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Research article

Ovi-protective mothers: exploring the proteomic profile of weevil (*Gonipterus*) egg capsules

Natalia M. Souza [a], Tianfang Wang [b,c], Saowaros Suwansa-ard [b], Helen F. Nahrung [d], Scott F. Cummins [b,c,*]

[a] Tropical Fruit and Market Access RD&E, Horticulture and Forestry Science, Department of Agriculture and Fisheries, Portsmith, QLD 4870, Australia
[b] Centre for Bioinnovation, University of the Sunshine Coast, Maroochydore 4558, QLD, Australia
[c] School of Science, Technology and Engineering, University of the Sunshine Coast, Maroochydore 4558, QLD, Australia
[d] Forest Research Institute, University of the Sunshine Coast, Maroochydore, QLD 4558, Australia

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ABSTRACT

Insects of different orders produce elaborate structures to protect their eggs from the many threats they may face from the environment and natural enemies. In the weevil genus *Gonipterus*, their dark, hardened egg capsule is possibly generated by a mixture of the insects' excrement and glandular substances. To test this hypothesis, this study focused on the elucidation of protein components present in the egg capsule cover and interrogated them through comparative analysis and gene expression to help infer potential functions. First, female *Gonipterus* sp. n. 2 reproductive and alimentary tissues were isolated to establish a reference transcriptome-derived protein database. Then, proteins from weevil frass (excrement) and egg capsule cover were identified through mass spectrometry proteomics. We found that certain egg capsule cover proteins were both exclusive and shared between frass and egg capsule cover, including those of plant origin (e.g. photosystem II protein) and others secreted by the weevil, primarily from reproductive tissue. Among them, a mucin/spidroin-like protein and novel proteins with repetitive units that likely play a structural role were identified. We have confirmed the dual origin of the egg capsule cover substance as a blend of the insects' frass and secretions. Novel proteins secreted by the weevils are key candidates for holding the egg case cover together.

1. Introduction

Egg laying (oviparity) is the most common mode of reproduction among insects, along with a diversity of strategies to protect this fragile life stage from the threats of natural enemies looking for a source of nutrition for themselves or their offspring, and adverse environmental factors. These ovi-defensive strategies may include adult behaviours, such as parental care (Smiseth, 2014), hiding of eggs with environment materials (Gan-Yu et al., 2019), deposition of toxic chemical compounds (Eisner et al., 2000; Blum and Hilker, 2002) and building protective structures from insect-borne substances (Li et al., 2008), among others. The protective structures, variously referred to as egg capsules, oothecae, egg cases, egg pods or egg packets, are found across several insect orders including Blattodea (cockroaches), Phasmatodea (stick insects), Mantodea (mantises) and Orthoptera (grasshoppers and locusts) (Goldberg et al., 2015), where they have been studied in more depth. In Coleoptera, chrysomelid beetles (Cassidinae) (Chapman, 1998b; Goldberg et al., 2015) and some weevils (Curculionidae: Gonipterini) (Oberprieler et al., 2014) produce egg capsules, but literature on the strategy within this order is scarce.

Formation of egg protective structures varies greatly in terms of shape, size, number of eggs, and substances used across the different insect species that produce them. This variation allows for distinction of insect groups for the general public (Eiseman et al., 2010) and in scientific studies: in mantises, for example, morphology of the egg capsule (ootheca) may be used for species differentiation (Song et al., 2020). Among the egg protection structures, as well as in egg adhesion substances, proteinaceous blends secreted by the female are usually involved (Gillott, 2008; Li et al., 2008; Betz, 2010), although other substances like calcium oxalate crystals and food-borne waxes and metabolites may also be present (Whitehead, 2011).

The Australian genus *Gonipterus* (Coleoptera: Curculionidae) produces egg capsules containing several eggs laid transversely along the length and covered by a malleable dark substance that hardens once

* Corresponding author.
E-mail address: scummins@usc.edu.au (S.F. Cummins).

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dried (Tooke, 1955; Sanches, 2000). Variation in the number of eggs and shape of the structure have been reported for the three species that have become serious pests of *Eucalyptus* plantations around the world, *Gonipterus* sp. n. 2, *G. platensis* and *G. pulverulentus* [*sensu* Mapondera et al. (2012)] (Tooke, 1955; Freitas, 1979; Sanches, 1993; Souza, 2016). Some authors have speculated that this substance may be composed of frass (insect excrement), glandular substances or an anal secretion (Tooke, 1955; Santolamazza-Carbone and Rivera, 2003, Oberprieler et al., 2014), but so far, the nature of the protective substance laid along with the eggs is unknown. Recent research has demonstrated that frass and egg capsules present different organic compound profiles, whereby some originate from their host plants, while others are the result of metabolism of plant compounds or produced by the weevils themselves (Souza, 2021). This supports the hypothesis for the presence of weevil-borne substances in the egg capsule and the dichotomy of frass and egg capsules.

This study aimed to identify the protein components that are present in frass and the egg capsule's dark protective cover produced by *Gonipterus* sp. n. 2. No genome or transcriptome had previously been established for this species, and therefore, we developed the first *de novo* reference transcriptomes that were used to provide a deduced protein database, which supported our proteomic analysis. Then, protein profiles of frass and the egg capsule's were assessed for their differences and similarities, with more in-depth analysis performed for the egg capsule cover.

2. Materials and methods

2.1. Insect origin and rearing

Collection of *Gonipterus* sp. n. 2 was carried out in October 2018 in commercially planted *Eucalyptus* located in southern Western Australia, where this species is invasive and occurs in large numbers during outbreaks (Loch, 2006; Mapondera et al., 2012). Insects were kept in cages at 20 ± 1 °C, 14 h photo-phase for at least four months prior to the beginning of the experiment and fed on fresh *E. dunnii* foliage. Males and females were maintained together, allowing for mating to occur freely. For collection of samples, 72 females were selected and placed into smaller perforated plastic cages with a maximum of 12 individuals each, containing a freshly cut *E. dunnii* branchlet with young leaves, adequate for oviposition. Whole egg capsules and frass were collected over two consecutive days and processed daily for storage.

2.2. Tissue collection, RNA isolation and reference transcriptome preparation

Fifteen female *Gonipterus* sp. n. 2 utilized for oviposition were randomly selected and placed in small containers with a moist cotton ball for 24 h starvation to clean their alimentary system of any ingested leaf pieces and faecal matter. After starvation, the insects were killed by freezing at −20 °C for 30 min and dissected. Dissection was performed under a dissection microscope (Leica MZ6) by opening the insect abdomen laterally and removing contents for selection of tissues as described previously (Goldson and Emberson, 1981; Pratt et al., 2018). For reproductive tissue analysis, tissue from the bursa copulatrix, median and lateral oviducts, calix and pedicels (Figure 1A) of seven parous (reproductively active) females (Figure 1B) were excised and placed into a microcentrifuge tube containing RNAlater® (Ambion). Likewise, for analysis of alimentary tissue, the rectum and final portion of hindgut were excised and collected into RNAlater®. All tissues were preserved in −80 °C until used for RNA extraction.

Total RNA from reproductive and alimentary tissues, combined from 7 females, were extracted. For homogenization, up to 100 mg tissue was finely sliced using a sterile scalpel blade, then disrupted in TRIzol™ Reagent (Thermo Fisher Scientific; Seventeen Mile Rocks, Aust.) using a sterile pestle with manual rotation. Further procedures for total RNA isolation followed the TRIzol™ Reagent manufacturer's instructions. RNA concentration was determined using the Nanodrop 2100 and integrity was assessed for RNA degradation with a Bioanalyzer RNA 6000 Nano mRNA kit (Agilent Technologies; Mulgrave, Aust.). Total RNA was then sent to Novogene (Hong Kong) for Illumina 2500 sequencing, then *de novo* assembled (combining reproductive and alimentary tissues) using their standard workflow (https://en.novogene.com/). This formed the reference transcriptome from which a protein database was established

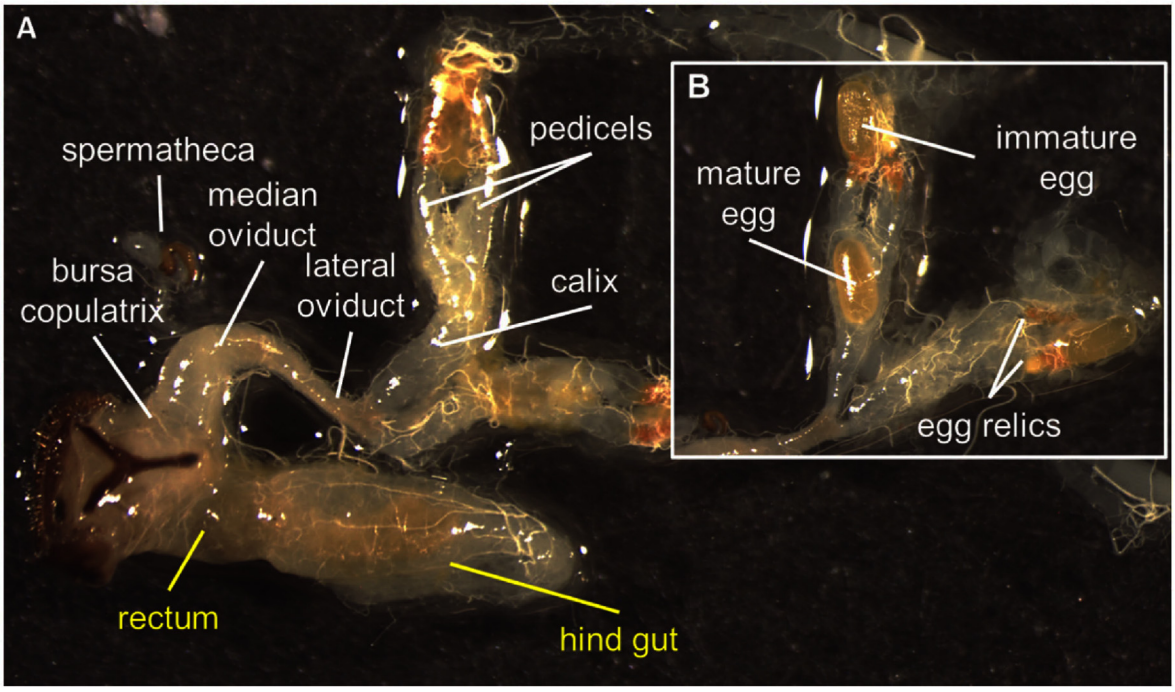

Figure 1. Photos of dissected *Gonipterus* sp. n. 2 female tissues. **(A)** Reproductive (white captions) and final portion of alimentary tissue (yellow captions). **(B)** Eggs within parous female tissues.

using the ORFpredictor (Robinson and Oshlack, 2010). Relative expression of genes for the two tissues was determined using RNA-seq reads mapped against the respective tissue *de novo* reference transcriptome, and presented based on transcripts per kilobase million (TPM), utilizing the *de novo* RNA-seq CLC Genomic Workbench 11 software (Qiagen; Clayton, Aust.).

2.3. Sample collection and protein extraction

Frass pellets dropped on the surface of leaves were removed with forceps and placed into sterile microcentrifuge tubes. Whole egg capsules were carefully detached from the leaves and placed separately into a compartmentalized acrylic box where they had 10 μL filtered water (MilliQ) added and left for 30 min to soften. Each capsule was then transferred to an excavated glass block for dissection and separation of eggs and capsule cover (Figure 2) under dissection microscope (Leica MZ6). Capsules with broken eggs were discarded to avoid contamination of capsule cover with egg contents. Egg capsule cover material and water used for softening were transferred into sterile microcentrifuge tubes. Samples of frass and capsule cover were stored at −80 °C until protein extraction. Utensils used for collection of samples and dissection were cleaned with sodium hypochlorite (2% m/v) and rinsed with filtered water (MilliQ) before and between samples.

The covers of 15 egg capsules were combined and homogenized before aliquots were collected for protein extraction; frass pellets from all cages were also combined and processed similarly. Protein extraction followed a methodology described previously (Whaite et al., 2018). Briefly, aliquots of capsule cover and frass were placed into new microcentrifuge tubes and homogenized in two different 2-D extraction buffers (GE Healthcare; Parramatta, Aust.): Buffer II–Urea and 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS) and Buffer IV–Urea, Thiourea, CHAPS and n-Decyl-N,N-dimethyl-3-ammonio-1--propanesulfonate (SB 3-10) (exact concentrations for each solution are confidential), prepared according to manufacturer's instructions and added to samples at approximately 10 μL/mg of sample. Homogenates were vortexed and pulse centrifuged several times to mix the solution and vortexed again for 90 s prior to incubation in a sonic bath at room temperature for 5 min. The homogenates were then centrifuged at 12, 000 × g for 8 min, then supernatant transferred into new tubes. Both supernatant and pellet were stored at −80 °C until used for either in-solution trypsin digestion or sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by in-gel trypsin digestion.

2.4. In-solution and in-gel trypsin protein digestion

In-solution trypsin digestion was undertaken using methods previously described (Ni et al., 2018). Briefly, concentrated total egg capsule cover proteins were resuspended in 6 M urea, vortexed and sonicated for 2 min followed by incubation in reducing reagent (200 mM dithiothreitol, 25 mM NH_4HCO_3) for 60 min at 37 °C. Egg capsule cover proteins were vortexed upon addition of alkylating reagent (200 mM iodoacetamide, 25 mM NH_4HCO_3) and the solution incubated for 60 min at room temperature. A further reduction step was undertaken for 60 min at room temperature. The solution was diluted by the addition of ddH_2O and vortexed before the addition of the enzyme. Trypsin was added in a 1:10 ratio, mixed thoroughly and incubated overnight at 37 °C. The enzyme was inhibited at pH < 3 by addition of 10% formic acid.

In-gel trypsin digestion was performed following SDS-PAGE. Frass and capsule cover proteins in supernatant and pellets were added to 2 × Laemmli Sample Buffer (Bio-Rad; Gladesville, Aust.) containing 2-mercaptoethanol (final concentration of 355 mM) (1:1) and incubated at approximately 100 °C for 5 min. Samples were run in duplicates in a precast 4–20 % polyacrylamide gradient gel (Amersham ECL Gel, GE Healthcare Life Sciences) for 10 min at 80 V and then 35 min at 120 V. Separated proteins were visualised by Coomassie Brilliant Blue (Bio-Rad) stain prepared with 0.25 g of Coomassie Brilliant Blue R-250 in 90 mL of methanol:H_2O (1:1, v/v) and 10 mL of glacial acetic acid, filtered through a Whatman No. 1 filter to remove any particulate matter, followed by destaining solution (10% acetic acid, 50% methanol, and 40% H_2O) for 12 h. Protein sizes were estimated using a Pierce Blue Molecular Weight marker (Thermo Scientific). All visible protein bands were excised and placed into tubes separately, then processed by in-gel trypsin digestion as described previously (Wang et al., 2016). The samples were reconstituted in 0.1% formic acid and stored at −20 °C until mass spectroscopy.

2.5. uHPLC tandem QTOFMS/MS analyses and protein identification

Egg capsule cover and frass proteins were analysed by LC-MS/MS on an ExionLC liquid chromatography system (AB SCIEX, Concord, Canada)

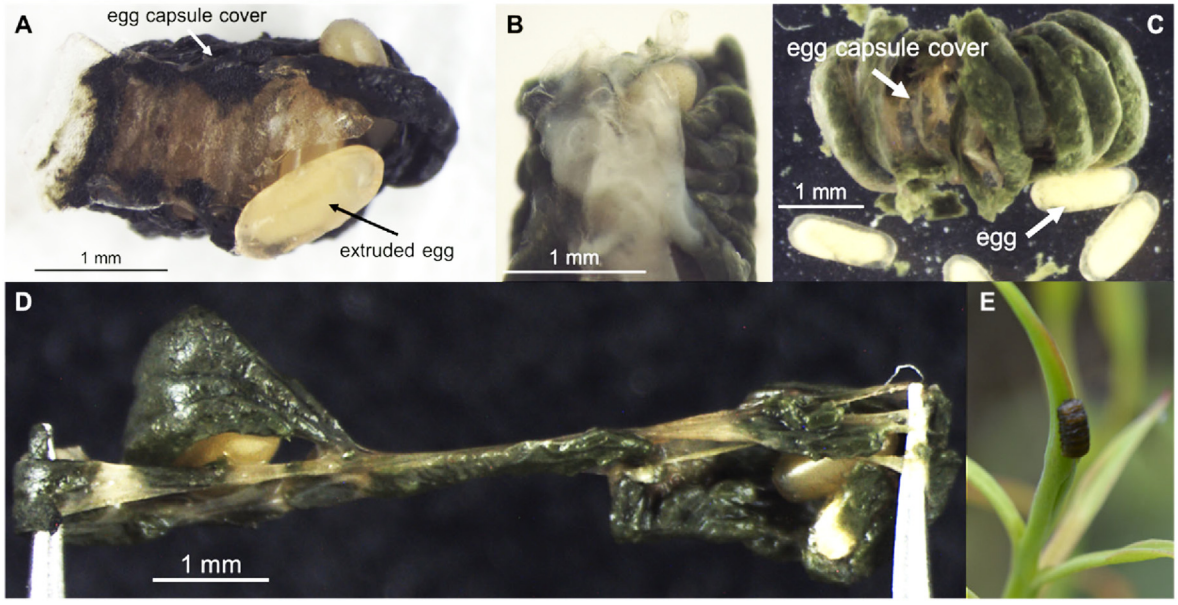

Figure 2. Egg capsules of *Gonipterus* sp. n. 2. **(A)** Intact egg capsule with extruded egg (view from the bottom of a detached capsule). **(B)** Egg capsule in water with softening cover (view from the bottom). **(C)** Egg capsule cover and eggs separated. **(D)** Re-hydrated egg capsule cover being stretched with tweezers. **(E)** Egg capsule in the field attached to a flushing leaf. Dissection microscope photos obtained with Nikon SMZ800N, Software TCCamera version 4.2, Tucson Photonics Co, Ltd.

coupled to a QTOF X500R mass spectrometer (AB SCIEX, Concord, Canada) equipped with an electrospray ion source. Each sample (20 µL) was injected onto a 100 mm × 1.7 µm Aeris PEPTIDE XB-C18 100 uHPLC column (Phenomenex, Sydney, Australia) equipped with a SecurityGuard column for mass spectrometry analysis. Solvent A consisted of 0.1% formic acid (aq.) and solvent B contained 90/10 acetonitrile with 0.1% formic acid (aq.). Linear gradients of 5–35% solvent B over 10 min at 400 µL/min flow rate, followed by a steeper gradient from 35% to 80% solvent B in 2 min and 80%–95% solvent B in 1 min were used for peptide elution. Solvent B was held at 95% for 1 min for washing the column and returned to 5% solvent B for equilibration prior to the next sample injection. The ion-spray voltage was set to 2400 V and the declustering potential set to 100 V. The interface heater was set to 150 °C and the flow rate of the curtain gas and nebulizer gas was set to 25 and 12 psi, respectively. The mass spectrometer acquired 500 ms full scan TOF-MS data that was followed by 20 by 50 ms full scan product ion data in an Information Dependent Acquisition mode. The TOF-MS full scan was set to cover the mass range between 350-1800 for product ions. Product ions that exceeded 100 counts with a charge state between +2 and +5 triggered the acquisition of data. This resulted in MS/MS spectra of the 20 most intense ions. The software used for data acquisition and processing was the Analyst TF 1.5.1 software (AB SCIEX, Concord, Canada).

The LC-MS/MS data were imported to the PEAKS studio (Bioinformatics Solutions Inc., Waterloo, ON, Canada, version 7.0) with the assistance of MS Converter module of ProteoWizard. *De novo* sequencing of peptides, database search and characterizing specific post translational modifications (PTMs) were used to analyse the raw data; false discovery rate was set to ≤1%, and [−10 * log(p)] was calculated accordingly where p is the probability that an observed match is a random event. The PEAKS used the following parameters: (i) precursor ion mass tolerance, 0.1 Da; (ii) fragment ion mass tolerance, 0.1 Da; (iii) tryptic enzyme specificity with two missed cleavages allowed; (iv) monoisotopic precursor mass and fragment ion mass; (v) a fixed modification of cysteine carbamidomethylation; and (vi) variable modifications including lysine acetylation, deamidation on asparagine and glutamine, oxidation of methionine and conversion of glutamic acid and glutamine to pyroglutamate. The database search included our own *Gonipterus* sp. n. 2 transcriptome-derived protein database and the *Eucalyptus* (https://eucg

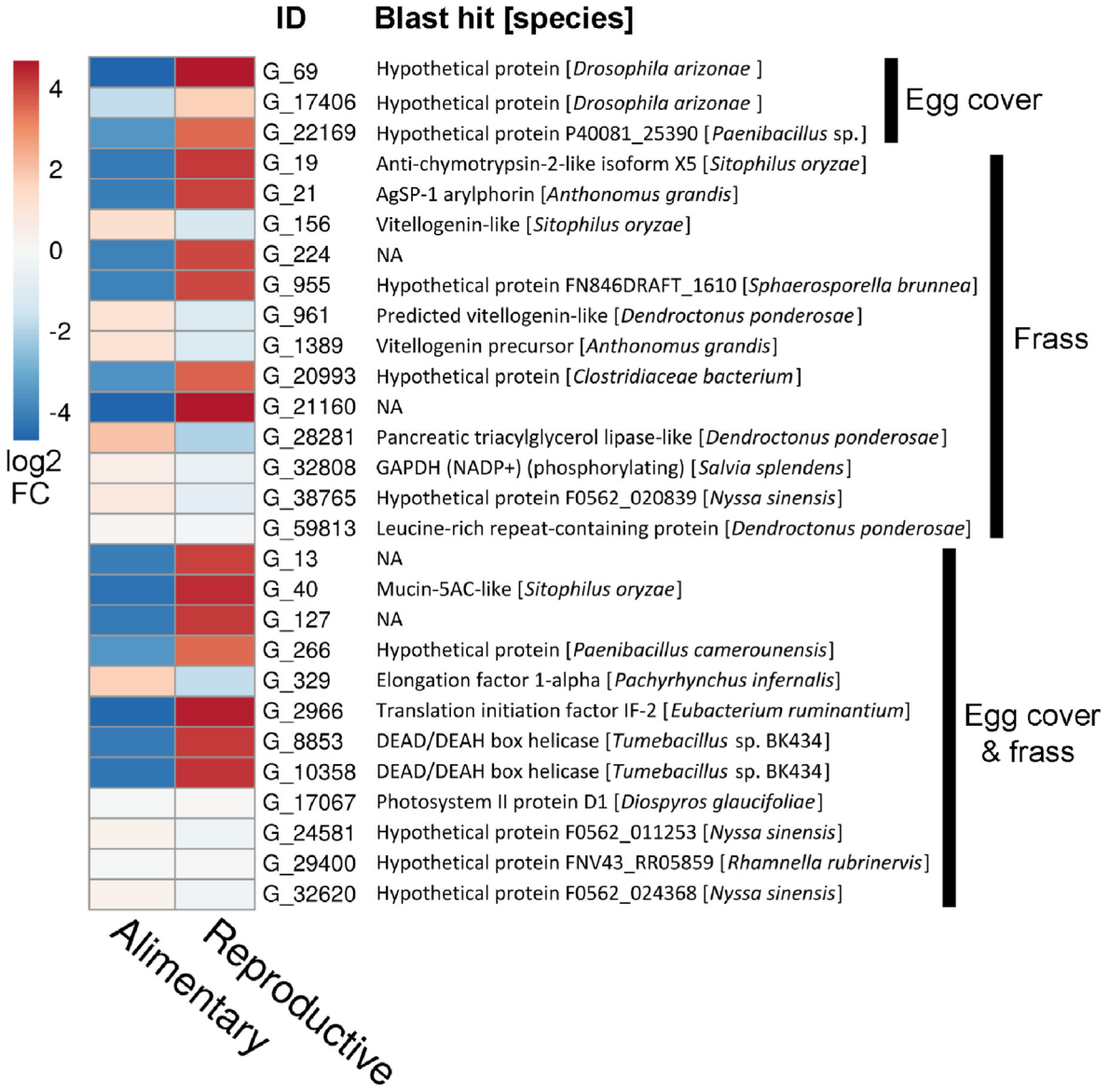

Figure 3. Identification of proteins isolated from *Gonipterus* sp. n. 2.egg cover, frass and egg cover/frass using in-solution trypsin digestion and mass spectrometry. Heatmap shows relative gene expression (log2 fold-change) in alimentary and reproductive tissue for each protein. Protein sequence details can be found in **File S1** and **File S2**.

Table 1. Proteins present in egg capsule cover and frass of *Gonipterus* sp. n. 2. TPM, transcripts per million.

Source	ID	Extract buffer[a]	−10l gP	No. of peptides	Unique peptides	Post-translational modification	Alimentary TPM	Reproductive TPM
egg cover proteins	G_69	E II	44.75	2	1		2.66	1774.86
	G_17406	E II	68.37	3	1		0.00	11.21
	G_22169	E II	89.4	3	2	Hydroxylation	230.63	28747.44
frass proteins	G_19	F IV	82.33	3	3		221.13	71863.12
	G_21	F IV	70.41	3	3		21.59	5952.68
	G_156	F IV	108.14	5	4		747.78	130.71
	G_224	F IV	71.28	4	3		2.83	670.40
	G_955	F IV	45.69	2	1		22.06	5208.22
	G_961	F IV	76.46	3	2	Acetylation (K); Methyl ester	107.62	22.80
	G_1389	F IV	80.59	3	3		371.26	78.59
	G_20993	F IV	68	4	2	Carbamidomethylation; Carbamidomethylation (DHKE X@N-term); Mutation	4.13	594.58
	G_21160	F IV	117.22	8	1	Acetylation (Protein N-term); Mutation	0.67	424.45
	G_28281	F IV	45.4	1	1		35.16	2.06
	G_32808	F IV	60.34	2	2		1.55	0.75
	G_38765	F II	45.76	1	1		1.89	0.65
		F IV	44.96	1	1			
	G_59813	F IV	24.16	1	1	Deamidation (NQ)	1.24	0.00
egg cover + frass proteins	G_13*	E II	112.21	4	4	Mutation	467.66	125908.59
		F IV	110.7	6	6			
	G_40	E II	103.97	8	8	Methylation; Mutation	9.93	4133.34
		F IV	187.51	21	21			
	G_127	E II	104.08	5	5	Hydroxylation; Methyl ester; Mutation	9.52	3042.64
		F IV	95.41	4	4			
	G_266	E II	91.27	3	1	Hydroxylation	25.75	3528.06
		F IV	140.28	10	4			
	G_329	E IV	39.88	1	1		126.15	11.69
		F IV	45.16	1	1			
	G_2966	E II	96.64	5	2		2.61	1424.36
		F IV	125.3	7	4			
	G_8853	E II	127.82	6	2	Mutation	21.50	7331.69
		F IV	139.89	7	2			
	G_10358	E II	102.48	6	2	Deamidation (NQ); Mutation	42.79	15481.68
		F IV	102.23	8	3			
	G_17067	E IV	67.91	2	2	Acetylation (Protein N-term); Carbamidomethylation (DHKE X@N-term)	1.75	1.79
		F II	96.58	4	4			
	G_24581	E IV	40.95	2	2		1.46	0.88
		F II	25.97	1	1			
	G_29400	E IV	66.05	3	3	Sodium adduct	0.86	0.93
		F II	28.85	1	1			
		F IV	81.44	4	4			
	G_32620	E IV	33.3	1	1		1.83	1.19
		F IV	35.1	1	1			

* no matches with database proteins.

[a] E: Egg capsule cover, F: frass, II: Extraction buffer II, IV: extraction buffer IV.

enie.org/). Protein matches were manually assessed by BLASTp (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins) to determine conservation and annotation. N-terminal signal peptides were predicted using SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP-3.0/) (Dyrløv Bendtsen et al., 2004). Prediction of tandem repetitive sequences in proteins was conducted using the online platform T-REKS (https://bioinfo.crbm.cnrs.fr/index.php?route=tools&tool=3) (Jorda and Kajava, 2009). Short tandem repeats alignment images were generated with Weblogo (https://weblogo.berkeley.edu/logo.cgi) (Schneider and Stephens, 1990; Crooks et al., 2004).

3. Results and discussion

The *Gonipterus* sp. n. 2 reference transcriptomes (combined reproductive and alimentary tissues; see Figure 1) were produced from 28,482,914 total paired-end reads (Genbank accession PRJNA737578), which could be assembled into 63,414 contiguous sequences with an average length of 636 nucleotides. Relative gene expression analysis revealed that 20,136 transcripts were expressed exclusively in the alimentary tissue, whereas 11,292 were exclusive to the reproductive tissue; the remaining (31,986) were shared by both. This represents a considerable increase to the genetic data about this species that, so far, was limited to only the mitochondria-encoded cytochrome c oxidase I (CO1) sequence, utilized for species identification within the genus (NCBI public database). This new data provides a key resource to explore several aspects of this weevil's physiology, including the detoxification of noxious compounds present in their hosts (Branco et al., 2019) and identification of egg case cover proteins (this study).

Our proteomic analysis using in-solution trypsin digestion of egg capsule cover and frass samples recovered 12 proteins in both, 3 exclusive to egg capsule cover and 13 exclusive to frass (Figure 3 and Table 1). Proteins identified exclusively in the egg capsule cover and in both samples were, with a few exceptions, expressed more abundantly in the reproductive tissue than in alimentary tissue. High confidence annotations were obtained for most proteins, and among those the best matches were matches from other weevils and from plants. Proteins derived from frass and egg capsule cover that matched with others involved in physiological processes in plants likely originated from the host, *Eucalyptus dunnii*, used as feed for the insects assessed in this study. Further analysis of MS peptides against a *Eucalyptus grandis* [a relative to *E. dunnii* (https://eucgenie.org/)] protein database derived from a transcriptome, resulted in 14 additional matches to plant proteins (Table S1). The presence of these plant proteins, as residues of plant digestion in both the frass and the egg capsule cover, provides strong evidence that the egg capsule cover is indeed partially formed by frass, but the presence of weevil-derived proteins further supports the addition of other physiological processes to form the structure as well as other compounds found previously.

Of the 3 proteins exclusive to the egg capsule cover, two were found to have similarity to *Drosophila arizonae* (Diptera: Drosophilidae) proteins, but with no functional annotation (Figure 3, Table 2), thus their roles in the egg capsule cover could not be inferred. *Post-hoc* analysis of the transcripts coding for these proteins showed that contig G_69 and G_17406 were partial-length sequences presenting imperfect tandem repeats of 15 amino acids (GDRQSRPEGQWQSIQ). Glutamine(Q)-rich regions have been associated with increased protein adhesion and complex formation (reviewed in Fan et al., 2014). The remaining egg capsule cover protein (encoded by G_22169), as well as many others also present in the frass, matched with a bacterial protein (Table S2), which does not necessarily mean these bacteria were present in the weevils' body or in the sample. E-values of most matches indicate that these are unlikely to be the same, although some homology was found, so we presume these are either similar proteins produced by the weevils or may be produced by gut bacteria, commonly found in insects (Yun et al., 2014).

Among those proteins identified only from the frass was the G_28281 (expressed primarily in the alimentary system; see Figure 3), which has high homology (E-value = 1.00E − 120; 56% identity) with a pancreatic triacylglycerol lipase-like protein found in the weevil *Dendroctonus ponderosae*. This enzyme is likely involved in breaking down lipids present in the weevils' host plant (Zibaee et al., 2014), such as the oils and waxes. The G_19, expressed 325-fold higher in the reproductive tissue, annotated to an anti-chymotrypsin-2-like isoform X5 from *Sitophilus oryzae*, also a weevil. This protein is in the serpin superfamily, a large group of proteins that are well-known as protease inhibitors in the immune response system, as well as contributing to some reproductive processes and interactions with pathogens in arthropods (Meekins et al., 2017). In summary, this indicates that this protein may be secreted by female *Gonipterus* sp. n. 2 to impede the establishment of pathogens on the eggs and/or inhibit the breakdown of the egg capsule cover. Gene expression data indicates generally high levels of transcript within the reproductive tract, although quantitative proteomics will be necessary to determine protein levels found in the egg capsule.

We additionally found that among the identified frass proteins, three vitellogenin-related proteins were annotated with similarity to other weevil species. Vitellogenins are precursors of vitellin, a major component of egg yolk that nourishes the developing embryo (Chapman, 1998b). We found the vitellogenin-related proteins exclusive to frass extracts, and their gene expression is highest in alimentary tissue. This class of proteins are commonly produced in the insects' (including Coleoptera) fat body, then secreted into the haemolymph and transported to the oocytes to be incorporated into the eggs (Trougakos and Margaritis, 2002; Tufail and Takeda, 2008). Given the ubiquity of fat body tissue in an insects' abdomen, we presume that the vitellogenin-like proteins detected in our study could have originated from a perivisceral

Table 2. Identification of proteins according to BLAST match (June 2020).

Contig	BLAST match description [species]	E-value
G_19	Anti-chymotrypsin-2-like isoform X5 [*Sitophilus oryzae*[a]]	3E − 150
G_21	AgSP-1 arylphorin [*Anthonomus grandis*[a]]	9.00E − 112
G_40	Mucin-5AC-like [*Sitophilus oryzae*[a]]	9E − 40
G_69	Hypothetical protein [*Drosophila arizonae*[b]]	2.00E − 09
G_127	Hypothetical protein J07HX64_02393 [halophilic archaeon J07HX64[c]]	9.9
G_156	Vitellogenin-like [*Sitophilus oryzae*[a]]	0.00
G_224	Translation initiation factor IF-2 [*Bacillus* sp.[c]]	0.52
G_266	Hypothetical protein [*Paenibacillus camerounensis*[c]]	9.00E − 08
G_329	Elongation factor 1-alpha [*Pachyrhynchus infernalis*[a]]	0.00
G_955	Hypothetical protein FN846DRAFT_1610 [*Sphaerosporella brunnea*[d]]	8.00E − 04
G_961	Predicted vitellogenin-like [*Dendroctonus ponderosae*[a]]	0.00
G_1389	Vitellogenin precursor [*Anthonomus grandis*[a]]	0.00
G_2966	Translation initiation factor IF-2 [*Eubacterium ruminantium*[c]]	2.00E − 05
G_8853	DEAD/DEAH box helicase [*Tumebacillus* sp.[c]]	7.00E − 11
G_10358	DEAD/DEAH box helicase [*Tumebacillus* sp.[c]]	6.00E − 05
G_17067	Photosystem II protein D1 [*Diospyros glaucifoliae*[f]]	0.00
G_17406	Hypothetical protein [*Drosophila arizonae*[b]]	1.00E − 06
G_20993	Hypothetical protein [*Clostridiaceae bacterium*[c]]	1.00E − 07
G_21160	Hypothetical protein [*Paenibacillus* sp.]	7.7
G_22169	Hypothetical protein P40081_25390 [*Paenibacillus* sp.[c]]	0.003
G_24581	Hypothetical protein F0562_011253 [*Nyssa sinensis*[e]]	4.00E − 132
G_28281	Predicted pancreatic triacylglycerol lipase-like [*Dendroctonus ponderosae*[a]]	1.00E − 120
G_29400	Hypothetical protein FNV43_RR05859 [*Rhamnella rubrinervis*[e]]	0.00
G_32620	Hypothetical protein F0562_024368 [*Nyssa sinensis*[e]]	5.00E − 140
G_32808	Glyceraldehyde-3-phosphate dehydrogenase (NADP+) (phosphorylating) [*Salvia splendens*[e]]	0.00
G_38765	Hypothetical protein F0562_020839 [*Nyssa sinensis*[e]]	8.00E − 103
G_59813	Predicted putative leucine-rich repeat-containing protein DDB_G0290503 [*Dendroctonus ponderosae*[a]]	5.00E − 06

[a] curculionid (Coleoptera: Curculionidae).
[b] fly (Diptera).
[c] bacteria.
[d] fungus.
[e] plant.

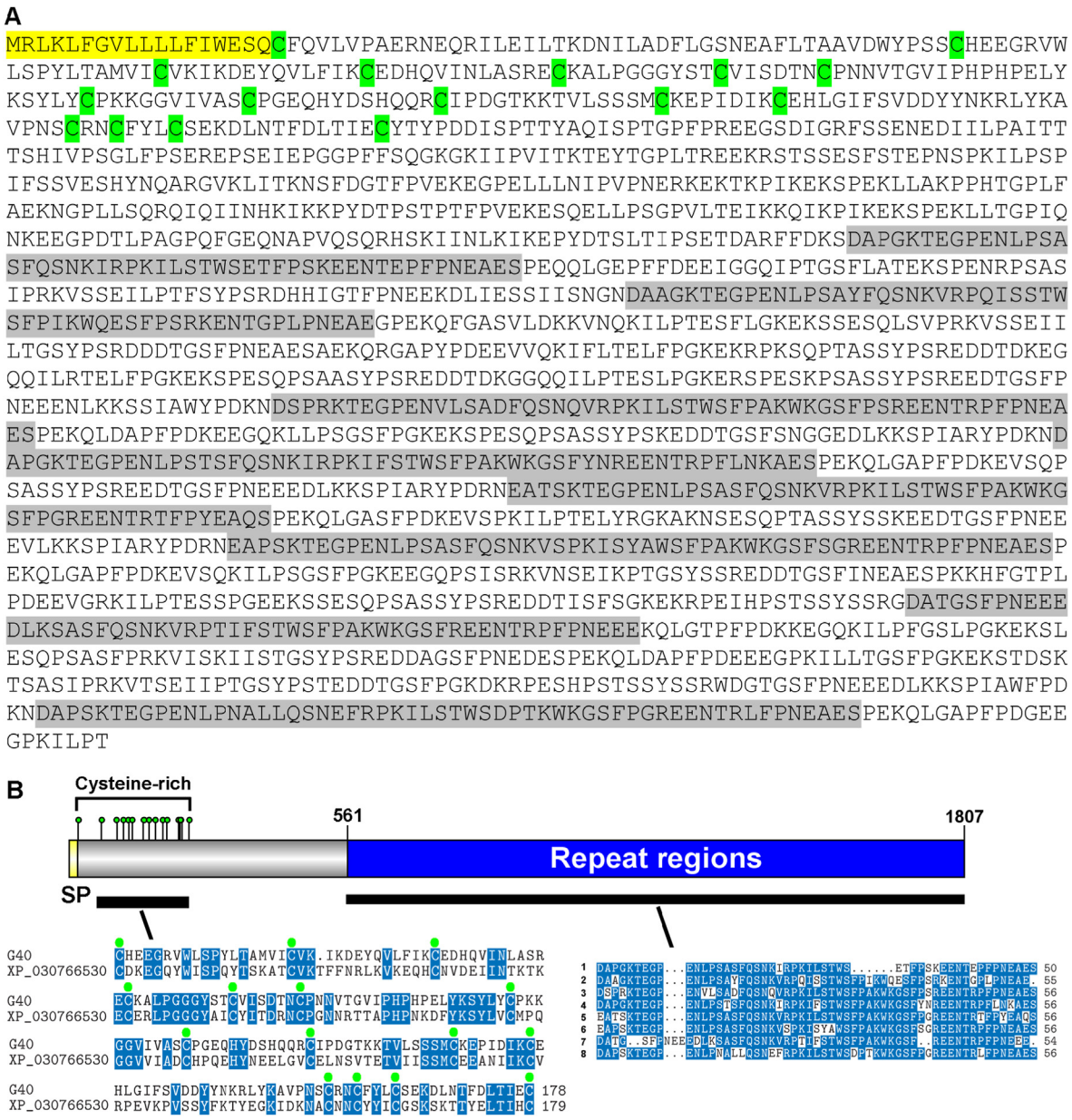

Figure 4. *Gonipterus* sp. n. 2 protein G_40. **(A)** Annotated protein sequence showing signal peptide (yellow), cysteines (green) and eight repeats (grey). **(B)** Schematic representation of G_40 showing location of signal peptide (SP), cysteine-rich region and repeat regions. Multiple sequence alignments show: cysteine-rich region with the N-terminal region of mucin-5AC-like protein of *Sitophilus oryzae* (XP_030766530) and repeats. Blue shading represents conservation, green circles denote position of cysteines. Protein sequences can be found in **File S1**.

fat body that surrounds the alimentary canal (Chapman, 1998a). Another interesting finding was the annotation of G_21 with an AgSP-1 arylphorin from *Anthonomus grandis* (Coleoptera: Curculionidae), which is an indicator of diapause in this species since it negatively correlates with vitellogenin (Lewis et al., 2002). Quantifying the abundance of these proteins over time may elucidate any similar function in *Gonipterus*: females have lengthy non-egg-laying periods (Souza et al., 2021) that may be physiologically analogous to diapause. Among the proteins found in both egg capsules and frass was the protein encoded by G_40 (highly expressed in reproductive tissue). *In silico* secretome analysis confirmed it to have characteristics of a secreted protein (i.e. presence of signal peptide) and contains repetitive protein repetitive units (Figure 4A). Within the N-terminal cysteine-rich region, it has most similarity with a weevil mucin-like protein (Figure 4B). Mucins are a family of proteins commonly found in vertebrates forming a protective gel-like substance [e.g. in humans, mucins are found covering the epithelium of the respiratory, digestive and reproductive systems (Wagner et al., 2018)] and, in insects, they are present in the peritrophic membrane of the midgut, forming a mucus layer that protects the cells from chemical and mechanical damages that may occur from digestion (Kramer and Muthukrishnan, 2005; Dias et al., 2018). In our study, however, this protein was expressed 400-fold more in the reproductive tissue compared to the alimentary tissue, indicating that although similar, this protein has a different function. The elucidation of an eggshell-related mucin-like protein in the brown planthopper, *Nilaparvata lugens* (Lou et al., 2019), supports the relevance of mucin-like proteins in *Gonipterus* egg capsules.

The protein repetitive units contained 8 highly conserved sequences between 50-56 amino acids in length (Figure 4B). This region has some similarity to the aggregate spidroin protein from the spider *Trichonephila clavipes* (Araneae: Araneidae) and a fibroin heavy chain-like protein from the crinoid *Anneissia japonica*. Spidroins are involved in the production of spider silk, of which the aggregate spidroins in particular, are part of an

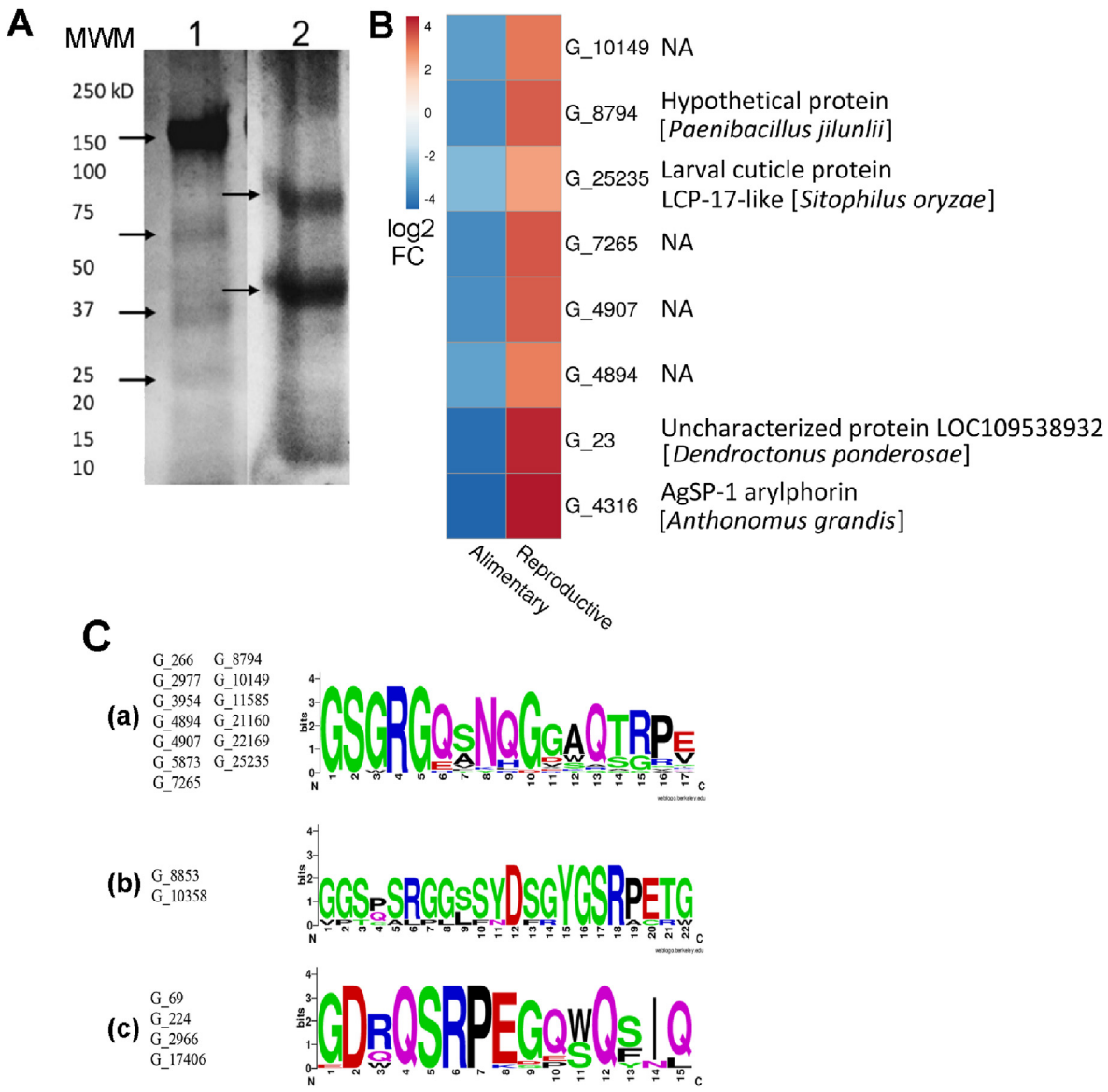

Figure 5. Identification of proteins isolated from *Gonipterus* sp. n. 2.egg cover using SDS-PAGE with in-gel trypsin digestion and mass spectrometry. **(A)** Coomassie blue stained SDS-PAGE gel. Lane 1 shows supernatant and Lane 2 shows insoluble pellet. Proteins were extracted using Buffer II (urea and CHAPS). MWM, molecular weight marker in kilodalton (kDa). Arrows indicate major protein bands that were excised. See **File S3** for raw gel images. **(B)** Heatmap shows relative gene expression in alimentary and reproductive tissue for each protein (not including those presented in Figure 3). **(C)** Sequence logo representation of 3 conserved repeat domains, denoted (a, b and c). Protein sequences can be found in **File S1**.

adhesive coating used by these organisms to capture their prey (Moon, 2018). These two comparative species matches strongly suggest a structural function for G_40, as a gel-like adhesive substance keeping the weevil egg capsule structure integral and attached to the host plant leaves.

Analysis of egg capsule cover proteins using SDS-PAGE with Coomassie stain, identified 6 major protein bands (Figure 5A). Major bands (at approximate MW 150, 75, 60, 40, 30 and 25 kDa) were excised for in-gel trypsin digestion and mass spectrometry, resulting in the identification of 24 proteins (Table 3 and File S2), eight of which we had previously identified from egg capsules and three from frass (Figure 5B). Six identified proteins had no BLAST match, and generally exhibited gene expression far higher in the reproductive tissue than alimentary tissue. Several proteins shared tandem repetitive motif sequences, termed as groups a, b and c, with highly conserved amino acid motifs (Figure 5C). Group (a) was characterised by 17 amino acids with a highly conserved N-terminal region consisting of G_1SGRG_5. Group (b) was characterized by 22 amino acids dominated by glycine and serine residues. Egg adhesive secretions of the gum moths *Opodiphthera eucalypti* and *O. helena* (Lepidoptera: Saturniidae) are over-represented by glycine and serine (Li et al., 2008), so our proteins could have a similar function. The repeat motif of group (c) was 15 amino acids that are generally rich in serine, arginine, proline and glutamic acid residues.

Collating the results from both extractions (in-solution and in-gel), we found proteins with significant matches to known proteins with possible functions, from structural to protease inhibitors. However, there were a number of proteins that could not be annotated or presented poor matches, including those expressed far more abundantly in the reproductive tissue (compared to alimentary tissue). These are novel proteins that could be necessary for the production of the egg capsule and protection of eggs.

Among the annotated proteins, once again those with better matches presented homology with other known weevil proteins. Another match with the previously discussed AgSP1 arylphorin was found on the egg

Table 3. Proteins present in egg capsule cover of *Gonipterus* sp. n. 2 derived from SDS-PAGE. TPM, transcripts per million.

ID	Motif repeat group[a]	−10l gP	No. of peptides	Unique peptides	Post-translational modification	Alimentary TPM	Reproductive TPM
G_13*		45.71	1	1	-	467.66	125908.59
G_19		110.4	3	3	-	221.13	71863.12
G_23		33.87	1	1	-	22.37	8056.73
G_224	C	27.45	1	1	-	2.83	670.4
G_266	A	139.54	11	3	Deamidation (NQ); Dehydration; Carbamidomethylation (DHKE X@N-term); HexNAc1dHex2; Mutation	25.75	3528.06
G_955		92.68	7	3	-	22.06	5208.22
G_2966	C	111.25	5	2	Pyro-glu from Q; Carbamidomethylation (DHKE X@N-term)	2.61	1424.36
G_2977*	A	100.51	2	2	-	4.08	310.29
G_3954*	A	105.56	8	1	Deamidation (NQ); Carbamidomethylation (DHKE X@N-term)	10.73	2314.04
G_4316		38.05	1	1	-	0.36	179.12
G_4894	A	100.51	2	2	-	14.33	1010.72
G_4907	A	74.05	1	1	-	1.02	140.12
G_5873*	A	100.51	2	2	-	29.99	4006.79
G_7265	A	100.51	2	2	-	8.7	1388.38
G_8794	A	129.61	10	1	Deamidation (NQ); Carbamidomethylation (DHKE X@N-term); HexNAc1dHex2; Mutation	15.79	2107.53
G_8853	B	106.17	4	1	Mutation	21.5	7331.69
G_10149	A	100.51	2	2	-	39.65	3282.36
G_10358	B	93.88	4	1	Mutation	42.79	15481.68
G_11585*	A	100.51	2	2	-	0	4.97
G_17406	C	84.69	3	1	Pyro-glu from Q; Carbamidomethylation (DHKE X@N-term)	0	11.21
G_22169	A	100.51	2	2	-	230.63	28747.44
G_24581		87.02	2	2	Carbamidomethylation; Oxidation (M)	1.46	0.88
G_25235	A	74.05	1	1	-	0.52	16.96
G_28020*		20.43	1	1	-	1.13	0.91

* no BLAST matches.
[a] Based on amino acid sequence conservation.

capsule cover (G_4316) albeit with a less confident E-value (1.00E-48), more highly expressed in the reproductive system. This could be a fragment of G_21, found only in the in-solution extract of frass, which also deposits on the egg capsule cover. Additionally, we found a match with the LCP 17-like (larval cuticle) from *Sitophilus oryzae*, which is part of a family of proteins that, together with chitin, forms the exoskeleton (cuticle) of insects (Noh et al., 2016) and has important structural function attributing physical properties (such as elasticity) to the cuticle (Qiao et al., 2014). These proteins are known to present repetitive tandem motifs (Willis, 2010) and, in our case, G_25235 aligned with several others that also present the motif common to Group (a). Our proteins in this group contain notably different sequences than those commonly associated with cuticular proteins, yet the match suggests these could have a similar structural role in the egg capsule cover.

We conclude that the weevil *Gonipterus* n. sp. 2 egg capsule cover is composed of a proteinaceous blend that is largely secreted by female reproductive tissue. Frass is also part of the structure, as evidenced by the presence of plant proteins in our analysis, confirming a previous hypothesis that this egg capsule cover is a combination of frass and weevil glandular products, as well as plant secondary compounds and metabolites (Souza, 2021). Among the major proteins present are mucin/spidroin-like and novel proteins containing glycine-, glutamic acid- and serine-rich tandem repeats that suggest a putative structural function in binding the capsule together. Further studies to isolate and purify these proteins could help elucidate their physical properties and function in *Gonipterus* weevils.

Declarations

Author contribution statement

Natalia M. Souza: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Tianfang Wang: Analyzed and interpreted the data; Wrote the paper.

Saowaros Suwansa-ard: Performed the experiments; Wrote the paper.

Helen F. Nahrung: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

Scott F.Cummins: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Data availability statement

Data associated with this study has been deposited at NBCI Genbank under the accession number PRJNA737578.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

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