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Comparative proteomic analysis of *Trioza erytreae* nymphs developed on *Citrus ×limon* and *Citrus ×sinensis* host plants

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Abstract

Trioza erytreae is a vector of Huanglongbing (HLB), a highly damaging citrus disease. Lemon plants (*Citrus ×limon*) are the preferred host for *T. erytreae*, although the underlying mechanisms behind this remain to be fully elucidated. A comparative proteomic analysis of *T. erytreae* nymphs in their fourth and fifth instars that were fed either lemon or sweet orange (SwO) was carried out to investigate the interaction with its hosts. A 24-hour sucrose feeding assay was conducted to understand proteomic responses to a nutrient-poor diet. Proteomic profiling using nanoscale liquid chromatography coupled to tandem mass spectrometry (nanoLC-MS/MS) identified a total of 1,477 psyllid proteins with high confidence. Oviposition and nymphal development were also evaluated across citrus hosts, revealing higher numbers of nymphs developing on lemon than on SwO. Feeding on SwO enriched pathways related to "transmission across chemical synapses" and "metabolism of proteins". Responses observed under a 24-hour sucrose-only diet enriched the biological processes "response to external stimulus", "response to stress" and "cytoskeleton organization". In contrast, these enrichments were absent on lemon host, suggesting that lemon provides a more favourable environment for psyllid development. In addition, nymphs developed on lemon exhibited enhanced energy metabolism and an increase in translation initiation factors. Overall, the results demonstrate that development strongly depends on host plant species, with SwO impairing optimal growth and lemon promoting successful nymphal development.

Keywords African citrus psyllid · Huanglongbing · Insect plant interaction · Sap-feeding insects

Introduction

The phloem-restricted bacterium *Candidatus* Liberibacter spp. is the causal agent of Huanglongbing (HLB), a highly destructive citrus disease transmitted by the psyllids *Trioza erytreae* Del Guercio, 1918, and *Diaphorina citri* Kuwayama, 1908 (Bové 2006). Europe is one of the few continents free of HLB (Alquézar et al. 2022). Since 2015, Spain and Portugal have reported the presence of *T. erytreae* in their continental territories (Paiva et al. 2020; EPPO

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2021, 2022 and 2023; FAO 2021; Nunes et al. 2023; Duarte et al. 2024). Fortunately, no *C.* Liberibacter bacteria have been detected in Europe to date. Currently, there is no effective treatment for HLB, so management strategies focus on vector control (before and after the introduction of the bacteria) and eliminating infected plants (Alquézar et al. 2022). Analysing the interaction between the vector and the host plant may assist in identifying the key factors that control the psyllid vector (Magalhães et al. 2025).

Trioza erytreae and *D. citri* are hemimetabolous insects, classified within the order Hemiptera. Their lifecycle comprises five nymphal instars, while subsisting on sap (Aubert 1987; Mito et al. 2010). Both psyllid species require young leaves and the emergence of young flushes for oviposition and nymphal development (Catling 1969; Cifuentes-Arenas et al. 2018). *Trioza erytreae* causes the formation of pit galls on infested leaves, with one nymph developing in each pit gall (Van den Berg 1990).

The primary hosts for *T. erytreae* belong to the Rutaceae family, particularly the subfamily Aurantioideae, which includes the genus *Citrus* (Aubert 1987). The susceptibility exhibited by various citrus species to *T. erytreae* varies considerably. According to Aubert (1987), the sweet orange (SwO) [*Citrus* × *sinensis* (L.) Osbeck] is a common host. Lemon plants [*Citrus* ×*limon* (L.) Osbeck] have been identified as a preferred host for *T. erytreae* (Aidoo et al. 2019a; Benhadi-Marín et al. 2021; Magalhães et al. 2025). The development of *T. erytreae* on the highly suitable lemon host yields larger nymphs and adults when compared to alternative hosts (Aidoo et al. 2019b). The volatile profile and flushing rhythm of lemon plants are the primary determinants affecting the host selection by adult *T. erytreae* (Catling 1969; Antwi-Agyakwa et al. 2019). Nonetheless, the molecular mechanisms that drive the psyllid´s development on lemon plants remain to be elucidated.

Nutritional intake and dietary habits affect insect development (Delisle and Hardy 1997; Layalle et al. 2008). Nutritional-rich diets impact the insect's proteome, leading to increased energy metabolism, upregulation of translation initiation factors and protein synthesis, and enhancement of its growth potential (Arrese and Soulages 2010; Nagarajan and Grewal 2014). Proteomics studies enable the identification of essential molecular interactions between insects and their plant hosts. For instance, a patent, based on omics studies, has been issued for a method that silences *D. citri*'s trehalase activity, resulting in psyllid development arrest when feeding on transgenic citrus plants (Hunter et al. 2017). This patent was based on omics studies that highlighted the importance of the trehalase gene and its impact on *D. citri* development (Shukla et al. 2015; Yang et al. 2020; Liu et al. 2020).

As demonstrated by Franco et al. (2020) and Killiny (2017), different citrus species exhibit distinct sap composition and respond differently to sap-feeding insects (Leimu and Koricheva 2006). Some hosts show a resistant response, characterised by escalating secondary metabolite production, while others adopt a tolerant response, avoiding resource depletion and promoting growth (Leimu and Koricheva 2006). The composition of the phloem sap is indicative of the plant's defence systems, and this can vary depending on how the plant reacts to the invasive agent (Kehr 2006; Thompson and Goggin 2006).

As a defence mechanism, plants produce reactive oxygen species and protein kinases, in addition to relocating micro- and macronutrients to uninfected organs. This process usually serves to reduce the available nutrient supplies to insects (Kehr 2006; Thompson and Goggin 2006). Sweet orange plants have shown resistance responses to *D. citri* feeding, exhibiting the activation of defence pathways such as phenylpropanoid biosynthesis and alpha-linolenic acid metabolism (Sun et al. 2022). The citrus pummelo tree [*Citrus maxima* (Burm.) Merril] shows an increased production of amino acids, sugar acids, xylose, and other sugars in its sap as a tolerant response to *D. citri* infestation (Shugart et al. 2019).

Sap-feeding insects have evolved several strategies to evade plant defences. These include the production of salivary effectors to alter plant metabolism to supress or to inhibit plant defence compounds (Walling 2008; Will et al. 2013). To facilitate penetration and mitigate plant defence responses, certain insects secrete gelling saliva that serves to create a salivary sheath, which protects, stabilises, and lubricates the stylet. This process facilitates stylet penetration, sap extraction, and digestion (Walling 2008; Yu and Killiny 2018).

Artificial diets have proven to be instrumental in the identification of dietary components influencing insect development (Chen et al. 2017a; Catalani et al. 2021). However, research on psyllid vectors of HLB remains limited. Although research has been conducted on artificial diets for *D. citri* (Hall et al. 2010; Russell and Pelz-Stelinski 2015), to the best of our knowledge, no studies have been undertaken on *T. erytreae*. Insects undergo molecular adaptation when transitioning from a protein-rich diet to a more restrictive one (Carsten et al. 2005; Chen et al. 2017a). Sugar-only diets and low-nitrogen source diets have a detrimental impact on insect fertility and development, due to a metabolic shift towards survival strategies (Chen et al. 2017b; Wu et al. 2020). Thus, acute metabolic shifts triggered by transitions to nutritionally deficient diets may reveal key aspects of the initial diet's quality.

Omics studies have been successfully employed to elucidate the intrincate interactions between sap-feeding insects

and their plant hosts (Oates et al. 2016; Zogli et al. 2020). As indicated by previous studies, SwO exhibits a distinct response to *T. erytreae* infestation compared to lemon plants, resulting in a greater number of differentially abundant proteins (DAPs) in SwO hosts (Magalhães et al. 2024). In terms of the proteomics response of the psyllid to the plant host, *D. citri* exhibited a higher number of DAPs when switching from *Murraya paniculata* (L.) Jack to *Citrus macrophylla* Wester, compared to the reverse transition (Ramsey et al. 2022). *C. macrophylla* exhibited a higher level of nymphal colonization compared to *M. paniculata* (Westbrook et al. 2011). Proteomic studies have yielded insights into the developmental biology of insects (Gundappa et al. 2014; Si et al. 2020). The fruit fly *Drosophila melanogaster* Meigen, 1830, seems to be the optimal ortholog model for the functional analysis of insect development (Roberts 2006; Casas-Vila et al. 2017). Although the fruit fly undergoes holometabolism, while *T. erytreae* exhibits hemimetabolism (Mito et al. 2010), and their development differs, a number of metabolic pathways, such as the "hedgehog (Hh) signalling pathway", are conserved as these are essential to insect development (Yamanaka et al. 2013; Villarreal et al. 2015; Lin and Smagghe 2019).

The present study is a proteomic analysis using nanoscale liquid chromatography coupled to tandem mass spectrometry (nanoLC-MS/MS). The objective of this study was to understand host-related effects on the development of *T. erytreae* nymphs. To achieve this objective, a comparative proteomic analysis was conducted on *T. erytreae* nymphs in their fourth and fifth instars, which were fed either lemon or SwO. The oviposition, infestation behaviour and development of *T. erytreae* on these two hosts were monitored over time. Furthermore, a 24-hour sucrose-feeding treatment was conducted to ascertain proteomic responses to a nutrient-poor diet. This approach has the potential to elucidate the richness of the original diets on lemon and SwO plant hosts. The experiment was based on the hypothesis that a more significant metabolic adjustment may be indicative of a more favourable original diet.

Materials and methods

Insect origin and rearing

Adults of *Trioza erytreae* were collected using a handheld aspirator from pesticide-free lemon orchards in Caracoi, Porto district, Portugal (41°18'46.4"N 8°38'09.7"W), in 2021. The captured psyllids were transferred to conical centrifuge tubes (50 ml) and subsequently released onto lemon and sour orange (*C. aurantium* L.) plants within acrylic cages (40 × 30 × 43 cm) covered with insect-proof nets. The rearing cages were maintained within a climate chamber at 21 °C ± 1 °C with a relative humidity of 50 ± 5% and a photoperiod of 16:8 h of light: dark (L: D).

Infestation and psyllid development

Adult psyllid specimens from the rearing cages were used to infest 'Valencia Midknight Seedless' sweet orange [*C. ×sinensis* (L.) Osbeck] (SwO) and 'Eureka' lemon [*C. ×limon* (L.) Osbeck] (lemon) plants, both grafted onto 'Carrizo' citrange (*C. trifoliata* × *C. ×sinensis*) rootstock. A total of 16 plants, 8 lemon and 8 SwO specimens were used in the study. These 2-year-old plants, with 0.8 to 1 m in height, were acquired from a certified nursery accompanied by a phytosanitary passport. All plants were potted in 5 L tall pots (19 cm diameter and 25 cm high) containing pine bark and coconut fibre (50:50). Plants were maintained in a climate chamber with controlled conditions of temperature (23.5 ± 1 °C), humidity (79 ± 5%) and photoperiod (14:10 h, L:D). Three weeks before the infestation, the citrus plants were pruned to stimulate new shoot formation, and the plants selected for the experiment exhibited a comparable number of new shoots. Plants were isolated within a cylindrical insect-proof net, which was fixed above the canopy with a wooden structure and secured to the tree trunk above the pot, enveloping the entire canopy. *Trioza erytreae* mature adults were aspirated from the rearing cages with a handheld aspirator and collected into a conical centrifuge tube (50 ml). The psyllids were then introduced onto the netted citrus hosts using the conical centrifugal tubes. Ten *T. erytreae* mature adults of mixed age (five males and five females) were used per plant.

The intensity of egg-laying per leaf was categorised using the following scale: AO- no eggs; BO- 1 to 10 eggs; CO- 11 to 20 eggs; DO- 21 to 40 eggs; EO- 41 to 100 eggs; FO- more than 100 eggs. The intensity of nymphs per leaf was categorised using the following scale: A- no nymphs; B- 1 to 10 nymphs; C- 11 to 20 nymphs; D- 21 to 40 nymphs; E- 41 to 100 nymphs; F- more than 100 nymphs. The egg-laying and nymphal intensity were evaluated two days after infestation (DAI) and then at three-day intervals throughout the experiment. Plant hosts were observed daily, and the first appearance of each developmental stage was recorded, from first instar nymphs to the first new adult emergence.

All nymphs were removed from the leaves of each plant once the first adult emerged, to ensure the collection of the maximum number of nymphs per plant. The removed nymphs were in the fourth and fifth instar developmental stage. The nymphs were divided into two groups: one for protein extraction, and one that underwent sucrose feeding, followed by protein extraction. As the nymphs were removed from the leaves, they were counted, along with

the pit galls that had formed on the leaves. Dead nymphs attached to the leaves were not counted or used in the aforementioned protocols.

This work was carried out in Portugal, an area free from HLB. Therefore, HLB infection was not considered in this study to avoid any risk of citrus orchard infection.

Experimental groups and sucrose feeding

The study comprised four distinct experimental groups: "Lemon" nymphs exclusively developed on lemon; "SwO" nymphs exclusively developed on sweet orange; "LemonSuc" nymphs that underwent a 24-h sucrose feeding diet after being detached from the lemon plants; "SwOSuc" nymphs that underwent a 24-h sucrose feeding diet after being detached from the SwO plants. Each experimental group comprised four biological replicates ($n=4$), with each replicate consisting of 100 whole-body nymphs. The nymphs of each biological replicate were randomly collected from two distinct plants within the same group, and the plants were not used for any other replicate.

The fourth and fifth instar nymphs from the "Lemon" and "SwO" experimental groups were removed from the leaves, weighed in 2 ml microcentrifuge tubes, promptly frozen in liquid nitrogen and stored at − 80 °C until the protein extraction procedure. 50% of the fourth and fifth instar nymphs collected from the "Lemon" and "SwO" were allocated to the "LemonSuc" and "SwOSuc" 24-h sucrose feeding diet treatments, respectively (Fig. 1).

In the 24-h sucrose feeding diet, the nymphs were removed from their hosts and transferred to a Petri dish lined with filter paper (Whatman Grade 1) soaked in 1 ml of a 0.5 M sucrose solution. The nymphs were maintained in the Petri dish for a period of 24-h, under the same climatic conditions that were used for the infestation of host plants and nymphal development. Nymphs were observed to quickly settle and new honeydew excretions were observed, which indicated active feeding (Van den Berg et al. 1991b; Urbaneja-Bernat et al. 2023). After the 24-h sucrose diet, the nymphs, amounting to 100 individuals, were removed from the Petri dishes, weighed in 2 ml microcentrifuge tubes, frozen in liquid nitrogen and stored at − 80 °C until the protein extraction procedure.

The period of 24-h for the sucrose feeding diet was selected on the basis of preliminary studies, which demonstrated a nymphal survival rate of 94%, while after 48 h it decreased to 82%. After a period of sucrose feeding lasting for a minimum of 48 h, the majority of surviving nymphs emerged as new adults. In fact, after 72 h period, an average of 60% of the nymphs were either dead or morphed into adults.

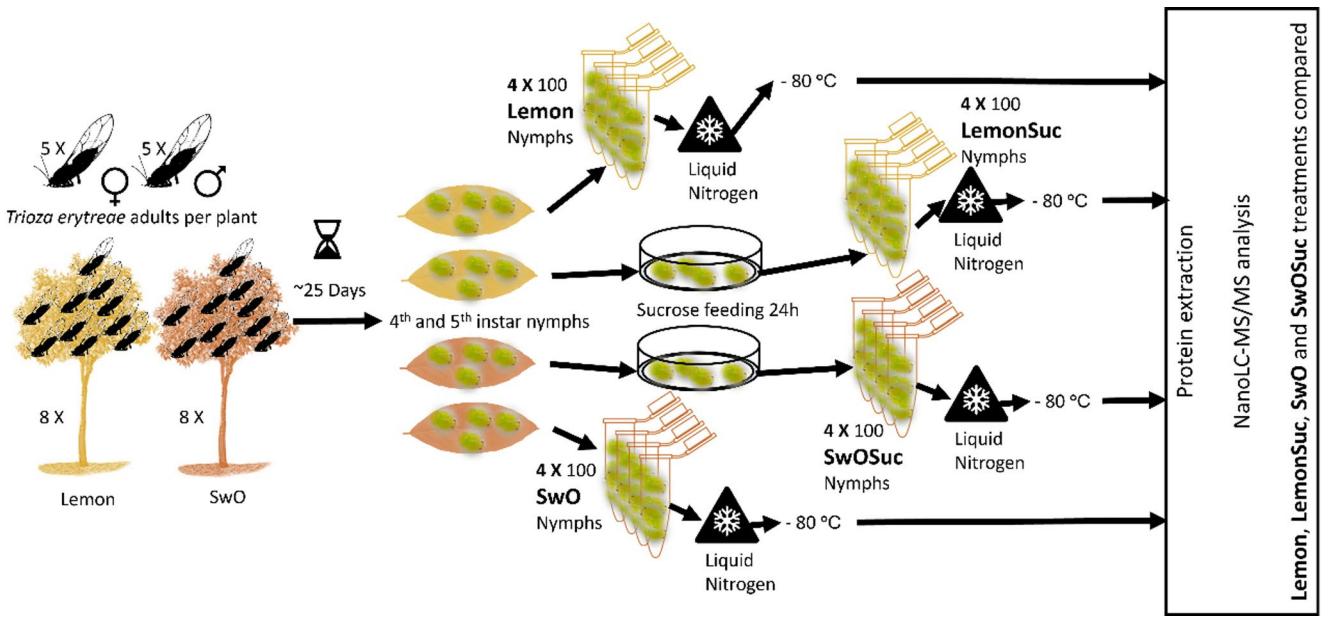

Fig. 1 An overview of the experimental workflow: infestation of citrus hosts with *Trioza erytreae*, nymphal development and sucrose feeding. "Lemon": an experimental group formed by nymphs developed exclusively on lemon plants. "LemonSuc": an experimental group composed of nymphs developed on lemon plants and then fed for 24-h on sucrose. "SwO": an experimental group formed by nymphs that developed exclusively on sweet orange (SwO). "SwOSuc": an experimental group composed of nymphs developed on SwO plants and then fed for 24-h on sucrose. The following comparisons were made SwO vs. Lemon; LemonSuc vs. Lemon; SwOSuc vs. SwO and SwOSuc vs. LemonSuc

Trioza erytreae nymphs' protein extraction and analysis

Total protein was extracted from the fourth and fifth instar nymphs collected from the four experimental groups: "Lemon", "SwO", "LemonSuc" and "SwOSuc" (Fig. 1). The protein extraction procedure was adapted from Cilia et al. (2009) using the TCA-acetone method for protein extraction from aphids. Modifications were made to the homogenisation procedure and centrifugation force, based on the *D. citri* protein extraction protocol from Ramsey et al. (2015). Briefly, frozen samples of 100 nymphs each, were homogenised using a 3 mm stainless steel ball in a TissueLyser Mixer Mill 400 (Retsch, Haan, Germany). The initial cycle was set to last for 1 min and was conducted without the addition of a buffer (dry cycle) to ensure the complete destruction of the samples. Subsequently, 500 µl of protein extraction buffer (10% TCA with 2% β-mercaptoethanol in acetone) was added to each sample. Four homogenization cycles were performed, each with a duration of 1 min, with the exception of the second cycle, which was 2 min in duration. Each cycle was preceded by a 5 min incubation on ice. All cycles (dry and wet) were configured at 30 Hz. The steel balls were removed, and the samples were vortexed, stored at − 80 °C for 5 h and centrifuged at 14,000 g for 30 min at 4 °C. The resulting pellets were washed with 1 ml of acetone at 4 °C and centrifuged at 14,000 g for 5 min at 4 °C. The washing step was repeated two more times. The pellets were then dried, resuspended in 200 µl of protein solubilization buffer (7 M urea, 2 M thiourea, 4% CHAPS) and stored at − 20 °C overnight. Samples were centrifuged at 14,000 g for 20 min at 4 °C and the soluble protein fraction was recovered as the final extraction product (Fig. 1) and was stored at − 80 °C. The total soluble protein extracted from *T. erytreae* nymphs was quantified using a Genesys 1Q-S spectrophotometer (Thermo Electron Corporation, Bremen, Germany) with a Quick Start™ Bradford Protein Assay Kit (Bio-Rad, Hercules, USA), according to the manufacturer's instructions and using bovine serum albumin (BSA) as the standard. To analyse the quality of the nymph protein extracts, they were subject to electrophoresis (30 µg of total soluble protein per sample) using 12% sodium dodecyl-sulphate polyacrylamide gels (SDS-PAGE), according to the Laemmli method (Laemmli 1970), and stained with Coomassie blue. Prior to nanoLC-MS/MS proteomic analysis, 50 µg of soluble protein from each sample underwent a solid-phase-enhanced sample-preparation (SP3) protocol (Hughes et al. 2018), followed by overnight enzymatic digestion with trypsin/LysC (2 µg) at 37 °C and 1000 rpm. The concentration of the resulting peptides was then quantified using fluorescence.

Proteomic analysis of nymphs

Proteomics data acquisition

The proteome of the nymphs from the four experimental conditions (Lemon, LemonSuc, SwO and SwOSuc, $n=4$ samples of 100 nymphs per condition) was obtained based on the protocol described by Osório et al. (2021). Protein identification and quantification were carried out using a nanoscale liquid chromatography coupled with tandem mass spectrometry (nanoLC-MS/MS) on an *Ultimate* 3000 liquid chromatography system coupled to a Q-Exactive Hybrid Quadrupole-Orbitrap mass spectrometer (Thermo Scientific, Bremen, Germany), by an external service provider (Proteomics Scientific Platform of i3S, Ipatimup, Porto, Portugal).

In summary, 500 ng of trypsin/LysC-digested samples were loaded into a trapping cartridge (Acclaim PepMap C18 100 Å, 5 mm × 300 µm i.d., 160454, Thermo Scientific, Bremen, Germany) in a mobile phase of 2% acetonitrile (ACN), and 0.1% formic acid (FA) at a flow rate of 10 µL/min. Following a 3 min loading interval, the trap column was switched in-line to a 50 cm × 75 µm inner diameter EASY-Spray column (ES803, PepMap RSLC, C18, 2 µm, Thermo Scientific, Bremen, Germany) at a flow rate of 250 nL/min. The separation was achieved by employing a solvent mixture of A: 0.1% FA and B: 80% ACN, 0.1% FA with a gradient elution program: 5 min (2.5% B to 10% B), 120 min (10% B to 30% B), 20 min (30% B to 50% B), 5 min (50% B to 99% B), and 10 min (hold 99% B). Subsequently, the column was subjected to a 17 min equilibration period with 2.5% B. Data acquisition was controlled by Xcalibur 4.0 and Tune 2.9 software (Thermo Scientific, Bremen, Germany).

The mass spectrometer was operated in data-dependent (dd) positive acquisition mode, alternating between a full scan (m/z 380–1580) and subsequent higher-energy collisional dissociation tandem mass spectrometry (HCD MS/MS). This method was established for the 10 most intense peaks from a full scan (normalized collision energy of 27%). The electrospray ionization (ESI) spray voltage was set to 1.9 kV, with the global settings configured as follows: optimal lock mass (m/z 445.12003), lock mass injection, full MS, and chromatographic peak width at a full width half maximum (FWHM) of 15 s. The full scan settings were as follows: 70 k resolution (m/z 200), automatic gain control (AGC) target 3×10^6, maximum injection time 120 ms; dd settings: minimum AGC target 8×10^3, intensity threshold 7.3×10^4, charge exclusion: unassigned, 1, 8, >8, peptide matches preferred, exclude isotopes on, and dynamic exclusion 45 s. The MS2 settings were as follows: microscans − 1, resolution − 35 k (m/z 200), AGC target − 2×10^5, maximum

injection time − 110 ms, isolation window − 2.0 *m/z*, isolation offset − 0.0 *m/z*, dynamic first mass and spectrum data type profile.

Data processing, protein-label-free quantification and identification

The mass spectrometry (MS) raw data were processed using Proteome Discoverer 2.5.0.400 software (Thermo Scientific, Bremen, Germany). Protein identification searches were conducted using the UniProt protein sequence database for *D. citri* (taxon ID 121845, 21,517 entries) and for *C.* ×*sinensis* (taxon ID 2711, 44,601 entries) and a common contaminant database from MaxQuant (version1.6.2.6, Max Planck Institute of Biochemistry, Munich, Germany). The psyllid *T. erytreae* currently lacks a publicly available assembled genome, hence *D. citri* was selected as the reference. *Diaphorina citri* was selected due to its classification within the superfamily Psylloidea, exhibiting a similar development, comparable host plants, and serving as a vector for the same citrus disease transmitted by *T. erytreae*. Shared developmental traits between *D. citri* and *T. erytreae* increase the plausibility of orthology. However, it is important to note that interspecies database searches may introduce specific biases, such as sequence divergence, which could hinder the identification of species-specific peptides and single amino-acid differences that may diminish peptide-spectrum match scores or lead to misassignments. In order to mitigate the aforementioned risks, a stringent peptide-level False Discovery Rate (FDR) control was applied. The Sequest HT tandem mass spectrometry peptide database search program was employed to identify tryptic peptides, with an ion mass tolerance of 10 ppm for precursors and 0.02 Da for fragmented ions and missing cleavage sites was set as 2. Cysteine carbamidomethylation was defined as a constant modification. Methionine oxidation, asparagine, and glutamine deamidation, peptide N-terminus Gln-> pyro-Glut, protein N-terminus acetylation, and loss of methionine and Met-loss+Acetyl were defined as variable modifications. The peptide confidence was set to high and the Inferys rescoring node was considered for this analysis. The Percolator processing node was enabled with the following settings: maximum Delta Correlation (deltaCn) 0.05; decoy database search target FDR 1%; validation based on the *q*-value.

Protein-label-free quantification was conducted using the Minora feature detector node at the processing stage. The following parameters were employed for precursor ion quantification: (1) Unique peptides plus razor; (2) Precursor abundance was determined based on intensity; (3) Normalization mode was based on total peptide amount; (4) The minimum number of replicate files was set to 50% in each sample group; (5) Pairwise protein ratio calculation and hypothesis testing were based on a t-test (background based). The Feature Mapper node in Proteome Discoverer software was employed to generate features from distinctive peptide-specific peaks within a narrow retention-time and mass range (a maximum shift of 10 min and 10 ppm of mass tolerance was permitted for the mapping of features from different sample files). For the purpose of feature linking and mapping, the signal to noise (S/N) threshold was set at 5 for each comparison. Libraries of proteins and peptides confidently identified and quantified for each experimental condition were generated and the mass spectrometry proteomics data were deposited in the ProteomeXchange Consortium via the PRIDE (www.ebi.ac.uk/pride) (Perez-Riverol et al. 2022) partner repository with the dataset identifier PXD059807 and https://doi.org/10.6019/PXD059807. Fold-change in protein abundance was calculated in each established comparison (SwO vs. Lemon; LemonSuc vs. Lemon; SwOSuc vs. SwO and SwOSuc vs. LemonSuc) using the medians (log2-transformed) between relative protein abundance in the experimental groups and differentially abundant proteins (DAPs) were considered.

Bioinformatics analysis

Functional analysis of the proteome was performed using *Drosophila melanogaster* Meigen, 1830, orthologues of the identified proteins, obtained with the STRING webtool version 12.0 (https://string-db.org/) (Szklarczyk et al. 2023). Enrichment analysis was performed using the webtool PANGEA, PAthway, Network and Gene-set Enrichment Analysis, a multi-species enrichment tool, version 1 beta 1 (https://www.flyrnai.org/tools/pangea/) (Hu et al. 2023). A hypergeometric test/Fisher's exact test was conducted with a Benjamin and Hochberg (Benjamini and Hochberg 1995) FDR correction, considering an adjusted *p*-value of < 0.05. The following databases were used for all the enrichment analysis: Gene Ontology (Ashburner et al. 2000; Aleksander et al. 2023), FlyBase (Öztürk-Çolak et al. 2024) and Reactome (Milacic et al. 2024). Bubble plots were created using R software (R Core Team 2020) with the ggplot2 package (Wickham 2016). Venn diagrams were created using the Venny 2.1 web tool (https://bioinfogp.cnb.csic.es/tools/venny/) (Oliveros 2015).

Statistical analysis

Statistical tests were performed using RStudio software (R Core Team 2020), unless otherwise specified in this section. The normality of all data was assessed using a Shapiro–Wilk test, while homoscedasticity was evaluated with Levene's

test for normally distributed data and the Brown–Forsythe test for non-normally distributed data.

A Welch's *t*-test was employed to compare the mean number of flushes per plant between SwO and lemon plants ($n=8$). A Wilcoxon–Mann–Whitney test was employed to compare the average development time of *T. erytreae* on the two hosts. A χ^2 (Chi-squared) test was used to compare leaves with eggs and those devoid of eggs in the two citrus hosts. Each egg intensity class on the oviposited leaves was compared among citrus hosts using a Fisher's exact test. The mean number of nymphs and pit galls per plant was compared between SwO and lemon plants, using a Student's *t*-test. In proteomic analysis and to determine the DAP's, the following criteria were considered: (1) a minimum number of biological samples in which a protein was detected in an experimental group was set to 75% (e.g. 3 out of 4); (2) the identification of at least two unique peptides for protein assignment; (3) a stringent FDR with the *q*-value set at <0.01. Protein intensities were log2-transformed and were normalized based on the quantile method. Protein-wise linear models for paired samples combined with empirical Bayesian statistics were employed for differential abundance analysis, using the R Bioconductor package limma (Ritchie et al. 2015). FDR correction was achieved using the Benjamini-Hochberg (BH) method, and DAPs were identified with an adjusted p-value of <0.05.

Results

Trioza erytreae exhibited a citrus host-specific oviposition pattern and nymphs developed better on lemon than on SwO plants

The psyllids and the citrus hosts exhibited signs of growth and development throughout the course of the experiment. With regard to the host plants, at the onset of the experiment, the mean number of new flushes per plant was 4.6 for lemon and 6.4 for the SwO, values that are not significantly different (Welch's *t*-test, $P=0.15$). The increase in the number of new leaves from 5 DAI to 23 DAI was 9.4% for lemon and 9.6% for SwO plants (Table 1), indicating that both hosts exhibited a similar growth pattern.

Henceforth, the term "available leaves" refers to the new leaves from the growth induced by pruning. It is a prerequisite for a successful infestation by the psyllid that young leaves are available. The first indications of oviposition were recorded at 2.6 and 2.5 DAI on average in the lemon and SwO plants, respectively. The period of maximum oviposition on the lemon host was recorded at 5 DAI, with 13.8% of the available leaves infested with eggs. In contrast, over the same 5 DAI, a more dispersed pattern was observed on SwO, with 42.2% of the available leaves having eggs (Table 1). The maximum number of eggs laid on SwO, recorded at 8 DAI, were on 45.7% of the available leaves. In contrast, in the lemon host, egg deposition at 8 DAI was recorded on 12.5% of the available leaves. In conclusion, the citrus host influenced the proportion of available leaves containing eggs ($P<0.05$, Chi squared test) during all the experiment except at 23 DAI.

The egg intensity, defined as the number of eggs per available leaf, was categorised as AO (no eggs per leaf), BO (1–10 eggs per leaf), EO (41–100 eggs per leaf) and FO (more than 100 eggs per leaf). The categorization was affected by the citrus host ($P<0.05$) at both 5 and 8 DAI, although EO was only significantly influenced by the citrus host at 8 DAI. The present study revealed higher levels of oviposition intensity per leaf on lemon, compared to SwO. At 5 DAI, 52% of the leaves with eggs in lemon plants exhibited more than 20 eggs, and 24% had over 100 eggs (Fig. 2A). Conversely, at 5 DAI the SwO plants had a low egg intensity, and 27.4% of leaves with eggs had more than 20, and 5.7% had over 100 eggs (Fig. 2A). A difference in egg intensity between the citrus hosts was also observed at 8 DAI. In the case of lemon, 66.67% of leaves exhibiting egg-laying had more than 20 eggs, while in SwO, this value decreased to 28.19%.

The quantity of infested leaves increased significantly from 5 DAI to 23 DAI, with a more notable increase in the lemon plants (412%) compared to the SwO plants (49.1%).

Table 1 Total number of oviposited and infested leaves of citrus plants. Number of oviposited and infested leaves of citrus plants at 5 DAI and 23 DAI, respectively. Increase in the number of new leaves and new infested leaves of citrus plants from 5 DAI to 23 DAI. "Available leaves" refers to the quantity of new leaves from the growth induced by pruning, which were the ones that were available for the initial infestation. "Oviposited leaves" denotes the number of leaves harbouring *Trioza erytreae* eggs, with the percentage of these leaves in relation to the "Available leaves" indicated in brackets. "Infested leaves" denotes the number of leaves containing *T. erytreae* nymphs, with the percentage of these leaves in relation to the "Available leaves" indicated in brackets. The emergence of "New leaves" was determined by calculating the difference between the "Available leaves" at 5 DAI and the "Available leaves" at 23 DAI, with the percentage increase in new leaves indicated in brackets. Similarly, the calculation of "New infested leaves" involved the subtraction of "Oviposited leaves" at 5 DAI from "Infested leaves" at 23 DAI, with the percentage increase of new infested leaves indicated in brackets. "SwO" refers to sweet orange plants

Host citrus	5 DAI		23 DAI		Increase from 5 DAI to 23 DAI	
	Available leaves	Oviposited leaves	Available leaves	Infested leaves	New leaves	New infested leaves
SwO	251	106 (42.2%)	275	158 (57.5%)	24 (9.6%)	52 (49.1%)
Lemon	181	25 (13.8%)	198	128 (64.6%)	17 (9.3%)	103 (412%)

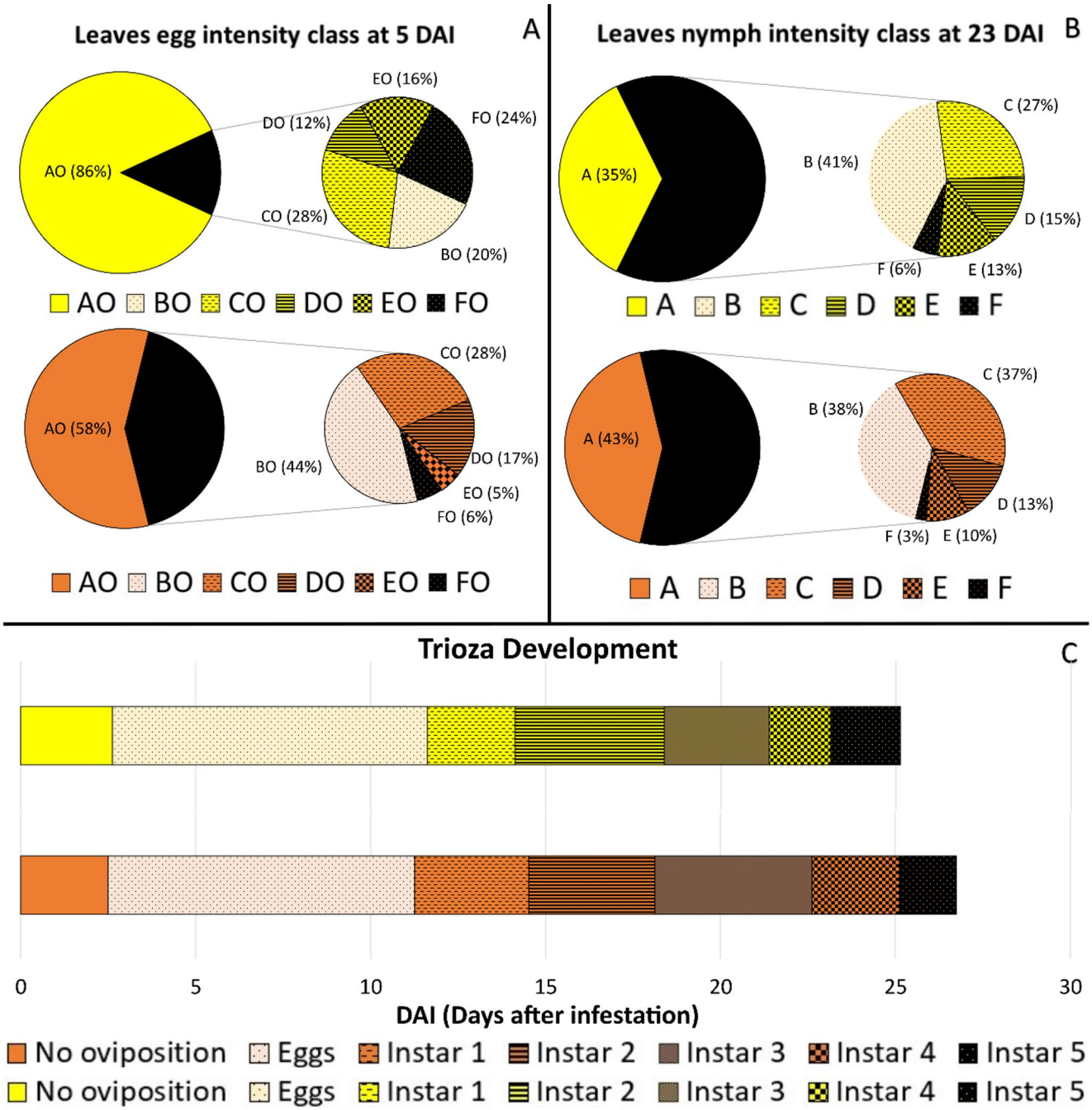

Fig. 2 Evaluation of *Trioza erytreae* infestation. **A** Pie charts depicting the presence and intensity of *Trioza erytreae* eggs deposited per leaf five days after infestation (DAI); **B** Pie charts illustrating the presence and intensity of *Trioza erytreae* nymphs per leaf at 23 DAI; **C** First observation of each psyllid developmental stage in each host. The leaf egg-laying intensity scale is as follows: AO- no eggs; BO- 1 to 10 eggs; CO- 11 to 20 eggs; DO- 21 to 40 eggs; EO- 41 to 100 eggs; FO- more than 100 eggs. The nymph intensity scale is as follows: A- no nymphs; B- 1 to 10 nymphs; C- 11 to 20 nymphs; D- 21 to 40 nymphs; E- 41 to 100 nymphs; F- more than 100 nymphs. The yellow background represents data recorded from the lemon plants. The orange colour represents data recorded from the sweet orange plants

At 23 DAI, a similar number of infested leaves was recorded in both hosts, with 128 on lemon and 158 on SwO plants (Table 1). The evaluation of infestation intensity at 23 DAI showed that the distribution of nymph intensity classes was independent of the host plant ($P>0.05$, Chi squared test). Despite these results, a higher percentage of infested leaves in the lemon (5.5%) contained over 100 nymphs, in contrast to the infested leaves of SwO (2.5%) (Fig. 2B).

The average number of pit galls per plant exhibited no significant variation among plant hosts ($P=0.16$, Students

t-test), with values of 330.1 [(±47.5 standard error of the mean (SEM)] for lemon and 246.8 (±30.8 SEM) for SwO plants. A significantly higher number of nymphs at the fourth and fifth instar developed on lemon hosts compared to SwO hosts ($p<0.05$, Student *t*-test). The average number of fourth and fifth instar nymphs per plant was 318.5 (±47.3 SEM) for lemon plants and 99.3 (±27.6 SEM) for SwO plants. The duration of the psyllid development was similar in the two citrus hosts (Fig. 2C). However, the time required for the initial fifth instar nymph to mature ($P=0.013$) was significantly longer in SwO (25.1 DAI±1.46 standard deviation of the mean SDM), compared to the lemon plants (23.1 DAI±0.64 SDM). Additionally, the time required for the first adult emergence is also longer on SwO (26.8 DAI±1.28 SDM) than on lemon (25.1 DAI±0.64 SDM) ($P=0.018$).

Protein identification from *Trioza erytreae* nymphs

The soluble protein extracts of the fourth- and fifth-instar nymphs from the four experimental groups were resolved by Coomassie blue with one-dimensional SDS-PAGE and showed consistent high-quality protein extracts (Supplementary Fig. A). A visual inspection of the gels revealed that the protein band profiles of the four different experimental groups were similar.

Protein libraries that were confidently identified/quantified in the nymphs at the fourth and fifth instar by nanoLC-MS/MS, were profiled and compared between the nymph samples that developed on lemon and those that developed on SwO, with and without sucrose treatment. A total of 2,777 proteins were identified in the nymph samples, of which 2,617 were assigned to *D. citri*, and 161 were assigned to *C. ×sinensis*. Following the exclusion of 25 proteins classified as contaminants, a total of 1,500 proteins confidently identified/quantified were filtered based on the following rigorous criteria's: (a) proteins with a high FDR (q-value<0.01); (b) more than two unique peptides and (c) proteins identified in at least 75% of the samples from each independent treatment group. Of these, 1,477 were assigned to *D. citri*, and 23 were assigned to *C. ×sinensis* (Fig. 3A and Supplementary Table A).

The global comparative proteomic analysis exhibited substantial overlap among the detected proteins in the four experimental groups. Among the 23 citrus host plant proteins and 1477 psyllid proteins identified, 15 and 1410, respectively, were common across all four experimental groups (Fig. 3A).

Citrus hosts proteins identified in *Trioza erytreae* nymphs

Fifteen common citrus host plant proteins were identified in the nymphs from all the treatment groups (Fig. 3A), where five of these were related to proteina metabolism, namely A0A067GTS6 (KH type-2 domain-containing protein), A0A067EVG3 (26 S protease regulatory subunit 6B), A0A067DE71 (Isoleucine–tRNA ligase), A0A067DP37 (60 S ribosomal protein L23), and A0A067EFK3 (Peptidyl-prolyl cis-trans isomerase) (Supplementary Table A). Four proteins were associated with the plant stress response, comprising two heat shock proteins A0A067FWR4 (Heat shock cognate 70 kDa protein) and H9NHJ9 (Hsp90). In addition, two proteins were associated with the oxidative stress response: A0A067F9T7 (Peroxidase) and A0A067DDL8 (Superoxide dismutase family protein) (Supplementary Table A). Two citrus host proteins associated with antioxidant activity and plant stress response were identified: one exclusively found in nymphs feeding on lemon (A0A067H2F2, Catalase) and the other exclusively found in nymphs feeding on SwO (A0A067H6D4, Peroxidase) (Supplementary Table A).

Differentially abundant proteins identified in nymphs from the four experimental groups

A total of 173 DAPs were identified across the four comparisons of the four experimental groups ($P<0.05$, Limma, with Benjamin-Hochberg correction). The SwO vs. Lemon comparison yielded the highest number of DAPs (101), followed by the LemonSuc vs. Lemon comparison (92 DAPs), the SwOSuc vs. SwO comparison (26 DAPs), and the SwOSuc vs. LemonSuc comparison (1 DAP) (Figs. 3B and 4, Supplementary Table B).

The citrus host effect: lemon plants modify nymph energy metabolism while SwO plants hinder fertility and development

A comparison of SwO vs. Lemon revealed that 81 of the 101 DAPs were more abundant in SwO nymphs, while 20 were more abundant in Lemon nymphs (Fig. 4A). The 20 proteins identified where mainly related to energy metabolism, and enriched pathways were "generation of precursor metabolites and energy", "carbohydrate metabolism process", "formation of ATP by chemiosmotic coupling", "mitochondrial fatty acid beta-oxidation", among other energy-related pathways (Fig. 5).

In the SwO vs. Lemon comparison, nymphs that developed on SwO exhibited an enrichment of the semi-sterile phenotype groups. These phenotype groups are defined in

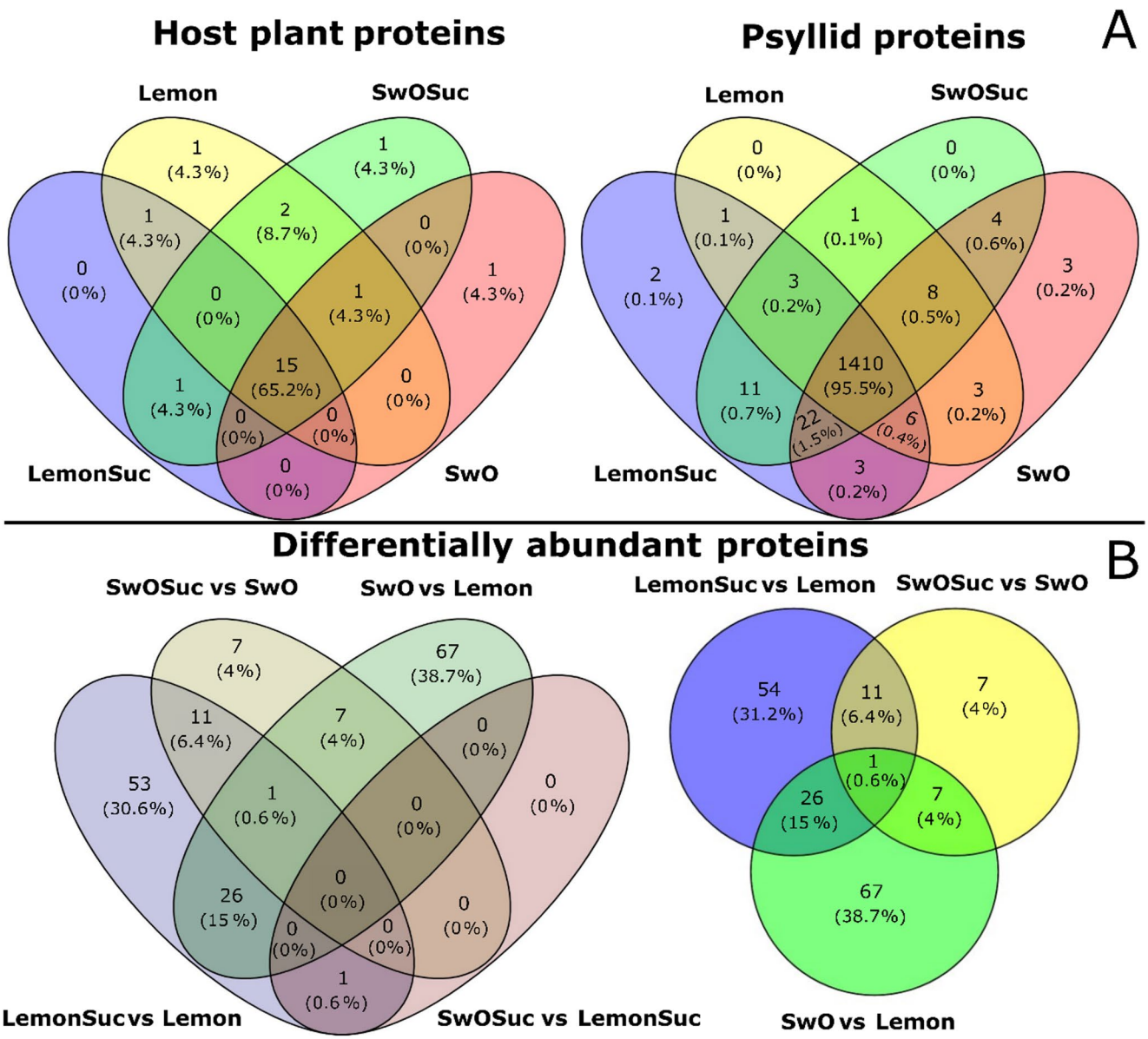

Fig. 3 Venn diagrams illustrating the comparison of the number of proteins identified in *Trioza erytreae* between the four experimental groups. **A** Confidently identified/quantified "host plant proteins" are proteins identified as belonging to the *Citrus ×sinensis*, according to the UniProt protein sequence database, while "psyllid proteins" are proteins assigned as belonging to the *Diaphorina citri* UniProt protein sequence database. **B** Differentially abundant proteins (DAP´s, $p<0.05$) between the specified comparisons: "Lemon" refers to the nymphs that developed on lemon plants; "LemonSuc" refers to the nymphs developed on lemon plants followed by 24-h sucrose feeding; "SwO" refers to the nymphs that developed on sweet orange (SwO); "SwOSuc" refers to the nymphs developed on SwO plants followed by 24-h sucrose feeding. The Venn diagrams were generated using the webtool https://bioinfogp.cnb.csic.es/tools/venny/index.html

the Flybase phenotype database (https://flybase.org/) as *D. melanogaster* mutant phenotypes that have a decreased (by at least 50%) capacity to produce fertilised eggs compared to the wild-type (Fig. 5). Two proteins that contributed to this enrichment were: "CkIIbeta" (Casein kinase II beta subunit) and "Abi" (Abelson interacting protein) (Supplementary Table B). The enrichment of these semi-sterile phenotype groups may indicate an effect on *T. erytreae* adult fertility, however functional assays to validate this were not performed in the present study.

Other abundant proteins identified in SwO were associated with the biological processes "actin cytoskeleton organisation", "signal transduction" and "transmission across chemical synapses" pathways (Fig. 5). This finding was coherent with the higher abundance of "Syn" (synapsin-1) and "Sls" (titin) proteins identified in SwO nymphs (Supplementary Table B). These two proteins also enriched

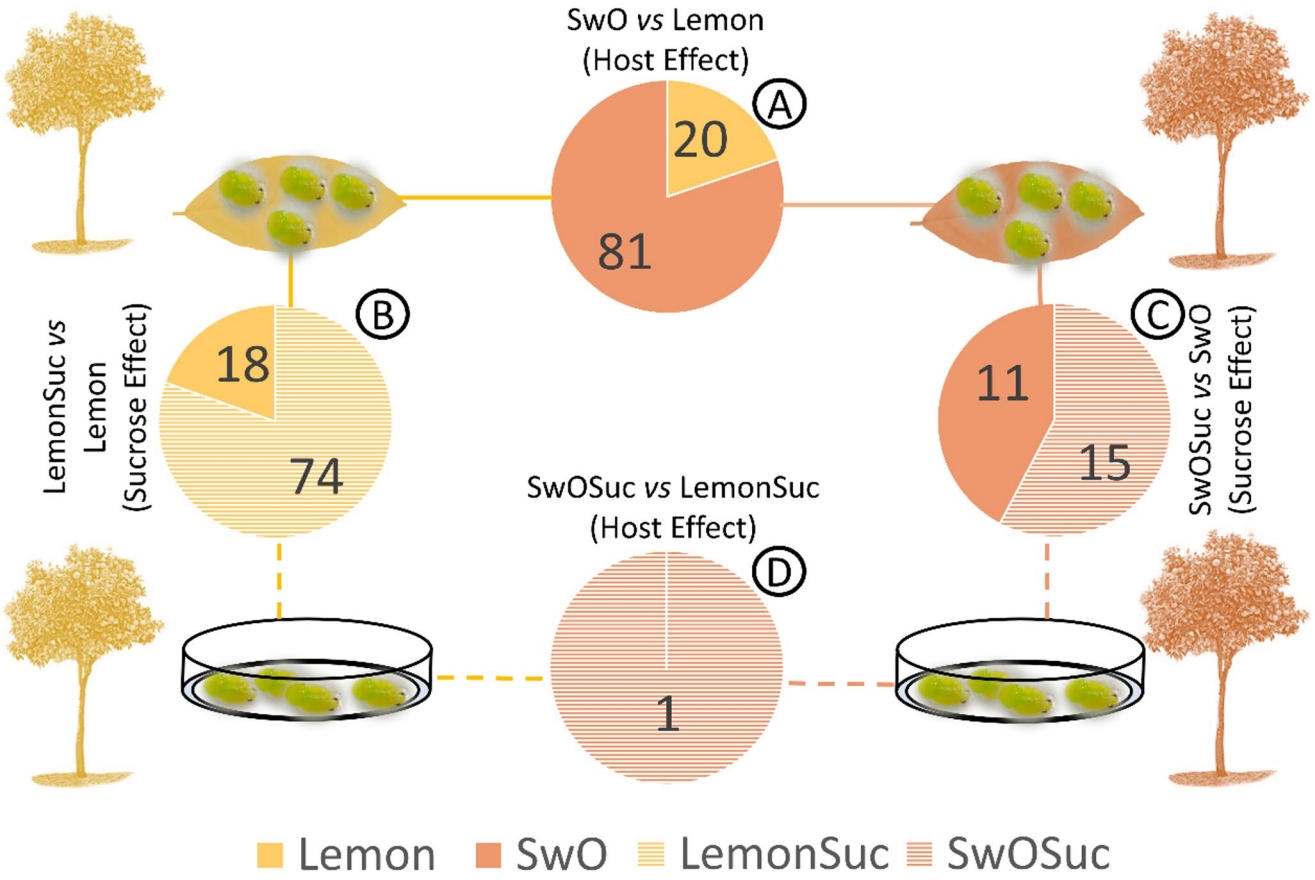

Fig. 4 Differentially abundant proteins (DAP) identified in *Trioza erytreae* nymphs from the four experimental groups. Each pie chart represents one of the 4 comparisons performed and the numbers inside the pie charts represent the DAPs that were most abundant in the different experimental groups. The yellow colour denotes the lemon plant experimental group. The orange colour denotes the sweet orange (SwO) plant experimental group. Dashed lines and striped colouring in pie charts represent nymphs that were fed sucrose (24-h). Solid lines and solid colours in pie charts represent nymphs that were not exposed to sucrose feeding. "Lemon": nymphs that developed exclusively on lemon plants. "LemonSuc": nymphs that developed on lemon plants and were then fed sucrose for 24-h before sampling. "SwO": nymphs exclusively developed on SwO. "SwOSuc": nymphs that developed on SwO plants and were then fed sucrose for 24-h before sampling. **A** SwO vs. Lemon, **B** LemonSuc vs. Lemon, **C** SwOSuc vs. SwO, **D** SwOSuc vs. LemonSuc

the phenotype groups "paralytic" and "abnormal locomotor behaviour". Additionally, the phenotype groups "abnormal neuroanatomy" and "abnormal neurophysiology" were enriched by "Dap160" (dynamin associated protein 160) and "CadN" (neural-cadherin) (Supplementary Table B).

A comparison of the SwO and lemon bubble plots (Fig. 5) revealed a significant disparity in the enriched pathways between the nymphs that grew in the two infested citrus hosts. The nymphs from the lemon plants exhibited enriched pathways related to growth, development and energy metabolism, including "organelle biogenesis and maintenance", "mitochondrial biogenesis" and "cuticle development" (Fig. 5). Two specific ATP synthases were identified, namely "blw" (ATP synthase subunit alpha, mitochondrial) and "ATPsynbeta" (ATP synthase subunit beta, mitochondrial) (Supplementary Table B). In contrast, none of these pathways were enriched in the SwO nymphs, which had enriched pathways linked to abnormal development (Fig. 5).

Sucrose feeding had a higher impact on nymphs that developed on lemon plants, with the hedgehog signaling pathway affected

The proteomic analysis identified 11 common proteins in nymphs fed sucrose irrespective of the plant host (LemonSuc and SwOSuc). The list comprises "MRP" (Multidrug-Resistance like protein 1, isoform A), "Mi-2" (Chromodomain-helicase-DNA-binding protein Mi-2 homolog), "SMC1" (Structural maintenance of chromosomes 1), "bt" (Bent, isoform F), "Nedd4" (E3 ubiquitin-protein ligase Nedd-4), and "wrd" (Well-rounded, isoform B) (Supplementary Table A). Three DAPs were assigned to the "Hh signalling pathway" in both sucrose feeding treatments, which were "Tnpo" (Transportin, isoform A), "wdb" (Widerborst), and

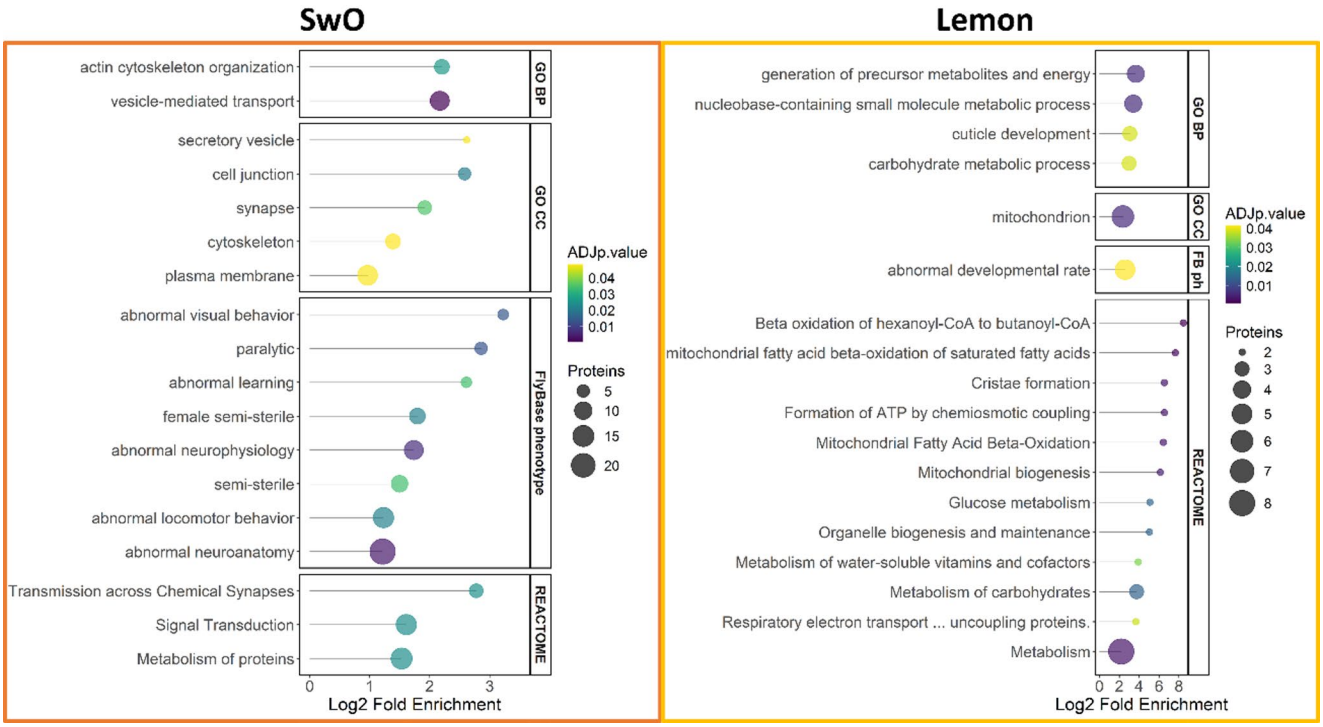

Fig. 5 Bubble plots of the enriched parameters identified when differentially abundant proteins (DAPs) in *Trioza erytreae* nymphs from the SwO and Lemon comparison were used to interrogate different databases (FDR adjusted *p*-value threshold of 0.05). The size of the bubbles indicates the number of proteins in each pathway, whereas the colour denotes the FDR corrected *p*-value and fold enrichment. The *Drosophila melanogaster* proteome was used as the reference. "Lemon": nymphs that developed exclusively on lemon plants. "SwO": nymphs exclusively developed on SwO. GO BP: Gene ontology biological process database; GO CC: Gene ontology cellular component database (https://geneontology.org/); FlyBase phenotype or FB ph: Flybase phenotype database (https://flybase.org/) terms refer to ortholog annotations and not to observed psyllid phenotypes; REACTOME: reactome pathway database (https://reactome.org/)

"CG5504" (Protein tumorous imaginal discs, mitochondrial) (Supplementary Table B). The semi-sterile phenotype groups were also enriched with the more abundant proteins in the SwOSuc (Fig. 6) and LemonSuc (Fig. 7) treatment groups.

A comparison of the SwOSuc vs. SwO treatment groups identified 15 proteins that were more abundant in SwOSuc nymphs and 11 that were more abundant in SwO nymphs. Four proteins were identified in both SwO and SwOSuc, which were absent in lemon-fed nymphs (Fig. 3A), namely "bw" (Brown), "CG13185" (Midasin), and "Klp61F" (kinesin-like protein Klp61F) (Supplementary Table A).

In the LemonSuc vs. Lemon comparison, 74 proteins were identified as being more abundant in the LemonSuc nymphs, whereas 18 were more abundant in the Lemon nymphs (Fig. 4B). The 18 proteins with increased abundance were predominantly associated with translation-related functions, including "translational regulator activity", "translation initiation complex formation", and "ribosomal scanning and start codon recognition", among others (Fig. 7). The proteins assigned to these pathways included "eIF3b" (Eukaryotic translation initiation factor 3 subunit B) and "RpS28b" (40 S ribosomal protein S28) (Supplementary Table B). With regard to biological processes associated with responses to external stimuli, chemicals and stress, these were enriched in the LemonSuc nymphs in the LemonSuc vs. Lemon comparison (Fig. 7). The proteins "Hrb87F" (heterogeneous nuclear ribonucleoprotein at 87 F), "Letm1" (mitochondrial proton/calcium exchanger protein), "coro" (coronin), and "NUCB1" (calcium-binding protein) were found to be more abundant (Supplementary Table B). Additionally, abnormal phenotype groups were enriched in LemonSuc nymphs including "abnormal behaviour", "abnormal neurophysiology" and "abnormal neuroanatomy" pathways (Fig. 7). The proteins that were assigned to the abnormal and the paralytic phenotype groups included "shot" (short stop), "twz" (Tiwaz, isoform B), "tau" (Microtubule-associated protein), the previously mentioned "Dap160" and "Syn". The proteins of the LemonSuc nymphs that enriched the "Hh signalling pathway" were "UbcE2M" (Nedd8-conjugating enzyme UbcE2M), "flw" (Serine/threonine-protein phosphatase beta isoform) and "Gbeta76C" (Guanine nucleotide-binding protein subunit beta-2) (Fig. 7 and Supplementary Table B).

The enriched biological processes identified in the transition of *T. erytreae* nymphs from lemon plants to a sucrose-only diet in the LemonSuc vs. Lemon comparison,

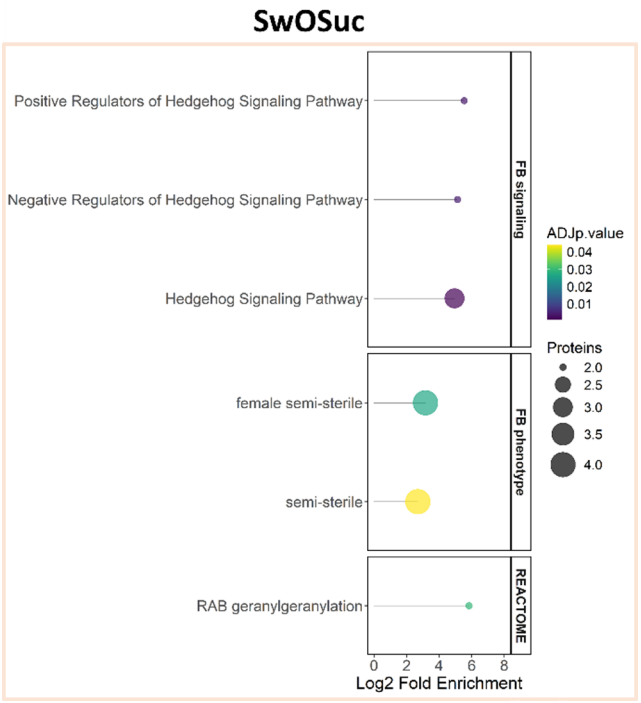

Fig. 6 Bubble plot of the enriched parameters identified when differentially abundant proteins (DAPs) in *Trioza erytreae* nymphs from the SwOSuc and SwO comparison were used to interrogate different databases (FDR adjusted *p*-value threshold of 0.05). The size of the bubbles is indicative of the number of proteins in each pathway, whereas the colour denotes the FDR corrected *p*-value and fold enrichment. The proteome of *Drosophila melanogaster* was used as the reference. "SwO": nymphs exclusively developed on SwO. "SwOSuc": nymphs that developed on SwO plants and were then fed sucrose for 24-h before sampling. FB signaling: Flybase signaling pathways database (https://flybase.org/); FB phenotype: Flybase phenotype database (https://flybase.org/) terms refer to ortholog annotations and not to observed psyllid phenotypes; REACTOME: reactome pathway database (https://reactome.org/)

were suggestive of a reactive adaptation. The aforementioned pathways included "response to external stimulus", "response to chemical", "response to stress", "actin cytoskeleton organization" and "microtubule cytoskeleton organization" (Fig. 7).

Discussion

This section will examine possible explanations for the diminished success of nymphal development on SwO plants compared to lemon plants. The greater proteome adjustment observed in lemon-developed nymphs in response to a dietary shift towards sucrose is a complement to our understanding of this plant-insect interaction.

Trioza erytreae exhibited a citrus host-specific oviposition pattern and nymphs developed better on lemon than on SwO plants

The presence of young flushes and their tender leaves is imperative for oviposition by *T. erytreae* (Catling 1969). The uniformity observed in the initial number of flushes and new leaf growth among the two hosts in the present study indicates that flushing intensity per se should not serve as a distinguishing criterion between the citrus hosts. The life cycle duration of *T. erytreae* observed in the present study (25 to 27 days) (Fig. 2C) is closely aligned with the optimal development time (23.9 days) identified by Pérez-Otero et al. (2024). This study confirmed the findings of Moran (1968), who showed that the host plant influences the development time of *T. erytreae*.

The oviposition behaviour exhibited by adult females of *T. erytreae* differed markedly when infesting lemon or SwO plants. A more dispersed oviposition pattern, characterised by a reduced egg count per leaf, was observed on SwO plants. Conversely, a denser pattern with a high number of eggs per leaf was observed on lemon plants (Table 1; Fig. 2A). The number of *T. erytreae* eggs laid per shoot differs according to the host plant from which they feed and probe, as was demonstrated in previous studies (Aidoo et al. 2019a; Hernández-Suárez et al. 2021). The oviposition patterns on SwO suggest an initial oviposition of a few eggs on a leaf, followed by a search for alternative leaves. This behaviour appears to indicate that the initial leaf is suboptimal for oviposition. Conversely, *T. erytreae* deposited a significant number of eggs on a single leaf of lemon plants, indicating that the leaf is perceived as optimal.

The substantial increase in the number of newly infested leaves observed on lemon plants from 5 to 23 DAI (Table 1) is probably related to the concentrated oviposition pattern observed in this host (Table 1). *Trioza erytreae* disperses at a higher rate when encountering infested flushes (Van den Berg et al. 1991a). Therefore, in order to reach full maturity, the emerging nymphs appeared to be compelled to seek out fresh foliage, where there was less competition from other nymphs and sufficient space.

The increased number of vacant galls and dead fourth and fifth instar nymphs, that remained attached to the leaves of SwO plants, suggested that a high proportion of the nymphs were unable to complete their development. Tamesse (2000) substantiated this observation by documenting a comparable number of *T. erytreae* in the initial stages (eggs and first instar nymphs) per flush in 'Eureka' lemon and 'Valencia' SwO, alongside a significantly lower number of fourth and fifth instar nymphs on 'Valencia' SwO.

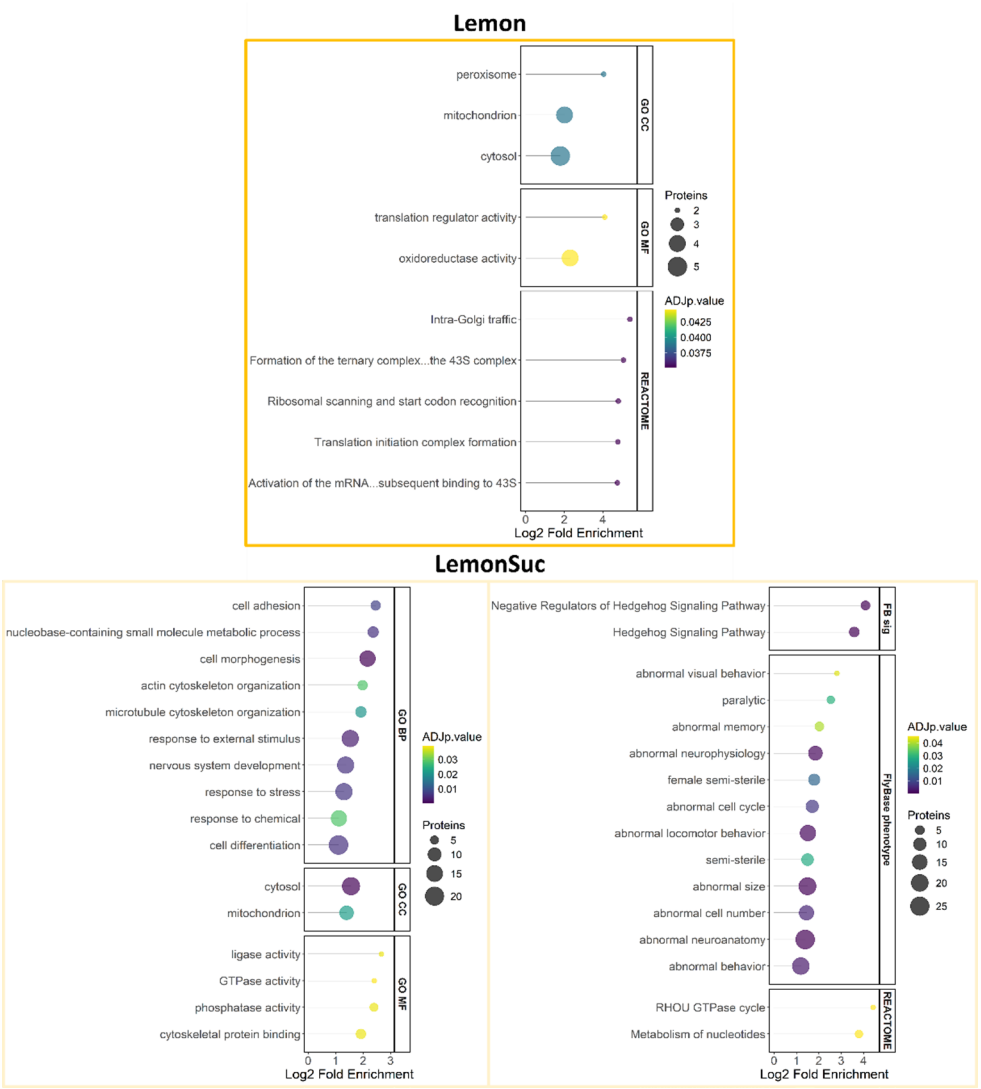

Fig. 7 Bubble plots of the enriched parameters identified when differentially abundant proteins (DAPs) in *Trioza erytreae* nymphs from the SwO and Lemon comparison were used to interrogate different databases (FDR adjusted *p*-value threshold of 0.05). The size of the bubbles indicates the number of proteins in each pathway, whereas the colour denotes the FDR corrected *p*-value and fold enrichment. The *Drosophila melanogaster* proteome was used as the reference. "Lemon": nymphs that developed exclusively on lemon plants. "LemonSuc": nymphs that developed on lemon plants and were then fed sucrose for 24-h before sampling. GO BP: Gene ontology biological process database; GO CC: Gene ontology cellular component database; GO MF Gene ontology molecular function database(https://geneontology.org/); FlyBase phenotype or FB ph: Flybase phenotype database (terms refer to ortholog annotations and not to observed psyllid phenotypes) FB sig: Flybase signaling pathways database (https://flybase.org/); REACTOME: reactome pathway database (https://reactome.org/)

Citrus host plant affected the types of proteins identified in nymphs

The present study identified citrus host plant proteins in *T. erytreae* nymphs, suggesting their potential location within the insect gut or as a result of ingestion and subsequent retention by the nymphs. A comparable number of total plant proteins was identified in nymphs developing in both citrus hosts (Fig. 3A). This finding is consistent with the understanding that insects ingest, metabolise, and modify protein conformation, leading to the preservation of only a limited number of highly stable plant proteins (Salvucci et al. 1998; Chen et al. 2007). The identified host plant proteins were heat shock proteins, proteins related to the oxidative stress response, and to protein metabolism (Wang and Constabel 2004; Shafqat et al. 2020). These proteins have been identified as part of a general response in plant metabolism that escalates following infestation by phloem-feeding insects, as was previously identified in the response of SwO to *T. erytreae* (Du et al. 2015; Wu et al. 2019; Magalhães et al. 2024). Heat shock proteins are a class of proteins described to play a role in the stress response of SwO (Shafqat et al. 2020). Several proteins involved in oxidative metabolism have the capacity to inhibit the digestive processes of insects (Wang and Constabel 2004). Two enzymes, catalase (A0A067H2F2) and a peroxidase (A0A067H6D4), were uniquely identified in nymphs developing on lemon and SwO, respectively (Supplementary Table A). The two enzymes were found to be induced in tomato plants (*Solanum lycopersicum* L.) infested by phloem-feeding insects, with catalase activity exhibiting a positive correlation with the host's resistance to aphids (Zhao et al. 2016). The presence of peroxidase in nymphs fed on SwO, an enzyme involved in oxidative metabolism, may indicate a plant response to insect feeding and has the potential to influence insect digestion and gut physiology (Wang and Constabel

2004; Zhao et al. 2016). Barbehenn et al. (2010) suggest that plant proteins may interact with insect digestive enzymes or gut microbiota to negatively impact nutrient assimilation or detoxification processes, thereby lowering the rate of insect development. The two identified plant proteins may serve as markers for feeding events and plant responses that could be affecting the insect's digestion.

Three of the four proteins that were uniquely identified in the nymphs that developed on SwO plants (SwO and SwOSuc, see Sect. 4.4), namely "bw", "CG13185" and "Klp61F" (Supplementary Table A), are associated with the molecular function of ATP binding (Mackenzie et al. 1999; Garbarino and Gibbons 2002; Van den Wildenberg et al. 2008). These findings provide a foundation for further research investigating plant-host responses to psyllid feeding.

The proteome of the nymph is contingent on the species of citrus host

It is anticipated that the use of the *D. citri* UniProt proteome as a reference will result in conservative identification of proteomic profiles due to the absence of species-specific *T. erytreae* sequences in a reference database. This approach limits the identification of species-specific proteins related to host-insect interaction. As an example, salivary proteins, also referred to as effectors, frequently display substantial sequence divergence, even among closely related species, as a consequence of strong selective pressures from host plant immune systems (Bos et al. 2010; Hogenhout and Bos 2011). Consequently, they are likely to be under-represented in the findings of the present study.

The greater number of DAPs identified in SwO nymphs compared to Lemon nymphs (101 DAPs) (Fig. 4) is indicative of the greater impact of the citrus host on the *T. erytreae* proteome, as opposed to the impact of dietary shift to sucrose-only diet (see Sect. 4.4). A comparable outcome was observed in a study on *D. citri* adults, wherein psyllids nourished on distinct diets without dietary shifts exhibited greater proteome differences between them, and afterward, upon exposure to dietary shifts, these psyllids displayed more similar proteomic profiles (Ramsey et al. 2022).

In the present study, the enriched pathways in nymphs from SwO were related to the "protein metabolism" pathway. Protein metabolism was also affected in insects experiencing nutritional deprivation, according to Chen et al. (2017b). The enriched pathways identified in the nymphs developed on lemon plants were related to growth, development, and energy metabolism (Fig. 5). These pathways are vital for the effective development of insects (Arrese and Soulages 2010; Fraga et al. 2013). *D. citri* exhibited a greater number of ATP synthases during its development on a highly suitable plant host, in contrast to a less suitable host (Ramsey et al. 2022). The present study indicates that the increased levels of ATP synthases ("blw" and "ATPsyn-beta") and the induction of energy-related pathways in nymphs that developed on lemon hosts suggest that their development was enhanced on this host compared to SwO.

The nutritional composition of the diet exerts a significant influence on psyllid development (Shugart et al. 2019). The feeding of psyllids on pumelo trees resulted in a higher population of *D. citri* psyllids compared to their feeding on mandarin trees. The diet from the pummelo tree had higher levels of nutrients, including sugar acids, xylose, and other sugars, along with the amino acid serine (Shugart et al. 2019). Previous studies revealed that the macro- and micronutrient profiles of lemon and SwO plant leaves differ (Galvez-Sola et al. 2015), suggesting that psyllids have a distinct diet when feeding on each citrus host. Indeed, a greater proteomic modification was identified in the leaf-enriched vascular sap of SwO in response to *T. erytreae* infestation compared to lemon plants, which may be related to enhanced resistance (Magalhães et al. 2024).

In the SwO vs. Lemon comparison, the proteomic analysis of nymphs that developed on SwO plants exhibited enrichment in the proteins "CkIIbeta" and "Abi" (Supplementary Table B). These proteins may be related to the semi-sterile phenotype groups (Fig. 5), as they affect oogenesis and may further impact the fertility of the ensuing adults (Wong et al. 2011; Squarr et al. 2016). Alternatively, the same proteins may have a role in adjustments in signal transduction, cytoskeletal dynamics, and cell cycle progression. The detection of proteins in insect nymphs related to semi-sterile phenotypes is unclear, as these proteins pertain to adult phenotypes. In other studies, insects feeding on lower quality diets had reduced fertility rates (Wang et al. 2013; Chen et al. 2017b), probably caused by a prioritisation of survival over reproduction (Parthasarathy and Palli 2011). However, it should be noted that assays on adult psyllids were not performed in the present study. The present finding is consistent with the life history theory, that predicts trade-offs between current survival and future reproduction when resources are limited. Studies in insects indicate that nymphs raised on restricted diets yielded adults that laid fewer eggs with lower hatch rates, while inadequate diets across successive generations reduced oviposition and fertility in subsequent adults (Wittmeyer et al. 2001; Coudron and Kim 2004). In a similar vein, the influence of the plant host on the fertility rates of psyllids was demonstrated for *Bactericera cockerelli* (Šulc) (Hemiptera: Triozidae) (Mustafa et al. 2015). The "Abi" is known to be an adaptor protein, that links Abelson (Abl) signalling to actin cytoskeleton regulation and cell cycle control, including modulation of cyclin-dependent kinase 1 (CDK1) activity, cyclin

A and B levels, and cytoskeleton-dependent events during the G2 phase of the cell cycle (Lin et al. 2004; Kondylis et al. 2007; Lim et al. 2018). An increase in "Abi" abundance could indicate alterations in checkpoint regulation and cytoskeletal remodelling under nutritionally suboptimal conditions. Similarly, "CkIIbeta" is known to function as the regulatory subunit of the casein kinase II holoenzyme, influencing kinase specificity and phosphorylation of substrates involved in cell cycle progression, stress responses, and developmental signalling in insects (Kalmykova et al. 2002; Bandyopadhyay et al. 2016). Consequently, elevated "CkIIbeta" may serve as an indicator of compensatory modulation of signalling and checkpoint control in response to dietary stress. Together, the higher abundance of "CkIIbeta" and "Abi" in nymphs developing on sweet orange suggest a coordinated cellular response to nutritional limitation, involving adjustments in signal transduction, cytoskeletal dynamics, and cell cycle timing. Further investigation is required for a comprehensive analysis of the "CkIIbeta" and "Abi" proteins in nymphs to understand their role on *T. erytreae* fertility and cell cycle modulation to identify potential control strategies.

In SwO developed nymphs, the "signal transduction" and "transmission across chemical synapses" pathways were enriched (Fig. 5) by "Syn", a protein implicated in the modulation of locomotor behaviour (Godenschwege et al. 2004), and "Sls", a protein related to muscle development (Burkart et al. 2007) (Supplementary Table B). The latter protein was identified in higher abundance in *D. citri* raised on a host where psyllids had narrower wings (Paris et al. 2016; Ramsey et al. 2022). This finding suggests that feeding on a specific host could have detrimental effects on the development of the nymphs. The present study suggests that SwO exerts such a deleterious effect. The impact of the host on psyllids at the adult stage was not evaluated. Furthermore, the proteins "Dap160", which induces endocytosis (Tang et al. 2005), and "CadN", which is essential for insect development (Hummel and Zipursky 2004) (Supplementary Table B), were more abundant in SwO nymphs. This observation may be indicative of an increased requirement for synaptic vesicle recycling and cell–cell adhesion processes in response to the less suitable nutritional conditions.

The observation that nymph development success was three times lower on SwO compared to lemon plants, coupled with the findings from proteomic analysis, suggests that the differing nutritional profiles of the two citrus hosts may have induced a stress effect on the nymphs developed on SwO. Overall, the results of the present study indicate that the nutrition supplied by a lemon plant-based diet is more conducive to the development of *T. erytreae* than the SwO diet.

The sucrose feeding treatment significantly impacted the proteome of nymphs raised on lemon hosts

50% of the nymphs that developed on citrus hosts were subsequently subjected to a sucrose feeding diet. The sucrose feeding treatment had a more pronounced impact on the proteome of nymphs that developed on lemon plants (92 DAPs), compared to those that developed on SwO plants (26 DAPs) (Fig. 4). This pattern may be interpreted in two different ways in the context of a stress response to a SwO plant diet: (i) it may be indicative of stress priming enhancing preparedness of the nymphs towards the sucrose stress, lowering the need of a proteome responsiveness; (ii) it may be a result of chronic stress limiting the proteome responsiveness to a new stress (higher metabolic rigidity). As demonstrated by Weldon et al. (2019) and Kawecki et al. (2021), fruit flies exhibited a reduced capacity to withstand starvation when previously subjected to nutritional stress. Furthermore, the study undertaken with the fruit fly *Bactrocera dorsalis* demonstrated that a period of dietary deprivation resulted in an enhanced level of resilience to nutritional stress (Chen et al. 2017a and b).

The objective of this step was to infer the impact of the prior diet on citrus hosts by comparing the variations in the nymph proteome resulting from the transition to a sucrose-only diet. Nymphs exposed to a sucrose-feeding diet on LemonSuc and SwOSuc synthesised four proteins associated with ATP binding, namely "MRP", "Mi-2", "SMC1" and "bt" (Bent, isoform F) (Supplementary Table A) (Dege and Hagman 2014; Cole 2014; Yi et al. 2017). ATP-binding proteins are essential for the development and the resilience of nymphs against xenobiotic stress (Broehan et al. 2013). However, the presence of ATP-binding proteins in nymphs that matured on lemon plants was not observed. This finding suggests that lemon host plants do not elicit xenobiotic stress in nymphs or that the stress may have been of lesser intensity compared to the other three groups. The unique proteins identified in the sucrose-fed nymphs (SwOSuc and LemonSuc), "Nedd4" and "wrd", are required for proper synaptic growth (Viquez et al. 2006; Zhong et al. 2011). Sucrose diets have been shown to initially increase synaptic currents in *D. melanogaster* embryos (Suzuki et al. 2002), which may provide a potential explanation for the death of nymphs when feeding on sucrose diet longer than 24 h.

The present study identified three common DAPs in both SwOSuc and LemonSuc nymphs that enriched the "Hh signaling pathway". The identified proteins exhibit different regulatory roles: "Tnpo" functions as a positive regulator, "wdb" as a context-dependent regulator and "CG5504" (Supplementary Table B) as a negative regulator (Canamasas et al. 2003; Jia et al. 2009; Su et al. 2011; Shi et al.

2014). The proteome of SwOSuc nymphs exhibited an equilibrium of positive and negative regulators of the "Hh signalling pathway" (Fig. 6). In contrast, the proteome of Lemon nymphs was dominated by negative regulators of the "Hh signaling pathway" including "UbcE2M" and "Gbeta76C" (Fig. 7 and Supplementary Table B) (Du et al. 2011; Li et al. 2018). Both negative and positive regulators of the "Hh signalling pathway" can induce developmental malformations in insects, as observed in the red flour beetle [*Tribolium castaneum* Herbst, 1797] (Villarreal et al. 2015) and in the fruit fly (Goodman et al. 2021). The proteomic findings of nymphs after 24 h of sucrose consumption suggest possible changes in the nymphs that would affect the adult psyllid.

Proteomic analysis revealed a higher abundance of the "eIF3b" and "RpS28b" proteins in Lemon compared to LemonSuc (Supplementary Table B). These two proteins related with translation are vital for cellular proliferation and division (Dong and Zhang 2006; Marygold et al. 2007). Similarly, a significant reduction in the number of translation transcripts was observed in *D. melanogaster* larvae when they were shifted from a complete food diet to a starvation diet, or a diet comprising only sugar (Nagarajan and Grewal 2014). The sucrose feeding treatment had a pronounced influence on the proteome of nymphs matured on lemon, enhancing their active responses to stress, chemicals and external stimuli (Fig. 7). The protein "Hrb87F", which is present in higher abundance, was found to be associated with active responses and enhanced starvation tolerance (Singh and Lakhotia 2012). Other highly abundant proteins linked to biotic and abiotic stress responses were observed in LemonSuc nymphs, namely "Letm1", "coro" (coronin) and "NUCB1" (Supplementary Table B) (Lee et al. 2008; Jin et al. 2008; Berkey et al. 2009), suggesting that the dietary shift from lemon plants to a sucrose only diet triggers a stress response in *T. erytreae* nymphs.

It has been shown that insects subjected to a more restricted diet exhibit greater resilience to external stressors, a phenomenon attributed to the priming of stress responses (Chen et al. 2017a and b). This may explain the reduced necessity for adaptation of the SwO developed nymphs when transitioning to a sucrose diet. Conversely, it is conceivable that nymphs developing on SwO are metabolically constrained rather than primed. Prolonged exposure to a nutritionally poor or stressful host might reduce metabolic reserves and adaptability, constraining the ability to initiate extensive proteomic or transcriptional responses to novel stressors (Weldon et al. 2019; Kawecki et al. 2021). The present study suggests that sucrose feeding treatment elicits stress responses, which can lead to developmental impairments and behavioural alterations in *T. erytreae*. Further functional studies are required to elucidate the impact of sucrose-induced proteins on *T. erytreae* homeostasis and adult development.

Conclusion

The present findings indicated that *T. erytreae* exhibited divergent oviposition and infestation behaviour patterns on SwO and lemon plants. The developmental time was found to be slightly prolonged for the nymphs that had fed on SwO. The number of nymphs that successfully developed into the fourth and fifth instars was three times higher in lemon plants when compared to SwO plants. The proteome enrichment analysis of nymphs developed on lemon plants seemed to be closely related to a greater enrichment in developmental and energy-metabolism related pathways, namely the "generation of precursor metabolites and energy", "formation of ATP by chemiosmotic coupling", "mitochondrial biogenesis" and "cuticle development". The SwOSuc and LemonSuc diets resulted in the enrichment of ATP-binding proteins associated with xenobiotic stress. The SwO diet enriched the "signal transduction" and "transmission across chemical synapses", possibly indicating neurodevelopmental adaptation needs. The lack of enrichment in these proteins and pathways on Lemon suggests that this host is more favourable for the effective development of *T. erytreae*. The observation that nymphs developed on Lemon exhibited a more substantial proteome adjustment in response to the sucrose feeding treatment than nymphs developed on SwO substantiates this hypothesis. Overall, proteomic approaches improve our understanding of the regulatory mechanisms governing biosynthetic pathways in *T. erytreae* in response to feeding on different citrus host species. This research is pertinent to the study of *T. erytreae* adaptations and the identification of potential targets for its control. Future work incorporating behavioural assays and neuroanatomical analyses will be required to determine whether these proteomic signatures correspond to measurable behavioural or morphological changes in *T. erytreae*.

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Data availability The mass spectrometry proteomics data were deposited in the ProteomeXchange Consortium via the PRIDE (www.ebi.ac.uk/pride) (Perez-Riverol et al. 2022) partner repository with the dataset identifier PXD059807 and http://doi.org/10.6019/PXD059807.

Declarations

Conflict of interest The authors declare no competing interests.

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