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ORIGINAL PAPER

Changes of feeding behavior and salivary proteome of Brown Marmorated Stink Bug when exposed to insect-induced plant defenses

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Abstract

The invasive Brown Marmorated Stink Bug (BMSB), *Halyomorpha halys* Stål, has dispersed widely throughout North America and Europe, negatively impacting agro-ecosystems and urban areas. This species is phytophagous and highly gregarious at all developmental stages. Therefore, it is important to determine how the congeners react to plant defenses induced by first infestation. Lipoxygenase activity was found to be enhanced in faba bean (*Vicia faba* L.) leaves by BMSB feeding or its salivary compounds. We analyzed BMSB feeding behavior by comparison with our previously published EPG waveform library for that pest, and identified some EPG variables associated with test probes, stylets pathway, and sustained ingestion. We demonstrated that, on elicited plants, BMSB probes were delayed, with sustained ingestion events being shorter. Moreover, significant changes in salivary gland proteins involved in plant allelochemical detoxification were detected when BMSB was exposed to plant defenses. Our results confirmed that this polyphagous invasive Heteroptera has the ability to detect plant defenses and to adapt its feeding strategies in consequence.

Keywords *Halyomorpha halys* · *Vicia faba* · Lipoxygenase · Electropenetrography · Salivary glands · LC/MS–MS

Introduction

Halyomorpha halys Stål (Hemiptera: Heteroptera: Pentatomidae), the Brown Marmorated Stink Bug (BMSB), is native to Eastern Asia, where it feeds on a large diversity of host plants (Lee et al. 2013). This species was first recorded outside Asia in the mid-1990s in the USA. Since then, BMSB has spread widely throughout the USA and is now considered an invasive species. BMSB was also accidentally introduced into Switzerland (in Europe), and was first detected in 2007 (Wermelinger et al. 2008). A decade later, BMSB is well established in several countries, especially across Southern Europe. Recent genetic studies suggested that the European populations of this species are the result of multiple introductions from Eastern Asia and/or from North America (Gariepy et al. 2014; Cesari et al. 2015; Gariepy et al. 2015). Europe presents ideal conditions for the establishment and spread of this pest, due to the climate, suitable and varied agricultural landscapes, and dense human activity. This pest will likely colonize a large area of Europe over the next few decades (Zhu et al. 2012; Wallner et al. 2014).

BMSB is phytophagous, feeding on various plant organs but with fruit preference (Rice et al. 2014). It is highly polyphagous in its native region, with most host plants also being present in Europe (Lee et al. 2013; Maistrello et al. 2016; Musolin et al. 2018). Therefore, BMSB can easily find woody hosts, crop fields, or perennial herbaceous plants in areas where it was introduced. Polyphagy likely contributes to its successful colonization process globally, as it is a characteristic of many other invasive species (Kirkendall and Faccoli 2010; Kenis et al. 2016). Pentatomids adjust their

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feeding strategies according to the plant tissue. On seeds, they use a cell rupturing strategy, whereas they secrete a salivary sheath on leaves and stems to facilitate the penetration of the stylets (Backus 1988; Backus et al. 2005; Lucini et al. 2016). Direct current (DC) electropenetrography allows researchers to analyze precise feeding behaviors, such as pathway, salivation, and phloem or xylem ingestion phases. A first description of EPG waveforms for BMSB provided the necessary basis for comparative studies focusing on BMSB feeding behavior (Serteyn et al. accepted).

Plants have developed a panel of complex defensive strategies in response to the feeding activity of insects. These responses may be constitutive or inducible by a variety of eliciting compounds from insects to be applied on plants during feeding. These defenses could be expressed locally (i.e., specifically at the site where the plant is attacked) or systemically, with metabolic pathways and secondary metabolites acting as signal molecules. Both local and systemic defenses lead to the production of toxic/antifeeding molecules or compounds involved in interspecies communication. Insect-borne elicitors are called HAMPS (Herbivore Associated Molecular Patterns) and induce a defense cascade in combination with molecules produced during mechanical injury (Wu and Baldwin 2010). Increasing numbers of studies demonstrated that some compounds in insect saliva act as elicitors (Browse and Howe 2008; Howe and Jander 2008; Mithöfer and Boland 2012). Regarding its feeding strategies, BMSB would mainly induce the jasmonic acid (JA) pathway (Conti et al. 2008; Pérez-Hedo et al. 2015). Lipoxygenase is one of the key enzymes in that pathway, frequently studied as an indicator of plant defense elicitation (Gosset et al. 2009). Saliva of insect plays many roles in insect–plant interactions, as effectors to facilitate feeding or elicitors that induce plant defense mechanisms (Walling 2000; Felton and Tumlinson 2008; Hogenhout and Bos 2011). Several examples showed that generalist insects adapt their salivary compounds according to the host plant species (Acevedo et al. 2017; Rivera-Vega et al. 2018), and some evidences supported that insects are able to rapidly adapt their salivary secretions when exposed to oxidative plant metabolites (Celorio-Mancera et al. 2015).

Prado and Tjallingii (2007) and Dugravot et al. (2007) examined how plant-colonizing aphids cope with the defenses induced by their own feeding. They found that systemic resistance factors were located in the phloem with local resistance only having a minor impact on the probing behavior of aphids. A similar concept could be applied to the less-studied BMSB, as its nymphal stages are wingless and gregarious on plants, but likely perform very different feeding behaviors that would be subjected to different plant defense pathways. We hypothesized that BMSB invasiveness and its wide range of hosts are related to its ability to overcome plant defenses induced by the insect itself. Therefore, we aimed to elucidate the interactions between BMSB and one of its host plants, the broad (or faba) bean. Specifically, we focused on (1) validating the hypothesis that the JA pathway is induced by the feeding activity of BMSB and/or its salivary compounds, both locally and systemically; (2) assessing whether other BMSB individuals are subsequently able to detect the response and adapt their feeding strategies, in terms of behavior and salivary compounds (as salivation in plant always accompanies sap ingestion). Our results are expected to provide insights on the localization of plant defensive responses when attacked by such a polyphagous invasive Heteroptera, as well as the ability of these insects to overcome plant defenses to feed successfully.

Materials and methods

Plants and insects

Brown Marmorated Stink Bugs (BMSB) were collected from East China, the native area of this species, and were maintained inside cages in a high-security rearing room in Belgium (16 h light, 23 ± 2 °C). The insects were fed young broad bean plants (*Vicia faba* L. cv. "Grosse Ordinaire") and sunflower seeds (*Helianthus annuus* L. cv. unknown), which were replaced every two weeks with new ones.

For the experiments, 2-week-old broad beans with four fully developed leaves were individually transplanted into pots filled with loam (La Plaine Chassart, Belgium), while fifth-instar nymphs were isolated and starved with ad libitum access to water.

Insect dissection

Insects were first chilled on ice for a few minutes, and then dissected in Ringer solution (9.000 g/L NaCl, 0.146 g/L KCl, 0.200 g/L $CaCl_2$, 0.010 g/L $NaHCO_3$; in distilled water; autoclaved) to collect whole salivary glands. The salivary glands were carefully detached by cutting the salivary duct, without perforating the glands. Accessory and principal glands (described by Peiffer and Felton 2014) were kept together.

Plant treatments

The first experiment focused on examining defense induction caused by the feeding activity of insects. Two nymphs were starved for 3 days, and were then restrained on the two youngest leaves in a tulle bag and allowed to feed for 24 h ("Ins" treatment). The control consisted of an empty tulle bag on a similar-aged plant ("Ck" treatment).

The second experiment aimed to verify the role of insect salivary compounds in inducing plant defenses. Salivary

gland extract (SGE) was applied to the plants by mimicking stink bug attack. To prepare that solution, 98 salivary glands from 61 fifth-instar larvae were rinsed for a few minutes in a drop of phosphate buffer saline (PBS) at pH 7. The glands were crushed in 100 µL PBS, and centrifuged at 16,000 g for 5 min at 4 °C. The supernatant proteins were quantified with an RC-DC kit (Bio-Rad, USA) and the concentration of the sample was adjusted to 20 µg/µL in PBS. To be consistent with the work of Peiffer and Felton (2014), 10 µg salivary proteins were applied on plants. Therefore, 0.5 µL of the SGE was injected to the main vein of the two youngest leaves. The vein was perforated using a glass capillary of 0.1 mm diameter, which is the diameter of the stylets of fifth-instar BMSB larvae (personal observation). Then, the SGE was applied to the hole and pushed into the vein with a needleless syringe ("Injury + SGE" treatment). For controls, only the pressure of the syringe was applied ("Ck" treatment), which could be accompanied by an injury made by the capillary alone ("Injury" treatment) or along with the application of PBS ("Injury + PBS" treatment). This process was repeated 10 times over a 24-h period on the same two leaves, based on the observation of the BMSB feeding cycle by Wiman et al. (2014).

Plant defensive response: lipoxygenase activity as an indicator

Twenty-four hours after the beginning of the treatment, the treated leaves, the untreated leaves (i.e., the first two leaves that formed on the plant, which were not exposed to any of the stated treatments), and the roots of six plants per treatment were separately crushed in liquid nitrogen to obtain a homogeneous thin powder, which was stored at − 80 °C. Six hundred milliliters of PBS 100 mM pH 7 was added to 0.2 g plant powder, 40 mg $Na_2S_20_5$, 29 mg EDTA, and 250 mg Tween 20. The solution was incubated for 1 h on ice, vortexed every 10 min, and then centrifuged twice at 20,000×*g* at 4 °C for 12 min. The supernatant was stored at − 80 °C until further use for photospectrometry.

To tubes containing 930 µL oxygenated PBS, 16 µL of the supernatant was added, along with 36 µL of an emulsion of 140 mg linoleic acid with 18 mg Tween 80 in 50 mL deoxygenized water, as a substrate for the lipoxygenase enzyme (LOX). The products of this reaction were measured at 234 nm every 5 s during 10 min at 35 °C. Enzymatic activity was calculated on the linear part of the curve, and expressed in Unit of enzyme Activity (UA) per fresh weight, where 1UA is defined as the oxidation of 120 nmol of linoleic acid per minute. The calculation was based on the variation of absorbance in 1 min and on the molar extinction coefficient of 25,000 M^{-1} cm^{-1}. LOX activity was compared between the treatments by *F* test in one-way analysis of variance, with a significance threshold of 0.05.

Electropenetrography recording

Before each DC electropenetrography (EPG) recording, fifth-instar BMSB were starved for 24 h. Each insect was wired following the methods of Lucini and Panizzi (2016). Insects were connected to a Giga-8d basic DC-EPG system, with an input resistance of 10^9 Ohm (EPG Systems, Wageningen, The Netherlands), as described in details in our previous study (Serteyn et al. accepted). Each insect was then placed and restrained on the upper side of the treated leaf of a broad bean plant, directly after the 24-h period of nymphal feeding ("Ins" treatment in experiment 1) or control treatment ("Ck" treatment in experiment 1). Immediately after, a 6-h recording was launched using the software Stylet$^+$d (EPG Systems). Twenty-two and twenty-four replicates were obtained, respectively, for "Ck" and "Ins" treatments, after exclusion of insects that did not probe or that detached themselves before the end of the recording. Each individual was recorded only once, with a new plant for each insect.

The recorded waveform output was analyzed using the software Stylet$^+$a (EPG Systems), using our previous waveforms characterization (Serteyn et al. accepted). Dozens of EPG variables were calculated on a Microsoft Excel worksheet. Out of these variables, the most pertinent and non-redundant variables were selected, if they were represented by sufficient replicates to be statistically relevant. Most of the 63 selected variables were related to probing and salivation behavior and could be used as indicators of plant suitability for the pest, especially concerning recognition, tasting, and sustained ingestion acceptance. BMSB behaviors on the two treatments were compared using the Kruskal–Wallis test for each variable separately. Significance was set at 0.05.

Quantitative proteomics on salivary glands

Among the individuals recorded on EPG, respectively, 13 and 11 individuals were dissected for Control and Insect treatment, allowing us to collect 19 and 14 whole salivary glands. Final step of dissection and preparation of samples for proteomics (extraction, quantification, reduction/alkylation, and digestion) were conducted following the protocols of Serteyn and Francis (2019). After the protein quantification step, each sample was divided into four technical replicates of 20 µg each. The protein digests were independently analyzed by liquid chromatography (nano 2D Acquity; Waters, USA) coupled with an ESI-ion trap (Q Exactive Plus; Thermo Fisher Scientific, USA) in positive ion mode (LC–ESI–MS/MS).

Spectra were treated using Maxquant vs 1.5.2.8. Database searches were performed on NCBI database restricted to Hemiptera taxonomies. Carbamidomethyl of cysteines (resulting from alkylation before digestion) and oxidation of methionine were set as variable modifications, with an

FTMS tolerance of 10 ppm. Identifications of proteins were registered when score was higher than 15, with at least 1 unique peptide. Each protein hit was then quantified, and expressed in LFQ (label-free quantification) intensities. To improve the identification of uncharacterized proteins, BLAST analyses were performed against NCBI Arthropoda taxonomies.

Using the software Perseus vs 1.6.2, contaminants were removed from dataset, intensities were $\log_2(x)$ transformed, and samples were grouped according to the treatment. Proteins were considered present in a treatment when at least 2 out of the 4 replicates showed an MS signal, and proteins were considered absent in a treatment when none of the 4 replicated showed a signal. To complete the qualitative differences, two-sample *t* test with a 95% confidence level (p value ≤ 0.05) was performed when at least 3 out of the 4 replicates showed a signal. Only protein hits being significantly different between treatments were clustered according to their intensities. Every differential protein was associated with a category of biological process, based on Swiss-Prot/TrEMBL, gene ontology databases, and more widely with KEGG pathways. Their amino acid sequence was also searched for potential secretion signal, using the website http://www.cbs.dtu.dk/services/SignalP-5.0/.

Results

LOX as an indicator of plant defense response

With fifth-instar BMSB larvae on broad bean plants for 24 h, lipoxygenase activity increased in both treated ($p = 0.014$) and untreated distal leaves ($p = 0.027$) (Fig. 1a). This response was not observed in roots. Higher LOX activity was observed in leaves treated with salivary gland extract (SGE) compared to untreated plants ($p = 0.022$), while the injury by itself did not affect this pathway (Fig. 1b). Despite a slight increase in LOX activity, the application of salivary compounds was not sufficient to induce a significant systemic response in untreated distal leaf ($p = 0.170$) (Fig. 1b).

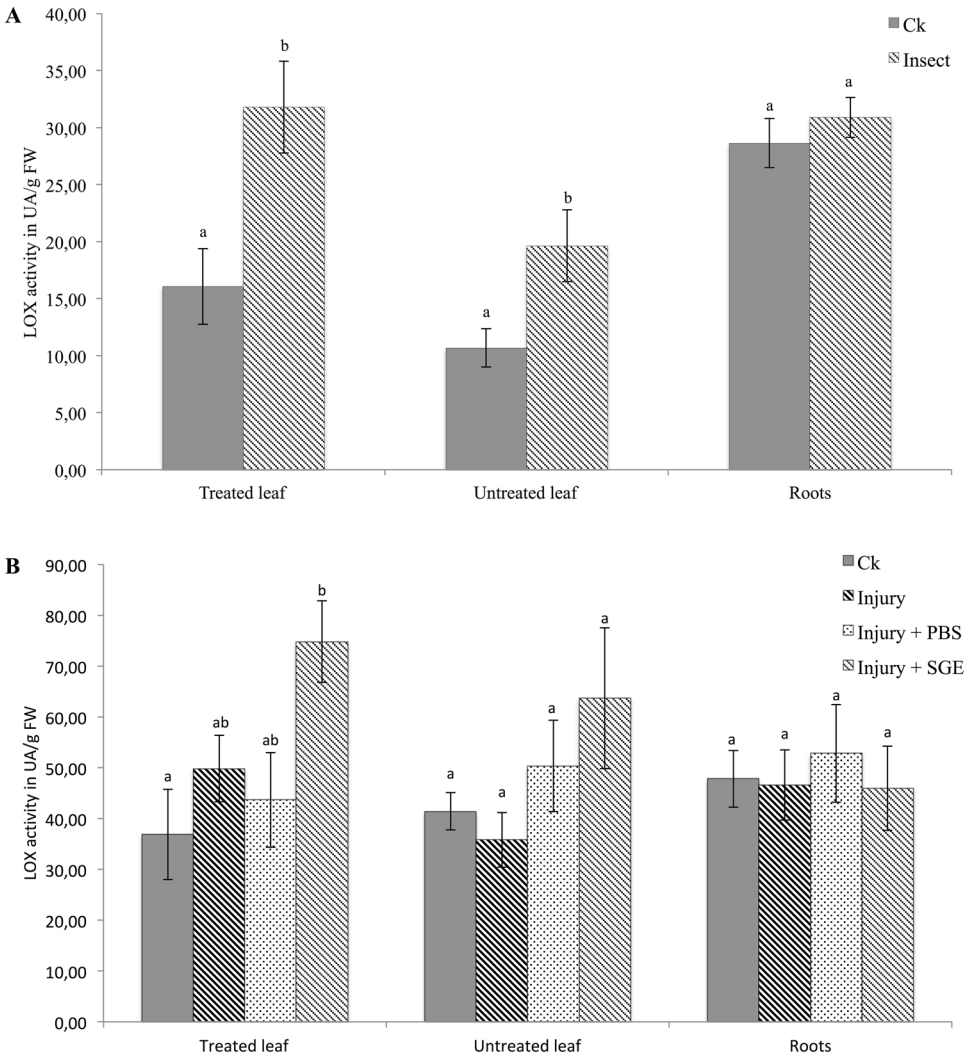

Fig. 1 Lipoxygenase activity in treated leaves, untreated distal leaves and roots of faba bean **a** directly after the 24-h-long insect feeding and **b** 24 h after first salivary gland extracts injection. "Ck": untouched, healthy plants; "Ins": 24-h-long feeding by two BMSB; "Injury": piercing by a capillary; "Injury + PBS": piercing by a capillary with injection of PBS; "Injury + SGE": piercing by a capillary with injection of salivary glands extract diluted in PBS. "Treated leaf": leaf exposed to the treatment; "Untreated leaf": distal leaf, not exposed to the treatment. Statistical analyses were performed separately within each plant tissue. Different letters indicate significant differences ($p < 0.05$). Error bars represent standard error of the means

Feeding behavior

To analyze how BMSB individuals react to induced plant defense, feeding behavior was recorded on the upper side of treated leaves (i.e., leaves that had been previously exposed to insect feeding) or unexposed control leaves of a broad bean plant. Six-hour-long recording was considered sufficient for insects to acclimate to their new conditions and to start probing. Several sustained ingestion events could be recorded during this period. The EPG waveforms were grouped into three main phases: non-probing (standing still or movement, contact between the leaf and the labium), pathway (penetration of the stylets through plant tissues), and ingestion (either xylem or putative phloem sap).

The generated variables were compared according to plant treatment (Supplementary Table S1). Insects exposed to a plant that had been previously fed on took longer to start probing (Fig. 2a–c). When these insects finally fed sustainably, the duration was shorter compared to insects that fed on naive plants (Fig. 2d–f). Xylem was the most frequent feeding site. No difference in pathway phases was identified, even with respect to X-wave events.

Quantitative proteomics on salivary glands

Quantitative gel-free proteomics was performed on salivary glands from insects that were EPG-recorded. In total, 1058 proteins were identified in BMSB salivary glands. Among them, 28 were only identified in control treatment, and 34 were expressed only when BMSB was exposed to activated plant defenses (Fig. 3a). Out of the 1058 total proteins, 718 could be associated with both treatments, 77 proteins were differentially expressed between treatments, and 21 were up-regulated in insects exposed to plant defenses (Fig. 3a). These proteins were hierarchically clustered according to their expression profile, but no trend related to protein functions could be reliably deduced from the clusters (Fig. 3b). Therefore, profiles of KEGG pathways were determined for each treatment, taking the qualitative and quantitative differences altogether (Fig. 3c). In comparison with the control, up-regulated proteins in "Ins" treatment were associated with general metabolism and cellular processes, while proteins of organismal systems and genetic information processing were down-regulated. Only differential proteins with putative roles in plant–insect interactions are presented in

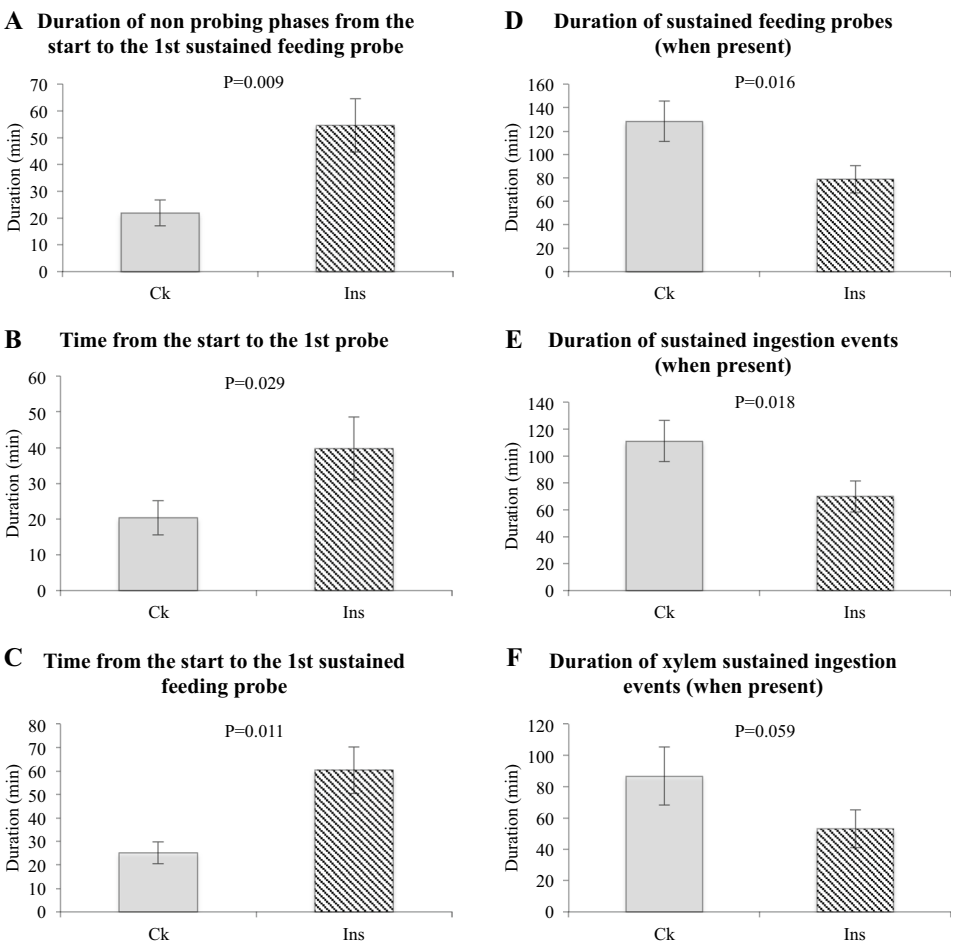

Fig. 2 Calculated electropenetrography variables according to the plant treatment. "Ck": untouched, healthy plants; "Ins": 24-h-long feeding by two BMSB. Asterisks indicate significant difference at $p < 0.05$. Error bars represent the standard error of the mean (SEM)

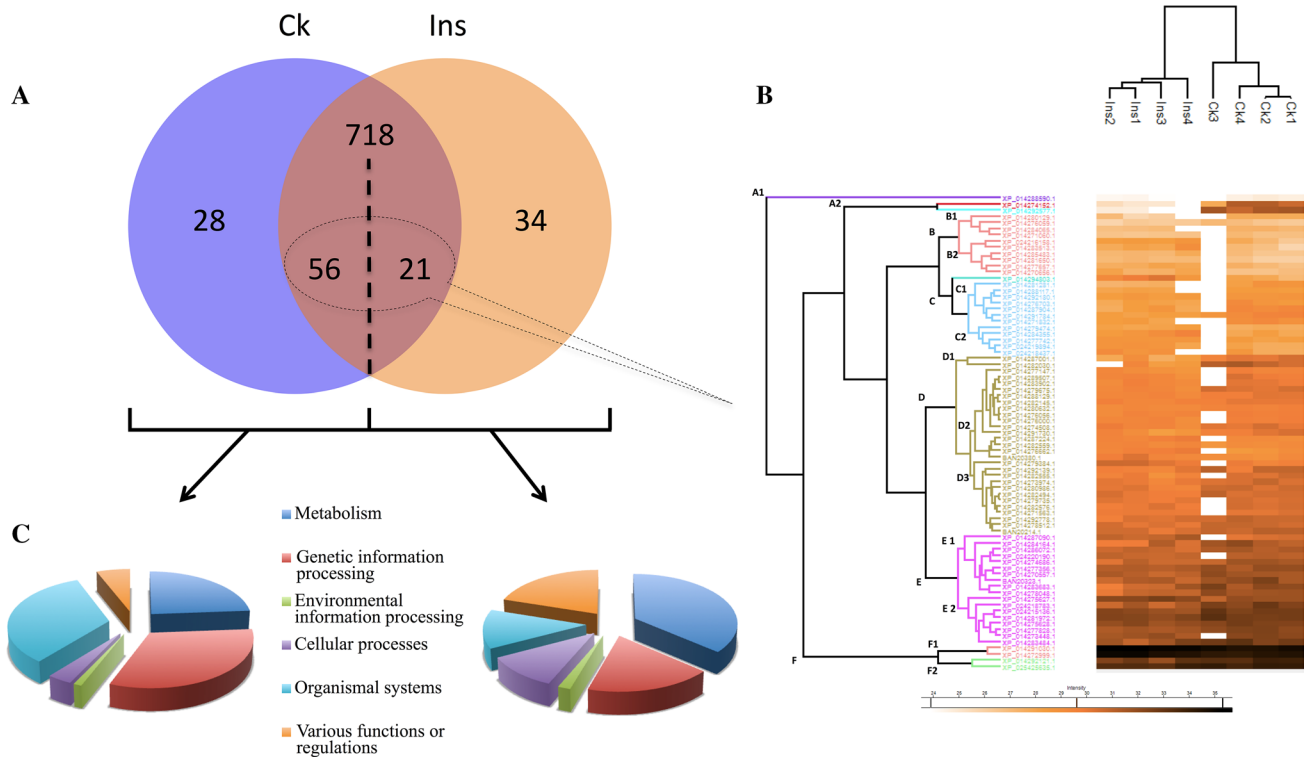

Fig. 3 **a** Venn diagram, distributing proteins of *Halyomorpha halys* salivary glands according to the plant treatment ("Ck": untreated leaves; "Ins": 24-h feeding by 2 BMSB); **b** Heatmap showing the LFQ intensities and hierarchical clustering of differentially expressed proteins; **c** KEGG pathways associated with qualitative and quantitative differences according to the treatment

Table 1. See Supplementary Table S2 for details on all differential proteins.

Discussion

This study provided the first comparative and quantitative analysis of the feeding behavior and salivary compounds of the pentatomid *H. halys* in response to local plant defenses induced by the presence of previous insects. We showed that (1) direct feeding by BMSB or salivary compound application on plants enhanced lipoxygenase activity in faba bean leaves; (2) probing by BMSB individuals was delayed on plants previously damaged by insect feeding with subsequent sustained ingestion events being shorter, compared to that on naive plants; and (3) saliva composition changed according to the elicitation of plant defenses.

LOX as an indicator of plant defense response

This study did not elucidate the whole mechanism of plant defenses triggered by BMSB. This aim would have required a more complete analysis of the metabolites produced by plants or the defense genes that were expressed. We focused on LOX expression as a marker of plant defense that was elicited by the presence of insects within a 24-h period. Therefore, the observed increase of LOX activity allowed us to use EPG experiments on elicited plants. Of note, LOX activity was measured at only one time point, preventing us from drawing conclusive inferences, as we may have missed the peak in enzyme activity. Also, activity kinetics might vary between actual insect attack and artificial application of saliva. Nonetheless, our results support the hypothesis that injected salivary compounds have a key role in inducing plant defenses (Rodriguez-Saona et al. 2002; Moraes et al. 2005), while a similar experiment on *Nezara viridula* L. showed that salivary compounds and mechanical injuries caused by stylets activities work together to induce plant volatiles (Williams et al. 2005). We also noticed necrotic spots when a real attack occurred, as well as when salivary gland extract was applied. BMSB feeding triggered, at least, defensive pathways that are closely related to the lipoxygenase enzyme. These pathways probably produce further antifeedant or toxic secondary metabolites and volatile organic compounds (Howe and Jander 2008; Griffiths 2015), as previously observed for other Heteroptera (Moraes et al. 2005; Williams et al. 2005; Degenhardt et al. 2012). Among other roles, LOX is situated upstream of an oxidation cascade from free fatty acids to JA synthesis (Griffiths 2015), and is a signal molecule that is deeply involved in plant–insect

Table 1 Differentially expressed (presence/absence or statistically different) proteins in *Halyomorpha halys* salivary glands with putative role(s) in plant–insect interactions

Fasta headers[a]	Secretion signal peptide?[b]	Mol. weight [kDa]	Score	Mean LFQ intensity			Log2(mean LFQ intensity)			Biological process[d]	Cluster	Putative role in plant-insect interactions[e]
				Ck	Ins	Fold change	Ck	Ins	P value[c]			
Down-regulated in "Ins" treatment												
Qualitative differences												
>XP_014291184.1/AQM58360.1 venom protein family 2 protein 3 [*Pristhesancus plagipennis]*	Yes	21.136	36.0	4.99E+08	NS		28.89	NS		Secretome		Toxin activity
>XP_014286982.1 endophilin-B1 isoform X4 [*Halyomorpha halys*]	No	31.863	20.2	3.31E+07	NS		24.98	NS		Various regulations		Cellular response to starvation
Quantitative differences												
>XP_014283902.1 glutathione S-transferase isoform X2 [*Halyomorpha halys*]	No	23.863	112.8	1.09E+09	7.00E+08	0.64	30.02	29.38	0.010	Stress response	D2	Response to oxidative stress
>XP_014278512.1/ATU82686.1 venom protein family 10 protein 2 [*Pristhesancus plagipennis*]	Yes	16.327	215.4	1.81E+09	1.29E+09	0.71	30.75	30.26	0.011	Secretome	D3	Toxin activity
>XP_014281281.1/ATU82691.1 venom protein family 12 protein 1b [*Pristhesancus plagipennis*]	Yes	20.929	42.3	4.15E+08	1.84E+08	0.44	28.63	27.46	0.007	Secretome	C1	Toxin activity
>XP_014280129.1 vasotab-like [*Halyomorpha halys*]	Yes	9.050	174.5	2.29E+08	8.94E+07	0.39	27.77	26.41	0.020	Secretome	B1	Toxin activity
*>XP_014292139.1 probable GPI-anchored adhesin-like protein PGA18 isoform X2 [*Halyomorpha halys*]	Yes	24.010	28.3	1.80E+09	9.03E+08	0.50	30.74	29.75	0.038	Structural molecule	D3	Pathogenesis
Up-regulated in "Ins" treatment												
Qualitative differences												
>XP_014276614.1 carbonyl reductase [NADPH] 3-like [*Halyomorpha halys*]	No	33.074	32.8	NS	7.46E+07		NS	26.15		Carbohydrate metabolism		Xenobiotic metabolic process
>XP_014280077.1 glycogen [starch] synthase [*Halyomorpha halys*]	No	78.288	32.0	NS	4.01E+07		NS	25.26		Carbohydrate metabolism		Cellular response to starvation
>XP_024218988.1 guanine deaminase isoform X2 [*Halyomorpha halys*]	No	48.127	28.1	NS	1.39E+08		NS	27.05		Carbohydrate metabolism		Production of xanthine

Table 1 (continued)

Fasta headers[a]	Secretion signal peptide?[b]	Mol. weight [kDa]	Score	Mean LFQ intensity			Log2(mean LFQ intensity)			Biological process[d]	Cluster	Putative role in plant-insect interactions[e]
				Ck	Ins	Fold change	Ck	Ins	P value[c]			
*>XP_014278522.1 carboxypeptidase Q-like [*Halyomorpha halys*]	Yes	53.168	21.3	NS	6.53E+07		NS	25.96		Protein metabolism		Sensory perception of smell
>BAN20936.1 peroxiredoxin [*Riptortus pedestris*]	Yes	28.500	20.3	NS	3.10E+08		NS	28.21		Stress response		Response to oxidative stress
>XP_024214585.1 alpha,alpha-trehalose-phosphate synthase [UDP-forming] [*Halyomorpha halys*]	No	87.015	35.4	NS	2.23E+07		NS	24.41		Stress response		Cellular response to oxidative stress
>XP_024217780.1/KOC60175.1 UDP-glucuronosyltransferase 1-6 [*Habropoda laboriosa*]	No	141.100	51.1	NS	2.24E+08		NS	27.74		Stress response		Xenobiotic metabolic process
>XP_014288771.1 neural/ectodermal development factor IMP-L2 [*Halyomorpha halys*]	Yes	28.855	46.2	NS	4.03E+07		NS	25.27		Biologial rhythm		Response to starvation
>XP_024214976.1 cubilin-like [*Halyomorpha halys*]	Yes	412.340	40.1	NS	2.57E+07		NS	24.61		Various functions		Hyperosmotic response
>XP_014282104.1 flotillin-1 [*Halyomorpha halys*]	No	47.955	36.3	NS	1.39E+07		NS	23.73		Various regulations		Cellular response to exogenous dsRNA
>XP_014283980.1 protein AATF-like [*Halyomorpha halys*]	No	60.116	33.1	NS	5.43E+06		NS	22.37		Various regulations		Negative regulation of reactive oxygen species metabolic process and of superoxide anion generation
Quantitative differences												
*>XP_024219894.1 esterase FE4-like, partial [*Halyomorpha halys*]	No	26.712	100.9	2.41E+08	4.18E+08	1.74	27.84	28.64	0.034	Amino acid metabolism	C2	Resistance to organophosphate insecticides
>XP_014281650.1 ATP-binding cassette sub-family F member 1 [*Halyomorpha halys*]	No	115.660	133.4	8.44E+07	1.46E+08	1.73	26.33	27.12	0.006	Protein metabolism	B2	Inflammatory response
>XP_014283513.1 xanthine dehydrogenase/oxidase-like isoform X1 [*Halyomorpha halys*]	No	141.520	175.9	1.06E+08	3.31E+08	3.12	26.66	28.30	0.016	Various regulations	B2	Positive regulation of reactive oxygen species metabolic process

NS no signal

*Identified in secreted saliva of BMSB, according to Peiffer and Felton (2014)

[a]Obtained from Maxquant or BLAST

[b]Determined by the website: http://www.cbs.dtu.dk/services/SignalP/

[c]Obtained from two-sample *T* test (ANOVA)

[d]According to gene ontology on Swiss-Prot/TrEMBL

[e]According to literature (references in text) and Swiss-Prot/TrEMBL

interactions (Howe and Jander 2008). Therefore, the induction of LOX by BMSB feeding supports the observation of a JA-dependent pathway primed by BMSB oviposition on faba bean plants (Rondoni et al. 2018), leading to the production of volatiles that attract parasitoid wasps (Rondoni et al. 2017). Moreover, BMSB restrained on one leaf triggered a lipoxygenase-related response both at the damaged site and in distal leaves, which is a systemic reaction that has been previously observed following chewing activity by *Spodoptera exigua* larvae (Pare and Tumlinson 1998). Then, some signaling processes might exist inside vascular ducts, such as signaling molecules in phloem (Rodriguez-Saona et al. 2002) or wound-induced pressure changes in xylem [reviewed by Wu and Baldwin (2010) and Farmer et al. (2014)].

How induced defense impacted feeding behavior

As presented in our previous EPG study with BMSB (Serteyn et al. accepted), the use of DC-EPG with fixed Ri of 10^9 Ohm is not recommended for large insects like stink bugs (Backus et al. 2018). Indeed, BMSB has probably suffered from the plant voltage, which could have highly impacted its feeding behavior, causing insect prostration, with delayed and shortened test and ingestion probes. The manually changeable input voltage is therefore a source of heterogeneity, and we unfortunately did not write down the voltage in the few cases that we had to modify it (from 50 to 100 mV for the most extreme cases). The likely effect of this hypothesis would be a greatly reduced number of significant probing differences between "Ck" and "Ins" treatments. Then, we could have missed valuable and subtle information in our comparative study. Only the variables most highly impacted by the treatments were still significantly different, and will be discussed hereafter.

Among about twice more temptations of recordings, the remaining 22 and 24 replicates were the least voltage-impacted individuals, which usually started to probe within the two first hours and successfully ingested plant sap. We proposed a list of EPG variables that were consistent with feeding behaviors that have the greatest impact on plant yield and fruit quality, such as test probes, pathway phases, and sap uptake. These variables form part of a long list that is widely used in experiments on aphids (Sarria et al. 2009; Giordanengo 2014). However, we could not use the same waveform nomenclature, nor Sarria's Excel workbook, because the feeding behaviors of aphids and stink bugs are different.

It was expected that such a gregarious pest would not be negatively impacted by the plant defenses that they induce, as with aphids for which ingestion phases are not perturbed by local defense (Dugravot et al. 2007; Prado and Tjallingii 2007). However, fifth-instar BMSB individuals were impacted by the previous feeding activity of congeners and resulting plant defense. Firstly, delayed probing might be caused by semiochemicals applied to the leaf surface by previous insects or by plant volatiles resulting from LOX-involving pathways, such as C6 and C9 aldehydes or methyl-jasmonate (Howe and Jander 2008; Gosset et al. 2009; Griffiths 2015). Secondly, we did not observe perturbations in X-waves preceding sustained ingestion, even though they are critical phases of feeding site acceptance, allowing the insect to test the content and overcome plant defense systems (Backus et al. 2009). Finally, the shortened sustained feeding events imply that some antifeedant or toxic compounds (such as terpenoids, protease inhibitors, oxidative enzymes) were released in vascular ducts, and were then detected by the insect during feeding. Besides the direct effect of plant defenses, the fitness of BMSB could also decrease because of the reduction of feeding time.

How induced defense impacted salivary proteome

The strongest point of this study was that BMSB individuals used for proteomics were the same as the ones recorded with EPG. Therefore, feeding behavior was directly associated with salivary proteome investigation in response to plant treatment. Firstly, the observation of delayed probes due to plant defenses was consistent with the proteomic data, which suggested a reluctance of feeding and a switch of physiological priorities. Secondly, we were able to correlate the general reduction of ingestion duration with the down-regulation of proteins involved in pathogenicity or more largely in insect normal development. Like some generalist insects that can adapt their salivary compounds following a host switch or exposure to plant defensive metabolites (Celorio-Mancera et al. 2015; Acevedo et al. 2017; Rivera-Vega et al. 2018), proteome of BMSB's salivary glands was modified by insect-induced plant defenses.

According to our results, BMSB's salivary gland proteomes were very similar between treatments (activated defenses or not), with only a few qualitative differences, suggesting very subtle changes in insect's physiology due to its exposure to plant defenses. Nonetheless, when we added the quantitative differences in the equation, interesting trends could be deduced. The down-regulation of proteins of organismal systems and genetic information processes suggested that the insect allocated its resources towards other priorities than its regular feeding and development, in favor of metabolic pathways, struggling for toxic compound deactivation. Therefore, the following discussion focuses on proteins with putative roles in plant–insect interactions.

Several down-regulated proteins in "Ins" treatment could be attributed to a toxin activity or a pathogenesis role. Some of them were found to be close to venom proteins of the predatory bug *Pristhesancus plagipennis* (Reduviidae),

which are essential to bug feeding (Walker et al. 2017). These proteins presented a secretion signal and could be injected in plant along with BMSB saliva. Therefore, down-regulation of such toxic proteins in insect exposed to elicited plant suggests that plant defenses directly decreased the negative impact of insect feeding. Glutathione-S-transferase (GST) was the only detoxifying enzyme down-regulated in insect treatment. Among many other examples (Ahmad et al. 1986), it has been found in salivary glands of *Lygus lineolaris* (Zhu et al. 2016), but never in secreted saliva, to our knowledge. Indeed, GST does not present a secretory signal, and it is known to play an intracellular role in resistance to various xenobiotics and insecticides (Ahmad et al. 1986; Sharma et al. 2014). GST activity was induced in the generalist aphid *Myzus persicae* feeding on Brassicaceae plants, as a response to glucosinolate ingestion (Francis et al. 2005). Moreover, in the case of the generalist caterpillar *Spodoptera frugiperda*, induction level of GST highly depended on the host plant and its glucosinolate concentration (Yu 1982). To explain the unexpected down-regulation of GST in BMSB exposed to *V. faba* defenses, we hypothesize that these defenses involved other plant allelochemicals than glucosinolates, and BMSB allocated its resources to more efficient detoxifying enzymes than GST.

In the up-regulated metabolism section, several proteins could be associated with response to stress due to oxidative or hyperosmotic compounds, xenobiotics, or exogenous dsRNA. Firstly, UDP-glucuronosyltransferase belongs to UDP-glycosyltransferase family, known to be induced in tissues of insects exposed to plant secondary compounds, such as flavonoids and phenolic acids (Després et al. 2007). Its gene expression had been positively correlated with the exposure of the grasshopper *Oedaleus asiaticus* to such plant metabolites (Huang et al. 2017). Secondly, esterase FE4 provided resistance to insecticides for aphids (Tang et al. 2017) and to adverse environmental conditions for bees (Ma et al. 2018). More generally, it could have a role in the response to oxidative stress caused by plant defenses. Even if it does not present a secretion signal, this enzyme had also been identified in secreted watery saliva of BMSB (Peiffer and Felton 2014). Thirdly, xanthine dehydrogenase had already been observed in secreted saliva of *Lygus hesperus*, and because of its oxidoreductase activity, it was probably involved in detoxification of plant defenses (Cooper et al. 2013). However, this enzyme lacked a secretion signal, which suggests that detoxification activity occurs in insect cells, after allelochemical ingestion. Xanthine dehydrogenase was also detected in our previous proteomic study on BMSB salivary glands (Serteyn and Francis 2019). Therefore, this new observation supports our first hypothesis, stating that xanthine dehydrogenase is an interesting candidate for BMSB adaptation to various host plants. Finally, the identification of secretion signals allowed a step forward in our results discussion, by identifying effector proteins that could be injected into the plant tissues. Among them, peroxiredoxin is a largely distributed protein in plant pest taxa. Because of its presence in secreted saliva, it allows insects to overcome early defensive signals by reducing hydrogen peroxide (Guiguet et al. 2016). Indeed, insect feeding generates a burst of reactive oxygen species in damaged plant cells, which is part of early signaling events (reviewed by Wu and Baldwin 2010). Up-regulation of peroxiredoxin strongly supports that BMSB detected local induced plant defenses and adapted accordingly its salivary compounds in the first hours of exposure. Also, a carboxypeptidase had previously been identified in BMSB saliva (Peiffer and Felton 2014), and could be associated with the metalloprotease family (Carolan et al. 2009). This family had been identified in saliva of the aphid *Acyrthosiphon pisum* and was speculated to target plant defense proteins (Carolan et al. 2011).

In the light of these results, it seemed clear that BMSB was able to rapidly (within the first hours) respond physiologically to plant defense compounds, and secrete modified saliva that would allow BMSB to counteract plant defenses.

Conclusions

This is the first time that a comparative study associates insect's feeding behavior and its salivary compound investigation, even if both aspects cannot be separated from each other. With this approach, we were able to lessen the hypotheses resulting from each aspect separately, and draw stronger conclusions. Our results suggest that BMSB is able to recognize plant defenses and rapidly adapt its salivary compounds, which would allow a remarkable plasticity of host plants. However, despite the gregarious behavior of BMSB, individuals seemed to be negatively impacted by plant defense induced by their own presence. They struggled to counteract allelochemicals, adapting their feeding behavior and their salivary compounds. Therefore, while foraging for food in nature, they would tend to avoid previously infested plants, which would lead to greater damage and propagation of the invasive pest. In any case, this study identified behavioral and physiological traits of this new pest species, providing novel insights on how it interacts with host plants.

Authors' contributions LS, LP, MLF, and FF conceived and designed research. LS, LP, and MS conducted experiments and analyzed data. LS wrote the manuscript. All authors read and approved the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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