A Novel Method for Estimating Soybean Herbivory in Western Corn Rootworm (Coleoptera: Chrysomelidae)

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ABSTRACT The western corn rootworm, *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae), is the key pest of corn, *Zea* mays L., in North America. The western corn rootworm variant is a strain found in some parts of the United States that oviposits in soybean, *Glycine max* (L.) Merr., thereby circumventing crop rotation. Soybean herbivory is closely associated with oviposition; therefore, evidence of herbivory could serve as a proxy for rotation resistance. A digital image analysis method based on the characteristic green abdominal coloration of rootworm adults with soybean foliage in their guts was developed to estimate soybean herbivory rates of adult females. Image analysis software was used to develop and apply threshold limits that allowed only colors within the range that is characteristic of soybean herbivory to be displayed. When this method was applied to adult females swept from soybean fields in an area with high levels of rotation resistance, 54.3 ± 2.1% were estimated to have fed on soybean. This is similar to a previously reported estimate of 54.8%. Results when laboratory-generated negative controls were analyzed showed an acceptably low frequency of false positives. This method could be developed into a management tool if user-friendly software were developed for its implementation. In addition, researchers may find the method useful as a rapid, standardized screen for measuring frequencies of soybean herbivory.

KEY WORDS western corn rootworm, soybean herbivory, image analysis, crop rotation

The western corn rootworm, *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae), is a serious pest of corn, *Zea mays* (L.), throughout the midwestern United States. The larvae feed primarily on corn, although they can complete development on some additional grass species (Branson and Ortman 1970), including several common grass weed species (Wilson and Hibbard 2004). As adults, the beetles feed primarily on corn silks, pollen, and exposed kernels (Ball 1957), although they will feed upon leaves and pollen of other plants (Ludwig and Hill 1975). In some areas, adults readily migrate to, and subsequently feed upon the foliage of, soybean, *Glycine max* (L.) Merr. Soybean is a poor dietary choice for western corn rootworm adults (Mabry and Spencer 2003); the resulting dietary stress has been shown to induce gravid females to oviposit (Mabry et al. 2004). This feeding and oviposition in soybean fields has allowed the western corn rootworm to circumvent crop rotation as a management strategy in some parts of the Corn Belt.

Initially discovered in eastern Illinois in 1987 (Levine and Oloumi-Sadeghi 1996), the prevalence of western corn rootworm damage to first-year corn has increased over time; the range now covers parts of several Corn Belt states, including Indiana, Iowa, Michigan, Missouri, Ohio, Wisconsin, and Ontario, Canada (O'Neal et al. 2002; Gray et al. 2009). After an early study suggested that rotation-resistant, or "variant," western corn rootworms were attracted to soybean as an oviposition site (Sammons et al. 1997), wind-tunnel (Spencer et al. 1999) and electroantennogram (Hibbard et al. 2002) studies found no evidence of attraction. This evidence, combined with subsequent observations of oviposition in oat, *Advena sativa* L., stubble; alfalfa (Rondon and Gray 2003), *Advena sativa* L.; and winter wheat, *Triticum aestivum* L. (Schroeder et al. 2005), suggests that a general lack of fidelity to corn as an oviposition site (exacerbated by stress-induced oviposition in soybean) is the mechanism of rotation resistance.

Rotation-resistant western corn rootworms pose a substantial threat across a large portion of the Corn Belt; yet, in any particular location the magnitude of the threat is variable. The high degree of mixing of genetically indistinguishable wild-type and rotation-resistant individuals (Miller et al. 2006) is an obstacle to predicting the threat of rotation resistance on a site-specific basis. Consequently, tools to improve the forecasting of the rootworm risk would be valuable to researchers, consultants, and their clientele. Because all major treatment options for western corn rootworm larval injury (primarily soil insecticides, seed

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coat insecticides, and *Bacillus thuringiensis* [Bt] corn hybrids) must be implemented at planting time, and sampling methods for eggs and larvae in the soil are normally impractical, sampling methods for rotation-resistant western corn rootworm currently focus on the adults present in the soybean field the previous year. Adoption of Pherocon AM sticky traps, the most commonly recommended sampling method for rotation-resistant western corn rootworm (O'Neal et al. 2001), has been poor (Rondon and Gray 2003) due to several key disadvantages. Of particular concern is the messy, labor-intensive nature of these traps (Levine and Gray 1994) and their propensity to over-represent males (Kuhar and Youngman 1995). Field sweeps allow the user to sample for western corn rootworm and other soybean insects simultaneously, and they are useful for measuring regional differences in suspected rotation-resistant rootworm abundance, but their primary drawbacks include the amount of labor required and their inability to compensate for diel differences in western corn rootworm activity and abundance in soybean (Isard et al. 2000).

Soybean is widely planted (usually in rotation with corn) throughout Indiana (≈2.2 million ha planted in 2009) and the other states that make up the current core of the variant zone (≈12.5 million ha total, planted in Illinois, Indiana, Iowa, Michigan, and Ohio in 2009; NASS 2009). The modeling work of Onstad et al. (1999) used 10–20 western corn rootworm adults per 100 sweeps in soybean as a threshold for the presence of rotation resistance. Western corn rootworm adults collected from these areas often contain soybean foliage within their gut contents (Spencer et al. 1999), despite the fact that soybean foliage is not a suitable diet for rotation-resistant or wild-type western corn rootworm adults (Mabry and Spencer 2003). Rotation-resistant adults' relative insensitivity to dietary switches may allow them to remain in soybean fields long enough after feeding on foliage to be induced to oviposit by the resulting dietary stress (Mabry et al. 2004).

As outlined above, collection of adults from soybean fields is a viable approach to estimating rotation-resistance pressure levels. Furthermore, western corn rootworm individuals from areas with high levels of rotation resistance are more likely to have soybean foliage in their guts (Spencer et al. 1999), as a close association exists between soybean herbivory and oviposition (Mabry and Spencer 2003; Mabry et al. 2004; Pierce and Gray 2006). It follows that the level of soybean herbivory observed in a sample of adult female western corn rootworms could serve as a proxy for the level of rotation-resistant rootworm activity (i.e., oviposition in soybean). A dark green coloration of the ventral abdomen due to the presence of ingested soybean foliage in the gut (Fig. 1) has been used previously to identify soybean-feeding western corn rootworm adults (Mabry and Spencer 2003). This coloration of the gut contents is externally visible through the translucent integument of the ventral abdomen.

Fig. 1. Close-up image of female western corn rootworm adults that have fed on soybean foliage (left) and corn tissue (right). Soybean tissue is visible through the integument of the ventral abdomen. (Photo: John Obermeyer). (Online figure in color.)

Admittedly, visual analysis of this green coloration is a subjective categorization. A technique capable of reducing the subjectivity of this characterization could be a useful management tool to connect this visible diagnostic character to oviposition behavior, and the frequency of soybean herbivory among females could be estimated. If herbivory frequencies were then correlated with damage to first-year corn grown in rotation with soybean, this technique could then be further developed into a predictive method for management applications.

Computer software is frequently used to perform colorimetric analyses of digital images produced by digital camera or other equipment (Hong et al. 2001), and such analyses have been applied widely in the biological and physical sciences. Here, we describe the development, validation, and potential applications of a colorimetric assay capable of estimating soybean herbivory rates among adult female western corn rootworm beetles.

Materials and Methods

Insects. *Soybean Sweeps.* To create a threshold relevant to field-captured western corn rootworms, all threshold development and validation steps were performed using adult females collected from soybean fields. Sweeps were conducted in R5- and R6-stage soybean fields at the Purdue University Agronomy Center for Research and Education (ACRE; West Lafayette, IN) from 21 August 2009 through 9 September 2009 to collect a large sample of soybean foliage-feeding western corn rootworms. Western corn rootworms feeding upon soybean were commonly observed while conducting these sweeps. Our collection area (northwestern Indiana) is close to the area where rotation resistance was initially found, and the area has exhibited high rates of rotation-resistant rootworm activity for approximately two decades (Gray et al. 2009). Insects collected during sweeps were transferred to plastic bags and temporarily stored in a cooler packed with dry ice. The sex of captured western corn rootworm beetles was determined by examination of the abdominal apex, where females lack an additional sclerite that is present on males (White 1977); females were stored at −80°C, whereas males, other insects, and plant material were discarded.

Negative Controls. A laboratory feeding assay was used to generate adult western corn rootworm females with no soybean material in their gut contents; these females were used as negative controls. These beetles were collected in cornfields (DKC61-73, Dekalb, St. Louis, MO) at ACRE from 14 July 2008 through 25 July 2008. Because female western corn rootworms are induced to disperse from cornfields by the progression of corn phenology (Pierce and Gray 2006), beetles were collected early in the season to reduce the likelihood of previous exposure to soybean. Beetles were collected using a mouth aspirator, returned to the laboratory, and then reared in groups of ≈ 50–100 on corn foliage, tassels, and silks collected from the beetles' source field in 1.025-liter plastic cups for a period of 72 h, a period of time long enough to ensure throughput of previously consumed material (Spencer et al. 2003). After the rearing period was complete, these beetles were killed by adding cucurbitacin baits laced with 3.9% carbaryl insecticide to their rearing cups and then stored at −80°C.

Threshold Development. Threshold limits for digital images were established to display only the green coloration present in the abdomens of soybean feeders by filtering out the normal coloration of nonsoybean-feeding western corn rootworm adults on a computer output. Female soybean-swept beetles and negative controls were photographed using a Nikon D40X digital SLR camera (Nikon, Tokyo, Japan) with a Nikkor 18-70 mm AF-S DX zoom lens (Nikon). To hold the insects in place for photography, a 12 by 12 array of regularly spaced ovals was created using Microsoft Office Word 2003 (Microsoft, Redmond, WA) and printed on sturdy cardstock; the ovals were subsequently cut out. The overall size of the template (13.3 by 9.5 cm) was chosen to correspond with the viewfinder of the camera/lens combination at maximum zoom; oval size (7.94 by 4.76 mm) was chosen to fit an average-sized beetle. This template was placed over white Play-Doh modeling compound (Hasbro, Providence, RI), which held the beetles in place. For each photograph, a group of beetles was centered within the template. Each individual beetle was positioned so that its ventral side was exposed through an oval opening and faced up toward the camera; the beetle head was oriented to the left. The metathoracic legs were removed from each beetle to avoid obstruction of the dorsal abdomen. Photographs were taken under fluorescent lighting; no flash was used. A constant internal focal length of 70 mm was used at a constant focusing distance of 0.38 m, which was the maximum possible zoom using this camera/lens combination. Images of groups of up to 90 individuals of 851 negative control beetles (15 images) and 569 soybean-swept beetles (13 images) were collected and stored in JPEG format (Fig. 2a).

Not all beetles swept from soybean fields show the green abdominal coloration that was the target of this analysis. Soybean-swept beetles that showed green coloration in the ventral abdomen were used in threshold development and will be referred to as "soy feeders." Uniform digital image samples of the ventral abdomens were taken from a random subset of both these soy feeders and the negative control beetles using GIMP 2.6.7 (www.gimp.org; link verified 5 February 2010) open-source image processing software. A 36 by 44 pixel oval selection was taken from the region just anterior to the apical sternum and saved as a separate image. This area corresponds to the region of the abdomen in which the green coloration of the soybean-containing beetle gut contents underneath is most clearly visible. The 36 by 44 pixel area was chosen to correspond with the area of interest on an average-sized beetle abdomen at the image size used. Samples of the ventral abdomens of 110 total beetles (55 negative controls, 55 soy feeders) were taken in this manner.

Abdominal image samples were analyzed using ImageJ (Rasband 1997-2008; http://rsb.info.nih.gov/ij/), a public-domain image processing and analysis program provided as a free download online by the National Institutes of Health (Bethesda, MD). Color analyses were performed using values from the Commission Internationale de l'Eclairage (International Commission on Illumination; CIE) L*a*b* color space (CIE LAB), which was designed to represent visual assessments of color differences mathematically (McLaren 1976). In CIE LAB, each color is assigned a multivariate numerical value consisting of three quantities: L*, a*, and b*. Each quantity falls within a range from 0 to 255 and represents a different component of perceived color: The L* quantity represents the range from dark (0) to light (255); likewise, the a* quantity ranges from green (0) to red (255), and b* ranges from blue (0) to yellow (255). The combination of values from these three channels results in a unique, descriptive color "signature" with high levels of discriminatory ability for a selected image.

Threshold Color version 1.9 (Landini 2008; http://www.dentistry.bham.ac.uk/landinig/software/software.html), a downloadable plug-in for ImageJ, was used to display three histograms of the CIE LAB values (L*, a*, and b*) of all pixels for each beetle abdomen JPG image. The peak of the histogram for each quantity was determined and considered as a measure of central tendency for the image of each female. Using these peaks, a three-coordinate variable of peak values was recorded for each image.

Cluster analysis (centroid linkage, Euclidean distance) was performed using MINITAB version 15.1 statistical software (Minitab Inc. 2006). In this analysis, multivariate points were described by the three CIE LAB peak quantities for the image of each soybean-feeding ($n = 55$) or negative control ($n = 55$) female. The number of clusters to be considered was

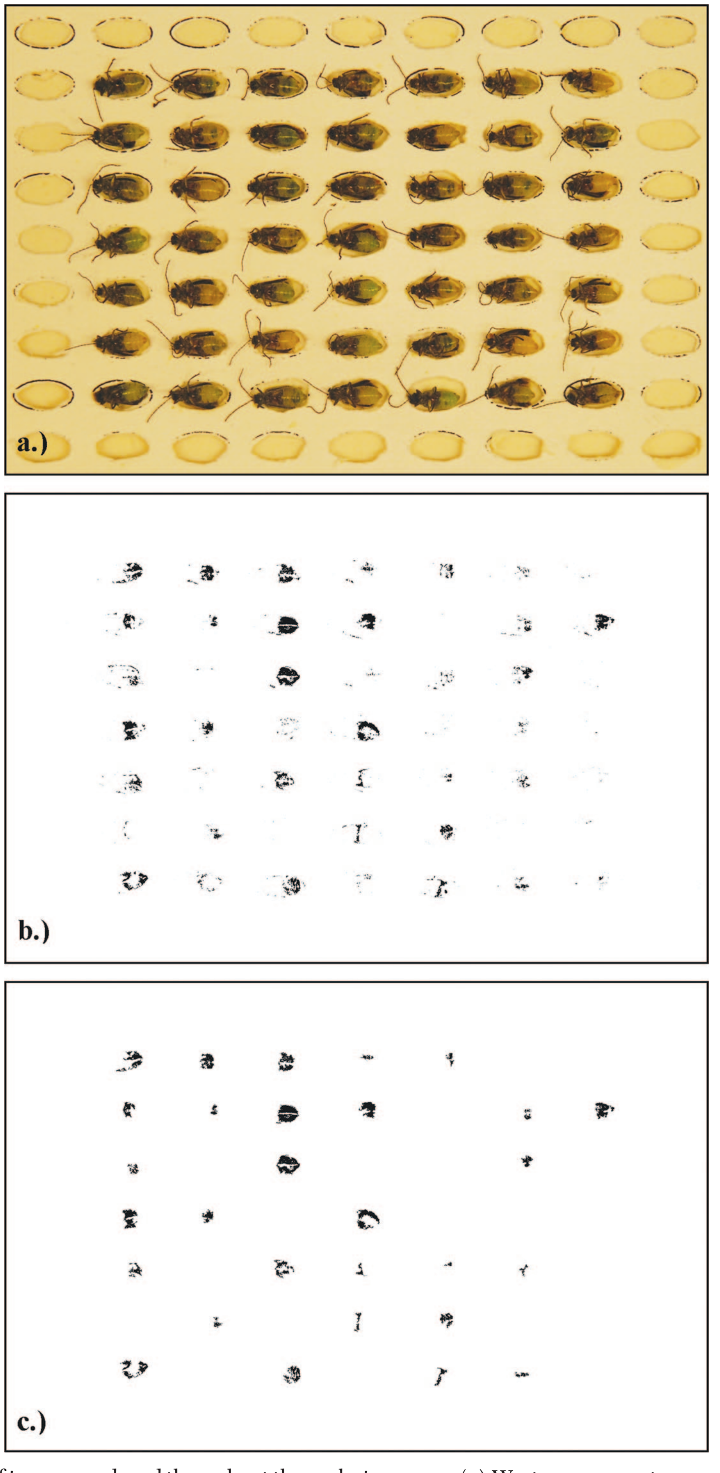

Fig. 2. Examples of images produced throughout the analysis process. (a) Western corn rootworm adult females arranged on a template and photographed. These beetles were swept from an R6-stage soybean field. (b) The resulting image with threshold limits applied using the Threshold Color plug-in for ImageJ. Note that some extraneous background shading remains. (c) The result after a lower particle limit of 100 pixels was applied to the image shown in b by using ImageJ. This is the final image from which soybean feeding status was determined; any beetle showing abdominal shading at this stage was considered positive for consumption of soybean foliage. (Online figure in color.)

chosen using the scree method (Cattell 1966). The separation between clusters was then compared with the separation based on category using a dendrogram. This analysis was done to determine which CIE LAB quantity or combination of quantities resulted in the clearest differentiation between the soybean-swept and negative control beetle groups. Based on the results of cluster analysis, an analysis of similarities (ANOSIM) was performed using Primer version 5.2.9 statistical software (Primer-E Ltd., Plymouth, United Kingdom) on the peak values from the a* and b* quantities to determine whether the difference between the two beetle groups was significant. An ANOSIM is comparable with a multivariate version of the analysis of variance (ANOVA) test; the statistic is tested using a null distribution created through randomization of the data (Clarke and Gorley 2001).

Individual color peaks for soy feeders were used to establish a range of CIE LAB values (mean peak ± 2 SDs) characteristic of soybean-feeding rootworms. The ranges obtained from the soy feeders (L*, 98–138; a*, 114–124; and b*, 173–188; see Results) were applied as threshold limits to all soybean-swept beetles (irrespective of green coloration) and negative control beetles using the Threshold Color plug-in. After threshold limits were applied, only pixels whose values were within these threshold ranges for "soybean green" were displayed (Fig. 2b). The resulting image was transformed so that pixels within the threshold ranges were black, whereas background pixels were white.

In some cases (see Fig. 2b), extraneous background shading introduced subjectivity to the determination of "positive" or "negative" soybean feeding status. The particle analysis feature of ImageJ was used to objectively filter out this extraneous shading. When using this method to filter the image, only adjacent groups of pixels, or "particles," greater than a set minimum size, or "particle limit," were displayed by the computer output. Each image was analyzed with the particle limit set at three arbitrarily chosen, progressively increasing levels: 100, 250, and 500 pixels. Each beetle with shaded areas remaining on its abdomen after particle analysis was scored as positive (Fig. 2c). The proportions of females testing positive for soybean consumption in each category were compared separately at each particle limit setting using a two-sample proportions test.

Gut Content Dissections. To verify the presence of soybean foliage in the guts of beetles showing the characteristic green abdominal coloration, gut content dissections were performed on 25 randomly selected soy feeders (soybean-swept females showing green abdominal coloration) and 25 randomly selected negative controls that had been photographed previously. Gut contents were removed by inserting dissecting forceps through the anal opening and firmly grasping the rectal tube. The hindgut was gently pulled out of the beetle through the anal opening and set aside. The abdomen was separated from the thorax and discarded, leaving the remaining alimentary tract intact and attached to the head and thorax. After the midgut was detached and set aside, the thoracic exoskeleton and attached musculature were removed and discarded. The remainder of the alimentary canal was then detached from the head.

The three separated sections composing the alimentary canal were submerged in distilled water. Ingested plant material was forced along the digestive tract with forceps until it was expelled. This consumed material, along with 10–100 μl of distilled water, was selectively imbibed using a micropipette and dispensed onto a glass microscope slide; a coverglass was used. Slide-mounted gut contents were immediately examined under a compound microscope at 40×, 100×, and 400× magnification, and any material recognized as consumed plant tissue was identified and recorded. (Because soybean pollen was not present in the field at the time samples were collected, and adults are known to feed on pollen from multiple sources [Ludwig and Hill 1975], no effort was made to identify the source of any pollen that was found.) Several permanent reference slides of the gut contents of soybean-swept and negative control beetles were prepared as identification aids.

To observe the presence or absence (and relative quantity based on visual estimation) of soybean foliage within the guts of soybean-swept females that did not show green abdominal coloration, 25 such individuals were randomly selected and dissected as described above. The gut contents of these beetles were examined under a dissecting microscope at 10–45× magnification and analyzed for presence and relative quantity (based on observations) of soybean foliage.

Blind Assay. A "blind" assay was conducted to examine how image analysis results compared with visual determination of the soybean-feeding status of beetles. Image analysis was performed by personnel that were not directly involved in the assay development process. Three groups of beetles with randomly assigned percentages of soybean feeders (20% [5/25], 36% [9/25], and 84% [21/25]) were prepared using a randomly selected subsample of the soy feeders and negative control beetles that had been used for assay development. Each beetle in a sample was randomly assigned a number from 1 to 25 to facilitate comparison of the results; the feeding status corresponding to each beetle was not revealed to the technicians performing the analyses. Two laboratory technicians each performed an independent analysis of these beetle groups using written instructions describing how to arrange the beetles, take photographs, and analyze the resulting images. Each image they produced (i.e., one for each sample) was analyzed using three different particle limits (the minimum area of shading required for a positive determination as described above): 100, 250, and 500 pixels. The results were recorded and compared with the known percentages in each case.

Results

Threshold Development. Cluster analysis using all three color channels did not yield a clear separation between the soy feeders and negative controls; how-

Table 1. Mean ± SE peaks of the CIE LAB pixel histograms from digital image samples of the ventral abdomens of western corn rootworm[a]

CIE LAB quantity	Soybean feeders	Negative control beetles
L*	117.7 ± 1.34	127.9 ± 1.60
a*	118.9 ± 0.36	133.7 ± 0.45
b*	180.7 ± 0.50	184.9 ± 0.64

[a] The abdomens of females swept from soybean showing green abdominal coloration (soybean feeders) and females reared in the laboratory on corn foliage, silks, and tassels (negative controls) were analyzed using the Threshold Color plug-in for ImageJ to determine peak values of CIE LAB quantities.

ever, when only two of the three channels (a* and b*) were considered, the groups were cleanly separated at a cutoff of three clusters: one cluster contained all of the soy feeders, one contained all but two of the negative controls, and the third contained the other two negative controls. An ANOSIM revealed a significant difference between the two groups in these same channels (global $R = 0.664$; $P < 0.001$; 999 permutations). Peak values (Table 1) for the selected soybean-swept western corn rootworm abdominal samples were determined to be distributed normally for all three CIE LAB quantities based on the Ryan–Joiner test ($P > 0.100$). Threshold ranges of L* 98–138, a* 114–124, and b* 173–188 were calculated to represent the limits containing the peak color values of 95% of the soybean-feeding population (Fig. 3). When these threshold ranges were applied to soybean-swept and negative control beetles, the proportion of beetles testing positive for soybean was significantly ($P < 0.001$) greater for soybean-swept beetles than for negative control beetles at each particle limit (Table 2).

Gut Content Dissections. Soybean foliage was abundant and readily identifiable in all 25 soybean-swept beetles showing green abdominal coloration

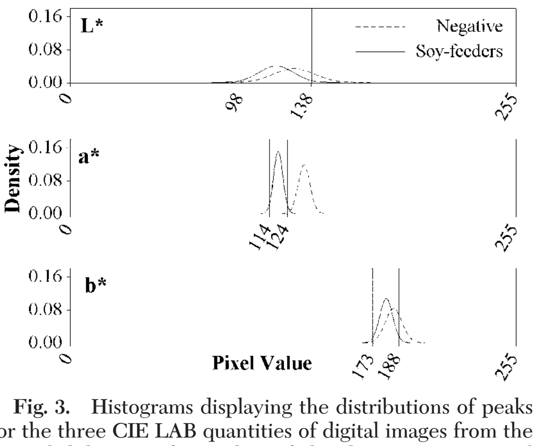

Fig. 3. Histograms displaying the distributions of peaks for the three CIE LAB quantities of digital images from the ventral abdomens of 55 soybean-fed and 55 negative control western corn rootworm females. Peaks were determined using the Threshold Color plug-in for ImageJ. Threshold limits (mean peak values ± 2 SDs define the threshold limit ranges) are marked and labeled.

Table 2. Total proportions ± SE of female western corn rootworms that tested positive for soybean foliage[a]

Particle limit	Soybean-swept beetles	Negative control beetles
100	0.543 ± 0.021	0.048 ± 0.007
250	0.446 ± 0.021	0.011 ± 0.004
500	0.357 ± 0.020	0.005 ± 0.002

[a] Threshold limits were applied to digital images of females swept from soybeans in the field (soybean-swept) and reared in the laboratory on corn foliage, silks, and tassels (negative control) by using the Threshold Color plug-in for ImageJ. Proportion testing positive for soybean-swept beetles was significantly greater than for negative control beetles for all particle limits based on two-sample proportions test ($P < 0.001$ for all three particle limits).

(Fig. 4). Soybean foliage was the dominant material present in these beetles; in addition to soybean foliage, 40% (10/25) of the beetles contained pollen (probably from corn or weeds), whereas 16% (4/25) contained corn silk or tassel material. At 100× and 400× magnification, the characteristic cell structure of the palisade tissues of soybean foliage (long, narrow, rounded cells arranged side-by-side) was observed to be intact. There was little evidence of digestion in the beetle gut: soybean foliage material found in the hindguts of these beetles was similar in structure, arrangement, and condition to that found in the fore- and midguts. Corn material was found in the guts of dissected negative controls as follows: 76% (19/25) of beetle guts contained silks; 64% (16/25) contained tassel material; 52% (13/25) contained pollen; 16% (4/25) contained foliage; and 16% (4/25) contained no recognizable corn tissue. In contrast to soybean foliage material, corn material seemed much more highly digested and was difficult to identify; cell structure and arrangement were not easily observed. Of the 25 soybean-swept beetles not showing green abdominal coloration, 44% (11/25) contained at least a trace amount of soybean foliage; however, the quantity of soybean foliage material consumed was much less than that found in beetles that showed green abdominal coloration.

Blind Assay. Each technician took roughly 2 h to complete image acquisition and analysis of the selected beetles. At each particle limit, there were 150 opportunities (two technicians × three samples × 25 beetles per sample) for the results of an individual beetle to differ from the results based on visual analysis (Table 3). For a particle limit of 100 pixels, four observations of individual beetles differed from the expected results (2.7%); two of these were false positives and two were false negatives. At a particle limit of 250 pixels, nine observations differed from the expected results (6.0%) and at a particle limit of 500 pixels, 18 observations differed from the expected results (12.0%); all conflicts between observed and expected results for 250- and 500-pixel particle limits were false negatives as additional beetles were eliminated based on the area of shading. Slight variations in the images due to slightly different zoom levels used by the two technicians caused these deviations.

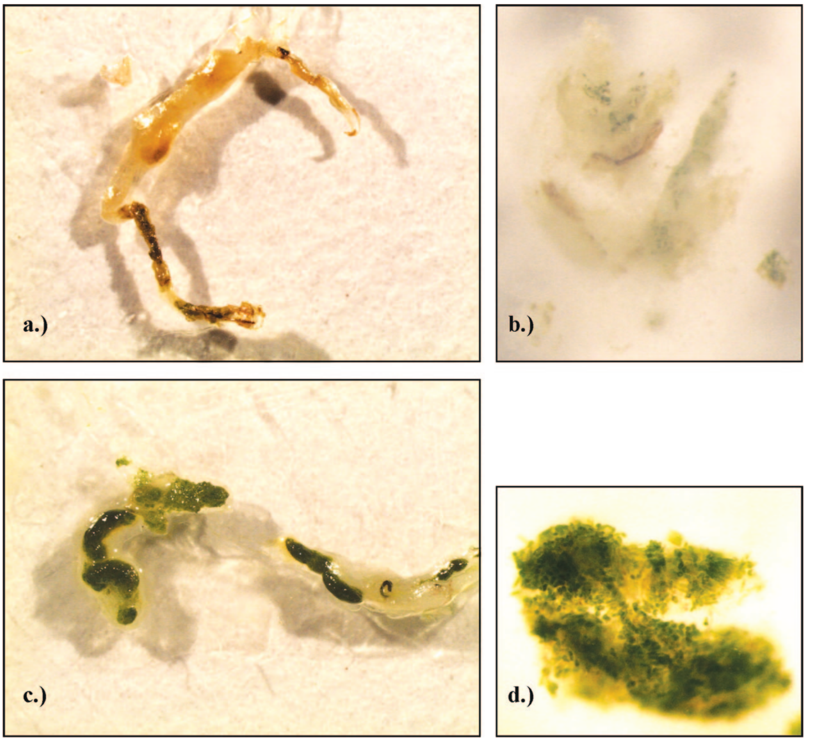

Fig. 4. Dissected guts and gut-contents of adult western corn rootworm females. Entire gut (63× magnification; a) and a sample of the gut contents (100× magnification; b) are shown for an individual that was reared on corn foliage, silks, and tassels in the laboratory. Likewise, the entire gut (63× magnification; c) and a sample of the gut contents (100× magnification; d) are shown for an individual showing the characteristic green coloration that was swept from an R6-stage soybean field. Images were acquired using a model M125 stereomicroscope (Leica Microsystems, Wetzlar, Germany), in combination with a model EC3 digital microscopy camera (Leica Microsystems). (Online figure in color.)

Discussion

Our results demonstrate that the digital image analysis technique described here has potential utility as part of a sampling-based approach to management of the rotation-resistant western corn rootworm. Although the rate of spread of the rotation-resistant strain of western corn rootworm has been estimated at 10–30 km/yr by using modeling (Onstad et al. 1999), this rate may be slowed as the level of landscape diversity increases (Onstad et al. 2003). The result has been an uneven distribution of the rotation-resistant strain and therefore sporadic occurrences of rotation resistance throughout many parts of the Midwest. An overreliance on anecdotal information, coupled with significant uncertainty among growers and consultants as to the effectiveness of crop rotation in a given area, often results in unnecessary treatments, as most producers are not inclined to risk losses. This use of pest management treatments as "insurance" often results in costs and frequencies of applications that are disproportionate to actual need. For example, Gray et al. (1993) were able to document that in 1990 and 1991, 26 of 58 producers (45%) had rootworm damage at or above the economic injury index, whereas in the same area >88% of the cornfields were treated for rootworm control. Little has changed in the years since that study was published. There remains a need for a strategy that gives growers confidence about making management decisions that can be implemented on a farm-by-farm basis. Simple, accessible tools to more accurately assess the risk of larval damage to rotated corn would help remove confusion about local risk in the areas where rotation resistance is found.

Table 3. Soybean herbivory percentages measured by two naïve system users in blind assays of three 25-beetle samples containing different percentages of soybean-feeding and negative control western corn rootworm females

Actual % of soybean feeders	Particle limit (pixels)	% based on blind assay[a]	
		Technician 1	Technician 2
20 (5/25)	100	20 (5/25)	20 (5/25)
	250	20 (5/25)	20 (5/25)
	500	16 (4/25)	20 (5/25)
36 (9/25)	100	32 (8/25)	40 (10/25)
	250	32 (8/25)	36 (9/25)
	500	24 (6/25)	36 (9/25)
84 (21/25)	100	80 (20/25)	88 (22/25)
	250	68 (17/25)	68 (17/25)
	500	52 (13/25)	60 (15/25)

[a] Technicians independently evaluated these unknown samples at 100, 250, and 500 pixel particle limits.

Digital image analysis techniques have been used for a variety of past entomological studies; in particular, thresholds have been used to separate leaf tissue damaged by insect herbivory from healthy (background) tissue (James and Newcombe 2000; Lee et al. 2005). However, threshold analysis of the dark green abdominal coloration that results from soybean foliar feeding is a more complex problem. Because the green coloration normally lacks clear edge effects, and these color differences may seem superficially similar to the normal yellow abdominal coloration of nonsoybean-fed western corn rootworms, a more quantitative approach to threshold determination was needed. It is important to note that, although the initial determination of threshold limits was mathematically complex, implementation is much simpler and involves nothing more than knowing where and how to open the images and enter the predetermined threshold limits into the computer software. The materials required (digital camera, computer with internet access for downloading free software) are already available and widely used by pest management researchers and consultants, the target audience for this application.

Levine et al. (2002) identified soybean foliage in the dissected gut contents of 54.8% of female western corn rootworm adults collected from soybean in east central Illinois. Our results when the lowest particle limit setting (100 pixels) was used were similar, as 54.3 ± 2.1% of beetles swept from soybean in nearby west central Indiana tested positive for soybean consumption. As the particle limit (the minimum area of shading required to yield a positive result) was increased, the percentage of beetles testing positive for soybean foliage decreased (to 44.6 ± 2.1% at a particle limit of 250 pixels and 35.7 ± 2.0% at a particle limit of 500). The variable particle limit setting would allow a user to alter the precision level of the assay. For example, if the user wanted to minimize the type I error rate (false positives), a higher particle limit would be used; likewise, if the user wanted to minimize the type II error rate (false negatives), a lower limit would be desirable. Management applications would probably fall into the latter category to minimize the risk of a grower failing to make a necessary treatment.

The results of the blind assay demonstrate that novel users can be taught to conduct this image analysis method relatively quickly. Use of a particle limit with the Analyze Particles function in ImageJ effectively eliminates the need to make subjective judgments when interpreting the results. The lower limit of 100 pixels resulted in the closest approximation of the expected results; however, it is noteworthy that all false positives were eliminated when a limit of 250 or 500 pixels was used. It is important to emphasize that differences in the results of the analysis between the two technicians occurred not during the computer analysis itself, but during image acquisition. More detailed written instructions for potential users clearly specifying the internal focal length and external focusing distance (i.e., the zoom level), lighting conditions, and other camera parameters would minimize the human error rate.

The abundance of soybean foliage found within the guts of female western corn rootworms identified as soybean feeders by their green coloration validates the use of this coloration as an indicator of soybean herbivory. The poor digestion of soybean foliage material is consistent with observations that this represents a suboptimal diet for western corn rootworm adults (Mabry and Spencer 2003). In contrast, corn foliage, tassels, silks, and pollen seemed much more highly digested. When soybean-swept individuals not showing the green coloration were dissected, 44% contained some soybean foliage; in each of these cases, the amount of soybean foliage consumed was <50% of the amount of foliage present in beetles showing green coloration, and normally consisted of only a few isolated groups of cells. The presence or absence of coloration is clearly related to the amount of soybean foliage consumed. Siegfried and Mullin (1990) showed that the rate of oviposition increases as the suitability of host plant material decreases (i.e., the level of dietary stress increases). Although no published studies have examined the link between the quantity of soybean foliage consumed and the degree of dietary stress that occurs, there is probably some threshold level of herbivory that must be exceeded before there is enough stress to produce a stimulatory effect.

As outlined above, current sampling methods for rotation-resistant western corn rootworm have key weaknesses that limit their usefulness and, consequently, their adoption by growers. In its current form, the image analysis method could be used in combination with traditional sampling methods (sweeps, sticky traps) to improve their predictive value, making their use more attractive to growers. Fresh, intact beetles are required for the image analysis method, meaning that trapping intervals would need to be short enough to prevent widespread beetle decay and desiccation (likely a maximum of 48 h).

One obvious next step in this work is developing a link between image analysis data and the damage levels to first-year corn in the same area the following year, by using methods similar to those used by O'Neal et al. (2001) to develop an economic injury level based on Pherocon AM traps in soybean. In the long-term, this technology could be used to provide an improved regional "rotation-resistance risk map" by using samples collected by individuals in a variety of areas/states. These data could be analyzed/collated by, for example, extension entomologists and then distributed to clientele through many of the usual, established media (e.g., factsheets, grower talks, websites). These types of site-specific and informative data would represent a marked improvement on the present state of monitoring for this behavioral variant.

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