Instar- and Stage-Specific Photoperiodic Diapause Response of *Lygus hesperus* (Hemiptera: Miridae)

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Abstract

The western tarnished plant bug (*Lygus hesperus* Knight) (Hemiptera: Miridae) is a polyphagous pest of numerous western crops. This pest overwinters in a relatively short duration adult diapause, but many details regarding diapause induction and maintenance remain unstudied. Instar-specific responses to a switch from short (10-h) to long (14-h) days, long to short days, and a temporary (4-d) switch from short to long days were compared with responses of insects maintained under respective short- and long-day photoperiods. Influences of short days received during the nymphal stage on subsequent adult reproductive development under long days were also examined. Substantial diapause responses were observed only for insects switched to short days by 4th instar. Few insects that switched from short to long days exhibited diapause characters by Day 10 of adulthood. When 2nd instars were temporarily switched from short to long days no effect on the diapause response was observed, but the response was diminished when the switch occurred beginning at 4th instar. Reproductive development of adults reared under short days and switched to long days was modestly delayed compared with insects reared from egg to adult under long days. These results indicate the diapause response is elicited by short day cues received prior to the 5th instar, and that continued exposure to short days is necessary to maintain the adult diapause. These findings, combined with earlier reports of diapause termination in the field, suggest that southern populations of *L. hesperus* may express a more dynamic and complex overwintering strategy than was previously recognized.

Key words: western tarnished plant bug, photoperiod, overwintering, development

The western tarnished plant bug, *Lygus hesperus* Knight (Hemiptera: Miridae), is a polyphagous pest attacking a wide variety of crops in the western United States. It is also a key pest of western cotton (*Gossypium* spp.) (Malvales: Malvaceae). Management of *L. hesperus* has traditionally been accomplished through application of insecticides, although recent advances in the use of cultural controls have shown promise in high-value crops such as organic strawberry (*Fragaria × ananassa*) (Weston) Duchesne ex Rozier, Rosales: Roseaceae (Swezey et al. 2007, 2013, 2014). Although trap cropping has been shown effective for reducing *L. hesperus* populations in cotton (Stern et al. 1967, Stern 1969, Sevacherian and Stern 1974), its adoption in low value field crops has been limited. Nevertheless, in some areas, improved management (Ellsworth 1998) and use of reduced-risk insecticides (Anonymous 2013) have reduced the economic impact of *L. hesperus* to the point that further progress in its management will require a more astute understanding of its ecology and basic biology.

McGuire et al. (2006) suggested management of *Lygus* might be improved by targeting overwintering populations before they infest crops that are susceptible to injury. However, this approach will require a more complete understanding of the mechanisms and dynamics governing overwintering populations. *L. hesperus* is widely considered to overwinter in a relatively short duration adult diapause induced by short daylengths (Beards and Strong 1966, Leigh 1966, Strong et al. 1970). However, recent reports of low-temperature biology include dramatically extended egg (Cooper and Spurgeon 2013) and nymph development (Cooper and Spurgeon 2012), and prolonged host-free survival of non-diapausing adults (Cooper and Spurgeon 2015). In addition, Spurgeon (2012) showed that *L. hesperus* exhibits considerable plasticity in its diapause response, which is subject to rapid selection in laboratory culture, and Spurgeon and Brent (2015) found that a substantial portion of the *L. hesperus* population from Arizona does not enter diapause in response to short days. Finally, adults and nymphs are observed on alfalfa (*Medicago sativa* L. Fabales: Fabaceae) or weed hosts throughout most or all of the winter and early spring in the southern San Joaquin Valley of California and in Central Arizona (D.W.S.,...
unpublished observation). These combined observations and reports suggest the dynamics of *L. hesperus* overwintering is more complex than generally recognized.

One poorly studied aspect of overwintering ecology in *L. hesperus* involves the diapause responses of specific instars to daylength. Most information on this topic comes from the report by Beards and Strong (1966). They reported that exposure of nymphs to short days induces diapause but the instar(s) most responsive were not identified. They also reported induction in a small but substantial percentage (20%) of bugs switched from a 16-h photophase to a 10-h photophase as young adults. Based on their studies of late-season diapause, which used small and unreplicated samples of field-collected nymphs, they concluded that nymphs occurring in the field in October and November probably do not enter diapause. Regarding termination of the diapause, Beards and Strong (1966) reported that exposure to long daylengths could nullify previously induced diapause, but the extent of such nullification depended on ’environmental conditioning’ received by the early instars. In addition, the diapause could be terminated in adult females by exposure to a 16-h photophase, but less so by exposure to a 13.5-h photophase. In contrast, Leigh (1966) reported that most diapausing adults exposed to a 13.5-h photophase for 2 wk had terminated diapause. Regardless of these inconsistencies, it is generally accepted that diapause in field populations of *L. hesperus* terminates in late-fall to early-winter (Beards and Strong 1966, Strong et al. 1970). However, collections from alfalfa in early-March in Arizona, when daylength is <12 h, often include female *L. hesperus* that exhibit diapause characters but initiate reproduction within 7–10 d when placed in the laboratory colony maintained under a 14-h photophase (D.W.S., unpublished observation). From these reports it is apparent that a better understanding of diapause in *L. hesperus* should include additional information on stage or instar-specific responses to daylength. The objectives of this work were to determine i) the extent to which specific instars exhibit a diapause response to short (10-h) days, ii) the influence of long (14-h) days on the incidence of diapause in *L. hesperus* previously exposed to short days, and iii) whether temporary interruption of the short-day photoperiodic regime influences subsequent diapause response.

### Materials and Methods

Four experiments were conducted to address the objectives. Two experiments examined the responses of insects to reciprocal photoperiod switches (switched from short to long days and switched from long to short days). One experiment examined the influence of a 4-d interruption in the short-day (10-h) photoperiod program. A fourth experiment used serial dissections to examine the temporal pattern of reproductive development of diapause-induced and non-diapause adults. In all cases, experimental insects were obtained as F₀ or F₁ progeny of adults collected from fields of alfalfa near Maricopa, AZ. The various collections were made throughout the calendar year (October 2015, March, April, June, October, and December 2016, and March 2017).

Field-collected adults were confined within 0.03-m³ screened cages containing shredded paper where they were provided pods of green bean (*Phaseolus vulgaris* L. Fabaceae: Fabaceae) for feeding and oviposition. Each cage also contained a cotton pad saturated with water, and raw seeds of sunflower (*Helianthus annuus* L. Asteraceae: Asteraceae). Adult rearing cages were maintained at about 27°C under a 14:10 (L:D) h photoperiod.

Eggs for the experiments were obtained by exposing eight bean pods to the adults for 6–8 h. The bean pods were then cut in half, the ends were sealed with melted paraffin, and each bean half was enclosed within a 100 × 15-mm Petri plate lined with a filter paper disk and sealed with Parafilm M (Pachining Plastic Packaging, Chicago, IL). Bean halves were randomly assigned to environmental chambers (130-BLL, Percival Scientific, Perry, IA) maintained at 26.7 ± 0.5°C with either a 14:10 (L:D) or 10:14 (L:D) h photoperiod. The two halves of a given bean were assigned to different photoperiods. During the 6–7 d between oviposition and egg hatch, the plates were inspected periodically for condensation, which was blotted away to minimize mold growth. Upon hatch, the nymphs were individually placed within 18-ml plastic vials (Thornton Plastics, Salt Lake City, UT) with a section of green bean pod waxed on the cut ends. Each vial was closed with a snap-cap lid that was screened with nylon organy. Throughout each experiment, the bean sections were replaced thrice weekly. Also, in each repetition of each experiment, the insects represented a different field collection, and experimental treatments were re-assigned to different environmental chambers.

In the first three experiments, diapause response was determined by dissection of 10-d-old adults. Dissections were performed under 0.7% NaCl (wt:vol) as described by Spurgeon (2012). Females exhibiting a hypertrophied fat body and lacking evidence of vitellogenic oocytes or follicular relics were classified as diapausers. Males with a hypertrophied fat body and poorly developed medial accessory glands were classified as diapausers. These criteria were described by Spurgeon and Brent (2010) and they were applied as recommended by Brent and Spurgeon (2011).

### Reciprocal Photoperiod Switching of Early Instars and Adults

The experiment included eight treatments; four in which the insects were initially exposed to short days (10-h photophase) and four with initial exposure to long days (14-h photophase). Treatments within each photoperiod group included i) not switched (maintained under the original photoperiod until dissection as adult), ii) switched at 1st instar (at hatch), iii) switched at 3rd instar, and iv) switched at adult eclosion. These treatments were selected based on a preliminary experiment that suggested a low diapause response of insects exposed to the short days beginning at 3rd instar. In each of the four experimental repetitions, 25 newly hatched nymphs were allocated to each treatment. In the latter two treatments, insects molting to the instar preceding the photoperiod switch were checked twice daily with the last check between 15:00 and 16:00 h to ensure they were switched as early as possible in the appropriate stadium. The insects in all treatments were similarly observed as 5th instars to ensure adult age was accurately identified. Insect sex was recorded at the time of adult eclosion.

The incidence of diapause was examined among the eight treatments by a conditional, generalized linear mixed model using events/trials syntax, a binomial distribution, and Laplace estimation (PROC GLIMMIX; SAS Institute 2012). A means model was used with fixed effects of photoperiod switching treatment, bug sex, and their interaction. Repetition of the experiment was a random effect, as was the repetition × treatment interaction which served as the error term for the test of photoperiod treatment. Significance of comparisons among treatments was adjusted for multiplicity using the SIMULATE option and differences were declared when the adjusted P-value was <0.05. Diapause was not observed for some treatment combinations and the initial analyses failed to converge because of an infinite likelihood. Convergence was achieved by rescaling the number of diapause events for treatment combinations where diapause was not observed (from zero to 0.5). This rescaling had
negligible effect on estimates or comparisons of treatment combinations that were originally non-zero.

Reciprocal Photoperiod Switching of Late Instars
Results of the photoperiod switching experiment using early instars were not completely consistent with results of the preliminary experiment, so a second experiment was conducted using late (4th and 5th) instars. The experiment included three treatments: i) not switched (exposed to short days from egg through adult); ii) maintained under long days until the molt to 4th instar, then switched to short days; and iii) maintained under long days until the molt to 5th instar, then switched to short days. Procedures were exactly as described for the previous experiment except for sample size; 30 insects were allocated to each treatment in each of three experimental repetitions. The data were analyzed using the same statistical approach and model as in the study of early instars and adults.

Interruption of Short Days Beginning with 2nd or 4th Instar
The experiment involved three treatments: i) exposed to short days from egg to dissection as an adult; ii) exposed to short days except for a 4-d period of exposure to long days beginning at the molt to 2nd instar; and iii) exposed to short days except for a 4-d period of exposure to long days beginning at the molt to 4th instar. Procedures were the same as those previously described, including daily observation of the nymphs during the stadium immediately before switching photoperiods. Thirty insects were established in each treatment of each of three experimental repetitions. Analyses, and the ANOVA model, were the same as those used in the previous experiments except no rescaling was needed to avoid problems with separation (no treatment exhibited zero diapause response). In addition, the diapause response of nymphs switched during 4th instar that remained 4th instars until they were switched back to short days was compared with the response of nymphs molting to 5th instar during the interruption period. These comparisons were made in a contingency table with instar at the switch back short days as rows, days beginning at the molt to 4th instar as columns, and repetitions of the experiment as strata. Inferences were based on the Mantel–Haenszel non-zero correlation statistic ($Q_{\text{SMH}}$, Stokes et al. 2012).

Influence of Photoperiod Switching on Reproductive Development of Diapausin Adults
The experiment included two initial photoperiods (short day, 10-h photophase; long day, 14-h photophase) and three adult ages at dissection (2, 4, and 6 d). These ages were selected based on a preliminary experiment that indicated an inability to distinguish the photoperiod treatments at adult ages ≥ 9 d. Eggs were maintained until hatch under the same photoperiod as the initial nymphal photoperiod. Thirty newly hatched nymphs were assigned to each combination of photoperiod treatment and age at dissection. Insects developing to adulthood under short days were switched to the long day chamber on the day of adult eclosion. At dissection, female ovaries were classified as exhibiting no development, pre-vitellogenic oocytes, vitellogenic oocytes, or eggs, as described by Spurgeon and Cooper (2012). Assessments of male reproductive development were based on conditions of the medial accessory glands and the seminal vesicles. Mediul accessory glands and seminal vesicles were classified as empty (‘undeveloped’ in Spurgeon and Cooper 2012), filling, or filled using the descriptions of Spurgeon and Cooper (2012). Three repetitions of the experiment were conducted. Data corresponding to females and males were analyzed separately. The pattern of occurrence of reproductive classes for a given organ within adult age was compared between photoperiod treatments in contingency tables (PROC FREQ, SAS Institute 2012). For each reproductive character, photoperiod treatments constituted rows in the tables, ordered classifications of reproductive development were columns, and repetitions of the experiment were strata analogous to blocks in an ANOVA (Stokes et al. 2012). Associations between distributions of counts among the reproductive classes and the photoperiod treatments were evaluated using the Mantel–Haenszel row-mean-score test ($Q_{\text{SMH}}$, Stokes et al. 2012), and standardized mid-ranks (SCORES = MODRIDIT option). Where the Mantel–Fleiss criterion indicated marginal totals were too small for asymptotic tests, exact tests were requested (the COMOR option of the EXACT statement). Agresti–Coull asymmetrical 95% confidence limits (CL) were calculated from marginal totals for each combination of photoperiod treatment, insect sex, and age at dissection.

Results
Reciprocal Photoperiod Switching of Early Instars and Adults
Analyses of the incidence of diapause did not indicate an effect of insect sex or an interaction between sex and photoperiod treatment (Table 1). However, photoperiod treatment influenced the estimated probability of diapause occurrence (Table 1; Fig. 1a, b). The probability of diapause was not different for insects maintained under

Table 1. Tests of model effects assessing the probability of diapause exhibited by 10-d-old L. hesperus adults after switching from short (10-h) to long (14-h) days or from long to short days in three experiments

| Experiment                        | Model effect | F    | df  | P      |
|-----------------------------------|--------------|------|-----|--------|
| Switched as early-instar or adult  | Sex          | 1.97 | 1, 24 | 0.173  |
|                                   | Treatment*   | 16.02 | 7, 21 | <0.001 |
|                                   | Sex × Treatment | 0.66 | 7, 24 | 0.706  |
| Switched as late instar           | Sex          | 0.21 | 1, 6 | 0.661  |
|                                   | Treatment*   | 16.19 | 2, 4 | 0.012  |
|                                   | Sex × Treatment | 0.37 | 2, 6 | 0.702  |
| Switched for 4 d as 2nd or 4th instar | Sex       | 0.22 | 1, 6 | 0.656  |
|                                   | Treatment*   | 9.88  | 2, 4 | 0.028  |
|                                   | Sex × Treatment | 0.69 | 2, 6 | 0.538  |

Analyses for each experiment used a conditional mixed model with a binomial distribution.
*The eight treatments comprised insects switched from short to long days at 1st or 3rd instar or adult, switched from long to short days at 1st or 3rd instar or adult, and maintained continuously under short or long days.
*The three treatments comprised insects maintained under short days, or switched from long to short days at 4th or 5th instar.
*The three treatments comprised insects maintained under short days, or temporarily switched from short to long days for 4 d as 2nd or 4th instars.

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the short days (not switched) compared with those switched from long to short days at 1st or 3rd instar (adjusted-\(P \geq 0.574\); Fig. 1a). Likewise, the diapause response of insects switched from short days to long days, irrespective of the instar that was switched, or switched from long to short days at adulthood, did not differ (adjusted-\(P \geq 0.839\)). The diapause response of insects maintained under short days or switched to short days at 1st or 3rd instar was greater compared with insects switched to short days as adults, or switched from short to long days at any instar (adjusted-\(P \leq 0.022\); Fig. 1a,b).

**Reciprocal Photoperiod Switching of Late Instars**

The incidence of diapause was influenced by photoperiod treatment but not by insect sex (Table 1). The treatment by sex interaction indicated the treatment response was similar for both sexes. Comparisons among treatments indicated that when 4th instars were switched from long to short days their diapause response was similar to insects maintained under short days from egg to 10-d-old adult (adjusted-\(P = 0.180\); Fig. 1c). The probability of diapause for nymphs switched to short days as 5th instars was lower compared with those maintained under short days throughout (adjusted-\(P = 0.011\)) or those switched to short days as 4th instars (adjusted-\(P = 0.021\); Fig. 1c). Only a single insect (male) switched from long to short days at the 5th instar exhibited the characters of diapause.

**Interruption of Short Days Beginning with 2nd or 4th Instar**

All of the nymphs temporarily switched from long to short days for 4 d at 2nd instar were 3rd instars when they were returned to short days. More than 80% of the insects temporarily switched from short to long days at 4th instar became 5th instars during the 4-d period under long days. Analyses of the diapause responses indicated a significant effect of photoperiod treatment (not switched, switched at 2nd instar, switched at 4th instar) but not of insect sex or the sex by treatment interaction (Table 1). Comparisons among treatments indicated the temporary photoperiod switch occurring during 2nd instar did not influence the diapause response compared with insects maintained continuously under short days (adjusted-\(P = 0.878\); Fig. 2). In contrast, the probability of diapause for insects temporarily switched from short to long photoperiods at 4th instar was lower compared with those maintained under short days or those switched at 2nd instar (adjusted-\(P \leq 0.047\); Fig. 2). The diapause response of insects temporarily held under long days beginning at 4th instar was numerically higher for individuals that did not molt to 5th instar during the short day interruption (62%) compared with those molting to 5th instar during the 4-d interruption period (32%). However, the number of insects remaining in 4th instar at the end of the interruption period (13) was small, and the numerical difference was not statistically significant (\(Q_{\text{CSMH}} = 3.57, \text{df} = 1, P = 0.059\)).

**Influence of Photoperiod Switching on Reproductive Development of Diapausing Adults**

Associations between photoperiod treatment (maintained under long days until dissection, switched from short to long days at adulthood) and distributions of accessory gland classes were observed at each interval of male age (Table 2; Fig. 3). Overall, medial accessory gland development of males switched from short (10-h) to long (14-h) days at adulthood appeared delayed by 2–3 d compared with males exposed only to long days. However, a small proportion (\(\approx 0.15\)) of males switched from short to long days exhibited a lack of accessory gland development by 6 d of adult age (Fig. 3). Compared with the medial accessory glands, development of the seminal vesicles was less responsive to photoperiod treatment.
Table 2. Mantel–Haenszel row-mean-score ($Q_{SMH}$) tests of association between development class and adult age for female (ovary) and male (medial accessory gland, seminal vesicle) reproductive organs of *L. hesperus* exposed continuously to long (14-h) days or switched from short (10-h) to long days at adult eclosion

| Reproductive organ | Adult age (d) | $Q_{SMH}$ | df | $P$  |
|--------------------|--------------|-----------|----|------|
| Medial accessory gland | 2 | 12.42 | 1 | <0.001 |
|                  | 4 | 15.92 | 1 | <0.001 |
|                  | 6 | 18.25 | 1 | <0.001 |
| Seminal vesicle | 2 | 0.87 | 1 | 0.352 |
|                  | 4 | 2.10 | 1 | 0.147 |
|                  | 6 | 12.90 | 1 | <0.001 |
| Ovary | 2 | 6.80 | 1 | 0.009 |
|                  | 4 | 19.45 | 1 | <0.001 |
|                  | 6 | 22.08 | 1 | <0.001 |

*Development classes were (i) empty, (ii) filling, or (iii) filled.

*Development classes were (i) absence of oocytes, or presence of (ii) previtellogenic oocytes, (iii) vitellogenic oocytes, or (iv) eggs.

**Discussion**

Beards and Strong (1966) reported that nymphs, and about 20% of adult *L. hesperus*, responded to short days by entering adult diapause. Results of the work reported herein indicates only egg through 4th instars are substantially responsive to short days, and induction of diapause was not observed for individuals exposed to short days beginning at adult eclosion. The inductive effects of prior exposure to short days during the entire nymphal stage were largely nullified after a 10-d exposure to long days as adults. Further, exposure of an early instar (2nd) to temporary interruption of the short day program by long days did not influence the incidence of diapause. These findings suggest that short days are necessary to maintain as well as induce diapause in *L. hesperus*, and that length of the photophase during the 4th instar should be the primary cue governing the incidence of diapause in the field.

The molecular or physiological mechanisms driving or maintaining diapause in *L. hesperus* are not known. However, the reduction in diapause incidence for 4th instars experiencing a temporary interruption of the short day program, even into the otherwise unresponsive 5th stadium, suggests a lag in the processes by which long days diminish or nullify the response to short days. Whether these processes in the nymphal stage are the same as those terminating diapause in the adult is not known.

Beards and Strong (1966) also reported that whether long days terminated diapause in adult *L. hesperus* was dependent on the length of the photophase and the environment experienced by the nymphs. In contrast, Leigh (1966) reported relatively rapid termination of diapause for adults exposed to a 13.5-h photophase. Results of the current study and personal experience (previously cited unpublished observation) are generally consistent with the report of Leigh (1966). Although a small proportion of both sexes in this study failed to initiate reproductive development by 6 d of adult age, a trend for a rapidly diminishing diapausing population in response to long days (14 h) was apparent. The diapause response of the population of *L. hesperus* from central Arizona is incomplete and variable (Spurgeon and Brent 2015), and methods of distinguishing diapausing from reproductive adults at earlier ages will be necessary for more precise investigation of diapause termination. In addition, the delay in seminal vesicle development during diapause termination was subtle compared with the delay in accessory gland or ovary development. This delay in seminal vesicle development during diapause terminated diapause in adult *L. hesperus* was dependent on the length of the photophase and the environment experienced by the nymphs. In contrast, Leigh (1966) reported relatively rapid termination of diapause for adults exposed to a 13.5-h photophase. Results of the current study and personal experience (previously cited unpublished observation) are generally consistent with the report of Leigh (1966). Although a small proportion of both sexes in this study failed to initiate reproductive development by 6 d of adult age, a trend for a rapidly diminishing diapausing population in response to long days (14 h) was apparent. The diapause response of the population of *L. hesperus* from central Arizona is incomplete and variable (Spurgeon and Brent 2015), and methods of distinguishing diapausing from reproductive adults at earlier ages will be necessary for more precise investigation of diapause termination. In addition, the delay in seminal vesicle development during diapause termination was subtle compared with the delay in accessory gland or ovary development. This delay in seminal vesicle development was also unexpected; although seminal vesicle condition has been used as a criterion for diapause (Leigh 1966, Strong et al. 1970), more recent reports have found no association between seminal vesicle condition and diapause (Spurgeon and Brent 2010). Therefore, information to
infer the mechanisms leading to the delay in seminal vesicle development, or associated ecological implications, is not available.

It is widely accepted that duration of the diapause in *L. hesperus* is relatively short and that most of the field population terminates diapause in late fall or early winter (Beards and Strong 1966, Strong et al. 1970). However, two of the current studies (response of late instars, termination in adults) each included a cohort of insects derived from field collections made in December. Responses in those experimental repetitions were not notably different from those of the other repetitions. The experiments of Spurgeon and Brent (2015) included *L. hesperus* derived from a collection during November, and Spurgeon (2012) included insects obtained from multiple field collections in December. Neither of those studies indicated a different response from the progeny of adult *L. hesperus* collected at a time when diapause should be terminating. These observations, combined with observations of diapausing females in the field in March (D.W.S., unpublished observation) raise the question of whether *L. hesperus* might exhibit more than one diapause generation, or overlapping generations, in the southern portions of its range. It is not clear why, provided temperatures are high enough to permit development, that *L. hesperus* maturing to adulthood in December–February would not also enter diapause in response to short days. Nor is it clear why Beards and Strong (1966) supposed that nymphs in the field in October–November would not enter diapause. Alternatively, the incomplete diapause response of southern *L. hesperus* populations, and the relatively short duration of diapause in the field, may constitute a bet-hedging strategy that ensures overlapping generations of diapausing *L. hesperus*. Such generational overlap could ensure population survival of short disruptions to the availability of feeding hosts, such as those produced by an occasional freeze. This ecological strategy would be more flexible and adaptive, and possibly more subject to selection, than a strategy relying on survival of a single generation of diapausing adults to the exclusion of non-diapausing individuals. This hypothesis would also accommodate other proposed mechanisms of overwinter survival, such as extended host-free longevity of reproductive adults at low temperatures (Cooper and Spurgeon 2015).

Results of this study clearly demonstrate instar-specific differences in the ability of *L. hesperus* to exhibit the diapause response to short days. The findings also indicate the importance of short days in maintaining diapause in the adult. Further advances in understanding the mechanisms governing *L. hesperus*, and their implications to field populations, will require expanded knowledge of the termination of diapause under short-day conditions and the potential limits to host-free survival afforded by diapause.

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