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Leafy Spurge Biological Control Using Black Dot Flea Beetles and Deleterious Rhizobacteria: Final Report Submitted to South Dakota Department of Agriculture Weed and Pest Control

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LEAFY SPURGE BIOLOGICAL CONTROL USING BLACK

DOT FLEA BEETLES AND DELETERIOUS RHIZOBACTERIA

Final Report

Submitted to South Dakota Department of Agriculture Weed and Pest Control

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SUMMARY AND RECOMMENDATIONS

Size of leafy spurge (*Euphorbia esula* L.) roots and their location in the soil profile are important factors relating to survival of first-instar black dot flea beetle (*Aphthona nigriscutis* Foudras) larvae. First-instar larvae must find leafy spurge roots between 1 to 4 mm diam in the first 2 days after hatching from eggs for survival. In field studies, we determined that most flea beetle larvae reside within 7.6 cm of the soil surface. Their location in the upper areas of the soil profile allows accessibility to desirable size roots, however the larvae are more susceptible to freezing temperatures in this region. Overall, there were fewer black dot flea beetle larvae in the soil at the Pollock site in 1997 compared to 1995 and 1996. In 1997, harsh winter weather conditions may have resulted in high mortality of larvae.

We studied adult reproductive factors in black dot flea beetle collection for distribution efforts. Sex ratios of adults from South Dakota and North Dakota sites were highly skewed in favor of females. In mid-July (about 30 d following adult emergence), female insemination was higher than 40%. Oocyte (unlaid eggs within the female) maturation ratings were about half of the average values observed on the last sampling date. In the past few years, black dot flea beetles were collected from the Pollock site for distribution to new areas and these activities were conducted in early July. Usually, a few hundred adults were released to each new site. Since males are rare, we recommend that collections be taken in mid-July to capture larger numbers of inseminated females. By releasing females that have already mated, reproduction in the new area may be increased. In addition, other researchers have found that establishment of biological control agents was greatly improved by releasing 1,000 or more individuals to each site.

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We conducted genetic analysis of black dot flea beetles from North American and Hungarian populations to determine the potential effects of inbreeding on genetic variability. Factors that contribute to inbreeding include female-biased sex ratios, lack of migration between established sites, and founder effects (the flea beetles in a population are all descendent from a few founding individuals). Surprisingly, there were several alleles present in the North American populations that were not detected in the Hungarian population. Thus, we concluded that genetic variability was not lower in the North American populations.

We examined the influence of deleterious rhizobacteria isolated from 2 leafy spurge populations in the field. We saw promising results in 2 years of research. The first was that live deleterious rhizobacteria (*Pseudomonas fluorescens*) cells were detected on leafy spurge roots 1 yr following treatments. A second was that root carbohydrate levels in deleterious rhizobacteria treated areas were reduced by about 20% compared to control (blank) treatments. Roots from areas treated with isolate 1 (Montana strain) contained slightly lower root carbohydrate concentration than roots treated with isolate 2 (South Dakota strain). Results from these deleterious rhizobacteria studies indicate that deleterious rhizobacteria interfere with the ability of leafy spurge to store carbohydrates in roots. These findings are encouraging because winterkill due to freezing temperatures may be higher for leafy spurge roots with lower carbohydrate content. However, additional research is needed in the field, greenhouse, and laboratory before using these isolates for leafy spurge control.

INTRODUCTION

Leafy spurge (*Euphorbia esula* L.) is a deep-rooted noxious weed that reproduces asexually from root and crown buds, and from seed (Luster and Farrell 1996). Leafy spurge reduces rangeland productivity by competing with desirable forage and causing illness in livestock. Estimated annual losses due to leafy spurge have ranged from $76 to 120 million (Bangsund 1991, Watson 1985, respectively). Herbicide and mowing treatments are difficult and expensive, and retreatments are usually necessary over several years. Also, conventional methods may not be feasible in ecologically sensitive areas, hillsides, and highly eroded areas. Leafy spurge biological control has primarily focused on root-feeding insects and several species of *Aphthona* flea beetle have been introduced to North America for biological suppression of the weed (Julien 1987). Black dot flea beetles (*A. nigriscutis* Foudras) are native to Europe and North American populations are descendant from Hungarian stock (Andre Gassmann, personal communication). Black dot flea beetles prefer dry sites with coarse soils and have become established in many areas of the northern Great Plains (Rees et al. 1996). Females emerge in spring, mate, and lay eggs in the soil near leafy spurge (Fornasari 1996). Adults die in late summer and larvae overwinter in the soil (Fornasari 1996). Subsequent to hatching from the egg, larvae must locate leafy spurge roots for survival and development. Larvae are very mobile and experience 3 instars prior to pupation. Adults feed on leafy spurge foliage, but larvae cause the most damage by feeding on roots. Currently, there is little information on the root-feeding habits of larvae.

In order to expedite the distribution of flea beetles, adults have been collected yearly from an established site near Pollock, South Dakota for distribution to leafy spurge infested areas. The numbers of adults released to each site has ranged from 100 to 1000. The choice of

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collection date has been based on the availability of large numbers of adults. Typically, adults are collected in early July. Between 1988 and 1992, black dot flea beetles were introduced to 46 sites in South Dakota (Clay and Scholes 1993). Adult beetles were recovered at 45% of those sites in the summer following each release (Clay and Scholes 1993). In past years, the reproductive status of adults collected for distribution was unknown. The potential for black dot flea beetle reproduction in a new area may be improved by releasing adults that have already mated.

Natural populations usually contain a large amount of genetic variation (Judson 1995, Marinkovic and Ayala 1975, Smith et al. 1983, Hartl and Clark 1989) and these genetic resources allow populations to adapt to variable environments. However, the current strategy of collecting and distributing relatively small numbers of flea beetles into isolated habitats may be potentially detrimental to genetic variability in the prospective populations. Capula (1994) found reduced genetic variability in introduced island populations of lizards, compared to that of mainland populations. Capula (1994) attributed the decreased genetic variability to the founder effect (the original introduced individuals possessed only a subsample of tne genetic resources present in the parent population). Extensive inbreeding may result (Swart et al. 1996, Latter and Robertson 1962) and population growth may be impaired (Marinkovic and Ayala 1975). Nowierski (1996) used isozyme analysis to differentiate 4 species of *Aphthona* (from Europe), but genetic studies of North American *A. nigriscutis* are needed to determine potential founder effects on variability within introduced populations.

Deleterious rhizobacteria (*Pseudomonas fluorescens*) colonize leafy spurge roots and have been isolated from leafy spurge in South Dakota and Montana (Souissi and Kremer 1994). Deleterious rhizobacteria are host-specific and cause damage to leafy spurge by producing

harmful toxins that are absorbed by the roots (Souissi and Kremer 1994). In order for deleterious rhizobacteria to harm leafy spurge, they must come in contact with roots (Kloepper and Beauchamp 1992). Insect feeding activity may facilitate bacterial contamination of roots, but this has not been investigated for flea beetles and leafy spurge. Perennial weeds that experience stress to root systems are more susceptible to winterkill and sugar content of plant tissue is proportional to its freezing tolerance (Lym and Messersmith 1993).

The 2 primary objectives of this research were: 1) to study the biology of black dot flea beetles, and 2) to determine effects of black dot flea beetles and deleterious rhizobacteria on leafy spurge root carbohydrates. There were several aspects of black dot flea beetle biology that were investigated in field and laboratory studies. Those include: adult sex ratios, female insemination rates, oocyte development, larval root-size preference, larval survivability, larval densities in soil, and genetic variability within adult populations. Studies of black dot flea beetle biology provide information that may improve the efficiency of collection and distribution of the insect and may increase establishment potential in leafy spurge infested areas.

BLACK DOT FLEA BEETLE BIOLOGY

Larval flea beetle survivability and root size preference

Materials and Methods

Black dot flea beetle eggs were obtained from laboratory colonies maintained at the USDA-ARS Northern Grain Insect Research Laboratory in Brookings, South Dakota. Thirty hatching larvae were individually placed on moist Whatman # 1 filter paper in 25 cm^2 plastic containers and were incubated at 25° C. Larvae were checked every 6 hr and mortality was recorded. Larvae that did not respond to tactile stimulation were considered dead.

Leafy spurge roots for the root size preference studies were obtained from undisturbed infestations in Marshall County, South Dakota. Roots of differing diameters were cut into 1.3 cm sections and the ends were sealed with candle wax. Thirty hatching larvae were individually placed on <1, 1, 2, 3, 4, or 5 mm diam roots (180 total larvae and root sections). Each root with larva was placed on moist soil in 25 cm^2 plastic containers and were incubated at 25° C. Larvae were monitored until they initiated feeding or died.

Results and Discussion

Larvae that were denied food began expiring after the first 6 hr (Fig. 1). At 18 hr, mortality was 10%. However, at 24 hr after hatching, larval mortality had risen to 43%. Mortality steadily increased in the subsequent time periods until all larvae were dead at 54 hr. About 60% of larvae on 1, 2, 3, and 4 mm diam roots fed or burrowed into the respective root sections (Fig. 2). Larvae on roots < 1 mm diam did not initiate feeding and subsequently died. Only 7% of larvae were observed feeding on 5 mm diam roots. Results from these studies suggest that black dot flea beetle larvae must locate 1 to 4 mm diam. leafy spurge roots within 24 hr of hatching from the egg for survival. Root hairs may not provide a suitable epidermal cell layer for larval attachment, and first instar larvae may not be able to penetrate the thickened epidermal cells of roots 5 mm diam or larger.

Densities of soil-borne flea beetle larvae

Materials and Methods

The density of black dot flea beetle larvae in the soil was determined by collecting soil samples from the Pollock, South Dakota site (T128N, R79W, Sec 21, NW ¼, Campbell County). The point of original release of black dot flea beetles was marked with a metal post in 1990. Three transects were delineated in leafy spurge patches at the Pollock site (Fig. 3). The center

Fig. 1. Percent cumulutive mortality of newly hatched black dot flea beetle larvae in the absence of food.

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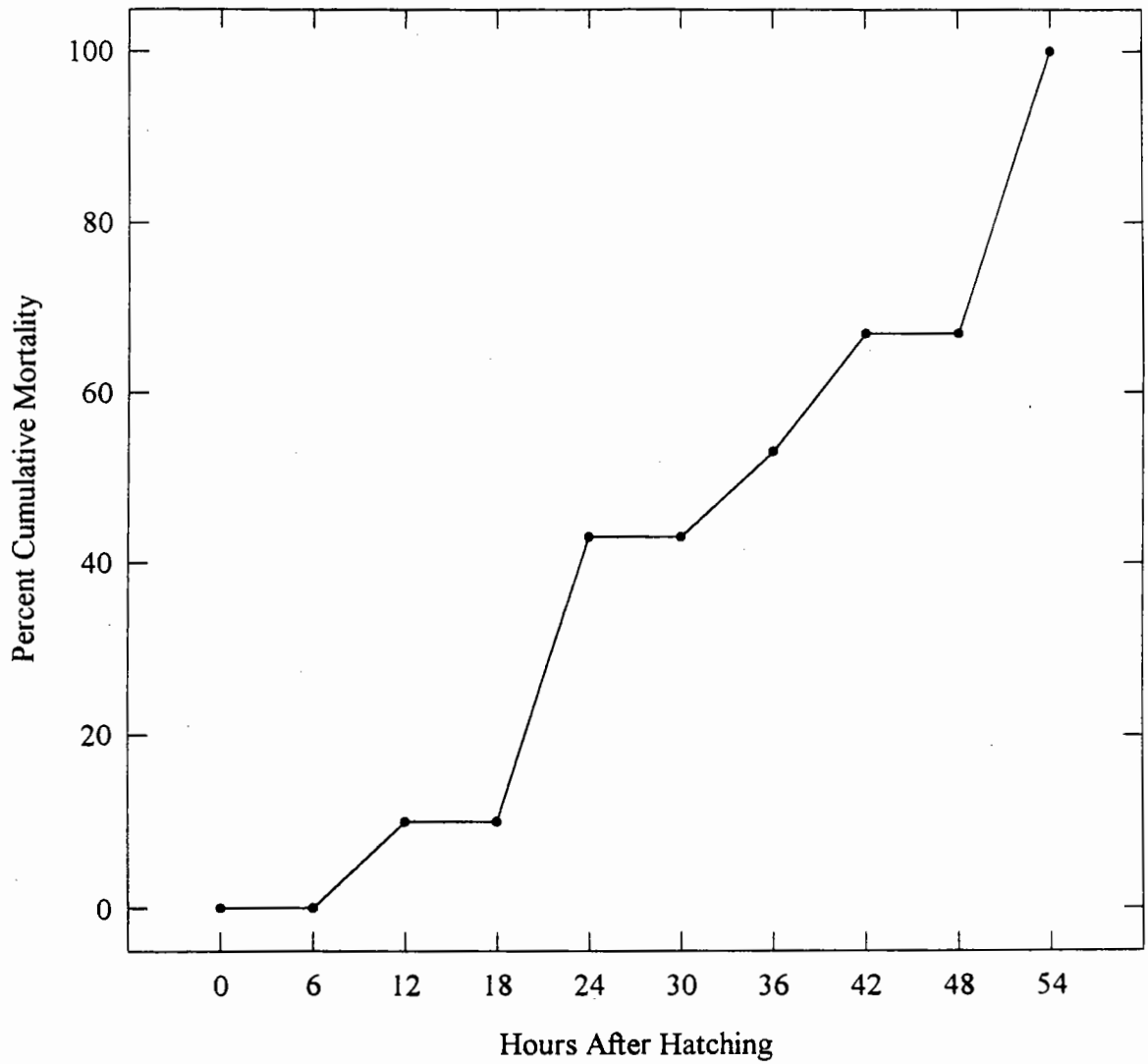
Fig. 2. Percentage of black dot flea beetles that initiated feeding on leafy spurge roots of differing diameters.
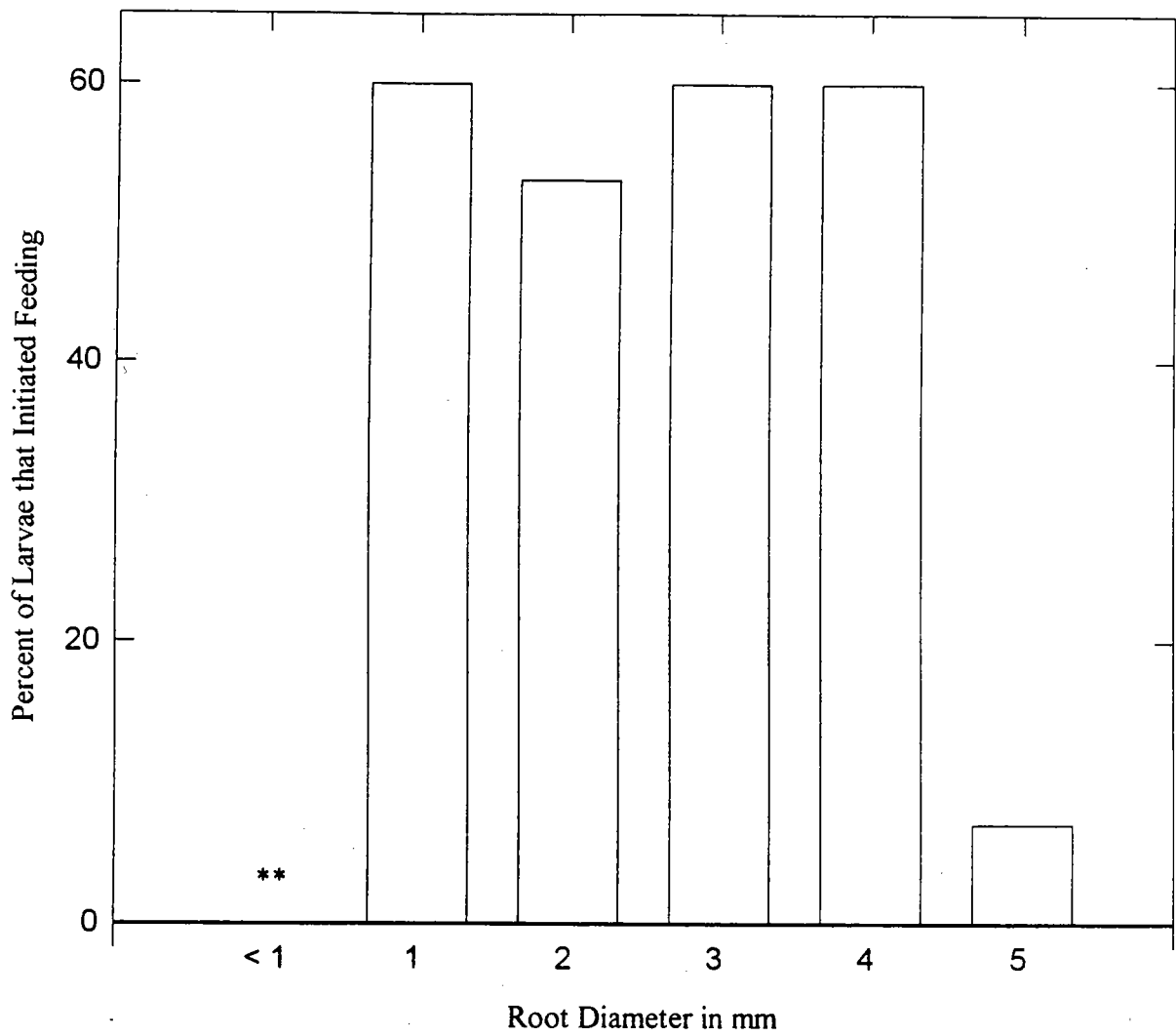
** larvae did not feed on roots less than 1 mm diam.

Fig. 3. Layout of transects at the Pollock, South Dakota study site. Transect point D in Transect Two is the original point of black dot flea beetle release in 1990.

Transect Two

• J   Transect Two   • P   Transect Three

 • I   • O   • M

  • H   • N   • L

• • • * D • • •   ← 40 m →   • K

A B C   E F G

    • M   • • • • D • • •

                A B C   E F G

    • L   • J

                |←→|

    • K   2.5 m   • I

                     • H

↑

18 m

↓

• J   Transect One   • M

 • I   • L

  • H   • K

• • • • • • •

A B C D E F G

N ↑

12
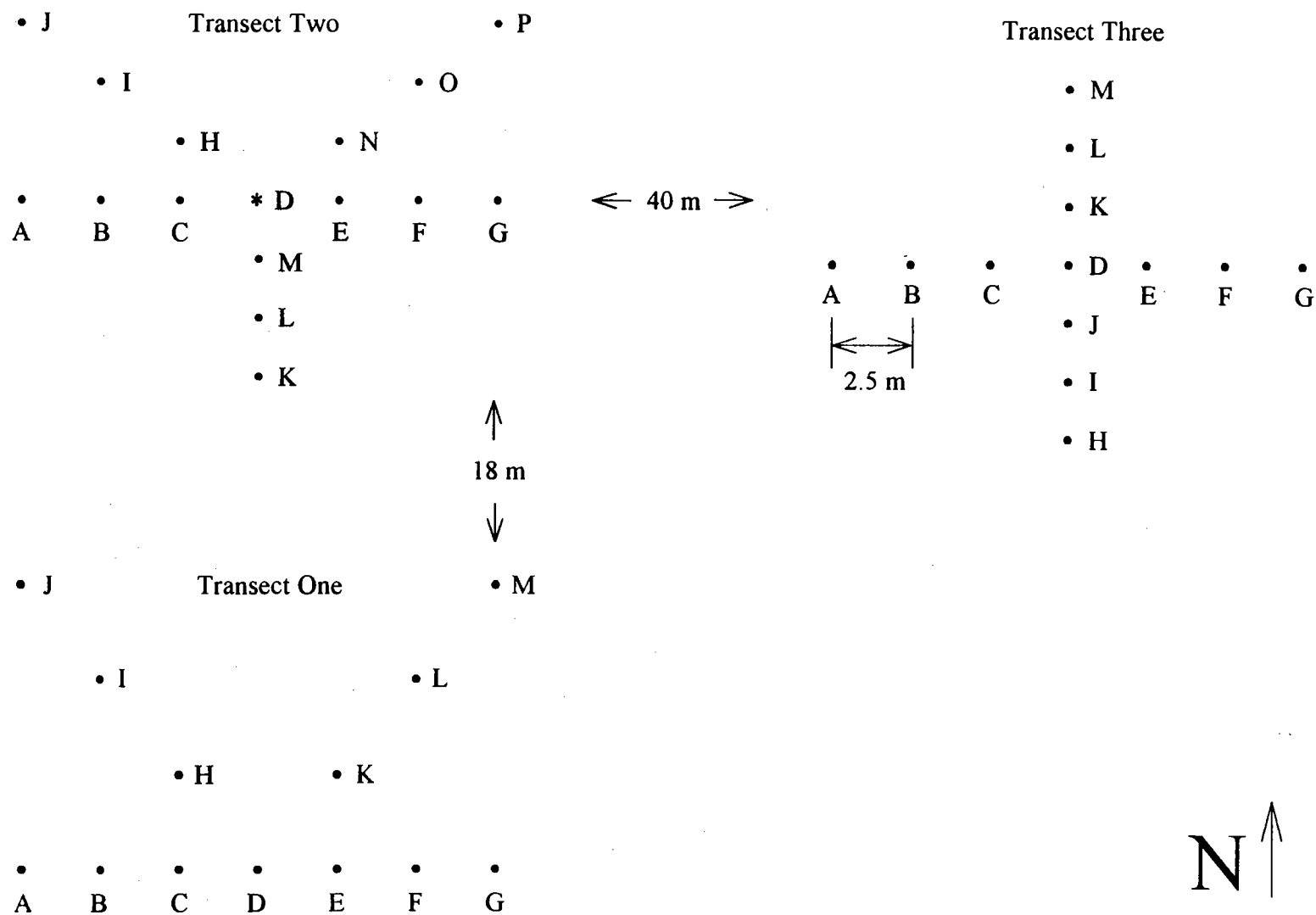
point of Transect Two was placed within 0.25 m of the metal post marker. The center point of Transect One was 18 m south of the original point of black dot flea beetle release. Transect Three's center was 40 m east of the original point of release. The distance between each point within transects was 2.5 m.

Each spring, senescent leafy spurge shoots were located at points in transects and a cup cutter was used to extract soil and roots from surface to 7.6 cm and 7.6 to 10.2 cm depth. In 1995, soil was collected from the surface to 15.0 cm depth using a 10.5 cm diam cup cutter and was evaluated for particle size and pH. Individual core samples were placed in a 3.8 liter plastic bags. Each year, 13 core samples were taken from Transect One, 16 from Transect Two, and 13 from Transect Three. Core samples were collected on 22 May 1995, 19 April 1996, and 29 April 1997. In 1996 and 1997, samples were collected within 0.3 m from original (1995) transect points.

In the laboratory, soil core samples were broken into smaller fractions by hand. Leafy spurge roots were removed from samples and inspected for the presence of larvae. Soil was then sifted through a sieve (2 mm opening, No. 10 USA Standard Testing Sieve, Fisher Scientific Company). Larvae were visually located and picked from soil using a microforceps. Black dot flea beetle larval identification was confirmed by comparison with laboratory-reared specimens.

Results and Discussion

The leafy spurge infestation at the Pollock site covered about 20 ha. Soil classification was Sully silt loam with a pH of 7.4. Soil was comprised of 20.8% sand, 61.9% silt, and 17.3% clay. In 1995, 42 larvae were found in core samples collected from surface to 7.6 cm depth (Table 1), while only 3 larvae were found in core samples taken from below 7.6 cm depth.

Table 1. Number of black dot flea beetle larvae collected from transect points at the Pollock, South Dakota site.

Transect One	1995	1996	1997	Transect Two	1995	1996	1997
A	1	3	2		0	4	0
B	0	0	0		2	2	0
C	2	3	0		2	1	0
D	0	0	0	*	2	2	1
E	0	0	0		5	0	0
F	0	1	0		9	0	0
G	10**	1	3		0	0	0
H	1	1	0		3	0	0
I	0	2	10		0	0	0
J	0	0	2		3	0	0
K	0	2	0		1	0	0
L	0	0	3		0	1	0
M	0	0	1		2	2	0
N					0	0	0
O					0	0	0
P					0	0	0

Transect Three	1995	1996	1997
A	0	0	0
B	1	5	0
C	0	0	1
D	0	5	0
E	0	0	0
F	0	0	0
G	0	0	1
H	0	13	1
I	1	28	0
J	0	2	2
K	0	1	0
L	0	0	1
M	0	0	0

* Original point of black dot flea beetle release in 1990.
** 3 of the 10 larvae collected in 1995 were located at 7.6 to 10.2 cm depth.

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Consequently, in 1996 and 1997, soil core samples were taken only from the surface to 7.6 cm depth.

Average larval densities were 124 per m^2 in 1995, 217 per m^2 in 1996, and 74 per m^2 in 1997 (Table 2). In 1995, Transect Two contained relatively high densities (209 per m^2) of larvae. However, the density of larvae was 41% lower in 1996 (Table 2). Only 1 larva was found in all of the soil cores collected from Transect Two in 1997. Densities of larvae within Transect One ranged from 116 to 187 per m^2 between 1995 and 1997. Transect Three was farthest from the original point of release and relatively few larvae were retrieved from soil cores in 1995 and 1997. In 1996, 2 transect points within Transect Three contained 13 or more larvae. Since adult dispersal is minimal, the highest densities of larvae would be expected in the leafy spurge patch closest to the original point of release. This was true 5 years after introduction, but not in 1996 or 1997, even though living leafy spurge shoots were present in the transect area. Larval densities fluctuated between transect points and transects during the study.

Variability of larval densities in transects over time may be due to changing biological and environmental factors. Attractiveness of patches as prospective flea beetle oviposition sites may have changed from year to year. In patches with high larval densities, the root structure of plants may be altered as a result of extensive feeding. Additionally, harsh winter weather conditions in 1997 may have resulted in high mortality of soil-borne larvae.

Adult flea beetle sex ratios, female insemination, and oocyte development

Materials and Methods

Adult black dot flea beetles were collected from the Pollock site in 1995, and Pollock, Valley City, North Dakota (T139N, R58W, Sec. 34, NE ¼, Barnes County) and Theodore

Table 2. Average number of black dot flea beetle larvae per $m^2 \pm$ SEM (standard error of the mean) in transects at the Pollock, South Dakota site.

	#/m^2		
Transect	1995	1996	1997
One	124 ± 88	116 ± 37	187 ± 89
Two	209 ± 70	87 ± 34	7 ± 7
Three	18 ± 12	480 ± 259	53 ± 21
Site Average	124 ± 39	217 ± 84	74 ± 30

Roosevelt National Park (T140N, R102W, Sec. 22, NW ¼, Billings County, North Dakota) sites

in 1996 for the reproductive status studies. A 38.1 cm diam. heavy duty sweep net (BioQuip

Products, Gardena CA) was used to collect samples. In 1995, 200 adults were collected on each

sampling date at Pollock. The targeted sample size on each sampling date in 1996 at South

Dakota and North Dakota sites was 100. Samples were obtained by walking and sweeping the

net opening back and forth through dense areas of leafy spurge shoots. Each sweep consisted of

an arc of 180°. The sweep net was checked periodically for beetles and sampling continued until

the approximate number of adults was captured, or until 15 min had passed. Contents of the

sweep net were placed in a 9 cm diam $\times$ 17.5 cm ht cardboard container with a bouquet of leafy

spurge shoots. Each cardboard container was placed on 2 ice packs (to immobilize beetles)

within an insulated cooler and shipped overnight to South Dakota State University. Specimens

were examined immediately upon arrival. Samples were collected on 5 dates each year from the

Pollock site (28 June, 6 July, 18 July, 25 July, and 3 August in 1995, and on 21 June, 9 July, 15

July, 25 July, and 30 July in 1996). Samples were collected on 5 dates in 1996 from the Valley

City site (14 June, 24 June, 2 July, 10 July, and 26 July) and from Theodore Roosevelt National

Park (26 June, 2 July, 10 July, 17 July, and 25 July).

In the laboratory, adults were picked randomly from containers without regard to size or

activity, and were placed under a dissection microscope. Abdominal characteristics described by

McDaniel et al. (1992) were used to determine the sex of adults. Twenty-five females from each

sample were dissected and analyzed for insemination and oocyte (unlaid eggs within the female

abdomen) development. Insemination of females was determined by removing the spermatheca,

crush-mounting it between glass slide and cover slip, and determining the presence (inseminated)

or absence (not inseminated) of spermatazoa using a compound microscope. Development of the

oocyte was evaluated using a rating scale similar to that of Sherwood and Levine (1993). Oocyte characteristics that were used to determine development included: size, color, rigidity, and condition of the chorion. Females that had no oocyte development were given a rating of 0. Stage 1 females contained oocytes that were relatively small and milky white. Stage 2 females contained larger yellow oocytes (than in stage 1 females). Females with yellow, pliable oocytes were categorized as stage 3. Stage 4 females contained larger, yellow, rigid oocytes with sculptured outer membranes.

Results and Discussion

In 1995, adult emergence began in late June and was preceded by about a 1 wk period of low temperatures $\geq 8^{\circ}$ C. Temperatures were as high as 31° C during that same wk. Total precipitation in 1995 between 22 May and 13 August was 14.9 cm. Adults could not be collected with sweep net sampling after 3 August, but their rarity did not appear to be weather related.

The average percentage of males collected in 1995 samples was 2.5 (Fig. 4a) and female insemination averaged 32% (Fig. 4a). Female-biased sex ratios in arthropod populations are not unusual (Hamilton 1967), but insemination of females did not exceed 50% during the sampling season (Fig. 4a). Average oocyte maturation gradually increased from late June to 25 July (Fig. 5a).

In 1996, adult emergence at the three sites began in mid to late June following about 1 wk of low temperatures $\geq 7^{\circ}$ C. High temperatures at the study sites for this same 1 wk period prior to emergence were $\geq 28^{\circ}$ C. Total precipitation at Pollock, Valley City, and Theodore Roosevelt National Park between 22 May and 13 Aug was 11.1, 19.2, and 18.0 cm, respectively. Adults

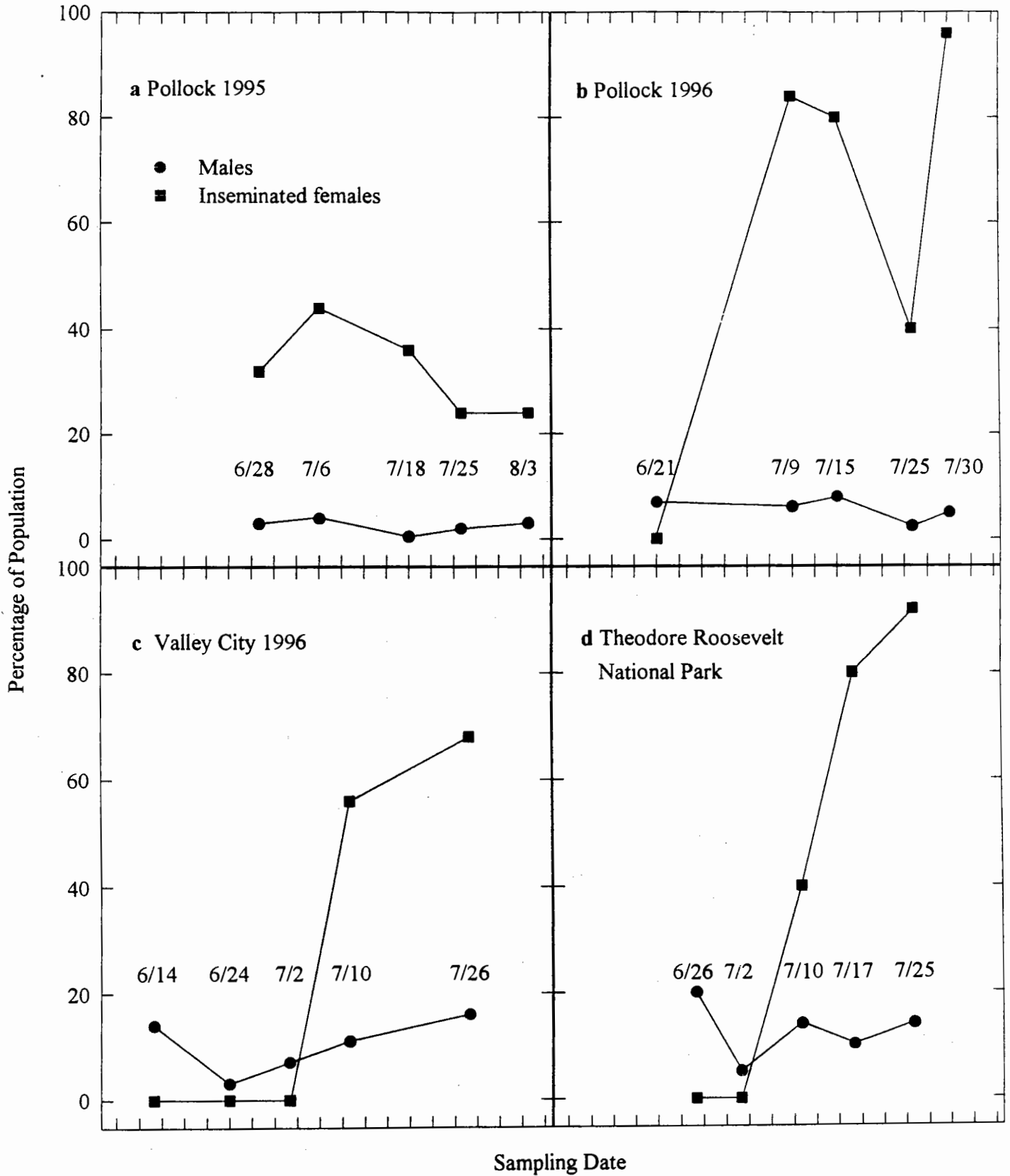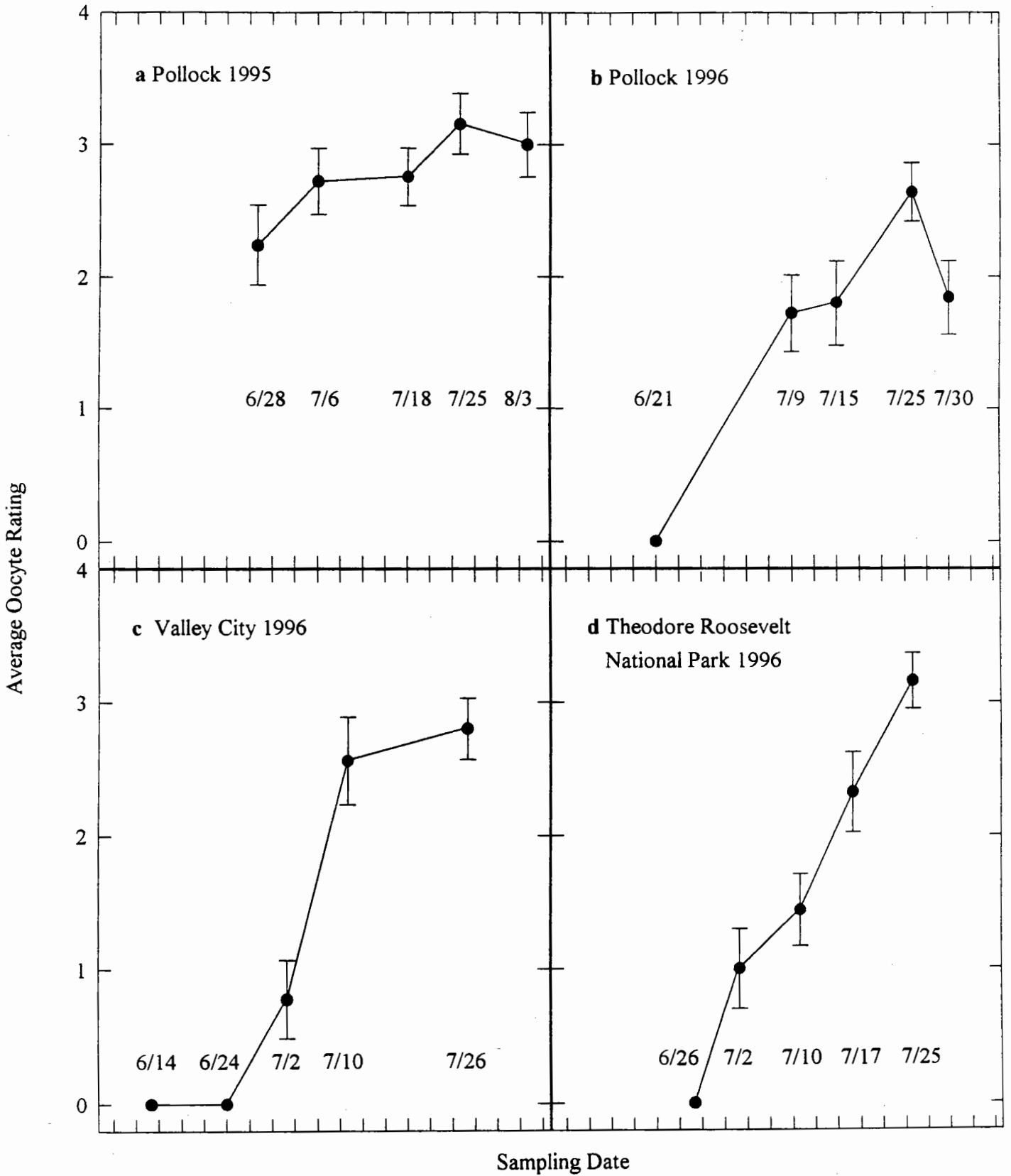Fig. 4. Percentages of males and inseminated females at study sites.

Fig. 5. Average oocyte rating of female black dot flea beetles from study sites.

could not be captured at the study sites in August. The collection period for adults using sweep net sampling was less than 2 mo long.

Average percentages of males in Pollock, Valley City, and Theodore Roosevelt National Park samples were 5.6, 10.2, and 12.6, respectively (Fig. 4b–d). Males comprised 16% of sampled adults at the Valley City site on 26 July (Fig. 4c). The percentage of males at the Theodore Roosevelt National Park site on 26 June was 20% (Fig. 4d). These data agree with Jackson's (1997), who observed emergence of 229 and 283 adults from Valley City soil cores under controlled conditions. Jackson (1997) reported that males comprised 16–22% of adults from soil cores collected in 1994 and 1995. The sex ratio of adult black dot flea beetles in a Hungarian sample was 1:4 (male: female)(Andre Gassmann, personal communication). Sommer and Maw (1982) studied *A. cyparissiae* (Koch) and *A. flava* Guill. collected from Europe and found sex ratios of about 1:1. According to Nunney (1993) sex ratios have a major influence on the effective size of populations and highly polygynous mating systems can produce low values of effective population size.

In 1996, insemination and oocyte development in females (from the three sites) was not detected until after 2 July. Average insemination of females for the last 3 sampling dates was 72.0% at Pollock, 41.3% at Valley City, and 70.7% at Theodore Roosevelt National Park. Oocyte development was similar at the 3 sites and appeared to follow trends of increasing female insemination (Figs. 4 and 5). Sherwood and Levine (1993) reported that mating increased oocyte growth and egg-laying in the western corn rootworm (*Diabrotica virgifera virgifera* LeConte). The stimulus associated with mating that appears to be necessary to sustain ovarian development is the presence of spermatazoa in the spermatheca (Sherwood and Levine, 1993).

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Mating success of black dot flea beetles released to an uncolonized area may be lower than that observed at established sites, especially if there is male mortality en route. Eckstrand and Seiger (1975) concluded that insect mating frequency declines with lower population densities. Hopper and Roush (1993) advised release of over 1000 individuals at a single site and time to improve establishment of insect species used for biological control.

In past years, collections of black dot flea beetles for distribution to new areas were taken in early July. During the first week of July, female insemination and oocyte development were relatively low. Results from our studies suggest that collections for distribution taken in mid-July (about 30 d following adult emergence) would contain >40% inseminated females. Additionally, mid-July oocyte maturation ratings had progressed to about half of the average values observed on the last sampling date. Oocyte development indicates when oviposition will take place during the summer season (Sherwood and Levine, 1993) and collections for distribution taken in mid-July would not interfere with a majority of oviposition activity. Finally, a strategy that times collections with high female insemination rates and oocyte maturation would improve the potential for reproduction at the new release site.

Genetic analysis of black dot flea beetles

Materials and Methods

Adult black dot flea beetles were collected from Pollock, Valley City, and Theodore Roosevelt National Park study sites for genetic analysis between 14 and 26 June, 1996. In 1997, adults were collected from the 3 North American sites between 9 and 16 July. Live specimens were shipped overnight in insulated containers with ice packs to South Dakota State University and then stored in an ultracold unit at -80° C. Hungarian specimens were collected near Baja on 17 June, 1997 and were frozen and shipped in an insulated container with dry ice. Forty-eight

individuals from each North American site were genetically analyzed each year. Genetic tests

were conducted on 96 individuals from Hungary. Genetic variability was determined using

cellulose acetate electrophoresis methodologies described by Hebert and Beaton (1993). The

enzyme stains used were malate dehydrogenase (MDH) and phosphoglucomutase (PGM).

Because of the large number of samples, alleles were differentiated by measuring mobility and

width to the nearest mm. Alleles were designated letters based on mobility and frequencies were

determined. Allelic frequency data from the 4 populations were used in calculating Nei's genetic

identity and Nei's genetic distance matrices (Nei 1972). Genetic identity and genetic distance

calculations provide comparisons of populations based on allelic frequency data (Nei 1972).

Results and Discussion

For PGM, D was the most common allele in the Hungarian and Valley City populations

(Table 3). This allele was found at frequencies of 0.18 and 0.09 at Pollock and Theodore

Roosevelt National Park, respectively. The E allele was more prevalent at Pollock and Theodore

Roosevelt National Park and occurred at a frequency of about 0.27. There were 2 alleles that

were unique to populations. The B allele was observed in Valley City, but was not found in the

other sampled populations (Table 3). Similarly, the J allele was present in Theodore Roosevelt

National Park, and was not detected in Pollock, Valley City, or Hungary. Valley City exhibited

the greatest number of different alleles at PGM, while the fewest were observed in Pollock and

Hungarian samples (Table 3). Pollock specimens did not exhibit the C allele which was present

in the 3 other populations. In addition, there were 2 alleles (H and I) that were present in all 3

North American populations, but not detected in Hungarian samples. Frequencies of

heterozygotes were 0.42 and 0.40 at Pollock and Theodore Roosevelt National Park,

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Table 3. Allele frequencies at PGM and MDH for *Aphthona nigriscutis* populations from Baja, Hungary, Pollock, South Dakota, Valley City, North Dakota, and Theodore Roosevelt National Park (T.R.N.P.).

Gene Locus	Alleles	Population			
		Hungary	Pollock	Valley City	T.R.N.P.
PGM	A	0.06	0.00	0.01	0.00
	B	0.00	0.00	0.07	0.00
	C	0.17	0.00	0.09	0.02
	D	0.44	0.18	0.21	0.09
	E	0.11	0.28	0.15	0.27
	F	0.14	0.15	0.13	0.23
	G	0.08	0.13	0.18	0.10
	H	0.00	0.19	0.13	0.08
	I	0.00	0.08	0.02	0.16
	J	0.00	0.00	0.00	0.04
	Heterozygotes	0.17	0.42	0.21	0.40
MDH	A	0.01	0.01	0.01	0.00
	B	0.00	0.02	0.06	0.00
	C	0.00	0.03	0.10	0.01
	D	0.14	0.06	0.00	0.06
	E	0.45	0.22	0.06	0.21
	F	0.33	0.38	0.37	0.23
	G	0.05	0.17	0.30	0.22
	H	0.02	0.08	0.12	0.15
	I	0.00	0.02	0.01	0.12
	J	0.00	0.02	0.00	0.01
	Heterozygotes	0.68	0.61	0.35	0.66

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respectively. The occurrence of heterozygotes was considerably lower in the Hungarian and Valley City samples (Table 3).

The most common allele at MDH in the North American populations was F and occurred at frequencies of 0.23–0.38. Thirty-three percent of Hungarian individuals possessed this allele, but individuals exhibiting E were more prevalent (Table 3). For MDH, none of the alleles were unique to a single population. However, the North American populations (combined) exhibited 4 alleles that were not detected in Hungarian samples (Table 3). Valley City lacked D and J, and Theodore Roosevelt National Park lacked A and B. Pollock possessed the largest number of different alleles at MDH. Valley City had the lowest frequency of heterozygotes (Table 3). In Hungary, Pollock, and Theodore Roosevelt National Park samples, 61–68% of individuals exhibited heterozygosity.

The North American populations were more similar to each other with respect to allele frequencies (as indicated by genetic distance calculations) than each was to the Hungarian population. Comparisons of the Pollock, Valley City, and Theodore Roosevelt National Park populations to each other using Nei's (1972) formulas generated genetic distance values of less than 0.097 (Table 4). Genetic distances between the Hungarian population and the North American populations were 0.112 (Pollock), 0.286 (Valley City), and 0.146 (Theodore Roosevelt National Park). However, these values are relatively small considering that Nowierski et al. (1996) determined a genetic distance value of 0.205 for populations of *A. nigriscutis* from Batmonaster, Hungary and Lobau, Austria. Also, genetic variability was not lower in the North American populations compared to the Hungarian population. Instead, there were several electrophoretic variants present in the North American populations that were not detected in the Hungarian population.

Table 4. Matrix of Nei's genetic identity (above diagonal lines) and Nei's genetic distance (below diagonal lines; Nei 1972) for *Aphthona nigriscutis* populations from Baja Hungary, Pollock, South Dakota, Valley City, North Dakota, and Theodore Roosevelt National Park (North Dakota). The genetic identity value is 1.000 when 2 populations have the same alleles in identical frequencies, while it is 0.000 when they have no common alleles. Genetic distance measures the accumulated number of gene substitutions per locus.

Population	Hungary	Pollock	Valley City	Theodore Roosevelt National Park
Hungary	———	0.773	0.517	0.714
Pollock	0.112	———	0.885	0.885
Valley City	0.286	0.053	———	0.799
Theodore Roosevelt National Park	0.146	0.053	0.097	———

Hamilton (1967) determined that whenever there is brother-sister mating among small arthropods, there is usually a restriction in the production of males. In addition, as long as males are sufficiently numerous to fertilize all the females, the population will show accelerated expansion (Hamilton 1967). These conditions may have occurred in the North American populations, but whether rapid population growth was sufficient to produce the alleles (through mutation) that were not observed in the Hungarian population is unknown.

INFLUENCE OF DELETERIOUS RHIZOBACTERIA
ON LEAFY SPURGE ROOT CARBOHYDRATES

Materials and Methods

Deleterious rhizobacteria experiments were conducted at 3 leafy spurge infested sites in South Dakota. Abraham Lake and Olson sites (Abraham Lake site, T127N, R54W, Sec 2, SE ¼; Olson site, T127N, R53W, Sec 20, NW ¼, Marshall County) were located in the dry subhumid region of eastern South Dakota, while the Pollock site was located in the north central part of the state at the margins of dry subhumid and semi-arid regions. Black dot flea beetles were first introduced to the Pollock site in 1990 and have become established. No *Aphthona* flea beetles had been introduced to the Abraham Lake or Olson sites.

Ring structures cut from PVC pipe (15.2 cm diam × 10.2 cm ht) were placed around leafy spurge shoots at the study sites. Each ring was inserted into the ground using a wooden block and a sledgehammer and the ring was hammered until the rim was level with the soil surface. A total of 339 ring treatments were applied to the three sites between 19 May, 1995 and 14 June, 1996. Treatment dates, number of rings treated, and retrieval dates for each study site are presented in Tables 5–8. Rings were randomly assigned treatments of 2 g. starched-based granules containing no deleterious rhizobacteria (blank controls), or strains of deleterious

Table 5. Deleterious rhizobactera treatments and treatment dates for the Pollock site.

Treatment	No. of treated rings	No. of rings retrieved		No. of treated rings	No. of rings retrieved	
	12 June 1995	19 Oct. 1995	4 June 1996	3 Oct. 1996	4 June 1996	3 Oct. 1996
Control	36	12	12	12	12	12
Isolate 1	36	12	12	12	12	12
Isolate 2	36	12	12	12	12	12

Table 6. Deleterious rhizobactera treatments and treatment dates for the Abraham Lake site.

	No. of treated rings	No. of rings retrieved		No. of treated rings	No. of rings retrieved
	20 May 1995	20 Sep. 1995	14 June 1996	14 June 1996	4 Oct. 1996
Control	12	6	6	12	12
Isolate 1	18	9	9	12	12
Isolate 2	18	9	9	12	12

Table 7. Deleterious rhizobactera treatments and treatment dates for the Olson site.

		No. of treated rings	No. of rings retrieved		No. of treated rings	No. of rings retrieved
		19 May 1995	20 Sep. 1995	14 June 1996	14 June 1996	4 Oct. 1996
Olson	Control	12	6	6	12	12
	Isolate 1	18	9	9	12	12
	Isolate 2	18	9	9	12	12

Table 8. Additional deleterious rhizobacteria treatments and treatment dates for the Olson site.

		No. of treated rings			No. of rings retrieved
		11 Sep., 1995	20 Oct., 1995	3 June, 1996	4 Oct., 1996
Olson	Control	3	3	3	9
	Isolate 1	3	3	3	9
	Isolate 2	3	3	3	9

rhizobacteria isolate 1 (Montana isolate), or isolate 2 (South Dakota isolate). Granulated treatments were incorporated into the soil using a gardening claw tool. Samples were collected by extracting the ring and all of the contents (soil, roots, shoots) with a spade. Ring samples were placed in 3.8 liter plastic bags and stored at 4° C.

Isolates of deleterious rhizobacteria had been selected for spontaneous antibiotic resistance prior to treatments. This provided a marker for detection and introduced deleterious rhizobacteria were distinguished from indigenous soil bacteria by culturing samples on media containing antibiotics. Roots from rings were weighed and the number of buds on roots was recorded. A 3.5 cm section of leafy spurge root and 20 ml soil was removed from each ring sample and tested for presence of live deleterious rhizobacteria.

Carbohydrate analysis was conducted on root samples from all rings that were treated in spring 1995, and on 3-5 root samples from each ring treatment that were retrieved on 3 and 4 Oct., 1996 (Tables 5-8). A 5 cm section of leafy spurge root was removed from each sample and washed. The root section was cut into ≤ 2 mm pieces and dried for 24 hr at 70° C. Root segments were then fine ground using mortar and pestle. A 0.1 g sample was weighed and carbohydrate content was determined using chemical analysis procedures described by Scholes (1996).

Results and Discussion

Leafy spurge carbohydrate levels varied over time. Roots removed in spring 1996 contained 30% less total nonstructural carbohydrates than roots removed in fall 1995 or fall 1996 (Fig. 6). Lym and Messersmith (1987) studied seasonal variation in leafy spurge root carbohydrates and also found that levels were lowest in early spring. Changes in air temperature

were determined by Lym and Messersmith (1987) to be an important influence on root carbohydrate content.

Live deleterious rhizobacteria were detected on leafy spurge roots but were not found in soil within rings. The number of strain 1 bacterial cells per root segment ranged from 50 to 185,000. The number of strain 2 bacterial cells per root segment ranged from 100 to 150. Leafy spurge root weight and numbers of root buds were lower in deleterious rhizobacteria treatment rings than in blank-treated control rings (Figs. 7 and 8, respectively).

Average carbohydrate content in roots treated with blanks was 220.70 mg/g and was higher than in leafy spurge roots treated with deleterious rhizobacteria (Fig. 9). Combined data for deleterious rhizobacteria treatments showed 20% lower carbohydrate levels than blank treatments. Roots from areas treated with isolate 1 had slightly lower root carbohydrate concentration than roots treated with isolate 2. Average carbohydrate content in roots treated with isolate 1 and isolate 2 was 170.10 and 183.97 mg/g, respectively (Fig. 9). Root carbohydrate levels were higher in Pollock blank-treated rings containing black dot flea beetle larvae than in blank-treated rings at Olson and Abraham Lake sites (Fig. 10). However, there were no significant differences between sites for root carbohydrates in deleterious rhizobacteria treatments.

These results indicate that black dot flea beetle feeding activity did not contribute to deleterious rhizobacteria reductions in root carbohydrates, and in the absence of deleterious rhizobacteria may have actually stimulated carbohydrate storage in roots. Leafy spurge shoots may have compensated for larval root-feeding by directing photosynthate to injured roots. Many plant species are able to regenerate and compensate for damage caused by herbivores. In a study by Peschken and Wilkinson (1981), Canada thistle (*Cirsium arvense* [L.] Scop.) stems mined by

Fig. 6. Amount of total nonstructural carbohydrates in leafy spurge roots removed from the 3 study sites in fall 1995, spring 1996, and fall 1996.
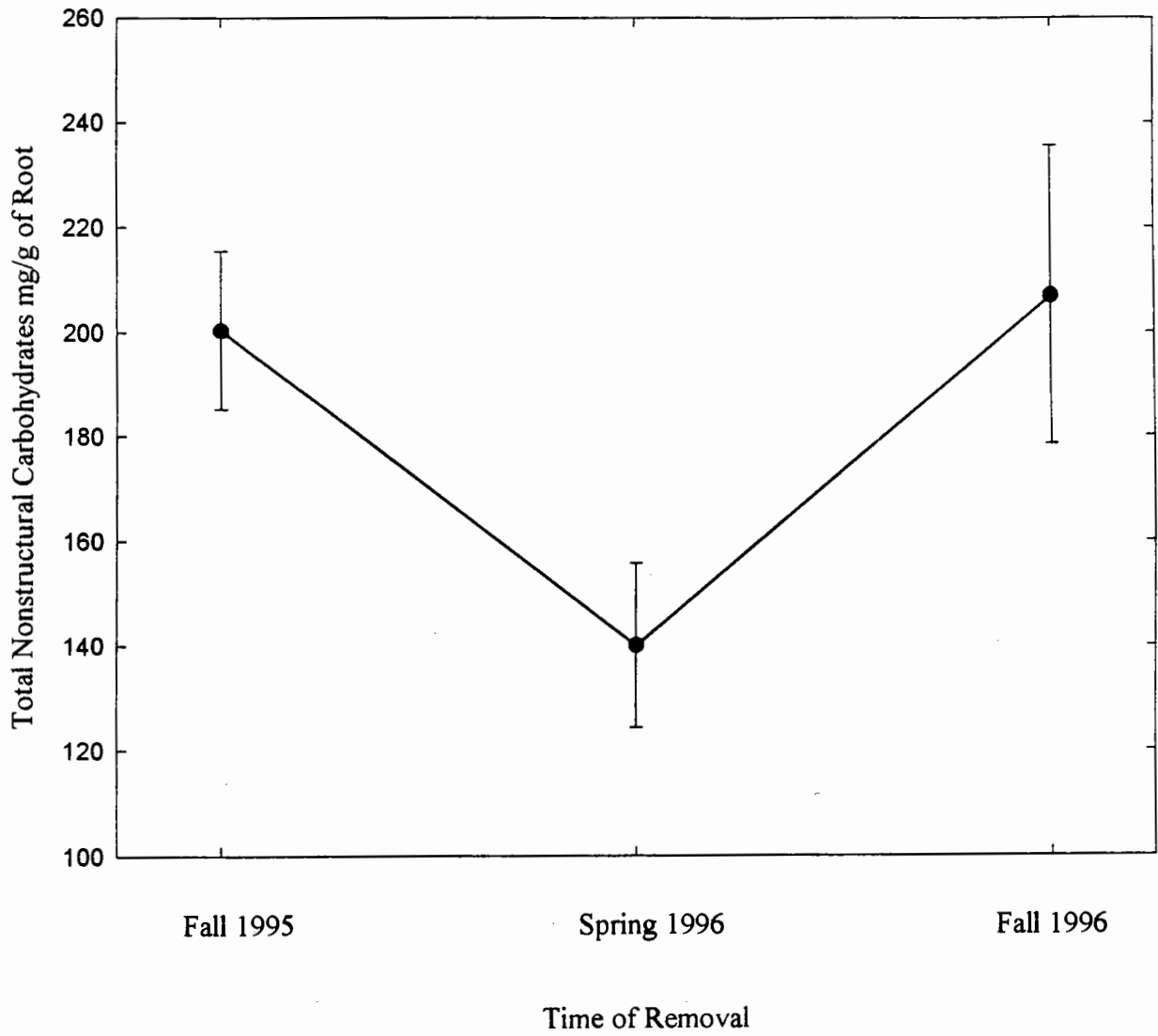
Fig. 7. Average weight (g) of leafy spurge roots treated with starch granules (blank controls), deleterious rhizobacteria strain 1, or strain 2.
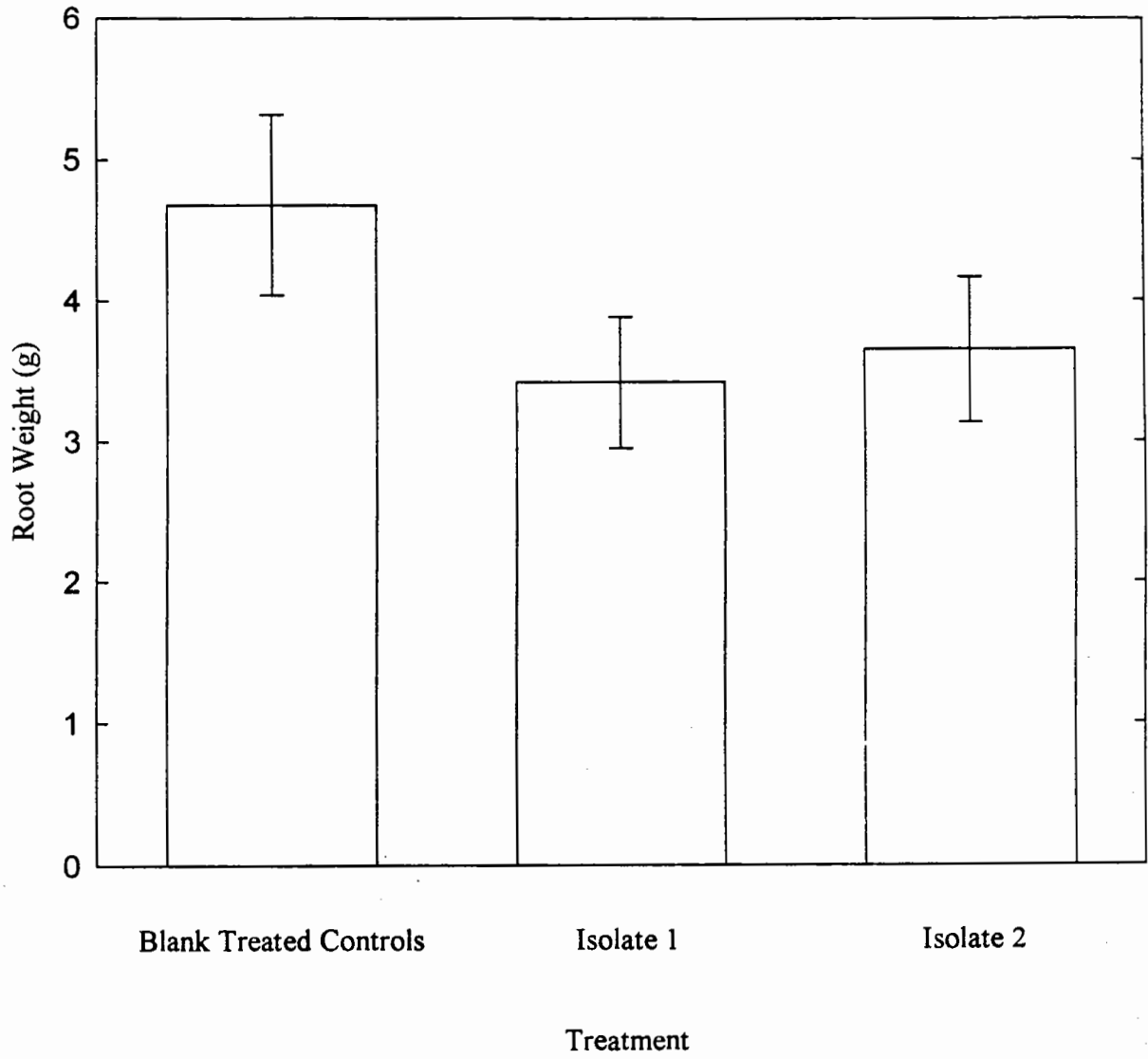
Fig. 8. Average number of buds on leafy spurge roots treated with starch granules (blank controls), deleterious rhizobacteria strain 1, or strain 2.
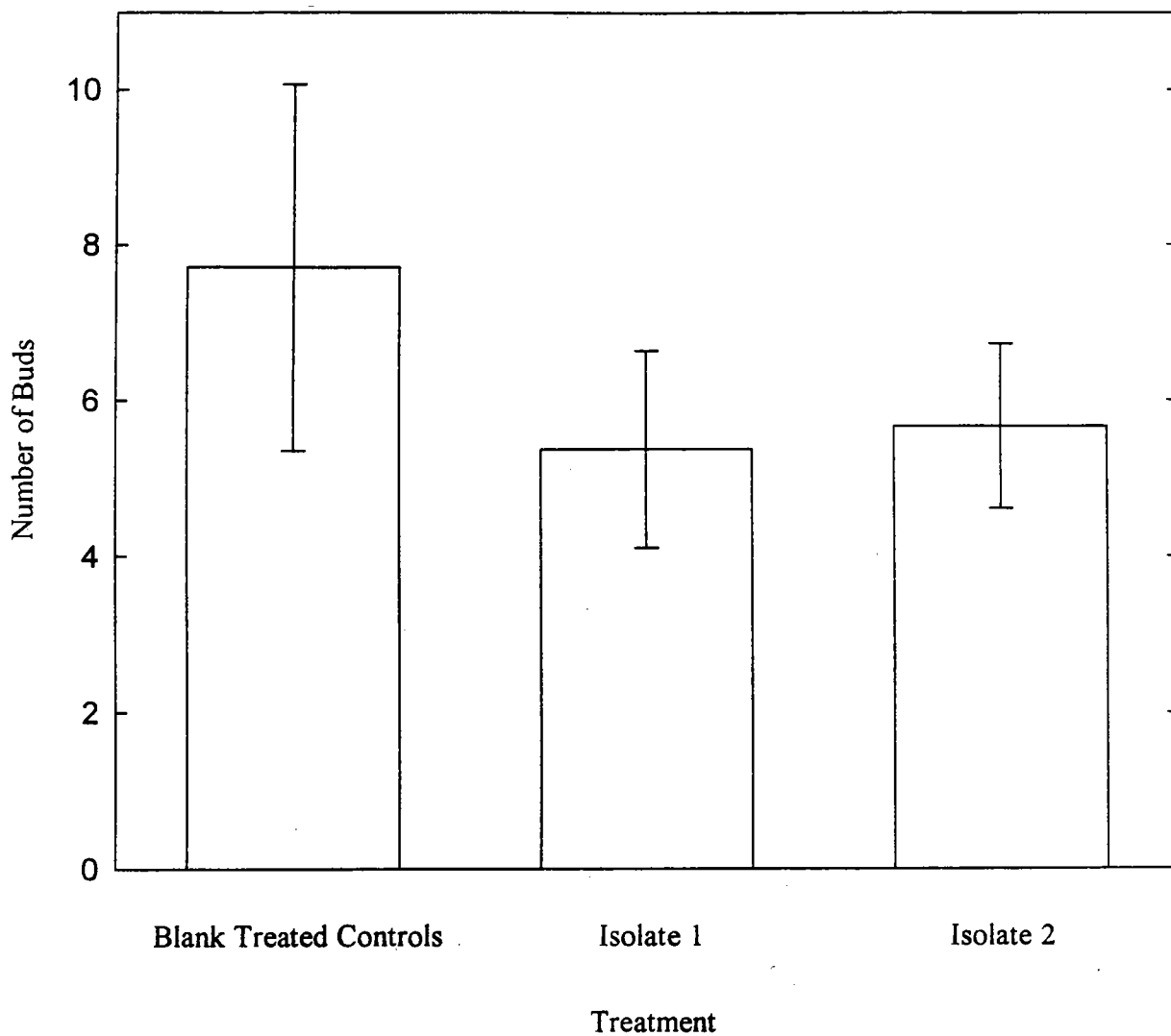
Fig. 9. Total nonstructural carbohydrates in leafy spurge roots treated with starch granules (blank controls), deleterious rhizobacteria strain 1, or strain 2.
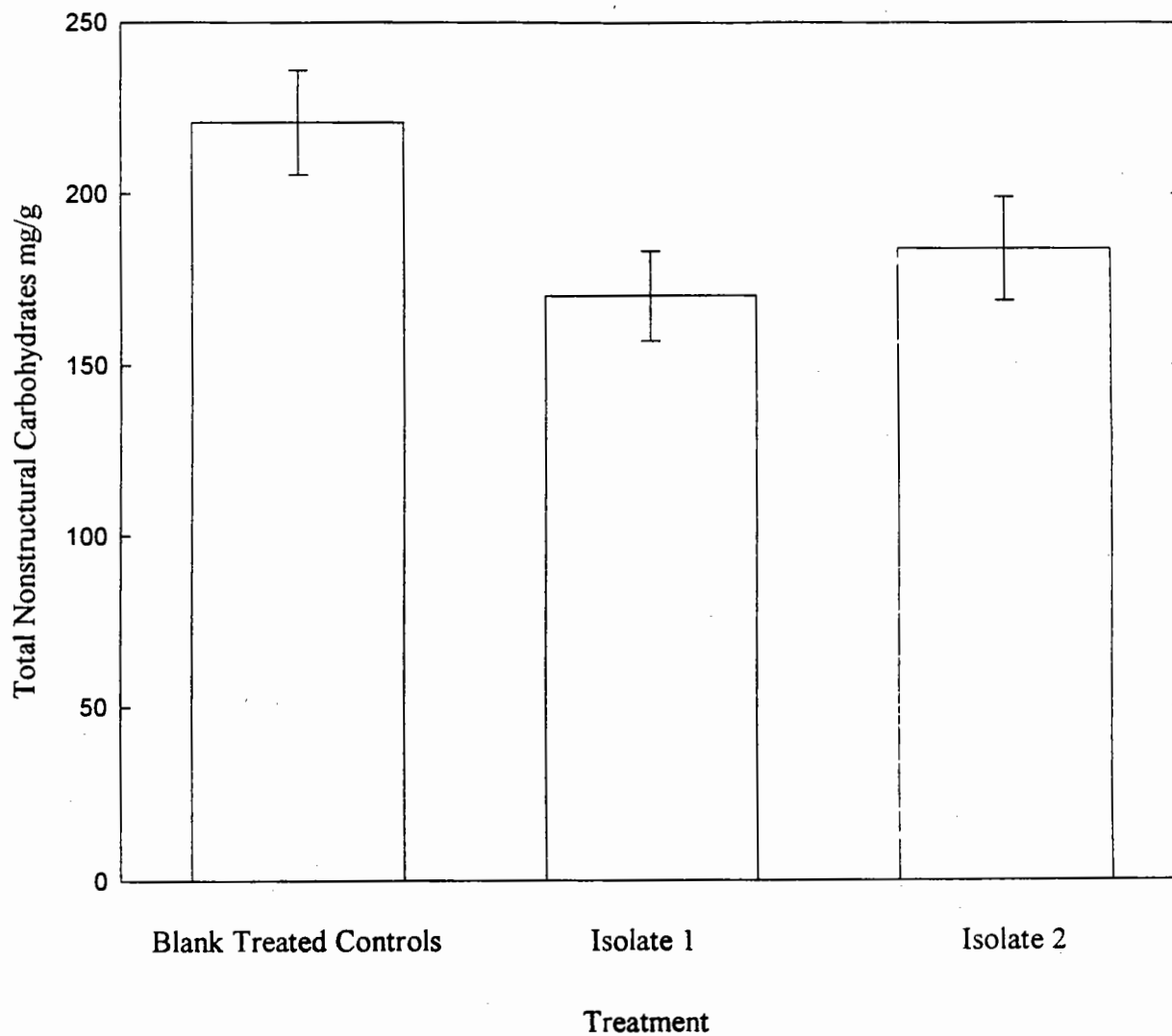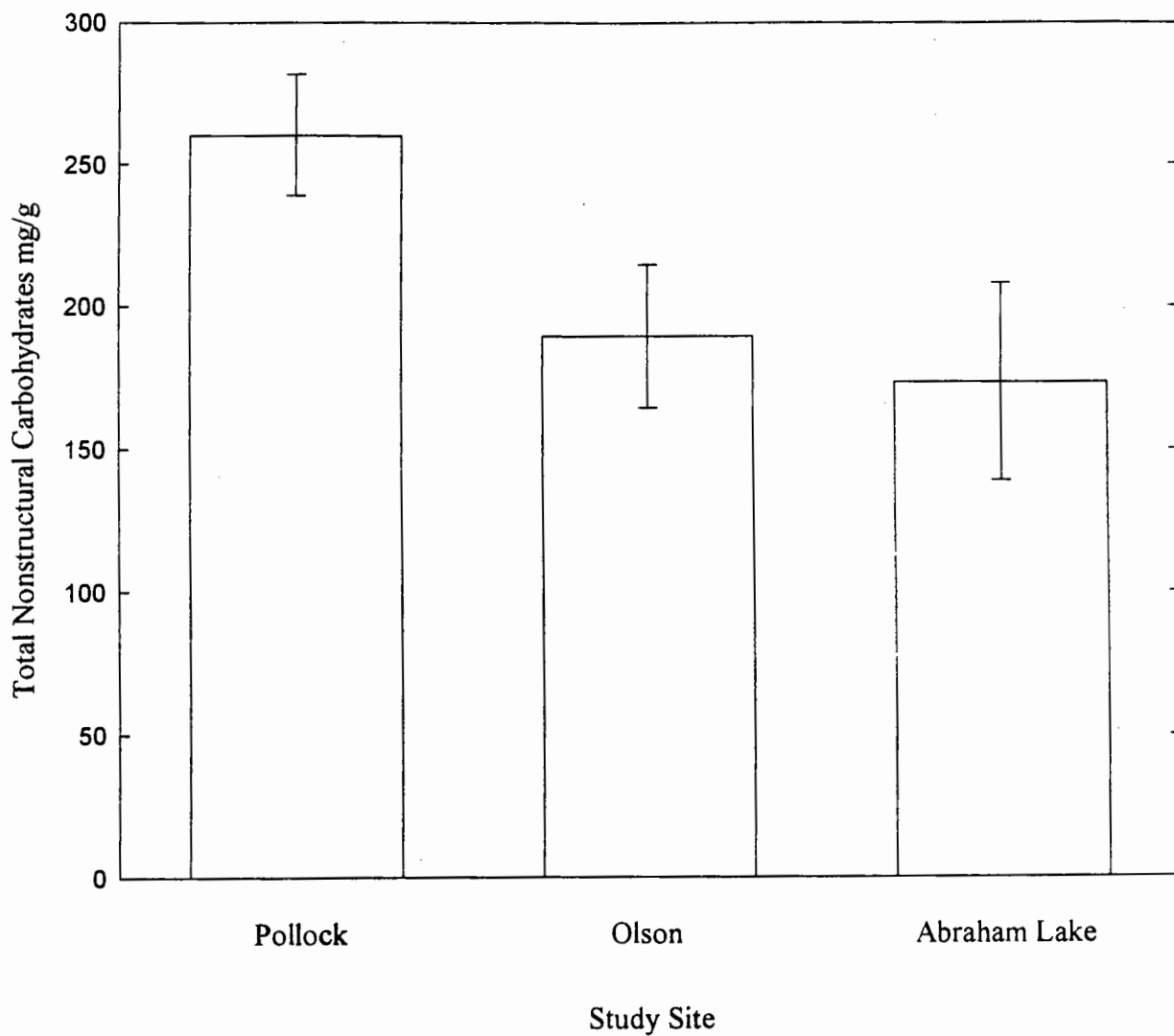
Fig. 10. Total nonstructural carbohydrates in leafy spurge roots treated with starch granules (blank controls) only. Samples were taken from rings at the Pollock, Olson, and Abraham Lake sites.

Ceutorhynchus litura (F.) grew taller than unmined stems. Also, Saner and Muller-Scharer
(1994) found more vegetative shoots on common toadflax (*Linaria vulgaris* Mill.) attacked by
Eteobalea spp. than on undamaged clones. Our study integrated black dot flea beetle root
feeding with deleterious rhizobacteria to reduce leafy spurge root carbohydrates. Although root
carbohydrates were reduced by deleterious rhizobacteria, the density of black dot flea beetle
larvae in soil may not have been high enough in the 2 years of our study to provide large
synergistic reductions in total nonstructural root carbohydrates.

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