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Effect of host plant and immune challenge on the levels of chemosensory and odorant-binding proteins in caterpillar salivary glands

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ABSTRACT

More than half of the proteome from mandibular glands in caterpillars is represented by chemosensory proteins. Based on sequence similarity, these proteins are putative transporters of ligands to gustatory receptors in sensory organs of insects. We sought to determine whether these proteins are inducible by comparing, both qualitatively and quantitatively, the salivary (mandibular and labial) proteomes from caterpillars (*Vanessa cardui*) reared on different plants and artificial diet containing either bacteria or bacterial cell-walls. We included a treatment where the caterpillars were switched from feeding on artificial diet to plant material at some point in their development. Additionally, we evaluated the degree of overlap between the proteomes in the hemolymph-filled coelom and salivary glands of caterpillars reared on plant material. We found that the quality and quantity of the identified proteins differed clearly between hemolymph-filled coelome, labial and mandibular glands. Our results indicated that even after molting and two-day feeding on a new diet, protein production is affected by the previous food source used by the caterpillar. Candidate proteins involved in chemosensory perception by insects were detected: three chemosensory (CSPs) and two odorant-binding proteins (OBPs). Using the relative amounts of these proteins across tissues and treatments as criteria for their classification, we detected hemolymph- and mandibular gland-specific CSPs and observed that their levels were affected by caterpillar diet. Moreover, we could compare the protein and transcript levels across tissues and treatment for at least one CSP and one OBP. Therefore, we have identified specific isoforms for testing the role of CSPs and OBPs in plant and pathogen recognition. We detected catalase, immune-related protein and serine proteases and their inhibitors in high relative levels in the mandibular glands in comparison to the labial glands. These findings suggest that the mandibular glands of caterpillars may play an important role protecting the caterpillar from oxidative stress, pathogens and aiding in digestion. Contamination with hemolymph proteins during dissection of salivary glands from caterpillars may occur but it is not substantial since the proteomes from hemolymph, mandibular and labial glands were easily discriminated from each other by principal component analysis of proteomic data.

1. Introduction

Molecules detected in caterpillar saliva and other insect secretions have been found to mediate the interaction between plants and herbivores (Mithofer and Boland, 2008) by either suppressing (Weech et al., 2008) or activating plant defenses (Schmelz et al., 2009). These molecules have been defined as herbivore-

Abbreviations: ACN, acetonitrile; BGRP, beta-glucan recognition protein; CSP(s), chemosensory protein(s); EST(s), expressed sequence tag(s); HAMPs, herbivore-associated molecular patterns; LC-MS/MS, liquid chromatography tandem mass spectrometry; MRSP, methionine-rich storage protein; OBP(s), odorant-binding protein(s).

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associated molecular patterns (HAMPs). HAMPs such as glucose oxidase (Musser et al., 2005a; Tian et al., 2012) and caeliferin (sulfated fatty acid) (Schmelz et al., 2009) produce a burst of jasmonic acid in the host plant which in turn activates a defense response cascade against herbivory. Moreover, it has been observed that HAMPs in labial saliva of caterpillars could reduce the infectivity of bacterial pathogens (Musser et al., 2005b). The importance of HAMPs has drawn interest into the study of the composition of insect saliva, aiming to understand the evolutionary relationship between herbivores and their hosts (Musser, 2005).

A single type of chemosensory protein (CSP) represents about half of all soluble proteins in the salivary glands of caterpillars by proportion of total protein mass (Celorio-Mancera et al., 2012). The biological function of most CSPs in Arthropoda remains elusive regardless of their high level of conservation. CSPs seem to transport chemical cues within insect sensilla while others may be associated with completely different processes, such as organ regeneration (Pelosi et al., 2006). In addition, this type of small protein has been identified in both sensory and non-sensory tissues (Gong et al., 2007; Jacquin-Joly et al., 2001; Liu et al., 2010; Picimbon et al., 2001). Therefore, the identification of CSPs through sequence similarity barely suggests the discovery of putative proteins belonging to a multifunctional gene family of hydrophobic-ligand carriers (Pelosi et al., 2006). Nevertheless, it has been proposed that CSPs sequester or store plant-derived chemicals in the insect body as a strategy against predation (The Heliconius Genome Consortium, 2012).

Caterpillar CSPs secreted during feeding are possible factors determining acceptability or rejection of a plant. We consider that the larval stage of herbivorous insects makes the ultimate host-plant selection. Tasting is critical for the acceptability or rejection of food by insect herbivores (Chapman, 2003). Larvae can often move between hosts and locate new feeding sites (Bernklau et al., 2009; Cunningham et al., 2011; Jones and Coaker, 1977) perceiving with their sensilla in the maxillae and eipipharynx (Hansson, 1995; Schoonhoven and van Loon, 2002) chemicals in the leaf surface (Chapman and Bernays, 1989). Moreover, it has been observed that leaf and root herbivores are even able to exploit those herbivore-induced plant volatiles for host plant location (Carroll et al., 2008; Robert et al., 2012).

It is necessary to determine whether the function of CSPs determines the behavior of caterpillars in response towards chemicals in the environment. Investigations regarding the location and function of CSPs in insect larvae are few. To date, expression of genes encoding CSPs has been studied on few species of Lepidoptera and mostly on adult specimens; few have conducted studies on the larval stage and if so, never in a tissue-specific manner (Gong et al., 2007; Jacquin-Joly et al., 2001; Liu et al., 2010; Picimbon et al., 2001). So far, the mutation of a member of the odorant-binding protein family (OBP), chemically similar to the CSP family, has been found to alter the sensitivity in the fruit fly towards a pheromone (Ebbs and Amrein, 2007; Su et al., 2009). Therefore, it is feasible that CSPs and OBPs bind nutrients, phagostimulants or may trigger immune defense mechanisms to protect the larvae against pathogens on the plant surface. Yet, it may well be, according to Dasmahapatra and collaborators (The Heliconius Genome Consortium, 2012), that this kind of proteins shuttle toxins from the plant for their accumulation in the insect body. Alternatively, they may represent additional HAMPs involved in the activation or deterrence of plant defenses against herbivory.

We have speculated that CSPs may recognize plants and/or microorganisms on the leaf surface (Celorio-Mancera et al., 2012). On the quest to challenge our hypothesis, we used a proteomics approach to test whether the levels of chemosensory proteins in salivary glands of *Vanessa cardui* (painted lady) caterpillars change depending on a variety of diet treatments. Since we aimed for the identification and relative quantification of all the proteins in the caterpillar saliva, we also paid particular attention on additional proteins involved in chemoreception, immunity and digestion, which appeared to be relevant factors due to their relative quantities in labial and mandibular glands of caterpillars (Celorio-Mancera et al., 2012). Therefore, we conducted a set of experiments to assess whether the protein levels between labial and mandibular glands changed due to: a) host plant b) a switch from artificial diet to plant material in a particular time of larval development, and c) bacteria or bacterial cells walls in the diet. In addition, since contamination of salivary gland samples with hemolymph is highly possible during dissection of these organs we considered it important to compare the proteomes of hemolymph, mandibular and labial glands from larvae reared on the same food source.

2. Methods

2.1. Insect rearing

Butterfly eggs of the species *V. cardui* were obtained from a laboratory colony and two commercial suppliers (World Wide Butterflies [www.wwb.co.uk] and Heart of England Butterflies [www.heartofenglandbutteflies.com]). Males and females were marked and placed in mating cages without including both sexes from the same origin in a given cage. Two generations were reared from this population under laboratory conditions (25 °C; LD 18:6) avoiding full-sib mating. The progeny obtained from five mating pairs from the second generation was subjected to three rearing diets following a split-brood design (Fig. 1). That is, 10 to 20 neonates from each family were transferred immediately after hatching to individual plastic cups containing either 1) soybean/wheat-based artificial diet (AD) (Stonefly "Heliothis" diet, product 38-0600, WARD's) prepared following the manufacturer's instructions, 2) leaves of marsh thistle (*Cirsium palustre*) or 3) leaves of stinging nettle (*Urtica dioica*). Leaves of either host were kept moist using a wet cotton ball at their base and replaced as needed.

2.2. Diet treatments and sample collection

Fig. 1 summarizes how the experiment was designed and conducted. In the host treatments, caterpillars fed either thistle (T) or nettle (N) from neonate stage until the time for their dissection. In treatment "AD-T", caterpillars molting into their 5th larval stage or shortly after were transferred to thistle leaves. The rest of the caterpillars reared on AD were also allocated randomly to the other three immunity treatments during or just after molting into their 5th larval stage. The control diet for the immunity treatments consisted of 3 g of artificial diet spiked with 200 μl of Luria-Bertani (LB) medium four hours before provided to the caterpillars. The treatments containing either bacterial walls (Pep) or live bacteria (Bac) were prepared exactly as the control diet but the LB medium contained peptidoglycan from Bacillus subtilis (0.08 μg/μl; Sigma−Aldrich, product no. 69554) or *Escherichia coli* bacteria (OD600 = 0.95; Invitrogen, product no. K4500). After two days of feeding, the labial and mandibular glands of 5th-instar larvae were dissected following the protocol previously described (Celorio-Mancera et al., 2012) and pooled according to gland type and biological replicate. Each feeding-treatment consisted of four biological replicates and each biological replicate per gland type (labial or mandibular) was represented by gland pairs from five individuals, one per butterfly family. The soluble protein fraction (luminal proteins) obtained per biological replicate was transferred to new tubes and further processed as described below. In order to assess

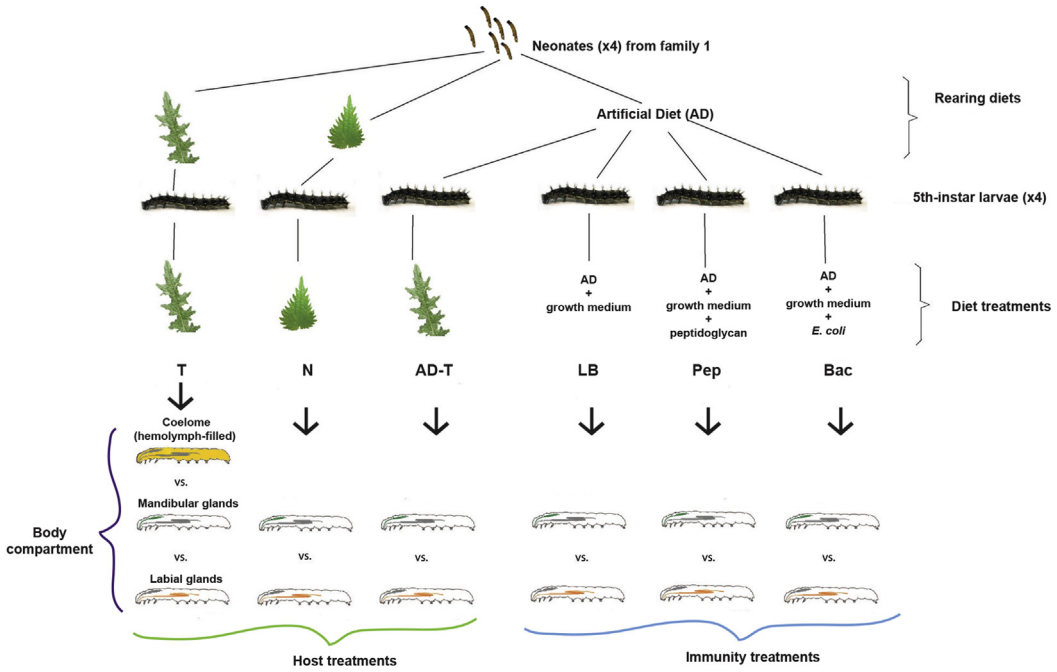

Fig. 1. Experimental design to test the effect of host plant and immune challenge on salivary proteomes of caterpillars. Protein location in the insect body and the effect of diet switch on salivary proteomes of caterpillars was also addressed. Neonates from five butterfly families were split randomly and reared on three different diets: 1) thistle (T); 2) nettle (N) and 3) soybean/wheat-based artificial diet (AD). During molting into their 5th larval instar those larvae from diets 1 and 2 continued on their corresponding host plants while larvae reared on AD were transferred to four different treatments: thistle (AD-T), AD containing growth medium (LB), AD spiked with peptidoglycan dissolved in growth medium (Pep) and AD spiked with growth medium containing *E. coli* (Bac). This experimental design was replicated four times per butterfly family. The experimental unit consisted of five individuals combined, one per family, following a split-brood design.

the degree of overlap between proteins from different locations in the insect body, i. e. proteomes from the hemolymph-filled coelom (He), labial (LG) and mandibular glands (MG), we obtained a droplet (approximately 20 μl) of hemolymph from each caterpillar fed with thistle (Fig. 1). The hemolymph samples, which were also pooled according biological replicate, were obtained by piercing the dorsal area of the fifth segment in the caterpillar bodies with a pin.

2.3. Proteomics

2.3.1. Preparation of protein extracts in solution

The harvested samples were mixed well and after centrifugation at 13,000 rpm for 10 min at 4 °C the protein concentration was determined using the Pierce BCA protein assay kit (Thermo Fisher, USA) on the Epoch microplate spectrophotometer (BioTek, Winooski, USA). From each sample 3 μg of protein were dissolved in a total volume of 90 μl buffer containing 2 M Urea, 0.1% surfactant ProteaseMax (Promega, Wisconsin, USA), 50 mM ammonium bicarbonate (Sigma, Munich, Germany) and 10% acetonitrile (Fisher, Loughborough, UK). The resulting protein solutions were incubated at 50 °C for 30 min while shaking followed by bath sonication for 10 min at room temperature. Samples were centrifuged at 13,000 rpm for 5 min and directly subjected to a tryptic digestion program carried out by a liquid handling robot (MultiProbe II, Perkin Elmer, Waltham, USA). This included protein reduction in 5 mM dithiothreitol (Sigma, Munich, Germany) at 56 °C and alkylation in 15 mM iodacetamide (Sigma, Munich, Germany) for 30 min at room temperature. Trypsin was added in an enzyme to protein ratio of 1:30 and digestion was carried out over night at 37 °C.

2.3.2. Nano-LC-MS/MS analysis

After tryptic digestion, the samples were acidified and cleaned with C18 StageTips according to the manufacturers' description (Thermo Fisher Scientific Inc, Waltham, USA). Eluted peptides were dried and re-suspended in 3% ACN and 0.2% formic acid. nLC-MS/MS analyses were performed on an Easy-nLC system (Thermo Fisher Scientific, Bremen, Germany) in-line coupled to a QExactive MS (Thermo Fisher Scientific, Germany). From each sample, 0.5 μg were injected from a cooled auto sampler onto the nLC 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: 3–35% ACN in 89 min, 35–95% ACN in 5 min and 95% ACN for 8 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 1650 and with a resolution of R = 70,000 at m/z 400, followed by data-dependent HCD fragmentation scans (MS2) from maximum ten most intense precursor ions with a charge state ≥2. MS2 scans were acquired with a resolution of R = 17,500, a target value of 2e5, isolation width was set to 4 and normalized collision energy to 26. For all sequencing events dynamic exclusion of 90 s was enabled. The instrument was calibrated externally according to the manufacturer's instructions and all samples were acquired using internal lock mass calibration on m/z 429.08874 and 445.12003.

All samples were processed and analyzed in a randomized manner with blank runs between each sample in order to avoid cross contamination.

2.3.3. Peptide identification and protein quantification

Mass lists were extracted from the raw data using Raw2MGF v2.1.3 and combined into one file using Cluster MGF v2.1.1, programs part of the Quanti work-flow described below. Since *V. cardui* is not one of the model organisms with well-defined databases, the data was searched against a nucleotide database (251,184 sequences and 50,638,116 residues) compiled from contigs of the *V. cardui* transcriptome-assembly generated and annotated

previously (Celorio-Mancera et al., 2012). To prevent misidentifications from trypsin and keratin, the data was first searched against a SwissProt database (downloaded in April 2013 containing 539,829 sequences and 191,670,831 residues), and the queries matching these contaminants were removed. Mascot v2.4.1 (Matrix Science Ltd., London, UK) was used for both searches using the following parameters: tryptic digestion (maximum two miscleavages); carbamidomethylation (C) as fixed modification; pyroglutamate (Q) and oxidation (M) as variable modifications; a precursor tolerance of 5 ppm; a fragment tolerance of 0.01 Da; instrument set to ESI-TRAP and the option for target-decoy. Additionally, the search against SwissProt included all taxa. Since Mascot is translating and searching nucleotide databases in six frames, the option for decoy database was instead of searching a concatenated database. The decoy hits were then used to calculate a peptide threshold for 1% FDR, which in this case was 16. The quantification of the data was done using the Quanti work-flow, quantification software that uses extracted ion chromatograms (Lyutvinskiy et al., 2013). In short, after searching the combined mgf files against the nucleotide database, the resulting dat file and the 52 raw files (representing 4 biological replicates of 13 samples, with single replicates of each) were uploaded into Quanti v2.5.4.3. The following parameters were used: score threshold 16; mass tolerance 10 ppm; minimum peptides/protein 2; maximum allowed deviation in rt 3% and 5 min. The quantitative values were further processed by multiplying the values with the reference abundance and normalizing each sample to the median of the summed intensities for all the samples. The values were finally log10-transformed.

2.3.4. Statistical analysis

Coefficients of variation (four replicates) in percent were calculated for all treatments. Pair comparisons between treatments were performed using the log10-transformed of the normalized protein intensities, the log2 ratios were calculated from the medians of the two treatments compared, and Student's t-test was used to estimate the p-value for the comparison. Estimation of false positives was done by calculating the expectation value (E) and using Bonferroni correction to adjust the level of significance. The q-values (FDR corrected p-values) were also calculated (Benjamini and Hochberg, 1995). Since it is impossible to avoid leakage of biofluids from neighboring tissues during dissection, the level of cross-contamination of these fluids and the salivary origin of the proteins was assessed by comparing the hemolymph and salivary glands from larvae reared on thistle. Proteins with a higher abundance in at least one salivary gland were considered to be salivary. The cases where proteins were missing from all 4 replicates in on tissue, while present in all four in the other were consider being present in the second tissue. In this case, E-values below 1 were considered to be significant. Principal component analysis (PCA) was performed using SIMCA 13.0.3 (Umetrics, Umeå, Sweden). Default settings were used with the exception of using Par scaling. Model performance was reported as cumulative correlation coefficients for the model (R2X[cum]) and predictive performance based on seven-fold cross validation calculations (Q2[cum]). By default, proteins with missing values in 50% of the comparisons were removed. Otherwise the whole data set quantified by Quanti was used.

2.4. Transcript quantification

Total RNA from all biological replicates across diet treatments for each body compartment was prepared according to the method described previously (Celorio-Mancera et al., 2013). A total of three hundred and fifty nanograms of DNA-free total RNA was converted into single-stranded cDNA using mixture of random hexamer and oligo (dT)18 primers according to the Maxima first strand cDNA synthesis kit for real time quantitative PCR protocol (ThermoScientific, USA). The elongation factor 1-alpha (EF1a) and the glyceraldehyde-3-phosphate dehydrogenase (G3PD) were used as endogenous control genes, (EF1a: forward primer: 5′CACAAAGAACAAAGCCAGGAG and reverse primer: 5′GGGAAAAGTTGAAGCAGGAAC; G3PD: forward primer: 5′ACCCAGAAGACAGTTGATGGA and reverse primer: 5′CCAAGACGGACAGTTAGGTCA). Gene-specific primers were designed on the basis of sequence obtained for Vca_contig33997 (CSP-like protein, called CSP1 henceforth with forward primer: 5′CGAAGGATGTGCCAAGTGTA and reverse primer: 5′ACGACTGGTAGCCTTCTGGA). Primers were also designed to quantify the expression of two additional genes on the basis of sequence obtained for Vca_contig36359 (OBP-like protein, called OBP2 henceforth with forward primer: 5′TAATCATGAACTGCGCCAAG and reverse primer: 5′TGATCGTCCATCATTCCTGA) and Vca_contig32439 (CSP-like protein, named CSP3 henceforth with forward primer: 5′GTCGAAACAGTGTGCGGTAA and reverse primer: 5′TTAACGAGCTCCTCCCATTG). Quantitative RT-PCR was done in optical 96-well plates on a StepOnePlus System (Applied Biosystems by Life Technologies) using the Maxima SYBR qPCR green Mix (ThermoScientific, USA) to monitor double-stranded DNA synthesis in combination with ROX as a passive reference dye included in the PCR master mix. The cycling conditions consisted of 3 min holding stage at 95 °C, forty 2-step cycles at 95 °C for 3 s and 60 °C for 30 s for the denaturation and annealing correspondingly and a 3-step melt curve stage using default settings. All primer efficiencies were around 60% including reference genes.

3. Results

More than a thousand predicted proteins in hemolymph and salivary glands were identified and quantified across feeding treatments (Additional files 1–13). A quantitative comparison between the protein levels in the three tissues, shows that they are significantly different both on proteome and single-protein level (Fig. 2; Additional files 1–13). Out of 1129 quantified proteins, 640 were preferentially localized to one or more tissues (518 can distinguished between hemolymph and salivary luminal proteins). The number of proteins originated from hemolymph was 167, while 350 originated from salivary glands. Of these, 203 were labial proteins and 80 were mandibular proteins. We focused our attention to a list of proteins which were previously detected as important factors in salivary glands of caterpillars (Celorio-Mancera et al., 2012). This list included proteins predicted to be involved in chemoreception (chemosensory and odorant-binding proteins) immune-related proteins (beta-glucan recognition [BGRP2], immune related [hdd11], serpin, methionine-rich storage protein [MRSP], arylphorin and apolipophorin) and digestive and detoxifying enzymes (esterase, catalase and serine protease). We identified several isoforms for each kind of protein in this list except for catalase. Serine proteases and serine-protease inhibitors were the protein categories represented by the highest number of isoforms (23 and 20 respectively). Table 1 provides the location (body compartment) where the signal intensities of peptides for an identified protein were highest and statistically significant. Note that the additional files 1 to 13 provide the statistical significance of the levels of each protein identified when comparing two treatments of interest. We considered fibroin as a control for our proteomic study since it is a well-described silk protein from labial glands (Akai et al., 2003). We found the highest signal intensities for fibroin light chain (Vca_contig40182) in the labial glands

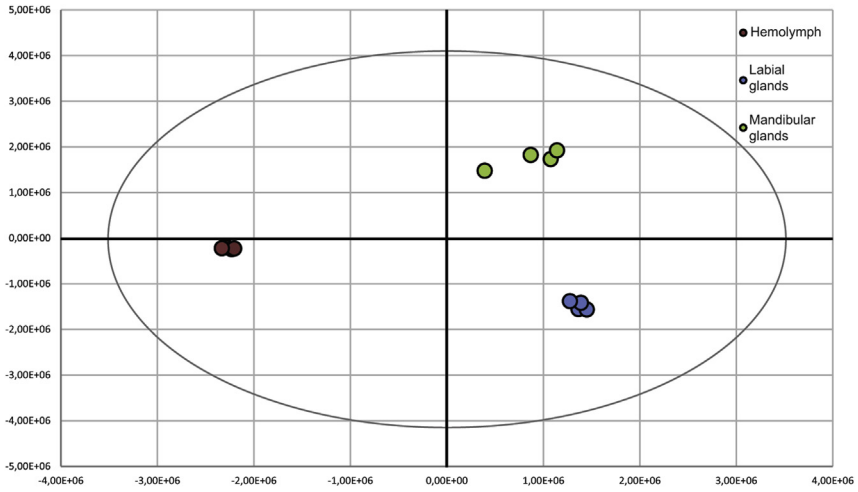

Fig. 2. Principal component analysis of the hemolymph-filled coelome (He), labial (LG) and mandibular glands (MG) proteomes from caterpillars fed with thistle (four replicates per body compartment). Model performance: R2X (cum) = 0.841 and Q2(cum) = 0.737.

(Table 1) and no change on the levels of this protein in response of diet treatment (Additional files 4–13). It was not possible to assess the location for the heavy-chain type of fibroin heavy chain (Vca_contig33943). The peptide intensities corresponding to two isoforms of chemosensory protein, referred hereafter as CSP1 and CSP2 (Vca_contig33997 and Vca_contig34071 respectively) and OBP1 (Vca_contig20692) were highest in the hemolymph-filled coelom (Table 1). However the levels of CSP1 changed in response to the caterpillar diet in the mandibular glands (Additional files 9 and 10; graphed in Fig. 3A) and expression of its transcript was significantly higher in this glands when compared to hemolymph cells and labial glands (Additional file 14A). CSP1 was induced when the insect fed on thistle in comparison to nettle. Peptidoglycan in the diet had an inhibitory effect on the production of CSP1 and this response of CSP1 was restricted to the mandibular glands of caterpillars (Fig. 3A; Additional files 9–13). In contrast, CSP2 abundances changed in both labial and mandibular glands. In the labial glands, CSP2 levels were highest in response to nettle when compared to the thistle treatment (Additional files 4 and 5). In the mandibular glands, CSP2 was induced upon a switch to thistle, when compared to the thistle treatment and when the caterpillars fed the artificial diet containing live bacteria in comparison to the control (Additional files 10 and 12). Although the highest peptide intensities for CSP3 (Vca_contig32439) were found in the mandibular glands, we could not detect statistical differences in the levels of this protein in response to caterpillar diet (Additional files 4–13). The signal intensities for OBP1 peptides were significantly higher in the hemolymph than in either kind of salivary gland (Table 1). Although OBP1 was detected in the salivary glands we found no significant change across diet treatments (Additional files 4–13). Contrasting the findings for OBP1, the levels of OBP2 (Vca_contig36359) in the mandibular glands were highest when the insects were switched from artificial diet to thistle and differed statistically from the levels detected when the caterpillars were reared on nettle (Fig. 3B). However, the level of this protein was not found to be statistically different from that in the labial glands or in hemolymph (Table 1). Gene expression studies demonstrated that the transcript encoding OBP2 was indeed expressed in hemolymph cells and both labial and mandibular glands in relative similar levels (Additional file 14B).

Four isoforms of arylphorin and five of apolipophorin were highest in the hemolymph-filled coelom (Table 1). Also, all MRSPs, four isoforms of each BGRP2 and serine protease and six serine protease inhibitors were most abundant in this body compartment. Although more abundant in the mandibular glands, we detected statistical differences among treatments for the levels of hdd11 (Vca_contig12851) in labial glands (Table 1, Fig. 4A). This protein was induced upon the presence of bacteria in the diet in comparison to the control but was not different from the diet containing bacterial cell-wall fragments (Additional file 7 and 8, Fig. 4A). Mass spectrometric signals for esterase (Vca_contig21946) were strongest in labial glands and for catalase (Vca_contig12291) and serine protease (Vca_contig12996) in the mandibular glands (Table 1). The relative levels of all these proteins were affected by the diet treatments in the body compartment where they were detected as most abundant. Esterase was induced by the peptidoglycan-containing diet (Additional file 6). Catalase was more abundant when the caterpillars fed nettle in comparison to thistle and when switched from artificial diet to thistle (Additional file 9 and 10 respectively; Fig. 4B). Serine protease was higher in response to thistle (compared to nettle) and inhibited by the peptidoglycan-containing diet (Additional files 9, 11–13; Fig. 4C).

4. Discussion

4.1. Proteome overlap and contamination

We have previously discussed the possibility of finding hemolymph proteins in samples prepared from salivary glands (Celorio-Mancera et al., 2012). The sensitivity of the proteomics methodology is such that detection of contaminants even at low levels is possible. Therefore, we considered it important to determine the degree of overlap between the proteomes from hemolymph, labial and mandibular glands. Indeed, the hemolymph and salivary gland proteomes were clearly distinct and the highest levels of proteins characteristic of the hemolymph such as BGRP2, arylphorin, MRSP and apolipophorin were detected in the hemolymph as previously observed (Celorio-Mancera et al., 2012). Gene expression analysis is required for those isoforms for which the location could not be assigned. We performed such analysis to verify the location of OBP2, but transcription was similar across the body compartments. Contrasting this result, our protein statistics assigned the location of hemolymph to CSP1, but the gene expression analysis revealed that the transcript for this protein is mostly expressed in the mandibular glands. These results present the possibility of protein circulation across the insect body, especially for those small proteins involved in chemoreception.

Table 1
Selection of proteins identified in hemolymph and salivary glands from caterpillars reared on thistle. Pair comparisons using Student's t-test between body compartments were performed on log10-transformed data (normalized protein intensities). The non-significant comparisons with a p-value higher than 0.05 are denoted by cells filled with gray color. To determine the most likely origin of the protein the statistics from Additional files 1–3 hemolymph, labial glands and mandibular glands reared on thistle were used. Only proteins showing significant differences (E < 1) in the comparisons and proteins that were completely absent in all four replicates in a sample, while present in all four replicates in the other, were used. The log2 of the ratio of the medians determined the most likely compartment, indicated in the three columns: He (hemolymph-filled coelome)/LG (labial glands), He/MG (mandibular glands) and MG/LG.

Protein ID	Description	Contig IDs	Peptides	log2 (He/LG)	He vs LG p-value	E	log2 (He/MG)	He vs MG p-value	E	log2 (MG/LG)	MG vs LG p-value	E	Body compartment
VCA_contig39740	arylphorin	VCA_contig39740	2	−1.23	7.79E-01	7.32E+02	1.81	6.78E-01	5.90E+02	−3.04	4.09E-01	3.83E+02	n.d.
VCA_contig22995	arylphorin	VCA_contig3935; VCA_contig22995	4	−3.44	7.34E-01	6.90E+02	−2.91	4.77E-01	4.15E+02	−0.53	5.29E-01	4.96E+02	n.d.
VCA_contig27971	arylphorin	VCA_contig27971; VCA_contig32358; VCA_contig36407	3	0.73	4.91E-01	4.61E+02	1.08	4.36E-01	3.79E+02	−0.35	8.61E-01	8.08E+02	n.d.
VCA_contig28220	arylphorin	VCA_contig28220	8	4.33	2.23E-06	2.09E-03	6.04	2.70E-02	2.35E+01	−1.71	5.63E-01	5.28E+02	He
VCA_contig29305	arylphorin	VCA_contig29305; VCA_contig34178; VCA_contig34647	14	3.70	3.69E-05	3.47E-02	3.52	5.52E-03	4.81E+00	0.18	8.48E-01	7.95E+02	He
VCA_contig29351	arylphorin	VCA_contig29351; VCA_contig34933	7	6.30	1.51E-07	1.42E-04	7.82	9.04E-03	7.86E+00	−1.52	3.25E-01	3.05E+02	He
VCA_contig30259	arylphorin	VCA_contig30259; VCA_contig33121	2	4.04	2.29E-01	2.15E+02	7.77	4.15E-02	3.61E+01	−3.73	6.42E-02	6.02E+01	n.d.
VCA_contig30825	arylphorin	VCA_contig30825; VCA_contig28539; VCA_contig39269	7	4.63	1.83E-05	1.72E-02	4.06	1.90E-03	1.66E+00	0.56	5.13E-01	4.81E+02	He
VCA_contig20805	apolipophorin-1/2	VCA_contig20805	7	2.06	6.39E-04	6.01E-01	4.49	2.01E-04	1.75E-01	−2.43	4.08E-03	3.83E+00	He
VCA_contig27649	apolipophorin-1/2	VCA_contig27649; VCA_contig5026; VCA_contig34062; VCA_contig33705	123	2.85	3.63E-05	3.42E-02	5.08	1.59E-04	1.39E-01	−2.24	1.52E-02	1.43E+01	He
VCA_contig27729	apolipophorin-1/2	VCA_contig27729	8	3.32	3.84E-04	3.61E-01	7.39	3.36E-04	2.92E-01	−4.07	2.59E-02	2.43E+01	He
VCA_contig38968	apolipophorin-1/2	VCA_contig38968; VCA_contig27735; VCA_contig7401; VCA_contig10678	137	2.95	6.05E-05	5.69E-02	5.03	1.57E-04	1.37E-01	−2.08	1.56E-02	1.46E+01	He
VCA_contig40180	apolipophorin-1/2	VCA_contig40180	6	1.80	4.93E-03	4.63E+00	3.74	3.36E-03	2.92E+00	−1.94	5.19E-02	4.87E+01	
VCA_contig39990	apolipophorin-iii	VCA_contig29523; VCA_contig39990; VCA_contig29023; VCA_contig35718; VCA_contig37259; VCA_contig31639	31	6.62	4.73E-08	4.44E-05	5.33	9.20E-03	8.00E+00	1.29	9.88E-02	9.27E+01	He
VCA_contig32439	chemosensory protein (CSP3)	VCA_contig32439	3	0.70	4.18E-01	3.93E+02	−7.04	7.00E-04	6.09E-01	7.74	2.25E-04	2.11E-01	MG
VCA_contig33997	chemosensory protein (CSP1	VCA_contig33997	11	5.95	1.76E-04	1.66E-01	−1.22	1.28E-02	1.11E+01	7.17	4.85E-07	4.55E-04	He
VCA_contig34071	chemosensory protein (CSP2)	VCA_contig34071	3	7.23	3.60E-05	3.38E-02	1.60	8.36E-03	7.27E+00	5.64	1.65E-04	1.55E-01	He
VCA_contig12291	catalase	VCA_contig12291	23	0.26	9.22E-01	8.66E+02	−2.86	7.04E-04	6.13E-01	3.11	7.36E-03	6.91E+00	MG
VCA_contig12308	antitrypsin isoform 3	VCA_contig12308	10	−5.62	2.44E-05	2.29E-02	−5.98	2.63E-06	2.29E-03	0.35	3.47E-01	3.25E+02	LG/MG
VCA_contig13152	antitrypsin isoform 3	VCA_contig13152; VCA_contig41663	11	−3.40	1.40E-04	1.31E-01	−3.43	2.46E-03	2.14E+00	0.02	5.15E-01	4.83E+02	LG/MG
VCA_contig7796	serine protease serpin	VCA_contig7796	2				2.98	9.38E-03	8.16E+00				n.d.
VCA_contig12839	serine protease inhibitor	VCA_contig12839	19	−7.04	2.21E-04	2.08E-01	−1.57	8.55E-02	7.44E+01	−5.48	4.47E-05	4.19E-02	LG
VCA_contig13837		VCA_contig13837	10	5.31	2.18E-05	2.05E-02	0.05	5.63E-01	4.90E+02	5.26	7.85E-05	7.36E-02	He

(*continued on next page*)

Table 1 (*continued*)

Protein ID	Description	Contig IDs	Peptides	log2 (He/LG)	He vs LG p-value	E	log2 (He/MG)	He vs MG p-value	E	log2 (MG/LG)	MG vs LG p-value	E	Body compartment
	serine protease inhibitor												
VCA_contig14919	serine protease inhibitor	VCA_contig14919	5	3.06	2.20E-04	2.06E-01	−0.78	9.81E-02	8.54E+01	3.84	9.75E-05	9.14E-02	He
VCA_contig21783	serine protease inhibitor 2	VCA_contig21783	2	−5.10	3.58E-03	3.37E+00	−9.13	6.46E-05	5.62E-02	4.03	4.52E-03	4.24E+00	MG
VCA_contig7464	serine protease inhibitor dipetalogastin	VCA_contig7464	2	6.11	5.22E-04	4.90E-01	−2.62	1.52E-02	1.33E+01	8.73	2.41E-04	2.26E-01	He
VCA_contig38411	serine protease serpin	VCA_contig38411	3				1.49	2.02E-01	1.76E+02				n.d.
VCA_contig12303	serpin 1	VCA_contig12303	19	4.69	3.33E-06	3.13E-03	3.47	2.40E-04	2.09E-01	1.22	2.25E-02	2.11E+01	He
VCA_contig14348	serpin 1	VCA_contig14348	2										n.d.
VCA_contig20847	serpin 1	VCA_contig20847	4	1.41	1.57E-01	1.48E+02	2.04	4.33E-02	3.77E+01	−0.63	6.34E-01	5.94E+02	n.d.
VCA_contig28372	serpin 1	VCA_contig28372	2				2.15	4.43E-01	3.85E+02				n.d.
VCA_contig29859	serpin 1	VCA_contig28278; VCA_contig29859	2				−2.46	1.25E-01	1.09E+02				n.d.
VCA_contig33924	serpin 1	VCA_contig33924	8	8.51	2.32E-05	2.19E-02	−0.87	1.14E-01	9.88E+01	9.38	1.59E-05	1.49E-02	He
VCA_contig39987	serpin 1	VCA_contig39987	20	8.66	3.01E-04	2.83E-01	−2.26	1.03E-04	8.94E-02	10.91	2.76E-07	2.59E-04	MG
VCA_contig14104	serpin 2	VCA_contig14104	21	5.26	1.78E-05	1.68E-02	3.98	1.70E-03	1.48E+00	1.28	2.38E-01	2.23E+02	He
VCA_contig26821	serpin b6	VCA_contig26821	5	−4.27	1.66E-01	1.56E+02	−8.10	2.40E-03	2.09E+00	3.83	2.45E-02	2.29E+01	n.d.
VCA_contig12632	serpin isoform a	VCA_contig12632	2	5.73	2.54E-02	2.39E+01	−0.94	7.38E-01	6.42E+02	6.67	3.68E-03	3.46E+00	n.d.
VCA_contig11754	serpin peptidase clade b member like 1	VCA_contig11754	4	−4.09	8.80E-03	8.28E+00							n.d.
VCA_contig12851	immune-related hdd11	VCA_contig12851	8	0.20	7.96E-01	7.48E+02	−9.34	4.10E-05	3.56E-02	9.54	2.39E-05	2.25E-02	MG
VCA_contig20692	odorant binding protein (OBP1)	VCA_contig20692	10	4.36	1.86E-05	1.75E-02	3.26	4.32E-05	3.76E-02	1.10	3.21E-02	3.01E+01	He
VCA_contig36359	odorant binding protein (OBP2)	VCA_contig36359	8	0.26	8.17E-01	7.68E+02	−1.53	7.94E-02	6.91E+01	1.79	6.13E-02	5.75E+01	n.d.
VCA_contig28251	methionine-rich storage protein	VCA_contig28251	2	9.79	1.22E-04	1.15E-01	8.06	3.48E-02	3.03E+01	1.73	4.85E-01	4.55E+02	He
VCA_contig33841	methionine-rich storage protein	VCA_contig33841; VCA_contig21265; VCA_contig33785	21	6.02	7.88E-05	7.41E-02	5.93	3.30E-06	2.87E-03	0.09	8.17E-01	7.66E+02	He
VCA_contig33852	methionine-rich storage protein	VCA_contig33852; VCA_contig31693; VCA_contig31814; VCA_contig24662; VCA_contig28936	87	7.17	3.07E-06	2.88E-03	8.33	2.19E-06	1.90E-03	−1.16	9.35E-02	8.77E+01	He
VCA_contig35316	methionine-rich storage protein	VCA_contig35316	11	6.18	8.22E-04	7.73E-01	4.38	4.82E-03	4.20E+00	1.80	8.33E-02	7.81E+01	He
VCA_contig33943	fibroin heavy chain	VCA_contig33943	9	−10.67	1.26E-03	1.19E+00	−3.71	2.37E-01	2.06E+02	−6.96	2.73E-02	2.56E+01	n.d.
VCA_contig40182	fibroin light chain	VCA_contig40182; VCA_contig31270; VCA_contig32222; VCA_contig29086	14	−13.81	4.87E-06	4.58E-03	−3.52	2.79E-02	2.43E+01	−10.29	5.98E-05	5.61E-02	LG
VCA_contig7984	beta-glucan recognition protein 2	VCA_contig7984	10	7.20	1.00E-04	9.44E-02	1.45	3.80E-02	3.30E+01	5.75	2.58E-04	2.42E-01	He
VCA_contig8469	beta-glucan recognition protein 2	VCA_contig8469	7	2.28	1.28E-02	1.20E+01	1.01	7.22E-02	6.28E+01	1.26	1.28E-01	1.20E+02	n.d.
VCA_contig847	beta-glucan recognition protein 2	VCA_contig847	11	6.67	7.72E-09	7.25E-06	4.07	2.04E-04	1.78E-01	2.60	5.00E-02	4.69E+01	He
VCA_contig38867	beta-glucan recognition protein 3	VCA_contig38867	15	3.98	5.75E-04	5.41E-01	3.74	4.44E-04	3.87E-01	0.24	9.79E-01	9.18E+02	He

VCA_contig506	beta-glucan recognition protein 3	VCA_contig506	13	4.42	3.86E-03	3.63E+00	2.98	2.19E-04	1.90E-01	1.44	4.46E-01	4.18E+02	He
VCA_contig21032	serine (or cysteine) peptidase clade member 6b	VCA_contig21032	3				4.79	4.82E-01	4.19E+02				n.d.
VCA_contig11623	serine protease	VCA_contig11623	11	2.87	8.50E-02	7.99E+01	1.91	2.63E-01	2.29E+02	0.96	1.17E-01	1.10E+02	n.d.
VCA_contig12550	serine protease	VCA_contig12550	8	2.51	1.50E-01	1.41E+02	0.34	9.95E-01	8.66E+02	2.16	4.21E-02	3.95E+01	n.d.
VCA_contig12996	serine protease	VCA_contig12996; VCA_contig21373; VCA_contig21648	6	4.60	1.90E-03	1.79E+00	−3.39	3.36E-04	2.92E-01	7.99	8.12E-05	7.62E-02	MG
VCA_contig13104	serine protease	VCA_contig13104	2	4.82	3.87E-05	3.64E-02	−2.31	1.60E-02	1.39E+01	7.13	4.14E-05	3.89E-02	He
VCA_contig13692	serine protease	VCA_contig13692	5	0.47	5.65E-01	5.31E+02	−3.70	7.78E-03	6.77E+00	4.18	2.62E-03	2.46E+00	n.d.
VCA_contig14705	serine protease	VCA_contig14705	2				2.29	8.41E-02	7.32E+01				n.d.
VCA_contig21395	serine protease	VCA_contig21395; VCA_contig5306; VCA_contig8213; VCA_contig8972	6	2.53	6.65E-02	6.25E+01	1.33	3.67E-02	3.19E+01	1.20	3.66E-01	3.43E+02	n.d.
VCA_contig27728	serine protease	VCA_contig27728	3	−2.36	4.77E-01	4.48E+02	−1.99	4.69E-01	4.08E+02	−0.37	9.63E-01	9.03E+02	n.d.
VCA_contig28043	serine protease	VCA_contig28043	4	1.01	6.57E-01	6.18E+02	−4.44	1.38E-03	1.20E+00	5.45	5.99E-04	5.62E-01	MG
VCA_contig28512	serine protease	VCA_contig28512; VCA_contig33871	2	−1.43	1.85E-01	1.74E+02	−2.99	4.89E-02	4.26E+01	1.56	4.92E-01	4.62E+02	n.d.
VCA_contig31682	serine protease	VCA_contig31682	12	5.09	4.23E-04	3.98E-01	5.26	1.52E-03	1.32E+00	−0.18	7.45E-01	6.99E+02	He
VCA_contig32587	serine protease	VCA_contig32587	7	0.77	1.04E-01	9.82E+01	−0.42	1.56E-01	1.36E+02	1.19	3.53E-02	3.31E+01	n.d.
VCA_contig34035	serine protease	VCA_contig34035	2										n.d.
VCA_contig34082	serine protease	VCA_contig34082	4	1.97	3.56E-02	3.35E+01	1.92	1.08E-01	9.43E+01	0.05	7.63E-01	7.15E+02	
VCA_contig34115	serine protease	VCA_contig34115	10	1.72	4.85E-02	4.56E+01	−2.93	2.40E-03	2.09E+00	4.64	7.37E-04	6.92E-01	MG
VCA_contig34245	serine protease	VCA_contig34245	2	−2.26	8.81E-01	8.28E+02	−4.30	2.43E-01	2.12E+02	2.04	1.87E-02	1.76E+01	n.d.
VCA_contig7946	serine protease	VCA_contig7946	3	4.50	1.18E-03	1.11E+00	−3.23	2.78E-04	2.42E-01	7.73	6.24E-05	5.85E-02	MG
VCA_contig12625	serine protease 33	VCA_contig12625; VCA_contig14186	6	−0.29	8.64E-01	8.12E+02	0.72	5.58E-02	4.86E+01	−1.00	1.05E-01	9.80E+01	n.d.
VCA_contig8026	serine protease isoform a	VCA_contig8026	15	5.99	4.66E-03	4.38E+00	2.90	1.60E-05	1.39E-02	3.09	3.60E-02	3.37E+01	He
VCA_contig31840	serine protease snake	VCA_contig31840	9	0.78	9.63E-01	9.05E+02	−2.60	1.62E-02	1.41E+01	3.38	1.41E-01	1.33E+02	n.d.
VCA_contig33890	serine proteinase	VCA_contig33890	4	−8.34	6.18E-04	5.81E-01	−10.68	5.64E-05	4.91E-02	2.33	4.56E-03	4.28E+00	LG/MG
VCA_contig14266	serine proteinase-like protein 1	VCA_contig14266	3	6.18	6.12E-05	5.76E-02	3.32	1.87E-02	1.63E+01	2.86	1.18E-01	1.11E+02	He
VCA_contig21946	esterase	VCA_contig21584; VCA_contig21946	2							−5.83	5.16E-04	4.84E-01	LG
VCA_contig8642	esterase	VCA_contig8642	5	−2.86	3.37E-01	3.17E+02	−1.41	9.90E-01	8.62E+02	−1.45	5.96E-03	5.59E+00	n.d.

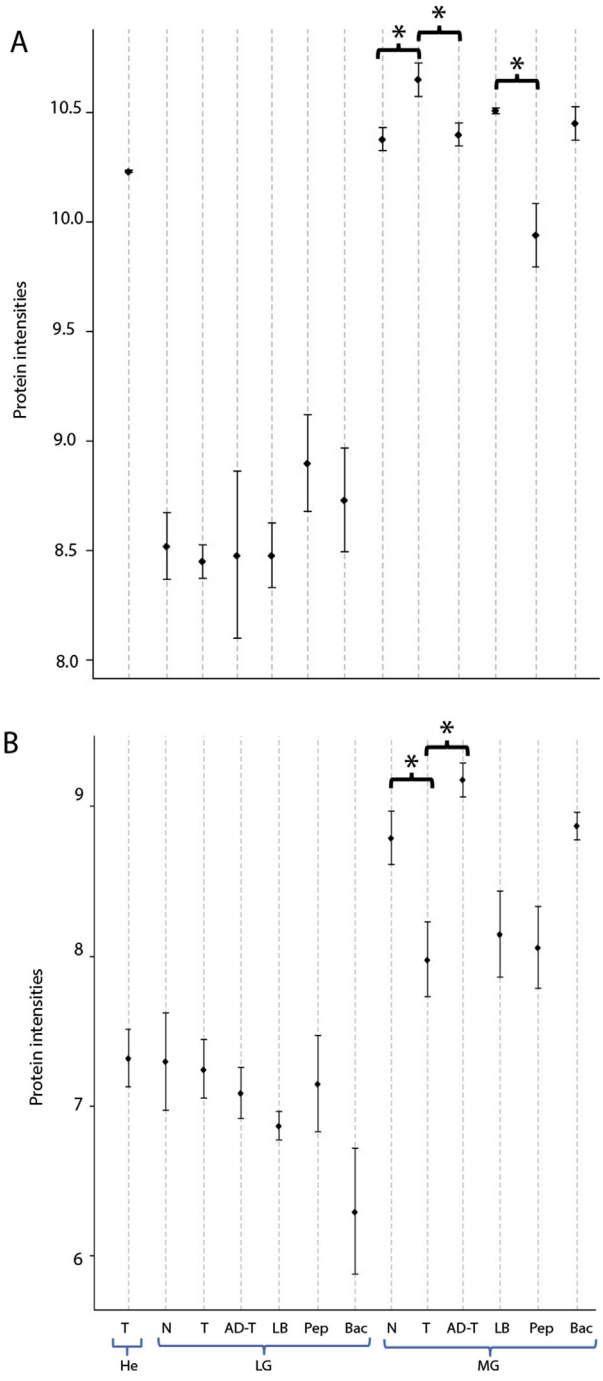

Fig. 3. Plots of protein levels in hemolymph-filled coelome and salivary glands of caterpillars across diet treatments (host and immunity). A) Chemosensory protein CSP1 (Vca_contig33997); B) Odorant binding protein OBP2 (Vca_contig36359). The signal intensities per protein were log10-transformed and normalized. Pair comparisons of treatments marked with an asterisk (*) are statistically significant (Student's t-test, p-value cut off = 0.05). Diet treatments: thistle = T; nettle = N; switch from artificial diet to thistle = AD-T; artificial diet and growth medium = LB; artificial diet, growth medium and peptidoglycan = Pep; artificial diet, growth medium and *E. coli* bacteria = Bac. Each plot depicts the average and standard error (4 replicates) per treatment. He = hemolymph-filled coelome; LG = labial glands; MG = mandibular glands.

4.2. Chemoreception

Three CSPs and two OBPs according to protein name were detected in hemolymph and salivary glands. Only CSP3 was detected at highest levels in the mandibular glands, but equally abundant across diet treatments. The other two were classified as "hemolymph" proteins, but their levels changed in response to the host and immune challenges. For one of them, CSP1, we could verify high transcription levels in the mandibular glands indicating the tag "hemolymph" may more reflect the mobility of the protein in the insect body than its origin. To investigate the movement of the protein in more detail different tissues should be involved in the analysis. We consider CSP1 a good candidate for further functional analysis since its gene is transcribed in the mandibular glands and the protein changes in response to host plant and immune challenge. CSP1, inducible when the caterpillars fed thistle or when switched to this host, may be involved in host recognition or in the identification of plant molecules. Alternatively, CSP1 in caterpillars may be part of the mechanism of perception of both pathogenic and non-pathogenic microbiota at the surface of thistle leaves. These speculations are also supported by additional observations made by other researchers: an aphid effector similar to CSPs from other insects including a CSP5 from *Anopheles gambiae* was found to trigger plant defenses (Bos et al., 2010) and genes encoding chemosensory proteins have been observed among those differentially expressed when comparing two populations of *Nilaparvata lugens* (rice brown plant hopper) with different virulence traits (Ji et al., 2013). We cannot discard CSP3 as a candidate mediating the caterpillar–plant interaction. Our diet treatments included only two hosts in the plant repertoire of the polyphagous *V. cardui*. Regarding CSP2, we could propose to test whether it functions as a carrier of pigments or other hydrophobic chemicals to the epidermis based on its high sequence similarity to the predicted CSP from epidermis of *P. xuthus* larvae. We consider necessary to address the possibility of contamination of the epidermis samples with mandibular gland tissue. Again, in situ hybridization studies or antibody-based detection of CSPs will contribute to clarify the location of these proteins in the insect body.

Based on our mass spectrometric quantification, the two OBP isoforms are very different from each other. OBP1 is a hemolymph protein while the location of OBP2 could not be determined and its gene expression was tissue-unspecific. However, OBP2 levels fluctuated significantly only in the mandibular glands in response to the host treatments. We speculate that OBP1 is a carrier of hydrophobic substances in the hemolymph-filled coelom while OBP2 may be involved in recognition of plant- or pathogen-derived compounds encountered by the caterpillar when feeding. Moreover, previous observations in other insect systems also indicate that OBPs may be involved in gustatory perception; inhibition of expression of a obp gene in a Drosophila line led to higher intakes of bitter compounds by these flies (Swarup et al., 2014). The olfactory and post-ingestive effects contributing to this behavior remain to be studied along with the molecular mechanism behind the detection of bitter compounds by OBPs. In our study system, we would like to understand the reasons causing the difference in relative quantities of OBP2 across treatments. Although the immune challenge treatments were not statistically different from each other, the distribution of the data reveals a tendency for higher levels of OBPs in response to the bacteria-containing diet and not to peptidoglycan. Higher number of biological and technical replicates and the inclusion of more treatments could be improvements for future experimental designs in order to assess whether OBP2 quantities are dependent on the concentration of bacteria in the diet. It is worth noting that our interpretation assumes that CSPs and OBPs are shuttling molecules from the diet, although they may also shuttle endogenous molecules produced indirectly due to the diet effect.

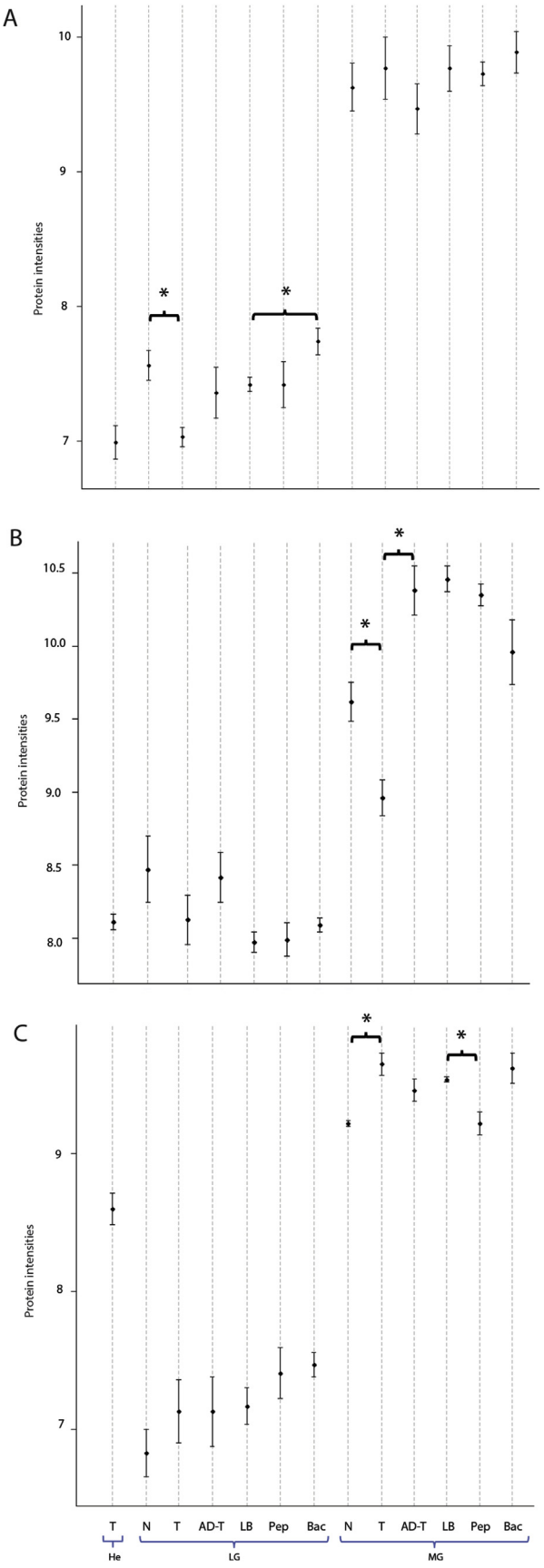

Fig. 4. Plots of protein levels in hemolymph-filled coelome and salivary glands of caterpillars across diet treatments (host and immunity). A) Hdd11 (Vca_contig12851); B) Catalase (Vca_contig12291); C) Serine protease (Vca_contig12996). The signal intensities per protein were log10-transformed and normalized. Pair comparisons of treatments marked with an asterisk (*) are statistically significant (Student's t-test, p-value cut off = 0.05). Diet treatments: thistle = T; nettle = N; switch from artificial diet to thistle = AD-T; artificial diet and growth medium = LB; artificial diet, growth medium and peptidoglycan = Pep; artificial diet, growth medium and *E. coli* bacteria = Bac. Each plot depicts the average and standard error (4 replicates) per treatment. He = hemolymph-filled coelome; LG = labial glands; MG = mandibular glands.

4.3. Immunity

Arylphorin, MRSP and apolipophorin were found composing approximately a third of both labial and mandibular proteomes in two *Vanessa* species (Celorio-Mancera et al., 2012). We corroborated that most isoforms of these proteins, which have been previously characterized as "hemolymph proteins" (Kanost MR et al., 1990), were found in caterpillar hemolymph and not in the salivary glands. Several isoforms of serine proteases were found in our samples and serpin was previously found as an important factor in the mandibular gland proteome (Celorio-Mancera et al., 2012). However, our present study reveals that there are isoforms in high abundance in both hemolymph and mandibular glands. Again, gene expression analyses are necessary to find where transcription occurs and understand better how mobile the protein is in the insect body. Two other immune-related proteins, hdd11 and BGRP (four isoforms), were found in highest levels in mandibular glands and hemolymph respectively. We expected that the relative levels of these proteins would have been affected depending on the immunity treatment. Indeed, the protein similar to hdd11 was induced when the caterpillars were exposed to pathogenic bacteria. However, such induction of the hdd11-like protein occurred in the labial glands where lower levels of hdd11 were detected. The lack of induction or suppression of the hdd11-like identified protein in the mandibular glands suggests the possibility of an alternative function for this protein. Other reasons for the lack of response towards our diet treatments in the mandibular glands could be that the artificial diet may have contained low concentrations of bacteria making this study a rather conservative test of the impact of *E. coli* on the salivary gland proteomes of caterpillars.

4.4. Digestion and detoxification

Serine proteases are important enzymes for insect digestion and their regulation is particularly plastic in generalist herbivores. Polyphagous caterpillars produce different isoforms of serine proteases and regulate their level of expression depending on the food source (Celorio-Mancera et al., 2013). In this study, we detected several isoforms of serine proteases where some were abundant in the salivary glands and some in the hemolymph. Most of the isoforms change in relative protein levels in response to diet. We interpret these results as an indication of the importance of serine protease in caterpillar digestion and its induction depending on the food source. Inducibility of serine protease in the mandibular glands stresses its importance in digestion probably already taking place during shearing of plant tissue and in the foregut.

Esterases are also a complex group of enzymes that can be involved in odor/pheromone degradation or insecticide detoxification (Teese et al., 2010). Although we detected a putative carboxylesterase in most abundant quantities in the labial glands, this protein responded only to the peptidoglycan-containing diet.

Protection from oxidative radicals in insects is achieved by antioxidative enzymes such as catalase. It has been proposed that catalase from caterpillar saliva may contribute to ameliorate oxidative stress in the foregut of caterpillars (Krishnan and

Kodrik, 2006). Our results indicated the importance of the mandibular glands as organs of production of catalase. The intensities for catalase were highest in larvae exposed to the switch in diet supposing a stress for the caterpillar. Pro-oxidant metabolites from the plant tissue may have induced an antioxidant defense response in the larvae. Although catalase levels are similar between the immune-challenge treatments and the diet-switch treatment, there is more variation in the samples from the diet-switch and bacteria-containing treatments suggesting a differential response to oxidative stress under these conditions.

5. Conclusion

We obtained distinct proteomes for hemolymph and for each salivary gland type in caterpillars indicating that contamination during dissection, although possible, is negligible. The next question to address is the mobility of proteins across the insect body which can be tackled by gene expression analysis and tissue localization of genes and proteins. We corroborated the presence of CSPs and OBPs in caterpillar salivary glands, identified by sequence-similarity. The relative levels of these proteins changed in response to caterpillar diet. Therefore, we have identified protein isoforms for testing the role of CSPs and OBPs in plant and pathogen recognition. The change in protein levels of one of the CSP isoforms in response to host and to a diet containing bacterial cell walls provided us with initial evidence supporting our hypothesis that CSPs may play a role in plant recognition and/or immunity. Since plants harbor a complex microbiota on their leaf surfaces, it is necessary to clarify the action of CSP1 in host and/or microorganism recognition. It would also be very informative to test whether CSPs from caterpillar saliva trigger defensive responses in plants. We detected catalase, immune-related protein and serine proteases and their inhibitors in high relative levels in the mandibular glands in comparison to the labial glands. These findings suggest that the mandibular glands of caterpillars may play an important role protecting the caterpillar from oxidative stress, pathogens and aiding in digestion. In global terms, the proteomes of salivary glands from caterpillars exposed to the different hosts and the diet switch were different from each other. This means that even after molting and two-day feeding on a new diet, protein production is affected by the previous food source used by the caterpillar.

Data accessibility

Final *V. cardui* transcriptome-assembly has been deposited at the Dryad Digital Repository: http://dx.doi.org/10.5061/dryad.0g6m2.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ibmb.2015.04.006.

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