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Using Microscopy to Assess Chorion Structural Integrity and Parasitoid Oviposition Sites on Stink Bug (Hemiptera: Pentatomidae) Eggs

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Abstract: Previous efficacy studies found that many insecticides used by growers could be having an adverse effect on egg parasitoids (*Telenomus podisi*) developing in the eggs of the brown stink bug (*Euschistus servus*), while unhatched stink bugs experienced lower levels of mortality. One plausible explanation for this was that insecticides might enter parasitized eggs more readily via oviposition wounds. Parasitized *E. servus* eggs, as well as nonparasitized stink bug (*Acrosternum hilare*, *E. servus*, *Murgantia histrionica*, and *Podisus maculiventris*) eggs, were examined using electron microscopy. Egg response to perforation by a tungsten probe served as a control. Microscopy images depicted the chorion surface as characterized by a matrix of ridges and micropylar processes in a ring around the margin of the operculum. Observations of oviposition sites showed a "scab" formed where the ovipositor penetrated the chorion, and at sites penetrated by the probe. These formations appeared to be the result of fluids from inside the egg leaking out, drying, and hardening after oviposition or probe perforation, suggesting that the response was not due to substances secreted by the parasitoid. Further, no open wounds or holes were seen to increase the possibility of insecticides entering parasitized eggs.

Key words: Pentatomidae, egg chorion structure, parasitoid oviposition, scanning electron microscopy

Introduction

In Virginia, major stink bug (Hemiptera: Pentatomidae) pests are the brown stink bug, *Euschistus servus* (Say), and the green stink bug, *Acrosternum hilare* (Say), which are bivoltine. They are polyphagous, feeding on the fruiting bodies of shrubs, trees, grasses, row crops, fruiting vegetables, and fruit, and can survive on weedy hosts (McPherson & McPherson, 2000). Insecticide applications targeting stink bugs often kill beneficial parasitoids and predators along with the target pests (Croft & Brown, 1975). Stink bug egg parasitoids such as *Telenomus podisi* Ashmead (Hymenoptera: Scelionidae) are present throughout the summer, parasitizing up to 50% of stink bug egg masses (Koppel et al., 2009) in southeastern Virginia, and could be subject to insecticide sprays as adults, or while developing inside stink bug eggs.

Previous studies demonstrated that applications of acephate, λ-cyhalothrin, spinosad, and thiamethoxam result in significantly greater mortality for parasitoids developing in stink bug eggs than developing stink bugs, but there is little explanation in the literature for why this occurs. A possibility is that the process of oviposition changes the physical structure of the egg chorion, leaving a large hole for insecticides to pass through.

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The general structure of pentatomid eggs is well described (Esselbaugh, 1946; Bundy & McPherson, 2000). The egg shell is referred to as the chorion and is composed of sclerotized proteins (Nation, 2002) that may form spinose, reticulate, or triangular patterns, which vary among stink bug species (Bundy & McPherson, 2000). At the top of the egg is a circular lid, the operculum, which is loosely attached to the stink bug egg and provides an opening for emergent nymphs (Esselbaugh, 1946). Micropyles are tube-like hollow protrusions of the chorion that are arranged in a circle around the operculum (Esselbaugh, 1946). Beament (1948) studied the properties of *Rhodnius prolixus* Stål (Hemiptera: Reduviidae) eggs, stating that it would serve as a "type specimen" for hemipterous pest-species' eggs, and determined that micropyles, which penetrate through all the layers of the chorion, are crucial to the transport of insecticides into eggs because chorion is impermeable to ovicidal or toxic substances (Beament, 1952). When the micropylar regions of eggs were submerged in ovicide, it was found that many more eggs were killed than when only the rear of the egg was submerged. After exposure to stained ovicides, eggs containing dead insects were observed to have at least one stained micropyle (Beament, 1948).

Scanning electron microscopy was utilized to closely examine the chorion and micropylar processes of different species of pentatomid eggs, some of which were punctured with a tungsten probe. Parasitized egg chorion was also examined for evidence of physical damage caused by oviposition.

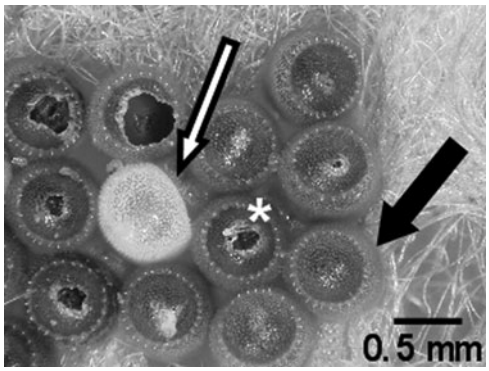

Figure 1. One nonparasitized (white arrow on light-colored egg) and 11 parasitized (black arrow one of the dark-colored eggs) eggs of *E. servus*. Eggs in which parasitoids have already hatched are characterized by an exit hole (asterisk on an example egg), and no oviposition sites are visible at this magnification. This light micrograph was taken using a Wild MP 400 Stereo Microscope.

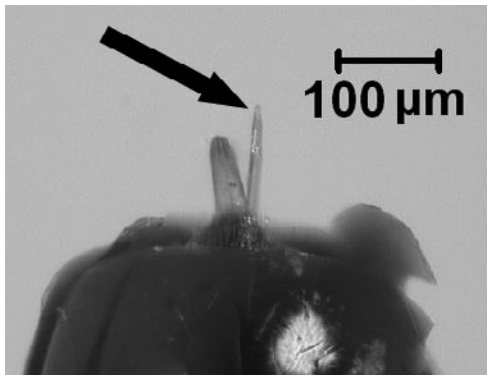

Figure 2. Abdomen (anterior) and ovipositor (posterior, arrow) of the egg parasitoid *T. podisi*. Image taken using a Wild MP 400 Stereo Microscope.

Materials and Methods

Nonparasitized and Parasitized Eggs

All nonparasitized eggs were obtained from a laboratory-reared colony of stink bugs using methods described by Koppel et al. (2009), except for eggs of the spined soldier bug, *Podisus maculiventris* (Say), which were found in a soybean field located at the Virginia Tech Tidewater Agricultural Research and Extension Center (TAREC), Suffolk, VA, USA. To obtain parasitized eggs of *E. servus*, *T. podisi* founders were field collected. Fresh *E. servus* egg masses, obtained from the laboratory, were pinned to the undersides of soybean leaves and on stems of host plants, which were located on the margins of fields, on sunny days with light wind. Eggs were exposed to partial sunlight and darkness for 48 h, there was no rain during this time, and no observable evidence of predation. The egg masses were collected and returned to a growth chamber [24.4°C, 85% R.H., 14:10 (L:D)] in the laboratory. Approximately 6 days later, the eggs changed color to black, signaling parasitization (Fig. 1). Parasitized *A. hilare* eggs were recovered from a soybean field located at the Virginia Tech TAREC and also stored in a growth chamber. All eggs were cultured in growth chambers within a 14 day period and then immediately stored at 5°C to optimize preservation.

Punctured Eggs

Nonparasitized eggs from the colony, all of which were no more than 7 days old, were punctured with a tungsten probe to create an artificial oviposition wound. To determine an appropriate probe diameter, a female *T. podisi* adult was dissected under a Wild MP 400 Stereo Microscope. The ovipositor sheath was separated from the ovipositor, and multiple stereo microscope images were edited into one, using Syncroscopy Automontage software (Synoptics Ltd., Frederick, MD, USA). The same software was also used to measure the diameter of the ovipositor, which was approximately 10 μm across (Fig. 2). A Picoprobe® Model T-4 Series tungsten probe tip with a shaft diameter of 10 μm was gently pressed against the egg chorion while being observed with a Wild MP 400 Stereo Microscope. The egg was considered to be punctured after the probe broke the outer shell of the chorion and had penetrated approximately a millimeter into the egg. After penetration, the probe was removed from the egg, cleaned using a damp tissue, and observed under the stereo microscope to confirm cleanliness. The eggs were allowed to remain at room temperature for 24 h before being placed in a 5°C refrigerator. *E. servus*, *A. hilare*, and harlequin bug, *Murgantia histrionica* (Hahn), eggs were punctured. Although nonparasitized *P. maculiventris* eggs were observed, the number of available eggs was severely limited, and none were punctured for this study.

All egg masses observed in this study contained between 10 and 15 eggs. There were five replicates each of nonparasitized, parasitized, and punctured *E. servus* and *A. hilare* egg masses, five replicates of nonparasitized and punctured *M. histrionica* egg masses, and five replicated of nonparasitized *P. maculiventris* observed using scanning electron microscopy.

Microscopy

Initial microscopy was performed at the Virginia Tech Institute for Critical Technology and Applied Science (Blacksburg, VA) using a FEI Quanta 600 FEG (FEI Company, Hillsboro, OR, USA) environmental scanning electron microscope (SEM), used in standard SEM mode. Eggs were attached to small aluminum stages with carbon tape and sputter coated with Au/Pd at 5 nm thickness while rotating and tilted 30° from horizontal. They were loaded into the microscope and observed under a vacuum of 10^{-5} torr at an accelerating voltage of 5 keV, a beam current spot size of 4, and a working distance of 10 mm. Further analysis was performed at Virginia State University (Petersburg, VA, USA) using a TM-1000 tabletop variable pressure SEM (Hitachi High Technologies America, Inc., Santa Clara, CA, USA). Eggs viewed in the TM-1000 SEM were attached to acetone-cleaned aluminum stubs with double-sided carbon tape. The samples were then loaded into the microscope and

observed under low vacuum at an accelerating voltage of 15 kV and an emission current of 44.5 mA.

Egg diameter and micropyle length were measured manually from each digital image. The micron mark generated with each digital image was the reference.

Results

Nonparasitized Eggs

The nonparasitized eggs of *E. servus*, *A. hilare*, *P. maculiventris*, and *M. histrionica* were observed. Observations about the eggs of the former three species are consistent with those of Bundy and McPherson (2000). *E. servus* eggs (Fig. 3a) were about 900 ± 11 μm in diameter with a length of 50 ± 5 μm per micropyle (Fig. 3b). There was an average of 33 ± 2 micropyles per egg. *A. hilare* eggs (Fig. 3c) were comparatively large and averaged 1,000 ± 8 μm in diameter. There was an average of 50 ± 9 micropyles (Fig. 3c) per egg, each of which was approximately 50 ± 9 μm in length (Fig. 3d). *P. maculiventris* eggs (Fig. 3e) were about 300 ± 15 μm in diameter and had 14 ± 2 micropyles per egg. The micropyles were the longest of the four stink bug species, about 280 ± 15 μm in length (Fig. 3f). The egg of the final stink bug species, *M. histrionica*, measured approximately 950 ± 10 μm in diameter (Fig. 3g). Each egg had 25 ± 2 micropyles, each measuring about 35 ± 5 μm in length (Fig. 3h). These micropyles seemed to be recessed in the egg chorion. *Murgantia histrionica* observations were in agreement with Esselbaugh (1946).

Parasitized and Punctured Eggs

Parasitized *E. servus* (Fig. 4a) and *A. hilare* (Fig. 4b) eggs featured a scab-like protrusion at most oviposition sites. All of the oviposition sites scabbed over, with an average scab diameter of 92 ± 7.18 μm and 66 ± 6.88 μm in length. Protrusions were distinct from the surface of the egg chorion. Eggs that were punctured with a tungsten probe also formed "scabs" (Fig. 4c–e), although there was only a small hole surrounded by solidified fluid from within the egg at two puncture sites. The diameter of scabs that formed on different species of stink bug eggs varied greatly, from 100–600 μm, averaging 318 ± 24.82 μm. The height was more consistent, averaging 81 ± 8.57 μm. Scabs apparently formed within 24 h on both parasitized and punctured eggs, oviposited on or punctured.

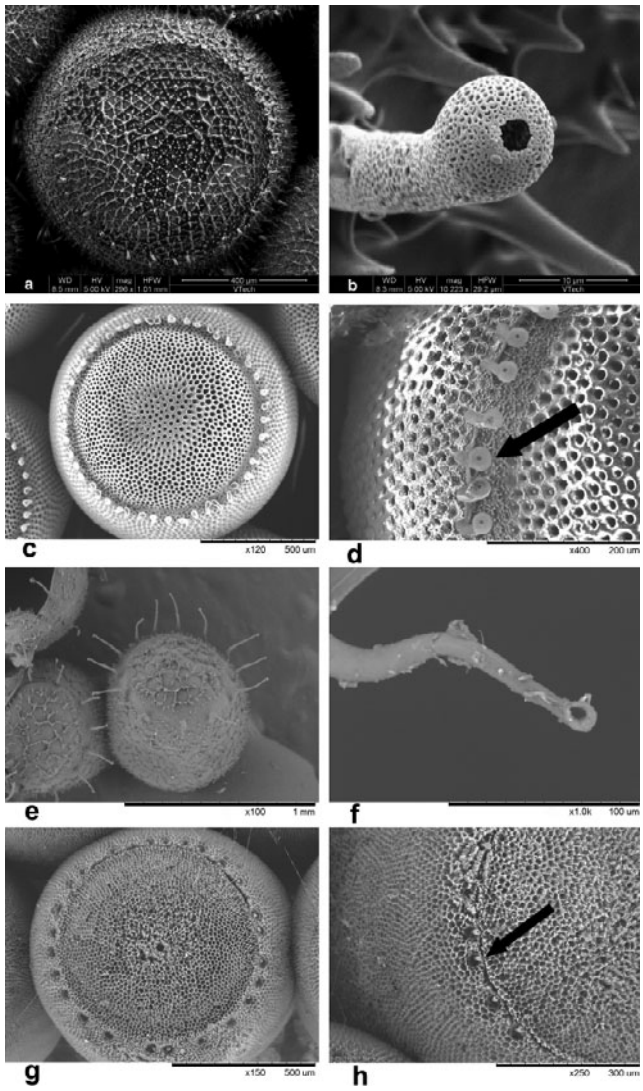

Figure 3. Nonparasitized pentatomid eggs and micropylar processes. Note that micrographs of eggs and micropyles were selected for this article based on overall image quality and may not show eggs or structures at an angle suited to measure exact dimensions. **a:** *E. servus* egg (bar: 400 μm). **b:** Micropylar process of an *E. servus* egg (bar: 10 μm). **c:** *A. hilare* egg (bar: 500 μm). **d:** Micropylar processes (arrow) of an *A. hilare* egg (bar: 200 μm). **e:** *P. maculiventris* egg (bar: 1 mm). **f:** Micropylar process of a *P. maculiventris* egg (bar: 100 μm). **g:** *M. histrionica* egg (bar: 500 μm). **h:** Micropylar processes (arrow) of an *M. histrionica* egg (bar: 300 μm).

Discussion

All images of eggs are representative of the total population analyzed in this study. As previously stated, our measurements of egg diameters, micropyle lengths, and number of micropyles per egg are consistent with those previously published by Bundy and McPherson (2000) and Esselbaugh (1946). There is no previously reported data for the diameter of the *T. podisi* ovipositor, including in Johnson's (1984) systematic description of the species.

Observations suggest that when nonparasitized *E. servus* and *A. hilare* eggs are punctured by the ovipositor of a parasitoid, there is no permanent hole in the chorion. Rather, a scab forms over the oviposition wound. The surface of the egg chorion and appearance of scabs remained consistent regardless of where the stink bug egg masses were parasitized in the field. When punctured with a probe, there is a similar hardening of internal egg fluids on the surface of the egg. Since the scab forms as a result of oviposition and a probe wound, the scabbing process is most likely a result of egg physiology, rather than substances secreted by the parasitoid.

The scabs that formed from probe wounds were more fluid and less set in appearance than those formed via oviposition. A human hand puncturing an egg with a probe

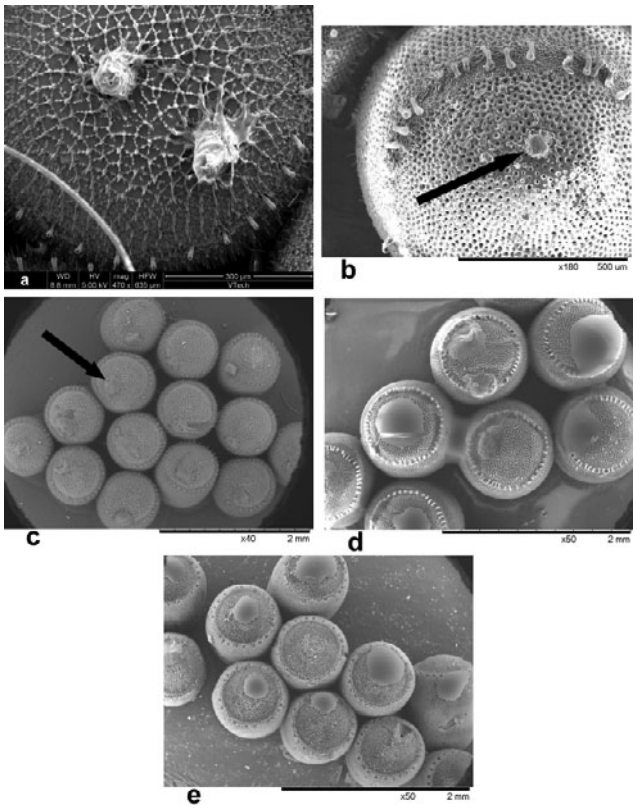

Figure 4. Scabs formed after egg parasitoid oviposition or puncturing eggs with a tungsten probe. Note that micrographs of eggs and micropyles were selected for this article based on overall image quality and may not show eggs or structures at an angle suited to measure exact dimensions. **a:** Scabs on the chorion of an *E. servus* egg formed after oviposition (bar: 200 μm). **b:** Scabs on the chorion of an *A. hilare* egg formed after oviposition (bar: 500 μm). **c:** Scabs (arrow) on the chorion of *E. servus* eggs formed on all pictured eggs after puncturing eggs with a probe (bar: 1 mm). **d:** Scabs on the chorion of *A. hilare* eggs formed on all pictured eggs after puncturing eggs with a probe (bar: 1 mm). **e:** Scabs on the chorion of all pictured *M. histrionica* eggs formed after puncturing eggs with a probe (bar: 1 mm).

does so with less finesse than a parasitoid using its ovipositor, making it likely that the ovipositor caused a cleaner wound, despite being the same diameter as the probe. Further, although parasitized *M. histrionica* eggs were not found or successfully reared for observation, a scab formed over probe wounds. It is likely that a scab also forms after parasitization, as it did for *E. servus* and *A. hilare*.

Overall, it is suggested that while oviposition causes obvious physical differences between nonparasitized and parasitized stink bug eggs, there are no open wounds or holes that would allow for increased entry of insecticides. However, further research could be performed to determine the exact nature of how long it takes for scabs to form and solidify, and if those scabs are permeable to insecticides while they are forming.

A further study was performed after microscopy concluded to evaluate the permeability of parasitized and nonparasitized *E. servus* eggs. ^{14}C-radiolabeled insecticides in three classes, commonly used in stink bug control, were utilized in these studies.

Conclusions

When *E. servus* and *A. hilare* eggs were punctured by the ovipositor of a parasitoid, a scab formed over the wound, and there was no permanent hole in the chorion. When punctured with a probe, there was a similar hardening of internal egg fluids on the surface of the egg. Since the scab formed in response to both oviposition and the probe wound, the scabbing process is most likely a result of egg physiology, rather than substances secreted by the parasitoid. Overall, while oviposition causes obvious physical differences between nonparasitized and parasitized stink bug eggs, there are no open wounds or holes, which could allow for increased movement of insecticides into eggs. Thus, the scabs would not seem to account for differences in insect mortality during insecticide efficacy testing.

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