

Digestive Enzymes and Stylet Morphology of *Deraeocoris nebulosus* (Hemiptera: Miridae), a Predacious Plant Bug

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ABSTRACT Mixed-feeding habits, such as zoophytophagy, make the ecological roles of many species of insects, especially hemipterans, difficult to assess. To understand the feeding adaptations of the predacious plant bug *Deraeocoris nebulosus* (Uhler), the digestive enzymes from the salivary glands and anterior midgut were analyzed, and the mouthpart stylets were investigated with scanning electron microscopy. Evidence of trypsin-like enzyme, α -glucosidase, and pectinase were found in the salivary glands. Low levels of trypsin-like, chymotrypsin-like, elastase-like, and pectinase activity, with high levels of α -amylase and α -glucosidase activity, were found in the anterior midgut. The insect's right maxillary stylet has two rows of at least six recurved barbs on the inner surface pointing away from the head. This plant bug is equipped mainly for zoophagy but has enzymes that would allow some degree of phytophagy.

KEY WORDS *Deraeocoris nebulosus*, digestive enzymes, Miridae, predator, stylet morphology, omnivory

FEEDING HABITS OF the Heteroptera range from strict phytophagy to strict zoophagy (Schaefer and Panizzi 2000). Several families contain omnivores whose mixed-feeding habits have been termed zoophytophagous or phytozoophagous, depending on the relative degree of animal versus plant consumption (Alomar and Wiedenmann 1996). The origin of feeding habits among the Heteroptera remains controversial (Sweet 1979, Cobben 1979, Cohen 1990, Schaefer 1997, Wheeler 2001). The diverse trophic habits of plant bugs make the Miridae ideal for studies of feeding strategies, including digestive enzyme composition and mouthpart morphology.

Mirids, as well as all other heteropterans, employ macerate (or lacerate) and flush feeding (Miles 1972, Hori 2000, Wheeler 2001) that incorporates piercing/sucking mouthparts and watery saliva from the salivary gland complex. Mirids feed in a manner that is typical of heteropterans, piercing and cutting tissues with their stylets while injecting digestive enzymes through the salivary canal to liquefy food into a nutrient-rich slurry. The food slurry is ingested through the food canal and passed into the alimentary canal where it is further digested and absorbed (Cohen 2000).

A theoretical and practical grasp of the role that heteropterans play in natural and agricultural systems requires a thorough understanding of their feeding habits. Such knowledge, however, is difficult to obtain by direct means, largely because of the cryptic nature of the feeding process and the amorphous nature of the ingested food. Heteropteran workers lament the lack of a thorough understanding of heteropteran feeding habits, especially for the Miridae (Wheeler 2001).

A consumer's ability to use plant or animal materials for food is indicated by the presence of specific digestive enzymes and by mouthpart morphology (Baptist 1941; Adams and McAllan 1956; Strong and Kruitwagen 1968; Miles 1972; Cohen 1990, 1995, 1996, 1998a, 1998b, 2000; Agustí and Cohen 2000; Hori 2000; Zeng and Cohen 2000a, 2000b). Digestive enzymes specific for zoophagy include proteases (e.g., trypsin, chymotrypsin, cathepsin), hyaluronidase, and phospholipase (Cohen 1998b, 2000). Specific digestive enzymes for phytophagy include amylase and pectinase (Cohen 1996).

Morphological comparisons of both mandibular and maxillary stylets reveal differences between heteropteran phytophages and zoophages. Cobben (1978) showed that the right maxillary stylets of predacious heteropterans (e.g., Nabidae, Anthocoridae) are more deeply serrated than those of phytophagous heteropterans (e.g., Tingidae). Cohen (1996) showed that the mandibular stylets of phytophagous and predacious pentatomids varied in relation to the direction of the barbs; those of phytophagous species point away from

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the head, whereas the barbs of predacious species point toward the head. The barbs on the mandibular stylets of predacious heteropteran families (e.g., Reduviidae) are more numerous than the barbs on the mandibular stylets of phytophagous heteropteran families (e.g., Lygaeidae, sensu lato) (Cohen 1990).

Deraeocoris nebulosus (Uhler) is a predator of plant-feeding arthropods including aphids, mites, scale insects, and whiteflies (Gillette 1908, Smith 1923, Wheeler et al. 1975, Jones and Snodgrass 1998). Phytophagy is unknown in *D. nebulosus*, but other species in this predatory genus feed facultatively on plants (McMullen and Jong 1967, Razafimahatratra 1981, Wheeler 2001). One of us (D.W.B.) has observed *D. nebulosus* with its stylets inserted into sweet potato leaves and cabbage, but whether this behavior involves the uptake of nutrients or just water is not known.

The objectives of this study were to determine the presence of certain digestive enzymes in the salivary gland complex and anterior midgut of *D. nebulosus* and to analyze its stylet morphology. We also evaluated this predacious mirid's ability to obtain nutrients from animals and plants.

Materials and Methods

Insects. A colony was established with *D. nebulosus* collected from oaks (e.g., *Quercus alba* L., *Q. stellata* Wang, and *Q. falcata* Michaux) in Greenville County and Pickens County, SC, during the summer of 1999 and was maintained at $25 \pm 2^\circ\text{C}$, $50 \pm 10\%$ RH, and a photoperiod of 14:10 (L:D) h. *Deraeocoris nebulosus* was reared on eggs of *Ephesthia kuehniella* Zeller (Lepidoptera: Pyralidae) (Beneficial Insectary, Redding CA, USA) at the Cherry Farm Insectaries, Clemson University, Clemson, SC, USA. Voucher specimens were placed in the Clemson University Arthropod Collection.

Sample Preparation. Enzyme samples were prepared by the method of Cohen (1993) with modification. Only adult female insects were used in these tests. The mirids were starved for 24 h before dissections to standardize the insects and to allow an accumulation of digestive enzymes. The insects were placed at -20°C for 4 min and then dissected in ice-cold phosphate buffer saline (pH 7.4) under a dissecting microscope. The salivary gland complex, including all lobes, accessory glands, and tubules, was exposed by holding the abdomen with fine forceps and pulling the head and prothorax away from the abdomen with another pair of fine forceps. The anterior midgut was exposed by holding the body with forceps and pulling the ovipositor and last three or four segments of the abdomen away from the rest of the abdomen with another pair of forceps.

The salivary glands of 25 insects were removed and placed in 1 ml of phosphate buffer for all assays except pectinase and hyaluronidase assays, where 10 insects were used, and the tissues were placed in 400 μl of buffer. The tissues were homogenized and then centrifuged at $15,294 \times g$ for 10 min at 4°C . The super-

natant was placed in a 1.5-ml centrifuge tube and kept at 4°C until use (within 48 h). The anterior midguts of the same 25 or 10 insects were treated as were the salivary glands. Protein concentrations of all enzyme samples were determined by bicinchoninic acid protein assay (Pierce, Rockford, IL), using bovine serum albumin as the standard. Three samples from 25 or 10 insects were used for each tissue.

α -Amylase Assay. Amylase activity in the salivary glands and anterior midgut was determined with a diagnostic kit (No. 577-3, Sigma, St. Louis, MO) following modifications of Zeng and Cohen (2000a, 2000b, 2000c). The substrate was 4,6 ethyldiene (G7)-*p*-nitrophenyl (G1)- α , D-maltoheptaside. Enzyme extracts (10 μl) were added to wells in an ELISA plate. The substrate was equilibrated to 37°C for 10 min, and 200 μl was added to each well. The plate was shaken for five s and incubated at 37°C for 30 min. Absorbance was read at 405 nm in a plate reader (SPECTRA MAX Plus, Molecular Devices Corporation, Sunnyvale, CA). Absorbance is directly related to amylase activity. Authentic α -amylase from barley malt (Sigma A-2771) was used as a positive control (1 mg/ml, 1 U/mg solid), and buffer and substrate only were used as a negative control. The relative amylase activity was calculated as absorbance units per milligram of protein. The assay was performed three times for each sample.

α -Glucosidase Assay. α -Glucosidase activity was tested from both salivary gland and anterior midgut extracts, following Agustí and Cohen (2000), by adding 100 μl of extract to 100 μl of 10-mM solution of *p*-nitrophenyl α -D-glucopyranoside (Sigma, N-1377) in an ELISA plate and incubating at 37°C for 1 h. The reaction was stopped by adding 100 μl of 15% Na_2CO_3 . Invertase from bakers yeast (Sigma I-4504) was used as a positive control (1 mg/ml, 400 U/mg solid), and buffer and substrate only were used as a negative control. Absorbance was read at 405 nm in a plate reader, and the relative α -glucosidase activity was calculated as absorbance units per milligram of protein. The assay was replicated three times.

General Protease Assay. General protease activity was determined using the EnzChek fluorescence protease assay kit (E-6638, Molecular Probes, Eugene, OR) following modifications of Zeng and Cohen (2000a). Extract samples were diluted by adding 10 μl of extract to 90 μl of the reaction buffer in an ELISA plate well, and then 100 μl of substrate solution buffer (casein derivatives heavily labeled with the pH-insensitive BODIPY FL-dye) was added. The assay plate was incubated and protected from light for 1 h at room temperature ($\approx 25^\circ\text{C}$). Fluorescence, which is proportional to protease activity, was measured in a fluorescent plate reader (SPECTRA MAX Plus, Molecular Devices Corporation, Sunnyvale, CA), equipped with standard fluorescein filters, at excitation/emission, 485/538 nm. Authentic trypsin from bovine pancreas (Sigma T-8003) and buffer and substrate only were used as positive (1 ml/mg, 10,000 BAEE units/mg protein) and negative controls, respectively. Relative protease activity was calculated as fluorescence units

per milligram of protein. The assay was performed three times for each sample.

Specific Protease Assays. Trypsin-like, elastase-like, and chymotrypsin-like enzymes were analyzed from salivary glands and anterior midguts, following the methods of Agustí and Cohen (2000), using 10 μM of specific nitroanilides for trypsin, elastase, and chymotrypsin, respectively. The substrates for trypsin and elastase, $\text{N}\alpha$ -benzoyl-L-arginine-*p*-nitroanilide (Sigma B-3133) and succinyl-alanyl-alanyl-alanyl-*p*-nitroanilide (Sigma S-4760), respectively, were dissolved in dimethylsulfoxide (DMSO) and added to phosphate buffer to make a 10- μM solution. The substrate for chymotrypsin, benzoyl-L-tryosine-*p*-nitroanilide (Sigma B-6760), was dissolved in dimethylformamide (DMF) and added to phosphate buffer to make a 10 μM solution. In ELISA plate wells 50 μl of each extract was added to 200 μl each of the three substrates. Trypsin from bovine pancreas (Sigma T-8003), elastase from porcine pancreas (Sigma E-0258), and α -chymotrypsin from bovine pancreas (Sigma C-7762) were used as the positive controls (1 mg/ml; 10,000 BAEE units, 3–6 U, and 40–60 U/mg protein, respectively) and buffer and substrate only was used as a negative control. After a 20-h incubation at 37°C, absorbance was read at 405 nm in a plate reader and the relative protease activity for each substrate was calculated as absorbance units per milligram of protein. The assays were performed three times for each sample.

Pectinase Assay. Pectinase activity was tested both from salivary gland and anterior midgut extracts following Agustí and Cohen (2000) with slight modification. Extract consisting of 100 μl was added to 4 ml of 0.5% pectin from citrus fruits (Sigma P-9135) in phosphate buffer saline (pH 7.4) and incubated at 37°C for 20 h. The pectin-extract solution was passed through a #150 Cannon-Fenske routine type viscometer (Cannon Instrument, State College, PA) at 25°C. Heat denatured extract was used as a negative control. Pectinase activity was measured as the reciprocal of viscosity (1/centistokes) per milligram of protein, because pectinase activity is indicated by viscosity reduction rather than a direct reading of viscosity.

Hyaluronidase Assay. Hyaluronidase activity was tested from both salivary gland and anterior midgut extracts similarly to pectinase. A 0.2% solution of hyaluronic acid from rooster comb (Sigma H-5388) in phosphate buffer saline (pH 7.4) was used as the substrate. The reciprocal of viscosity was used to express enzyme activity.

Scanning Electron Microscopy Preparation. Sixteen heads, including mouthparts, of *D. nebulosus* were placed in 95% ethanol for at least 24 h and then air dried. Dry heads were placed, labium up, on aluminum stubs with double-sided tape. Stylets were removed from the labium with an insect pin. Stylet bundles were kept together or were separated into their mandibular and maxillary stylets by placing a minuten pin at the base of the stylets and moving it toward the apex or by sliding forceps gently over the stylets from the base to the apex. Specimens were gold

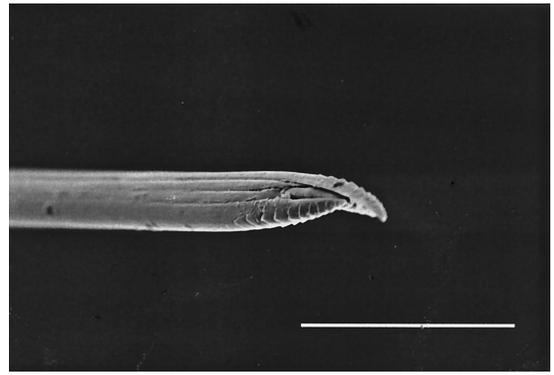


Fig. 1. Anterolateral view of the stylet bundle of *Deraeocoris nebulosus*. The two mandibular stylets are outside the two maxillary stylets. Scale bar = 50 μm .

coated with a sputtering device and viewed in secondary emission mode in a Hitachi 3500 scanning electron microscope at 10 kV.

Results

Digestive Enzymes. Amylase activity was detected in the anterior midgut (0.85 ± 0.05 SE, $n = 9$) but not in the salivary gland complex, and glucosidase activity was found in the anterior midgut (0.91 ± 0.04 SE, $n = 3$) and salivary gland complex (0.11 ± 0.13 SE, $n = 3$). General protease activity was observed from the anterior midgut (99.89 ± 12.29 SE, $n = 9$) and salivary gland complex ($4,491.44 \pm 304.13$ SE, $n = 9$).

Trypsin-like activity was detected in the salivary gland complex (1.90 ± 0.44 SE, $n = 3$) and anterior midgut (0.04 ± 0.01 SE, $n = 3$). Elastase-like and chymotrypsin-like activities were found from the anterior midgut (0.07 ± 0.04 SE, $n = 3$ and 0.03 ± 0.02 SE, $n = 3$, respectively) but not from the salivary gland complex.

Pectinase activity was found in the salivary gland complex (0.74 ± 0.02 SE, $n = 3$) and in the anterior midgut (0.18 ± 0.04 SE, $n = 3$). Hyaluronidase was not detected in either the salivary gland complex or the anterior midgut.

Mouthpart Morphology. The stylet bundle of *D. nebulosus* has two maxillary stylets inside two mandibular stylets (Fig. 1). The insect's right maxillary stylet has two rows of at least six recurved barbs on the inner surface pointing away from the head and the barbs are longer closer to the tip (Fig. 2).

Discussion

Proteases. The presence of protease activity in the salivary gland complex of this predator was expected. Trypsin-like protease was the only protease detected in the salivary gland complex, but the anterior midgut had low levels of trypsin-like, elastase-like, and chymotrypsin-like proteases. The presence of trypsin-like enzymes demonstrates the insect's ability to access structural or other insoluble proteins (Cohen 1993,

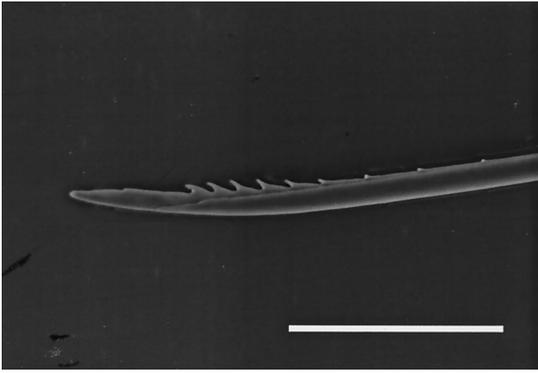


Fig. 2. Lateral view of the right maxillary stylet of *Deraeocoris nebulosus*. Scale bar = 50 μ m.

1998a, 2000). Possession of trypsin-like enzymes and the lack of elastase-like and chymotrypsin-like enzymes in the salivary gland complex is common for heteropteran predators (Cohen 1993, 1998a, 2000; Agustí and Cohen 2000). Trypsin-like enzymes are endoproteases that attack proteins at residues of arginine and lysine, but chymotrypsin-like enzymes, also endoproteases, attack proteins at aromatic residues (e.g., tryptophan). Elastase-like protease has been identified only in the salivary glands of one mirid, *Lygus hesperus* Knight (Zeng and Cohen 2001) and in the salivary glands of the reduviid, *Zelus renardii* Kolenati (A.C.C., unpublished data). These authors found more elastase activity in field-caught insects than those reared in the laboratory. Individuals of *D. nebulosus* in this study were reared in the laboratory, but another species, *D. nigritulus* Knight, was field collected and in a similar study also had no detectable elastase activity (D.W.B., unpublished data).

The strictly phytophagous mirid *Poecilocapsus lineatus* (F.) lacks detectable digestive proteases in its salivary gland complex (Cohen and Wheeler 1998), indicating an inability to use animal protein. However, the phytozoophagous mirids *Lygus lineolaris* (Palisot de Beauvois) and *L. hesperus* have trypsin-like and elastase-like enzymes but not chymotrypsin-like enzymes in their salivary gland complexes (Agustí and Cohen 2000), demonstrating their ability to use animal protein for nutrients. *Lygus hesperus* produces more elastase when given an elastin-spiked artificial diet (Zeng and Cohen 2001). The green mirid *Creontiades dilutus* (Stål) was the first mirid shown to have chymotrypsin-like activity in the salivary glands (Colebatch et al. 2001). The trypsin-like enzyme secreted into the food from the salivary glands through the salivary canal is thought to be ingested by the predator along with food and employed in the gut to continue breakdown of proteins (Cohen 1998b).

Low levels of activity of all three specific proteases—trypsin-like, elastase-like, and chymotrypsin-like—in the anterior midgut indicate either the ability of *D. nebulosus* to synthesize proteases in the gut or the presence of symbiotic microorganisms in the gut. These alkaline endoproteases help break down pro-

teins once they are inside the gut, making the proteins absorbable after further hydrolysis by exopeptidases such as aminopeptidase and carboxypeptidase.

Amylase. The salivary gland complex has no detectable amylase activity. The anterior midgut has approximately equal levels of α -amylase and α -glucosidase activity. *Deraeocoris nebulosus* might ingest intact starch granules, with starch digestion occurring completely in the midgut. Also, the ingestion of soluble glycogen from prey may be an alternative or additional explanation for the presence of amylase in the gut and its absence in the salivary glands. The distribution of amylase in either or both the salivary glands or guts of heteropterans in various ecological niches remains to be clarified. Conversely, plant-feeding mirids usually have high levels of amylase in the salivary glands (e.g., Agustí and Cohen 2000). These amylase secretions are thought to be ingested by the mirid along with partially digested starches to be used in the midgut to continue the breakdown of starch (Hori 1973, Takanona and Hori 1974, Wheeler 2001). The lack of detectable amylase in the salivary glands of *D. nebulosus* indicates that plant material is not a large part of its diet; this fundamental enzyme is found in the salivary glands of phytophagous heteropterans (Hori 2000). *Deraeocoris pulchellus* (Reuter) [as *D. punctulatus* (Fallén); see Kerzhner and Josifov (1999)] has "very low activity" of amylase in its salivary glands (Hori 1972). *Deraeocoris nigritulus*, a predator found on *Pinus virginiana* Miller has no detectable amylase activity in its salivary glands (D.W.B., unpublished data).

The mainly predacious geocorid, *Geocoris punctipes* (Say), which uses plant material for nutrients, has amylase activity in its salivary glands, indicating its ability to digest starches of plants before ingestion (Zeng and Cohen 2000a). *Orius insidiosus* (Say), a predacious anthocorid, has amylase activity, detected by whole-organism homogenization, demonstrating its ability to digest starch (Zeng and Cohen 2000b). Cohen (1996) showed that the predators *Nabis alternatus* Parshley (Nabidae) and *Sinea confusa* Caudell (Reduviidae) lack amylase activity in their salivary glands; another reduviid, *Zelus renardii*, shows amylase activity in its salivary glands. Although closely related species often have similar enzymes, each species should be studied individually for the presence or absence of enzymes.

α -Glucosidase. The presence of α -glucosidase in the salivary glands suggests that *D. nebulosus* can break down the sugary water from plants before ingestion, unless this enzyme is a nonsecretable cellular enzyme of the salivary glands as suggested by some heteropteran studies (Hori 2000). Twelve other mirids were found to have low levels of glucosidase in the salivary glands (Agustí and Cohen 2000, Hori 2000). Because the substrates of α -glucosidase are water soluble and commonly found might explain why only low levels of this enzyme are found in the salivary glands of many heteropterans. One notable exception is the coreids, which have strong glucosidase activity (Taylor and Miles 1994, Hori 2000).

Pectinase. The presence of pectinase in the salivary glands and midguts indicates an ability to feed on plants by attacking cells held together with pectin complexes. This enzyme, supposedly characteristic of the Miridae (Takanona and Hori 1974, Wheeler 2001), might indicate that *D. nebulosus* has evolved from a phytophagous species. Hori (2000) suggested that, among the Heteroptera, pectinase is a fundamental enzyme only in the Miridae. Pectinase might play a significant role in egg-laying behavior. Nearly all known mirids lay their eggs in plant material; some deposit eggs on plants (Wheeler 2001). Preoviposition behavior includes probing the plant with the rostrum—sometimes for more than 10 min (Knight 1941)—before placing the ovipositor in the plant material (Ferran et al. 1996, Wheeler 2001). Hori and Miles (1993) found “strong” pectinase in saliva discharged without stimulation from the tip of the stylets of *Creontiades dilutus* (Stål) (Hori 2000). Pectinase found in mirid zoophages, such as *D. nebulosus* and *D. nigrifulvus* (D.W.B., unpublished data), might be used to soften the plant material before oviposition. This possible function merits further study.

Hyaluronidase. Hyaluronidase is a common component of the venoms of scorpions, spiders, and at least one reduviid, *Platymeris rhadamantus* Gaerst (Edwards 1961). As a component of venom, it disrupts extracellular matrix and basement membranes, allowing venoms to disperse throughout organs and tissues. Hyaluronidase is used to disrupt basement membranes of organs and tissues inside the prey, acting as a tissue-macerating enzyme (Cohen 1998a, 1998b). The absence of this enzyme, therefore, raises questions about how *D. nebulosus* gains access to nutrients from the tissue matrix. Other types of tissue-macerating enzymes might be employed by this species, including chondroitinases or collagenase, or *D. nebulosus* might use predominantly mechanical means of tissue maceration. The presence of a deeply serrated rasp on the right maxillary stylet of *D. nebulosus* (Fig. 2, see discussion below) might compensate for the lack of hyaluronidase. The rasp might mechanically disrupt the organs and tissues, while the enzymes the mirid does have (e.g., trypsin) further macerate the tissue chemically (Cohen 2000).

Stylets. The mandibular stylets of *D. nebulosus* (Fig. 1) are typical of the Miridae whether phytophagous or carnivorous (Cobben 1978, Cohen 1996, Boyd 2001, Wheeler 2001). Many other heteropterans produce a salivary flange that is used as a fulcrum, among other functions, for stylet movement (Cohen 1998b, 2000). Because mirids do not produce a salivary flange, the apical serrations on the mandibular stylets are considered adaptive in holding onto tissues below the outer layer of the prey or host plant and in producing a fulcrum for movement of the maxillary stylets (Cobben 1978, Cohen 2000, Wheeler 2001).

Cobben (1978) suggested that the inner surface of the right maxillary stylets of heteropterans ranged from moderately serrated in predatory families (e.g., Anthocoridae, Nabidae) to smooth in strictly phytophagous families (e.g., Tingidae), with the Miridae

representing an intermediate condition (Wheeler 2001). Boyd (2001) observed maxillary stylets from 20 mirids, ranging from strictly phytophagous to strictly zoophagous, and suggested that the serrations of the right maxillary stylets are deeper for predators than for herbivores. The deep serrations in the right maxillary stylet of *D. nebulosus* (Fig. 2), which are similar to those of *D. olivaceus* (F.) (Cobben 1978), probably are used to disrupt prey by ripping and tearing tissues (Cohen 2000). The barbs point away from the head which indicate that the cutting action occurs when the stylet is thrust forward, unlike predacious pentatomids which have barbs on the mandibular stylets pointing toward the head (Cohen 1996).

Conclusions. *Deraeocoris nebulosus* has an arsenal of digestive enzymes suitable for zoophagy. The salivary enzymes include trypsin-like protease, which is essential in breaking down otherwise insoluble proteins in the prey. These enzymes and the deep serrations of the maxillary stylets enable this predator to break down tissues of arthropods mechanically and chemically (Cohen 2000). The presence of α -glucosidase and pectinase in the salivary glands indicates the ability of *D. nebulosus* to use plant material for more than just water uptake.

The presence of α -amylase activity in the midgut indicates the ability of *D. nebulosus* to digest starch from plants. α -Glucosidase in the midgut enables the bug to break down sugar obtained from starch or soluble glycogen from prey into monomeric units for absorption. The ability to use plant material might enable *D. nebulosus* to survive in the absence of prey for short periods of time, which would enhance the potential of this bug as a biological control agent (Naranjo and Gibson 1996).

Deraeocoris nebulosus is equipped mainly for zoophagy but has enzymes that would allow some degree of phytophagy. This mirid is a voracious predator of many species of arthropods and might be able to sustain itself on plant material for short periods of time. Its further evaluation as a biological control agent is warranted.

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