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Inheritance and World Variation in Thermal Requirements for Egg Hatch in *Lymantria dispar* (Lepidoptera: Erebidae)

M. A. Keena[1]

USDA Forest Service, Northern Research Station, Hamden, CT 06514 (mkeena@fs.fed.us) and [1]Corresponding author, e-mail: mkeena@fs.fed.us

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Abstract

Mode of inheritance of hatch traits in *Lymantria dispar* L. was determined by crossing populations nearly fixed for the phenotypic extremes. The nondiapausing phenotype was inherited via a single recessive gene and the phenotype with reduced low temperature exposure requirements before hatch was inherited via a single dominant gene. There was no evidence for sex-linkage or cytoplasmic effects with either gene. Eggs from 43 geographic populations were evaluated for hatch characteristics after being held for 60 d at 5°C followed by incubation at 25°C. There was considerable variation both within and among the populations in the proportion able to hatch, time to first hatch, and average time to hatch. Egg masses with reduced requirement for low temperatures before the eggs were ready to hatch were present in all subspecies of *L. dispar* and the phenotype was not fixed in most populations. The populations clustered into three distinct groups, and climatic variables were found to be rough predictors of those groups. Variation in hatch phenotypes between populations is likely an adaptation to local climate and within a population provides a bet-hedging strategy to ensure that at least some hatch synchronizes with host leaf-out. Continued vigilance to prevent movement of populations both within and between countries is warranted, because some of the alleles that confer nondiapause or reduced low temperature requirements before egg hatch are not present in all populations and their introduction would increase variation in egg hatch within a population.

Key words: egg diapause, hybrid, *Lymantria dispar*, gypsy moth, inheritance

Synchrony between egg-hatch and host bud-burst phenology is critical for spring-feeding Lepidoptera like gypsy moth, *Lymantria dispar* L. (Lepidoptera: Erebidae). If gypsy moth larvae hatch before host bud-break, they can starve to death in as little as 5 d if they can't find a suitable host by passively ballooning on the wind (Capinera and Barbosa 1976). Both increased mortality and dispersal have been documented in field populations when phenology with their hosts is asynchronous (Erelli and Elkinton 2000, Hunter and Elkinton 2000). Since gypsy moths are generalists and can survive and reproduce on hundreds of hosts (Liebhold et al. 1995), there is both a temporal and spatial aspect to the host phenology. Deciduous tree species consistently leaf out in a predictable order (Crawley and Akhteruzzaman 1988), so in a forest with many potential host species, gypsy moth first instars may be able to disperse and find suitable hosts even when hatch phenology does not line up with the most preferred hosts. However, if larvae hatch after all suitable host bud-break has occurred, the available leaves will be tougher and contain more defensive compounds, which can negatively affect early instar survival and development.

Gypsy moth is a univoltine species throughout its range and spends 8–9 mo/yr diapausing as a mature embryonic larva within the egg (Goldschmidt 1934, Leonard 1968). This egg diapause increases survival during the winter, and the satisfaction of diapause requirements renders the egg responsive to spring temperatures and allows it to complete postdiapause synchronizing hatch with bud-burst of hosts. Goldschmidt (1934) showed that gypsy moths from different geographic areas had heritable differences in diapause and hypothesized that the differences were adaptations to deal with the climatic conditions where they were found. Differences in timing and percentage hatch of eggs exposed to different temperatures and durations of low temperature have been documented for populations from the far east of Russia and the United States (Keena 1996). Eggs from the Russian populations required less exposure to low temperature to satisfy diapause requirements than did eggs from a North American population, allowing them to complete post diapause more quickly and hatch when moved to warmer temperatures. No evaluation of egg chill requirements of gypsy moth populations from around the world under controlled conditions has been done, although there have been some evaluations of different population responses to temperature, most often using field-collected eggs with unknown diapause states (e.g., Masaki 1956, Pantyukhov 1962, Lyons and Lysyk 1988, Tauber et al.1990, Wei et al. 2014). There is a concern that Asian gypsy moth populations may all possess this reduced requirement for egg chill to break diapause, which could

increase the risk of introduction and potential establishment. The ability to forecast egg hatch is also critical to timing control programs and predicting the ability of these populations to adapt to the range of climates found in North America.

Since the early 1990s, multiple introductions of gypsy moth populations with females that are capable of strong directed flight have occurred in the United States (U.S. Department of Agriculture–Animal and Plant Health Inspection Service–Plant Protection and Quarantine [USDA-APHIS-PPQ] 2014). Most of these introductions occurred because the flying female gypsy moths were attracted to lights at night in Asian ports where they laid their egg masses on ships or their cargo (Wallner et al. 1995). The egg masses were subsequently transported to North American ports where they hatched, dispersed, and established. Eradication protocols were immediately initiated in the areas where they established and in each case the eradication was deemed successful. This drastic response was considered warranted for multiple reasons: the Asian population females could disperse (up to 100 km, Rozhkov and Vasilyeva 1982), while the females of the population already present in the United States are flightless, and larvae from the Asian populations feed on a broader range of hosts (including some conifers) than the established population (Baranchikov 1989, Turova 1992).

Determining the inheritance of the differences in chill requirements to break diapause and in the requirement for egg diapause will distinguish the source of interpopulation variation (genetic or environmental) and determine the consequences of hybridization on diapause-related traits. The inheritance of three traits associated with egg diapause or postdiapause have been examined for gypsy moth: the number of days from egg laying to first hatch, the number of days from the end of chill to first hatch, and the duration of the hatch period. Days to first hatch was shown to be sex-linked and to vary depending on the environment when a North American nondiapause-selected population was crossed with a diapausing population (Lynch and Hoy 1978). However, when a Yugoslavian nondiapause-selected population was used, days to first hatch was determined to be polygenically inherited (Marović 1981). Work by several different groups on many geographical populations suggests that days from the end of chill to first hatch and duration of hatch are both polygenically inherited (Marović 1981; Goldschmidt 1932, 1933, 1934; Keena 1994).

In this paper, I assessed the mode of inheritance, and tested for cytoplasmic effects and sex linkage for gypsy moth diapause and related traits (e.g., days to first hatch, duration of hatch, and percentage hatch). To accomplish this, I crossed individuals from a North American population with almost no egg hatch after 60 d of chill at 5°C with both a Russian population selected for nondiapause and with a Russian population that > 80% of the eggs hatch after only 60 d of chill at 5°C. Time (d) to first hatch, average time to hatch, duration of hatch, and percentage of eggs hatching after 60 d of chill at 5°C followed by incubation at 25°C were determined for parental (north American and Russian), reciprocal F_1 hybrids, reciprocal backcrosses to both parental populations, and double reciprocal F_2 hybrids. The same traits were evaluated for crosses between the nondiapause and the diapausing populations with 0 and 30 d at 5°C followed by incubation at 25°C. Additionally, time (d) to first hatch, average time to hatch, duration of hatch, and percentage of eggs hatching after 60 d of chill at 5°C followed by incubation at 25°C were determined for eggs from 43 different populations from across the gypsy moth geographical range. The most likely mode of inheritance for each diapause trait, possible collection site climatic conditions that might predict diapause traits of a population, and implications of the findings for management programs are discussed.

Materials and Methods

Gypsy Moth Populations and Rearing

Information on the source location and collection of the 43 gypsy moth geographical populations used in these studies are given in Table 1. Based on the recent review of *Lymantria* (Pogue and Schaefer 2007), the Japanese populations (JN and JI) are the *japonica* subspecies, the other Japanese population (JS) and all Russian, South Korean, and Chinese populations are the *asiatica* subspecies, and all remaining populations should be the *dispar* subspecies. All populations were transported under permit to the USDA-Forest Service quarantine facility in Ansonia, CT. Voucher specimens for each population were deposited at the Entomology Division, Yale Peabody Museum of Natural History, New Haven, CT.

All populations were reared for at least one generation in the laboratory before use in the studies. Larvae to produce the egg masses used in the studies were reared in walk-in environmental chambers maintained at 25°C, 60% relative humidity (RH), and a photoperiod of 16:8 (L:D) h. Larvae were reared, in cohorts of 8–10, as described in Keena (1996). The high wheat germ artificial diet (Bell et al. 1981) was optimized for the individual populations using Wesson salt mix without iron and adding 0.27 g (Asian populations) or 0.10 g (European and North American populations) amorphous $FePO_4$ per liter of diet. The F1, F2, and back crosses were all reared on diet containing 0.12 g of amorphous FePO4 per liter of diet to reduce maternal effects in the generation tested and to expose all genotypes to the same environment. Pupae were harvested, sexed, and stored by sex, egg mass (family), and population. Adults were removed daily and held for 24 h before mating as individual pairs. Matings were random within population except that sibling matings were avoided. Ten days after mating, egg masses were harvested by scraping each egg mass from the inside of the 473.2-ml paper mating cup.

Selection of a Nondiapause Population and Evaluation of the Inheritance of Egg Diapause

To produce a nondiapausing population, precocious hatch (eggs that hatched without any exposure to cooler temperatures) from the second laboratory generation of the RM Russian population were reared and mated. In this first generation of the NDRM (nondiapausing RM) population, the 24 individual larvae that hatched from the 510 RM egg masses between the time they were laid and the time they were chilled (~40-d period) were reared. The resulting adults produced eight egg masses which were allowed to hatch (>80% of the eggs hatched) without being chilled and about 2,000 of the 3,698 larvae that hatched were reared. Twenty-nine to thirty-five egg masses each of the NDRM parental (second generation), the UM population from Massachusetts in the United States (parental diapausing population), and reciprocal hybrids between the two were produced through single-pair matings. The resulting egg masses were held intact in separate plastic petri dishes (100 by 15 mm, diam × height) with loose fitting lids and the dishes were then placed in covered, clear plastic boxes (30 long by 15.5 wide by 9 cm high, Wilson, Sunbury, PA). Relative humidity in the box was maintained at ~90–95% by placing water in the box; petri dishes with egg masses were placed on a wire screen (0.63-cm gauge) above the water. The egg masses were held at 25°C for hatch until there had been no larvae hatch in the last 7 d (the most recent laid egg masses were 38 d old at the time and the oldest

Table 1. Approximate location (latitude and longitude) of source populations, climatic information, and designations for populations of gypsy moth evaluated in this study, arranged by population designation

Population	Country	Closest city, region	Collection date[a]	Egg masses received	Latitude	Longitude
AM	Austria	Mistelbach, Vienna	Sept. 1993	39 individual	48.35° N	16.35° E
BS	Bulgaria	Sofia, Sofia	Feb. 1995	12 individual	42.51° N	23.40° E
CB	China	Beijing, Beijing	Aug. 1992 (Oct. 1994)	30 individual	39.53° N	116.23° E
CH	China	Qianan, Hebei	Aug. 1992 (Oct. 1994)	30 individual	40.00° N	118.41° E
CJ	China	Yanzikou, Beijng	Aug. 2011 (Oct. 2012)	4 individual	40.32° N	116.15° E
CL	China	Niuzhuang, Liaoning	Aug. 1992 (Oct. 1994)	30 individual	41.03° N	122.30° E
CN	China	Sandeli, Liaoning	Sept. 2011 (Oct. 2012)	4 individual	41.51° N	122.37° E
CR	China	Harbin, Heilongjiang	Aug. 2012 (Oct. 2012)	6 individual	45.78° N	126.61° E
CS	China	Huangdao, Shangdong	Aug. 1992 (Oct. 1994)	30 individual	35.52° N	120.02° E
FB	France	Benon, Charente-Maritime	Nov. 1994	6 individual	46.13° N	00.47° E
FR	France	Brumath, Touraine	Nov. 1993	25 individual	48.44° N	07.43° E
FV	France	Verneuil -s Indre, Touraine	Sept. 1993	29 individual	47.03° N	01.02° E
GE	Germany	Heilbronn, Baden-Württemberg	Sept. 1993	5 individual off buildings	49.08° N	09.14° E
GG	Germany	Gündlingen, Baden-Württemberg	Sept. 1993	25 individual	48.01° N	07.38° E
GH	Germany	Heilbronn, Baden-Württemberg	Sept. 1993	33 individual	49.08° N	09.16° E
GI	Germany	Heidelberg, Baden-Württemberg	Sept. 1993	22 individual off buildings	49.29° N	08.40° E
GK	Germany	Königsberg in Bayer, Bavaria	Sept. 1993	14 individual off buildings	50.10° N	10.34° E
GL	Germany	Lampertheim, Ofenheim	July 1994	30 individual	49.36° N	08.32° E
GW	Germany	Leingarten, Baden-Württemberg	Sept. 1993	32 individual	49.07° N	09.05° E
JI	Japan	Takizawa, Morika, Nishine Hachimantai City, Northern Iwate District, Honshu	Nov. 2014 (Oct. 2005)	15 individual	39.73° N	141.08° E
JN	Japan	Nagoya, Honshu	Mar. 1996	4 individual	35.15° N	137.08° E
JS	Japan	Sapporo, Hokkaido	May 1992 (Aug. 1994)	30 individual	43.00° N	141.30° E
KG	Greece	Kavála, Macedonia	Feb. 1997	58 individual	41.00° N	24.25° E
LJ	Lithuania	Juodkrante, Kuzsin Nezijos	Aug. 1994	47 individual	55.31° N	21.06° E
NJ	United States	Blairstown, Warren County, NJ	Nov. 1967	unknown	40.98° N	74.96° W
PP	Portugal	Ponte de Sor, Portalegro	Aug. 1994	2 individual	39.15° N	08.06° W
RA	Russia	Nakhodka, Russia (Vancouver, BC)[b]	Sept. 1991	>100 mixed off ship	42.82° N	132.88° E
RB	Russia	Bellyk, Krasnoyarsk	Dec. 1992	>30 mixed off rocks	54.30° N	91.18° E
RM	Russia	Mineralni, Primorski	Aug. 1992	20 individual	44.10° N	133.15° E
RS	Russia	Shira, Khakassia	Aug. 1994	6 individual	54.41° N	90.00° E
SC	Switzerland	Convento, Tessin	Sept. 1993	32 individual	46.04° N	08.57° E
SK	Korea	Samhwa-Dong, Gangovon-Do	Nov. 2014 (Aug. 2009)	16 individual	37.49° N	129.06° E
TC	Croatia	Losinj, Cres Island	Mar. 1995	11 individual	44.32° N	14.28° E
TL	Croatia	Lirovljani, Slovenia	Mar. 1995	5 individual	45.23° N	16.55° E
TN	Croatia	Novska, Slovenia	Mar. 1995	7 individual	45.20° N	16.59° E
TO	Croatia	Otok, Slovenia	Mar. 1995	10 individual	45.07° N	18.53° E
TS	Croatia	Strizovojha, Slovenia	Mar. 1995	5 individual	45.13° N	18.28° E
UC	United States	Bethany, New Haven County, CT	Mar. 1994	12 individual	41.25° N	73.00° W
UM	United States	Wrentham, Norfolk County, MA	Feb. 1992 (Nov. 1993)	25 individual	42.05° N	71.20° W
UN	United States	Coinjock, Currituck County, NC	May 1992 (Nov. 1993)	15 individual	36.18° N	75.57° W
UW	United States	Morgantown, Monongalia, WV	Jan. 1994	20 individual	39.37° N	79.55° W
VN	Slovakia	Nitra, Bratislava	Jan. 1995	12 individual	48.20° N	18.06° E
WP	Poland	Wroclaw, Piaski Forest, Poznan	Mar. 1995	30 indivdual	51.53° N	16.59° E

[a] Date in parentheses is when the population was received at the Ansonia facility.

[b] Egg masses were scraped in Vancouver, British Columbia, from the superstructure of the Russian grain ship, Allalinhorn, which had just arrived from the Far East Russian port of Nakhodka.

The JI population was started using a combination of egg masses from three locations.

were 67 d old), then all the egg masses were chilled for 30 d at 5°C. After 30-d chill the egg masses were returned to 25°C to allow any additional larvae to hatch that could. The study was ended when there had been no hatch from any egg mass for at least 2 wk.

Evaluation of the Inheritance of Reduced Thermal Requirements for Egg Hatch

Egg masses from the same crosses as used in Keena et al. (2007) were used to evaluate inheritance of egg hatch after exposure to 60 d at 5°C. The parentals (RM population and UN a population form North Carolina, Untied States) were crossed to create reciprocal F_1 hybrids and the first generation F_1 hybrids were crossed with the parentals to create the eight backcrosses (RMUN × UN, UNRM × UN, UN × RMUN, UN × UNRM, RMUN × RM, UNRM × RM, RM × RMUN, and RM × UNRM; female parentage always listed first) and with each other to create the four double reciprocal F_2 hybrids (RMUN × RMUN, RMUN × UNRM, UNRM × UNRM, and UNRM × RMUN). After 7 d (from the mating date) the egg masses were harvested and a single 100–200 egg sample from each of the

29–32 egg masses for each cross was put in separate petri dish (50 by 9 mm, diam × height) with tightly fitting lid (Falcon, BD Biomedicals, Bedford, MA). Egg samples in petri dishes were then placed in the plastic boxes (same as the NDRM population) over water and held an additional 32 d in the rearing chamber to ensure prediapause was completed (Gray et al. 1991) before the studies began. The egg samples were moved to 5°C for 60 d then moved back to 25°C to hatch. The remainder of each egg mass was cut in half longitudinally and held separately for later hatch at 25°after optimal chill (133 - or 140-d chill at 5°C), so the larvae could be used in the Keena et al. (2007) studies. The study ended when no larvae had hatched in the last 2 wk for both the 60 and 133 or 140 d groups of eggs.

Evaluation of World Variation in Thermal Requirements for Egg Hatch

Fourteen to sixty egg masses were chosen randomly from each geographic population for use in these studies, depending on availability. Two samples of 100–200 eggs from each egg mass were put in separate petri dishes (50 by 9 mm, diam × height) with tightly fitting lids. The Petri dishes with the egg samples were held the same way those from the RM by UN crosses. One sample from each egg mass was held for 60 d and the other for 150 d at 5°C before they were incubated at 25°C for hatch. Sixty days at 5°C was chosen because it produced the greatest differences in means to first hatch and percentage hatch of embryonated eggs between populations with different thermal requirements for completing diapause and proceeding to hatch (Keena 1996). The inclusion of the 150 d at 5°C provided an estimate the maximum eggs from each population capable of hatching. This estimate could be used to adjust the 60 d data if needed for significant differences in egg hatch between the populations. The study ended when no additional larvae had hatched from any egg mass within the last 2 wk for both chill lengths.

Data Collection and Statistical Analyses

Egg samples or masses were checked daily as much as resources allowed, but at a minimum Monday, Wednesday, and Friday (generally when few eggs were hatching). Time (d) to first hatch (time from incubation at 25°C to first hatch), average time to hatch, and duration of hatch (number of days from first to last hatch) were calculated for each egg sample. First hatch was either the number of days from incubation at 25°C or from the day the egg mass was laid, in the case of the ND population crosses, until the first egg hatched. Following the termination of each of these studies, eggs were dehaired and examined to determine embryonation. Unembryonated eggs appeared yellow (often flattened) while embryonated eggs appeared dark and a fully formed larvae was generally visible. The number of each type of egg was recorded and the percent hatch of embryonated eggs was calculated. There was a significant difference between geographical populations in the percentage hatch of the eggs after the longer chill period (150 d) so the percentage hatch at 60 d was divided by the percentage hatch at 150 d to adjust for those differences. This was not done in the other studies because there were no significant differences between the crosses. When full egg masses were assessed the NDRM population crosses, the fecundity was also determined.

The fit of the data for each parameter that was measured to various distributions was evaluated using PROC UNIVARIATE (SAS Institute 2004). The Shapiro–Wilk and the Anderson–Darling test were used to assess normality. The following dependent continuous variables were analyzed in PROC GLIMMIX (SAS Institute 2004): days to 1st hatch, average days to hatch, percentage hatch of embryonated eggs, percentage unembryonated eggs, and fecundity. A completely randomized design was used with population or cross as the fixed effect. The gamma distribution with a log link was used for days to first hatch for the crosses and a normal distribution with an identity link was used for both days to first hatch and average days to hatch in the geographic populations and fecundity in the NDRM crosses. The beta distribution with a logit link was used for the percentage hatch of embryonated eggs and percentage unembryonated eggs data sets. Zero percent was replaced with 0.0001 and 100 percent was replaced with 0.9999, as all values must be between 0 and 1 for the Beta distribution. For each model, residuals were evaluated for normality, and the homogeneity of variance was assessed using Levene's test. Differences among means were determined by the least-squares means test with $\alpha = 0.05$ and a conservative Tukey–Kramer grouping (SAS Institute 2004).

PROC CLUSTER (SAS Institute 2004) using a complete linkage analysis was used to determine if the geographic populations segregated into distinct groups based on hatch traits after exposure to 5°C for 60 d: adjusted percentage hatch of embryonated eggs, days to first hatch, and percentage of egg masses with 0, 10, and 90% hatch. Collection site climatic variables (obtained for the closest city, unless altitude was substantially different, to the collection site that had climatic data reported on this web site http://www.weatherbase.com) and latitude were then evaluated using a classification tree algorithm, GUIDE (Loh 2015), to determine if any of the variables were good predictors for partitioning the populations into the three identified clusters. The best classification tree was determined based on the lowest cross-validation mean costs when using univariate splits based on unit misclassification costs and assuming equal prior probability of each of the three identified clusters. The climatic variables included were: annual average temperature, average monthly temperatures, average low and high monthly temperatures, the average number of d/yr above 32°C, the average number of d/yr below 0°C, and annual growing degree days (cumulative average daily temperature minus 10°C) (see Suppl Table 3 [online only] for select climatic variables).

Cytoplasmic Effects and Sex-Linked Genes

Differences between reciprocal F_1s and between backcross populations may be caused either by maternal effects or by sex-linked genes, or both. In Lepidoptera, the females are the heterogametic sex (ZW) and males are the homogametic sex (ZZ); thus, effects of loci on the W-chromosome are confounded with maternal effects, so for the purposes of this paper both will be referred to as "cytoplasmic effects." If the reciprocal crosses are significantly different and resemble the parental lines then Z-linkage is indicated. Two sets of the backcrosses provide an opportunity to assess cytoplasmic effects. The females of the RMUN × UN and UNRM × UN backcrosses both received their W-chromosome from the N father, but they received different cytoplasms and Z-chromosomes. Likewise the UNRM × RM and RMUN × RM females both received their W-chromosome from the R father, but different cytoplasms and Z-chromosomes. If significant differences between the backcrosses in each set exist then cytoplasmic effects are indicated. The mean percentage hatch of embryonated eggs for each set was compared using Yate's Corrected χ^2 (Statistix 2013).

Tests for Dominance and Fit to Gene Models

To determine if dominance was present in either the inheritance of egg diapause or the thermal requirements for hatch, the F_1 mean

percentage hatch of embryonated eggs was compared to that of the mid-parent (the average of the trait value of father and mother) using a Yate's Corrected χ^2 (Statistix 2013). If there is a significant difference then dominance is indicated (Falconer 1989).

The fit of a single gene model for inheritance (with or without dominance depending on the results of the previous test) of egg diapause or the thermal requirements for hatch were also evaluated using the pooled cross type (F_1, F_2, BN, and BR) means. To determine how well the single gene model fit the data, the expected and observed frequencies for each cross and phenotype combination were compared using PROC FREQ with the TESTF = () option (SAS 1999). Yate's corrected χ^2 (Statistix 2013) was used when the expected and observed values for a single cross and phenotype combination were compared.

Results

The Nondiapause Population and Inheritance of Egg Diapause

There was hatch from all the NDRM egg masses (third generation) and on average 79% of the eggs hatched with no exposure to low temperature (Table 2). None of the UM population eggs hatched and only about 2% of the F_1 hybrid eggs hatched with no chill. Exposing the remaining eggs to 5°C for 30 d apparently did not satisfy the chill requirements for diapause and subsequent hatch of most of the remaining eggs, only about 2% of the UM eggs and less than 1% of the additional NDRM or F_1 eggs hatched when re-incubated at 25°C. The percentage of unembryonated eggs was significantly higher in the NDRM and F_1 crosses than in the UM population with which it was crossed. Fecundity was also significantly lower in the NDRM and the NDRM × UM hybrid than in the UM parental population. No significant Z-linkage was found ($\chi^2 = 0.0000$, $P = 1.0000$). The mean percentage hatch of embryonated eggs without chill of the F_1 (2%) was significantly different from the mid-parent value (41%, $\chi^2 = 42.78$, $P = 0.0000$), indicating that there was dominance involved in the inheritance of this trait. A test for cytoplasmic effects could not be performed since no backcrosses could be created. Since the mean of the F_1 was not significantly different from that of the UM parental and dominance was indicated, a single recessive gene inheritance for nondiapause is suspected.

Evaluation and Inheritance of Reduced Thermal Requirements for Egg Hatch

The UN × UNRM cross had significantly higher percentage hatch of embryonated eggs after 133 or 140 d at 5°C than all the other crosses (Suppl Table 1 [online only]). It took significantly longer for the first larvae to hatch from the UN parental group egg masses than for the RM parental, backcrosses to the RM, or the UNRM × UNRM F_2 cross egg masses (Supp Table 1 [online only]). Percentage hatch of embryonated eggs varied significantly by cross ($F = 13.73$; df = 15, 464; $P < 0.0001$; Fig. 1). A significantly smaller percentage of the UN parental embryonated eggs hatched than did those from all the other crosses except the UN × RMUN backcross. The percentage hatch of embryonated eggs of the RM parental was not significantly different from that of the F_1, F_2, or backcrosses to the RM, but was significantly higher than that of the UN parental and backcrosses to it. Average time to egg hatch varied significantly by cross ($F = 34.55$; df = 15, 444; $P < 0.0001$). The UN parental eggs on average took significantly longer to hatch than those of any of the crosses or the RM parental (Fig. 2). The average number of days for backcrosses to the RM and RM parental eggs to hatch was

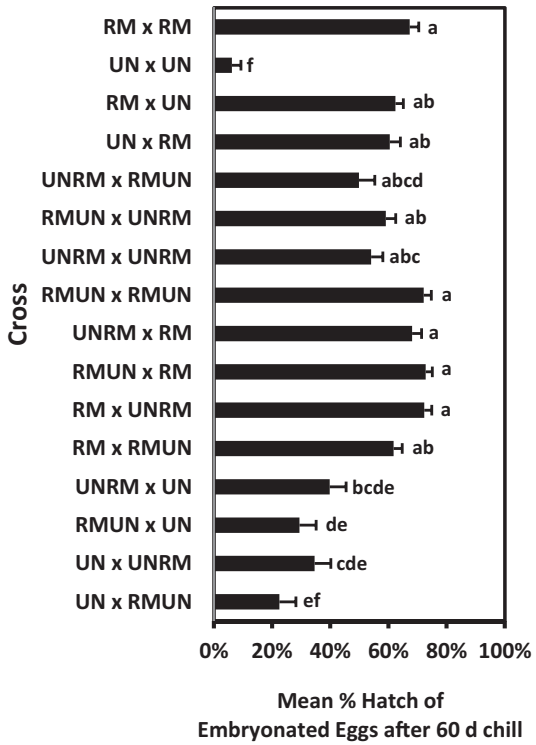

Fig. 1. Percentage hatch of *L. dispar* eggs from various crosses between a Russian population (RM) and a North American population (UN) after 60 d chill at 5°C followed by incubation at 25°C. The female parent is given first and values followed by the same letter are not significantly different.

Table 2. Hatch characteristics (mean ± SE) of *L. dispar* egg masses from various crosses between a nondiapausing Russian population (NDRM) and a North American population (UM); the female parent is given first

Population	Fecundity	% Unembryonated eggs	Days to first hatch after 0 d at 5°C	% Hatch of embryonated eggs after 0 d at 5°C	Cumulative % hatch of embryonated eggs after 30 d at 5°C	% Egg masses with hatch after 30 d at 5°C	*n*
NDRM × NDRM	734 ± 46 b	7.6 ± 1.3 a	23.9 ± 0.5 b	79.4 ± 2.5 a	79.7 ± 2.4 a	100.0	35
NDRM × UM	734 ± 50 b	6.7 ± 1.2 a	33.7 ± 2.6 a	2.5 ± 0.6 b	2.9 ± 0.7 b	37.9	29
UM × NDRM	855 ± 46ab	7.1 ± 1.2 a	20.7 ± 1.6 b	2.4 ± 0.5 b	2.7 ± 0.6 b	25.7	35
UM × UM	946 ± 48 a	3.5 ± 0.7 b	No hatch	0.0 ± 0.0	2.3 ± 0.5 b	3.1	32
Statistics	$F = 4.69$	$F = 4.76$	$F = 11.2$	$F = 120.13$	$F = 122.54$		
	df = 3,127	df = 3, 127	df = 2, 38	df = 3, 126	df = 3, 126		
	$P = 0.0039$	$P = 0.0035$	$P = 0.0002$	$P < 0.0001$	$P < 0.0001$		

Means in the same column followed by the same letter are not significantly different when analyzed by PROC GIMMIX (SAS Institute 2004) followed by Tukey–Kramer Least Squares Mean test with $\alpha = 0.05$.

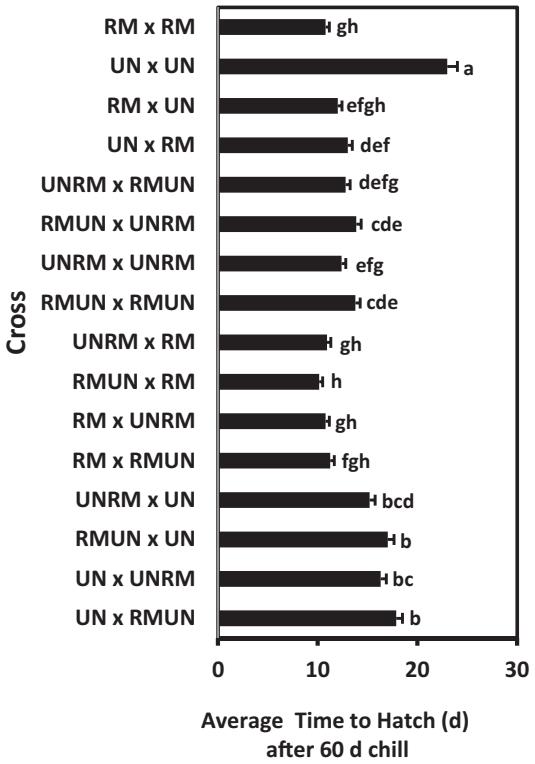

Fig. 2. Average time (d) to *L. dispar* egg hatch for various crosses between a Russian population (RM) and a North American population (UN) after 60 d chill at 5°C followed by incubation at 25°C. The female parent is given first and values followed by the same letter are not significantly different.

not significantly different, but the average days to hatch for the backcross to the UN parental was significantly different from that of the two parentals.

The pooled F1 mean percentage hatch of embryonated eggs was significantly different from that of the mid-parent (36%, $\chi^2 = 11.64$, $P = 0.0006$), indicating that there was dominance involved in the inheritance of this trait. There were no significant cytoplasmic effects (RMUN × RM = UNRM × RM, χ^2 0.22, $P = 0.6395$; RMUN × UN = UNRM × UN, χ^2 1.81, $P = 0.1780$) or Z-linkage (χ^2 0.02, $P = 0.8838$), indicating that the trait is autosomally inherited. Inheritance of reduced thermal requirements for egg hatch fit a single gene with dominance model well (χ^2 4.63, $P = 0.9476$).

World Variation in Thermal Requirements for Egg Hatch

Mean percentage hatch of embryonated eggs after 150 d at 5°C followed by incubation at 25°C varied significantly by geographical population (Suppl Table 2 [online only]). Therefore, the mean percentage hatch of embryonated eggs after 60 d at 5°C was adjusted for this variation in viable eggs by dividing the 60 d mean by the 150 d mean for each population. The adjusted mean percentage hatch of embryonated eggs after 60 d at 5°C followed by incubation at 25°C varied significantly by geographical population ($F = 17.58$; df = 42, 1116; $P < 0.0001$). The highest relative percentage hatch of embryonated eggs after 60 d at 5°C (57.7–74.4%) was in the two Russian populations from the Far East (RM and RA), the UC and NJ populations originating from the United States, and a single population from China (CJ; Fig. 3). The following populations had low percentage hatch of embryonated eggs after 60 d at 5°C (0.0–12.3%): the populations from Poland, Greece, Austria, Slovakia, Switzerland, and Croatia; four of the seven German populations

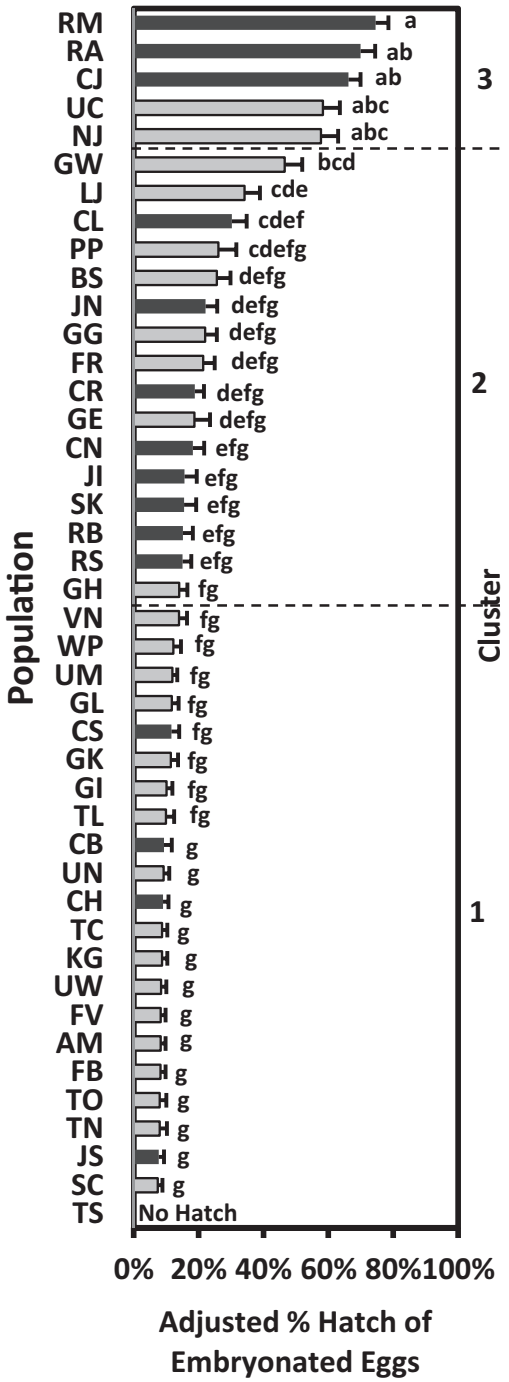

Fig. 3. Adjusted mean (± SE) percentage hatch of *L. dispar* embryonated eggs from various populations after 60 d chill at 5°C followed by incubation at 25°C, arranged by assigned cluster. Black bars are populations from Asia and grey bars are populations from Europe or North America. Bars followed by the same letter are not significantly different. Population abbreviations are given in Table 1. The percentage hatch after 60 d at 5°C was divided by the percentage hatch from the same egg mass after 150 d (optimal for maximum hatch) to adjust for the eggs that were not viable. Cluster assignment was based on PROC CLUSTER (SAS Institute 2004) analysis using population hatch traits.

(GK, GL, GI, and GH); two populations from France (FV and FB); half the populations from China (CH, CB, CS); three of the five populations from the United States (UM, UW, and UN); and the Japanese JS population. The remaining populations had

intermediate percentage hatch of embryonated eggs after 60 d at 5°C that ranged from 15.1 to 46.5%. The two populations collected from near Beijing China (CB 1992 and CJ 2012) had significantly different percentage hatch after 60 d. There was considerable variation in thermal requirements for egg hatch within each subspecies of *L. dispar* (e.g., Asian populations had between 8 and 74% hatch of embryonated eggs) and between egg masses from the same geographical population, as evidenced by the variation in the percentage of egg masses with hatch after 60 d at 5°C across the populations.

The mean time to first egg hatch varied significantly by population ($F = 20.19$; df = 41, 658; $P < 0.0001$) and was the longest, > 33 d, in three populations: one from Lithuania (LJ), one from Bulgaria (BS), and one from France (FB; Fig. 4). Mean days to first egg hatch was ≤ 14 d for seven populations, including all five populations that had the highest percentage hatch of embryonated eggs: three from China (CR, CN, and CJ), two from Russia (RM and RA), one from Europe (BS), and the laboratory standard strain originally collected in New Jersey, United States (NJ). The average time to egg hatch, after 60 d at 5°C, varied from 10.7 to 45.6 d among the populations (Suppl Table 2 [online only]). The eggs from five populations (one Russian [RM], three European [BS, KG, and PP], and the laboratory standard strain [NJ]) on average hatched faster than the eggs from one Russian (RS), five German (GK, GI, GH, GW, and GG), two United States (UC and UN), and three Chinese (CL, CH, and CS) populations, and the eggs of the other populations were intermediate in average time to hatch. There was considerable variation in time to egg hatch both within populations and *L. dispar* subspecies (e.g., mean days to first hatch in the Asian populations varied from 11.5 to 36.7 d).

The geographic populations segregated into three distinct clusters based on hatch traits after 60 d at 5°C followed by incubation at 25°C. The cluster each population was assigned to is given in Figs. 3 and 4 (also Suppl Table 2 [online only]) and Table 3 provides the mean values for each hatch trait for the clusters. The best classification tree using collection site climatic variables as predictors for partitioning the populations into the three identified clusters is given in Fig. 5. This tree misclassified 10 of the 42 geographic populations (cross-validation mean cost 0.41 ± 0.07). Six (three Chinese [CL, CN, and CR], two Japanese [JI and JS], and two Russian [RB and RS]) of the 10 errors were populations that belonged in cluster two being classified in cluster three; all of these populations have females that are capable of full flight.

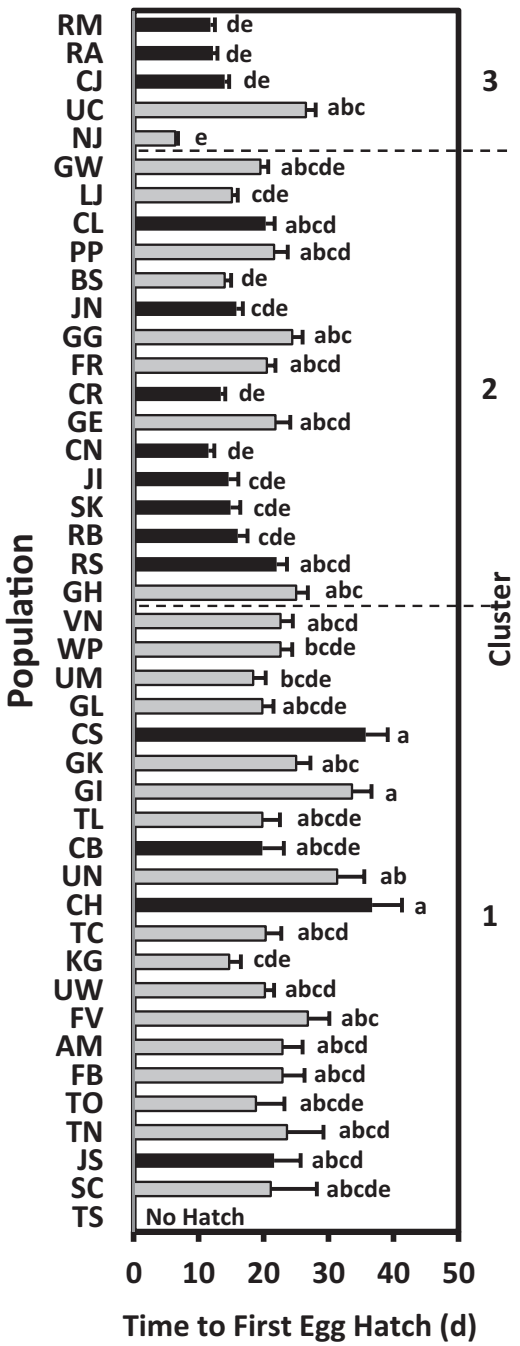

Fig. 4. Time to first hatch (± SE) of *L. dispar* eggs from various populations after 60 d chill at 5°C followed by incubation at 25°C, arranged by assigned cluster and percentage hatch of embryonated eggs (see Fig. 3). Black bars are populations from Asia and grey bars are populations from Europe or North America. Bars followed by the same letter are not significantly different. Population abbreviations are given in Table 1. Cluster assignment was based on PROC CLUSTER (SAS Institute 2004) analysis using population hatch traits.

Discussion

There was considerable variation both within and among the populations from different world areas in the proportion of embryonated eggs that were able to hatch and time to first hatch after eggs were held for 60 d at 5°C followed by incubation at 25°C. The populations with the highest percentage hatch originated from Russia (RM and RA), China (CJ), and the northeastern part of the United States (UC and NJ); all from between 40 and 44°N latitude. The NJ population is a population that has been in culture many generations and was inadvertently selected for this trait over time (Bell 1989). All the egg masses from the RM, RA, and NJ populations, and all but one of the UC and two of the CJ egg masses had at least some eggs that were capable of hatching after 60 d at 5°C. In addition, more than half of the egg masses from each of these populations had>30% of the eggs hatch. There were an additional six populations that had a quarter of the egg masses with>30% of the eggs hatch, two from China (CL and CN), and four from Europe (BS, GW, LJ, and PP). Thus, the phenotype that needs less exposure to low temperatures to satisfy diapause requirements, allowing eggs to complete post diapause and proceed to hatch more quickly when temperatures warm in *L. dispar* is not restricted to *asiatica* or *japonica* subspecies populations, and the phenotype is not fixed in most populations.

The inheritance of no requirement for chill in the eggs of the nondiapause population developed from precocious hatch of the

Table 3. Cluster means (±SD) for hatch traits (after exposure to 5°C for 60 d) used to segregate the geographic populations

Cluster	n^a	Adjusted[b] % hatch of embryonated eggs	Days to first hatch	Percentage of egg masses			
				No hatch	≥10% hatch	≥50% hatch	≥90% hatch
1	21	9.0 + 2.5	23.3 + 6.3	70.3 + 17.6	8.5 + 7.4	1.1 + 2.3	0.2 + 0.8
2	17	21.9 + 8.6	18.4 + 4.2	20.2 + 10.7	44.9 + 19.0	8.9 + 9.1	3.8 + 5.4
3	5	65.3 + 7.3	14.1 + 7.4	0.7 + 1.5	96.6 + 4.8	64.0 + 19.5	20.2 + 7.0
Statistics		$F = 168.1$	$F = 6.82$	$F = 83.3$	$F = 101.5$	$F = 109.9$	$F = 48.0$
		df = 2, 42	df = 2, 42	df = 2, 42	df = 2, 42	df = 2, 42	df = 2, 42
		$P < 0.0001$	$P = 0.0028$	$P < 0.0001$	$P < 0.0001$	$P < 0.0001$	$P < 0.0001$

[a] Number of populations in the cluster.

[b] The percentage hatch after 60 d at 5°C was divided by the percentage hatch from the same egg mass after 150 d (optimal for maximum hatch) to adjust for the eggs that were not viable.

PROC CLUSTER (SAS Institute 2004) using a complete linkage analysis was used to determine the clusters for the geographic populations. Means and standard deviations for the clusters were determined using PROC GLM.

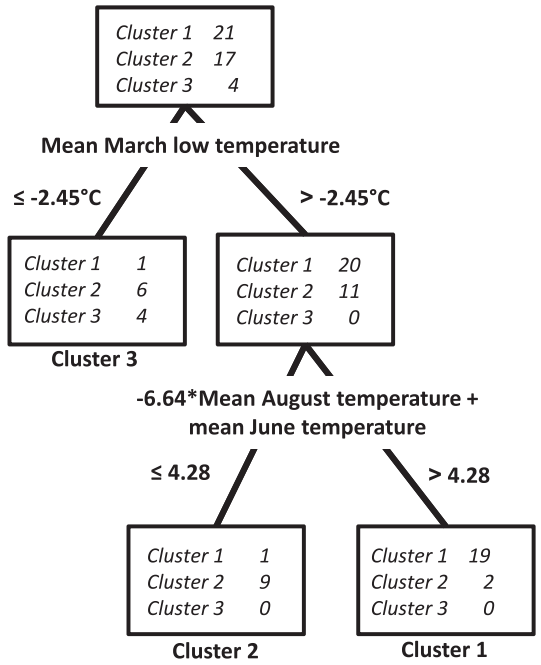

Fig. 5. Classification tree based on collection site climatic variables to predict which of the three clusters (segregated by hatch characteristics) a geographic population is most likely to belong. True classes of the geographic populations that were predicted by this tree are provided inside each box and the predicted class is given below the terminal nodes.

RM population was found to be inherited via a single recessive gene with no Z-linkage. This is in agreement with what Lynch and Hoy (1978) found for the inheritance of the selected Connecticut population when exposed to no chill but different from their results when 30 d of chill had been applied to the eggs. The apparent polygenetic inheritance after limited chill was also seen in a nondiapause population selected from Yugoslavia (Marović 1981). The reason that both these other nondiapause populations had this apparent environmentally specific phenotypic expression may have to do with the way the populations were selected. These other nondiapause populations required 8–12 generations of selection to achieve the >80% hatch without chill and the larvae retained, at least in the initial generations, hatched up to about 100 d after mating rather than at the time that diapause normally begins (diapause begins at 21 d at 25°C in NJ population, [Bell 1989]) (Hoy 1977; Marović and Vasic 1978). The NDRM population used only those that hatched within the first 40 d (likely the homozygous recessive individuals) and achieved >80% hatch in the second generation. Thus, the NDRM population apparently only selected for the one gene while the other two nondiapause populations selected for multiple genes, which is further evidenced by Z-linkage being absent with 0 d chill and confirmed when 30 d chill was applied to the Connecticut nondiapause population (Lynch and Hoy 1978).

The RM phenotype that needed less exposure to low temperatures to satisfy diapause requirements, allowing the egg to complete post diapause and proceed to hatch more quickly when temperatures warmed was found to be inherited via a single dominant gene with no evidence of Z-linkage or cytoplasmic effects. This single dominant gene appears to control both the satisfaction of diapause by 60 d chill at 5°C and the how fast the egg hatches after exposure to 25°C, although further study is needed to confirm this. Given the dominance of this phenotype it would tend to be maintained in a population unless there is strong selection against it and would be retained after hybridization with a population that does not express this phenotype. While the nondiapause phenotype, being recessive, is less likely to predominate in a population unless there is strong selection for it and would be at least initially not expressed after hybridization with a diapausing population. In addition, there is likely fairly strong selection against the nondiapause phenotype since individuals processing it would be less likely to hatch and find suitable foliage and environmental conditions to survive and complete development. However, work with the Connecticut nondiapause population found that although most of the eggs would hatch and not complete development under Connecticut conditions (they might under different conditions) that 9% of the eggs from this population overwintered and hatched in synchrony with budbreak the next spring (Hoy and Knop 1978).

Phenotypic variation can provide a bet–hedging mechanism for dealing with environmental unpredictability (Kaplan and Cooper 1984). Temporal differences between gypsy moth hatch and leaf-out phenology of larval hosts is predicted to severely limit larval survival in 5 out of 11 yr (Foster et al. 2013). Gypsy moth could have evolved a bet-hedging strategy for dealing with this unpredictability. In addition, there are substantial climatic differences across the geographical range of gypsy moth. These factors explain the high degree of both within and between population variability in egg hatch phenotypes that are observed. It also demonstrates why a dominant phenotype that has the ability to fulfill diapause requirements with a shorter exposure to low temperatures and proceed to complete post diapause and hatch quickly after temperatures warm would be advantageous

in environments with shorter growing seasons or rapid onset of spring conditions, since the individuals are ready to hatch as soon as the conditions become favorable. This emphasizes the risk from additional introductions of different phenotypes (both within and between countries) that could improve gypsy moth's ability to deal with environmental uncertainty from year to year in the timing of host leaf–out by adding additional variation in egg hatch traits. However, the ultimate result of introductions on the expressed phenotypes in a freely hybridizing population over time would depend on several additional factors, including initial ratio of the phenotypes, costs verses fitness of the phenotypes in the particular environment, and propensity of different phenotypes to mate. Environmental factors can also add to phenotypic variation, for example it has been shown that the parental larval host affects egg provisioning and better provisioned eggs hatch earlier (Rossiter et al. 1993).

The cluster analysis indicated that the geographic populations broke out into three distinct groups based on hatch traits after the eggs had been exposed to 60 d at 5°C then incubated at 25°C. One of the clusters was composed of populations which most of the egg masses exhibited the RM phenotype and another cluster contained populations that the majority of the egg masses had few if any eggs that were able to hatch after that limited number of days of chill. The final cluster was intermediate with a wide range percentage hatch of eggs among the egg masses. This may indicate that in parts of the range there is strong selection against the RM phenotype (reduced chill needed to meet egg diapause requirements) or that other genes may be involved, such as the polygenic genes described previously that confer lack of diapause in a Yugoslavian *L. dispar* population (Marović 1981).

A classification tree using climatic variables roughly predicted the three clusters of geographic populations. When average March low temperatures were ≤ -2.45°C the cluster in which the RM phenotype was exhibited by most eggs masses was predicted and the misidentified populations that also fit this criteria were all Asian populations (CL, CN, CR, JI, JS, RB, and RS), all but one from the intermediate cluster. Although it would seem that areas that have shorter winters might favor the RM phenotype, such as would be present in North Carolina where the UN population is from, that is not the case. Instead this analysis indicates that cooler spring temperatures, such as are found in Connecticut where the UC population is from, may favor maintenance of this phenotype in the population if present and that the phenotype has not spread to all of these areas yet. This also emphasizes the need to consider daily extremes and not just averages when assessing the responses of populations to temperature, which has been shown to be important in other Lepidoptera (Scriber and Sonke 2011). Additional work is need to understand and model the diapause and post diapause of the phenotype with reduced chill needed to meet egg diapause requirements is also needed to provide an accurate way of predicting the timing of hatch of this phenotype which is used to predict when specific stages appropriate for management treatments would be present. Finally, continued vigilance to prevent the movement of gypsy moth populations from one area to another is also warranted given the potential advantages of increased variation in hatch phenotypes present in a population could have for gypsy moths.

Supplementary Data

Supplementary data are available at *Environmental Entomology* online.

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