 (Andrews, 2010). Clean data were assembled into a reference transcriptome using Trinity v2.8.4 (Grabherr et al., 2011) with a k-mer size of 23. Subsequently, resulting contigs were scanned for putative coding regions of minimum 100 aa length by TransDecoder v5.5.0 (Haas & Papanicolaou, 2017). To address the completeness and quality of the inferred in silico proteome, a search for conserved protein domains was performed using DOGMA v3.4 (Dohmen et al., 2016), followed by a search for single-copy orthologs via BUSCO v3.1.0 (Simão et al., 2015; Waterhouse et al., 2018) using an insect dataset. We used BLAST+ v2.9.0 (Altschul et al., 1990; Camacho et al., 2009) with Swiss-Prot/UniProtKB database (Release 2019_07) and an e-value cutoff of 1e−5 to identify orthologs.

2.5 | Generation of the spectral ion library

The eight IDA files of cricket samples (described above) were simultaneously analyzed in ProteinPilot (Version 5.0.1, AB Sciex) with the Paragon algorithm (Shilov et al., 2007) against the constructed reference proteome complemented by egg and milk protein sequences downloaded from UniProtKB in January 2019 (207,051 entries). The same was done for the two IDA files of diet samples but only against egg and milk proteins (16 entries). Search parameters are available in Table S2 in the supplementary material.

2.6 | SWATH data processing

SWATH data were processed using the SWATH Acquisition MicroApp (Version 2.0.1, AB Sciex) in PeakView (Version 2.2, AB Sciex). The generated ProteinPilot "group" file was imported by setting the maximum number of proteins to be imported at 880 (recorded from the FDR report: 1% Global FDR) for crickets and at 16 for diets without importing peptides shared by more than one protein. Eleven endogenous peptides from *A. domesticus* were manually
selected and used for retention time calibration in cricket samples, whereas fifteen milk and egg peptides were used in diet samples (see Figures S1 and S2 in the supplementary material). Processing settings for peak extraction are available in Table S3 in the supplementary material. All proteins included in the dataset were identified with one to six peptides (Gupta & Pevzner, 2009) (see Table S4 in the supplementary material). We successfully assigned 517 unique Uniprot ID’s (some of them exhibiting several isoforms in the dataset), while 81 remained unidentified (see the raw data in the supplementary material).

### 2.7 | Statistical analysis

Samples were subjected to normalization using the library proBatch (Čuklina et al., 2018) on Bioconductor (Gentleman et al., 2004) as explained below. First, proteins from the diet (see the raw data in the supplementary material) were excluded from the dataset as their areas had likely been influenced by gut content at the moment of sample collection. Additionally, two biological replicates (one from every treatment, 3:1-Solitary-Female and 3:1-Solitary-Male) had to be excluded from the dataset because of evident anomalies in protein profiles after data acquisition likely due to problems during sample preparation (e.g., extremely high values for some proteins and absence of values for others). Subsequently, protein areas were Log$_2$-transformed, and quantile normalization was performed using the "normalize_data_dm" function. With this normalization method, the distribution of raw signal intensities is homogenized across samples (i.e., the distributions of individual samples is set to the same quantiles); this method assumes that protein areas are constant among samples and the distribution would be similar in consequence (Čuklina et al., 2018). Posteriorly, the protein areas of technical replicates were averaged using arithmetic means (two technical replicates for every sample, the mean Pearson correlation coefficient between technical replicates was 0.928 ± 0.015 (x̅ ± SD), see Table S5 in the supplementary material). Exploratory data analysis was done using a principal component analysis (PCA) with the library factoextra ("prcomp" and "fviz_pca_ind" functions) (Kassambara & Mundt, 2017), and a hierarchical clustering heatmap using Euclidean distance with the library proBatch ("plot_heatmap_diagnostic" function) (Čuklina et al., 2018). In both cases (PCA and heatmap), we used the Log$_2$-transformed and quantile-normalized data.

The differential protein expression (DPE) analysis was performed using the tool DiCoExpress (Lambert et al., 2020), which integrates functions from the library edgeR (Robinson et al., 2010) for this purpose. Differential expression was tested using generalized linear models based on the negative binomial distribution where the experimental factors (described above) and the interaction of them were included in the models. The p-values were adjusted to control the false discovery rate by the Benjamini–Hochberg procedure, and an alpha (FDR) of 0.05 was considered as the threshold for significance. Additionally, in order to conduct enrichment analysis, we retrieved gene ontology (GO) terms (Ashburner et al., 2000) and metabolic pathways (Kanehisa & Goto, 2000) associated with the proteins identified in our dataset. GO terms were retrieved from UniprotKB for all proteins using the library UniprotR (Soudy et al., 2020), and metabolic pathways were retrieved from the Kyoto Encyclopedia of Genes and Genomes (KEGG) in a two-step process. First, KEGG Orthology (KO) terms were assigned to our list of proteins using the tool Retrieve/ID mapping from the UniprotKB website (Pundir et al., 2016). Subsequently, the KO terms were used to retrieve the pathways using the tool KEGG Mapper (Kanehisa & Sato, 2020). The enrichment analysis was performed using hypergeometric tests with the function "phyper" integrated into DiCoExpress (Lambert et al., 2020) for three different GO domains (i.e., biological processes, molecular functions, and cellular components), as well as for the KEGG pathways, using a subset of proteins differentially expressed according to the DPE analysis described above. The complete set of proteins and their respective annotations were used as the reference list. We used an alpha (p-value) of 0.05 to classify GO terms and KEGG pathways as overrepresented. All data analysis was performed in R 4.0.2 (R Core Team, 2019) using RStudio (RStudio Team, 2015). All data files (protein areas, GO terms, and KEGG pathways) and scripts can be accessed in the FigShare repository (https://doi.org/10.6084/m9.figshare.13341680.v1). Mass spectrometry data are available in the ProteomeXchange repository with the identifier PXD025535.

### 3 | RESULTS

#### 3.1 | Reference and identified proteome of Acheta domesticus

The in silico assembly of the A. domesticus reference transcriptome resulted in 1,450,907 contigs with a mean contig size of 422 nt. Subsequent prediction of putative coding regions with TransDecoder resulted in 207,035 open reading frames (ORFs; minimum 100 aa; reduced to 116,072 ORFs when omitting multiple ORFs per transcript), with the longest being 8,086 and a mean size of 234 amino acids. The in silico proteome of A. domesticus constructed in this study appears to be of high completeness; DOGMA found 93.5% (n = 4,431) of all insect Conserved Domain Arrangements and 98.3% of all insect BUSCO orthologs (n = 1,658) were represented in either complete (97.2%) or fragmented (1.1%) form in the proteome. However, it is worth mentioning that the in silico proteome was not screened for duplicates; therefore, several ORFs might be redundant. After processing the SWATH data, the final proteome dataset of the house cricket A. domesticus contained a list of 685 proteins (without considering 16 proteins from the diet that were excluded from the dataset for the data analysis. Table S4 in the supplementary material). From these, 517 proteins (and in some cases, up to six isoforms of the same proteins) were identified using the UniprotKB (at an e-value cutoff of 1e$^{-5}$), and 81 proteins remained unidentified. In
**FIGURE 1** Hierarchical clustering heatmap showing the relative protein abundance and clustering of biological samples according to the experimental factors. The color code on the bottom right corner indicates the Log$_2$-transformed protein area. The labels in the x-axis indicate the sample identity (e.g., cricket 1.1GrF.3) by naming the diet (1.1 or 3.1, protein to carbohydrates ratio), social environment (Sol and Gr, for solitary and group), sex (F and M, for female and male), and replicate number (1–5).
our dataset, all proteins can be found in all samples, that is to say, no protein was exclusively expressed by particular sex or under a specific experimental condition.

3.2 | Data quality and exploratory analysis

The diagnostic analysis of the samples revealed that there were no batch effects (i.e., unwanted variation) after data normalization (Figure S3 in the supplementary material), but rather the experimental factors (i.e., sex, diet, and social environment) were driving the clustering of the samples. On the one hand, the hierarchical clustering heatmap (Figure 1) showed that the factor “sex” explains the clustering pattern at a considerable extent. Conversely, the factors “diet” and “social environment” did not exhibit an apparent clustering among biological replicates, thus suggesting that these factors elicited changes in a lower number of proteins (in comparison with that of sex). This same result pattern is confirmed by the PCA analysis (Figure S4 in the supplementary material), in which a clear differentiation between sexes can be seen. Nevertheless, some subtle differences among experimental conditions can be discerned in male crickets, but not in female crickets. From the initial data exploration, it can also be inferred that protein quantity exhibited a considerable variation (Figure 1), but only a few proteins have remarkably high values. Nevertheless, this observation is not surprising as, in this study, we performed a proteome-wide assessment of whole insects.

3.3 | Differentially expressed proteins

The differential expression analysis revealed that a substantial number of proteins had different relative abundances depending on the experimental factors. The factor “sex” led to differential expression of 280 proteins (40.87% of the proteins identified in this study) (Figure 2). Female crickets overexpressed 148 proteins (21.60%) while 132 proteins (19.27%) were overexpressed in male *A. domesticus* across experimental conditions (see complete protein list and statistical results in Table S6 in the supplementary material). Due to this striking difference between sexes, we studied the effects of diet and social environment on male and female cricket protein expression separately for a more precise interpretation of the results.

Male *A. domesticus* protein expression profile was affected by diet composition (Figure 3). The balanced diet induced overexpression of polyubiquitin (fold-change $\log_2 = 2.03$, Likelihood ratio = 37.085, FDR < 0.001, Figure 3a), while the protein-rich diet led to overexpression of dehydrogenase/reductase SDR family member 11 (DHRS11) (fold-change $\log_2 = 1.76$, Likelihood ratio = 21.71, FDR < 0.001, Figure 3b), phosphoenolpyruvate carboxykinase (PEPCK) (fold-change $\log_2 = 1.37$, Likelihood ratio = 16.94, FDR = 0.009, Figure 3c), adult-specific rigid cuticular protein 15.5 (ACP15.5) (fold-change $\log_2 = 1.29$, Likelihood ratio = 13.22, FDR = 0.039, Figure 3d), and pupal cuticle protein C1B (CUC1B) (fold-change $\log_2 = 0.82$, Likelihood ratio = 13.19, FDR = 0.039, Figure 3e) in male crickets. Nonetheless, neither the social environment alone nor the interaction between diet and social environment influenced the relative abundance of any of the proteins of male *A. domesticus*.

In like manner, female crickets were as well affected by diet composition (Figure 4). In this case, the balanced diet elicited overexpression of betaine-homocysteine S-methyltransferase 1 (BHMT) (fold-change $\log_2 = 1.61$, Likelihood ratio = 18.00, FDR = 0.014, Figure 4a), dolichyl-diphosphooligosaccharide–protein glycosyltransferase (DDOST) (fold-change $\log_2 = 1.61$, Likelihood ratio = 15.47, FDR = 0.017, Figure 4b), 40S ribosomal protein S15a (RPS15A) (fold-change $\log_2 = 1.29$, Likelihood ratio = 15.10, FDR = 0.017, Figure 4c) and 6-phosphogluconate dehydrogenase (6PGD) (fold-change $\log_2 = 0.90$, Likelihood ratio = 16.84, FDR = 0.014, Figure 4d), while the protein-rich diet did not induce a significant overexpression in any protein of female *A. domesticus*. Furthermore, the interaction of sex and social environment caused an interesting pattern of protein expression. Specifically, female crickets living in groups but fed with different diets had overexpression of PDZ and LIM domain protein Zasp (Zasp52) (fold-change $\log_2 = 1.54$, Likelihood ratio = 15.80, FDR = 0.039, Figure 4f) when fed with the protein-rich diet, while delta-1-pyrroline-5-carboxylate synthase (P5CS) (fold-change $\log_2 = 1.42$, Likelihood ratio = 13.72, FDR = 0.048, Figure 4f) was overexpressed when female crickets received the balanced diet. Similar as for male crickets, social environment alone did not influence the relative abundance of any of the proteins of female *A. domesticus*.

![Figure 2](image-url) Proteins that exhibited significant differential expression when comparing male and female *Acheta domesticus*. For a complete list of proteins and their corresponding FDR values, please see Table S4 in the supplementary material.
and its child-term “protein homodimerization activity” did not re-nobiotics, and the molecular function “identical protein binding” and actin-binding. Furthermore, the cellular component that held functions that supported such features were hydrolase activity with an increase in metabolism and locomotion, the molecular hormone pathway), and specifically, energy (i.e., oxidative phosphorylation) and lipid metabolism (the latter was mainly related to fatty acids). Moreover, males had increased representation of locomotion and associated muscle traits (i.e., muscle development and homeostasis, cGMP-PKG signaling pathway). In accordance with an increase in metabolism and locomotion, the molecular functions that supported such features were hydrolase activity and actin-binding. Furthermore, the cellular component that held the overexpressed proteins was mainly the mitochondrion. Yet, the overrepresentation of biodegradation and metabolism of xenobiotics, and the molecular function “identical protein binding” and its child-term “protein homodimerization activity” did not relate straightforwardly to the aforementioned enriched pathways or terms.

### 3.4 Enrichment analysis

We identified 5,126 GO terms associated with 512, out of the 685, proteins in our cricket proteome dataset. From these terms, 2,221 annotations corresponded to biological processes, 1,389 to molecular functions, and 1,526 to cellular components. Additionally, we mapped 325 KO ID’s for 344 proteins, while 341 remained without any associated KO ID. Using the KEGG mapper, we retrieved a total of 271 pathway annotations. Enrichment analysis was performed for the set of proteins with differential expression caused by the factor “sex”. In both cases (i.e., male and female crickets), a considerable overlap can be noticed between the enriched KEGG pathways and GO terms. Furthermore, a tight association among GO domains (i.e., biological processes, molecular functions, and cellular components) is evidenced in our analysis.

In the first place, male crickets (Figure 5a) exhibited an overrepresentation of metabolic pathways (i.e., thermogenesis, thyroid hormone pathway), and specifically, energy (i.e., oxidative phosphorylation) and lipid metabolism (the latter was mainly related to fatty acids). Moreover, males had increased representation of locomotion and associated muscle traits (i.e., muscle development and homeostasis, cGMP-PKG signaling pathway). In accordance with an increase in metabolism and locomotion, the molecular functions that supported such features were hydrolase activity and actin-binding. Furthermore, the cellular component that held the overexpressed proteins was mainly the mitochondrion. Yet, the overrepresentation of biodegradation and metabolism of xenobiotics, and the molecular function “identical protein binding” and its child-term “protein homodimerization activity” did not relate straightforwardly to the aforementioned enriched pathways or terms.

In contrast, female crickets (Figure 5b) had an overrepresentation of processes related to genetic information such as translation (i.e., enriched ribosome activity) and protein processing in the endoplasmic reticulum. Additionally, female crickets had a higher representation of glucose metabolism and catabolism of proteins and lipids. Likewise, enrichment of the lysosome was likely associated with catabolic processes of macromolecules. The molecular functions overrepresented were mainly related to protein synthesis (i.e., rRNA binding and ribosome) and metabolism (i.e., lipid transporter activity and nutrient reservoir activity). Besides, the cellular components enriched in female crickets were the extracellular region, ribosome (both large and small subunits), lysosome, and nucleolus.

The complete enrichment analysis results for both sexes are available in Table S7 in the supplementary material. Enrichment analysis was not conducted for the other subsets of proteins as those lists were too short (i.e., less than seven proteins affected by diet and social environment in male and female crickets). Yet, we manually linked the GO terms (focusing mainly on biological processes) and KEGG pathways for the subsets of proteins overexpressed in every experimental condition (Figure 6). First, the differentially expressed proteins in male crickets in response to diet constitution were related to the biosynthesis of hormones, cuticle structure, carbohydrate metabolism, and several signaling pathways (Figure 6a). The lack of annotations for polyubiquitin in all consulted databases precludes the possibility to link this protein with any biological process in male A. domesticus. In contrast, the differentially expressed proteins in female crickets in response to diet constitution, and the interaction of diet and social environment, were associated with carbohydrate metabolism, several phases of the protein synthesis process (i.e., amino acid metabolism, translation, and protein folding), and development of muscle structure (Figure 6b).
In this study, we mapped the proteome of age-standardized crickets *A. domesticus* subjected to a range of experimental conditions utilizing high-throughput MS-based techniques. We used a highly reproducible method for protein identification and quantitation (Collins et al., 2017), and a recently developed framework for the multifactorial analysis of "omics" datasets (Lambert et al., 2020). We found that sex alone explained a significant proportion of the differentially expressed proteins and their associated biological processes and pathways. Moreover, we were able to directly link effects of contrasting experimental conditions (i.e., different diet composition and social environments) to changes in protein expression and performed a functional analysis of the differentially expressed proteins.

**FIGURE 4** Changes in protein expression elicited by the experimental conditions in female *Acheta domesticus*. Diet composition affected the expression of betaine-homocysteine S-methyltransferase 1 (a), dolichyl-diphosphooligosaccharide–protein glycosyltransferase (b), 40S ribosomal protein S15a (c), and 6-phosphogluconate dehydrogenase (d). The interaction of diet and social environment induced differential expression of PDZ and LIM domain protein Zasp (e) and Delta-1-pyrroline-5-carboxylate synthase (f). Protein to carbohydrates ratios 3:1 and 1:1 stand for the protein-rich and the balance diet, respectively. FDR stands for false discovery rate, a method for adjusting p-values and controlling type I errors.
making use of comprehensive functional annotations databases (i.e., KEGG and GO).

4.1 Different proteome profile in male and female crickets

A substantial portion of the proteome of the cricket *A. domesticus* exhibited a remarkable variation in protein abundance due to sex. This finding supports our first hypothesis and is of particular relevance given that in our previous study, we had found that females *A. domesticus* possessed a higher protein content (bulk) than males. However, the relative content was similar among sexes (Gutiérrez, Fresch, et al., 2020).

In this study, we demonstrated that divergent life strategies in males and females of a single species might be explained by the differences in the relative abundance of particular proteins. Enriched KEGG pathways and GO terms suggest that female crickets exhibited an increase in protein and lipid catabolism and glucose metabolism—a phenomenon that is likely related to the heavy investment in oogenesis that female crickets undergo in early adulthood (Gutiérrez, Fresch, et al., 2020; Smykal & Raikhel, 2015). Contrarily, male crickets were characterized by pathways and processes that suggest a more active life and a higher metabolic activity. Enrichment of proteins related to locomotion may be motivated by multiple factors such as foraging, intraspecific aggression, or finding mating partners (Hack, 1997; Nosil, 2002). Furthermore, the overrepresentation of processes related to movement and metabolism in male crickets may imply higher metabolic rates and oxidative damage (caused by reactive oxygen species) in these individuals. This possibility would partially explain the relatively shorter life span of males in this species (Visanuvimol & Bertram, 2011). Additionally, male crickets apparently used thermogenesis (at least to a greater extent than females) as a mechanism for energy dissipation (Simpson & Raubenheimer, 2009).

4.2 Effects of diet composition on male cricket proteome

Male *A. domesticus* had the relative abundance of five proteins affected by the experimental conditions imposed in our study. Even though previous studies suggested that diet composition would induce major changes in gene expression (Zou et al., 2013), we did not find a large set of proteins with noticeable changes in their expression in response to altered diet composition. However, the differentially expressed proteins detected here are involved in several significant pathways and processes. In four out of the five differentially expressed proteins (i.e., PEPCK, DHR11, ACP15.5, and CUC1B), overexpression was triggered by the protein-rich diet. The remaining protein, polyubiquitin, was overexpressed when males were fed with a balanced diet. The function of polyubiquitin was not yet determined in the database consulted (i.e., KEGG and GO), but this protein is involved in selective autophagy to avoid the accumulation of cytotoxic aggregates (Morimoto et al., 2015; Nguyen et al., 2014). Polyubiquitin serves as well as a recognition signal for the proteasome (i.e., multi-subunit enzymes responsible for proteolysis in the cytosol) for protein degradation processes (Li, 2018). This suggests that a higher abundance of this protein in male crickets fed with a

![FIGURE 5](image-url)
balanced diet would increase their ability to avoid cell damage (comparatively with male crickets fed with the protein-rich diet).

The enzyme PEPCK is involved in regulating gluconeogenesis and glyceroneogenesis (Yang et al., 2009), which may influence the synthesis of triglycerides in a cascading effect (Okamura et al., 2007). Additionally, PEPCK is also associated with several signaling pathways. For instance, AMPK (AMP-activated protein kinase), which increases catabolism upon activation (i.e., breakdown of macromolecules to produce energy), thus stimulating ATP production (Herzig & Shaw, 2018). Also, the insulin pathway, which is involved in the biosynthesis and control of hormones (e.g., juvenile hormone and ecdysone) (Smykal & Raikhel, 2015) and plays an important role in the regulation of carbohydrate and lipid metabolism (Saltiel & Kahn, 2001). This increased abundance of PEPCK may partially explain why crickets fed with distinct diets achieved similar whole-body lipid content (Gutiérrez, Fresch, et al., 2020). While the balanced diet would supply enough carbohydrates to build their lipid reservoir (Simpson & Raubenheimer, 2012), the crickets fed with a protein-rich diet would make use of a different pathway to convert excess protein into lipids. For instance, gluconeogenesis via gluconogenic amino acids (Schutz, 2011).

Additionally, the enzyme DHR11 is involved in the biosynthesis of steroid hormones (Endo et al., 2016, 2019). Therefore, we would expect that a higher expression of this protein (i.e., DHR11) in male crickets fed with the protein-rich diet would influence the production of ecdysone. In insects, ecdysone is the primary steroid hormone and has important postdevelopmental roles in stress resistance and longevity (Tatar et al., 2003; Uryu et al., 2015). Furthermore, the proteins ACP15.5 and CUC1B are structural constituents of cuticle in arthropods (Andersen et al., 1997; Norup et al., 1996). A higher abundance of these proteins in male crickets fed with the protein-rich diet suggests the potential use of the

![Figure 6](image_url)
cuticle as a repository of excess proteins, which partially agrees with our second hypothesis. In other insect species, cuticle structure and thickness have been suggested to be related to the nutritional status of the organism and the food quality they can access (Nalepa, 2011; Peeters et al., 2017) as cuticle structure depends largely on nitrogen availability (Andersen, 1979). However, these proteins (i.e., ACP15.5 and CUC1B) were not differentially expressed in female A. domestici cus fed with the protein-rich diet.

The lack of effect of social environment on the proteome profile of male A. domesticus may suggest that intense interaction with conspecifics, which may suppose competition for resources and the interaction with diet composition affected several phenotypic traits of male crickets. However, the underlying mechanisms for such changes remain to be elucidated in future studies.

### 4.3 Effects of diet composition and social environment on female cricket proteome

Female crickets also exhibited overexpression of a small number of proteins in response to the experimental conditions of our study. In this case, both the diet composition and the social environment had a significant effect on the relative abundance of specific proteins. Four proteins (i.e., BHMT, 6PGD, DDOST, and RPS15A) were affected by diet constitution only, being overexpressed when female A. domesticus were fed with the balanced diet. The remaining proteins, Zasp52 and P5CS, were affected by the interaction of diet composition and social environment.

All proteins that were responsive to diet constitution in female crickets are involved in several protein synthesis steps such as amino acid metabolism, translation, and protein folding. In our previous study, we demonstrated that female crickets fed with a balanced diet were characterized by a longer life span and reduced fecundity, in comparison with females fed with the protein-rich diet (Gutiérrez, Fresch, et al., 2020). While the inhibition of protein synthesis in other species has been related to life span extension (Pan et al., 2007), in our results, we found a seemingly contradicting pattern. Females fed with a balanced diet showed overexpression of proteins involved in the metabolism of amino acids. In a balanced diet, crickets would find a lower quantity of amino acids when compared with the protein-rich diet, and accordingly, protein biosynthesis would be limited. As a response, crickets would need to increase the expression of proteins involved in amino acids synthesis and metabolism, and in a later stage, increase protein biosynthesis.

Regarding our fourth hypothesis, we did not find changes in the expression of proteins related to the TOR pathway. Contrarily, the functions of the overexpressed proteins in females fed with a balanced diet suggest that a longer life span and reduced fecundity may result from a higher energy investment in preventing cell damages. For instance, BHMT is an enzyme with an important role in the metabolism of amino acids (Pérez-Miguelanz et al., 2017) and the maintenance of the cellular osmolytic equilibrium (Picha et al., 2013). RPS15A is a structural component of the ribosomes, the organelles that catalyze protein synthesis (Anger et al., 2013), and DDOST is an essential subunit of the N-oligosaccharyl transferase (OST) complex for protein modification (i.e., N-glycosylation) (Pfeffer et al., 2014; Roboti & High, 2012). Even though 6PGD is an enzyme primarily involved in carbohydrate metabolism (pentose phosphate pathway) for the production of NADPH (Hisar et al., 2009), it is also required for structural integrity and protein secretion (H. Li et al., 2019; Yang et al., 2016). Additionally, P5CS is as well an enzyme involved in the metabolism of amino acids; when overexpressed, it can cause accumulation of proline, usually as a protective mechanism in response to environmental stress (Yang et al., 2020). However, this protein was overexpressed only when grouped females were fed with a balanced diet and not in their solitary counterparts.

Conversely, the protein Zasp52 was only overexpressed when grouped female crickets were fed with a protein-rich diet. This protein is involved in muscle structure; it is localized in the Z-disks and is explicitly required for muscle attachment and myofibril assembly (Katzemich et al., 2011; Liao et al., 2020). The aforementioned suggests that investment into muscle tissue was higher when females were under these specific experimental conditions (i.e., grouped and fed with a balanced diet). We did not find overexpression of storage proteins in crickets (male or female) fed with the protein-rich diet as we initially hypothesized (second hypothesis). The excess of protein was instead metabolized or deposited in different tissues in other forms as storage proteins usually are more advantageous for insects in their early developmental phase (Hauerland, 1996). Furthermore, females living in groups did not exhibit differential expression of proteins linked to lipid deposition as we initially hypothesized (third hypothesis) according to our previous findings (Gutiérrez, Fresch, et al., 2020).

### 4.4 Caveats

In the development of this study, we identified two issues that might restrict the scope of our results. First, we extracted the proteins from the whole body of the insects. While this can be considered to be a positive point as in this manner, our dataset would include the major fraction of the expressed proteome of the house cricket A. domesticus. Tissue- and organ-specific processes might be obscured by considering the relative abundances of proteins at the organism scale. In previous investigations using "omics" technologies in ecological studies, tissue-specific analysis seems to be the rule rather than the exception (Diz & Calvete, 2016). In our study, we decided to extract proteins from whole-body samples as we regard our experimental treatments (and insect age) to be highly standardized. Therefore, we consider our samples to be truly comparable (e.g., with slight intra-treatment variation). Additionally, we aimed to trace the entire protein profile without a preconception (or bias) toward particular organs. Thus, we are confident that our study could detect...
even minor differences in proteins due to the high standardization of treatments.

Secondly, the limited amount of information about our model species, the cricket *A. domesticus*, may at least partially preclude a more complete identification of proteins and their physiological roles (Bräutigam et al., 2008; Heck & Neely, 2020; Oppert et al., 2020). Therefore, the functional annotations, although comprehensive, may not represent the full picture of physiological changes induced by the experimental factors manipulated in our study. Yet, it is worth mentioning that even though the functional annotation (i.e., GO terms) information is commonly acquired through experimental methods, most of the association of terms and proteins is done with computational methods and only a small fraction is manually curated (Rhee et al., 2008). In this study, we retrieved GO terms from UniprotKB, which is based mainly on manual annotations, and the potentially incidental annotations (e.g., contributes_to, colocalizes_with) are not considered.

Some critics may argue that 685 proteins are a relatively small number for an insect proteome, especially when compared with previous studies that mapped over 10 thousand proteins for a single species (e.g., Zdobnov et al., 2002). Nevertheless, it is worth noticing that the number of proteins identified in this study is similar to others using SWATH-MS (Fabre et al., 2019; Okada et al., 2016). We consider that number of quantified proteins in this study is the outcome of several entangled factors, yet they do not compromise the veracity of our results. First, we analyzed the proteome contained in the whole body of the insects; consequently, it is expected to capture the most abundant proteins at the organism level. Second, our sample-preparation protocol may have excluded low-abundance proteins as these usually require particular methods aiming to improve proteome coverage (Blankenburg et al., 2019). Third, by using a non-model organism, we faced a more remarkable lack of information than other studies (Bräutigam et al., 2008). And lastly, we ensure high specificity in the identification of the proteins to avoid false discoveries.

5 | CONCLUSIONS

All considered, our study showed that male and female *A. domesticus* exhibited unique responses to experimental conditions where diet and social environment were manipulated. Therefore, our findings support the initiative of assessing sex-specific responses in ecological and physiological studies (Goos et al., 2016; Maklakov et al., 2008; Morehouse et al., 2010). Furthermore, our results shed new light on the physiological mechanisms that allow for plasticity in this omnivorous insect species. The ability to modify the relative abundances of particular proteins would confer this species the capability to inhabit a considerable range of habitats and exploit different nutritional resources of distinct macronutrient ratios (Gutiérrez, Fresch, et al., 2020; Morales-Ramos et al., 2018; Visanuvimol & Bertram, 2011). While a few proteins were responsive to our experimental treatments, it is recognized that changes in single proteins may significantly impact the phenotypes (Baer & Millar, 2016), as proteins usually have multiple functions and may interact with other proteins and macromolecules in complex networks (Baer & Millar, 2016).

The treatments manipulated here (i.e., diet composition and social environment) induced sex-specific changes in a small set of proteins with particular roles. It was evident that diet had a greater influence on the proteome of the cricket *A. domesticus* than the social environment. Most changes evidenced in female crickets suggest an alteration of the protein synthesis processes, carbohydrate metabolism, and muscle structure development. Simultaneously, the proteins responsive to the diet in male crickets suggest changes in hormone production, carbohydrate metabolism, and apparently the deposition of excess protein in the cuticle.

Although many recent studies have addressed the changes in metabolites (including proteins and lipids) in specific tissues of a wide range of organisms, to our knowledge, this is the first endeavor to study the complete proteome of an invertebrate animal across several different experimental treatments but under highly controlled conditions and timing. Our approach allowed us to detect cryptic responses to experimental conditions (i.e., diet composition and social environment) that are not easily inferred from morphological or behavioral traits. In future studies, it would be ideal to produce a complete reference proteome of the cricket *A. domesticus* intended for proteomics analysis, which would improve the database quality. Additionally, the inclusion of complementary omics techniques (e.g., transcriptomics, lipidomics, metabolomics) and novel bioinformatics tools (e.g., coexpression analysis) would help to elucidate further physiological changes not evidenced by our proteomics analysis. On the other hand, the results herein presented could be confirmed through specific experiments using RNAi and CRISPR technologies to validate the roles of the proteins overexpressed under our experimental conditions.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

AUTHOR CONTRIBUTIONS

Yeisson Gutiérrez: Conceptualization (lead); Data curation (lead); Formal analysis (lead); Investigation (lead); Methodology (lead); Writing—original draft (lead); Writing—review & editing (lead). Marion Fresch: Conceptualization (lead); Data curation (lead); Formal analysis (equal); Investigation (lead); Methodology (lead); Software (lead); Writing—review & editing (equal). Sören L. Hellmann: Data curation (equal); Methodology (equal); Software (equal); Writing—review &
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This article has earned an Open Data Badge for making publicly available the digitally-shareable data necessary to reproduce the reported results. The data is available at [http://doi.org/10.6084/m9.figshare.13341680].

DATA AVAILABILITY STATEMENT
All data analyzed in this study and the R scripts are available in the FigShare repository https://doi.org/10.6084/m9.figshare.13341680.v1. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium with the dataset identifier PXD025535.

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