

The Effects of Photoperiod on Diapause Induction in *Hypena opulenta* (Lepidoptera: Erebidae), a Biological Control Agent Against Invasive Swallow-Worts in North America

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Abstract

Many insects exhibit a short-day diapause response, whereby diapause is induced when daylength falls below a critical threshold. This response is an adaptation to ensure synchrony between periods of insect activity, and the availability of resources, but it can cause problems when organisms are moved to new locations, where early or late-induced diapause can prove a barrier to establishment. We explored the role of photoperiod in diapause induction in *Hypena opulenta*, a recently introduced classical biological control agent for invasive swallow-worts in North America. We conducted four experimental cage releases as well as a growth chamber experiment to determine the threshold photoperiod for diapause induction in *H. opulenta*. We determined that the critical photoperiod for inducing diapause in 50% of *H. opulenta* is 15 h 35 min, which the moth only experiences in the Ottawa release site around summer solstice. This may lead to univoltinism, premature diapause, and poor establishment at some North American release sites. Our results can inform practical aspects of the biological control program for *H. opulenta*, such as fine-tuning methodologies for stockpiling diapausing pupae in the laboratory and narrowing down the optimal time window for releases at a given location. Additionally, our results will be important for the development of a temperature-based phenology model to more accurately predict voltinism in *H. opulenta* across the invasive range of swallow-worts in North America.

Key words: biological control, diapause, *Hypena opulenta*, swallow-worts, *Vincetoxicum*

Many organisms use photoperiod as a cue for phenological shifts, such as the termination of reproduction, dispersal, or the onset of diapause (Nelson et al. 2009). From the organism's perspective, using photoperiod to predict future environmental conditions is more reliable than using the physical conditions themselves because, while physical conditions can be erratic (i.e., a short, temporary window of favorable conditions in early spring), photoperiod correlates with a specific day of the year and therefore with more regular, long-term seasonal changes in conditions. Additionally, the use of photoperiod allows organisms time to undergo physiological changes before the onset of unfavorable conditions late in the season (Grevstad and Coop 2015).

Although photoperiod is useful for optimizing the phenology of organisms in their native ranges, it can cause problems when organisms are moved to new locations because photoperiod and the occurrence of favorable conditions vary geographically (Masaki 1999,

Bradshaw and Holzapfel 2007). Many insects exhibit a short-day diapause response, whereby diapause is induced when daylength reduces to a critical threshold (photoperiod that induces response in 50% of the population; Gill et al. 2017). This response ensures synchrony between periods of insect activity and the availability of resources (van Asch and Visser 2007); however, when insects are introduced to new environments this synchrony can be disrupted, and insects may enter diapause too early or too late (Grevstad and Coop 2015). Such asynchrony may be most problematic in feeding guilds that target more ephemeral resources, such as seed predators (Story et al. 1992); however, early or late diapause can be highly detrimental to the establishment of any weed biological control agent (Stiling 1993, Bean et al. 2007, Gupta et al. 2016). The Tamarisk leaf beetle, *Diorhabda carinulata* (Desbrochers) (Coleoptera: Chrysomelidae), for example, was introduced to the western United States for the control of *Tamarisk* spp., but initially failed to establish

at latitudes below 38°, because daylengths were too short to cue a second generation (Bean et al. 2007).

Hypena opulenta (Christoph) (Lepidoptera: Erebididae) was recently introduced into North America for the control of two invasive swallow-worts, *Vincetoxicum rossicum* (Kleopow) Barbar. (Gentianales: Apocynaceae) and *Vincetoxicum nigrum* (L.) Moench (Gentianales: Apocynaceae). The moth was first identified as a possible biological control agent during surveys in the Ukraine (Weed and Casagrande 2010), and extensive tests demonstrated that it is specific to European swallow-worts (Weed and Casagrande 2010, Hazlehurst et al. 2012). The native distribution of *H. opulenta* extends from Russia, east to Afghanistan, and south into Iran and Syria; it has also been found in Turkey and Turkmenistan (Fibiger et al. 2010). The petition to release *H. opulenta* in North America was submitted in 2011 (Casagrande et al. 2011), and releases in Canada began in 2013 (Young and Weed 2014) and in the United States in 2017 (Tewksbury, unpublished data). Although the agent has established in both Canada and the United States, dispersing in some cases over 2 km from release sites (Bourchier et al. 2019), population sizes remain low and levels of defoliation are insufficient to halt the spread of the weeds.

Adult *H. opulenta* emerge in the spring and females lay ca. 400 eggs, predominantly on the underside of leaves (Weed and Casagrande 2010). Larvae develop through four or five instars before pupating attached to leaves or, more commonly, in the soil. The moths overwinter as pupae in the soil, but diapause is facultative, so multiple generations in a single summer may be possible (Hazlehurst et al. 2012). If *H. opulenta* is to reach population densities sufficient to suppress invasive swallow-worts and prevent late-season recovery of the weeds, then undergoing a second generation may be critical for the successful control of these weeds. Understanding how and where it is possible for *H. opulenta* to undergo multiple generations can be the key to success for a biological control program against the invasive swallow-worts.

Photoperiod is thought to be the main cue for diapause induction in *H. opulenta* (Weed and Casagrande 2010). Therefore, we explored the possible effects of differences in photoperiod on the seasonal biology of the biological control agent. We conducted four experimental cage releases and a growth chamber experiment to determine the threshold photoperiod for diapause induction in *H. opulenta*. The results may help to determine whether photoperiod, in the moth's introduced range, represents an impediment to the efficacy of *H. opulenta* as a biological control agent.

Methods

Insect Production

All *H. opulenta* used in this study were from laboratory reared colonies maintained at the University of Toronto, ON, and Agriculture and Agri-Food Canada in Lethbridge, AB. The populations were descendants of insects originally collected in 2006 and 2012 from Donetsk, Ukraine, by CABI-Switzerland (A. S. Weed and A. Gassmann, unpublished data). The laboratory colony was reared in clear plastic containers at 20–25°C, 40–60% relative humidity (RH), and a 16:8 h (L:D) diel period. For diapause induction, eggs were moved to a growth chamber with the same conditions as described above but a 12:12 h (L:D) diel period, where they were reared until pupation. Approximately 3 wk after pupation, pupae were then moved to plastic containers bedded with moist cotton or vermiculate and gradually cooled down over 2 wk before they were stored in a permanently dark growth chamber at 2–5°C for 3–5 mo. Additional details can be found in the *Hypena opulenta* rearing guide (Miller et al. 2015).

Experiment 1: The Influence of Release Date on Diapause Induction in Field Cages

In the summer of 2017, we conducted four caged releases of *H. opulenta* at a field site in Uxbridge, Ontario, Canada (44.088681, -79.106804) to determine how the timing of releases affected the moth's ability to undergo two generations in a single summer. The outdoor cages were sized 183 × 183 × 183 cm and covered with Lumite amber-colored screen (BioQuip Products, Rancho Dominguez, CA). The field site consisted of a mixed pine hardwood forest, the understory of which was dominated by *V. rossicum*; cages were placed at sites with close to 100% coverage of the invasive weed. The mean density (\pm SE) of *V. rossicum* within the cages was 124 \pm 28 stems per square-meter. All *H. opulenta* individuals used in this experiment were from an overwintering laboratory colony (see rearing conditions above). To break diapause, overwintering pupae were placed on moist vermiculite in clear plastic containers and held at room temperature until adult emergence. Eight adult moths (four males and four females) were released in each cage, and releases were staggered so that two releases took place before the summer solstice (on June 15 and 20) and two after the summer solstice (on June 23 and 28; Fig. 1). We revisited the release cages every week to observe the progress of moth development. When no more larvae were found (approximately 2 mo after each release), all *V. rossicum* stems within each cage were pulled and searched for pupae. The top layer of soil within each cage was also thoroughly searched for pupae. Pupae were then placed individually on moist cotton wool in plastic containers and stored in a growth chamber at 25°C, 75% RH, and a 16:8 h light:dark (L:D) diel period. Pupae were monitored daily for emergence and, after a period of 3 wk, pupae that were still alive but had not emerged were assumed to be in diapause based on a mean pupal developmental time of about 10 d at 25°C (Lukas Seehausen, unpublished data). Pupae were checked for viability by gently squeezing the abdomen with feather weight forceps causing pupae to wiggle if they were alive.

Experiment 2: The Influence of Photoperiod on Diapause Induction Under Laboratory Conditions

In order to determine the critical photoperiod for diapause induction in *H. opulenta*, newly emerged *H. opulenta* adults (five males and five females) were placed into plastic oviposition cages (55 × 40 × 22 cm) containing cut *V. rossicum* stems in June 2019. All *V. rossicum* stems used in the experiment were collected from the above described field site in Uxbridge, Ontario, Canada. All *H. opulenta* individuals used in this experiment were from an overwintering laboratory colony, as in experiment 1. Oviposition cages were kept in a large growth chamber at 25°C, 75% RH, and a 16:8 h (L:D) diel period. Plant

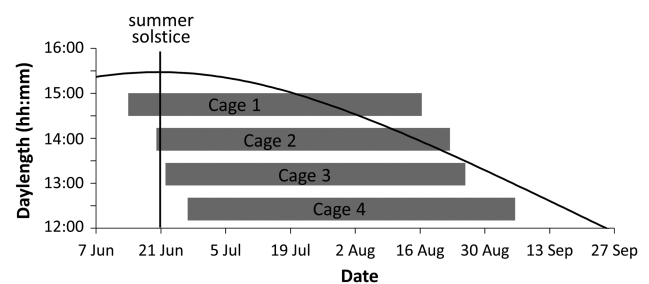


Fig. 1. Timing of releases (beginning of gray bars) and collection of pupae (end of gray bars) of *Hypena opulenta* in field cages (cage 1–4) at Uxbridge, Ontario around the summer solstice (21 June 2018 vertical line). The black line indicates the daylength over the duration of the field experiment.

material was replaced every day and leaves with freshly laid eggs were distributed between five identical plastic cages containing no adult moths. These cages were placed in five different growth chambers, all set at 25°C and 75% RH but with a range of different diel periods: 16:8 h; 15:9 h; 14:10 h; 13:11 h; and 12:12 h (L:D). Fresh *V. rossicum* stems were added to each cage every two days to provide food for emerging larvae. Once larvae had reached the second instar, they were transferred individually to small plastic cups containing a disk of moistened tissue paper in the base, and two to three leaves of *V. rossicum*. Cups were cleaned every 2 d to remove frass and leaves were replaced as necessary until pupation. Once pupae were fully formed, *V. rossicum* leaves were removed and pupae were retained in their cups on a layer of moist cotton wool. The experiment was continued until at least 35 pupae had been generated in each chamber. Pupae were returned to their respective growth chambers to be monitored daily for emergence. After a period of 3 wk, pupae that were alive but had not emerged were assumed to be in diapause.

Statistical Analyses and Geographic Analysis

The influence of release date (for the field experiment), and hours of photoperiod, on the induction of diapause were analyzed in separate logistic regressions using the *glm* function of the 'stats' package in R (R Core Team 2019).

To investigate the maximum daylength *H. opulenta* can experience in its area of origin and its introduced range, we calculated daylengths at summer solstice for different latitudes. Daylengths were calculated based on the most commonly applied definition in the United States, with sunrise and sunset starting and ending when the top of the sun is apparently level with the horizon. In each case, the sun is, in fact, 0.8333° below the horizon (see Forsythe et al. 1995 for the formula). The results were then plotted on a world map, together with coordinates of the initial collection site of *H. opulenta* close to Donetsk, Ukraine, and a selection of swallow-wort infested sites that cover the latitudinal extremes of the weeds invasive range in North America (Bourchier et al. 2019, EDDmaps 2019). At each of these North American sites we calculated the number of days with daylengths >15 h (see above definition) to evaluate the time period during which daylength remained long enough to allow for at least some individuals of a second generation (based on the results of experiment 2).

Results

Experiment 1: The Influence of Release Date on Diapause Induction in Field Cages

All pupae recovered from the *H. opulenta* cage release conducted on June 15 (10 pupae) emerged as adults. Conversely, the majority of pupae recovered from cage releases conducted on June 20 (29 pupae), June 23 (100 pupae), and June 28 (10 pupae) entered diapause (97%, 98%, and 100%, respectively). Release date significantly influenced diapause induction in *H. opulenta* ($\chi^2 = 68.41$; $df = 1$; $P < 0.0001$). The probability of diapause increased logarithmically with later release dates (Fig. 2A), approaching 100% when moths were released at or after the summer solstice at Uxbridge, Ontario (where the maximum daylength is 15 h 30 min; Fig. 2B).

Experiment 2: The Influence of Photoperiod on Diapause Induction Under Laboratory Conditions

The number of larvae successfully reared to the pupal stage was 35, 95, 73, 53, and 48 for a photoperiod of 12:12, 13:11, 14:10, 15:9, and 16:8 h L:D, respectively. Photoperiod length significantly

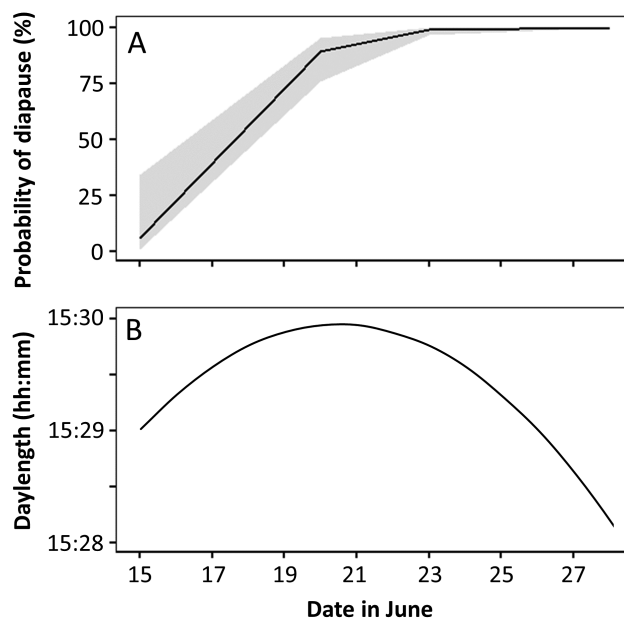


Fig. 2. (A) Probability ($\pm 95\%$ confidence interval) of diapause for offspring of *Hypena opulenta* adults released into field cages in Uxbridge, Ontario at different dates in June around the summer solstice (June 15, 20, 23, and 28) (experiment 1). (B) Daylength in Uxbridge, Ontario, during the release period.

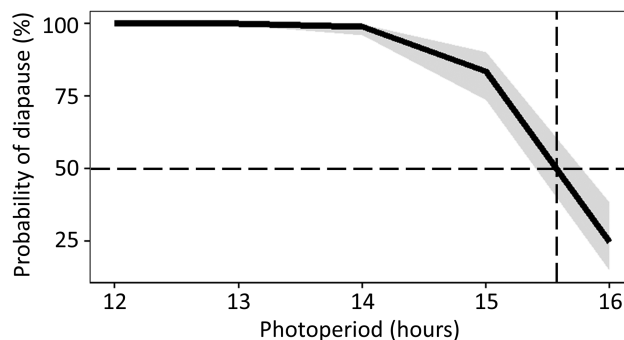


Fig. 3. Probability ($\pm 95\%$ confidence interval) of pupal diapause induction (A) for *Hypena opulenta* reared from egg to pupae at five photoperiods (12–16 h) (experiment 2). The intersection of the dashed lines indicates the critical photoperiod (15 h 35 min) where diapause was induced in 50% of individuals.

influenced diapause induction ($\chi^2 = 145.37$; $df = 1$; $P < 0.0001$). *Hypena opulenta* reared at a 16-h photoperiod largely avoided diapause, with 88% emerging for a second generation. Conversely, almost all *H. opulenta* reared at photoperiods of 15:9 h or below went into diapause (94–99%). This is also supported by the calculated probability of entering diapause, which is ca. 25% at a 16:8 h L:D photoperiod and >75% at photoperiods below 15 h (Fig. 3A). The threshold photoperiod for diapause induction in 50% of *H. opulenta* reared at 25°C was calculated to be 15 h 35 min.

Geographic Analyses

The major known sites of swallow-wort infestation in North America are situated at latitudes that receive between 15:9 and 16:8 L:D h daylight at summer solstice (Fig. 4). However, only the most northern site in Ottawa, Ontario actually receives >15 h 30 min of daylight. In comparison, daylength at summer solstice in Donetsk,

Ukraine, the site of origin for the *H. opulenta* populations introduced into North America, is slightly above 16:8 h L:D (Fig. 4).

The annual time period during which daylength is long enough (>15 h) to potentially allow for a second generation of *H. opulenta* in North America ranged from 69 d at the most northern site (Ottawa, Ontario; 45.38°N) to 15 d at the most southern site (Harrisburg, PA; 40.1°N). In contrast, there are 81 d with daylengths >15 h in Donetsk, Ukraine (Table 1).

Discussion

Increasing proportions of *H. opulenta* individuals enter diapause as daylengths decrease from 16 to 15 h, with the critical threshold photoperiod that induces diapause in 50% of the population being 15 h 35 min. This result is supported by both the field and the laboratory experiment, and suggests that univoltinism and premature diapause are likely at some North American release sites. *Hypena opulenta* is known to have a facultative diapause (Weed and Casagrande 2010) and at least two generations per year are possible in its native area of distribution (Fibiger et al. 2010). In North America, two generations have been observed under field conditions at release sites in Ottawa, Canada (Bourchier et al. 2019) and our results in field cage releases near Uxbridge, Canada (experiment 1) show that the earliest releases conducted before the solstice on June 15 resulted in a second generation of adults emerging in the field under natural conditions. While indicative, our results do not necessarily confirm that *H. opulenta* is bivoltine in Uxbridge because we released lab-reared adults and the timing of natural emergence in

Uxbridge remains unknown. The moth's voltinism in other parts of North America is also yet to be confirmed but as releases occur farther south, the shorter time intervals when days are longer than 15 h suggest that the moth may be primarily univoltine.

The maximum daylength at summer solstice in Uxbridge is 15 h 30 min. Almost all (97–100%) offspring of adult moths that were released at or after the summer solstice went into diapause. These individuals were highly unlikely to have experienced the maximum daylength because eggs were laid after the summer solstice. In contrast, 100% of the surviving offspring from adult moths released 1 wk before the summer solstice did not go into diapause and emerged. Thus, exposure of young individuals (eggs and larvae) to a 15 h 30 min daylength, or at least very close to this daylength, was sufficient for *H. opulenta* to not induce diapause under field conditions.

Knowledge of this critical photoperiod allowed us to analyze where this biological control agent might experience daylengths that are long enough to allow for emergence of adults. At the original collection site of *H. opulenta* in Donetsk, Ukraine, daylengths reach slightly longer than 16 h at the summer solstice. In our study, the most northern North American release site was located in Ottawa, Canada, where *H. opulenta* is known to have two generations (Bourchier et al. 2019); daylength in Ottawa at the summer solstice is 15 h 40 min. Thus, it is likely that the moth is also bivoltine in the Donetsk source area. Any site south of our field cage site (Uxbridge) receives <15 h 30 min of light at the summer solstice and thus, the possibility of bivoltinism will strongly depend on when adults emerge from overwintering sites in spring or when releases are done.

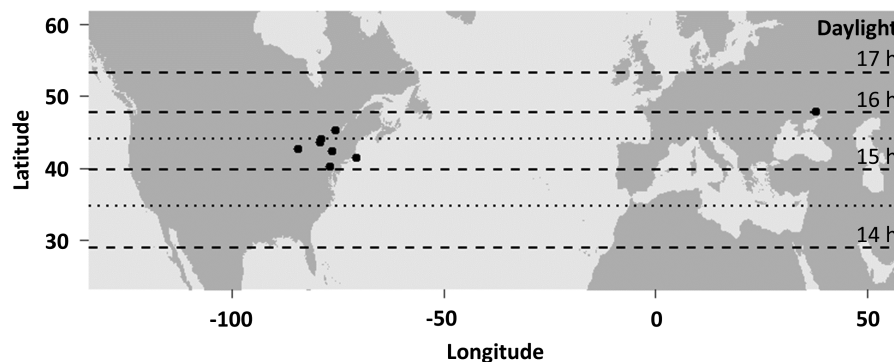


Fig. 4. Latitude (white horizontal lines) and hours of daylight (black horizontal lines) at summer solstice (June 21) for (black dots) the original collection site of *Hypena opulenta* in Europe (Donetsk, Ukraine) and different locations in North America where the moth was introduced as biological control agent against invasive swallow-worts.

Table 1. Site in the native range of *Hypena opulenta* (Donetsk, Ukraine) where released populations were originally collected and key swallow-wort infestation sites in North America, arranged from high to low latitude

Site	Latitude	Daylength (hh:mm) on June 21	Dates with daylength >15 h	Number of days with daylength >15 h
Donetsk (Ukraine)	47.94	16:02	May 13–Aug. 1	81
Ottawa, ON	45.38	15.40	May 19–July 26	69
Toronto, ON	43.65	15.26	May 25–July 20	57
East Lansing, MI	42.74	15.20	May 29–July 16	49
Ithaca NY	42.44	15.17	May 30–July 15	47
Nashon Island, MA	41.5	15.11	June 4–July 10	37
Harrisburg, PA	40.1	15.02	June 15–June 29	15

The final two columns indicate the date range, and the number of days for which daylengths are long enough (> 15 h) to prevent the onset of diapause. Daylength was calculated with sunrise and sunset starting and ending when the top of the sun is apparently level with the horizon (Forsythe et al. 1995). *Hypena opulenta* releases should be conducted such that the critical life stages occur during this window, in order to promote a second generation.

The laboratory experiment shows that even at a 16-h photoperiod, diapause is induced in at least some individuals (12% observed and 25% estimated probability of diapause). This phenomenon has been observed in a previous study (Weed and Casagrande 2010) and may be attributed to other environmental and physiological factors affecting diapause induction (Tauber et al. 1986, Danks 1987, Leather et al. 1993, Dautel and Knülle 1998, Gill et al. 2017) in addition to those examined here. For example, low temperature has also been shown to induce diapause (Beck 1982) and high temperatures can delay or eliminate diapause induction (Chippendale 1982). Although to date no influence of temperature on diapause induction has been shown for *H. opulenta* (Weed and Casagrande 2010), such an effect has been observed in a closely related species. For example, low temperatures have been shown to affect the proportion of individuals going into diapause at a short photoperiod in *Helicoverpa punctigera* (Hübner) (Lepidoptera: Noctuidae) (Cullen and Browning 1978). In addition to temperature, nutritional status of insects or the quality of available food sources can be factors leading to diapause induction (Hunter and McNeil 1997). It has been suggested that seasonal changes in host plant quality due to senescence of *Vincetoxicum* plants in late summer and autumn affect diapause induction in *H. opulenta* (Weed and Casagrande 2010). Differences in the quality of foliage may therefore account for some of the variability in diapause induction in our experiments and warrants further investigation.

Other factors that may influence the critical photoperiod include previous photoperiod exposures (e.g., maternal exposures; Reznik et al. 2011) and sensitivity to twilight (Wang et al. 2014). Many insects perceive some portion of twilight as daylight (Beck 1980); so, the exact daylength at any given site is difficult to determine. We calculated daylengths to include a portion of twilight, but future work should try to elucidate how twilight is interpreted by *H. opulenta*. If the moths interpret daylength without twilight, then release windows will be even shorter than those calculated here. For example, swallow-wort infestations in Harrisburg, Pennsylvania, are at a latitude of 40.1, where civil twilight is upwards of 1 h in early summer (Beck 1980). If civil twilight is not perceived by *H. opulenta*, multivoltinism may be impossible at these sites because there are no days >15:9 h L:D, if civil twilight is not included. Finally, the critical photoperiod for diapause induction is phenotypically plastic, and varies geographically among populations (Bradshaw 1976, Masaki 1999, Grevstad et al. 2012), such that variations in diapause induction of recently introduced insects can occur along geographic gradients (Tanaka and Murata 2016). Therefore, populations of *H. opulenta* at lower latitudes within the native range might exhibit photoperiodic responses and other physiological traits that make them better adapted to southern North American release sites. However, diapause induction in a certain percentage of a population at even optimum conditions might be a bet hedging strategy of *H. opulenta* to increase fitness in an unknown future environment, as has been shown for other insects (Bradford and Roff 1993). Postrelease monitoring will therefore be crucial to determine the acclimatization of *H. opulenta* to conditions at actual release sites.

For the biological control program against swallow-worts in North America, the potential for *H. opulenta* to be univoltine and enter diapause early in the year is potentially problematic in several ways. First, in comparison to bivoltinism, univoltinism leads to slower population growth that will take much longer to reach densities capable of affecting the target weed (Tauber et al. 1996). Second, the absence of active *H. opulenta* later in the season would allow the swallow-worts to recover and compensate for earlier

damage. Late-season recovery by invasive plants has been shown to severely restrict the efficacy of biological control agents (Dudley and DeLoach 2004). Third, diapause is generally an adaptation for surviving long periods of cold (Bale and Hayward 2010); so, longer periods of warm temperature can be severely detrimental to the survival of diapausing insects, as increased metabolic rates lead to faster depletion of energy stores (Thompson and Davis 1981). Finally, early diapausing insects may be exposed to increased predation pressure, as many predators are more active earlier in the summer (Bean et al. 2007), and additionally, the pupal stage has few means of defense or escape and is therefore especially vulnerable to predation.

An additional factor influencing voltinism in *H. opulenta* will be the specific life stage(s) that are most sensitive to photoperiod. Preliminary results in the laboratory (Jones et al., unpublished data), as well as observations during mass rearing (Lisa Tewksbury, personal communication) suggest that early instar larvae are the sensitive stage in *H. opulenta*. This would enhance the chances of the moth achieving two generations, as individuals would only need to complete part of the larval stage before the critical photoperiod, in order for a second generation to be initiated.

In addition to the possible implication for the efficacy of *H. opulenta* to control swallow-worts in North America described above, our results can also inform practical aspects of the biological control program. For the mass-rearing of the moth and stockpiling of pupae, diapause can be induced by exposing larvae to any photoperiod $\leq 15:9$ h L:D. However, to ensure a maximum percentage of diapause induction, an exposure to a photoperiod between 12:12 and 14:10 h L:D beginning at the egg stage is recommended. Field releases (especially of adults) are best done before June 21 (summer solstice) to allow for the possibility of a second generation, but if the maximum daylength at summer solstice is between 15 h 00 min and 15 h 30 min, then *H. opulenta* may only have two generations if they are released in the time window of Table 1. Of course, bivoltinism at these sites may also depend on other environmental factors as described above, and future work should prioritize the effects of temperature on photoperiod response, and pupal development time to calculate adult emergence in spring. Additionally, our results will be important for the development of a temperature-based phenology model for *H. opulenta*, similar to that created for the looper beetle *Gallerucella calmariensis* L. (Coleoptera: Chrysomelidae) (Grevstad and Coop 2015). Such a model will allow us to more accurately map the probable number of *H. opulenta* generations across the invasive range of swallow-worts, and to inform biological control practitioners of the optimum time window for releases of a given life stage at a given location.

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