

Larval Sampling and Instar Determination in Field Populations of Northern and Western Corn Rootworm (Coleoptera: Chrysomelidae)

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ABSTRACT Abundance and head capsule width were measured for northern (*Diabrotica barberi* Smith & Lawrence) and western corn rootworm (*D. virgifera virgifera* LeConte) larvae recovered primarily from maize root systems but also from large soil cores each centered around a root system. Larvae for measurement derived from field populations under infestation and rotation regimes that allowed most specimens to be assigned to species. A frequency distribution of head capsule widths indicated three separate peaks for western corn rootworm, presumably representing frequency of the three larval instars, with no larvae measuring 280 or 420 μm in the valleys between peaks. Multiple normal curves fit to similar but partially overlapping peaks generated by northern corn rootworm suggested that division of first to second and second to third instar can best be made for this species at 267 and 406 μm , respectively (270 and 410 when measurements are made to the nearest 20 μm). These results implied that instar of individuals from mixed northern and western corn rootworm populations can be accurately judged from head capsule width without having to determine species. The relative abundance of western corn rootworm instars was similar in root systems removed from the center of 19-cm diameter \times 19-cm deep soil cores and in soil cores from which the root systems were removed. Furthermore, the number of larvae from root systems correlated significantly with that from the surrounding soil. These results indicated that the former and much more convenient sampling unit can be used to estimate population developmental stage and possibly density, at least early in the season when these tests were done and young larvae predominated.

KEY WORDS *Diabrotica barberi*, *Diabrotica virgifera virgifera*, corn rootworm, larval instar determination, larval sampling

THE NORTHERN CORN ROOTWORM, *Diabrotica barberi* Smith & Lawrence, and the western corn rootworm, *D. virgifera virgifera* LeConte, are important pests of maize, *Zea mays* L., that now occur together throughout much of the U.S. Corn Belt. Most crop damage is caused by root feeding by larvae, whose subterranean habits complicate population monitoring and the application of an integrated pest management approach to this important pest complex. These complications have undoubtedly contributed to increasing corn rootworm adaptation to current pest management options, which consist mainly of crop rotation and insecticide treatments, and may hinder detection of resistance should it develop in response to new management technologies such as transgenic maize (Krysan et al. 1986; Levine et al. 1992; Levine and Oloumi-Sadeghi 1996; Meinke et al. 1998; Onstad et al. 1999, 2001).

The best method of recovering corn rootworm larvae from soil is by sieving and flotation of soil cores held frozen before processing, a procedure shown to recover 70–100% of larvae, depending on size (Bergman et al. 1981). Because corn rootworm larvae aggregate close to the maize stem, sampling from sufficiently large soil cores can be used to estimate absolute pest population levels (Sechrist 1969, Fisher and Bergman 1986). However, the sieving/flotation technique and other standard but less efficient methods to estimate corn rootworm populations during the crop damaging stage, because they all involve handling of large soil volumes, are either too laborious for practical purposes (Bergman et al. 1981) or detect early stage larvae too inefficiently to provide enough time for intervention before significant root damage occurs (Weiss and Mayo 1983). Fromm et al. (1998) recently described an inexpensive funnel apparatus for rapidly and easily collecting live rootworm larvae from drying maize roots. Several early researchers working primarily with northern corn rootworm, likewise, avoided soil handling by limiting sampling to maize roots (Apple et al. 1969, Chiang et al. 1969,

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Gould 1971), but this approach has not been widely adopted or validated despite its greater ease.

Abundance data are necessary to monitor pest populations but difficult to interpret independently of data on pest developmental stage when attempting to predict the potential for subsequent crop injury. Insect molts clearly demarcate sequential developmental stages, and head capsule width has been used to separate the three larval instars typical of *Diabrotica* species (Sweetman 1925, Pitre and Kantack 1962). Although head capsule width appeared to be a reliable character to rate larval instar in western and southern corn rootworm, *D. undecimpunctata howardi* Barber, few individuals were sampled and most were from laboratory colonies not always reared on maize (Sweetman 1926, Arant 1929, George and Hintz 1966). Similar head capsule measurements are lacking for northern corn rootworm, and inability to morphologically distinguish northern from western corn rootworm during early larval stages, has undoubtedly contributed to the lack of size information for younger field populations of both species (Krysan 1986).

In ongoing rootworm sampling studies, we routinely measured larval head capsule width of western corn rootworms artificially established in the field with eggs (Sutter and Branson 1980) and feral northern corn rootworms surviving, as a result of an extended egg diapause (Krysan et al. 1986, Levine et al. 1992), in maize rotated annually with soybeans. These studies yielded large numbers of larvae under field conditions that let us assign most specimens to species, a process that was augmented by molecular means of distinguishing the two species (Roehrdanz 2003). Considering the often strong dependence of insect body size on larval nutrition and degree of crowding (e.g., Saunders and Bee 1995 and references therein), these studies offered the further advantage of host-reared specimens taken in multiple years over a wide range of larval densities.

In this study, we fit multiple curves to mostly single-species data sets depicting frequency of individuals falling within size categories and used these curves to further evaluate reliability of head capsule width as an indicator of larval instar for northern and western corn rootworm. We also compared abundance and head capsule width between larvae recovered behaviorally from maize root systems and those manually extracted from soil cores from which roots were removed to evaluate suitability of the former sampling method for estimating developmental stage and density of corn rootworm field populations.

Materials and Methods

Experimental Site and Designs. Our study was conducted yearly from 1999 to 2001 using Pioneer 5751 (1999 only) or 37H24 hybrid maize, *Zea mays* L., at the Eastern South Dakota Soil and Water Research Farm near Brookings, SD, on Barnes clay loam soil (fine-loamy, mixed, superactive, Calcic, Hapludoll). Maize rows were oriented east to west at a spacing of 76.3 cm

in each of two crop rotation and rootworm infestation regimes contributing to the study.

First, naturally occurring corn rootworm populations were sampled in 30.5- × 30.5-m subplots that were part of a long-term crop rotation, tillage, and nitrogen fertilization experiment established in 1990 and maintained as described by Pikul et al. (2001). Second, we sampled within 30.5- × 91.4-m field plots of maize that were artificially infested with western corn rootworm eggs 1 d after planting, except as otherwise stated. The artificially infested plots had been planted to soybeans or wheat during the previous two growing seasons to reduce contamination with feral northern corn rootworms often abundant in 2-yr maize rotations. Neither test employed insecticides for rootworm control.

In the experiment with naturally occurring rootworms, maize plots were planted 12, 3, and 14 May 1999, 2000, and 2001 at the rate of 77,000, 72,000, and 82,000 seeds per ha, respectively. Each whole plot was split into three subplots in which nitrogen fertilization was applied at high, intermediate, or low levels. For this study, rootworm sampling was limited to the three high nitrogen subplots within chisel-tilled whole plots planted to maize every other year after soybeans (Pikul et al. 2001).

The naturally occurring corn rootworm larvae were sampled each year from 12 plants per subplot at 5- to 8-d intervals from late May or early June through late June or early July. Sampling was limited to two corn rows out of a total of 40 per subplot: 15 and 31 in 1999, 18 and 34 in 2000, and 7 and 27 in 2001. Three plants were randomly chosen per subplot quadrant and digging date within sample rows. In addition to larval sampling, cumulative adult emergence was estimated by species using four cages that were placed one per subplot quadrant within sampling rows. Each cage (61 cm long × 76 cm wide) was embedded in the soil and positioned so as to trap adult beetles emerging from the root systems of three maize plants (Hammack 2003). The cages were emptied twice weekly through peak emergence and then weekly during September.

The artificial infestations were established in a randomized complete block design in maize planted 12, 13, and 11 May 1999, 2000, and 2001 at the rate of 77,000, 72,000, and 82,000 seeds per ha, respectively. The eggs used for infestation were from a locally collected, western corn rootworm colony maintained at the Northern Grain Insects Research Laboratory since 1987 (Jackson 1986) and shown comparable to native populations with respect to crop damage potential (Hibbard et al. 1999). Infestation methods were those of Sutter and Branson (1980).

Each of six blocks placed in the first 2 yr of the experiment consisted of 16 adjacent 30.5-m long rows, with alternate rows left untreated to provide a spacial buffer between egg treatment levels of 0, 200, 400, 600, 800, 1000, 1600, and 2400 per 30.5-cm length of row. The root systems of six adjacent plants within each treatment row were dug and sampled for larvae on 15 and 22 June 1999, 27 June 2000, and 5 July 2000. Head capsule widths were measured on larvae recovered

from treatments established with 200, 400, and 1600 eggs per 30.5 cm of row to provide specimens from both low- and high-density populations. Peak adult populations were previously reported from infestation rates of 1200 eggs per foot of row, with fewer recovered from emergence cages at higher doses (Branson et al. 1980, Sutter et al. 1981). Three emergence cages per treatment row, each covering three maize plants at locations separated by ≈ 6 m, provided estimates of adult emergence in a manner cited above for the naturally occurring populations.

A series of 81 soil cores 19 cm in diameter and depth and centered on maize plants was taken to compare the head size and abundance of larvae recovered from the root system with those of specimens taken from the surrounding soil mass without root system. Because corn rootworms tend to aggregate near the maize stem, 19-cm-diameter cores are large enough to estimate absolute rootworm densities when plant densities are also known (Sechriest 1969, Fisher and Bergman 1986). The 19-cm soil cores were taken within the field plots artificially infested, as already described, with eight densities of western corn rootworm eggs in 1999 ($N = 40$, 4–6 per density except for three cores at the 0 egg dose) and 2000 ($N = 29$, 3–4 per density except for two cores at the 0 dose). Sampling was continued in 2001 ($N = 12$, one at the 0 dose), but the western corn rootworm infestations were established manually and at only three densities (0, 400, and 1000 eggs per 30.5 of row) on 15 May in maize planted 11 May 2001. Artificial infestations in 2001 were otherwise designed as in 1999–2000. The 81 soil cores, each with enclosed plant, were taken 10–20, 21–29, and 22–27 June 1999, 2000, and 2001, respectively, when maize was 42.0% stage V6 (Ritchie et al. 1989). The rest of the sample was 3.7% V4, 25.9% each V5 and V7, and 2.5% V8.

Larval Recovery. The only method used to recover rootworm larvae from maize root systems was by drying individual root systems in darkness at room temperature above a water trap within a simple apparatus constructed from window screen and two 0.7-liter plastic drinking cups (Fromm et al. 1998). Plants dug in the field were returned to the laboratory, where soil was washed from individual root systems with a pressurized stream of water. We added this wash to the handling protocol of Fromm et al. (1998) to better standardize the sampling unit because the soil volume adhering around the roots after just gentle shaking varied greatly with soil conditions at digging. The wash was discarded, except as described in the next paragraph for root systems separated from surrounding 19-cm-diameter soil cores. Washed plants were staged (Ritchie et al. 1989), severed just above the roots, and their root systems transferred individually to cups for drying, a process that continued to yield larvae for 2–11 d depending on plant maturity. During drying, larvae were collected from the water traps below cups at daily intervals to ensure that most individuals were obtained alive. Ice water was used to temporarily immobilize larvae and enable measurement of head capsule widths to the nearest 20 μm using a dissecting

microscope capable of 50 \times magnification and fitted with an eyepiece reticle (100 subdivisions in 10 mm) calibrated with a stage micrometer. Measurement to 20 μm was as precise as this equipment permitted. Larvae recovered live from drying cups were stored individually at -70°C . Because of relatively large western corn rootworm populations occurring naturally in 2000, species of feral specimens collected in 2000 from the 2-yr maize-soybean rotation and stored at -70°C was confirmed using mitochondrial polymerase chain reaction (PCR) primers that distinguish northern and western corn rootworm (Roehrdanz 2003).

Whenever a 19-cm soil core was taken, the encircled root system was first separated from the rest of the core, washed free of soil over a sieve fine enough to retain all rootworms (180- μm openings), and then dried in the apparatus of Fromm et al. (1998), as described in the previous paragraph. Material retained by the sieve was pooled with the rest of the soil from the core before the combined soil sample was stored at approximately -20°C until processed. Larvae were recovered from each combined soil sample using the washing-sieving flotation procedure described for frozen samples by Bergman et al. (1981); however, our sieved soil samples contained sizable amounts of macro- and particulate organic matter that floated along with the rootworms. For efficient larval recovery, it was necessary to spread all material retained by the sieves on filter paper grids and examine the papers systematically with a dissecting microscope. Head capsules were measured as described for the root samples, except that cold immobilization was unnecessary with dead larvae.

Data Analysis. Data on larval head capsule widths were graphed as histograms showing the frequency of individuals observed within successively larger, 20- μm wide, size categories. Such histograms demonstrated three peaks, which were interpreted to represent peak frequency of the head capsule widths of the three larval instars. We assumed a normal distribution of head capsule widths and fit multiple Gaussian curves to the histograms applying a nonlinear least squares regression method based on the Levenberg-Marquardt algorithm and available in Origin 6.1 software (Moré 1978, OriginLab Corp. 1999). Once parameter values for overlapping normal curves were calculated, along with 95% upper and lower limits about parameter values, standard normal deviates were used to estimate the proportion of individuals of each instar that fell outside of selected size ranges (Zar 1996). Chi-square calculated from a 3×2 contingency table compared the relative abundance of the three instars between root systems and surrounding 19-cm soil samples (Zar 1996). Linear regression also available in Origin 6.1 software examined the relationship between larval recoveries from root and soil portions of the 19-cm soil cores.

Results and Discussion

Despite avoidance of maize planting in the previous two growing seasons, adult emergence data indicated

Table 1. Cumulative emergence per plant (mean ± SEM) of western and northern corn rootworm adults by year from first year maize treated with 0, 200, 400, and 1,600 western corn rootworm eggs per 30.5 cm of row in a 3-yr rotation

Species	Egg dose	6 July-10 Sept. 1999 ^a	13 July-19 Sept. 2000 ^b
Western	0	2.2 ± 0.2	1.2 ± 0.4
	200	9.0 ± 2.2	6.7 ± 0.7
	400	15.0 ± 2.1	9.7 ± 2.4
	1,600	21.7 ± 5.4	13.8 ± 1.9
Northern	0	1.2 ± 0.2	0.8 ± 0.3
	200	1.3 ± 0.6	0.7 ± 0.1
	400	2.2 ± 0.6	0.4 ± 0.2
	1,600	1.0 ± 0.3	0.5 ± 0.3

^a N = 5 blocks of 9 plants/block. All data from one block were omitted in 1999 because of higher northern corn rootworm recoveries in several rows near the block perimeter.

^b N = 6 blocks of 9 plants per block.

small numbers of feral northern corn rootworms within the plots that were artificially infested with western corn rootworm eggs (Table 1). Indeed, an entire block of data was disregarded in 1999 because of several border rows with higher than background emergence of northern corn rootworm. In no case did the number of emerging northern corn rootworm adults appear related to level of treatment with western corn rootworm eggs (Table 1). Therefore, data in Table 1 from both years and all egg density levels except western corn rootworm in untreated control rows were used to estimate an adult western to northern emergence ratio of 14:1 in the artificially infested plots.

Larvae recovered from infestations established with western corn rootworm eggs fell into three separate and distinct size categories based on head capsule widths (Fig. 1,WCR). These categories included individuals with head capsules that measured 200–260, 300–400, and 440–560 μm in width. Centers of fitted normal curves (95% lower, upper limit) were 216 (211, 218), 329 (326, 333), and 499 μm (493, 505), respectively, compared with mean values ± SEM of 216 ± 1, 332 ± 1, and 501 ± 1. These are similar to the 200, 325, and 500 μm mean values reported for a small number of first-, second-, and third-instar western corn rootworms reared in the laboratory on a squash diet and examined daily throughout larval development (George and Hintz 1966).

Naturally occurring adult populations emerging from the 2-yr rotated maize were 43:1, 8:1, and 116:1 northern: western corn rootworm in 1999, 2000, and 2001, respectively (Table 2). Polymerase chain reaction methodology was used to verify species of larval specimens only in 2000 because only the 8:1 ratio in 2000 suggested the possibility of significant numbers of western corn rootworm larvae in the rotated maize. Of larvae recovered live in 2000 from root systems of maize rotated annually with soybeans (122 live, 126 total), head capsule data were retained for only 103 individuals confirmed to be northern corn rootworm by PCR (Roehrdanz 2003). These individuals comprised 84.4% of the 2000 live sample. The disregarded

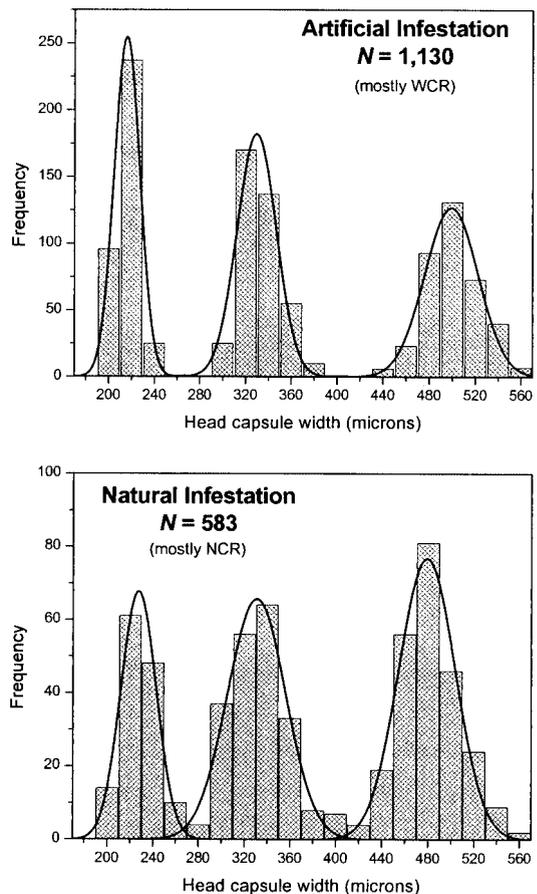


Fig. 1. Frequency of head capsule widths among corn rootworm larvae recovered from root systems of maize artificially infested with western corn rootworm (WCR) eggs or rotated annually with soybeans and naturally infested with primarily northern corn rootworm (NCR). Fitted normal curves are shown.

portion of the 2000 live sample was western corn rootworm (0.8%) or could not be assigned to species (14.8%), either because of sample condition or previously unrecognized variability at a primer binding site.

The frequency of head capsule widths among corn rootworm larvae recovered in the rotated maize also fell into three distinct groups although, in contrast with the western corn rootworm, these size categories

Table 2. Cumulative emergence per plant (mean ± SEM) of feral western and northern corn rootworm adults by year from maize rotated annually with soybean

Year	Collection dates	Northern	Western
1999	6 July–14 Sept.	13.0 ± 3.3	0.3 ± 0.3
2000	5 July–19 Sept.	9.3 ± 1.6	1.2 ± 0.8
2001	10 July–18 Sept.	34.8 ± 4.1	0.3 ± 0.2

N = 3 subplots yearly (12 plants per subplot).

Table 3. Parameter values and their 95% confidence limits for the three normal curves fitted to the Fig. 1, NCR histogram depicting the frequency of larval head capsule widths among corn rootworms sampled in maize rotated annually with soybeans

Parameter ^a	Value	± SEM	Lower limit	Upper limit
x_{c1}^b	227.27	1.36	224.14	230.30
w_1^c	31.03	3.06	23.79	38.29
A_1^d	2632.70	207.25	2182.82	3106.28
x_{c2}	330.69	1.82	326.61	334.76
w_2	50.83	3.65	43.48	59.68
A_2	4182.63	259.32	3622.38	4772.29
x_{c3}	479.07	1.52	475.68	482.55
w_3	48.77	3.04	41.68	56.99
A_3	4688.95	253.20	4114.18	5290.95

^a Equation for a normal curve: $y = y_0 + \frac{A}{w \cdot \sqrt{\frac{\pi}{2}}} e^{-2(x-x_c)^2/w^2}$

The baseline value, y_0 , was fixed at 0 for all curves.

^b Value of x at the center of curve 1.

^c Equals two standard deviations around the center of curve 1.

^d Total area under curve 1.

overlapped to some extent (Fig. 1, NCR). Despite significant deviation from the assumption of three overlapping normal curves ($\chi^2 = 240.7$, $df = 10$, $P < 0.001$), which was likely due at least in part to the relatively small number of comparatively broad 20- μm -width categories, this model accounted for most of the observed data variation ($r^2 = 0.978$). Fitted curves overlapped at head capsule widths of 266.6 and 405.8 μm . These data suggested that division between first, second, and third northern corn rootworm larval instars can best be made at 270 and 410 μm , respectively, when measuring to the nearest 20 μm .

The 95% upper and lower confidence limits about parameter values shown in Table 3 define curves with greater and lesser degrees of overlap than those fitted in Fig. 1, NCR. Curves defined by these confidence limits and standard normal deviates about curve centers (x_c) allow calculation of errors expected using the 270- and 410- μm values to differentiate instars. These calculations predict correct assignment of 99.7 (98.1–100), 99.1 (97.9–99.5), and 99.8% (99.5–99.9) of first, second, and third northern corn rootworm larval instars, respectively.

Table 4 compares soil and root samples with respect to developmental stage of recovered larvae using head

Table 4. Developmental stage of larvae recovered from maize root system compared with surrounding soil comprising 19- × 19-cm cores taken within 3-yr rotated maize infested with a range of western corn rootworm eggs densities, 1999, 2000, and 2001

Larval instar	Maize root system		Soil core less root system	
	No.	%	No.	%
First	264	63.8	1,735	66.9
Second	121	29.2	715	27.6
Third	29	7.0	142	5.5

$N = 81$ soil cores, each with encircled root system, although no larvae were found in the six cores dug in rows treated with 0 WCR eggs. A chi-square test showed no difference between sample sites in the relative abundance of larval instars ($P > 0.05$).

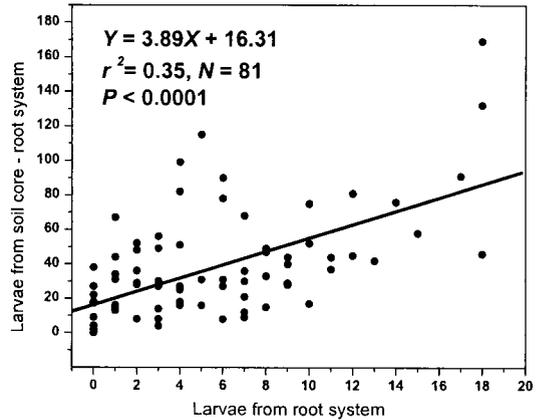


Fig. 2. Linear regression analysis of the relationship between abundance of larvae in root systems (X) and in surrounding soil (Y) within 19-cm-diameter and deep cores centered on maize plants artificially infested with western corn rootworm eggs. Standard errors about slope and intercept were 0.59 and 14.17, respectively, and residual mean square error was 648.88.

capsule widths of 270 and 410 μm to distinguish instars. There was a tendency toward proportionately more second and third instars in root compared with soil portion of 19-cm soil cores surrounding maize plants, perhaps because larvae crawled out of the drying roots over some days. Even so, 65.0, 82.6, 93.7, and 100% of larvae had crawled from roots by 1, 2, 3, and 9 d after roots were dug, respectively, and the observed tendency for older instars in root samples was not statistically meaningful ($\chi^2 = 2.35$, $df = 2$, $P > 0.25$). These data imply that sampling maize root systems produces an unbiased estimate of corn rootworm developmental stage compared with estimates obtained from large soil samples, at least relatively early in larval development when first instars were the most abundant stage and maize was predominantly in the 5- to 7-leaf stage.

Linear regression analysis also revealed a highly significant correlation between larval recoveries from root and soil ports of the above 19-cm-diameter soil cores centered on maize plants (Fig. 2). The r^2 value of 0.35 indicates considerable variation about the line generated by individual soil cores (Fig. 2), but substitution of mean larval recoveries from soil at each level of recovery from root systems nearly doubled the r^2 value to 0.68 ($Y = 3.80X + 15.82$, $N = 18$, $P < 0.0001$; SE about slope and intercept was 0.65 and 6.55, respectively; residual mean-square error was 216.14). Thus, because only 14 or fewer root systems were sampled at any level of larval recovery from roots, the root sampling method shows considerable potential for easier and faster estimation of western corn rootworm numbers before peak crop damage is inflicted. Whether root sampling will prove useful for assessing density of mixed-species populations will depend on how similarly northern and western corn rootworm are distributed between root and soil and on how

efficiently each species is extracted from each sampling unit. Soil core data similar to that collected here for western corn rootworm will be needed for northern corn rootworm to assess applicability to the mixed-species populations prevalent across much of the U.S. Corn Belt.

In conclusion, head capsule width proved to be a reliable indicator of larval instar for field populations of western corn rootworm developing in the northwestern U.S. Corn Belt. This conclusion, of course, assumes that genetic determinants of head capsule widths characterizing the western corn rootworm colony that supplied eggs for the artificial field infestations are representative of populations occurring naturally in the region. Size categories delineating each instar were completely distinct for western corn rootworm but overlapped slightly for northern corn rootworm occurring naturally in maize rotated with soybeans. Nevertheless, the data suggested that head capsule widths of 270 and 410 μm can be used to separate first from second and second from third northern corn rootworm instars, respectively, with >95% accuracy when larvae are measured to the nearest 20 μm . Furthermore, the 270- and 410- μm values were equally applicable to both northern and western corn rootworm, indicating that developmental stage of mixed-species populations can be accurately estimated without having to separate the larvae by species. Finally, the data indicated that the relative abundance of western corn rootworm larval instars and possibly their absolute density can be accurately estimated from populations recovered from maize root systems, a vastly easier process than recovery from large soil cores.

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