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Chemosensory proteins, major salivary factors in caterpillar mandibular glands

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ABSTRACT

Research in the field of insect–host plant interactions has indicated that constituents of insect saliva play an important role in digestion and affect host chemical defense responses. However, most efforts have focused on studying the composition and function of regurgitant or saliva produced in the labial glands. Acknowledging the need for understanding the role of the mandibular glands in herbivory, we sought to make a qualitative and semi-quantitative comparison of soluble luminal protein fractions between mandibular and labial glands of *Vanessa gonerilla* butterfly larvae. Amylase and lysozyme were inspected as possible major enzymatic activities in the mandibular glands aiding in pre-digestion and antimicrobial defense. Although detected, neither of these enzymatic activities was prominent in the luminal protein preparation of a particular type of gland. Proteins isolated from the glands were identified by mass spectrometry and by searching an EST-library database generated for four other nymphalid butterfly species, in addition to the public NCBI database. The identified proteins were also quantified from the data using "Quanty", an in-house program. The proteomic analysis detected chemosensory proteins as the most abundant luminal proteins in the mandibular glands. In comparison to these proteins, the relative amounts of amylase and lysozyme were much lower in both gland types. Therefore, we speculate that the primary role of the mandibular glands in Lepidopteran larvae is chemoreception which may include the detection of microorganisms on plant surfaces, host plant recognition and communication with conspecifics.

1. Introduction

Four pairs of glands are associated with the oral cavity of insects, namely the mandibular, maxillary, hypopharyngeal and labial glands. The general function of these glands is regarded as the production of secretions (saliva) for the lubrication of mouth parts and bolus formation (Walker, 2009). However, the presence of these glands varies among Insecta and depending on the developmental stage (Walker, 2009). Two of these gland pairs are present in Lepidopteran larvae: the "salivary" or mandibular glands and the "silk" or labial glands.

Although the mandibular glands are ascribed to the ground plan of lepidopterans (Vegliante and Hasenfuss, 2012), an assessment of their role in herbivory has been neglected, especially in diurnal butterflies while in moths a handful of studies have provided evidence of their digestive and defensive role. The enzymatic activities associated so far with these type of glands are alkaline phosphatase and esterase in the wax moth (*Galleria mellonella*), amylase and maltase in an ermine moth (*Atteva fabriciella*), and glucose oxidase in the corn earworm (*Helicoverpa zea*) (Eichenseer et al., 1999; Mall et al., 1978; Wroniszewska, 1966). Mandibular gland secretions of some goat moths contain antifungal compounds

Abbreviations: ACN, acetonitrile; Aur, *Aglais urticae*; Bmo, *Bombyx mori*; CSP(s), chemosensory protein(s); EST(s), expressed sequence tag(s); Gme, *Galleria mellonella*; GPR(s), 1,3 beta glucan recognition protein(s); LC–MS/MS, liquid chromatography–tandem mass spectrometry; NCBI, National Center for Biotechnology Information; OBP(s), odorant-binding protein(s); PBS + PI, phosphate buffered saline containing protease inhibitors; Pca, *Polygonia c-album*; PS, molecular weight protein standards; 1-D SDS–PAGE, one-dimensional sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Vgo, *Vanessa gonerilla*; Vca, *Vanessa cardui*; Vat, *Vanessa atalanta*.

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and in some pyralid moths, the larval secretions affect oviposition by adult con- and heterospecifics (Anderson and Löfqvist, 1996). Other observations include the expression of an amylase gene in the mandibular glands of the mulberry silkworm (*Bombyx mori*) (Parthasarathy and Gopinathan, 2005), possible expression of c-type (chicken or conventional type) and i-type (invertebrate type) lysozyme genes in the cotton bollworm (*Helicoverpa armigera*) (Celorio-Mancera et al., 2011) and the description of the consistency of their secretions as "oily" (Felton and Eichenseer, 1999). It has also been observed that in fact some of the mandibular gland constituents are neutral lipids and the mechanical removal of these glands considerably decreases the growth of wax moth larvae (Wroniszewska, 1966).

In contrast, research has been particularly focused on characterizing the protein fibers (silk) produced in the labial gland pair of bombycoid moths (Akai et al., 2003) and on labial saliva interference with plant inducible direct and indirect defense responses (Bede et al., 2006; Musser et al., 2005). More recently, even proteomic studies have been performed to describe the proteins in silk glands of certain moth species (Celorio-Mancera et al., 2011; Shimomura et al., 2009).

Hence, the aim of our study was to contribute to the current understanding of Lepidopteran mandibular gland function through both a biochemical and proteomic comparative analysis between the mandibular and the labial glands in diurnal butterfly larvae (i.e. New Zealand red admiral, *Vanessa gonerilla*).

Our biochemical analysis was focused on the inspection of two enzymatic activities we hypothesized to be of major importance in the mandibular glands, regardless herbivore diet breath: amylase and lysozyme. Amylase may aid in pre-digestion of starch, while lysozyme may play an important antibacterial and/or digestive role, as it has been indicated by studies in salivary glands in other insects (Callewaert and Michiels, 2010). The phyllosphere, the leaf surface of plants, is a habitat for a diverse array of microorganisms (Lindow and Brandl, 2003) which are ingested indiscriminately by the caterpillar during feeding. The ingested microorganisms may represent possible pathogens though at the same time could provide the insect a feasible source of energy.

We also sought to obtain a proteomic analysis of mandibular and labial gland samples in order to provide the first large-scale quantitative and qualitative comparative analysis of luminal proteins present in these glands. The proteins present in our samples were identified by searching both publicly available protein sequences and an EST database constructed from cDNA libraries generated for four additional brush-footed butterfly species: *Vanessa atalanta*, *Vanessa cardui*, *Polygonia c-album* and *Aglais urticae*.

The most significant result obtained was the detection of a single chemosensory protein comprising slightly more than half of the whole mandibular gland proteome in the New Zealand red admiral larvae. In contrast, this same predicted protein represented about 1% of the labial gland proteome. Recently, CSPs have been detected in mandibular glands of all bee castes and ages (Iovinella et al., 2011). CSPs are 4-cysteine low molecular weight proteins with a large tissue distribution in insects including mouth organs (Honson et al., 2005). According to ligand binding assays, CSPs are predicted to have affinity to highly hydrophobic short linear molecules such as pheromones and fatty acids. However, they have not been observed having exclusively olfactory functions but involved in different biological contexts including, for example, insect limb regeneration (Honson et al., 2005). Our findings represent one step ahead on the query to understand the relevance of mandibular glands and CSPs as evolutionary innovations in lepidopteran larvae.

2. Material and methods

2.1. Insect rearing

New Zealand red admiral (Vgo) eggs were obtained through a commercial supplier (World Wide Butterflies, www.wwb.co.uk). After hatching, 51 neonates were transferred to individual plastic cups and placed in a climate chamber (22 °C; LD 8:16). Fresh leaves of stinging nettle (*Urtica dioica*) were provided to 32 individuals and leaves of baby tears (*Soleirolia soleirolii*) were the food source for the remaining larvae. Leaves of either host were kept moist using a wet cotton ball at their base and exchanged as needed. All larvae were weighed five days after molting into fifth instar when they were dissected to remove their mandibular and labial glands. Surviving larvae were assigned in equal numbers to two biological replicates consisting of larvae of approximate similar weight. Painted lady (Vca) larvae were also obtained from the same supplier and reared under laboratory conditions (25 °C; LD 12:12). Larvae of the first generation in the laboratory were fed on nettle until ready for dissection as described above for Vgo.

2.2. One-dimensional separation of salivary gland proteins

Cold-anesthetized larvae were dissected in phosphate buffered saline buffer (pH 7.4, 10 mM) containing a chelator-free cocktail of protease inhibitors prepared following manufacturer's instructions (Roche, Mannheim). Dissected salivary glands were rinsed with cold PBS + PI, and placed in a droplet of 20 μl (mandibular) and 30 μl (labial) of cold PBS + PI per pair of glands on a Petri dish kept on ice. Thereafter, glands were cut in half inside the droplet under the microscope, and transferred along with the buffer solution to 1.5 ml plastic tubes. Glands of five individuals were pooled per biological replicate per gland type. The samples were spun using a centrifuge (Jouan S.A centri A14) for 5 min at 17,530 g. The supernatant was transferred to new tubes, containing soluble protein from the salivary gland lumen. An aliquot of PBS + PI (100 μl) was added to each tube containing the precipitated gland tissue. The tissue was homogenized and subjected to the same centrifugation step mentioned above. Again, the supernatant was removed and added to new plastic tubes, consisting of a cytosolic enriched soluble protein fraction. Vca hemolymph was collected by puncturing the dorsal side of the larvae with a needle and pipetting out about 20 μl of hemolymph from 5 individuals into a 1.5 ml plastic tube.

The total amount of protein in the samples was quantified by Bradford spectrophotometric assay (Bradford, 1976). Absorbance was measured at 595 nm using a 550 Perkin-Elmer spectrophotometer. An estimation of the complexity between the salivary protein produced in the hemolymph, labial and mandibular glands was made through separating the proteins using 12% bis–tris SDS–PAGE gels utilizing the NuPAGE Novex system (Invitrogen, Carlsbad). Samples were first standardized to contain 2.3 μg of total protein, acetone-precipitated and prepared under reducing conditions following manufacturer's instructions. In order to approximate the molecular weight of the proteins in the protein samples, an aliquot of Novex Sharp pre-Stained protein standards (Invitrogen, Carlsbad) were used. After electrophoresis (180 V, 45 min) gels were rinsed in ultrapure water 5 min (3×) and stained with SimplyBlue Safe Stain (Invitrogen, Carlsbad).

2.3. Biochemical assays

Amylase and lysozyme activities were measured using EnzChek Ultra Amylase and Lysozyme Assay Kits (Invitrogen, Eugene) according to manufacturer's instructions. The total protein content

used per sample for both enzymatic assays was standardized across labial and mandibular samples, using 2.3 μg (lumen) and 0.4 μg (tissue) of total protein for each glandular type. Blanks were made for buffer, substrate, enzymes, and experimental protein samples, in order to extract background fluorescence from gained results. Fluorescence was measured using a BMG polarStar OMEGA after 24 h of incubation at 37 °C (490–510 nm excitation/520 nm emission).

2.4. Brush-footed butterfly EST database

Next generation sequencing methods were applied to obtain ESTs for four brush-footed (nymphalid) butterfly species. RNA was extracted from species-specific pooled samples of whole larvae representing different developmental stages of Vat, Vca, Pca and Aur which fed on a variety of host plants. The protocols followed for RNA extraction, RNA quality control and preparation of non-normalized cDNA libraries have been previously described (Celorio-Mancera et al., 2011). Transcriptome sequencing for Vat, Vca and Aur was performed using mRNA-Seq assays on an Illumina HiSeq2000 Genome Analyzer platform. One cDNA library was generated per species (except for Pca) and sequencing was done in one lane to generate 100 bp single reads. Pca sequences were obtained through mRNAseq Illumina sequencing of 2nd and 4th instar larvae reared on different host plants. The library construction and sequencing was performed by a commercial service provider (MWG Eurofins, Germany). Various quality controls, including filtering of high-quality reads based on the score value given in fastq files, removal of reads containing primer/adaptor sequences and trimming of read length were done using the CLC Genomics Workbench software. The de novo transcriptome assembly was done using two different software packages: CLC Genomics Workbench and ABYSS Software. The de novo reference assemblies were annotated Blast searches were conducted on a local server using the National Center for Biotechnology Information (NCBI) blastall program. Homology searches (BLASTx and BLASTn) of unique sequences and functional annotation by gene ontology terms (GO; www.geneontology.org), InterPro terms (InterProScan, EBI), enzyme classification codes, and metabolic pathways were determined using the BLAST2GO software suite (Gotz et al., 2008). The sequence data generated in this study have been deposited at NCBI in the Short Read Archive database.

2.5. Proteomics

2.5.1. Preparation of protein extracts in solution

The protein samples from labial and mandibular glands were collected as described in Section 2.2. Protein extraction buffer was added to the samples to a final concentration of 0.3% ProteaseMax (Promega, Fitchburg, Wisconsin, USA) and 167 mM ammonium bicarbonate. The samples were sonicated on ice using a probe sonicator (Vibra-Cell™ CV18, Sonics & Materials, Newtown, USA) and after a quick spin, the lysates were incubated for 10 min while shaking. After centrifugation at 13,000 rpm for 5 min the protein concentration was determined and 5 μg of each sample were further diluted to a final concentration of 0.1% ProteaseMax, 50 mM ammonium bicarbonate and 8% ACN. The resulting gland protein solutions were incubated for 30 min at 50 °C followed by an additional bath sonication for 10 min at room temperature. Samples were centrifuged and directly subjected to a tryptic digestion protocol carried out by a liquid handling robot (MultiProbe II, Perkin Elmer). This included protein reduction in 5 mM dithiothreitol at 56 °C and alkylation in 15 mM iodoacetamide for 30 min at room temperature in the dark. Trypsin was added in an enzyme to protein ratio of 1:30 and digestion was carried out over night at 37 °C.

2.5.2. LC–MS/MS and quantification

While it is possible to use the peptide signal in MS to compare relative amounts of individual proteins, comparing the relative amounts of different proteins is more complicated. It has been shown that the sum of intensities of the most intense peptide ions is proportional to the protein molarity (Silva et al., 2006; de Godoy et al., 2008). Since we have not normalized the data using protein standards, here we are using semi-quantitative comparisons between proteins based on the normalized intensity.

After tryptic digestion, the samples were acidified and cleaned with C18 StageTips according to the manufacturers' description (Thermo Fisher Scientific Inc.). Eluted peptides were dried and resuspended in 3% ACN and 0.2% formic acid. LC–MS/MS analyses were performed on an Easy-nLC system (Thermo Scientific) directly on-line coupled to a hybrid LTQ Orbitrap Velos ETD mass spectrometer (Thermo Scientific, Bremen, Germany). From each sample, 0.5 μg were injected from a cooled auto sampler onto the LC column. The peptide separation was performed on a 10 cm long fused silica tip column (SilicaTips™ New Objective Inc.) packed in-house with 3 μm C18-AQ ReproSil-Pur® (Dr. Maisch GmbH, Germany). The chromatographic separation was achieved using an ACN/water solvent system containing 0.2% formic acid. The gradient was set up as following: 3–48% ACN in 50 min, 48–80% ACN in 3 min and 80% ACN for 7 min all at a flow rate of 300 nl/min. The MS acquisition method was comprised of one survey scan ranging from m/z 300 to m/z 2000 acquired in the FT-Orbitrap with a resolution of $R = 60{,}000$ at m/z 400, followed by two consecutive data-dependent MS/MS scans from the top five precursor ions with a charge state ≥ 2. For all sequencing events dynamic exclusion was enabled and unassigned charge states were rejected. The instrument was calibrated externally according to the manufacturer's instructions and all samples were acquired using internal lock mass calibration on m/z 429.088735 and 445.120025.

2.5.3. Peptide identification and protein quantification

Mass lists were extracted from the raw data using Raw2MGF, an in-house written program, and searched against two different databases using the Mascot search engine (Matrix Science Ltd., London, UK). In addition to the public non-redundant NCBI protein database, the data were also searched against a nucleotide database compiled from contigs of the sequenced EST mentioned above in Section 2.4. Results were combined by comparing the search results for each MS/MS and only keeping the highest scoring result for each spectrum. Only peptide identifications of a minimum Mascot score of 20 was used. The peptides were then grouped into protein groups according to shared peptides, each with subgroups of peptides matching specific accession combinations. Only peptides of a minimum length of 8 amino acids were used for the grouping. The subgroups were then quantified independently using the in-house developed quantification software Quanty (manuscript in preparation). To simplify the data where multiple subgroups existed, subgroups without overlapping accessions were chosen to represent the individual groups. The subgroup with the highest number of peptides was used and in cases with similar number of peptides, the subgroups that showed the lowest variation in the technical replicates were chosen. To verify the clustering and possibly generating new organism specific protein sequences, the accessions used for the identification of the peptides in each group were aligned and the peptides were mapped out onto the alignments. The butterfly nucleotide sequences that were identified were translated on the fly in 6 frames using the NCBI standard codons and only segments between stop codons that contain identified peptides were used for the alignments. ClustalW2 (v 2.09) was used to align the different protein groups (Larkin et al., 2007).

2.5.4. In-gel digestion and identification of mandibular proteins

Mandibular salivary protein samples from Vca and Vgo were separated using SDS–PAGE as described in Section 2.2. After staining using Coomassie, protein bands were excised from each lane, digested by trypsin using standard in-gel digestion protocols (Shevchenko et al., 1996) and peptides analyzed by mass spectrometry following the protocol in Section 2.5.2.

2.5.5. Bioinformatics tools and procedures

Translation of cDNA contigs and molecular weight predictions were performed using the "Proteomics" resource portal ExPASy (Gasteiger et al., 2003). Discrimination between putatively secretory and non-secretory proteins was achieved by using the program SignalP 4.0 (Petersen et al., 2011). Protein alignments were performed using the CLC Genomics Workbench (version 4.8) and molecular protein models were obtained through MODBASE (Pieper et al., 2011).

3. Results

3.1. Insect rearing

None of the New Zealand red admiral larvae survived to fifth instar on *S. soleirolii* and less than half of the larvae survived when using nettle as a host plant (62.5% fatality).

3.2. Protein separation using 1-D SDS–PAGE and "in-gel" proteomics

The protein profiles for each gland type were qualitatively consistent between biological replicates, at least at this level of detection (Fig. 1). The luminal protein profile of the mandibular glands is less complex in terms of number of protein bands than the corresponding one for the labial glands and in turn, the protein profile of the labial gland tissue was of higher complexity than in the luminal labial gland fraction (Fig. 1 and Fig. S1). Two proteins, one with a molecular weight of approximately 80 kDa and a second between 10 and 15 kDa were the most prominent in the mandibular gland protein profiles. However, the 80 kDa protein band was common to all samples examined (marked with an asterisk in Fig. 1 and Figs. S1 and S2) while the 10–15 kDa protein band was observed exclusively in the mandibular glands of both *Vanessa* species (position marked with two asterisks in Figs. S1 and S2).

Excluding trypsin, the enzyme used for the in-gel digestion of protein samples, the identification of the 80 kDa protein bands from Vgo and Vca mandibular gland protein profiles revealed storage proteins as the most abundant representing 38% and 77% respectively (Table S1). Chemosensory proteins were the most abundant proteins in both 10–15 kDa bands from Vgo and Vca mandibular gland luminal protein fractions representing almost 8% and 62% respectively (Table S1).

3.3. Amylase and lysozyme activities

Secreted active amylase and lysozyme activity were detected in the mandibular salivary glands of the phytophagous butterfly larvae used in this study (Fig. 2). The only activity statistically significantly different when comparing Vgo labial and mandibular glands luminal and tissue samples in a two-sampled *t*-test was amylase in the mandibular gland tissue ($df = 1$; $p = 0.03$).

3.4. "In solution" proteomics

A total of three hundred and seventy-six proteins were detected excluding common human-derived contaminants and added

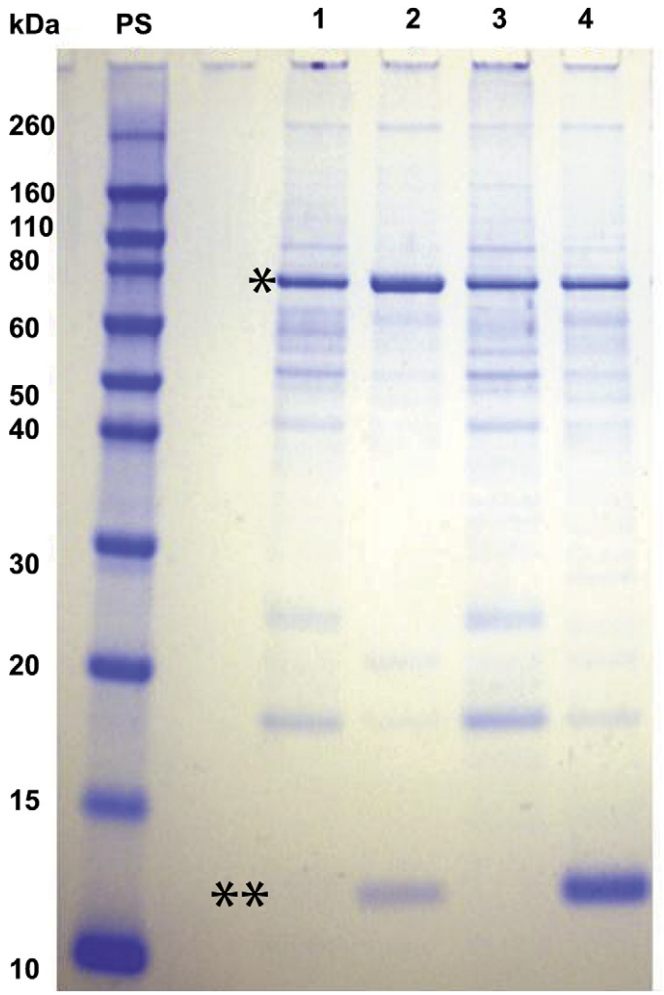


Fig. 1. 1-D gel protein profile comparing two biological replicates from both labial (lanes 1 and 3) and mandibular (lanes 2 and 4) gland luminal samples. PS = molecular weight protein standards. An asterisk denotes the 80 kDa protein band common to all samples and a double asterisk indicates the 10–15 kDa protein band appearing exclusively in the mandibular gland luminal samples.

protease inhibitors and protease (Table S2). Those translated nymphalid EST accessions matching the protein groups listed in Table S2 which were predicted to be secreted proteins are listed in Table S3 and we provide the corresponding alignment files for these predicted proteins and the detected matching peptides obtained from the Vgo samples (Supplementary alignment files).

The most abundant protein in the mandibular glands, based on our semi-quantitative analysis, was a chemosensory protein (henceforth Vgo CSPi) representing almost 51% of the mandibular gland proteome and 0.79% in the labial gland proteome (Table 1, Fig. 3A). The best hit for Vgo CSPi in our nymphalid butterfly EST database has a predicted molecular weight of 10.1 kDa after modeling the corresponding protein sequence encoded by contig Vat_30027 (Vat CSPi) (Fig. 4 and Fig. S3). The predicted molecular weight of this protein excluding its signal peptide and without modeling it after previously characterized CSPs was 11.6 kDa. Three other CSPs which appear to be variants of the same protein (Vgo CSPii) represented a much lower percentage of each salivary gland proteome (Fig. 3A). Among the 20 most abundant proteins we also found immune-related proteins in the mandibular gland, while proteins involved in cellular processes such as elongation factors, glycolytic proteins and a putative lysosomal glucocerebrosidase

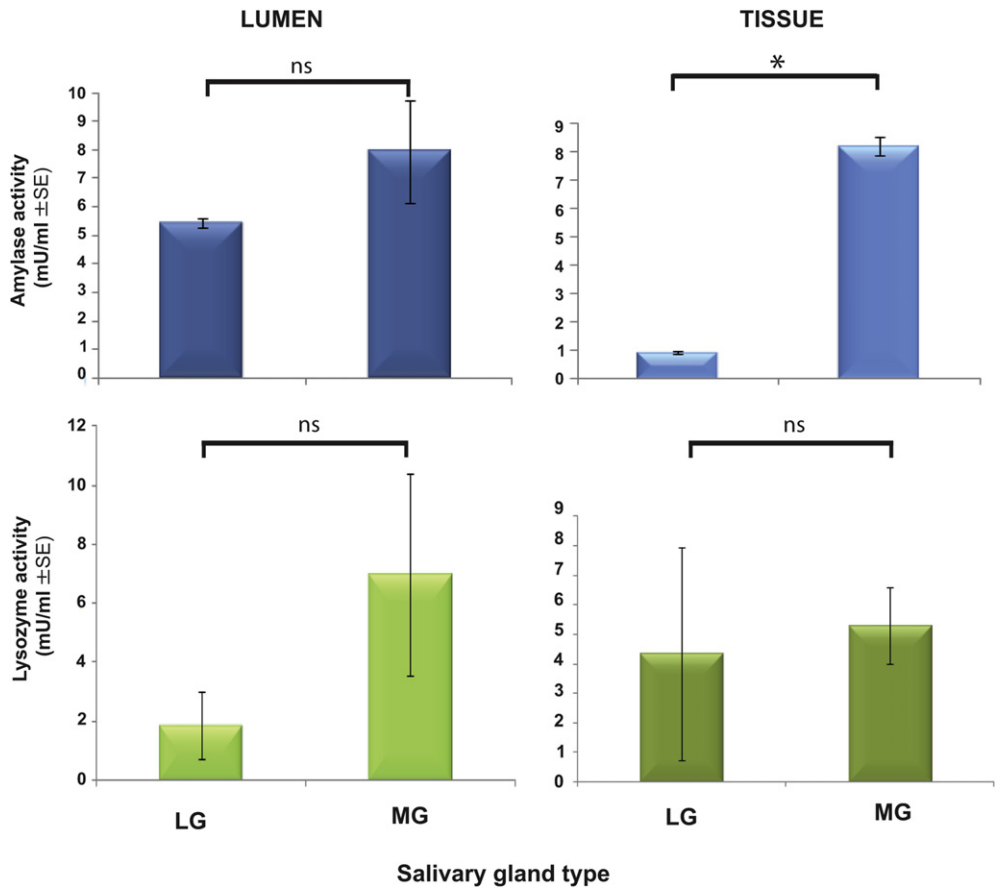
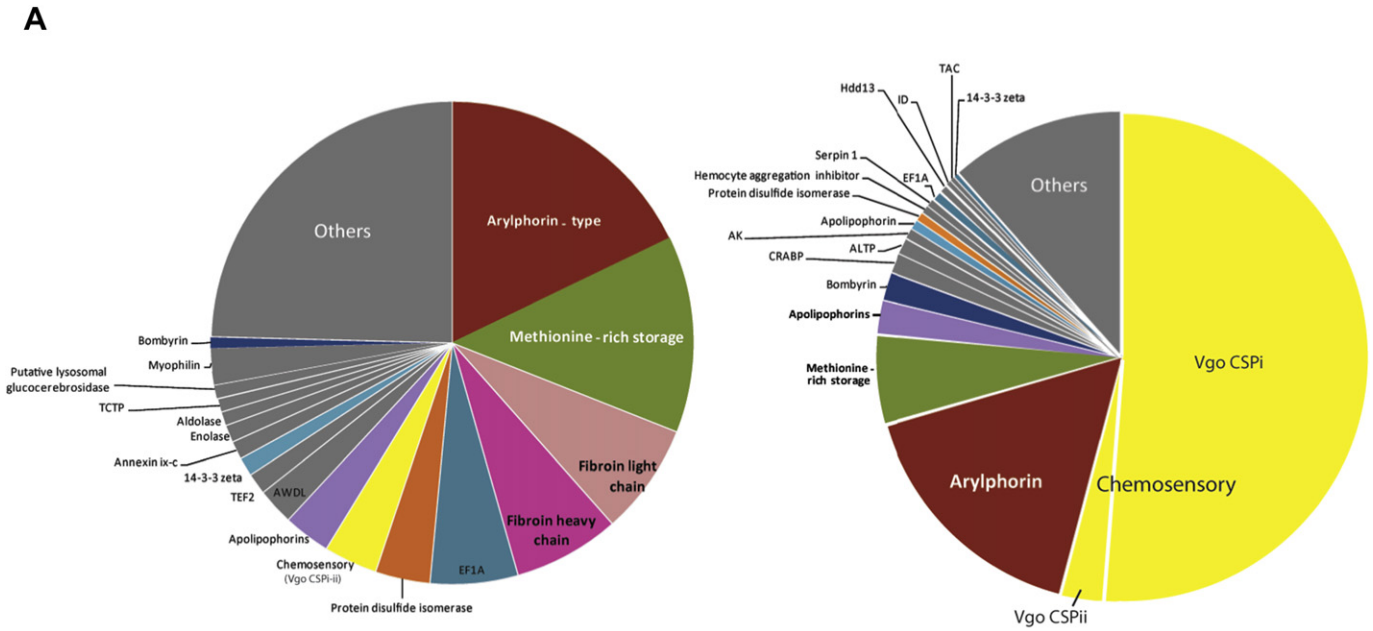


Fig. 2. Enzymatic activities in luminal and tissue protein samples after 24 h of incubation at 37 °C obtained from mandibular (MG) and labial (LG) salivary glands. Values significantly different from each other at $p < 0.05$ are denoted by an asterisk. Bars in columns indicate standard errors.

composed the rest of the labial gland proteome (Table 1). Although not in the top 20 most abundant proteins in our samples, a protein predicted to belong to the 4-cysteine class of OBPs (sericotropin) was also detected in relative abundance in the mandibular glands when compared to the labial glands (Fig. 3B, Table S4). The alignment of the sequences for the corresponding OBP contigs Vat_34343 and Vca_36359 with their best blast hit and two other insect sericotropins reveals the four conserved cysteines and the

Table 1
Top 20 best Uniprot blast hit for predicted proteins in the labial and mandibular gland luminal protein fractions. Ranking is based on protein percent abundance based on our semi-quantitation (the average of normalized and combined peptide signals per protein group from technical and biological replicates).

Rank	Grp	nr Pept	Description	LG (%)	Rank	Grp	nr Pept	Description	MG (%)
1	3	170	G6DKT4 – Arylphorin-type storage protein	17.86	1	148	9	G6CVL9 – Chemosensory protein	50.82
2	2	222	B7STX9 – Methionine-rich storage protein	13.15	2	3	170	G6DKT4 – Arylphorin-type storage protein	16.35
3	214	5	G6DJ37 – Fibroin light chain	7.46	3	2	222	B7STX9 – Methionine-rich storage protein	5.73
4	70	10	Q7Z010 – Fibroin heavy chain	7.15	4	1	194	G6DES6 – Apolipophorins	2.11
5	8	55	Elongation factor 1-alpha	5.87	5	314	2	Q8ITL3 – Chemosensory protein	1.90
6	11	36	G6DEL5 – Protein disulfide isomerase	3.67	6	341	3	Q2V8U9 – Chemosensory protein	1.88
7	39	16	Myophilin/isoform h	3.60	7	54	15	G6D4D8 – Bombyrin	1.87
8	1	194	G6DES6 – Apolipophorins	3.15	8	48	18	Cellular retinoic acid binding protein	1.33
9	51	17	Abnormal wing disc-like protein	2.45	9	68	10	Adhesion-like transmembrane protein	1.08
10	6	49	Translation elongation factor 2	1.42	10	10	34	Arginine kinase	0.70
11	15	29	14-3-3 zeta	1.26	11	88	16	G6CIC8 – Apolipophorin 3/A9XXC1 – Apolipophorin 3	0.66
12	28	25	Annexin ix-c	1.22	12	11	36	G6DEL5 – Protein disulfide isomerase	0.60
13	9	32	Enolase	1.17	13	24	23	G6CSZ4 – Hemocyte aggregation inhibitor protein	0.55
14	16	23	Aldolase	0.94	14	71	11	G6CU04 – Serpin 1/protease inhibitor	0.53
15	52	15	Translationally controlled tumor protein	0.90	15	8	55	Elongation factor 1-alpha	0.49
16	341	3	Q2V8U9 – Chemosensory protein	0.88	16	101	7	G6CQT8 – Chemosensory protein 4	0.44
17	181	6	G6D5I1 – Putative lysosomal glucocerebrosidase	0.87	17	141	6	O96383 – Immune-related Hdd13	0.38
18	314	2	Q8ITL3 – Chemosensory protein	0.81	18	21	21	Isocitrate dehydrogenase	0.37
19	148	9	G6CVL9 – Chemosensory protein	0.79	19	18	25	Tubulin alpha-1 chain	0.34
20	54	15	G6D4D8 – Bombyrin	0.78	20	15	29	14-3-3 zeta	0.33



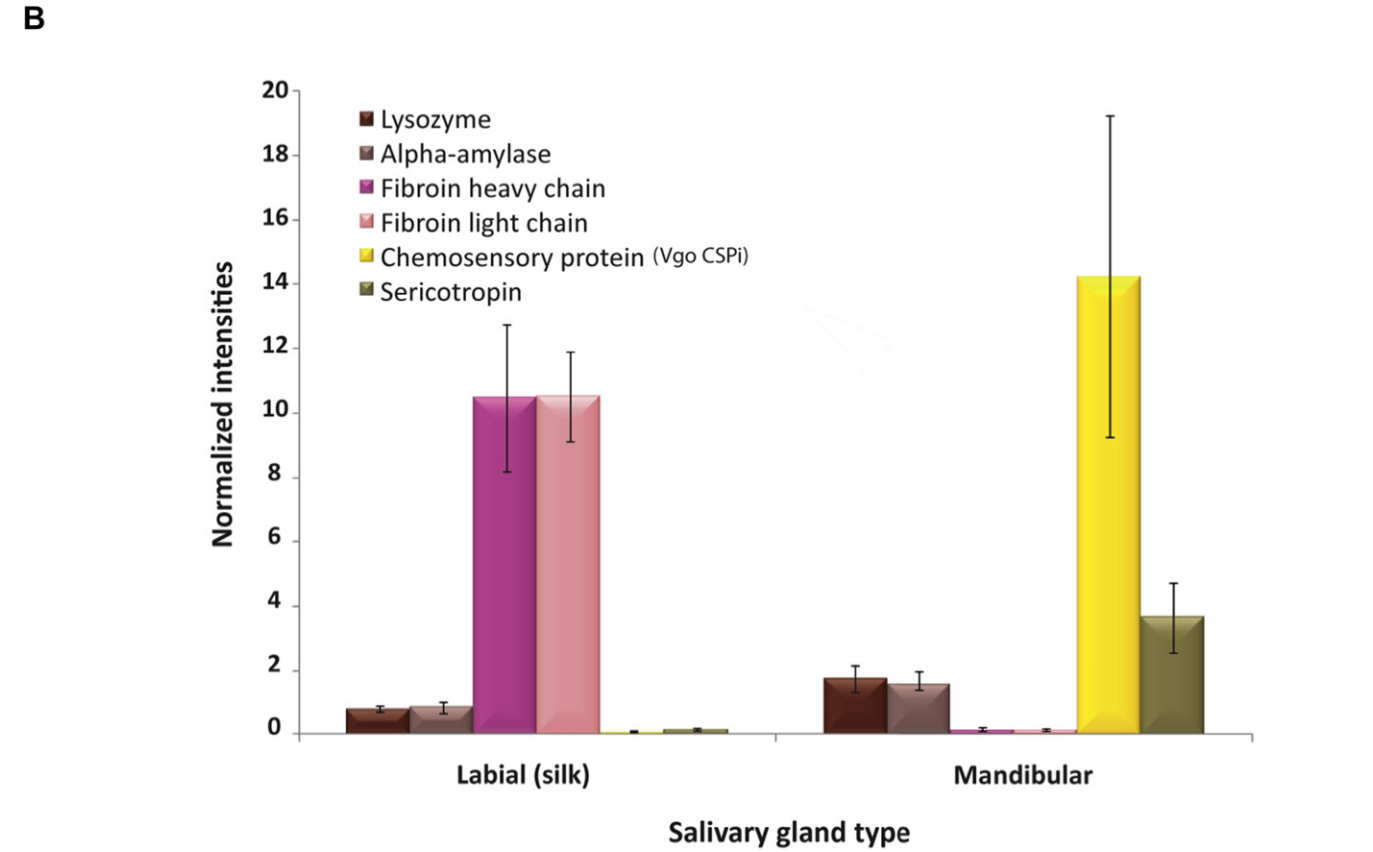


Fig. 3. Abundances of predicted proteins in mandibular and labial glands of New Zealand red admiral caterpillars. A. Pie charts depict the protein composition in percentage of labial (left chart) and mandibular (right chart) gland proteomes, based on semi-quantitative estimates of the protein abundances. B. Relative quantities of selected predicted proteins in both glandular types, based on label free quantification of peptide intensity in MS. Bars in columns indicate standard errors. Vgo CSPi = *Vanessa gonerilla* chemosensory protein "i" similar to G6CVL9, Vgo CSPii = group of Vgo chemosensory protein variants similar to G6CQT8, AK = arginine kinase ALT = Adhesion-like transmembrane AWDL = abnormal wing disc-like, CRAB = cellular retionoic acid binding, EF1A = elongation factor 1 beta, HAI = hemocyte aggregation inhibitor, Hdd13 = immune-related, ID = isocitrate dehydrogenase, TAC = tubulin alpha-1 chain, TCT = translationally controlled tumor, TEF2 = translation elongation factor 2, PLG = putative lysosomal glucocerebrosidase.

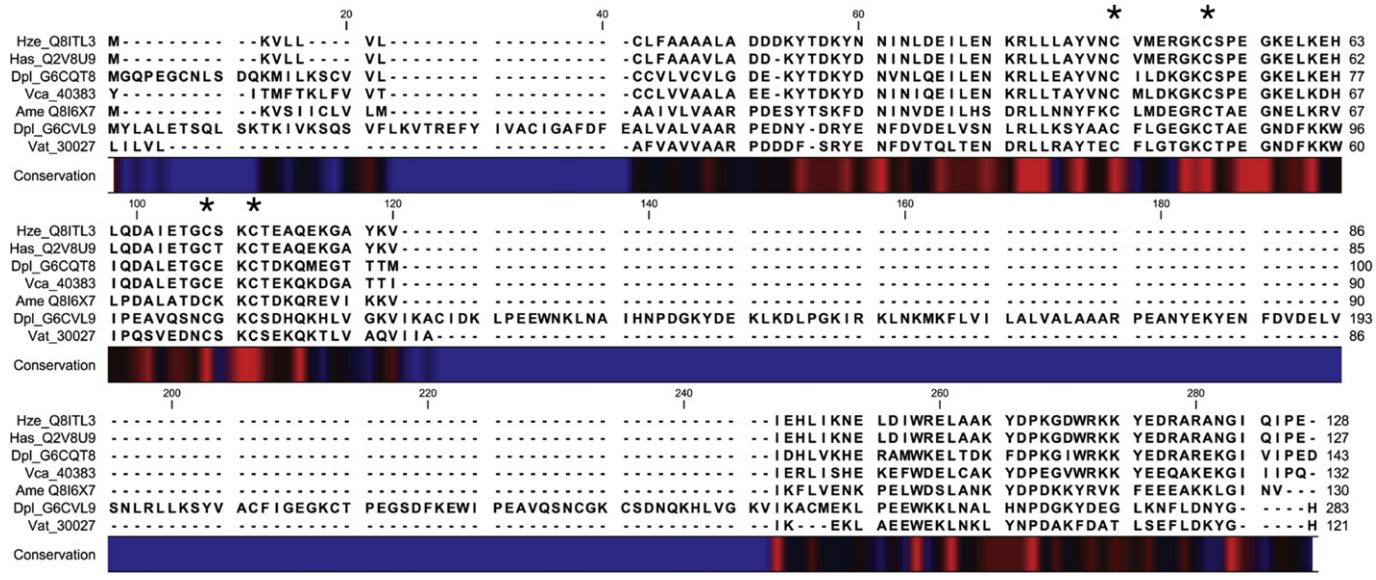


Fig. 4. Multiple alignments of CSPs. The degree of amino acid conservation is shown by a gradient of color underneath the alignment where 100% amino acid identity is represented by intense red color and 0% identity is depicted in blue. Conserved cysteines are indicated by asterisks. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

two OBP units in contig Vca_36359 (Fig. 5). The second most abundant group of proteins in the mandibular gland proteome was represented by storage proteins (i.e. methionine-rich and arylphorin-type). In contrast, this same group of proteins represented, along with fibroin, almost half of the soluble labial gland proteome. Lipid-binding proteins were also shared proteins between the two salivary gland proteomes.

Consistent to our enzymatic assays, amylase and lysozyme were detected in both salivary gland soluble protein extracts. However, these enzymes did not appear among the most abundant proteins and they occur in similar relative quantities in both salivary gland types (Fig. 3B, Table S4).

4. Discussion

4.1. Chemosensory proteins

The presence of CSPs in higher relative quantities in the mandibular gland soluble protein fraction opens new questions about their possible role in the larval body. The expression pattern of insect CSPs is unspecific and these proteins have been associated with other functions besides olfaction and taste (Jin et al., 2006; Pelosi et al., 2006). Therefore, the assignment of a putative molecular function based on sequence similarity is particularly difficult for the case of detected CSP-like proteins. At most, we can

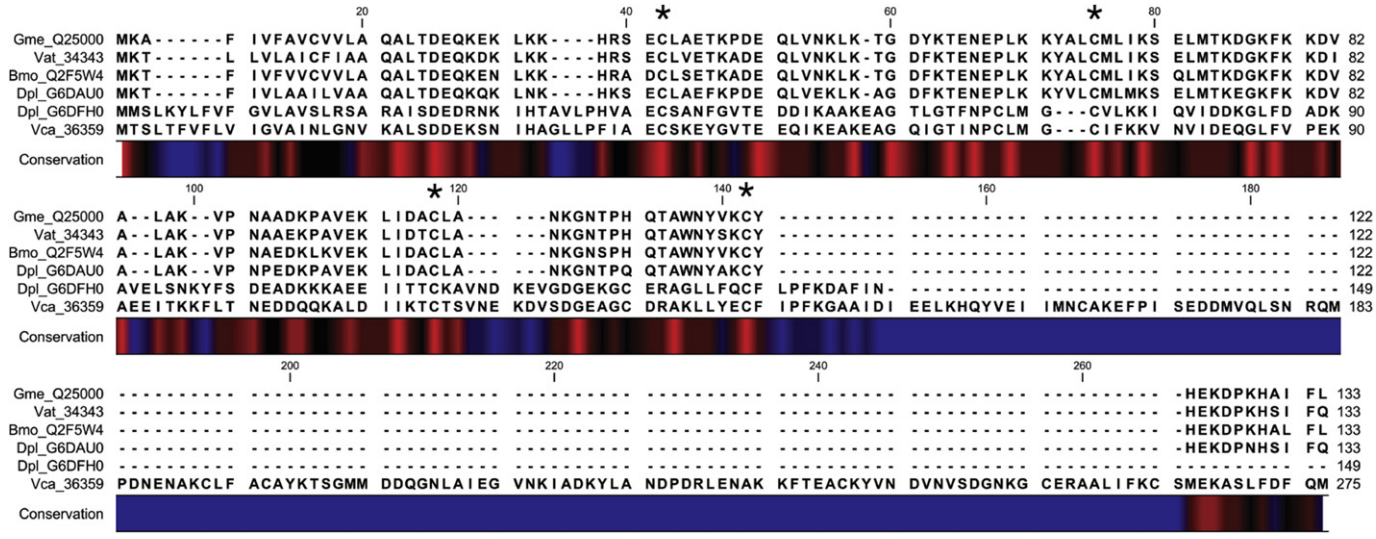


Fig. 5. Multiple alignments of OBPs. The degree of amino acid conservation is shown by a gradient of color underneath the alignment where 100% amino acid identity is represented by intense red color and 0% identity is depicted in blue. Conserved cysteines are indicated by an asterisk. Two additional OBP sequences corresponding to *B. mori* and *D. plexippus* were included in the alignment: Bmo_Q2F5W4 and Dpl_G6DAU0. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

only suspect that they move hydrophobic molecules across the caterpillar body. Saliva may be used as an interface where CSPs shuttle different kinds of compounds during herbivory providing information to the caterpillar about its environment. For now, the speculation of their role can be exhaustive; they could facilitate the communication not only between the caterpillar and its host plant by carrying phagostimulants/deterrents but also possibly binding molecules for the recognition of microorganisms on the leaf surface. The fact that one particular protein group, i.e. Vgo CSPi, predominated in the Vgo mandibular gland soluble proteome could be correlated to the low survivorship of the larvae feeding nettle. Since we presented a suboptimal host to a specialist caterpillar, this might have had an impact of its physiology. Although, the host plant of the New Zealand red admiral is the ongaonga (*Urtica ferox*) endemic to New Zealand (Gibbs, 1980), *V. gonerilla* larvae feeds on other species of the same genus including *Urtica aspera*, *Urtica incisa* and the introduced *U. dioica* (Barron et al., 2004) suggesting that this butterfly species might be a specialist on Urticaceae. It may be of interest to define more exhaustively the host range of *V. gonerilla* within the family Urticaceae and whether the high expression of a particular CSP in the mandibular glands may indicate a stress response.

The presence of 4-cysteine OBPs in our proteomic analysis may be explained by possible contamination since this type of OBPs have been previously detected in insect hemolymph (Graham et al., 2003; compare Furusawa et al., 2008 and Iovinella et al., 2011). Due to the divergence among isoforms and their unspecific tissue expression, 4-cysteine OBPs have been proposed to be proteins shuttling also small hydrophobic compounds in the insect body not without considering their possible involvement in other molecular functions (Graham et al., 2003). OBP genes consisting of two tandem, in-frame OBP-like sequences have been identified previously in other insect species and this class of genes, referred to as "double OBPs" by Lagarde et al., share sequence similarity to certain mosquito salivary proteins (Hekmat-Scafe et al., 2002; Lagarde et al., 2011). Analyzing the peptide alignment (Align_G6DFH0) corresponding to contig Vca_36359, we observed that only peptides covering the first CSP unit of the translated sequence were detected in our proteomic study.

Other proteins detected in our luminal samples were lipid (e.g. bombyrin) and iron-binding proteins predicted to be secretory and therefore, possibly involved in the transport of macromolecules, small molecules or ions into, out of or within cells. Some of these proteins along with those detected to be secretory and having chaperonine function may be contaminants from the cytosol.

4.2. Immune-related proteins

Storage proteins and apolipophorins characterized previously from insect hemolymph were detected consistently in both 1-D and shotgun proteomic analyses and they may represent contamination from hemocytes and/or hemolymph (Kanost et al., 1990; Kawooya et al., 1984; Paskewitz and Shi, 2005; Ryan et al., 1985). Alternatively, the mandibular and labial glands might produce these "hemolymph proteins" or they are able to circulate through the glands. An extensive overlap between plasma and salivary proteomes has been also observed in humans (Loo et al., 2010). We consider particularly intriguing the physiological role of salivary glands in the circulation/production of these proteins, especially since their abundance is greatly affected when the insect immune system is compromised (Freitak et al., 2007; Lourenĉo et al., 2009).

Among the 20 most abundant predicted proteins in the mandibular glands, the predicted one for Vat_33500 shared highest amino acid identity with the predicted gene product of cDNA clone Hdd13. This cDNA sequence was identified and characterized from immune-challenged fall webworm moth larvae (*Hyphantria cunea*) (Shin et al., 1998). It was found that this transcript was readily up-regulated and its expression was sustained even after 24 h post-injection of bacteria in the hemocoel of the larvae, displaying a similar expression pattern as the one for hemolin transcript. Unfortunately, there was no inspection of Hdd13 tissue-specific expression in the study. Another member of this immune-related group of transcripts, Hdd1, has been previously sequenced from a salivary gland cDNA library for *Triatoma infestans* (Assumpção et al., 2008).

Unique peptides corresponding to a 1,3 beta glucan recognition protein were also identified in our samples. GRPs are known to be involved in the recognition of bacteria and fungi, aggregating them and even eliciting prophenoloxidase activity and, as many other immune-related genes, they are expressed in different tissues, including the fat body and cuticle and display enzymatic activity aiding in digestion (Jiang et al., 2004; Pauchet et al., 2009). One major route of acquisition of microorganisms occurs during feeding and therefore, we suspect that the function of GRPs in caterpillar saliva deserves further attention. Moreover, a serine protease inhibitor (serpin) was observed among the most abundant proteins in the mandibular glands. Serpins are thought to protect insect from pathogen attack by inhibiting proteases produced by fungi and parasites among other functions (Kanost et al., 1990). Again, it is worth considering whether the presence of immune-related proteins in our analysis represents a stress response from the larvae toward its suboptimal host.

4.3. Digestive enzymes

We found peptides which matched contig sequences in our nymphalid EST databases which in turn share highest similarity to monarch butterfly amylase and lysozyme proteins. On average, both enzymes seem to be found in slightly higher quantities in the mandibular glands in comparison to the labial glands. However, at this level of detection, we also see high biological variability that prevents us from making strong conclusions. Our observation contradicts a previous one, where amylase activity in labial glands was higher than in mandibular glands on the basis of a qualitative assay for this enzyme in *A. fabriciella* larvae (Mall et al., 1978). The differential expression of a lysozyme gene inspected utilizing total RNA from *H. zea* labial glands in response to different diets (i.e. artificial, cotton, tobacco and tomato) has been observed previously (Liu et al., 2004). This observation led the authors to speculate that such differential expression may be due to different bacteria populations contained in each of the tested diets. Therefore, labial gland lysozyme has been suggested as a "pre-ingestive, ready-to-use antibacterial factor" (Liu et al., 2004). Based on literature review and our own results shown here, we suspect that the presence of hemocytes possibly aggregated to the gland tissue during dissection may confound the results. Indeed lysozymes, also known as muramidases, have been detected in these type of cells (Liu et al., 2004). Other observations, include the detection of lysozyme from *Manduca sexta* hemolymph with a predicted molecular weight of approximately 16 kDa (Furusawa et al., 2008) and the induction upon immune challenge of a 14.4 kDa lysozyme in *G. mellonella* also in hemolymph (Vogel et al., 2011). Lysozyme transcript is expressed in several *M. sexta* larval tissues including salivary (possibly labial) glands and hemocytes express lysozyme gene, although the level of the transcript is highest in the fat body (Mulnix and Dunn, 1994). Unfortunately, the data for the level of lysozyme transcript in hemocytes is not shown in the communication. The predicted enzyme for this gene (Uniprot number Q26363) belongs to the c-type lysozymes which are regarded as antibacterial proteins. These lysozymes can have digestive roles and display chitinase activity (Callewaert and Michiels, 2010).

Similarly to lysozyme, the biological meaning of amylase in insects remains unclear (Callewaert and Michiels, 2010). Amylases seem to be ubiquitous, inducible enzymes produced by many different organs, but it may as well be that their production is population or species-specific. For example, amylase has been detected in Lepidopteran hemolymph (Asadi et al., 2010) and it has been suggested that its function might be that of degrading fat body glycogen (Ngernyuang et al., 2011). In contrast, extensive proteomic and transcriptomic studies of hemolymph do not mention the detection of amylase in moth species including *B. mori* (Dawkar et al., 2011; Furusawa et al., 2008; Hou et al., 2010; Vogel et al., 2011). An amylase gene by in situ hybridization has been detected exclusively in *B. mori* mandibular glands (Parthasarathy and Gopinathan, 2005), while another independent study has found the expression of this same amylase gene restricted to the foregut of a multivoltine *B. mori* race from Thailand (Nanglai) (Ngernyuang et al., 2011).

The unique peptides matching Vca_12842 predicted protein were also detected in relative abundance in the labial glands. This sequence is most similar to monarch butterfly secretory glucocerebrosidase (G6D5I1). The annotation of the monarch butterfly sequence G6D5I1 is in turn, putative, based on the prediction of glycosyl hydrolase signature domains in its sequence. Therefore, we should be cautious on the interpretation of its presence in the labial gland. It may be of interest to investigate whether this predicted protein is strictly involved in glycolipid metabolism or has a role in digestion or host recognition.

Esterases were also detected in *V. gonerilla* mandibular and labial glands. Most of the secretory esterases detected in our samples have high homology to carboxyl/cholinesterase D5G3D3 from *H. armigera*. This protein, CCEOO1f, has been classified as a "larval midgut esterase of unknown function" in the cotton bollworm (Teese et al., 2010). Other carboxyl/esterase (Vat_7741) might be involved in pheromone degradation.

Two glycolytic enzymes were detected among the proteins in high relative abundance in the labial glands, namely, enolase and aldolase. In particular, enolase has been found differentially expressed regardless of the experiment conditions and tissue type analyzed in several proteomic studies in humans and rodents (Petrak et al., 2008). This recurrent identification of enolase, specifically in 2-DE-based studies, has generated speculations regarding its role as a possible universal sensor of cellular stress (Petrak et al., 2008).

Aut_8983 has 59 and 38% amino acid identity to the monarch butterfly protein G6CN51 and cotton bollworm protein B1NLD7 respectively. Neither of these proteins has been characterized yet. However, B1NLD7 has been detected in midgut and labial glands of the cotton bollworm larvae (Celorio-Mancera et al., 2011; Pauchet et al., 2008).

5. Conclusions

Chemosensory proteins were the major salivary factors in the mandibular glands of the butterfly larvae studied. The quality and quantity of the CSPs identified allowed the clear differentiation between caterpillar mandibular and labial glands and to a lesser extent, the presence of more abundant immune-related proteins in the mandibular glands. Differences between amylase and lysozyme quantities and activities did not aid discrimination between the two gland types. Similarly, storage and lipid-binding proteins appeared to be present in both mandibular and labial glands. A more strict functional analysis of CSPs will shed light on their possible involvement in host plant recognition or the insect immune response.

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Appendix A. Supplementary data

Supplementary data related to this article can be found online at http://dx.doi.org/10.1016/j.ibmb.2012.07.008.

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