

University of Nebraska - Lincoln
DigitalCommons@University of Nebraska - Lincoln

Faculty Publications: Department of Entomology

Entomology, Department of

2015

Morphology and Proteome Characterization of the Salivary Glands of the Western Chinch Bug (Hemiptera: Blissidae)

Crystal Ramm

University of Nebraska-Lincoln

Astri Wayadande

Oklahoma State University

Lisa Baird

University of San Diego, CA, Baird@sandiego.edu

Renu Nandakumar

University of Nebraska-Lincoln

Nandakumar Madayiputhiya

University of Nebraska-Lincoln

See next page for additional authors

Follow this and additional works at: <https://digitalcommons.unl.edu/entomologyfacpub>

 Part of the [Entomology Commons](#)

Ramm, Crystal; Wayadande, Astri; Baird, Lisa; Nandakumar, Renu; Madayiputhiya, Nandakumar; Amundsen, Keenan; Donze-Reiner, Teresa; Baxendale, Frederick; Sarath, Gautam; and Heng-Moss, Tiffany, "Morphology and Proteome Characterization of the Salivary Glands of the Western Chinch Bug (Hemiptera: Blissidae)" (2015). *Faculty Publications: Department of Entomology*. 415. <https://digitalcommons.unl.edu/entomologyfacpub/415>

This Article is brought to you for free and open access by the Entomology, Department of at DigitalCommons@University of Nebraska - Lincoln. It has been accepted for inclusion in Faculty Publications: Department of Entomology by an authorized administrator of DigitalCommons@University of Nebraska - Lincoln.

Authors

Crystal Ramm, Astri Wayadande, Lisa Baird, Renu Nandakumar, Nandakumar Madayiputhiya, Keenan Amundsen, Teresa Donze-Reiner, Frederick Baxendale, Gautam Sarath, and Tiffany Heng-Moss

Morphology and Proteome Characterization of the Salivary Glands of the Western Chinch Bug (Hemiptera: Blissidae)

CRYSTAL RAMM,¹ ASTRI WAYADANDE,² LISA BAIRD,³ RENU NANDAKUMAR,⁴
 NANDAKUMAR MADAYIPUTHIYA,⁴ KEENAN AMUNDSEN,⁵ TERESA DONZE-REINER,¹
 FREDERICK BAXENDALE,¹ GAUTAM SARATH,⁶ AND TIFFANY HENG-MOSS^{1,7}

J. Econ. Entomol. 108(4): 2055–2064 (2015); DOI: 10.1093/jee/tov149

ABSTRACT The western chinch bug, *Blissus occiduus* Barber, is a serious pest of buffalograss, *Buchloe dactyloides* (Nuttall) due to physical and chemical damage caused during the feeding process. Although previous work has investigated the feeding behaviors of chinch bugs in the *Blissus* complex, no study to date has explored salivary gland morphology and the associated salivary complex of this insect. Whole and sectioned *B. occiduus* salivary glands were visualized using light and scanning electron microscopy to determine overall structure and cell types of the salivary glands and their individual lobes. Microscopy revealed a pair of trilobed principal glands and a pair of tubular accessory glands of differing cellular types. To link structure with function, the salivary gland proteome was characterized using liquid chromatography tandem mass spectrometry. The salivary proteome analysis resulted in *B. occiduus* sequences matching 228 nonhomologous protein sequences of the pea aphid, *Acyrtosiphon pisum* (Harris), with many specific to the proteins present in the salivary proteome of *A. pisum*. A number of sequences were assigned the molecular function of hydrolase and oxido-reductase activity, with one specific protein sequence revealing a peroxidase-like function. This is the first study to characterize the salivary proteome of *B. occiduus* and the first of any species in the family Blissidae.

KEY WORDS salivary gland, Hemiptera, chinch bug, salivary proteome

Chinch bugs are a common pest known for damaging a variety of turfgrasses and field crops including perennial ryegrass, Kentucky bluegrass, zoysiagrass, fescue, and bentgrass; and sorghum, corn, wheat, and barley (Spike et al. 1994). The western chinch bug, *Blissus occiduus* Barber, is a serious pest of buffalograss, *Buchloe dactyloides* (Nuttall), a low maintenance turfgrass species with comparative freedom from arthropods and disease (Baxendale et al. 1999). In addition, *B. occiduus* has an extensive host range including turfgrasses such as green foxtail, Kentucky bluegrass, perennial ryegrass, and zoysiagrass and small grains such as corn, sugarcane, wheat, and barley (Ferris 1920, Bird and Mitchener 1950, Farstad and Staff 1951, Slater 1964, Baxendale et al. 1999, Eickhoff et al. 2004). Chinch bugs are categorized as salivary sheath feeders, lying down sheaths of gelling saliva that harden around the insect stylets as they advance toward the target

phloem tissue (Painter 1928, Backus et al. 2013). Chinch bug feeding occurs primarily within vascular tissues and bulliform cells (Anderson et al. 2006). Associated plant tissue injury is a result of direct stylet puncture, withdrawal of plant sap, and clogging of vascular transport systems via deposition of salivary sheath materials (Painter 1928). Buffalograss damage by *B. occiduus* is a result of feeding, as sap is withdrawn from tissues around the crown and stolon areas by the insect's piercing-sucking mouthparts. Feeding results in an initial reddish discoloration of plant tissue, followed by patches of yellowing or browning turf, and eventually plant death (Baxendale et al. 1999).

In recent years, increased attention has been given to insect saliva and its role in plant-insect interactions (Cherqui and Tjallingii 2000, Francischetti et al. 2007, Cooper et al. 2010, Carolan et al. 2011, DeLay et al. 2012, Nicholson et al. 2012, Will et al. 2012, Rao et al. 2013). Previous research has documented salivary gland morphology and the composition of salivary secretions in Hemipteran insects (Sogawa 1965, Wayadande et al. 1997, Madhusudhan and Miles 1998, Ni et al. 2000, Swart and Felgenhauer 2003, Carolan et al. 2011). Miles (1987, 1990) and Burd et al. (1998) suggested that associated damage and symptoms caused by insects with piercing-sucking mouthparts were due to the injection of salivary phytotoxins into plant tissues. Salivary secretion composition is quite diverse and consists of many different enzymes and metabolites depending upon the insect and the host plant

¹ Department of Entomology, University of Nebraska, Lincoln, NE 68583.

² Department of Entomology and Plant Pathology, Oklahoma State University, Stillwater, OK 74078.

³ Department of Biology, University of San Diego, CA 92110.

⁴ Department of Biochemistry, University of Nebraska, Lincoln, NE 68583.

⁵ Department of Agronomy and Horticulture, University of Nebraska, Lincoln, NE 68583.

⁶ Grain, Forage and Bioenergy Research Unit, USDA-ARS, Lincoln, NE 68583.

⁷ Corresponding author, e-mail: thengmoss2@unl.edu.

(Miles 1968). Hemipteran saliva has been documented to contain digestive enzymes such as amylase, protease, invertase, and lipase in the salivary glands of the milkweed bug (Bronskill 1958) and reductants, surfactants, cellulases, polyphenol oxidase, and peroxidase in aphid watery saliva (Miles 1999). Pectinases in saliva may help to not only break down the middle lamella of the plant cell wall but also function in gustatory exploration and overcoming plant resistance (Campbell and Dreyer 1985). The oxido-reductases peroxidase and polyphenol oxidase may allow these insects to detoxify plant defensive phytochemicals such as hydrogen peroxide (Ni et al. 2000). In addition, gelling sheath saliva may help with stylet support, lubrication, penetration, protection from bacteria and viruses, and suppression of wound signaling (Miles 1968, 1972, 1987, 1999; Cherqui and Tjallingii 2000).

Despite an increasing interest in the role of insect salivary secretions in plant feeding (Wayadande et al. 1997, Miles 1999, Cherqui and Tjallingii 2000, Francischetti et al. 2007, Cooper et al. 2010, Carolan et al. 2011, Will et al. 2012, Nicholson et al. 2012, DeLay et al. 2012, Rao et al. 2013), and the economic importance of the *Blissus* species (Mize and Wilde 1986, Potter 1998, Baxendale et al. 1999, Vittum et al. 1999, Eickhoff et al. 2004), little is known about the chinch bug salivary complex. The nonmodel status of this insect indicates that developing molecular and biochemical resources to understand the biology of the pest is going to be incremental and will take time. Here we focused on the salivary glands, as these tissues are likely to contain proteins that could inflict plant damage. The objectives of this research study were to identify and describe the salivary glands of *B. occidentus* using light (LM) and scanning electron microscopy (SEM) and characterize the salivary proteome of the salivary glands using liquid chromatography tandem mass spectrometry (LC-MS/MS).

Materials and Methods

Insect Samples. *B. occidentus* were collected from buffalograss research plots at the University of Nebraska-Lincoln East Campus by vacuuming the soil surface with a modified ECHO Shred N' Vac (Model #2400 ECHO Incorporated, Lake Zurich, IL). *B. occidentus* were sifted through a 2-mm mesh screen, and adult and fifth instars were collected with an aspirator. Voucher specimens were deposited in the University of Nebraska State Museum, Lincoln, NE.

LM of *B. occidentus* Salivary Glands. *B. occidentus* were cryoanesthetized, partially embedded ventral side down in wax and covered with a chilled solution of sodium phosphate buffer (0.1 mol/liter, pH 7.2). The exposed pronotum was removed with fine forceps (Fine Science Tools, Foster City, CA). Heads were carefully teased away from the bodies, leaving principal and accessory salivary glands intact. For salivary gland visualization and analysis, principal and accessory gland complexes were kept both intact with and freely isolated from the undisturbed head. Glands were transferred (via a 1,000- μ l pipettor) directly onto a glass

microscope slide, covered with a cover slip, and viewed under a phase contrast microscope (Olympus BX2 compound microscope with digital imaging). For histological cross-section analysis, principal and accessory glands were dissected out of chinch bugs in a chilled solution of 2% glutaraldehyde in 0.1 M Sorenson's sodium phosphate buffer (Clark et al. 1981), pH 7.2. Glands were transferred into Sorenson's fixative solution for 2 h. Glands were rinsed in buffer, dehydrated with a standard ethanol series, and embedded in JB-4 resin. Thick sections (4–6 μ m) were cut with a glass knife, mounted on glass slides, and stained with toluidine blue. Sections were visualized using an Olympus BX-51 light microscope.

For histological cross sections, confirmation of the accessory gland and the separate lobes of the principal gland was accomplished by separating the trilobed principal gland into the anterior, posterior, and lateral lobes. The individual lobes of the principal gland and the unilobed accessory gland were then processed following LM methods as previously described, sectioned, and observed by LM.

SEM of *B. occidentus* Salivary Glands. *B. occidentus* salivary glands were collected as described above in a chilled solution of 2% glutaraldehyde in 0.1 M Sorenson's sodium phosphate buffer, pH 7.2. Dissected glands were then prepared for SEM following similar methods as Johnson-Cicalese et al. (2011). Glands were visualized using a Hitachi 3400N scanning electron microscope operated at 15 kV.

LC-MS/MS of *B. occidentus* Salivary Glands. The salivary glands from 140 fifth-instar *B. occidentus* were pooled for proteome analysis. Glands were dissected from *B. occidentus*, as described above, into a chilled solution of potassium phosphate buffer (0.1 M, pH 7.5) containing 10% glycerol (Ni et al. 2000). Salivary glands (principal and accessory glands) were immediately transferred to a frozen microcentrifuge tube on dry ice and then stored in a -80°C freezer until protein extraction.

Protein was extracted from frozen salivary glands following a protocol modified from Heng-Moss et al. (2004) without the addition of added buffer and polyvinylpyrrolidone, but in the presence of a 1X protease inhibitor cocktail (Sigma P8340). Frozen glands were homogenized using a chilled polypropylene Pellet Pestle, 6.9 cm (Kimble-Chase Kontes). The homogenate was centrifuged at 14,000 \times g for 15 min at 4°C and the supernatant was collected. The crude homogenate was mixed 1:1 (v/v) with a sample loading buffer containing 0.5 M Tris-HCl (pH 6.8), 0.1% bromophenol blue, 25% glycerol, and 5% β -mercaptoethanol, heated at 95°C for 5 min, and loaded to a sample well. Salivary proteins were separated by SDS-PAGE using a 12% polyacrylamide gel (Criterion gel, Bio-Rad). Two samples (each containing homogenate from 70 individual salivary glands) were examined. Separated proteins were visualized using EZ blue staining solution (Sigma). The two gel lanes were divided into five transverse sections. The proteins in each gel section were subjected to in-gel trypsin digestion. The proteins were reduced with 10 mM (tris(2-carboxyethyl) phosphine)

(TCEP), alkylated with 55 mM iodoacetamide followed by digestion with trypsin (Roche; 1:50 trypsin: protein ratio) overnight at 37°C. The tryptic peptides were concentrated and re-suspended in 30 μ l 5% formic acid.

LC-MS/MS was performed with an Ultimate 3000 Dionex MDLC system (Dionex Corporation, Germering, Germany) integrated with a nano spray source and LCQ Fleet Ion Trap mass spectrometer (Thermo Finnigan, San Jose, CA) at the University of Nebraska Redox Biology proteomics core facility. LC-MS/MS included an online sample preconcentration and desalting using a monolithic C₁₈ trap column (Pep Map, 300 μ m I.D \times 5 mm, 100 μ , 5 μ m, Dionex). The sample was loaded onto the trap column at a flow rate of 40 μ l/min. The desalted peptides were then eluted and separated on a C18 Pep Map column (75 μ m I.D (X15 cm, 3 μ m, 100 μ , New Objective, Cambridge, MA) by applying an acetonitrile (ACN) gradient (ACN plus 0.1% formic acid, 90-min gradient at a flow rate of 250 nl/min) and were introduced into the mass spectrometer using the nano spray source. The LCQ Fleet mass spectrometer was operated with the following parameters: nano spray voltage, 2.0 kV; heated capillary temperature, 200°C; full scan m/z range, 400–2,000. The mass spectrometer

was operated in data-dependent mode with four MS/MS spectra for every full scan, five microscans averaged for full scans and MS/MS scans, a 3 m/z isolation width for MS/MS isolations, and 35% collision energy for collision-induced dissociation.

The MS/MS spectra were searched against *Acyrtosiphon pisum* (Harris) protein sequence database (International Aphid Genomics Consortium 2010), Version 2.00, NCBI, 17,695 sequences, using MASCOT (Version 2.2 Matrix Science, London, United Kingdom). Database search criteria were as follows: enzyme: Trypsin; missed cleavages: 2; mass: monoisotopic; fixed modification: carbamidomethyl (C); peptide tolerance: 1.5 Da; MS/MS fragment ion tolerance: 1 Da. Protein identifications were accepted with a statistically significant MASCOT protein score that corresponds to an error probability of $P < 0.05$.

Gene ontology (GO) was assigned to proteins using Blast2GO software (Conesa et al. 2005, <http://www.blast2go.org/>). Assigned GO terms for the predicted salivary proteome were categorized by molecular function (MF), biological process (BP), and cellular component (CC) to obtain information on the functional components of the salivary gland proteome.

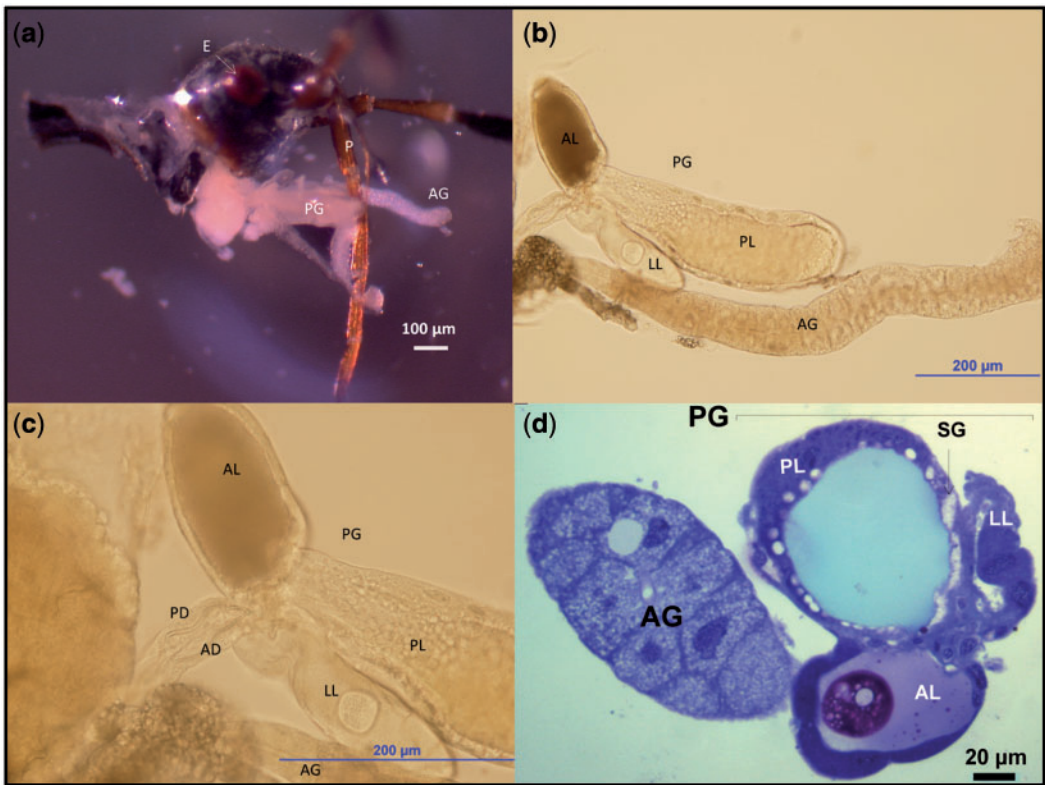


Fig. 1. Light micrographs of *B. occidius* salivary gland complex: (a) principal (PG) and accessory (AG) gland in proximity to *B. occidius* eye (E) and proboscis (P); (b) PG and the three lobes: anterior (AL), posterior (PL), and lateral (LL) lobes and AG; (c) PG and associated principal (PD) and accessory (AD) ducts; (d) histological cross section of entire salivary gland complex including the AG, PG, and the separate lobes (AL, PL, and LL) of the PG and numerous secretory granules (SG) gathered at the edge of the lumen. Scale bars are shown at the bottom right corner of each image.

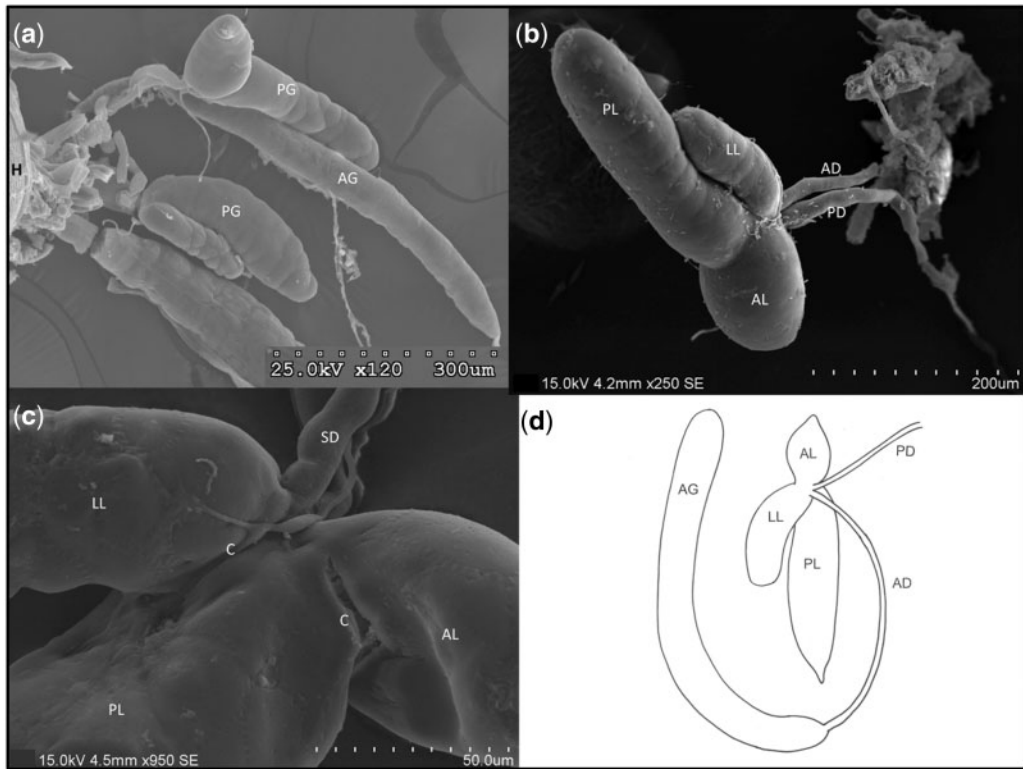


Fig. 2. Scanning electron micrograph images of the salivary glands of *B. occidius*: (a) complete salivary gland complex showing two principal glands (PG) and a connected accessory gland (AG) in proximity to the *B. occidius* head (H); (b) PG with the anterior (AL), posterior (PL), and lateral (LL) lobes and the associated principal (PD) and accessory gland (AD) ducts; (c) PG with AL, PL, and LL lobes and constrictions (C) between the lobes and associated salivary duct (SD). Scale bars are shown at the bottom right corner of each image. (d) Schematic drawing of one half of the *B. occidius* salivary gland pair. Principal gland is composed of the anterior lobe (AL), lateral lobe (LL), and posterior lobe (PL). Accessory gland (AG), accessory gland duct (AD), and principal gland duct (PD) are also shown.

Results and Discussion

LM and SEM of *B. occidius* Salivary Glands. Salivary glands were located and dissected from the prothoracic region of *B. occidius* as free organs attached to the head of the insect (Fig. 1a). Repeated dissection of chinch bug salivary glands revealed an isolated pair of iridescent bulbous sacs joined by cuticle-lined ducts (Figs. 1 and 2). The salivary gland complex of *B. occidius* consists of a pair of principal and accessory glands (Figs. 1 and 2). The principal gland is trilobed, consisting of an anterior (AL), posterior (PL), and lateral (LL) lobe that is located between the AL and PL (Figs. 1b, c and 2). The accessory gland is unilobed and tubular (Figs. 1b and 2a). Two side-by-side salivary ducts were observed leaving the principal gland (Figs. 1c and 2b). A strong constriction between all three lobes was observed in the principal gland (Figs. 1c and 2c).

Hemipteran salivary gland organization is morphologically diverse within different suborders but generally quite similar within a family. The morphological pattern of *B. occidius* salivary glands is consistent with that of other Hemipteran salivary glands, consisting of a pair of principal glands and a pair of accessory glands

(Dufour 1833, Baptist 1941, Southwood 1955, Bronskill 1958, Sogawa 1965, Miles 1972, Louis and Kumar 1973, Wayadande et al. 1997, Oliveira et al. 2006, Azevedo et al. 2007, Kumar and Sahayaraj 2012, Castro et al. 2013). In Hemiptera, the principal gland may be unilobed, bilobed, or multilobed while the accessory gland is always unilobed and vesicular or tubular. The principal gland of Heteroptera is most often further divided into at least two lobes, the anterior and posterior lobes (Goodchild 1966). Within the family Lygaeidae, several different species, *Gastrodes ferrugineus* and *Chilacis typhae*, have revealed a pair of trilobed principal glands (Baptist 1941). It is important to note that the chinch bug was identified first as a member of the family Blissidae and was recently moved into the family Lygaeidae. Similarities between the salivary gland organization of Lygaeidae and Blissidae further support the trilobed principal gland organization of *B. occidius*. Like *B. occidius*, the milkweed bug, *Oncopeltus fasciatus*, a species of Lygaeidae, has a trilobed principal gland and tubular accessory glands (Southwood 1955, Bronskill et al. 1958). The tubular accessory glands of *O. fasciatus* are slightly convoluted compared with the tubular accessory glands of *B. occidius* (Southwood

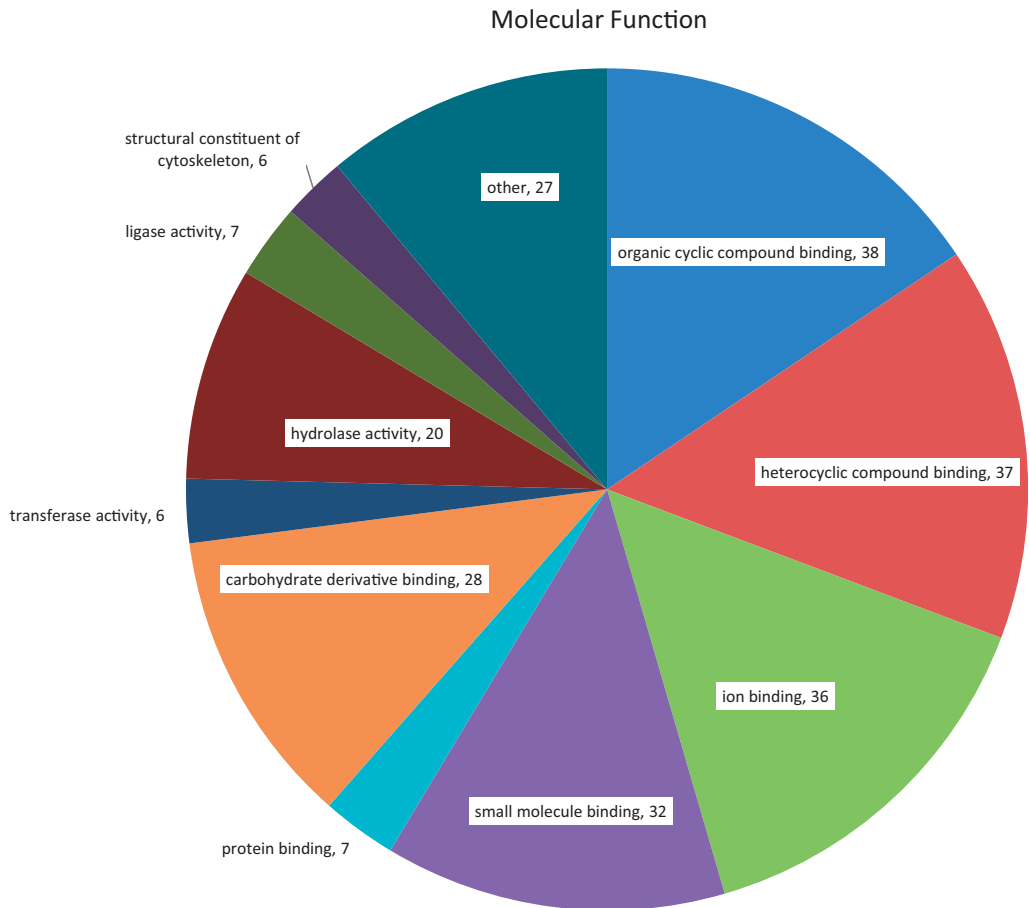


Fig. 3. Molecular Function (level 3) gene ontology terms for the *B. occiduus* salivary gland proteome.

1955). The trilobed principal gland and tubular accessory gland organization of the plant-feeding *B. occiduus* is like that of the predatory giant waterbug, *Belostomatia lutarium* (Hemiptera: Belostomatidae), although the principal glands of *B. lutarium* are acinuous (composed of many rosettes; Swart and Felgenhauer 2003). Two salivary ducts were observed leaving each principal gland of *B. occiduus*. Although the connection between the two different glands or the connection between the head (salivarium) and the glands of *B. occiduus* were never directly observed, we can make the assumption that the following analysis is correct based on the large volume of literature supporting the basic organizational structure of the Heteropteran salivary ducts. In *B. occiduus*, the duct closest to the posterior lobe and leading out of the principal gland is the accessory duct, which leads out into the tubular accessory gland (Baptist 1941, Southwood 1955, Oliveira et al. 2006, Azevedo et al. 2007; Figs. 1c and 2d). The duct closest to the anterior lobe, emerging from the principal gland, is the principal duct that extends toward the insect head and joins with the other principal duct to form the common salivary duct which leads into the salivarium emptying into the salivary canal of the insect stylet (Baptist 1941,

Southwood 1955, Oliveira et al. 2006, Azevedo et al. 2007; Figs. 1c and 2d).

Histological cross sections of salivary glands revealed binucleate principal and accessory glands as well as differences between the cellular contents of the different glands and between the three different lobes of the principal gland (Fig. 1d). This observation is supported by Miles (1972), indicating that plant feeders have distinct lobes of the principal gland, showing clearly distinct lumens. The posterior lobe of the principal gland clearly shows secretory granules of different sizes gathered at the edge of the lumen preparing to release contents (Fig. 1d). Histological evidence from differential staining of *B. occiduus* salivary gland cross sections provide evidence that it is very likely that each gland and the different lobes of the principal gland produce and secrete different products. According to Miles (1972), Hemipteran salivary glands and their lobes are composed of a variety of cells with different levels of activity and secretions. The multiple lobes of Hemiptera may function for the secretion of both watery and sheath saliva (Miles 1972, 1999). Watery saliva is most likely a combination of secretions by the principal gland and secretions from the accessory gland, with accessory

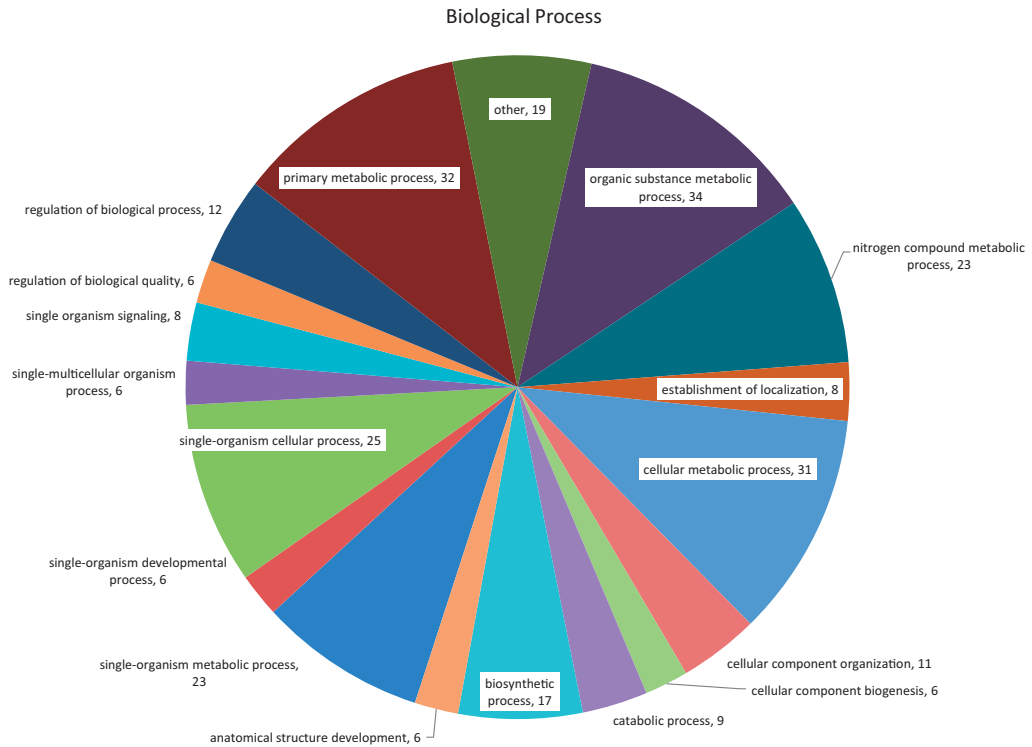


Fig. 4. Biological Process (level 3) gene ontology terms for the *B. occiduus* salivary gland proteome.

gland secretions diluting those of the principal gland as they are released from the insect stylet into plant tissues (Miles 1972, 1999). Results from Peiffer and Felton (2014) suggest that the watery and sheath saliva have much different protein profiles and may play different roles in eliciting plant responses.

Proteome Analysis of *B. occiduus* Salivary Glands. GO terms were assigned to a total of 191 *B. occiduus* proteins using Blast2GO. The GO terms included three main divisions: molecular function, biological processes, and cellular components. At level 3, 244 molecular function GO terms were assigned to these sequences. The majority of the molecular functions in the salivary gland proteome represented organic cyclic compound, heterocyclic compound, ion, and small molecule binding (143 terms) and hydrolase activity (20 terms; Fig. 3). Hydrolases are enzymes that catalyze the hydrolysis of chemical bonds, particularly pectic enzymes, degrade polysaccharides in the cell wall, and have been documented in Hemipteran saliva as being important in the development of necrotic symptoms (Ni et al. 2000, Miles 1990, Madhusudhan et al. 1994). *B. occiduus* sequences also annotated with four sequences with oxido-reductase function (Fig. 3). Oxido-reductases such as peroxidases are enzymes that interrupt the redox balance by the removal or generation of reactive oxygen species (ROS) and may additionally promote the gelling of the sheath saliva by enhancing disulphide bridge formation (Rao et al. 2013). Numerous studies have documented peroxidases in Hemipteran salivary secretions (Miles and Peng

1989, Madhusudhan and Miles 1998, Miles 1999, Cherqui and Tjallingii 2000, Rao et al. 2013, Vandermoten et al. 2014). A *B. occiduus* peptide annotated with a predicted peroxidase-like sequence from *A. pisum* (XP_003247028.1). The potential role of a salivary peroxidase and additional oxido-reductases may include the detoxification and control of ROS that are produced in response to insect pressure and stress (Miles and Oertli 1993). Hydrogen peroxide is an ROS that plays a key role as a signaling molecule in plant defense response pathways (Apel and Hirt 2004). Identification of a peroxidase-like protein in the salivary glands of *B. occiduus* may indicate this, along with other unidentified salivary peroxidases, potential role in suppressing the plant defense response and cellular signaling via the breakdown of hydrogen peroxide. The suppression of this stress-related defense response might help ensure uninterrupted feeding for a longer period of time.

There were 282 biological process GO terms in the salivary gland proteome, and the majority of these represented single-organism cellular and cellular metabolic processes (56 terms) and single-organism, primary, organic substance, and nitrogen compound metabolic processes (112 terms; Fig. 4). These results indicate that the cells within the salivary glands are highly metabolically active, correlating well with the indicated biological function of salivary gland tissue. There were 96 cellular component GO terms (Fig. 5). The majority of cellular component GO terms annotated with organelle (39 terms) and cell and membrane part (36). These

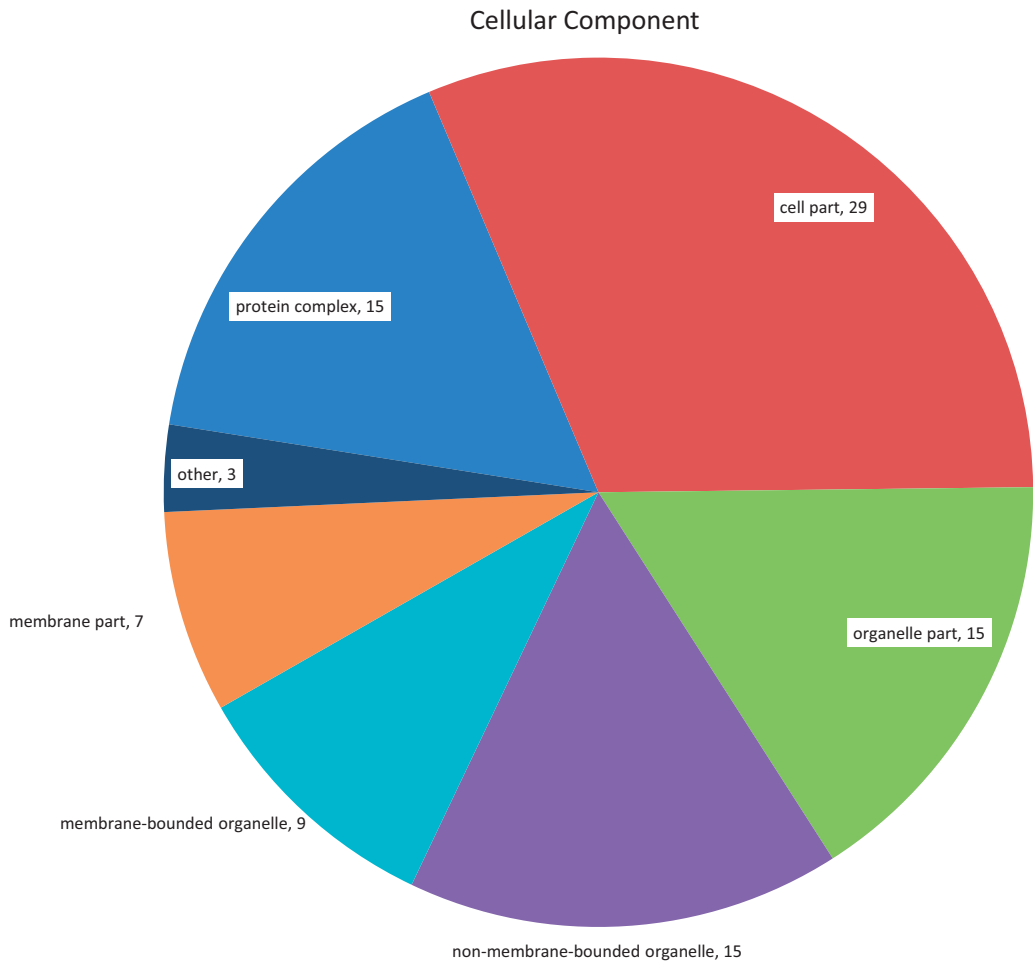


Fig. 5. Cellular Component (level 3) gene ontology terms for the *B. occiduus* salivary gland proteome.

results correspond positively with results from Delay et al. (2012), showing similar subcategories of gene ontology for the salivary transcriptome of the potato leafhopper, *Empoasca fabae*, for all the three main groups.

From all of the peptides obtained from in-gel digest of the *B. occiduus* salivary gland extracts, a total of 191 proteins were putatively identified following MS/MS searches against the NCBI *A. pisum* database. The *A. pisum* database was specifically selected to maximize discovery of chinch bug salivary sequences and to provide confidence to the overall interpretation of the data-set. Of these 191 sequences, 21 protein sequences matched specific proteins from the predicted salivary proteome of *A. pisum* (Table 1; Carolan et al. 2011). Of these 21 proteins, nine were categorized as hydrolases and two as oxido-reductases. Two other *B. occiduus* salivary protein sequences (gi|328698705 and gi|193662206) were also identified as having oxido-reductase activity from Blast2GO analysis (Supplement 1 [online only]). Peptides derived from chinch bug samples in addition matched to 170 other aphid proteins (Supplement 1 [online only]). These proteins did

not contain an apparent secretory signal based on a SignalP (www.ExPASy.org) analysis. Therefore, it is unclear if any of these 170 proteins are part of the chinch bug salivary proteome. However, these results potentially suggest a complexity to the salivary proteome that can be unraveled in the future. As shown in Supplement 1 (online only), many matches are to theoretical proteins annotated in the *A. pisum* genome, and deciphering the roles of these individual proteins is daunting. Nevertheless, these results provide good evidence that indeed chinch bug salivary glands had been dissected and yielded foundational data on the salivary proteome of this important insect pest. Of course, a tacit assumption was that if a peptide matched an *A. pisum* protein, it was scored as a protein of insect origin. The filtering (see methods) used for the searches largely eliminated uncertainty about the origin of the peptide (insect versus other organisms) and subsequently re-analysis of the 191 best matches to other related insect proteomes was not performed. Although it would have been impossible to predict a priori what proteins might have been present, the data demonstrated that the putative chinch bug salivary proteome was enriched in a

Table 1. LC-MS/MS results showing *B. occidius* peptide matches to specific proteins from the salivary proteome of *A. pisum* (Carolan et al. 2011)

Protein accession (gi)	Ref seq accession	Aphid base accession	Protein description	Prot. score	Prot. mass
gi 193603576	XP_001951233.1	ACYPI000474	PREDICTED: heat shock 70 kDa protein cognate 4-like isoform 2	147	71626
gi 193657105	XP_001951753.1	ACYPI0009530	PREDICTED: integral membrane protein 2B-like isoform 1	35	36020
gi 328723515	XP_001951336.2	ACYPI004656	PREDICTED: collagen alpha-1(IV) chain-like isoform 1	37	177972
gi 187109134	NP_001119672.1	ACYPI0000064	Actin-related protein 1	2555	42194
gi 193681197	XP_001948457.1	ACYPI006035	PREDICTED: actin-87E-like	2268	37534
gi 193591901	XP_001951517.1	ACYPI007027	PREDICTED: fructose-bisphosphate aldolase-like isoform 1	56	40189
gi 328713520	XP_003245102.1	ACYPI002182	PREDICTED: histone H2B 1/2-like	37	14046
gi 251823883	NP_001156510.1	ACYPI003154	14-3-3 protein zeta	805	28322
gi 328724395	XP_003248134.1	ACYPI006101	PREDICTED: plasma membrane calcium-transporting ATPase 3-like isoform 2	36	119914
gi 193596761	XP_001951915.1	ACYPI008923	PREDICTED: heat shock protein 70 B2-like [<i>Acyrtosiphon pisum</i>]	164	70180
Hydrolase function					
gi 242397408	NP_001156420.1	ACYPI007166	heat shock protein cognate 3	202	72993
gi 328718401	XP_001943129.2	ACYPI008535	PREDICTED: calcium-transporting ATPase sarcoplasmic/endoplasmic reticulum type-like isoform 1	78	111374
gi 193617621	XP_001949588.1	ACYPI002286	PREDICTED: transitional endoplasmic reticulum ATPase TER94-like	84	89914
gi 193690671	XP_001952242.1	ACYPI008923	PREDICTED: elongation factor 2-like	177	95558
gi 298676439	NP_001177327.1	ACYPI008874	tubulin beta-1	1332	50637
gi 193594183	XP_001948685.1	ACYPI010073	PREDICTED: tubulin alpha chain-like	717	50550
gi 328702459	XP_001943593.2	ACYPI007327	PREDICTED: tubulin beta-4 chain-like isoform 1	268	51350
gi 209915626	NP_001119645.2	ACYPI000061	ATP synthase subunit beta, mitochondrial	43	55777
gi 193666827	XP_001943349.1	ACYPI000622	PREDICTED: ATP synthase subunit alpha, mitochondrial-like	33	59986
Oxido-reductase function					
gi 193582510	XP_001951708.1	ACYPI010216	PREDICTED: glutamate dehydrogenase, mitochondrial-like	39	59929
gi 328715000	XP_003245510.1	ACYPI005939	PREDICTED: pyridoxine-5~phosphate oxidase-like	38	29242

Sequences matching to specific proteins from the salivary proteome of *A. pisum* with hydrolase and oxido-reductase function are indicated. Protein scores greater than 33 indicate sequence identity or homology ($P < 0.05$).

number of enzymes that might help the insects feed by overcoming plant defense responses. Future development of diagnostics, for example, antibodies, could be used to specifically address if a given protein was present in chinch bug salivary glands and in the saliva.

Additional proteins of interest that appeared to be part of *B. occidius* salivary glands include protein sequences with sequence identity to glucose dehydrogenase (gi|328715546) and lipase (gi|193624758) in Blast2GO (Supplement 1 [online only]). Glucose dehydrogenase has been reported in the salivary gland proteome of aphids and may play a role in detoxifying plant defense compounds (Harmel et al. 2008, Carolan et al. 2011, Nicholson et al. 2012). Previous studies have reported lipases in Hemipteran salivary glands, including the milkweed bug (Francischetti et al. 2007) and the froghopper (Hagley 1966). Lipases have been reported as aiding in ingestion and digestion by playing a role in the breakdown of plant cellular membranes (Francischetti et al. 2007). It has also been reported that salivary lipases may induce wound responses or signaling cascades that lead to the associated symptoms of insect feeding damages (Wang 1999).

Five *B. occidius* protein sequences (gi|328705984, gi|328707762, gi|193697416, gi|328714938, and gi|193643646) were predicted as having sequence identity with calcium ion-binding proteins in Blast2GO (Supplement 1 [online only]). Saliva contains enzymes that may interact with free calcium within plant tissue. As calcium is an important signaling compound in the salicylate, jasmonate, and ethylene signaling pathways,

these interactions might play a key role in plant feeding by avoiding eliciting plant defense responses and in the prevention of sieve tube closure, resulting in extended insect feeding (Will et al. 2007). Salivary secretions may not only play a role in preventing the wounding response of the host plant but may also play a role in eliciting the plant defense response. Gene expression studies have shown that phloem-feeding insects are able to change the physiology of their host plant, including secondary metabolism, source-sink relationships, and photosynthetic activity (Thompson and Goggin 2006). The *Blissus* species are salivary sheath feeders, secreting sheaths of gelling saliva that harden around the insect stylets as they advance intracellularly through plant tissue (Painter 1928, Backus et al. 2013). This sheath material may clog the sieve tubes and sequentially contribute to plant damage (Painter 1928). The phloem-associated probing behavior and phloem salivation of *B. occidius* feeding on buffalograss was recently documented by electrical penetration graph monitoring (Rangasamy 2008, Backus et al. 2013). Results showed that chinch bug feeding behaviors follow a stereotypical pattern, beginning with the formation of a salivary sheath, short periods of xylem ingestion, back to sheath formation, and then a transition (J wave/ X wave-like) to long-term phloem ingestion (Backus et al. 2013). These findings support the idea that chinch bugs may secrete different salivary enzymes while feeding from phloem sieve elements versus xylem tracheary elements to achieve different results. Studies such as Backus et al. (2013) will allow

for further understanding of chinch bug saliva and its potential role in the defense response of resistant and susceptible buffalograsses.

In conclusion, this was the first study to investigate the morphology and proteome of *B. occidius* salivary glands and the associated salivary proteome. The trilobed principal glands and tubular accessory glands and salivary proteins may be involved in *B. occidius* feeding and the overall plant–insect interaction. A number of *B. occidius* salivary proteins annotated with sequences from the *A. pisum* protein database, including hydrolase, oxido-reductase, glucose dehydrogenase, and calcium ion-binding proteins. Further studies are necessary to understand the role of these salivary proteins in the context of insect feeding and host-plant responses. Depending upon the host plant being fed upon, the resulting analysis of the insect salivary proteome may show a much different array of active proteins present at that specific point in time. Phloem-feeding insects are known for their ability to modify the composition of their salivary secretions in response to the defensive and nutritional content of their host plants (Peiffer and Felton 2005, Will et al. 2012). The proteins identified in this study will allow for future comparisons between the salivary gland proteome of chinch bug species differing in their interaction with host plants and resistance, and their abilities to cause economic and aesthetic damage. The future identification of *Blissus* salivary proteins playing a key role in the insect–plant interaction, such as an elicitor or suppressor of defenses, will help us gain an understanding for the molecular basis of these interactions. The identification of novel salivary proteins and compounds may be applied toward plant protection programs, aiding us in the understanding, selection, and development of plant cultivars resistant to *Blissus* feeding.

Supplementary Data

Supplementary data are available at *Journal of Economic Entomology* online.

Acknowledgments

This research was supported in part by the Nebraska Research Initiative Grant and the United States Golf Association.

References Cited

- Apel, K., and H. Hirt. 2004. Reactive oxygen species: metabolism, oxidative stress, and signal transduction. *Ann. Rev. Plant Biol.* 55: 373–399.
- Anderson, W. G., T. M. Heng-Moss, F. P. Baxendale, L. M. Baird, G. Sarath, and L. Higley. 2006. Chinch bug (Hemiptera: Blissidae) mouthpart morphology, probing frequencies, and locations on resistant and susceptible germplasm. *J. Econ. Entomol.* 99: 212–221.
- Azevedo, D.D.O., J. C. Zanuncio, J. S. Zanuncio Jr, G. F. Martins, S. Marques-Silva, M. F. Sossai, and J. E. Serrão. 2007. Biochemical and morphological aspects of salivary glands of the predator *Brontocoris tabidus* (Heteroptera: Pentatomidae). *Braz. Arch. Biol. Technol.* 50: 469–477.
- Backus, E. A., M. Rangasamy, M. Stamm, H. J. McAuslane, and R. Cherry. 2013. Waveform library for chinch bugs (Hemiptera: Heteroptera: Blissidae): Characterization of electrical penetration graph waveforms at multiple input impedances. *Ann. Entomol. Soc. Am.* 106: 524–539.
- Baptist, B. A. 1941. The morphology and physiology of the salivary glands of Hemiptera-Heteroptera. *Q. J. Microsc. Sci.* 2: 91–139.
- Baxendale, F. P., T. M. Heng-Moss, and T. P. Riordan. 1999. *Blissus occidius* Barber (Hemiptera: Lygaeidae): a new chinch bug pest of buffalograss turf. *J. Econ. Entomol.* 92: 1172–1176.
- Bird, R. D., and A. V. Mitchener. 1950. Insects of the season 1949 in Manitoba. *Can. Insect Pest Rev.* 28: 41.
- Bronskill, J. F., E. H. Salkeld, and W. G. Friend. 1958. Anatomy, histology, and secretions of salivary glands of the large milkweed bug *Oncopeltus fasciatus* (Dallas) (Hemiptera: Lygaeidae). *Can. J. Zool.* 36: 961–68.
- Burd, J. 1998. Seasonal development, overwintering biology, and host plant interactions of Russian wheat aphid (Homoptera: Aphididae) in North America (Revised to Show Published Title). Thomas Say Publications in Entomology, Annapolis, MD.
- Campbell, B. C., and D. L. Dreyer. 1985. Host plant resistance of sorghum: Differential hydrolysis of sorghum pectic substances by polysaccharases of greenbug biotypes (*Schizaphis graminum*, homoptera: Aphididae). *Arch. Insect Biochem.* 2: 203–215.
- Carolan, J. C., D. Caragea, K. T. Reardon, N. S. Mutti, N. Dittmer, K. Pappan, and O. R. Edwards. 2011. Predicted effector molecules in the salivary secretome of the pea aphid (*Acyrtosiphon pisum*): A dual transcriptomic/proteomic approach. *J. Proteome Res.* 10: 1505–1518.
- Castro, A.A.D., G.D.C. Canevari, T. G. Pikart, R. C. Ribeiro, J. E. Serrão, T. V. Zanuncio, and J. C. Zanuncio. 2013. Salivary gland histology of the predator *Supputius cincticeps* (Heteroptera: Pentatomidae). *Ann. Entomol. Soc. Am.* 106: 273–277.
- Cherqui, A., and W. F. Tjallingii. 2000. Salivary proteins of aphids, a pilot study on identification, separation and immunolocalisation. *J. Insect Physiol.* 46: 1177–1186.
- Clark, G., W. J. Doherty, and F. H. Kasten. 1981. General Methods, pp 1–103. In G. Clark (ed.), *Staining procedures*. Williams & Wilkins, Baltimore.
- Conesa, A., S. Götz, J. M. García-Gómez, J. Terol, M. Talón, and M. Robles. 2005. Blast2GO: A universal tool for annotation, visualization and analysis in functional genomics research. *Bioinformatics* 21: 3674–3676.
- Cooper, W. R., J. W. Dillwith, and G. J. Puterka. 2010. Salivary proteins of Russian wheat aphid (Hemiptera: Aphididae). *Environ. Entomol.* 39: 223–231.
- DeLay, B., P. Mamidala, A. Wijeratne, S. Wijeratne, O. Mitapalli, J. Wang, and W. Lamp. 2012. Transcriptome analysis of the salivary glands of potato leafhopper, *Empoasca fabae*. *J. Insect Physiol.* 8: 1626–1634.
- Dufour, L. 1833. Recherches anatomiques et physiologiques sur les Hémiptères, accompagnés de considérations relatives à l'histoire naturelle et à la classification de ces insectes. Mémoires des Savants étrangers de l'Académie Royale des Sciences de l'Institut de France Paris 4: 129–462.
- Eickhoff, T. E., F. P. Baxendale, T. M. Heng-Moss, and E. E. Blankenship. 2004. Turfgrass, crop, and weed hosts of *Blissus occidius* (Hemiptera: Lygaeidae). *J. Econ. Entomol.* 97: 67–73.
- Farstad, C. W., and A. Staff. 1951. Insects of the season in Alberta, 1950. *Can. Insect Pest Rev.* 29: 18.
- Ferris, G. F. 1920. Insects of economic importance in the cape region of lower California, Mexico. *J. Econ. Entomol.* 13: 463–467.

- Francischetti, I., A. H. Lopes, F. A. Dias, V. M. Pham, and J. Ribeiro. 2007.** An insight into the sialotranscriptome of the seed-feeding bug, *Oncopeltus fasciatus*. *Insect Biochem. Mol.* 37: 903–910.
- Goodchild, A.J.P. 1966.** Evolution of the alimentary canal in the Hemiptera. *Biol. Rev.* 41: 97–140.
- Hagley, E. 1966.** Studies on the aetiology of froghopper blight of sugarcane, pp.187–191. I. Symptom expression and development on sugarcane and other plants, vol. 183. II. Probable role of enzymes and amino acids in the salivary secretion of the adult froghopper. In: Proceedings of the 1966 Meeting of B.W.I. Sugar.
- Harmel, N., E. Létocart, A. Cherqui, P. Giordanengo, G. Mazzucchelli, F. Guillonnet, E. De Pauw, E. Haubruge, and F. Francis. 2008.** Identification of aphid salivary proteins: A proteomic investigation of *Myzus persicae*. *Insect Mol. Biol.* 17: 165–174.
- Heng-Moss, T., G. Sarath, F. Baxendale, D. Novak, S. Bose, X. Ni, and S. Quisenberry. 2004.** Characterization of oxidative enzyme changes in buffalograsses challenged by *Blissus occiduis*. *J. Econ. Entomol.* 97: 1086–1095.
- International Aphid Genomics Consortium. 2010.** Genome sequence of the pea aphid *Acyrtosiphon pisum*. *PLoS Biol.* 8: e1000313.
- Johnson-Cicalese, J., F. Baxendale, T. Riordan, T. Heng-Moss, and L. Baird. 2011.** Evaluation of buffalograss leaf pubescence and its effect on mealybug (Hemiptera: Pseudococcidae) host selection. *J. Kansas Entomol. Soc.* 84: 71–77.
- Kumar, S. M., and K. Sahayaraj. 2012.** Gross morphology and histology of head and salivary apparatus of the predatory bug, *Rhynocoris marginatus*. *J. Insect Sci.* 12: 19–19.
- Louis, D., and R. Kumar. 1973.** Morphology of the alimentary and reproductive organs in Reduviidae (Hemiptera: Heteroptera) with comments on interrelationships within the family. *Ann. Entomol. Soc. Am.* 66: 635–639.
- Madhusudhan, V. V., and P. W. Miles. 1998.** Mobility of salivary components as a possible reason for differences in the responses of alfalfa to the spotted alfalfa aphid and pea aphid. *Entomol. Exp. Appl.* 86: 25–39.
- Madhusudhan, V. V., G. S. Taylor, and P. W. Miles. 1994.** The detection of salivary enzymes of phytophagous Hemiptera: a compilation of methods. *Ann. Appl. Biol.* 124: 405–412.
- Miles, P. W. 1968.** Insect secretions in plants. *Annu. Rev. Phytopathol.* 6: 137–164.
- Miles, P. W. 1972.** The saliva of Hemiptera. *Adv. Insect Physiol.* 9: 183–255.
- Miles, P. W. 1987.** Feeding process of Aphidoidea in relation to effects on their food plants. *Aphids Biol. Nat. Enemies Control* 2: 321–339.
- Miles, P. W. 1990.** Aphid salivary secretion and their involvement in plant toxicoses, pp. 131–147. In R. K. Campbell and R. D. Eikenbary (eds.), *Aphid-plant genotype interactions*. Elsevier, Amsterdam.
- Miles, P. W. 1999.** Aphid saliva. *Biol. Rev. Camb. Philos.* 74: 41–85.
- Miles, P. W., and J. J. Oertli. 1993.** The significance of antioxidants in the aphid plant interaction: The redox hypothesis. *Entomol. Exp. Appl.* 67: 275–283.
- Miles, P. W., and Z. Peng. 1989.** Studies on the salivary physiology of plant bugs: Detoxification of phytochemicals by the salivary peroxidase of aphids. *J. Insect Physiol.* 35: 865–872.
- Mize, T. W., and G. Wilde. 1986.** New grain sorghum sources of antibiosis to the chinch bug (Heteroptera: Lygaeidae). *J. Econ. Entomol.* 79: 176–180.
- Ni, X., S. S. Quisenberry, S. Pornkulwat, J. L. Figarola, S. R. Skoda, and J. E. Foster. 2000.** Hydrolase and oxido-reductase activities in *Diuraphis noxia* and *Rhopalosiphum padi* (Hemiptera: Aphididae). *Ann. Entomol. Soc. Am.* 93: 595–601.
- Nicholson, S. J., S. D. Hartson, and G. J. Puterka. 2012.** Proteomic analysis of secreted saliva from Russian Wheat Aphid (*Diuraphis noxia* Kurd) biotypes that differ in virulence to wheat. *J. Proteomics.* 75: 2252–2268.
- Oliveira, J. A., M.G.A. Oliveira, R.N.C. Guedes, and M. J. Soares. 2006.** Morphology and preliminary enzyme characterization of the salivary glands from the predatory bug *Podisus nigrispinus* (Heteroptera: Pentatomidae). *B. Entomol. Res.* 96: 251–258.
- Painter, R. H. 1928.** Notes on the injury to plant cells by chinch bug feeding. *Ann. Entomol. Soc. Am.* 21: 232–242.
- Peiffer, M., and G. W. Felton. 2005.** The host plant as a factor in the synthesis and secretion of salivary glucose oxidase in larval *Helicoverpa zea*. *Arch. Insect Biochem.* 58: 106–113.
- Peiffer, M., and G. W. Felton. 2014.** Insights into the saliva of the brown marmorated stink bug, *Halyomorpha halys* (Hemiptera: Pentatomidae). *PLoS ONE* 9: e88483.
- Potter, D. A. 1998.** Destructive turfgrass insects: biology, diagnosis, and control. Ann Arbor Press, MI.
- Rangasamy, M. 2008.** Mechanisms of resistance to southern chinch bug, *Blissus insularis* Barber (Hemiptera: Blissidae), in St. Augustinegrass. Ph.D. dissertation, University of Florida, FL.
- Rao, S. A., J. C. Carolan, and T. L. Wilkinson. 2013.** Proteomic profiling of cereal aphid saliva reveals both ubiquitous and adaptive secreted proteins. *PLoS ONE* 8: e57413.
- Slater, J. A. 1964.** A catalogue of the Lygaeidae of the world. University of Connecticut, Storrs, CT.
- Sogawa, K. 1965.** Studies on the salivary glands of rice plant leaf-hoppers. I. Morphology and histology. *J. Appl. Entomol. Zool.* 9: 275–302.
- Spike, B. P., G. E. Wilde, T. W. Mize, R. J. Wright, and S. D. Danielson. 1994.** Bibliography of the chinch bug, *Blissus leucopterus leucopterus* (Say) (Heteroptera: Lygaeidae) since 1888. *J. Kans. Entomol. Soc.* 67: 116–125.
- Swart, C. C., and B. E. Felgenhauer. 2003.** Structure and function of the mouthparts and salivary gland complex of the giant waterbug, *Belostomatium laticornis* (Stål) (Hemiptera: Belostomatidae). *Ann. Entomol. Soc. Am.* 96: 870–882.
- Thompson, G. A., and F. L. Goggin. 2006.** Transcriptomics and functional genomics of plant defense induction by phloem-feeding insects. *J. Exp. Bot.* 57: 755–766.
- Vandermoten, S., N. Harmel, G. Mazzucchelli, E. De Pauw, E. Haubruge, and F. Francis. 2014.** Comparative analyses of salivary proteins from three aphid species. *Insect Mol. Biol.* 23: 67–77.
- Vittum, P. J., M. G. Villani, and H. Tashiro. 1999.** Turfgrass insects of the United States and Canada. Comstock Publishing Associates, Canada.
- Wang, X. 1999.** The role of phospholipase D in signaling cascades. *Plant Physiol.* 120: 645–651.
- Wayadande, A. C., G. R. Baker, and J. Fletcher. 1997.** Comparative ultrastructure of the salivary glands of two phytopathogen vectors, the beet leafhopper, *Circulifer tenellus* (Baker), and the corn leafhopper, *Dalbulus maidis* DeLong and Wolcott (Homoptera: Cicadellidae). *Int. J. Insect Morphol.* 26: 113–120.
- Will, T., W. F. Tjallingii, A. Thönnessen, and A. J. van Bel. 2007.** Molecular sabotage of plant defense by aphid saliva. *Proc. Natl. Acad. Sci.* 104: 10536–10541.
- Will, T., K. Steckbauer, M. Hardt, and A. J. van Bel. 2012.** Aphid gel saliva: sheath structure, protein composition and secretory dependence on stylet-tip milieu. *PLoS ONE* 7: e46903.