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# Embryonic death as a probable reason for the collapse of population densities in *Lymantria dispar* (Linnaeus, 1758) (Lepidoptera: Erebidae, Lymantriinae)

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**Abstract**

For the first time the causes of mass death of embryos in a population of *Lymantria dispar* (Linnaeus, 1758) with biochemical and molecular methods are investigated. In this study egg masses were collected in the territory of Russia from two populations, with low (the West Siberian population) and high (Trans-Ural) density and analyzed. Spring hatching of larvae from the eggs stored under constant laboratory conditions at 2º C was two times lower for insects of the Trans-Ural (TU) population compared to the West Siberian (WS) population (43 ± 6 % and 86 ± 7 % accordingly). The level of virus defined by PCR for TU and WS populations was 76±9 % and 36±6%, respectively. The major components of eggs were the same between populations. However, we found decreased concentrations of proteins important for embryogenesis in TU population, such as an arilforin-like molecule and no vitellin 2-like component. Field data were comparable to those of the laboratory, and in TU populations there was a decrease of density. The obtained results suggest that alteration in hormonal balance of the infected with nucleopolyhedrovirus (NPV) insects may lead to a decrease or absence of some key proteins of embryogenesis in eggs. It can influence hatching of larvae from eggs and, accordingly, population dynamics of the lepidopteran.
KEY WORDS: Lepidoptera, Erebidae, Lymantriinae, *Lymantria dispar*, population dynamics, embryonic death, nucleopolyhedrovirus.

**Muerte embrionaria como probable razón para el colapso de la densidad de población en *Lymantria dispar* (Linnaeus, 1758) (Lepidoptera: Erebidae, Lymantriinae)**

**Resumen**

Se investiga por primera vez con métodos bioquímicos y moleculares, las causas de la muerte masiva de embriones en una población de *Lymantria dispar* (Linnaeus1758). En este estudio se recogieron y analizaron masas de huevos de dos poblaciones en el territorio de Rusia: con baja (la población siberiana occidental) y alta densidad (Trans-Ural), respectivamente. La eclosión en primavera de las larvas de los huevos, guardados bajo condiciones contantes del laboratorio a 2º C, fue dos veces menor (la mitad) en las poblaciones de insectos de la Trans-Ural (TU) que en la del oeste siberiano (WS) (43 ± 6 % y 86 ± 7 % respectivamente). El nivel de virus definido por PCR para las poblaciones de TU y WS era de 76 ± 9 % y 36 ± 6 %, respectivamente. La mayoría de los componentes importantes de los huevos eran prácticamente los mismos entre las poblaciones. Sin embargo, encontramos una presencia reducida de proteínas importantes para la embriogénesis en la población de TU, como por ejemplo,una molécula parecida al pro-arilforin y una falta del componente pro-vitelín 2. Los datos de campo fueron comparables a éstos del laboratorio, y en las poblaciones de TU, había un decrecimiento de la densidad.

Los resultados obtenidos indican que la alteración en el balance hormonal de los insectos infectados con nucleopolihedrovirus (NPV) podría llevar a un disminucion o falta de algunas proteínas de la embriogénesis en huevos. Puede influir en el desarrollo de las larvas desde los huevos y, en consecuencia, en la dinámica de población del lepidóptero.
PALABRAS CLAVE: Lepidoptera, Erebidae, Lymantriinae, *Lymantria dispar*, dinámica de poblaciones, muerte embrionaria, nucleopolihedrovirus.

## Introduction

The gypsy moth is one of the most biologically and economically significant defoliator that periodically forms outbreaks in the territories of Eurasia, North America and North Africa (GIESE & SCHNEIDER, 1979; JOHNSON *et al*., 2005). Population dynamics of this insect can influence both abiotic and biotic factors, causing death of insects at various stages, including an egg phase. Insect eggs represent a self-sustaining system which provides the raw materials for building the larval body and the energy reserves for embryogenesis (SANDER *et al*., 1985). The development of the embryo is dependent upon the appropriate physiological and environmental conditions. The most important environmental condition for development of the embryo is favorable temperature and humidity (HAMILTON, 1950).

There are several studies of mass embryonic mortality of gypsy moth in natural populations (KONDAKOV, 1963; ILYINSKY & TROPIN, 1965; KOLTUNOV *et al*., 1998). This research demonstrates which abiotic or biotic factors cause embryonic death. Moreover, there are several observations about the unknown etiology of embryonic death. EGOROV (1958) demonstrated that in 1953 in the Altay territory there were up to 15 egg masses per tree. However, in the spring of 1954 a large portion of the embryos in the eggs were dead. That has led to the collapse of population density. Research of KONDAKOV (1963) in the Krasnoyarsk region in 1954 and 1955 has revealed a mass death of eggs without clear etiology. Laboratory cultivation of gypsy moth larvae from the Trans-Ural population in 1991, where the eggs were kept under 0º C and 60-70 % humidity, hatched in May at a rate of 5-10 % (KOLTUNOV *et al*., 1998). The same hatching rate authors observed in nature. Moreover, there is a study that in the Novosibirsk region decrease of gypsy moth density in 1997-1998 basically has been connected with mass death of embryos for unstated reasons (ILYINYKH, 2002).

It was shown that nucleopolyhedrovirus (NPV) could be one of the main factors of mortality in gypsy moth populations (ELKINTON, 1990; DWYER & ELKINTON, 1995; HOCH *et al*., 2001). Probably, NPV infections can be a factor resulting in the mortality of embryos (straight or indirectly). However, there are cases when the death rate from NPV at an embryo stage has still not been identified in our practice and in the literature. Moreover, nutrition content of the eggs could be one of the main reasons for successful development of embryos. It is known that lipid and carbohydrate reserves decrease as embryogenesis progresses (QUICKENDEN, 1970). Yolk proteins also take part in both energy storage and embryogenesis and they are critically important for development of the embryo (IZUMI *et al*., 1994).

Thus, in the known literature there are at least four studies about high mortality of embryos without clear etiology (EGOROV, 1958; KONDAKOV, 1963; KOLTUNOV *et al*., 1998; ILYINYKH, 2002). Therefore, in the present work the causes of this phenomenon on two populations of gypsy moth with low (WS population) and high (TU population) egg mortality are investigated using both biochemical and molecular methods.

## Material and Methods

### (a) Experimental design

The field and laboratory experiments were conducted during an outbreak of the gypsy moth in birch forests (*Betula pendula* Roth.) of Sverdlovsk (Trans-Ural population, Kamensk district) and

Novosibirsk (West Siberian population, Karasuksky district) regions of Russia in 2011-2012 (see figure 1). The investigations were performed at two stages of the outbreak: density increase in the West Siberian population (WS) and decrease in the Trans-Ural population (TU) (data from the Novosibirsk and Sverdlovsk Centers of Forest Protection).

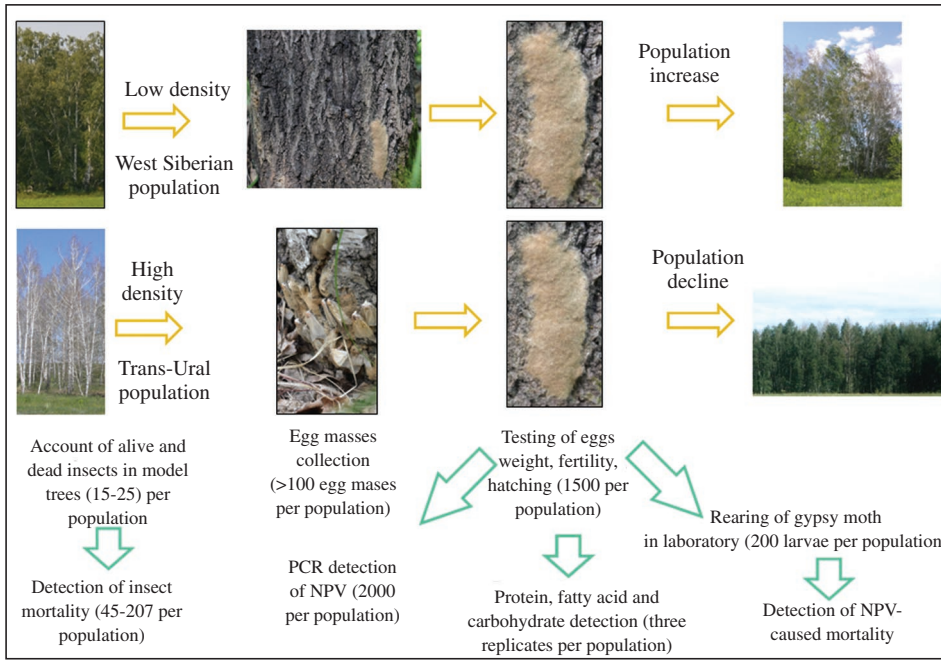


**Figure 1.–** Experimental design scheme (2011-2012).

For detection NPV-caused mortality, the number of studied plots per population varied from five to seven, and each 100 $m^2$ plot contained three to five model trees. The number of alive and dead insects was established at the larva and pupa stages by cutting branches from model trees under study, as described previously (ILYINYKH *et al*., 2004). To count the number of dead insects and to detect NPV in laboratory, three branches were cut per tree (one from low down, one from the middle and one from top of the crown). Cut branches fell on a parachute spread under the crown. The numbers of dead insects were counted on each branch and then this result was multiplied by the total number of branches in a tree (ILYINYKH *et al*., 2004). The cause of insect death (45-207 individuals per population) was determined by light microscopy (see next paragraph).

In 2011, more than 100 egg masses were collected in the third decade of September in both populations in studied plots. Eggs were stored at a temperature of 2º C prior to testing of hatching, fertility, mortality, weight, and virus with PCR. In January 2012 part of the eggs was stored at or below -20º C to test protein, fat, and carbohydrate content. Eggs from both populations were tested on fertility and weight (presented as mass per 100 eggs). Hatching and number of egg masses in field conditions for both populations were provided by the Centers of Forest Protection in 2011-2012. Moreover, the above- mentioned organizations provided data about the dynamics of the total areas of major defoliation (above 60 %) of birch forests caused by the gypsy moth for both populations in 2003-2014.

(b) Detection of nucleopolyhedrovirus

For detection of mortality from entomopathogens, 200 larvae from both populations were cultivated in May until reaching imago under conditions preventing exogenous virus on the artificial medium (AM) using the technique described by ILYINYKH (1997). Dead insects were examined on infection with light microscope (Biolam - R15; LOMO, Russia) using phase contrast.

Twenty cleared eggs from each of the studied egg masses were selected for PCR detection of NPV. Eggs were sterilized within 10 min in 0.25 % NaOH solution on a magnetic mixer, washed with sterile water and dried. Embryos were collected from eggs under sterile conditions (20 individuals per sample). Samples were stored at - 70º C until DNA was extracted.

Total DNA from samples of insects was extracted using the DNA Extraction kit ("MEDIGEN Laboratory", Russia) according to the protocol of the manufacturer. Detection of viral conjugation protein gene Ld130 was carried out in 20 l of buffer contained 10 l of PyroStartTM Fast PCR Master Mix (2X) ("Fermentas", USA); 0.1 l of forward (5' CGGGCATCATCCGCGGCC 3' (127651 - 127668)) and reverse (5 'CGCCCTCCAGCTCCGCGC 3' (127944 - 127927)) primers and 27.5 % of DNA on volume. Specific primers were designed using the full-genomic sequence (GenBank database at number NC_001973) of virus of gypsy moth. PCR carried out on a thermalcycler "DNA Engine Dyad® Peltier Thelmar Cycler" ("BIO-RAD", USA) using the following protocol (denaturation 30s with 94º C, annealing 30s with 68º C, synthesis 30 with 72º C (37 cycles)); synthesis of 7 min. - 72º C. The size of the detected gene fragment was 294 bp.

(c) Analysis

For protein, fatty acid and carbohydrate detection, 100 mg per sample of eggs from both populations were collected and homogenized using the FastPrep MP biomedicals (ICN) homogenizer and freeze dried. For testing of carbohydrate and protein concentrations frozen-dried samples were dissolved in distilled water. For determination of fatty acid concentration, the freeze-dried samples were dissolved in concentrated sulfuric acid.

Detection of fatty acid, carbohydrate and protein concentrations were carried out in triplicate using a spectrophotometer. Fat content was checked according to CHABROL & CHARONNAT (1937) with some modifications. The freeze-dried sample was incubated with 1 ml of sulfuric acid for 20 min at 100º C. The mix was cooled for 5 min and added to 500 l of vanillin (Sigma) and then dissolved in distilled water (13 mM). Optical density of the solution registered at 530 nm in 30 min. Concentration of fatty acids was determined with a calibration curve, using vegetable fatty acid (olive oil, 99 %) standards.

Detection of carbohydrates was carried out according to HANSEN & MOLLER (1975) with some modifications. We mixed 100 l of sample with 500 l of 0.5 % Antron (Sigma) solution in 72 % sulfuric acid and incubated for 11 min at 100º C. Then the mixture was quickly cooled to 0º C on ice. Optical density was detected at 630 nm in 60 min at 22º C. Concentration of carbohydrates were determined with a calibration curve, using starch (99 %, Sigma) standards. The protein concentration of samples was estimated by using the Bradford method (BRADFORD, 1976), using bovine serum albumin standards. Qualitative composition of proteins was detected with capillary electrophoresis (protein kit) (Agilent Bioanalazer).

Data are presented as mean ± the standard error. To check the data for normal distribution, the Wilk Shapiro W criterion was used. All results were assessed using one-way ANOVA, followed by Tukey's post-hoc tests to identify specific differences between means.

## Results

(a) Weight, fertility and mortality of eggs

We found that weight and fertility of eggs from both the WS (population increase) and TU

(population decline) populations did not differ (table 1). Moreover, the hatching of larvae in January was the same in both populations. However, in May (post-diapause) the hatching of larvae from eggs in the WS population was higher compared with the TU population (86±7 % and 43±6 % respectively, $p<0.01$) (table 1).

The same data was found in natural conditions during testing in the forest. Spring hatching in the TU population was about 30 % while in the WS population the hatching was about 85 %. The majority of larvae (approximately 90 %) that hatched from eggs in the TU population died of unclear reason at first and second instar. The study from the forest showed that the area around the TU population had low egg mass density: 21 ± 10 egg masses/ha (previous year 3650 ± 457 egg masses/ha). While the density of the egg masses in the WS population territory increased up 1240 ± 265 egg masses/ha (previous year 64 ± 23 egg masses/ha). Moreover, the total area of major defoliation (above 60 %) in the region increased from 28 hectares to 2500 while in the TU population this index decreased from 39945 hectares to 3. The data for both populations in 2003-2014 are shown on figure 2. These data demonstrate that the TU gypsy moth population (Sverdlovsk region) decreased, whilst the WS gypsy moth population (Novosibirsk region) increased.

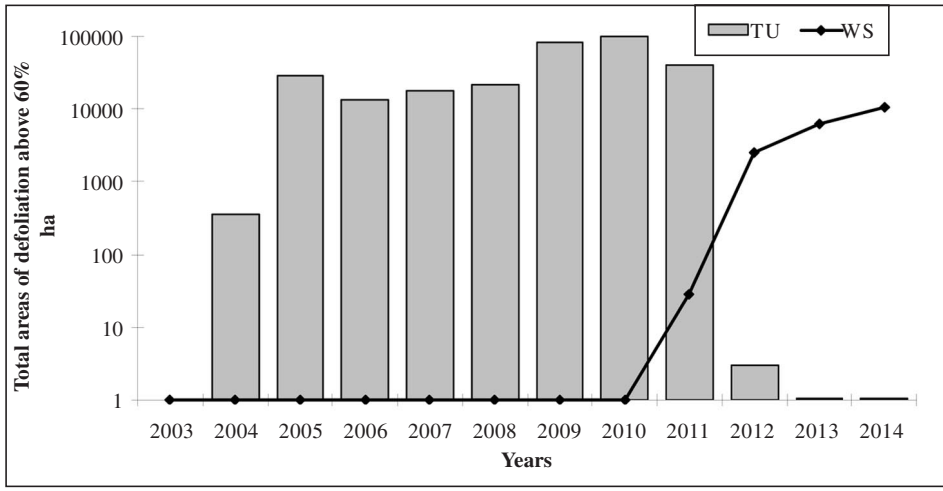


**Figure 2.–** The total areas of major defoliation (above 60 %) in the TU and WS populations in 2003-2014.

(b) NUTRITION CONTENT OF EGGS

The concentrations of proteins, lipids and carbohydrates in eggs were similar between both the TU and WS populations (figure 3). Concentration of lipids in eggs was on average 104 ± 13 mg/g, proteins averaged 16 ± 0.65 mg/g, and 1.93 ± 0.14 mg/g was the average concentration of carbohydrates. However, a qualitative protein assay showed that in eggs from the TU population, concentration of an arylphorin-like molecule (~ 75kDa) was 1.5 times lower ($p<0.001$) than in eggs from the WS population. Additionally, in eggs from the TU population, the vitellin 2-like component (~ 45kDa) was absent.

(c) INFECTIONS OF INSECTS

We did not find any infections, including NPV, in the embryos and hatched larvae with the light microscope. However, at cultivation on the artificial media, mortality of the TU population larvae from

NPV was 5±1% while larvae from the WS population did not die from virus ($p< 0.01$). The mortality of the larvae due to unstated reasons for the TU and WS populations was 92 ± 8 % and 12 ± 3 % ($p< 0.01$) respectively. The highest mortality (82 ± 7 %) for the TU population was observed in first instar larvae.

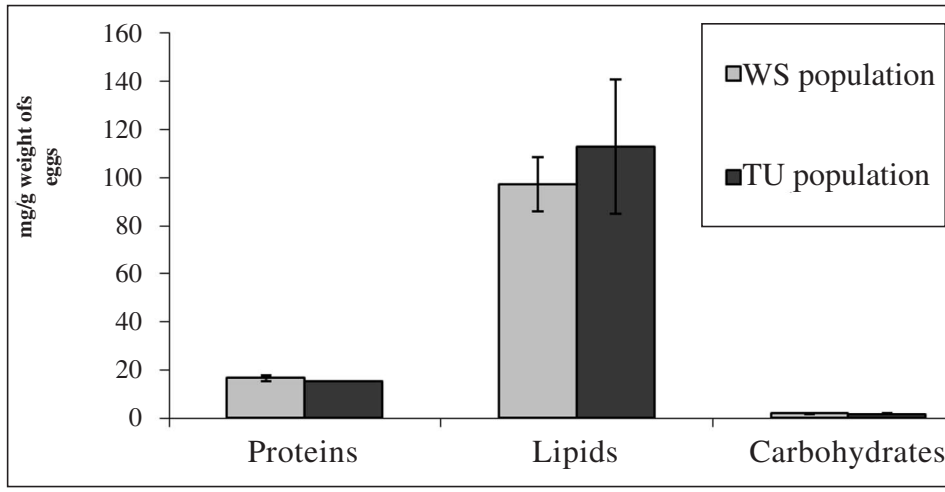


**Figure 3.–** Quantitative assay of nutrition in eggs from Trans-Ural (TU) and West Siberian (WS) populations of gypsy moth (January 2012).

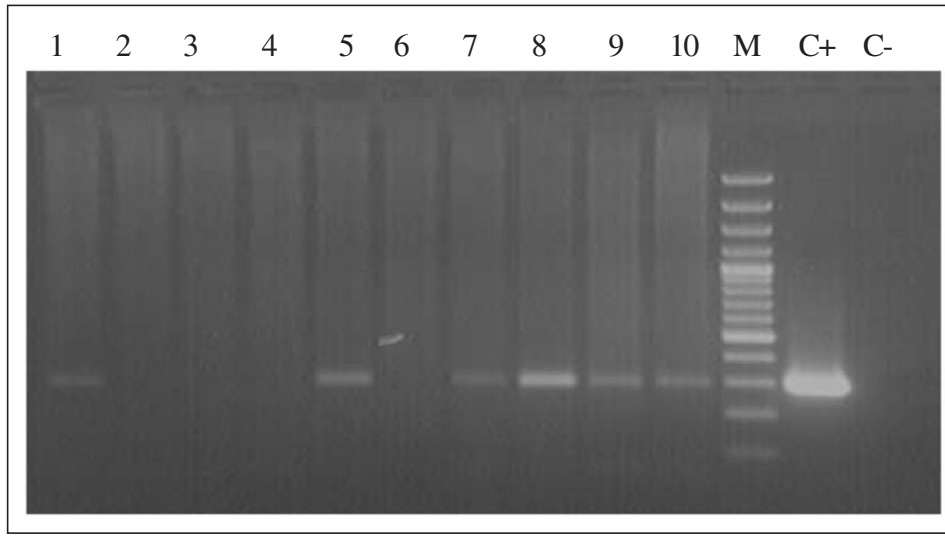


**Figure 4.–** PCR detection of virus LD 130 gene in embryos of the Trans-Ural gypsy moth population (2011). 1, 5, 7-10-positive at virus; 2-4, 6-negative at virus; M-ladder O'GeneRuler 100bp Plus DNA Ladder (FERMENTAS, USA); C+ - positive control, Ñ- - negative control.

In nature, the NPV carrying level (detected by PCR) among insects (embryos) from the TU population in 2011 was 76 ± 9 %, but mortality at the larva and pupa stages from NPV was 12 ± 5 %. The larvae from the WS population did not die from NPV in 2011; however, virus carrying quantity among embryos was 36 ± 6 % in the autumn. PCR detection of viral gene (LD 130) in embryos from the TU population showed 6 positive cases from 10 samples (figure 4). Data on the WS population aren't provided.

## Discussion

The TU population of gypsy moth was found to have increased mortality of embryos. This is probably the main factor of decline of population density in the area of the TU population. Several possible biotic reasons for embryonic death were analyzed in the study. Nutrition in eggs, an important factor of embryonic feeding, was similar for both the TU and WS populations. However, some key proteins of embryogenesis (arylphorin-like molecule and the vitellin 2-like component) were decreased or absent in eggs from the TU population. It is possible that the lowered survival of eggs from the TU population can be connected with the shortage of these substances.

It was demonstrated that NPV could be one of the main factors of mortality in population dynamics of the gypsy moth (DWYER & ELKINTON, 1995; HOCH *et al*., 2001; FULLER *et al*., 2012). At the same time, it is shown that the impact of this factor in various parts of gypsy moth areas can be different. In particular it is well known that in populations in the territory of Trans Ural and Western Siberia, NPVwas detected locally but at an insignificant level (about 10%) of larvae (ILYINYKH *et al*., 2004).

Baculoviruses infect over 600 species of insects (ROHRMANN, 2008), and in some cases, they were successfully used to control different insect pests (reviewed by INCEOGLU *et al*., 2006). Although horizontal route is thought to be the major pathway for baculovirus transmission (CORY & MYERS, 2003), some studies also reveal vertical transfer in field populations (reviewed by KUKAN, 1999; ZHOU *et al*., 2005; KOUASSI *et al*., 2009). Moreover, the vertical transmission of gypsy moth NPV was described earlier in our own investigations (ILYINYKH *et al*., 2004; ILYINYKH & POLENOGOVA, 2013). Individuals exposed to low doses of virus may acquire a non-fatal sublethal infection, but transmit the virus vertically to the next generation of insects (BURDEN *et al*., 2002; CABODEVILLA *et al*., 2011; MURILLLO *et al*., 2011). This may affect insect health, weight and fecundity (MYERS *et al*., 2000; VILAPLANA *et al*., 2008). Probably, NPV could be one of the reasons for embryo mortality in the TU population. The NPV is capable of affecting hormonal balance of the infected insects. In some baculoviruses the *egt*-gene was found, which is capable of coding the UDP-glucosyltransferase catalyzing binding of sugars by ekdisteroides (O'REILLY & MILLER, 1989; SLAVICEK *et al*., 1999). In particular infection of gypsy moth by NPV containing the *egt*-gene, led to abnormality of molting and growth of insects. Virus with a deletion of the *egt*-gene did not change the growth of insects (SLAVICEK *et al*., 1999).

Probably, alteration in hormonal balance of the infected insects (O'REILLY & MILLER, 1989; SLAVICEK *et al*., 1999) may lead to a decrease or absence of some key proteins of embryogenesis (arylphorin-like molecule and the vitellin 2-like component) in eggs from the TU population.

Perhaps, insects infected with NPV can demonstrate delay of development and lay eggs later in comparison with non-infected individuals or insects with smaller quantity of virus carriers. It can lead to a decrease in the sum of the effective temperatures necessary for normal embryogenesis of insects (especially in the conditions of a continental climate) and/or can change embryonic diapauses. It can influence hatching of larvae from eggs and, accordingly, population dynamics of the gypsy moth.

Possibly, in further research, the biochemical methods can be employed to diagnostics of a phase of depression in population dynamics of the gypsy moth.

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