The Spotted Lanternfly Contains High Concentrations of Plant Hormones in Its Salivary Glands: Implications in Host Plant Interactions

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Research Article

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Abstract

The spotted lanternfly (SLF), *Lycorma delicatula* is an invasive species in the United States that has emerged as a significant pest in vineyards. This polyphagous insect causes significant damage to grapevines and tree of heaven (TOH). SLF feeds voraciously on plant tissues using its piercing and sucking mouthparts through which it injects saliva and uptakes plant sap. Despite its impact, research on fundamental mechanisms mediating SLF interactions with their predominant hosts is limited. This study documents the morphology of salivary glands and quantifies plant hormones in salivary glands of SLF adults fed on grapevines and TOH using Liquid Chromatography-Mass Spectrometry (LC/MS). SLF adults have one pair of large salivary glands, ranging from 10–15 mm in length that extend from the insect's head to the last sections of the abdomen. The salivary glands of SLF contain salicylic acid (89 ng/g), abscisic acid (6.5 ng/g), 12-oxo-phytodienoic acid (5.7 ng/g), indole-3-acetic acid (2 ng/g), jasmonic acid (0.6 ng/g), jasmonic acid isoleucine (0.037 ng/g), and the cytokinin ribosides *trans*-zeatin (0.6 ng/g) and cis-zeatin (0.1 ng/g). While the concentrations of these hormones were similar in insects fed on grapevines and TOH, abscisic acid was more abundant in insects fed on grapevines, and jasmonic acid isoleucine was only detected in insects fed on grape. These results are discussed in the context of the possible implications that these hormones may have on the regulation of plant defenses. This study contributes to our understanding of the composition of SLF saliva and its potential role in plant immunity.

INTRODUCTION

The spotted lanternfly (SLF), *Lycorma delicatula* (Hemiptera: Fulgoridae) is an invasive species in the United States (Barringer et al. 2015; Dara et al. 2015). This insect is a highly polyphagous plant hopper that feeds on over 100 plant taxa worldwide (Barringer and Ciafré 2020). Though SLF has a wide host range, the tree of heaven (TOH, *Ailanthus altissima*), and grape (*Vitis* spp.) are considered preferred hosts (Liu 2019). These hosts are also highly susceptible to SLF feeding, with high and repeated insect infestations leading to plant death. It is known that SLF adults ingest large amounts of sap through the indirect observation of their secreted honeydew. However, the underlying mechanisms by which SLF may be affecting plant immunity are currently unknown.

SLF is an important pest of grapes that causes crop losses and vine decline. Using field-established Riesling grapevines, Harner et al. (2022) found that exposure to a large number of SLF adults resulted in a decrease in fruit-soluble sugars at harvest and a reduction in starch and nitrogen concentrations in roots. Extended exposure to SLF decreased grapevine transpiration, stomatal conductance, and leaf carbon assimilation (Harner et al. 2022). Furthermore, repeated infestations of SLF have caused grapevine death, but there is little information about the mechanisms involved or the number of insects and years of infestation that trigger this effect. An RNA-seq study in Marquette grapevines found that adult SLF feeding causes activation of protein kinases, phytohormones, and transcription factors. These signaling molecules trigger downstream target genes in charge of different metabolic processes and defensive mechanisms, including photosynthesis, stomata closure, cell wall reformation, and

detoxification (Islam et al. 2022). These studies provide valuable insights into the molecular and physiological responses of grapevines to infestation by SLF, but there is no knowledge of the insect-derived molecules that may be eliciting these responses.

SLF feeds on plant phloem and secretes saliva during its feeding activity. The morphology of the mouthparts has been documented, but the feeding mechanism is unknown. SLF has piercing-sucking mouthparts arranged into a stylet with a food canal that allows the uptake of plant sap, and a salivary canal that transports saliva from the salivary glands into plant tissues (Hao et al. 2016). The composition of SLF saliva is currently unknown but studies in other planthopper species have revealed that saliva plays a key role in their interaction with their host plants. Plant hoppers secrete gelling and watery saliva; the gelling saliva forms a salivary sheath that appears to help anchor the stylet and seal plant wounded cells during feeding, whereas the watery saliva contains proteins essential for feeding and modulation of plant signaling pathways (Huang et al. 2019). Proteins in planthopper saliva are involved in calcium binding, plant cell degradation, detoxification of secondary metabolites, extra-oral digestion, and plant defense regulation, among other functions (Huang et al. 2019). There is also evidence that saliva composition varies when planthoppers feed on hosts with different degrees of herbivore resistance (Huang et al. 2017), and therefore, may also vary when feeding on different host plants. Besides proteins, the salivary glands and saliva of herbivore insects also contain plant hormones and other small molecules with bioactive activity in plants (Acevedo et al. 2019; Ponce et al. 2021; Saraiva et al. 2021; Seng et al. 2023; Tooker and De Moraes 2006). However, plant hoppers have not yet been screened for the presence of these molecules.

Plant hormones or phytohormones, are signaling molecules that regulate various physiological processes in plants. These hormones play crucial roles in plant growth, development, and responses to abiotic and biotic stressors including insects and pathogens (Taiz et al. 2023). The key plant hormones involved in the regulation of plant immunity are Jasmonic acid (JA), salicylic acid (SA), and ethylene (ET), which are part of the octadecanoid, ethylene, and shikimate signal transduction pathways, respectively (Sakthi et al. 2021). JA is involved in the activation of defenses against chewing insects and necrotrophic pathogens, while SA regulates resistance against biotrophic pathogens (Erb et al. 2012; Zarate et al. 2007). Besides JA, SA, and ET, plants can activate other phytohormones in response to insect attacks, such as abscisic acid (ABA), cytokinins (CKs), and auxins (IAA), among others. The interactions of plant hormones, allow plants to adjust their defense mechanisms against a range of insects (Erb et al. 2012; Pieterse et al. 2012; Sakthi et al. 2021).

Treatment with synthetic phytohormones elicits plant defense responses and plant hormones have been quantified in various insect species (Tokuda et al. 2022; Tooker and De Moraes 2005). Because these molecules operate at low doses it may be reasonable to think that hormones present in insect secretions can modulate plant defenses. This study quantifies plant hormones in the salivary glands of SLF adults fed on grapevines and TOH using LC/MS. The hypothesis was that SLF adults would contain phytohormones in their salivary glands. This study is a step toward our understanding of the SLF saliva composition and its possible effect on plant immunity.

METHODS AND MATERIALS

SLF adults were collected from wild conditions in Alburtis, PA during early fall in 2021. The insects were placed inside mesh cages [(90 x 60 x 60 cm), Jinhua Quiangsheng

Outdoor Products, Zhejiang China] containing either grapevines (*Vitis labrusca* cv. Concord) or tree of heaven (*Ailanthus altissima*) plants. Plants were grown as explained in Laveaga et al (2023). There was a total of five cages per plant species, each one infested with 10 SLF adults (males and females). The cages were placed on weed barrier, and kept in field conditions in Alburtis, PA (coordinates 40° 26'43.368" N, 75° 37' 34.752" W) as explained previously (Laveaga et al. 2023). The insects were kept on their respective plant treatments for 27 days (Sep. 10 to Oct. 7). For dissections, the insects inside their treatment cages were transported to a laboratory in Penn State Berks (1801 Broadcasting Rd, Reading, PA 19610). The insects were chilled on ice (in a cooler with lid) for about 10 minutes and subsequently pinned on sylgard dissection dishes, the legs were removed with surgical scissors and the ventral side was cut open with a scalpel. The lateral sides of the insect's body were pinned, and the gut was removed to extract the two salivary glands that lay underneath. (See Fig. 1 for details). The salivary glands were picked up with fine forceps, quickly rinsed with ultrapure water (VWR Cat. 02-0201-1000), slightly dried in light-duty tissue paper (VWR Cat. 82003-820), placed in pre-weighed ice-cold 2 ml microfuge tubes (Eppendorf, Cat. # 022363352) and stored at -80°C until use. Each sample comprised five salivary gland pairs from five SLF individuals grown in the same cage. The fresh weight of salivary gland samples was obtained by subtracting the weight of tubes containing salivary glands from the weight of the tubes alone; samples were weighted in a precision scale (Mettler Toledo Excellence XSR, Columbus, OH, USA) with an accuracy of 0.1 mg. Four samples per plant treatment were sent to the Proteomics and Metabolomics Facility at the University of Nebraska-Lincoln for extraction and quantification of plant hormones.

Hormone extraction and LC/MS. Frozen salivary gland samples were first disrupted using stainless steel beads in a homogenizer (TissueLyserII, Qiagen) for three minutes at 20 Hz. Subsequently, 900 µl of cold methanol: acetonitrile (50:50 v/v) spiked with a mixture of deuterium-labeled internal standards of plant hormones [(D6-ABA (abscisic acid), D4-SA (salicylic acid), D2-JA (jasmonic acid), D5-IAA (indole-3-acetic acid), D5tZ (cytokinin trans-zeatin), D5tZR (trans-zeatin cytokinin riboside), D2-GA1 (gibberellin A_1) and D2-GA12 (gibberellin A_{12})] were added to the frozen samples for metabolite extraction in the Tissue Lyzer for five minutes at 20Hz. The samples were then centrifuged at 16,000 g, and the supernatants were collected into a new tube (the extraction with 900 µl of methanol: acetonitrile was repeated twice). The supernatants were pooled and dried down using a speed vac. The pellets were then re-dissolved in 15% methanol and run in a Sciex QTRAP 6500 + mass spectrometer equipped with a TurboIonSpray (TIS) electrospray ion source (Shimadzu, Columbia MD) using an MRM (Multiple Reaction Monitoring) mode, as previously detailed in Lopez-Guerrero et al (2022). Briefly LC separation was done on a ZORBAX Eclipse Plus C18 column (2.1 mm × 100 mm, Agilent) flowing at 0.45 mL/min. The gradient of the mobile phases A (0.1% formic acid) and B (0.1% formic acid/90% acetonitrile) was as follows: 5% B for 1 min, to 60% B in 4 min, to 100% B in 2 min, hold at 100% B for 3 min, to 5% B in 0.5 min. The instrument was set

up to acquire negative and positive ion modes. The Analyst software (version 1.6.3) was used to control sample acquisition and data analysis. The hormones were detected using MRM transitions that were optimized using standards. Quantification of hormones was carried out using an external standard curve prepared with a series of standard samples with varying concentrations of unlabeled hormones and fixed concentrations of the deuterium-labeled standards mixture (Lopez-Guerrero et al. 2022). Hormone concentrations are presented as ng/g of fresh weight.

Statistical analysis. Differences in phytohormone quantities from insects fed on grape and TOH were analyzed with a two-sample t-test at alpha = 0.05 using Minitab 21.1.0 (Minitab Inc., State College, PA, USA), and all graphs were generated in R version 4.3.2 (Foundation for Statistical Computing, Vienna, Austria).

RESULTS

SLF salivary glands. SLF adults contain one pair of large salivary glands whose length is approximately 15 mm in females and 10 mm in males. Each gland is comprised of one large acinus that extends from the head to the last segments of the abdomen, four small acini located close to the head, one accessory gland, and interconnected tubes that transport the glandular secretions to the mouthparts. Each acinus is comprised of a cluster of several white spherical secretory cells (Fig. 1). Each pair of glands accounts for about 2% of the total fresh body weight in females and 6% in males with some variation with adult maturity and weight gain.

Plant hormones in SLF salivary glands. We identified the presence of five plant hormones (SA, ABA, JA and its conjugate JA-ILE, IAA and its derivate methyl-IAA, and the cytokinin ribosides cZR and tZR) and the signaling molecule OPDA in salivary glands of SLF adults. The most abundant hormones were SA with an average concentration of 89 ng/g of fresh weight, followed by ABA (6.5 ng/g), OPDA (5.7 ng/g), and IAA (2 ng/g) across treatments (Fig. 2). Furthermore, the concentration of ABA was significantly higher in salivary glands of insects fed on grape compared with those fed on TOH ($t = 4.28$, $P = 0.023$). Traces of JA-ILE (on average 0.037 ng/g) were only identified in insects fed on grape, but the concentrations of the other hormones were similar for insects fed on grape and TOH (Fig. 2). OPDA was only identified in four out of the eight samples analyzed; it was present in one sample from insects fed on grape and in three samples from insects fed on TOH.

DISCUSSION

This study provides valuable insights into the morphology and composition of salivary glands in SLF adults. The presence of large salivary glands suggests the potential significance of these structures in SLF's feeding interactions with host plants. Large salivary glands may ensure a constant supply of saliva to sustain long or frequent insect feeding activity. Wild-collected SLF adults, further confined to single diets of grapevines and TOH contain several phytohormones, including SA, ABA, JA, JA-ILE, OPDA, IAA, methyl IAA, cZR, and tZR in their salivary glands. Notably, SA was found in substantial quantities,

followed by ABA, OPDA, and IAA. SLF individuals fed on grapevines had higher concentrations of ABA compared with those fed on TOH. Similarly, traces of JA-ILE were only found in the salivary glands of insects fed on grapevines, but the quantities of other hormones detected did not differ in insects fed on either host plant. Although this study doesn't directly demonstrate the secretion of these hormones into plants during insect feeding or their effect on plant immunity, the intriguing presence of these signaling molecules suggests they may be playing a significant role in regulating plant defenses as demonstrated in other insect and plant systems (Acevedo et al. 2019; Brütting et al. 2018).

SA has been identified in saliva, salivary glands, and eggs of different insect species (Acevedo et al. 2019; Saraiva et al. 2021; Tooker and De Moraes 2007). The role of SA in insect tissues has often been linked to the regulation of plant defenses and, more recently, to the suppression of insect immunity (Mollah et al. 2021). In plants, SA serves as a crucial signaling molecule, involved in responses against biotrophic pathogens, such as hypersensitive response (HR) and cell death (Ding and Ding 2020). SA accumulation in plants and downstream responses have also been reported after infestation with some sap-sucking insects (Deng et al. 2022). It has been demonstrated that some insect-derived effectors or molecules that suppress herbivore-induced defenses induce the SA pathway, which in turn suppresses JA-mediated herbivore resistance through an antagonistic interaction leading to enhanced herbivore performance (Cui et al. 2019; Ding and Ding 2020). SA-associated defenses are also induced by the exogenous application of SA in plants, which supports the hypothesis that SA in insect salivary glands can modulate plant defenses during insect feeding. The substantial quantities of SA in SLF, which remained consistent in insects fed on grape and TOH, suggest that the insect may rely on this signaling molecule to counter plant defenses in both host plants.

ABA is another hormone found in significant quantities in SLF salivary glands, especially when feeding on grapevines. ABA has been previously identified in the saliva of a Lepidoptera insect and nymphs (whole-body) of a Hemiptera species (Acevedo et al. 2019; Kai et al. 2017). In plants, this hormone is involved in plant growth and responses to abiotic and biotic stresses, including drought, cold stress, salinity, pathogens, and herbivore attacks (Erb et al. 2012; Li et al. 2022b; Singh et al. 2021). ABA is involved in the production and regulation of ROS (reactive oxygen species) molecules that play a critical role against pathogen infection and serve as signals in the activation of immune-related genes in plants (Li et al. 2022b). Increasing levels of ROS lead to higher Ca^{2+}concentration in guard cells, controlling stomatal closure (Li et al. 2022b). The closure of stomata reduces leaf transpiration and prevents water loss which benefits plants under drought conditions and may also be advantageous for insect herbivores (Lin et al. 2022; Nilson and Assmann 2007). For example, aphid infestation triggers the accumulation of ABA leading to stomatal closure, which enhances phloem feeding time and increases aphid populations (Sun et al. 2015). ABA also works synergistically with JA and antagonistically with SA likely influencing hormone crosstalk and specific plant immune responses (Pieterse et al. 2012). The presence of ABA in SLF salivary glands and its presumptive deposition into plant tissues may prevent host dehydration through stomata closure and could modulate defense responses to insect feeding.

The oxylipin 12-oxo-phytodienoic acid, commonly known as OPDA was also detected in SLF salivary glands. To the author's knowledge, this is the first time that OPDA has been detected in insect salivary glands. OPDA is commonly found in plants serving as a precursor of JA, and as a signaling molecule on its own (Jimenez Aleman et al. 2022). OPDA is accumulated in response to abiotic stress (flooding, heavy metals, salinity, drought) wounding, pathogen, and herbivore attacks (Archer et al. 2023; Kumar et al. 2019; Savchenko et al. 2014; Taki et al. 2005; Zhu et al. 2023). Similar to ABA, OPDA induces stomata closure (Savchenko et al. 2014) and is involved in the regulation of ROS (Taki-Nakano et al. 2014). OPDA is also involved in resistance to the phloem-feeding insects *Nilaparvata lugens* in rice and *Myzus persicae* in an *Arabidopsis* mutant (Archer et al. 2023; Guo et al. 2014). Although OPDA was detected in SLF fed on grape and TOH, it was more consistently detected in insects fed on TOH; speculatively, higher concentrations of this molecule in TOH may allow the insects to uptake and accumulate it in their salivary tissues.

Auxin (indole-3-acetic acid) or IAA and its nonpolar form methyl-IAA were detected in SLF salivary glands. IAA is a hormone commonly found in insects and other terrestrial arthropods, including those that don't feed on plants (Tokuda et al. 2022). The large concentration of this hormone in insect bodies, in some cases higher than those detected in plants, led to the hypothesis that insects were able to synthesize IAA de novo. It is now well known that various insect species can synthesize IAA from tryptophan (Suzuki et al. 2014; Yamaguchi et al. 2012), and an aldehyde synthase enzyme seems to catalyze this reaction in sawflies (Miyata et al. 2021). In plants, auxins have a variety of critical functions for growth, development, and immunity (Luo et al. 2018). Elevated auxin levels have been associated with enhanced disease susceptibility from biotrophic and hemibiotrophic pathogens (Kunkel and Johnson 2021), promoting auxin production seems to benefit pathogen infection due in part to the antagonistic crosstalk between auxin and SA (Erb et al. 2012). The role of auxins in response to insect infestation is less understood, but the hormone seems to accumulate in response to feeding by gall-inducing insects and by some chewing herbivores (Machado et al. 2016; Tooker and De Moraes 2011). The amounts of IAA found in SLF salivary glands were low (2ng/g FW on average) compared with those found in other organisms (Tokuda et al. 2022), and its influence on grape and TOH plants remains elusive.

Low amounts of JA (0.6 ng/g FW on average) were detected in SLF salivary glands from insects fed on grapevines and TOH, but traces (0.037 ng/g FW on average) of the bioactive form, JA-isoleucine (JA-ILE) were only detected in insects fed on grapevines. High concentrations of JA have been found in eggs and neonate larvae from several insect species (Tooker and De Moraes 2005; Tooker and De Moraes 2007), as well as in insect tissues such as the gut and salivary glands (Tooker and De Moraes 2006), and insect saliva (Acevedo et al. 2019). JA and its derivates are well-known molecules that get activated in plants in response to insect herbivory and wounding (Erb et al. 2012). These key signaling molecules induce the expression of herbivore defense-related genes, the production of toxic secondary metabolites, and the emission of volatile organic compounds that are used by herbivores and natural enemies to locate their hosts (Li et al. 2022a). However, some herbivores use effector molecules or microbes to activate the SA

pathway. This helps them thrive through the antagonistic interaction between SA and JA-regulated defense responses (Chung et al. 2013; Cui et al. 2019).

SLF salivary glands also contain low quantities of the cytokinin ribosides *trans*-zeatin (*t*ZR, average = 0.6 ng/g FW), and cis-zeatin (*c*ZR, average = 0.1 ng/g FW). CK ribosides have been previously identified in insect herbivores (Andreas et al. 2020), and larger amounts of this hormone are associated with insects that induce plant galls (Tokuda et al. 2022). Biosynthesis of CKs has not been demonstrated in insects, but bioinformatic analyses indicate that insects contain transcripts that encode proteins homologous to enzymes known to be involved in CK biosynthesis and metabolism (Mooi et al. 2024). CKs regulate nutrient allocation in plant tissues that can benefit some insect herbivores. For instance, CKs released in the saliva of the mirid bug *Tupiocoris notatus* alter the metabolism of tobacco plants to maintain their nutritional quality under herbivore attack. Feeding by *T. notatus* induces the accumulation of CKs in tobacco, which in turn promotes stable concentrations of glucose, fructose, and starch despite heavy herbivore damage (Brütting et al. 2018). Similarly, high concentrations of CKs were identified in green leaf sections (“green islands”) inhabited by mining insects in senescent apple leaves. The so-called “green islands” also had higher protein and sugar concentrations than the surrounding senescent tissues (Giron et al. 2007). CKs are also involved in the regulation of herbivore-induced defense responses in plants and interact with other signaling hormones, including auxins, ABA, JA, ethylene, etc (Schäfer et al. 2015).

Grapevine defense responses to SLF feeding have been previously investigated at the transcriptional level. Marquette grapevines infested with SLF had a high enrichment of genes associated with ABA, and auxin signaling, and equal induction of both JA and SA genes. There was also upregulation of genes associated with photosynthetic processes, biosynthesis of secondary metabolites, detoxification, and antioxidant activity, among others (Islam et al. 2022). The role that the identified phytohormones in SLF salivary glands may have on grapevine defense responses to SLF feeding remains elusive and deserves future investigation. It is also unknown if SLF or its associated symbionts synthesize plant hormones or if the insect accumulates them in their salivary glands after feeding on plants. The results of this study stimulate further investigation into the functional significance of phytohormones identified in SLF salivary glands on plant defense regulation.

In conclusion, this study provides significant insights into the morphology and composition of salivary glands in SLF adults. The presence of large salivary glands suggests their potential importance in SLF's feeding interactions with host plants. The detection of various phytohormones, including SA, ABA, JA, JA-ILE, OPDA, IAA, methyl IAA, cZR, and tZR in SLF salivary glands, raises intriguing questions about their role in regulating plant defenses. While this study does not directly demonstrate the secretion of these hormones into plants during feeding or their impact on plant immunity, it provides a foundation for further research in this area.

Declarations

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Author Contribution

F.E.A designed the study, conducted the experiments, analyzed the data, and wrote the manuscript.

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Figures

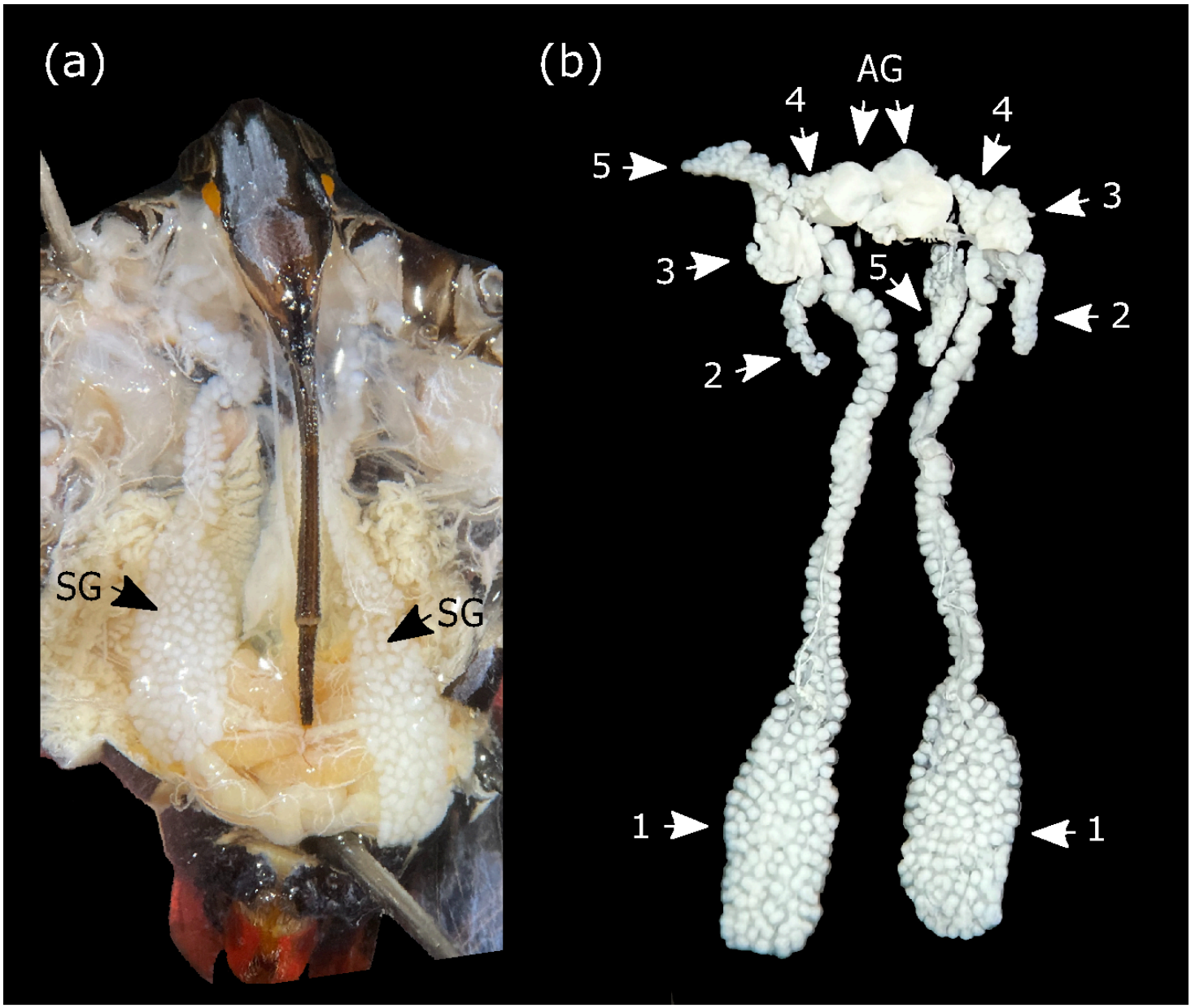

Figure 1

Salivary glands of adult spotted lanternflies (SLF). (a) dissection of an SLF female showing the paired salivary glands (SG) on each side of the insect's body. (b) Salivary glands of an adult female dissected out; numbers 1-5 indicate different acini types; AG are accessory glands.

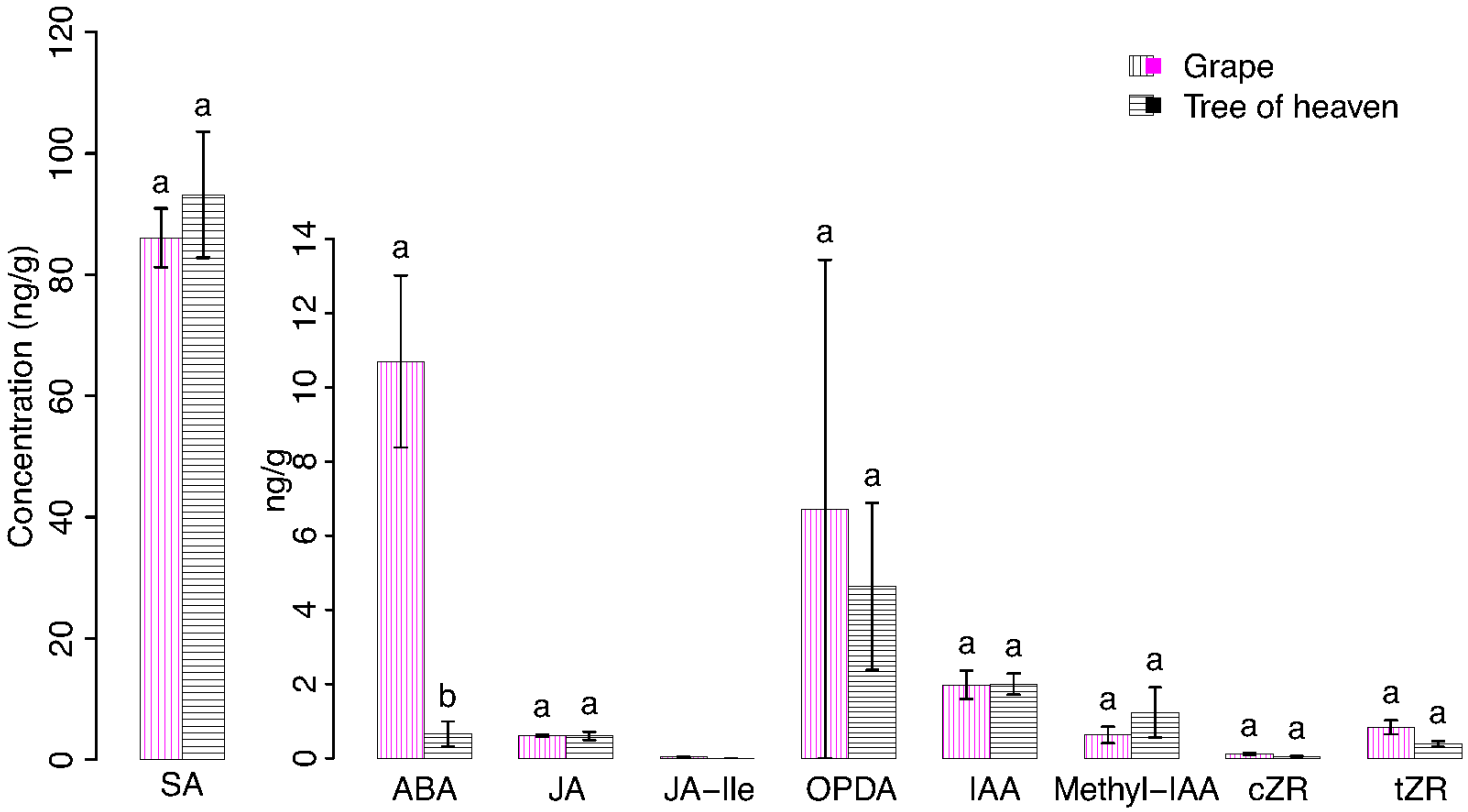

Figure 2

Phytohormones in SLF salivary glands. Bars represent untransformed means ± SEM. Different letters depict significant differences (α = 0.05) between insects fed on grapevines and TOH obtained with two sample *t*-tests. SA (salicylic acid), ABA (abscisic acid), JA (jasmonic acid), JA-Ile (Jasmonic Acid-Isoleucine Conjugate), OPDA (oxylipin 12-oxo-phytodienoic acid), IAA (indole-3-acetic acid), Methyl-IAA (Methyl-Indole-3-Acetic Acid), cZR (cytokinin riboside), tZR (trans-Zeatin cytokinin riboside).