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Insights into the insect salivary gland proteome: Diet-associated changes in caterpillar labial salivary proteins

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ABSTRACT

The primary functio of salivary glands is fluid and protein secretion during feeding. Compared to mammalian systems, little is known about salivary protein secretion processes and the effect of diet on the salivary proteome in insect models. Therefore, the effect of diet nutritional quality on caterpillar labial salivary gland proteins was investigated using an unbiased global proteomic approach by nanoLC/ESI/tandem MS. Caterpillars of the beet armyworm, *Spodoptera exigua* Hübner, were fed one of three diets: an artificial diet containing their self-selected protein to carbohydrate (p:c) ratio (22p:20c), an artificial diet containing a higher nutritional content but the same p:c ratio (33p:30c) or the plant *Medicago truncatula* Gaertn. As expected, most identified proteins were associated with secretory processes and not influenced by diet. However, some diet-specific differences were observed. Nutrient stress-associated proteins, such as peptidyl-propyl *cis–trans* isomerase and glucose-regulated protein94/endoplasmin, and glyceraldehyde 3-phosphate dehydrogenase were identified in the labial salivary glands of caterpillars fed nutritionally poor diets, suggesting a link between nutritional status and vesicular exocytosis. Heat shock proteins and proteins involved in endoplasmic reticulum-associated protein degradation were also abundant in the labial salivary glands of these caterpillars. In comparison, proteins associated with development, such as arylphorin, were found in labial salivary glands of caterpillars fed 33p:30c. These results suggest that caterpillars fed balanced or nutritionally-poor diets have accelerated secretion pathways compared to those fed a protein-rich diet.

1. Introduction

Recognition that insect oral secretions (saliva and/or regurgitant) modify host plant or animal responses has lead to proteomic investigation of the salivary gland (sialome) or associated secretions (secretome). In blood-feeding insects, often vectors of disease-causing parasites, identification of salivary proteins may lead to targets for the control of transmission and/or disease (Ribeiro and Francischetti, 2003; Valenzuela et al., 2003; Ribeiro et al., 2004; Kalume et al., 2005; Arcà et al., 2007). In phloem-feeding insects, salivary recognition factors responsible for the initiation of host plant defenses have been identified (Harmel et al., 2008; Carolan et al., 2009; Cooper et al., 2010). However, given the importance of caterpillar saliva as a potential source of enzymes for the digestion and detoxification of noxious compounds as well as effectors which suppress the induction of plant defenses, few studies have focused on the caterpillar labial salivary gland and proteins involved in secretory processes (Mathews et al., 1997;

Abbreviations: BiP, binding immunoglobulin protein/glucose regulated protein GRP78; COPI, coat protein complex I; COPII, coat protein complex II; EE, early endosome; ER, endoplasmic reticulum; ERAD, endoplasmic reticulum-associated degradation; ERGIC, endoplasmic reticulum Golgi intermediate compartment; GA3PDH, glyceraldehyde 3-phosphate dehydrogenase; GOX, glucose oxidase; GRP94, glucose regulated protein 94; HSP, heat shock protein; IDGF, imaginal disc growth factor; nanoLC/ESI/MS/MS, nanoliquid chromatography/electrospray ionization/mass spectroscopy/mass spectroscopy; LE, late endosome; NCBI, National Center for Biotechnology Information; P26S4, proteasome 26 subunit 4; p97/Npl4/Ufd1 complex, p97/nuclear protein localization 4/ubiquitin fusion degradation 1 complex; PDI, protein disulphide isomerase; PPI, peptidyl-propyl *cis–trans* isomerase; RPN, regulatory particle non-ATPase; TBP1, ATP-dependent TAT binding protein-1; TGN, *trans*–Golgi network; RACK1, receptor for activated protein C kinase; V-ATPase, vacuolar-ATPase.

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Eichenseer et al., 1999; Musser et al., 2002a; Weech et al., 2008; Zhou et al., 2008; de la Paz Celorio-Mancera et al., 2011, 2012).

Lepidopteran larvae possess two distinct salivary organs; a pair of labial salivary glands and a pair of mandibular salivary glands (House and Ginsborg, 1985). The watery secretions of the mandibular glands contain proteins, lipids, sterols and triglycerides but little is known about its full composition (Felton, 2008). Recent proteomic analysis of *Vanessa gonerilla* caterpillar mandibular glands identified key proteins, such as lysozyme, α-amylase, a putative chemosensory protein and sericotropin, associated with the mandibular salivary glands (de la Paz Celorio-Mancera et al., 2012). Even though the labial salivary glands of some caterpillar species, such as the silkworm, *Bombyx mori*, are specialized for silk production, these glands in many other caterpillar species are responsible for proteinaceous salivary secretions (Eichenseer et al., 2010). Structurally, caterpillar labial salivary glands are a pair of long, unicellular, tubular structures that fuse to form a common duct in the head region (House and Ginsborg, 1985; Parthasarathy and Gopinathan, 2005; Daimon et al., 2008). After secretory cells release enzymes, ions and water into the salivary gland lumen, ions are retaken up by the resorptive cells, and then the saliva is finally released from the labial salivary duct through a specialized organ, the spinneret (House and Ginsborg, 1985; Ali, 1997; Musser et al., 2002a). In caterpillar species whose labial glands mainly produce protein-rich saliva, important enzymes, including lysozyme, ascorbate peroxidase and glucose oxidase (GOX), in these secretions have been characterized (Mathews et al., 1997; Eichenseer et al., 1999; Liu et al., 2004; de la Paz Celorio-Mancera et al., 2011, 2012). However, in caterpillars, little is known about protein secretion processes in these glands.

Saliva formation and salivation are highly regulated processes. In human parotid cells, a number of pathways are implicated in proteinacous salivary secretion (Gorr et al., 2005). The classical secretory pathway is predominantly responsible for protein-rich secretions resulting from the exocytosis of large dense-core secretory vesicles, which may be constitutively secreted (minor) or stimulated in response to muscurinic-cholinergic and adrenergic signals. Pathways responsible for minor salivary secretions include a constitutive pathway where the secretory vesicles are derived from the *trans*–Golgi network and a regulated pathway where further maturation of the secretory granules occurs. In the main classical secretory pathway, proteins destined for salivary secretions through the *trans*–Golgi network and/or dense core secretory vesicles are synthesized by ribosomes and translocated into the ER lumen through the Sec translocon complex co-translationally or, less commonly, post-translationally (Kim et al., 2006; Gasser et al., 2008; Brunner et al., 2009). In the ER lumen, nascent polypeptide chains follow one of two major pathways (Braakman and Bulleid, 2011). One route involves the binding of the growing polypeptide chain to the Binding immunoglobulin Protein/Glucose-regulated Protein 78 kD/Heat Shock Protein 70 (BiP/GrP78/HSP70) followed by formation of disulfide bonds by a protein disulfide isomerase (PDI). For glycoproteins, *N*-linked monoglycans are recognized by the ERp57/calreticulin complex or canexin (Ellgaard and Helenius, 2003; Frickel et al., 2004). Only upon proper folding are proteins transported to the *trans*–Golgi network. Misfolded proteins remain bound to BiP which leads to retrograde translocation into the cytosol and proteasome-mediated protein degradation through the ER-associated degradation (ERAD) pathway (Nishikawa et al., 2001; Ryoo and Steller, 2007; Bagola et al., 2011). From the Golgi, a number of cellular pathways lead to secretory granular biogenesis and the extracellular release of proteinaceous material (Burgoyne and Morgan, 2003; Nashida et al., 2004; Gorr et al., 2005). The exact mechanism for the formation of dense-core granules from the *trans*–Golgi network is not fully understood but proposed models include the vesicular transport or the cisternal maturation or the cisternal progenitor models (Gorr et al., 2005; Pfeffer, 2010).

In response to adrenergic or muscarinic-cholinergic signals, secretory granules in salivary glands are guided to the plasma membrane on microtubule rails driven by kinesin protein motors (Hirokawa et al., 1998; Nashida et al., 2004). At the plasma membrane, granules transport then switches to an actin filament (microfilaments)/myosin system (Valentijn et al., 1999). Release of granule contents into the gland lumen may involve a rapid fusion and reclosure of the fusion pore (kiss-and-run model) or full fusion and emptying of the granule with the plasma membrane followed by retrieval involving a clathrin/dyamin-associated mechanism (Harata et al., 2006).

In diverse insect species, diet affects either salivary protein levels or activity or secretion. In female mosquitoes, blood-feeding rapidly results in dynamic changes to the salivary gland transcriptome (Das et al., 2010). Salivary secretions of the Russian wheat aphid, *Diuraphis noxia*, were also affected by diet (Cooper et al., 2010). In Noctuid caterpillars, a number of salivary enzymes have shown diet-specific gene expression and activity (Liu et al., 2004; Afshar et al., 2010). Mid-5th instar corn earworm, *Helicoverpa zea*, salivary glands have higher expression of the gene encoding lysozyme when caterpillars feed on cotton or tomato compared to tobacco plants (Liu et al., 2004). The effect of diet on caterpillar labial salivary GOX activity is of particular interest since GOX potentially negatively affects the plant's ability to mount an appropriate defense response (Eichenseer et al., 1999, 2010; Musser et al., 2002a; Weech et al., 2008). GOX catalyses the oxidation of glucose and the activity of this enzyme have been found to increase when caterpillars were fed carbohydrate-based diets, lending credibility to the idea that this enzyme may be a pre-ingestive mechanism to balance dietary sugar intake as excess carbohydrates have a negative impact on insect mortality (Felton, 1996; Warbrick-Smith et al., 2006; Babic et al., 2008). Caterpillars often exhibit a self-selective feeding behavior to balance their diet, in particular, the intake of protein to digestible carbohydrate (p:c) ratio, which leads to optimal performance (growth, development, fecundity) of the insect (Waldbauer and Friedman, 1991; Lee et al., 2002; Merkx-Jacques et al., 2008); the self-selected ratio of *Spodoptera exigua* Hübner caterpillars is 22p:20c (Merkx-Jacques et al., 2008). By altering the ratio and levels of p:c, Afshar et al. (2010) showed that labial salivary gland SeGOX gene expression reflects glucose levels, but enzyme activity is also influenced by the nutritional protein content such that enzyme activity was highest when caterpillars were fed a carbohydrate- and protein-rich diet (Afshar et al., 2010). This suggests that there is both transcriptional and post-translational regulation of GOX. However, an alternative explanation is that on the high carbohydrate diet, GOX protein levels and salivary secretions increase but on a high protein diet, a feedback loop inhibits secretory granule exocytosis leading to the accumulation of labial salivary digestive enzymes.

In many insect models, diet and, in particular, nutritional quality, alters salivary enzyme gene expression, activity and secretion (Liu et al., 2004; Merkx-Jacques and Bede, 2005; Hu et al., 2008; Afshar et al., 2010; Das et al., 2010). This study examined the role of diet on caterpillar salivary secretion machinery by using a global proteomic approach. Three diets were chosen: the plant *Medicago truncatula* L. and two artificial diets that varied in their protein and carbohydrate levels, while maintaining the same protein to carbohydrate (p:c) ratio. *M. truncatula* was chosen as it is eaten by caterpillars of the beet armyworm, *S. exigua*, a generalist Noctuid pest. The self-selected p:c ratio by these caterpillars is 22p:20c (Merkx-Jacques et al., 2008). Therefore, the two artificial diets (22p:20c and 33p:30c) represent this ratio; previous studies have shown SeGOX gene expression and enzyme activity is higher on 33p:30c (Afshar et al., 2010). *S. exigua* caterpillars were reared

from 3rd instars on the respective diets. Soluble proteins from labial salivary glands of actively feeding 4th instar caterpillars were subject to global proteomic analysis by nanoLC/ESI/tandem MS. Many of caterpillar labial salivary proteins identified play a role in protein secretion. Diet-specific differences were observed with nutrient-stress related proteins, such as peptidyl-propyl *cis–trans* isomerase (PPI) and glucose-regulated protein94 (GRP94/endoplasmin), identified in the nutrient-poor diets, 22p:20c and *M. truncatula* (Lee, 2001; Gupta and Tuteja, 2011). In comparison, proteins highly associated with nutritional allometry, such as imaginal disc growth factor (IDGF), and developmental processes were identified in the labial salivary glands of caterpillars fed the protein-rich diet, 33p:30c (Shingleton et al., 2008).

2. Materials and methods

2.1. Chemicals

Unless otherwise specified, all chemicals were obtained from Sigma–Aldrich Chemical Co.

2.2. Caterpillar colony

Beet armyworm, *S. exigua*, eggs were obtained from AgriPest and reared for multiple generations (>20) at 28.5 °C under a 16:8 (L:D) photoperiod. Colony caterpillars were reared on a meridic wheat-germ based diet (Bio-Serv). Adults were allowed to mate and lay eggs to maintain the colony.

2.3. Diets

2.3.1. Plant diet

Seeds of the barrel medic, *M. truncatula* (Gaertn.) cv. Jemalong A17, were scarified in sulfuric acid and germinated on moist filter paper. Germinated seedlings were planted in pasteurized soil (80 °C for 2 h) and grown in a phytorium chamber under the following conditions: 260 m Em^{-2} s^{-1}, 22 °C under a 16:8 (L:D) photoperiod. Plants were fertilized three times per week using dilute N:P:K fertilizer. Six-week old plants were used in the herbivore studies; these plants have an approximate p:c ratio of 16.6:27.7 (Merkx-Jacques, personnel comm.).

2.3.2. Artificial diet

Artificial diets with set p:c ratios were prepared according to Simpson and Abisgold (1985). The protein source was a 3:1:1 ratio of casein (bovine milk, Bio-Serv), peptone and albumen (egg-white, Bio-Serv) which represents an amino acid composition similar to wheat seedlings (Lee et al., 2003). The carbohydrate source was glucose and the non-digestible filler was cellulose. The diets made up such that the ratio of protein:glucose:cellulose was 22:20:44 for 22p:20c and 33:20:23 for 33p:30c. Components of all diets were: 0.55% cholesterol (Bio-Serv) solubilized in 0.5% linoleic acid, 2.5% Wesson's salts, 1% ascorbate (vitamin C), 0.5% choline chloride, 0.5% sorbic acid, 0.35% methyl paraben (Bio-Serv), 0.285% thiamine (vitamin B_1), 0.285% riboflavin (vitamin B_2), 1.14% nicotinamide (vitamin B_3), 0.285% pyridoxine (vitamin B_6), 0.285% folic acid (vitamin B_9), 2.85% *myo*-inositol, 0.57% calcium panthothenic acid (vitamin B_5), 0.285% *p*-aminobenzoic acid and 0.011% biotin (vitamin B_7) (Bio-Serv). Diets were prepared in 2% agar.

2.4. Experimental design

To ensure uniform growth and development, *S. exigua* caterpillars were reared until early 3rd instars on a wheat germ-based diet (Bio-Serv) and then transferred to the treatment diets: *M. truncatula* plants or AD 22p:20c or AD 33p:30c. Labial salivary glands were dissected from actively feeding mid-4th instar caterpillars. Cold anaesthetized insects were placed ventrally and the labial salivary glands removed and rinsed in sterile Nathanson's saline (150 mM NaCl, 3 mM KCl, 3 mM $CaCl_2$, 10 mM *N*-tris (hydroxymethyl) methyl-2-aminoethane sulfonic acid, 20 mM $MgCl_2$) containing 10% general proteinase inhibitor (Christensen et al., 1991). One hundred salivary glands were pooled and stored at −80 °C until homogenization. The experiment on each diet was independently repeated trice. Each analysis represents both the cellular and the salivary gland lumen content. Protein concentration in labial salivary gland homogenates were measured by the Bradford assay and protein concentration standardized across all samples (Bradford, 1976).

2.5. NanoLC–ESI–MS/MS

Proteins were separated by electrophoresis on a non-denaturing 4–15% gradient gel and for each lane, 3 gel pieces were removed for analysis by nanoLC–ESI–MS/MS. Following destaining of the gel slices in 50% methanol, the gel was placed in 10 mM dithiothreitol for 1 h at 56 °C to reduce protein disulfide groups. The gel was then incubated in 55 mM chloroacetamide for 1 h at room temperature to alkylate the proteins. After washing in 50 mM ammonium bicarbonate, gel pieces were placed in 100% acetonitrile (ACN). Proteins were digested using 1700 U trypsin in 50 mM ammonium biocarbonate for 8 h at 37 °C. Peptides were then extracted in 90% ACN in 0.5 M urea. Samples were dried in a speed-vac and then resolubilized in 5% ACN in 0.2% formic acid (FA). Separation of peptides was performed using a nano liquid chromatography-2-dimensional system (Eksingent) using a C_{18} column (150 μm × 10 cm). A gradient from 10 to 60% ACN in 0.2% FA over 1 h at a flow rate of 600 nL/min was used to elute peptides. After peptide separation by LC-chromatography, peptides entered the LTQ-Orbitrap mass spectrometer (ThermoScientific). Each full mass spectrum (MS) acquired at a resolution of 60,000 was followed by three MS/MS spectrum, where the three most abundantly multiple charged ions were selected for MS/MS sequencing. Peptide sequences were acquired using collision-induced dissociation in the linear ion trap.

2.6. Data analysis

Since the *S. exigua* genome has not been fully sequenced, protein identification was based on peptide comparison to the non-redundant NCBI dataset using Mascot version 2.2 (Matrix Science). Tolerance parameters were set at 0.03 and 0.5 Da for the precursor and fragment ions, respectively. As well, the following variable modifications were set: oxidation (M), deamination (NQ), phosphor (STY), nitroxyl (C), sulfide (C), carbamidomethyl, (C), 4-ONE (C) and sulfo (C). The high degree of redundancy present in the dataset which was reduced by NoRed, an algorithm identifies the optimal subset of proteins in the mass spectroscopy analysis to allow the minimum number of proteins to be identified (http://web-pages.mcgill.ca/staff/Group2/rsalav/web/Software/NoRed/NoRed.zip). Protein identification was based on the detection of two or more peptides.

Non-redundant proteins were quantified based on the protein score. The data was normalized to ensure that the total number of peptides identified in each mass specrophotometric run will not affect the final analysis. To be considered present in the salivary glands, a protein needed to be identified in all three independent biological replications (Table 1). Proteomic data is available in Supplemental Table 1 and deposited in the proteomics identification database (PRIDE; http://www.ebi.ac.uk/pride).

Table 1
S. exigua caterpillar labial salivary gland soluble proteins identified by nanoLC/ESI/MS/MS. Caterpillars were fed different diets: artificial diet containing 22% protein and 20% digestible carbohydrate (AD 22p:20c; **in orange**), artificial diet containing 33% protein and 30% digestible carbohydrate (AD 33p:30c; **in pink**), or the plant *M. truncatula* (plant; **in green**). Proteins were identified by searching the National Centre for Biotechnology Information (NCBI) nonredundant database. Proteins were identified based on the presence of >2 diagnostic peptides and needed to be present in all three independent experimental replications.

NCBI accession number	Protein description	Diet		
		22p:20c	33p:30c	*Medicago*
Protein secretion/exo- and endocytosis				
gi|12025459	Protein disulfide isomerase (*Bombyx mori*)	AD 22p:20c	AD 33p:30c	plant
gi|62241290	Protein disulfide isomerase (*B. mori*) ERp57	AD 22p:20c	AD 33p:30c	plant
gi|15077034	Calcineurin A (*B. mori*)	AD 22p:20c	AD 33p:30c	plant
gi|17826933	Calreticulin (*Galleria mellonella*)		AD 33p:30c	plant
gi|52630957	Probably transport protein Sec61 α-subunit (*Toxoptera citricida*)	AD 22p:20c	AD 33p:30c	plant
gi|7271162	Putative Sec23 protein (*Drosophila melanogaster*)	AD 22p:20c		plant
gi|2499773	FK506-binding nuclear protein; 46 kDa FK506-binding nuclear protein (Peptidyl-prolyl cis-trans isomerase) (PPIase) (Rotamase)			plant
gi|13991907	Receptor for activated protein kinase C (RACK1) (*Heliothis virescens*)	AD 22p:20c	AD 33p:30c	plant
gi|7722	Clathrin heavy chain (*D. melanogaster*)	AD 22p:20c		plant
gi|58864724	Putative annexin IX-A (*Manduca sexta*)		AD 33p:30c	
GTPase and associated proteins				
gi|17862730	LD46767p (*D. melanogaster*); GDI	AD 22p:20c	AD 33p:30c	plant
gi|53830716	Putative Rab7 (*Oncometopia nigricans*)		AD 33p:30c	
Heat shock proteins (HSP; chaperonins)				
gi|56378321	Small HSP 21.4 (*B. mori*)		AD 33p:30c	
gi|66547450	HSP 60, mitochondrial-associated (*Apis mellifera*)	AD 22p:20c	AD 33p:30c	plant
gi|2738077	HSP 60	AD 22p:20c	AD 33p:30c	plant
gi|27260894	HSP cognate 70 protein (*Spodoptera frugiperda*)	AD 22p:20c	AD 33p:30c	plant
gi|2921217	HSP 70 (*Beroe ovata*)	AD 22p:20c	AD 33p:30c	plant
gi|431201	HSP 70; heat shock protein HSP70 (*Pleurodeles waltl*)	AD 22p:20c	AD 33p:30c	plant
gi|2352599	HSP 82 Kd (*Drosophila persimilis*)	AD 22p:20c	AD 33p:30c	plant
gi|157018473	AGAP010691-PA (*Anopheles gambia*) HSP-90		AD 33p:30c	plant
gi|110758921	Endoplasmin-like isoform 1 (*A. mellifera*) GRP94	AD 22p:20c		
Protein degradation				
gi|159162145	Ubiquitin	AD 22p:20c	AD 33p:30c	plant
gi|463363	Ubiquitin (*Stephanodiscus yellowstonensis*)	AD 22p:20c	AD 33p:30c	plant

Table 1 (*continued*)

gi|4574740	ATP-dependent Tat-binding protein-1 (*D. melanogaster*) 26S proteasome regulatory subunit	AD 22p:20c		plant
gi|1066065	P26S4 Proteasome regulatory subunit 4-like protein (*D. melanogaster*)	AD 22p:20c		plant
gi|55233608	Proteome subunit RPN2 (*D. melanogaster*)	AD 22p:20c		plant
gi|312375012	Hypothetical protein AND_15223 (*Anopheles darlingi*) 26S proteasome regulatory complex	AD 22p:20c		plant
gi|157015594	AGAP005630-PA (*A. gambia*) Protein processing (p97/Ufd1/Np14) complex	AD 22p:20c		plant
V-type ATPases				
gi|12585497	Vacuolar-type proton ATPase subunit H	AD 22p:20c	AD 33p:30c	plant
gi|198149765	F0/F1 ATP synthase; GA 18845 (*Drosophila pseudoobscura*)	AD 22p:20c	AD 33p:30c	plant
gi|24583988	VHA 68-2 isoform C (*D. melanogaster*)	AD 22p:20c	AD 33p:30c	plant
gi|37781636	Vacuolar proton ATPase β-subunit (*Artemia fransiscana*)	AD 22p:20c		
gi|46909243	F1 ATP synthase β-subunit	AD 22p:20c	AD 33p:30c	plant
gi|12585498	V-type Vacuolar proton ATPase subunit C (*Manduca sexta*)	AD 22p:20c	AD 33p:30c	
14-3-3 proteins				
gi|52001207	14-3-3 protein (*Fundulus heteroclitus*)	AD 22p:20c		
gi|332814474	PREDICTED: similar to epsilon isoform of 14-3-3 protein (*Pan troglodytes*)	AD 22p:20c	AD 33p:30c	
Other				
gi|10801564	Imaginal disc growth factor-like protein (*B. mori*)	AD 22p:20c	AD 33p:30c	plant
gi|47607477	Imaginal disc growth factor (*Pieris rapae*)		AD 33p:30c	
gi|3318722	Trypsin inhibitor	AD 22p:20c	AD 33p:30c	plant
gi|44886002	Ribonuclease L inhibitor homolog (*B. mori*)	AD 22p:20c	AD 33p:30c	plant
gi|47216201	Unnamed protein product (*Tetraodon nigroviridis*)	AD 22p:20c	AD 33p:30c	plant
gi|56462182	Hypothetical protein 6 (*Lonomia obliqua*)	AD 22p:20c	AD 33p:30c	
gi|341904220	Hypothetical protein caebren_25396 (*Caenorhabditis brenneri*)		AD 33p:30c	
gi|198151038	GA28352 (*Drosophila pseudoobscura pseudoobscura*)	AD 22p:20c		
gi|5869989	Arylphorin subunit (*Spodoptera litura*)		AD 33p:30c	
gi|38260562	Thiol peroxiredoxin (*B.mori*)		AD 33p:30c	

(*continued on next page*)

Table 1 (*continued*)

Cytoskeleton				
gi|16517097	β-Tubulin	AD 22p:20c	AD 33p:30c	plant
gi|4140272	Tubulin	AD 22p:20c	AD 33p:30c	plant
gi|22450696	α-Tubulin IV	AD 22p:20c		
gi|87248487	Microtubule-assocated protein RP/EB family member 3 (*Bombyx mori*)		AD 33p:30c	
gi|62956591	β-actin: cell motility (*Vanessa cardui*)	AD 22p:20c	AD 33p:30c	plant
gi|61105584	β-actin: cell motility (*Palaemonetes pugio*)	AD 22p:20c	AD 33p:30c	
gi|3879477	Actin (*C. elegans*)		AD 33p:30c	
gi|10270	Kinesin heavy chain (Sea urchin)			plant
gi|76789674	Tropomyosin-1, isoforms 9A/A/B (*D. melanogaster*)		AD 33p:30c	
gi|328784401	Moesin/ezrin/radixin homolog 1 (*A. mellifera*)	AD 22p:20c	AD 33p:30c	plant
mRNA-associated				
gi|54635929	GA 18673 (*D. pseudoobscura pseudoobscura*)	AD 22p:20c		plant
gi|29841421	Similar to HLA-B-associated transcript nuclease		AD 33p:30c	plant
gi|38174030	HLA-B associated transcript 1: RNA transport spliceosome (*Xenopus tropicalis*)			plant
gi|17862978	SDO7045p (*D. melanogaster*) IGF-II mRNA binding protein			plant
gi|157019434	AGA P010876-PA (*A. gambia*) RNA degradation	AD 22p:20c	AD 33p:30c	plant
Protein synthesis				
gi|15081324	Ribosomal protein L18 (*Spodoptera frugiperda*)	AD 22p:20c	AD 33p:30c	plant
gi|15213760	Ribosomal protein L13 (*S. frugiperda*)	AD 22p:20c	AD 33p:30c	plant
gi|15213806	Ribosomal protein S7	AD 22p:20c	AD 33p:30c	plant
gi|15213808	Ribosomal protein S10 (*S.frugiperda*)	AD 22p:20c	AD 33p:30c	plant
gi|16566722	Ribosomal protein S3A (*S. frugiperda*)	AD 22p:20c	AD 33p:30c	plant
gi|16566725	Ribosomal protein S4 (*S. frugiperda*)	AD 22p:20c	AD 33p:30c	plant
gi|16566731	Ribosomal protein S6 (*S. frugiperda*)	AD 22p:20c	AD 33p:30c	plant
gi|16566734	Ribosomal protein S16 (*S. frugiperda*)	AD 22p:20c	AD 33p:30c	plant
gi|18253041	60S acidic ribosomal protein PO (*S. frugiperda*)	AD 22p:20c	AD 33p:30c	plant
gi|18253049	Ribosomal protein L7 (*S. frugiperda*)	AD 22p:20c	AD 33p:30c	plant
gi|27260896	Ribosomal protein S2 (*S. frugiperda*)	AD 22p:20c	AD 33p:30c	plant
gi|27462516	60S ribosomal protein L15 (*S. frugiperda*)	AD 22p:20c	AD 33p:30c	plant
gi|3282245	Ribosomal protein L5 (*B. mori*)	AD 22p:20c	AD 33p:30c	plant
gi|62083433	Ribosomal protein PO	AD 22p:20c	AD 33p:30c	plant
gi|18253045	60S acidic ribosomal protein P2 (*S. frugiperda*)	AD 22p:20c	AD 33p:30c	

Table 1 (*continued*)

gi|18253047	Ribosomal protein L3 (*S. frugiperda*)	AD 22p:20c		plant
gi|18253051	Ribosomal protein L18A (*S. frugiperda*)	AD 22p:20c		plant
gi|18253055	Ribosomal protein S8 (*S. frugiperda*)	AD 22p:20c		plant
gi|15213762	Ribosomal protein L13A (*S. frugiperda*)	AD 22p:20c		
gi|15213764	Ribosomal protein L14 (*S. frugiperda*)	AD 22p:20c		
gi|15213780	Ribosomal protein L30 (*S. frugiperda*)	AD 22p:20c		
gi|15213814	Ribosomal protein S13 (*S. frugiperda*)	AD 22p:20c		
gi|15213824	Ribosomal protein S18 (*S. frugiperda*)	AD 22p:20c		
gi|15213826	Ribosomal protein S19 (*S. frugiperda*)	AD 22p:20c		
gi|49532832	Ribosomal protein L8 (*Plutella xylostella*)	AD 22p:20c		plant
gi|54609281	Ribosomal protein 5A (*B. mori*)	AD 22p:20c		plant
gi|54609203	Ribosomal protein L7A (*B. mori*)			plant
gi|54609243	Ribosomal protein L16 (*B. mori*)			plant
gi|54609285	Ribosomal protein S3 (*B. mori*)			plant
gi|14994664	Ribosomal protein L9 (*S. frugiperda*)	AD 22p:20c		plant
gi|54609225	Ribosomal protein L17 (*B. mori*)	AD 22p:20c		
gi|56462224	Ribosomal protein 23 (*Lonomia obliqua*)	AD 22p:20c		
gi|66525285	60S ribosomal protein L4 isoform 1 (*A. melifora*)	AD 22p:20c		
gi|6469517	Translation initiation factor 5A (*S. frugiperda*)	AD 22p:20c	AD 33p:30c	
gi|51965706	Translation initiation factor 2g subunit	AD 22p:20c		plant
gi|28627569	Translation elongation factor 2 (*S. exigua*)	AD 22p:20c	AD 33p:30c	plant
gi|78190623	EF2; eukaryotic translation initiation factor 2 (*Suberites fuscus*)	AD 22p:20c	AD 33p:30c	plant
gi|27462592	eIF2a subunit (*S. frugiperda*)	AD 22p:20c	AD 33p:30c	
gi|17862358	eRF/ARF - translation factor (*D. melanogaster*) LD23157p	AD 22p:20c	AD 33p:30c	plant
gi|12328431	Elongation Factor 1a (*B. mori*)	AD 22p:20c	AD 33p:30c	plant
gi|28822215	Elongation Factor 1a	AD 22p:20c	AD 33p:30c	plant
gi|87248115	Elongation factor 1a (*B. mori*)	AD 22p:20c	AD 33p:30C	plant
gi|232030	Elongation factor 1b	AD 22p:20c	AD 33p:30c	
gi|56123349	Elongation Factor	AD 22p:20c	AD 33p:30c	plant
gi|135089	Alanyl t-RNA synthetase	AD 22p:20c	AD 33p:30c	plant
gi|1351153	Glycyl-tRNA synthetase	AD 22p:20c	AD 33p:30c	plant
gi|328787158	Valyl-tRNA synthetase isoform 1 (*A. mellifera*)		AD 33p:30c	
Metabolism				
gi|28971712	Mitochondrial aconitase A swivel domain. (*Antheraea yamamai*)	AD 22p:20c	AD 33p:30c	plant

(*continued on next page*)

Table 1 (*continued*)

gi|66508366	Inosine-5'-monophosphate dehydrogenase isoform 1 (*A. mellifora*)		AD 33p:30c	
gi|87248239	Transketolase (*B. mori*)	AD 22p:20c	AD 33p:30c	plant
gi|66523390	Glutamate dehydrogenase, mitochondrial isoform I (*A. mellifera*)	AD 22p:20c	AD 33p:30c	plant
gi|38371675	Pyruvate kinase (*B. mori*) glycolysis	AD 22p:20c	AD 33p:30c	
gi|53830714	Enolase (*Oncometopia nigricans*)	AD 22p:20c	AD 33p:30c	
gi|157013238	AGA PO12048-PA (*A. gambia*)	AD 22p:20c	AD 33p:30c	
gi|45330818	Fructose 1,6-bisphosphate aldolase (*Antheraea yanamai*)		AD 33p:30c	
gi|20151455	GH26789p (*D. melanogaster*) Glutamate synthase			plant
gi|34305305	5-Aminoimidazole-4-carboxamide ribonucleotide formyl tranferase/IMP cyclohydrolase (*Leptinotarsa decemlineata)*		AD 33p:30c	plant
gi|333468507	AGAP003168-PA (*A. gambia*) isocitrate/isopropyl malate dehydrogenase		AD 33p:30c	plant
gi|85062656	Asparagine synthetase (*B. mori*)		AD 33p:30c	plant
gi|87248157	Hydroxyacyl-coenzyme A dehydrogenase (*B. mori*)			plant
gi|51555846	Glycerol-3-phosphate dehydrogenase-1 (*B. mori*)	AD 22p:20c	AD 33p:30c	
gi|328777933	Nucleoside diphosphate kinase (*A. mellifera*)	AD 22p:20c	AD 33p:30c	
gi|82754585	Glyceraldehyde 3-phosphate dehydrogenase (*Colias eurytheme*)	AD 22p:20c	AD 33p:30c	
gi|157012452	AGAP001957-PA (*A. gambia*) lipid-binding site	AD 22p:20c	AD 33p:30c	
gi|28261391	ADP/ATP translocase (mitochondria) (*B. mori*)	AD 22p:20c	AD 33p:30c	plant
Keratin				
gi|119395750	Keratin, type II cytoskeletal I	AD 22p:20c	AD 33p:30c	plant
gi|181402	Epidermal cytokeratin 2	AD 22p:20c	AD 33p:30c	plant
gi|2443314	Keratin 14	AD 22p:20c	AD 33p:30c	plant
gi|435476	Cytokeratin	AD 22p:20c	AD 33p:30c	plant
gi|46399073	Type II α-keratin		AD 33p:30c	plant

3. Results

3.1. Proteomics

A conservative approach was taken to identify diet-dependent protein expression. Proteins were identified based on the presence of at least two peptides and a protein must be present in all of the independent biological replicates to be considered valid. Using this criteria, 131 soluble proteins were identified in labial salivary gland homogenates and the diet-specific expression characterized (Fig. 1, Table 1). This is a rigorous approach as one of the caveats to nanoLC/ESI/MS/MS is that most abundant peptides are detected and this could, in rare cases, lead to masking of proteins. Therefore, Supplemental Table 2 depicts the expressed proteins if a less stringent approach was used; proteins identified here are based on the presence of two or more peptides but the protein may be present in only two of the three biological replications. Proteins were categorized into the Gene Ontology functional and biological processes (Figs. 2 and 3). Using this approach, for a single protein multiple categories may be identified. Over 10% of the biological process classifications in the labial salivary glands are associated with protein secretion.

3.2. Proteins associated with protein secretion processes

Ribosomes association with the Sec61 translocon pore allows the growing polypeptide chain to enter the ER lumen during translation; this heteromeric, membrane-spanning protein complex is

comprised of Sec61α, β and γ (Greensfield and High, 1999). The Sec61 α-subunit was identified in *S. exigua* labial salivary glands (Table 1). Calnexin and Ca^2-dependent calreticulin recognize and bind to the GlcMan9GlcAc2 of newly synthesized glycoproteins (Michalak et al., 2009); calreticulin stabilizes the nascent polypeptide and acts as a docking protein for other enzymes, such as the protein disulfide isomerase (PDI) ERp57 (Baksh et al., 1995; Frickel et al., 2004; Pollock et al., 2004; Hosoda et al., 2010). Calreticulin has been identified in salivary secretions of numerous tick species including *Amblyomma americanum*, *Dermacentor variabilis*, *Haemaphysalis longicornis*, *Ixodes scapularis* and *Rhipicephalis sanguineus* (Jaworski et al., 1995; Ferreira et al., 2002; Nene et al., 2002; Xu et al., 2004, 2005). Calreticulin is also identified in the labial salivary glands of caterpillars which fed on plants or the 33p:30c artificial diet (Table 1) ER-associated PDIs act as chaperones by catalyzing the oxidation and isomerization of nascent polypeptides, stabilizing the protein structure and overexpression of PDI often results in increased heterologous protein secretion (Davis et al., 2000; Ellgaard and Helenius, 2003; Chung et al., 2004; Inan et al., 2006; Zhang et al., 2006; Mohan et al., 2007; Goo et al., 2008). PDIs have been identified in tick salivary glands and secretions (Knizetova et al., 2006; Liao et al., 2007). In fact, in response to blood feeding, Pdi gene expression increased in the tick *H. longicornis* (Liao et al., 2007). Protein levels also increased in *Helicoverpa armigera* midgut cells which had been chemically treated to induce apoptosis (Courtiade et al., 2011). Recently, de la Paz Celorio-Mancera et al. (2012) reported PDIs in both the labial and mandibular salivary glands of *V. gonerilla* caterpillars. Two protein disulfide isomerases, including ERp57, are identified in *S. exigua* caterpillar labial salivary glands (Table 1).

Peptidyl-propyl *cis–trans* isomerase (PPI) catalyzes a key rate-limiting step in protein folding, the conversion of the peptide bond of proline from the *cis* to *trans* isomer (Kay, 1996). As well, these proteins often act as cochaperones to Heat Shock Protein 90s (HSP90s) (Owens-Grillo et al., 1996). This protein was only identified in labial salivary glands of *S. exigua* caterpillars that fed on a plant diet (Table 1) and was also identified in *H. zea* salivary secretions (de la Paz Celorio-Mancera et al., 2011).

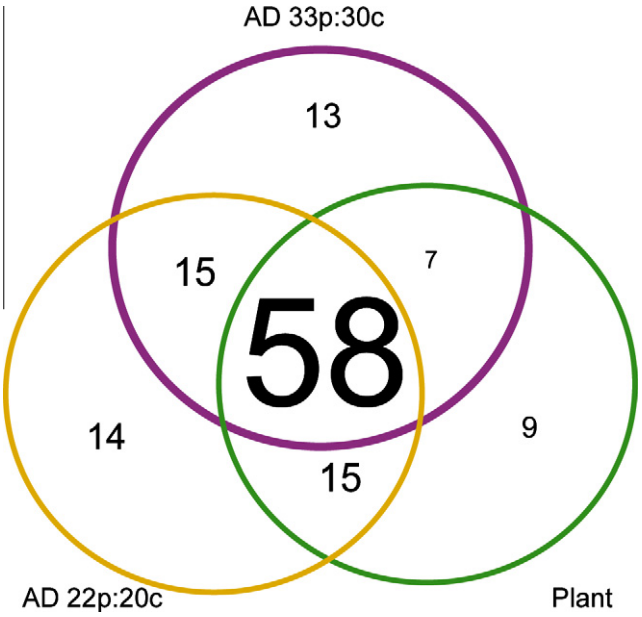

Fig. 1. Venn diagram depicting soluble proteins identified in the labial salivary glands of *S. exigua* caterpillars fed different diets: An artificial diet containing 22% protein and 20% digestible carbohydrate (AD 22p:20c), artificial diet containing 33% protein and 30% digestible carbohydrate (AD 33p:30c) or the plant *M. truncatula*. Numbers in the circles represent number of soluble proteins identified in triplicate experiments and the font size reflects these protein numbers. Numbers in overlapping circles indicate shared proteins; 58 proteins are found in caterpillar labial salivary glands independent of diet. Total proteins identified in labial salivary glands of caterpillars fed AD 22p:20c, AD 33p:30c and plant are 102, 93 and 89, respectively (see Table 1).

Cargo proteins destined for secretion then undergo anterograde transport to the Golgi apparatus for further sorting and processing. Sec23 forms an integral part of the Coat Protein Complex II (COP II) which is involved in the anterograde transport of secretory vesicles from the ER to the Golgi apparatus (Fromme et al., 2008). Specifically, Sec23 is involved in tethering and transporting the vesicle along microtubules to the Golgi. This protein was identified in caterpillars fed on the protein-poor diets; plant and 22p:20c (Table 1).

In vesicular trafficking, Rab GTPases are master regulators primarily involved in docking and vesicle fusion as well as protein quality control (Jordens et al., 2005; Grosshans et al., 2006; Schwartz et al., 2007; Fukuda, 2008; Stenmark, 2009; Diekmann et al., 2011; Lee et al., 2011). Specific Rab GTPases are associated with explicit transport vesicles and/or stages (Stein et al., 2003). For example, Rab7, a small GTPase identified in this proteomic analysis, is associated with retrograde trafficking between the early and late endosomal vesicles and, also, between late endosomal and lysosome fusion (Schwartz et al., 2007). Rab7 was only identified only in the labial salivary glands of caterpillars fed on the carbohydrate- and protein-rich diet, 33p:30c (Table 1).

These small RAB GTPases are negatively regulated through their association with guanine nucleotide dissociation inhibitors (GDIs) (Gilbert and Burd, 2001; Goody et al., 2005). After vesicle fusion, inactive GDP-bound Rab GTPases associate with GDP dissociation inhibitor (GDI) proteins that remove Rab proteins to recycle them back to donor membranes (Stein et al., 2003). Therefore, GDIs are involved in the regulation of GTPase activity and localization by delivery of Rab GTPases to the correct membrane compartments (Gilbert and Burd, 2001; Goody et al., 2005). The yeast gdi1 mutant leads to the loss of protein transport and Drosophila gdi mutants are lethal presumably affecting secretion processes associate with development (Garrett et al., 1994; Ricard et al., 2001). A GDI protein was found in the labial salivary glands of all caterpillars. Caterpillar labial salivary glands contain a member of the ezrin/radixin/moesin protein family which may act to disrupt this GDI-GTPase association (Table 1) (Takahashi et al., 1997). Of interest, HSP90 chaperones may also be involved in the interaction between GDI1 and target Rab GTPases (Raffaniello et al., 2009). In the exocrinic rat derived pancreatic cell line AR42J, HSP 90 co-localizes with Rab-GDI to stimulate vesicular secretion.

In caterpillar labial salivary glands, the intracellular receptor RACK-1 was identified in caterpillars reared on all diets (Table 1). When Rack-1 binds to activated protein kinase C (PKC), the receptor localizes to the appropriate membrane, stabilizing PKC and acting as a scaffold protein to facilitate interactions between PKC and downstream targets (Brandon et al., 2002; McCahill et al., 2002). In mammalian systems, PKC phosphorylation of target proteins such as 25 kDa synaptosome-associated protein (SNAP-25) and Munc18 leads to the activation of different exocytosis stages (Morgan et al., 2005). A putative link between RACK-1 and glyceraldehyde-3-phosphate dehydrogenase (GA3PDH) has been identified in the transcystosis of viral particles in aphid saliva (Seddas et al., 2004). Beet Western Yellows Virus particles must traverse aphid salivary gland epithelial membranes. In this case, transcytosis is believed to be mediated by interactions between viral particles and the host aphid proteins Rack-1 and GA3PDH. In eukaryotic glycolysis, GA3PDH catalyses the reversible conversion of glyceraldehyde-3-phosphate to 1,3-diphosphoglycerate generating NAD(P)H. However, when phosphorylated by PKC, this protein promotes microtubule formation and anchoring to vesicular tubular clusters that are necessary for the anterograde movement of coat protein

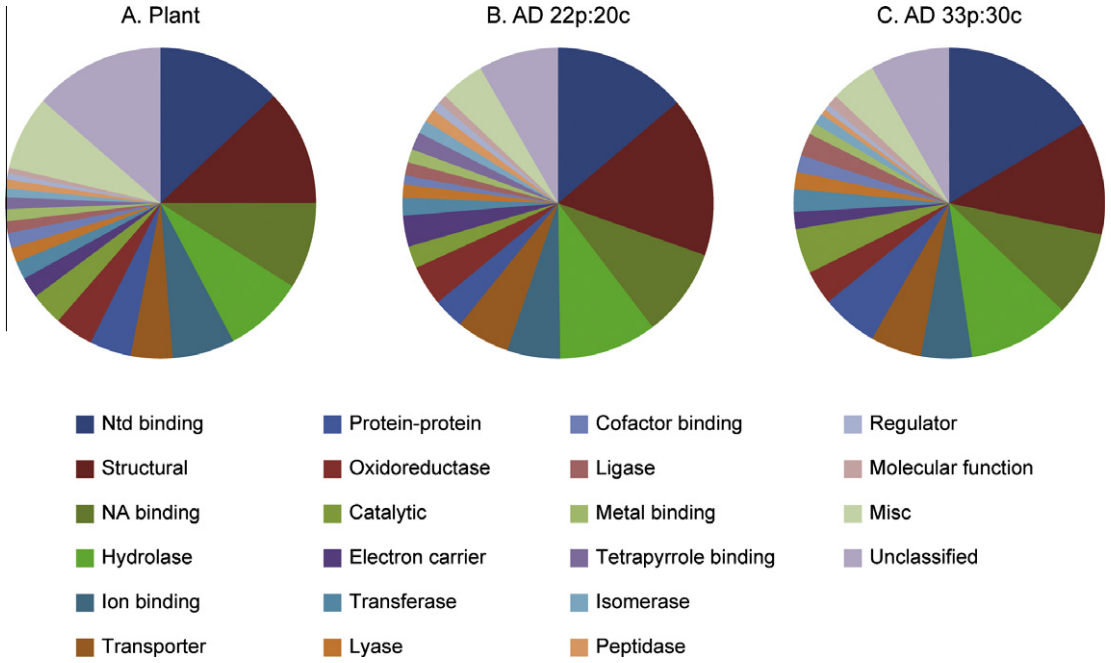

Fig. 2. Gene Ontology (GO) classification of soluble proteins expressed in *S. exigua* caterpillar labial salivary glands: Functional classification. The functional classification of labial salivary proteins from labial salivary glands of caterpillars fed (A) artificial diet containing 22% protein and 20% digestive carbohydrate (AD 22p:20c), (B) artificial diet containing 33% protein and 30% digestible carbohydrate (AD 33p:30c) or (C) the plant *M. truncatula*. Using this approach, a single protein may be classified under multiple categories. For simplicity, some categories were combined.

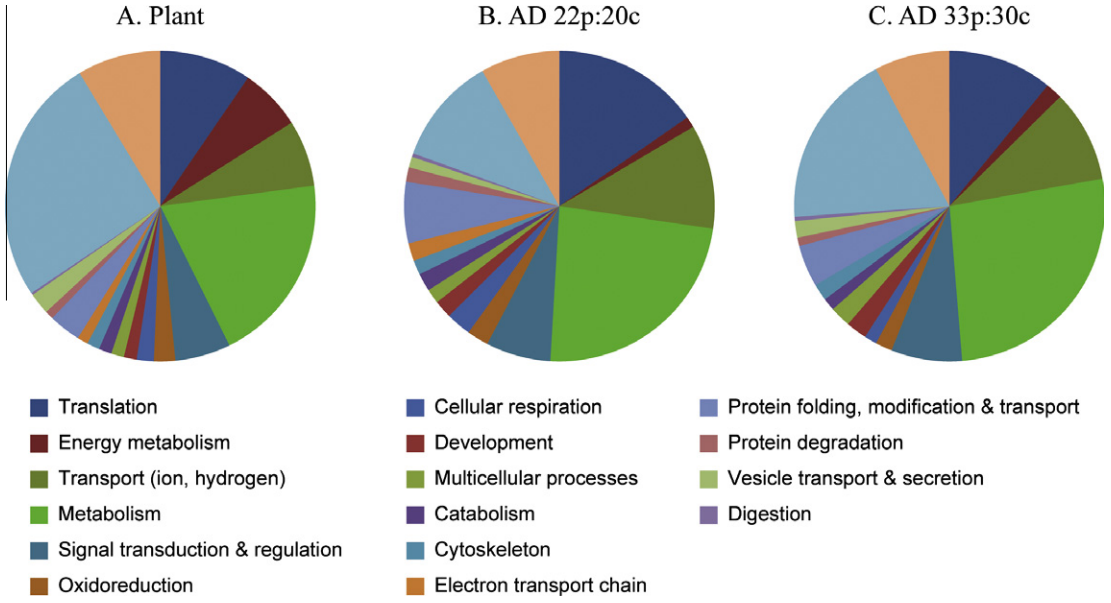

Fig. 3. Gene Ontology (GO) classification of soluble proteins expressed in *S. exigua* caterpillar labial salivary glands: biological process. The biological processes associated with soluble labial salivary gland proteins from caterpillars fed (A) artificial diet containing 22% protein and 20% digestive carbohydrate (AD 22p:20c), (B) artificial diet containing 33% protein and 30% digestible carbohydrate (AD 33p:30c) or (C) the plant *M. truncatula*. Using this approach, a single protein may be classified under multiple categories. For simplicity, some categories were combined.

complex I (COPI) vesicles (Csukai and Mochly-Rosen, 1999; Tisdale et al., 2009). Therefore, given these diverse biological roles, GA3PDH provides a potential link between glycolysis and cellular carbohydrate status with vesicular exocytosis (Glaser et al., 2002). GA3PDH was identified in the labial salivary glands of caterpillars fed artificial diets, both 22:20 and 33:30, but not on plant diet (Table 1). As well, this protein was identified in the salivary secretions of *H. zea* caterpillars reared on artificial diet (de la Paz Celorio-Mancera et al., 2011). RACK1 has also been implicated in targeting proteins for proteasome-mediated degradation (Ruan et al., 2009); this activity is regulated by the protein phosphatase calcineurin through the dephosphorylation of RACK1 (Liu et al., 2007).

3.3. Heat shock proteins

Heat shock proteins (HSPs) function in the cell as molecular chaperones that bind to nascent polypeptide chains and function

as holdases or foldases to ensure proper protein folding (Braakman and Bulleid, 2011). Initially identified and characterized based on their induction in response to heat stress, it is now recognized that HSPs may be constitutive and/or induced by a multitude of cellular stresses (Gupta and Tuteja, 2011). For example, HSP90 is constitutively expressed, comprising a major soluble ER protein, and only weakly induced by stress compared to HSP70 which has low endogenous levels but is a marker of ER-stress (Buchner, 1999; Landais et al., 2001). In response to stress, HSPs are crucial in protein refolding and preventing denaturation (Feder and Hofmann, 1999; Eletto et al., 2010).

Based on their molecular weight, HSPs are categorized into 5 major classes, small HSP (<30 kD), 60, 70, 90 and 100 (Sakano et al., 2006). Classes 60, 70 and 90 were identified in the labial salivary glands of caterpillars fed on all diets; in contrast, the small HSP 21.4 was only identified in caterpillars fed on the protein- and carbohydrate-rich diet, 33p:30c (Table 1). In *B. mori*, expression of shsp21.4 was high in the silk glands of this caterpillar (Li et al., 2009). In fat bodies, shsp21.4 levels are constitutive and downregulated after exposure to heat shock (Sakano et al., 2006).

Nutrient stress often results in the upregulation of HSPs, particularly glucose or amino acid deprivation (Lee, 2001; Abcouwer et al., 2002; Eletto et al., 2010). Grp79/BiP and Grp94/endoplasmin are constitutive but levels upregulated in response to nutrient stress (Heydari et al., 1993; Lee, 2001). BiP binds to nascent polypeptide chains and mediates ATP-dependent folding (Dudek et al., 2009); misfolded proteins bound to BiP for long periods are targeted for ERAD (Nishikawa et al., 2001; Ryoo and Steller, 2007; Bagola et al., 2011). Even though HSPs in the 70 and 90 class are constitutively expressed on all diets, Grp94/endoplasmin is present in the labial saliva of caterpillars fed on the artificial diet 22p:20c, suggesting nutrient stress on this diet (Table 1).

3.4. Endoplasmic reticulum-associated protein degradation

Misfolded proteins recognized by BiP, PDI and lectin sensors (EDEM1-3/Htmp/MnIIp/OS-9/Yos9P) are targeted for degradation through the ERAD system (Nishikawa et al., 2001; Ryoo and Steller, 2007; Mehnert et al., 2010; Bagola et al., 2011). Targeted proteins are uni- and di-ubiquitinated allowing their recognition by the Cdc48/p97 and ubiquitin fusion degradation 1/nuclear protein localization 4 complex (Ufd1/Npl4) complex which initiate their transport through Sec61 or derlin channels (Greensfield and High, 1999; Pye et al., 2007; Wolf and Stolz, 2012). After retrotranslocation from the ER, targeted proteins then assemble with E1-E3-ubiquitin conjugating enzymes for further addition of ubiquitin and proteasome-mediated degradation (Herrmann et al., 2007). Ubiquitin was identified in the labial salivary glands of all caterpillars, irrespective of diet (Table 1); however, many of the components of the transport machinery and the regulatory 26S proteasome protein complex were only identified in the salivary glands of insects fed on the plant and 22p:20c diets, including Ufd1/Npl4, the ATP-dependent Tat-binding protein1 (TBP1/Rpt5), 26S proteasome subunit 4 protein (P26S4/Rpt2), RPN2 (Table 1). TBP1/Rpt5 and P26S4/Rpt2 represent two of the six 19S-associated AAA^+ ATPases that provide energy for protein unfolding and translocation into the 26S proteasome through the 19S cap subcomplex. TBP1/Rpt5 has also been implicated in recruitment of targeted proteins through recognition of the polyubituitin tag and may be regulated through post-translational phosphorylation (Lam et al., 2002; Um et al., 2010). Both TBP1/Rpt5 and P26S4/Rpt2 are also implicated in the opening of the 20S proteasome subcomplex to allow entry of unfolded proteins (Smith et al., 2007; Park et al., 2010). Association between Rpn1 and Rpn2 forms a cone structure that links the 19S proteasome cap to the 20S proteolytic channel (Lander et al., 2012; Rosenzweig et al., 2012). As well, Rpn1 serves as a docking site for shuttle receptors that recognize ubiquitin-targeted proteins targeting them to the 26S proteasome (Gomez et al., 2011). Therefore, proteins associated with the ERAD system were identified in the labial salivary glands of nutritionally-stressed caterpillars.

3.5. Vacuolar type-ATPases

Epithelial cells of insect salivary glands have abundant H^+-ATPase (vacuolar ATP synthase (V-ATPase))/nH^+ cation (K^+ or Na^+) antiporter coupled systems that are involved in vesicular exocytosis and the ion and fluid reabsorption associated with salivary secretion (Nishi and Forgac, 2002; Wieczorek et al., 2009; Baumann and Walz, 2011). This highly conserved proton pump is comprised of two multimeric protein complexes, a membrane bound F0 complex and a F1 complex, which associate to form the active holoenzyme that hydrolyzes ATP to drive protons across a membrane generating a pH and chemiosmotic gradient (Sumner et al., 1995; Menzendorfer et al., 2000; Marshansky and Futai, 2008). A number of V-ATPase-dependent pathways are associated with vesicular trafficking (Marshansky and Futai, 2008). In mammals, isoform α2 is localized with the *cis*–Golgi or early endosomes; in these organelles, acidification of the lumen V-ATPase α2 results in pH-dependent enzyme or channel activation and/or recruits proteins, such as ADP ribosylation factors and GTPases, required for vesicular trafficking to the organelle (Marshansky and Futai, 2008). Isoform α1 is associated with the Golgi body and involved in vesicle fusion and secretion, particularly in synaptic cells (Marshansky and Futai, 2008). Acidification of the lumen of dense-core secretory vesicles also activates enzymes needed for protein processing (Kim et al., 2006).

Homologous roles of V-ATPases in insect secretory cells are being investigated, particularly their role in salivary fluid secretion (Wieczorek et al., 2009; Baumann and Walz, 2011). In this proteomic study, V-ATPases subunits (H, C, B) were abundant in *S. exigua* labial salivary glands (Table 1). In addition, calcineurin, a Ca^{2+}-dependent protein phosphatase, was identified in caterpillar labial salivary glands. Calcineurin is necessary for saliva production by blowfly salivary glands, but does not act directly on V-ATPase (Voss et al., 2010). Instead, calcineurin, activated through the IP_3 pathway, modulates the cAMP pathway by sensitizing the cAMP-pathway associated serotonin receptor, by activating cAMP production by adenylate cyclase and by inhibiting cAMP degradation by phosphodiesterase, thereby, integrating the two signal transduction pathways activated by serotonin.

There also are close connections between V-ATPase activity and glucose/cellular energy levels. In the caterpillar midgut, a strong correlation between feeding and goblet cell plasma membrane V-ATPase activity is observed (Wieczorek et al., 2000). In midgut goblet cells and also in yeast, glucose deprivation results in the phosphorylation of the F1 subunit C by protein kinase A, resulting in the reversible disassembly and inactivation of V-ATPase disassembly and inactivation (Sumner et al., 1995; Kane and Parra, 2000). In mammals, glucose regulation of the V-ATPase is further enhanced by the physical interaction and stabilization of the V0/V1 haloenzyme by the glycolytic enzyme fructose 1,6-biosphosphate aldolase (Lu et al., 2007). In the experiment described herein, fructose 1,6-bisphosphate aldolase was only identified in labial salivary glands of caterpillars fed on the carbohydrate- and protein-rich diet, 33p:30c (Table 1).

3.6. Non-classical protein secretion

14-3-3 proteins are a ubiquitous family of small 28–33 kDa regulatory proteins involved in many cellular roles, including vesicle trafficking and exocytosis by the classical secretion pathway

(Morrison, 1994; Chamberlain et al., 1995; Roth et al., 1999; Tzivion and Avruch, 2001). However, the ε-isoform of 14-3-3 proteins has also been implicated in a non-classical route of protein secretion that does not involve the ER-Golgi pathway (Carreño et al., 2005). In glioma cells, the ε-14-3-3 isoform interacts with phosphorylated endopeptidase 24.15 mediating extracellular secretion for neuropeptide degradation (Carreño et al., 2005). Two 14-3-3 isomers were detected in caterpillar labial salivary glands (Table 1); one isoform was detected only in caterpillars fed on the 22:20 artificial diet and the ε-isoform was identified in caterpillars fed the 22:20 and the 33:30 artificial diets, but not on the plant diet. Identification of the 14-3-3 ε-isoform suggests that this protein may be involved in mediating protein secretion by non-conventional routes.

3.7. Other proteins

3.7.1. Imaginal disc growth factor

Imaginal disc growth factors (IDGFs) were initially identified based on their potent activity on the cellular proliferation of imaginal discs, sac-like sheets of epithelial cells that develop into epidermal structures during metamorphosis to the adult (Bryant, 2001). Recent evidence suggests that IDGF is involved in nutritional signaling conveying information about the insect nutritional status to target tissues (Zhou et al., 2008; Wang et al., 2009). In 4th instar silkmoth, *B. mori*, haemolymph concentration of IDGF was approximately two times higher when caterpillars fed on artificial diet compared with lower nutritive value mulberry leaves (natural diet) (Zhou et al., 2008). *BmIDGF* expression in fat bodies of 4th instar silkworm *B. mori* caterpillars decreased in starved insects and sharply increased 24 h after re-feeding (Wang et al., 2009). Therefore, IDGF levels appear to be highly regulated by nutrition. Two homologues of IDGF were identified in *S. exigua* (Table 1). One was identified in the labial salivary gland of insects fed on all three diets. The second homologue was only identified in labial salivary glands of caterpillars fed on the 33:30 artificial diet. Therefore, IDGF levels in *S. exigua* caterpillar labial salivary glands also appear to be closely correlated with insect nutritional status.

3.7.2. Digestive enzyme inhibitors: ribonuclease L inhibitor homolog and trypsin inhibitor

Ribonucleases are often a component of saliva and may be a defensive strategy against ingested dsRNA viral pathogens or involved in digestion (Rampias et al., 2003; Maeda et al., 2005). In rat salivary parotid glands, ribonuclease L is found associated with an inhibitor which is removed before secretion (Robinovitch et al., 1968). Ribonuclease activity has been found in insect saliva and the presence of a ribonuclease L inhibitor identified in *S. exigua* labial saliva in this study suggests that a similar mechanism is present in caterpillar saliva (Table 1) (Musser et al., 2002b).

Trypsin inhibitor was identified in caterpillar labial salivary glands, regardless of the diet fed (Table 1). A Kunitz-type protease inhibitor has been identified as secreted from prepupal *Drosophila melanogaster* salivary glands where it possibly plays a role regulating proteolytic activities during metamorphosis (Kress et al., 2004). Serine proteinase inhibitors (Serpins) have been identified in the salivary glands of numerous blood-feeding arthropods, such as tick (*A. americanum* and *I. ricinus*) and the tse-tse fly (*Glossina morsitans*) (Attardo et al., 2006; Alves-Silva et al., 2010; Chalaire et al., 2011). Proteases, such as trypsin, have been identified in salivary glands and secretions of a number of arthropods (Zhu et al., 2003; Oliveira et al., 2006; Francischetti et al., 2007; Meiser et al., 2010); therefore, it is possible, that similar to the ribonuclease L inhibitor, this protein binds to and inhibits the salivary digestive enzyme trypsin when in the salivary gland and is removed upon salivary secretion.

3.7.3. Arylphorin

Arylphorins are aromatic amino acid-rich proteins, predominantly synthesized in the fat body but also in tissues such as muscle, epidermis and the salivary glands, which act as storage proteins and are implicated in gut-associated stem cell proliferation (Webb and Riddiford, 1988; Blackburn et al., 2004). This protein was only detected in labial salivary glands of caterpillars fed on the protein-rich diet (33:30), which reflects the availability of excess amino acids on this diet that can be stored as arylphorins (Table 1). Recently, this protein has also been identified as a major protein in the labial and mandibular salivary glands of *V. glonerilla* caterpillars (de la Paz Celorio-Mancera et al., 2012).

3.7.4. Thiol peroxiredoxin

Peroxiredoxin, or thioredoxin peroxidase, is an antioxidant enzyme that uses thiols as reducing equivalents to eliminate the cellular reactive oxygen species hydrogen peroxide (H_2O_2) (Hofmann et al., 2002). Recently, peroxiredoxins have also been shown to play important and diverse roles in redox cellular signaling. For example, in mammals, ER-associated peroxiredoxin IV oxidizes and further activates PDI, in the presence of H_2O_2, to accelerate protein folding (Zito et al., 2010). Peroxiredoxins are also involved in the redox regulation of V-type ATPases (Tavakoli et al., 2001). Peroxiredoxin is expressed in silk glands of *B. mori* caterpillars and fat body expression levels increased in response to temperature or pathogen stresses (Lee et al., 2005; Wang et al., 2008). Thiol peroxiredoxin was also identified in salivary secretions of *H. armigera* caterpillars (de la Paz Celorio-Mancera et al., 2011). In the study conducted here, peroxiredoxin was only identified in the *S. exigua* labial salivary glands of caterpillars fed on the protein-rich 33p:30c artificial diet, not in those fed on 22p:20c or plant diet (Table 1).

4. Discussion

This research investigated the soluble proteome of caterpillar labial salivary glands and the effect of diet, particularly the nutritional p:c ratio, focusing on proteins involved in protein secretory pathways. Proteins were identified by nanoLC/ESI/MS/MS and peptide comparison to the NCBI non-redundant dataset based on the presence of >2 peptides and only considered present if they were identified in all three independent biological replications (Table 1). Over 130 soluble proteins were identified and, as expected, most proteins (>40%) were identified in caterpillar labial salivary glands independently of diet (Fig. 1). Upon analysis of the Gene Ontology (GO) biological classifications, it was found that over 10% of the proteins identified in caterpillar labial salivary glands were associated with protein secretion (Figs. 2 and 3). As expected, a high degree of homology was seen when compared to well-characterized mammalian systems that allowed us to propose a basic model for protein export in insect models (Fig. 4).

In addition, diet-specific differences associated with the caterpillar sialome were observed (Fig. 1, Table 1). Plant nutritional quantity and quality (secondary metabolites, p:c quality, ratio and quantity) dramatically affects insect behaviour and physiology (Bede et al., 2007). Soluble labial salivary gland protein profile was compared in caterpillars fed one of three diets: 22p:20c, 30p:30c and the plant, *M. trunctula*. This includes two diets representing the self-selected p:c ratio of caterpillars of the beet armyworm, *S. exigua* (Merkx-Jacques et al., 2008): 22p:20c and 33p:30c where 22p:20c is of lower nutritional content than 33p:30c. Previous research in our laboratory has shown that dietary nutritional quality affects the gene expression and enzyme activity of the labial salivary gland-associated glucose oxidase (GOX) (Afshar et al., 2010). This is significant given that GOX activity has been implicated in the caterpillar's ability to prevent the induction of plant defense

responses (Musser et al., 2002a; Weech et al., 2008). Afshar et al. (2010) showed that SeGOX gene expression reflected changing dietary carbohydrate content, but GOX activity indicated that dietary protein also was involved in the regulation of enzyme activity; it was assumed that this suggested a post-transcriptional modification. However, the results of this study on the caterpillar labial salivary gland proteome sheds a different interpretation of these results. Perhaps, the lower GOX activity reflects active secretion processes occurring in caterpillars fed plants or 22p:20c. GA3PDH is associated with the salivary glands of caterpillars fed the protein-poor diets, plant or 22p:20c, suggesting links between glycolysis and protein secretion. Other nutrient stress-associated proteins, such as PPI and GRP94/endoplasmin, were also identified in these labial salivary glands (Lee, 2001; Gupta and Tuteja, 2011). Many proteins associated with ERAD and also stress-related chaperones, such as HSP90, were also identified in labial salivary glands of caterpillars fed on plant or the 22p:20c artificial diet. In comparison, proteins associated with retrograde transport, such as RAB7, and non-classical protein secretion, were identified in labial salivary glands of caterpillars fed 33p:30c. Proteins associated with

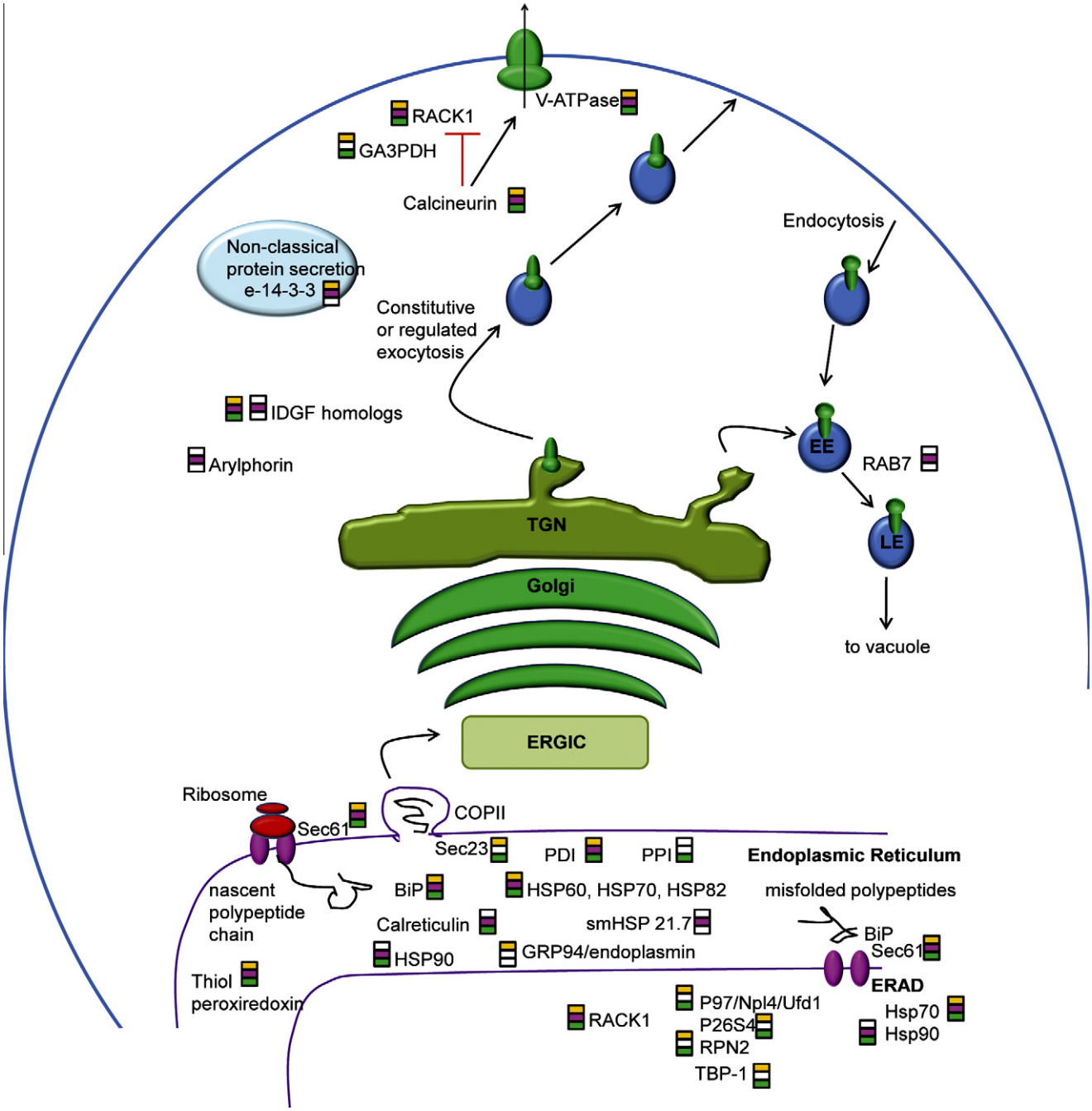

Fig. 4. Model of insect salivary protein secretion. Coloured bars indicate the diets associated with the presence of the protein. Caterpillars were fed on either 22p:20c artificial diet (orange) or 33p:30c artificial diet (pink) or plant *M. truncatula* diet (green) (see Table 1). *Abbreviations:* BiP, binding immunoglobulin protein/glucose regulated protein GRP78; EE, early endosome; ERAD, endoplasmic reticulum-associated degradation; ERGIC, endoplasmic reticulum Golgi intermediate compartment; GA3PDH, glyceraldehyde 3-phosphate dehydrogenase; GRP94, glucose regulated protein 94; HSP, heat shock protein; IDGF, imaginal disc growth factor; LE, late endosome; P26S4, proteasome 26 subunit 4; p97/Npl4/Ufd1 complex, p97/nuclear protein localization 4/ubiquitin fusion degradation 1; PDI, protein disulphide isomerase; PPI, peptidyl-propyl *cis-trans* isomerase; TBP1, ATP-dependent TAT binding protein-1;TGN, *trans*-Golgi network; RACK1, receptor for activated protein C kinase; RPN, regulatory particle non-ATPase; V-ATPase, vacuolar-ATPase. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

storage and nutritional signaling were also identified in these caterpillars fed a protein-rich diet (Webb and Riddiford, 1988; Shingleton et al., 2008). Therefore, based on the proteins associated with caterpillars reared on different dietary regimes, caterpillars fed nutritionally-poorer diets may have accelerated pathways leading to ER-mediated vesicular secretions compared to those fed 33p:30c (Table 1, Fig. 4).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jinsphys.2013.01.002.

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