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Abstract

The alfalfa snout beetle, *Otiorhynchus ligustici* L. (Coleoptera:Curculionidae), was introduced into the United States from Europe via wooden sailing ships carrying soil as ballast, was first reported in New York State in 1896 at the Port of Oswego and was first recorded as a pest of alfalfa when alfalfa was introduced into the area in the area. In subsequent years, this flightless and parthenogenetic insect has spread to nine Northern New York counties, infested over 200,000 hectares of cropland and has become the most serious pest of alfalfa in northern New York State. Research requires the availability of all life stages for an extended period of time. With a 2-year lifecycle and a mandatory diapause, the artificial rearing of a laboratory culture appears to be a non-viable option. Since the adults can be collected in the spring in large numbers, long term cold storage of adults would provide an extended window where all life stages would be available for laboratory and greenhouse research. A series of experimental storage regimes were developed and newly emerged beetles were placed under those regimes for extended periods of time. The impact of the storage conditions was measured by mortality and fecundity when compared to a control group. Generally, beetles survived better in storage at the colder temperatures. Beetles allowed to feed seven days before storage generally survived better than unfed beetles. In most cases, any length of storage reduced the number of eggs laid compared to the control group. Beetles survived for more than 300 days in storage and when warmed up, consumed food and laid viable eggs.

The alfalfa snout beetle, *Otiorhynchus ligustici* L. (Coleoptera: Curculionidae), was introduced into the United States from Europe via wooden sailing ships carrying soil as ballast (Lindroth 1957, York et al. 1971). The beetle was first recorded in New York State in 1896 at the Port of Oswego and was first recorded as a pest of alfalfa when alfalfa was introduced into the area in the 1920s (York et al. 1971). In subsequent years, this flightless and parthenogenetic insect has spread to nine Northern New York counties, infested over 200,000 hectares of cropland and has become the most serious pest of alfalfa in northern New York State (Shields et al. 2009).

The biology and life history of alfalfa snout beetle has been studied and described by several authors in Eurasia and North America (Lincoln and Palm 1941, Hanuss 1958, Nyilas 1962, York 1974, Jermy and K. Balázs 1990) and is very similar throughout Europe and Northern New York. Alfalfa snout beetle has a 2-year lifecycle. Larvae feed on the lateral roots and later on the tap roots of the host plants. Most larvae mature by late fall and move down in the soil to varying depths depending on soil type, temperature, and other factors. Mature larvae remain quiescent deep in the soil for ca. 8 months before pupation the

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following summer. After eclosion, adults remain in the pupal cells and only move to the soil surface the following spring after spending the second winter in the soil (Lincoln and Palm 1941). The factors controlling the mandatory diapause and the conditions trigging the breaking of diapause are unknown.

Research efforts focused on this insect require the availability of all life stages for an extended period of time; usually large numbers of individuals are needed. With a 2-year lifecycle and a mandatory diapause, the artificial rearing of a laboratory culture appears to be a non-viable option. However, the adults can be collected in the spring in large numbers and if the newly emerged adults can be stored for an extended period of time, the availability of all insect life stages will be greatly extended and allow for a long time period to conduct research in the laboratory and greenhouse environments (Shields et al. 2007).

Materials and Methods

The adult beetles used in these experiments were collected in Jefferson Co, New York near Great Bend, NY. Beetles were collected shortly after spring emergence on April 27, 1994 and April 28, 1995. In 1994, 2,000 beetles were collected and 5,300 beetles were collected in 1995. Collected beetles were placed in a cooler with ice packs and returned to the laboratory. In the laboratory, beetles were randomly assigned to each experimental regime. For the 1994 experiments, a total of 500 beetles were assigned to each of three storage regimes (total beetles 1500). In 1995, a total of 800 beetles were assigned to each of four storage regimes (3200 beetles). Each year, 15 beetles were randomly selected and placed immediately in egg laying containers with fresh food. These beetles were considered the control group.

Experimental storage temperatures were 1, 3 and, 5°C in 1994. All beetles were placed under one of these three temperatures immediately after return to the laboratory and being sorted. Most of the collected beetles had little chance to feed before collection and had been emerged from the soil less than two days. Beetles were placed in storage in the unfed condition. In 1995, the storage regimes were reduced to 1°C and 5°C. Two groups of beetles were placed in each temperature regime. The first group was placed directly into cold storage in the unfed condition while the second group was allowed to feed on alfalfa stems at ambient laboratory temperature for 7 days before being placed in cold storage. These beetles were fed in 60 cm × 60 cm × 60 cm screen cages with 400 beetles per cage. Fresh alfalfa cuttings were placed in the cages 3 times per week. Seven days was selected because the adults have ca. 21 day pre-oviposition period.

Prior to being placed in storage, groups of 30 beetles were placed together in 100 mm plastic petri dishes with a damp piece of filter paper on the bottom. The beetle filled petri plates were then placed into a Styrofoam ice chest with a two inch layer of moistened vermiculite in the bottom. The ice chest was then placed into a Foster™ upright freezer (model GL-20-AD-T(2)) adjusted to the desired storage temperature. Temperature probes were placed inside the ice chest and their leads were accessible from outside the freezer to monitor the temperature inside the ice chest. The ice chest was used to moderate the typical temperature variations within a freezer during the cooling cycles.

Approximately every 30 days, 15 live beetles/ experimental regime were removed from storage and placed individually in 130 ml glass bottles with screw-top lids. Each bottle contained a layer of approximately 1 cm of moist autoclaved soil to encourage oviposition. The soil was sifted through an 18 mesh screen (1 mm opening) to remove the large particles. Fresh alfalfa foliage was provided to the beetles every other day as food. All beetles were maintained at 23°C for the duration of this portion of the experiment in a large walk in chamber. Beetles were fed field collected alfalfa during May – September and greenhouse grown alfalfa during September – March.

Eggs were collected by washing each individual jar and counting each jar's contents. The soil from each bottle was rinsed in a 60-mesh screen (250 micron openings) to separate the eggs and large soil particles from the small soil particles. After the small soil particles were washed through the screen, the eggs and remaining soil particles were washed into a glass 100 mm petri dish. Eggs were counted and recorded. Jars with surviving beetles were supplied with new soil and fresh alfalfa. Dead beetles were recorded at the same time. Total and weekly egg production and time to mortality were analyzed by storage regime using SAS version 9.1 (SAS Institute 2006).

Results

1994 Study

Egg production. Eggs produced per beetle per week across all temperature treatment regimes were similar and some treatments were significantly different from the control group ($F = 4.62$, df = 20, $P = 0.05$) (Table 1). If the eggs produced per beetle per week per temperature is averaged across the storage duration, most levels of oviposition are significantly less than the control. The exception is 150 days of storage where there is no significant difference from the control. High variability within treatments makes it difficult to explain the treatment/regime differences. However, the total eggs laid in any storage regime was significantly lower ($F = 4.78$, df = 20, $P = 0.05$) than the control group. Cold storage of newly emerged beetles for a duration as short as 30 days reduced the total eggs production about 50%.

Mortality. Overall, beetle mortality in storage increased with time. The most rapid mortality was recorded under the 5°C temperature regime followed by the 1°C regime and then the 3°C regime. All beetles in 5°C were dead by 127 days in storage whereas the 1°C regime achieved 100 % mortality by 191 days. The 3°C regime recorded 100% mortality at 226 days in storage (Table 1). Even though all the 5°C beetles died in storage by 127 days, the study was able to proceed because numerous surplus beetles were also stored at 5°C and enough surviving beetles were available to continue the study to 226 days.

After removal from storage, the time to 50% mortality during the first 127 days of the study was not significantly different from the control group. However, time to 100% mortality was significantly shorter ($F = 3.97$, df = 20, $P = 0.05$) for the 3°C and 5°C groups compared to the control throughout the study. The exception occurred at 127 days. We believe this sudden increased lifespan for all temperature groups was due to the massive die off of weaker individuals in storage, leaving only the most robust individuals to be selected for the study (Table 1).

Beetles never ovipositing. The number of beetles never ovipositing before death after being removed from storage generally increased the longer beetles were in storage. In the control group, all beetles laid eggs. In contrast, after 180 days in storage at 1°C, the percent of beetles never laying eggs increased to a high of 45%. Under most of the temperature x storage duration regimes, the percentage of beetles not ovipositing varied between 10-20% (Table 1).

1995 Study

Egg Production. Egg production per beetle per week per experimental regime varied and the differences were significant ($F = 3.66$, df = 43, $P = 0.05$). Weekly egg totals through 140 days of storage were very similar across treatments, ranging between 57 and 24 eggs per beetle per week. The oviposition values at either end of the range are significantly different but there is substantial overlap of the ranges between treatments (Table 2). High levels of variability may be masking differences between groups, but high levels of variability in oviposition is characteristic with this parthenogenic insect.

Table 1. Effects of cold storage conditions on adult alfalfa snout beetle mortality and egg production, 1994-95.

Treatment	Total eggs per beetle	# Beetles not ovipositing	$\bar{X}$ eggs laid beetle/wk	50% egg production	90% egg production	50% mortality (wks)	100% mortality (wks)
Control	353.4 ± 35.2 a	0	29.5 ± 2.9 a	6.8	10.5	7.5	13
30d storage							
1° Unfed	142.7 ± 19.1 b	1	15.9 ± 2.2 b	4.5	8.1	8.1	11
3° Unfed	157.5 ± 18.5 b	0	22.5 ± 2.6 ab	3.5	6.3	6.8	9
5° Unfed	183.9 ± 25.0 b	3	23.0 ± 3.1 ab	4.0	7.2	7.9	11
60d storage							
1° Unfed	149.8 ± 14.5 b	0	21.4 ± 2.0 ab	3.5	6.3	8.1	11
3° Unfed	163.1 ± 21.5 b	2	27.2 ± 3.6 a	3.0	5.4	7.2	10
5° Unfed	194.1 ± 32.9 b	2	24.3 ± 4.1 ab	4.0	7.3	6.5	11
90d storage							
1° Unfed	184.7 ± 19.3 b	4	23.1 ± 2.4 ab	4.0	7.2	7.1	12
3° Unfed	178.9 ± 27.7 b	3	25.6 ± 4.0 ab	3.4	6.2	5.9	9
5° Unfed	200.0 ± 39.1 b	1	25.0 ± 4.9 ab	4.0	7.2	7.1	11
120d storage							
1° Unfed	180.5 ± 14.5 b	4	18.1 ± 1.4 b	5.0	9.0	9.3	13
3° Unfed	183.3 ± 19.9 b	3	26.2 ± 2.8 a	3.5	6.3	8.8	13
5° Unfed	158.2 ± 22.1 b	4	22.6 ± 3.2 ab	3.4	6.3	7.8	11
150d Storage							
1° Unfed	205.8 ± 23.8 b	3	20.6 ± 2.4 ab	4.9	8.8	8.9	13
3° Unfed	213.1 ± 33.6 b	3	26.6 ± 4.2 a	3.9	7.1	8.9	10
5° Unfed	223.2 ± 26.9 b	6	37.2 ± 4.5 a	3.0	5.4	3.8	9

Table 1. Continued.

Treatment	Total eggs per beetle	# Beetles not ovipositing	$\bar{X}$ eggs laid beetle/wk	50% egg production	90% egg production	50% mortality (wks)	100% mortality (wks)
180d Storage							
1° Unfed	173.6 ± 21.9 b	9	17.4 ± 2.1 b	5.1	9.2	1.0	13
3° Unfed	123.0 ± 2.9 b	3	20.5 ± 0.5 ab	2.9	5.3	6.8	10
5° Unfed	142.1 ± 20.2 b	4	15.8 ± 2.2 b	4.4	8.0	3.5	11
210d Storage							
3° Unfed	168.0 ± 21.7 b	5	24.0 ± 3.1 ab	3.5	6.3	6.5	10
5° Unfed	98.5 ± 13.2 b	4	16.4 ± 2.2 b	3.1	5.5	5.2	10
240d Storage							
5° Unfed	170.6 ± 18.0 b	3	28.4 ± 3.0 a	3.0	5.5	6.4	10

Table 2: Effects of cold storage conditions on adult alfalfa snout beetle mortality and egg production, 1995-96.

Treatment	Total eggs per beetle	# Beetles not ovipositing	$\bar{X}$ eggs laid per week	50% egg production	90% egg production	50% mortality (wks)	100% mortality (wks)
Control	317.4 ± 34.2 ab	1	28.9 ± 3.2 cde	5.5	9.8	9.0	13
29d storage							
1° Unfed	276.4 ± 42.8 ab	3	34.5 ± 5.4 cd	3.9	7.1	8.1	11
1° Fed	279.6 ± 31.8 ab	2	28.0 ± 3.4 cde	5.0	9.0	8.1	14
5° Unfed	250.2 ± 35.3 bc	1	35.8 ± 5.2 bc	3.5	6.3	7.0	9
5° Fed	272.1 ± 39.0 ab	1	34.0 ± 5.6 cd	4.0	7.2	8.2	11
56d storage							
1° Unfed	349.3 ± 39.5 a	1	34.9 ± 3.9 bc	5.0	9.0	8.2	14
1° Fed	181.1 ± 19.4 de	1	25.9 ± 2.8 de	3.5	6.3	8.3	10
5° Unfed	193.3 ± 13.5 cd	4	32.2 ± 2.2 cd	3.0	5.4	6.0	10
5° Fed	207.3 ± 21.4 cd	2	25.9 ± 2.7 de	4.0	7.2	8.5	10
84d storage							
1° Unfed	255.5 ± 27.7 bc	0	31.9 ± 3.5 cd	4.0	7.2	7.4	12
1° Fed	318.1 ± 29.8 ab	0	39.8 ± 3.8 bc	4.0	7.2	7.3	9
5° Unfed	237.8 ± 44.5 bc	2	29.7 ± 5.6 cd	4.0	7.1	5.6	8
5° Fed	295.8 ± 35.6 ab	0	40.0 ± 4.9 bc	3.7	6.6	8.5	11
112d storage							
1° Unfed	242.5 ± 33.2 bc	0	40.4 ± 5.5 bc	3.0	5.4	7.6	10
1° Fed	329.8 ± 45.1 ab	0	47.1 ± 6.5 ab	3.5	6.3	7.5	10
5° Unfed	286.8 ± 46.9 ab	4	36.5 ± 5.9 bc	4.0	7.0	5.3	10
5° Fed	340.9 ± 57.6 a	2	56.8 ± 9.6 a	3.0	5.5	6.5	10

Table 2: Continued.

Treatment	Total eggs per beetle	# Beetles not ovipositing	$\bar{X}$ eggs laid per week	50% egg production	90% egg production	50% mortality (wks)	100% mortality (wks)
140d Storage							
1° Unfed	170.5 ± 27.5 de	2	24.4 ± 3.9 de	3.5	6.4	6.6	9
1° Fed	169.9 ± 23.9 de	0	24.3 ± 3.4 de	3.5	6.4	6.4	9
5° Unfed	143.3 ± 16.2 ef	5	23.9 ± 2.7 de	3.0	5.4	3.0	8
5° Fed	259.7 ± 38.3 bc	5	37.1 ± 5.5 bc	3.5	6.3	4.0	9
169d Storage							
1° Unfed	76.9 ± 10.8 hi	3	12.8 ± 1.8 gh	3.0	5.3	5.0	9
1° Fed	81.7 ± 8.2 ghi	1	16.3 ± 1.6 fg	2.6	4.6	6.0	8
5° Unfed	73.3 ± 14.1 hi	9	18.3 ± 3.5 ef	2.0	3.7	3.0	7
5° Fed	129.0 ± 26.9 efg	11	32.3 ± 6.6 cd	2.0	3.6	2.5	6
197d Storage							
1° Unfed	121.7 ± 14.2 fg	7	20.3 ± 2.3 ef	3.1	5.5	3.0	9
1° Fed	100.2 ± 25.6 gh	8	25.1 ± 8.4 de	2.0	3.6	5.0	7
5° Unfed	91.6 ± 15.7 gh	5	22.9 ± 3.9 de	2.1	3.8	3.5	6
5° Fed	109.4 ± 24.0 gh	6	27.4 ± 6.0 cde	2.0	3.6	5.0	7
226d Storage							
1° Unfed	74.0 ± 25.2 hi	9	18.5 ± 1.7 ef	2.1	3.7	4.7	9
1° Fed	218.3 ± 23.7 cd	7	27.3 ± 3.0 cde	4.0	7.3	4.5	11
5° Unfed	79.5 ± 16.5 hi	13	13.3 ± 2.7 gh	3.1	5.5	1.0	10
5° Fed	52.5 ± 14.0 ij	11	10.5 ± 2.8 h	2.6	4.3	1.2	9
255d Storage							
1° Unfed	331.3 ± 35.4 a	4	55.2 ± 5.9 a	3.0	5.0	7.0	10
1° Fed	348.8 ± 41.1 a	3	49.8 ± 6.0 a	3.5	6.3	7.0	10
5° Unfed	279.2 ± 38.5 ab	8	46.5 ± 6.4 ab	3.0	5.5	2.1	8
5° Fed	292.9 ± 33.4 ab	1	58.6 ± 6.7 a	2.5	4.6	4.0	8

Table 2: Continued.

Treatment	Total eggs per beetle	# Beetles not ovipositing	$\bar{X}$ eggs laid per week	50% egg production	90% egg production	50% mortality (wks)	100% mortality (wks)
282d Storage							
1° Unfed	186.4 ± 28.9 de	2	31.1 ± 4.9 cd	3.0	5.4	5.4	9
1° Fed	169.8 ± 13.4 de	8	34.0 ± 2.8 cd	2.5	4.5	3.5	9
5° Unfed	98.0 ± 4.3 gh	13	49.0 ± 2.1 a	1.0	1.8	1.0	5
310d Storage							
1° Unfed	172.5 ± 33.2 de	5	43.1 ± 8.2 ab	2.0	3.6	3.5	7
1° Fed	207.9 ± 29.8 cd	5	34.7 ± 5.0 cd	3.0	5.3	5.2	8
5° Unfed	66.0 ± 0.0 ij	14	16.5 ± 0.0 fg	1.0	1.8	1.0	4
339d Storage							
1° Unfed	142.7 ± 4.8 ef	7	28.5 ± 0.9 cde	2.5	4.6	5.3	8
1° Fed	182.9 ± 12.4 de	8	36.6 ± 2.5 bc	2.5	4.6	6.1	8

During the first 29 days of storage, weekly egg production and total egg production for all treatment groups did not differ significantly from the control group. At 56 days of storage, the eggs per week were not different from the control but the total egg production for three of the storage regimes (1°C fed, 5°C unfed, 5°C fed) were significantly less ($F = 2.98$, df = 4, $P = 0.05$) than the control. After 84 days of storage, the weekly egg production per beetle continues to not be significantly different from the control group. In addition, total egg production per treatment regime was not significantly different from the control group. By 112 days in storage, the weekly egg production in two of the regimes (1°C fed, 5°C fed) were significantly higher ($F = 3.20$, df = 4, $P = 0.05$) than the control group. However, total egg production under all temperature regimes was not significantly different from the control. After 140 days in storage, weekly egg production averages were the same as the control, but total egg production in three of the regimes (1°C unfed, 1°C fed, 5°C unfed) was significantly less ($F = 2.76$, df = 4, $P = 0.05$).

Total egg production was significantly less ($F = 3.11$, df = 4, $P = 0.05$) for all storage regimes for 168 days, 196 days and 224 days. Weekly egg production was significantly different from the control for most regimes ($F = 3.27$, df = 4, $P = 0.05$) except 5°C fed, 5°C unfed – 169 days, 1°C fed and unfed, 5°C fed and unfed – 197 days and 1°C fed – 226 days. A big spike in egg production was recorded after 255 days in storage. Weekly egg production in all regimes was significantly better than the controls and the total egg production increased to a non-significant difference with the control group ($F = 2.87$, df = 4, $P = 0.05$). At 282 and 310 days in storage, all the experimental regimes were not represented due to beetle mortality in storage, and only 1°C fed and unfed, and 5°C unfed were available for testing. At 339 days in storage, only the two 1°C regimes were available for testing. In all cases, the total egg production in each regime was significantly less than the control. In contrast, most of the weekly egg production numbers were not different than the control with the exception of 5°C unfed – 282 days and 1°C unfed – 310 days ($F = 3.32$, df = 4, $P = 0.05$) (Table 2).

Mortality. A similar trend in overall mortality in storage was recorded in the second year of the study. At a given temperature, the "fed before storage" group survived better under the storage regime than the unfed group. All beetles were dead in the 5°C unfed group by 140 days whereas the 5°C fed group survived until 253 days. In the 1°C temperature, the fed group survived at a higher rate early in the study (56 d - 253 d) but both groups were equal in survival at the last three storage durations (300 d - 356 d) (Table 2).

5°C storage. Beetles removed from 5°C storage survived a similar time to the 50% mortality bench mark as the control group during oviposition through 84 days in storage. Unfed beetles recorded a higher numerical mortality rate when removed from storage than the fed beetles. By 112 days in storage, the unfed group had a numerically lower survival rate that the control group but remained similar to the fed group. Beetles removed from storage beyond 112 days had lower survival than the control group but were not different between the fed and unfed groups. Similar trends were observed in the 100% mortality bench mark although a slight increase in survival was noted at 224 days and 253 days ($F = 1.98$, df = 4, $P = 0.05$). The longer the beetles were in cold storage, the higher the number of beetles which never oviposited after being removed from storage. For example, after 29 days storage, only a single beetle failed to lay any eggs but after 226 days in storage, a majority of the beetles failed to lay eggs.

Overall, beetles survived better when stored at 1°C than at 5°C. However, no difference was noted in survival between the fed and unfed experimental groups at 1°C . Using the 50% mortality bench mark, survival at 1°C, during the oviposition period was not different from the control group through 112 days in storage, though numerically lower in most cases. At 140 days in storage, the fed group had a numerically lower survival rate to the 50% bench mark than

the control group but was not different from the unfed group. Storage for 168 d, 196 d, and 224 d resulted in lower survival rates than the control group. By 253 days in storage, the survival rate increased to the level of the control group but was reduced for the last three storage durations (282 d, 310 d, and 339 d). Similar trends were noted using the 100 % mortality bench mark. These data were more variable because a small group of long-lived individuals have a disproportionate impact on the data. The longer the beetles were in cold storage, the number of beetles which never oviposited after being removed from storage had an upward trend. However, the number of beetles not ovipositing after being stored at 1°C was lower than 5°C.

Discussion

Our results suggests that extended storage of adult beetles has a physiological cost at the temperatures tested. Beetles stored at temperatures between 1°C and 5°C remained inactive, but were still consuming fat reserves to remain alive. This is expressed by the reduction of total egg production when beetles are stored for a duration as short as 30 days (1994), increasing incidence of beetles which survive but do not lay any eggs as storage duration increases and increasing mortality of beetles in storage as the storage duration increases. These data suggest that the replenishing of depleted fat reserves is not completely reversible with feeding after the beetles are removed from storage. Pre-feeding the beetles for 7 days before storage allowed the beetles to replenish some of the fat reserves depleted during their 18 month diapause underground and allowed the beetles to survive longer in storage and positively influenced total egg production in some of the experimental regimes. If the beetles were allowed to feed for a longer duration after spring emergence before being placed in cold storage, the longer feeding period may allow for a greater accumulation of fat reserves, better survival and a great positive impact on total egg production. This insect has a ca. 3 week preoviposition period. During this time, food consumption is directed to building fat reserves prior to the onset of oviposition. Once oviposition is initiated, feeding by the beetle drops to a low level and the fat reserves are primarily utilized for egg production. Once the insect shifts from fat storage to oviposition, the question arises as to whether cold storage would have a larger negative impact on egg production than cold storage before the shift to ovipostion.

Oviposition data within each treatment was quite variable, but this high variability between individuals is characteristic of this insect. In Palm (1935), total egg production from caged beetles which were collected shortly after emergence ranged from 125 to 515 (330.7 ± 82.4). While cold storage reduced the total number of eggs laid by beetles compared to the control group, there was very little difference between many of the stored and control group in the rate of oviposition. As a result, the time benchmark of 50% and 90% oviposition was shorter for the cold stored beetle groups compared to the control group.

The results of this study effectively show that this insect can be stored under cold temperatures for extended periods of time. When needed, beetles can be removed from storage conditions and will lay viable eggs useful in research. For an insect which is very difficult to rear, the ability to store this insect for an extended length of time is very useful in extending the research season for this insect. Since this insect emerges in fairly large numbers over a rather short interval, large numbers of newly emerged adults can be collected and placed in storage for future research use (Shields et al. 2007).

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