Sperm Transfer in Gypsy Moths: Effect of Constant or Cyclic Temperature and Constant Light or Darkness during the Pupal Stage[1]

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ABSTRACT Sperm transfer and fertility of the gypsy moth, *Lymantria dispar* (L.), held as pupae under constant temperature and 24-h light, 24-h darkness, or a photoperiod of 16:8 (L:D) h were compared with males held in cyclic temperature and 24-h light, 24-h darkness, or a photoperiod of 16:8 (L:D) h. Constant temperature and constant light or darkness severely reduced its ability to transfer sperm. Males held under constant light were more severely affected than males held in darkness, but sperm transfer was not correlated with light intensity (between 80 and 3960 lux). Nearly all males held under constant light and most held under constant darkness were sterile or partially sterile. In either case, sterility was caused by poor sperm transfer. Females with a full complement of sperm were fertile regardless of holding condition of male pupae. The sterilizing effect of constant light or darkness was overridden by cyclic temperature of 28°C for 16 h and 22°C for 8 h. At a photoperiod of 16:8 h, pupae held in constant 28°C transferred less sperm than those held at 25°C. Pupae held at constant 30°C were sterile, but those held at 30°C for 16 h and 25°C for 8 h transferred similar quantities of sperm as males held at constant 28°C.

KEY WORDS *Lymantria dispar*, biorhythm, insect rearing, sterility

Holding the gypsy moth, *Lymantria dispar* (L.), under continuous light during the pupal stage disrupts movement of eupyrene sperm in adult males resulting in sterility (Giebultowicz et al. 1990). Recently, Proshold and Bernon (1994) observed that males held under constant light in an incubator with 40-w lamps were sterile, but some from an incubator with 20-w lamps transferred a full complement of fertile sperm. They hypothesized that light intensity may be an important factor in the induction of sterility. The objective of this study was to

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determine the importance of light intensity in causing sterility of males held under continuous light as pupae. Sperm transfer of males held as pupae in incubators with constant or cyclic temperature in conjunction with constant light or darkness was also studied.

Materials and Methods

Insects. Gypsy moth pupae were from a colony established in 1967 from eggs collected near Blairstown, NJ (ODell et al. 1984). The colony has been maintained for >40 generations by the U. S. Department of Agriculture, Animal and Plant Health Inspection Service, Otis Methods Development Center, Cape Cod, MA. Larvae were reared in 180-ml containers on 75 to 95 ml of wheat germ diet by the procedures of Bell et al. (1981) with modifications (Bernon[3]). Except for pupae held in various treatments, all stages were held in a rearing room at ≈25°C, 50 to 70% RH, and a photoperiod of 16:8 (L:D) h. Test insects were sexed as pharate pupae and placed in 280-ml containers with clear plastic lids. Males were transferred to experimental incubators while females were returned to the rearing room. The research developed through two phases in which male pupae were held under various light and temperature regimes.

Phase 1 – Light intensity and sperm transfer. Two tests were conducted. First, pupae were held in one of three incubators with 24-h light provided by 15, 20, or 40-w fluorescent lamps. The 15-w incubator (internal dimensions of 123 × 68 × 46 cm) was manufactured by Hotpack (model 352700, Philadelphia, PA). Light was provided by one lamp (model GE F15T8) located in top of the incubator causing a gradient of illumination from top to bottom. Thus, the incubator was divided into upper and lower levels.

The 20-w incubator (76 × 68 × 43 cm) was from Percival Manufacturing Co. (model 1-30 BLL, Boone, IA). Illumination was provided by 2 lamps, model GE F20T12.

The 40-w incubator was manufactured by Puffer-Hubbard Refrigeration (model PI28, Grand Haven, MI). The internal dimensions were tapered front to back from 147 to 127 cm and were 76 cm wide by 71 cm deep. One horseshoe-shaped lamp, model Phillips/GE FB40CW/6, provided illumination. All lamps were cool white.

Temperature in each incubator was set at 20°C. From 10 to 15 male, pharate pupae were placed in 280-ml containers with clear plastic lids. Two containers were placed in each incubator. For the 15-w incubator, one container was placed on the upper level and one on the lower level. Temperature of the upper level was slightly cooler than that of the lower level (19.9 ± 0.06°C, n = 76, and 22.5 ± 0.11°C, n = 51 [mean ± SEM], respectively). Temperature in the 20-w incubator was 20.0 ± 0.03°C, n = 98 and in the 40-w incubator was 19.8 ± 0.03°C, n = 81. Males remained in the 24-h incubators until 24 to 48 h after eclosion at which time they were caged with females < 48 h old. The insects were caged in single pairs in paper cartons (90 mm diam × 85 mm height) lined with brown paper for oviposition. The top of a clear-plastic Petri dish served as a lid. The cages were placed in the rearing room and observed at ≈20 min intervals between ≈1000 and 1500 h. If

[3] Standard Rearing Procedure for the Gypsy Moth. A manual available from G. L. Bernon. USDA-APHIS, Methods Development Center, Building 1398, Otis ANGB, MA 02542.

mating was observed, males were discarded after separation; if not, they were discarded the following morning. Females were dissected 5 to 7 d later, after they laid eggs. This procedure was repeated until > 75 pairs were set up with males from each incubator. Then the experiment was repeated with temperature of 25°C.

Males from the rearing room were studied as a control. These males were separated into two groups, one placed on a shelf ≈40 cm below the ceiling (upper level, temperature averaged 24.8 ± 0.07°C, n = 67) and the other ≈1.8 m below the ceiling (lower level, temperature averaged 24.4 ± 0.04°C, n = 48). Illumination was provided by 34-w, cool white, fluorescent lamps in ceiling fixtures.

In a second test, 280-ml containers with pharate pupae (7 to 9 cups per incubator, ≈17 males per container) were placed in the 24-h light incubators described previously. The containers were placed so that they would be exposed to a range of illumination. Light intensity (lux) was measured daily with a light meter (Extech Instruments, Waltham, MA) placed on top of each container. All containers were kept in the same location until eclosion. Then, males were paired with females as before. The following day, females were dissected. Of those that mated, the percentage with some eupyrene sperm was correlated with mean daily lux.

Phase 2 – Sperm transfer as affected by cyclic or constant light and temperature. First, a test was conducted to determine the lower limit for heat-induced sterility of pupae. Pharate pupae were placed in incubators with constant temperature of 25, 28, or 30°C and photoperiod of 16:18 (L:D) h. Sperm transfer by these males was compared with transfer in males held at 30°C during the light cycle and 25°C during the dark cycle. Heat units per 24 h would approximate that of constant temperature of 28°C.

Second, sperm transfer of male pupae held under 24-h light or darkness were compared with transfer in males from the rearing room. The 24-h-light males were held in one of the three incubators described previously. Pupae held in constant darkness were placed in 280-ml containers as pharate pupae. Then, each container was enclosed within a 4-L paper carton within two brown paper grocery bags. These bags were stapled closed and place in the rearing room or 24-h light incubator next to the clear-top containers with pupae exposed to light.

Third, a test was conducted to compare sperm transfer of males exposed to constant temperature (25°C) and 24-h light, 24-h darkness, or a photoperiod of 16:8 (L:D) h versus cyclic temperature and 24-h light, 24-h darkness, or a photoperiod of 16:8 (L:D) h. For cyclic temperature, males were held at 28°C for 16 h and at 22°C for 8 h. The total heat units per 24 h would approximate that of constant 25°C. Pupae held in darkness were placed at 4-L cartons inside brown paper bags as previously described. Test insects were obtained from the standard colony and assigned to the different conditions at random.

Light intensity determination. For each test, light intensity (lux) within each incubator was measured daily (Extech Instruments) ≥5X weekly during the pupal stage. As lux are based on the spectral sensitivity of human eyes and may not be suitable for insects (Young et al. 1987), illumination (micro-Einsteins per second per meter square ($\mu E\ s^{-1}\ m^{-2}$]) was measured with a 2 π Li-Cor quantum sensor (Li-Cor, Lincoln, NE) as a comparison. Ten to 20 readings were taken at each location over an 8-h period.

Sperm transfer and fertility assessment. The spermatheca was removed and prepared in a drop of Belar's saline on a microscope slide and viewed at 100X. The quantity of eupyrene sperm (fertilizing sperm) was scored as follows: (1) none, (2) low = spermatozoa scarce, (3) moderate = spermatozoa plentiful but could be viewed within one microscope field, and (4) high = spermatozoa numerous and the mass extended beyond a single microscope field. If eupyrene sperm were not present, the preparation was checked for apyrene (anucleate, nonfertile) sperm. Spermatheacae containing moderate or high levels of eupyrene sperm were considered to have received a full complement.

When females were removed for dissection, their egg masses were weighed and held at ≈25°C, 50 to 70% RH, and a photoperiod of 16:18 (L:D) h. After 35 d, subsamples from each egg mass (≈100 eggs) were dehaired by the method of Tardif and Secrest (1970), and the number of embryonated and nonembryonated eggs were counted.

Data analysis. Frequency data on sperm transfer were tested for homogeneity with a log likelihood ratio test (Sokal and Rohlf 1969). Data were pooled so that cell frequencies were ≥5. If cell frequencies could not be so combined, data were analyzed by Fisher's Exact Test (PROC, FREQ, SAS Institute 1989). Pearson's correlation coefficients were obtained for mean lux ($\log_e$ transformation) and percentage (arcsine [$\sqrt{\%/100}$]) of females with some eupyrene sperm (PROC CORR, SAS Institute 1989). Egg mass weight and percentage egg embryonation (arcsine [$\sqrt{\%/100}$]) were compared by an analysis of variance (PROC GLM, SAS Institute 1989).

Results and Discussion

Light intensity and sperm transfer. Of all locations, the 20-w incubator had the highest light intensity, followed by the 40-w incubator (Table 1). The lowest light intensity was in the lower level of the 15-w incubator. Light intensity of the rearing room was intermediate between the intensity of the lower and upper levels of the 15-w incubator. Pupae located on the upper level in the rearing room received 2 to 3X more light than those on the lower level. The same trend was observed for all locations whether measured in lux or µE.

Sperm transfer and fertility of males from the rearing room were similar regardless of whether they were held on the lower or upper levels as pupae. Of females caged with these males, 80 to 87% received a full complement of eupyrene sperm and for those, 98% of their eggs became embryonated (Table 2). The number of females with no sperm, or with low, moderate, or high quantities of eupyrene sperm were not significantly different whether males were from the lower or upper shelf $P = 0.31$, Fisher's Exact Test, $n = 78$).

Conversely, sperm transfer and fertility of males in constant 24-h light varied considerably among incubators. Nonetheless, > 93% of the males held under 24-h light as pupae mated regardless of light intensity. Sperm transfer was reduced in males from all 24-h light regimes but displayed considerable variation among incubators. Percentage of males that transferred a full complement of sperm varied from 2 to 56%. Of females with a full complement of sperm, nearly all were classified as having a moderate rather than a high quantity of eupyrene sperm. A three-way comparison of sperm transfer of males from the 15-w incubator (upper or lower

Table 1. Light intensity in lux (lumens m^{-2}) or micro-Einsteins per second per meter square ($\mu E\ s^{-1}\ m^{-2}$) in rearing chambers used to hold males of the gypsy moth during the pupal stage.

		Lumens m^{-2}				$\mu E\ s^{-1}\ m^{-2}$			
Incubator	Level	No.	mean	min	max	No.	mean	min	max
15-w	Lower	158	97	21	180	20	1.6	1.5	1.7
15-w	Upper	157	1339	270	3000	18	16.7	10.8	28.5
20-w		259	2396	370	3640	10	40.1	31.5	49.2
40-w		207	1960	920	2990	18	27.8	19.2	43.5
Rearing rm	Lower	108	217	35	354	20	4.1	3.7	4.6
Rearing rm	Upper	104	761	661	868	20	8.4	6.7	9.9

Table 2. Sperm transfer and percentage of eggs that were embryonated for gypsy moths with a full complement of eupyrene sperm (abundant) when females were paired with males held as pupae in incubators illuminated continuously with 15, 20, or 40-w lamps and provided a constant temperature of 20 or 25°C compared with pupae held at a photoperiod of 16:8 (L:D) h (rearing room).

Incubator	Level	Temperature (°C)	No.	% mating	♀♀ with sperm (%)	♀♀ with eupyrene sperm• (%)	♀♀ with eupyrene sperm• % embryonated eggd (mean ± SEM)
15-w	Lower	20	40	100	98	28	89 ± 5.1
15-w	Upper	20	40	95	92	12	98 ± 0.7
15-w	Lower	25	46	100	91	56	92 ± 3.4
15-w	Upper	25	75	95	55	3	46 ± 12.6
20-w		20	98	98	81	7	94 ± 1.6
20-w		25	92	100	92	22	91 ± 3.3
40-w		20	78	97	95	12	98 ± 1.3
40-w		25	104	93	53	2	71 ± 21.7
Rearing rm	Lower	25	41	98	93	80	98 ± 0.5
Rearing rm	Upper	25	39	97	92	87	98 ± 0.2

*These data are for ♀ ♀ with abundant eupyrene sperm only.

level by temperature by sperm class) was not consistent among temperature as indicated by a significant interaction ($G = 11.29$, df = 1, $P < 0.001$). Further, sperm transfer by males from the three incubators was not consistent between the two temperature regimes. Excluding males from the lower level of the 15-w incubator, a three-way log likelihood ratio test (incubator by temperature by sperm classification) gave a highly significant interaction ($G = 25.86$; df = 2; $P < 0.001$).

At 20°C, there was no significant difference in sperm classification of females mated to males from the upper or lower levels of the 15-w incubator ($P = 0.26$, Fisher's Exact Test, $n = 78$). Nor was there any significant difference in sperm classification of females mated to males in the 20 or 40-w incubator ($G = 3.76$, df = 2; $P > 0.1$. However, sperm transfer was significantly different among males from the three incubators ($G = 10.23$; df = 4; $P < 0.05$, log likelihood ratio test). From 12 to 28% of the males from the 15-w incubator transferred a full complement of sperm, or about twice the percentage of males transferring sperm from the other incubators. These data were consistent with the hypothesis that the sterilizing effect of 24-h light on pupae increases with light intensity.

At 25°C, few males from the 40-w incubator or upper level of the 15-w incubator transferred sperm. In contrast, more than half of the males from the lower level of the 15-w incubator transferred a full complement of sperm. Sperm transfer of males from the 20-w incubator was intermediate to that of males from the lower level of the 15-w incubator and those from the 40-w incubator. Light intensity of the 20-w incubator was greater than intensity in the 40-w incubator. Consequently, these data were not consistent with the above hypothesis.

Finally, there was no correlation between sperm transfer (range with some eupyrene sperm, 0 to 100%) and mean illumination during the pupal stage (80 ± 2.5 to 3960 ± 47.4 lux [mean ± SEM]). Correlation coefficients were not significant whether data were combined over all three incubators ($r = 0.32$, n = 24, $P = 0.13$) or whether data were analyzed separately for each incubator (r = -0.46 to 0.41, n = 7 to 9, $P \geq 0.25$). Thus, the stated hypothesis was rejected.

Sperm transfer as affected by cyclic or constant light and temperature. Males held at constant 30°C as pupae were sterile (Table 3). Only 1 of 30 transferred a small quantity of apyrene sperm. About half transferred a full complement of sperm when held as pupae at constant 28°C or 30°C for 16 h and 25°C for 8 h with no significant difference in sperm classification between the two groups ($P = 0.69$, Fisher's Exact Test, n = 40). However, these males transferred less sperm than males held at 25°C and a photoperiod of 16:8 (L:D) (control) $P = 2.58 \times 10^{-5}$, Fisher's Exact Test, n = 60). Females mated with control males received a full complement of sperm more often than females mated with males held at 28°C or at cyclic temperature of 30:25°C. Further, 71% of females that received a full complement of sperm from control males were given the highest sperm classification compared with <10% mated with males held at 28°C or cyclic temperature of 30:25°C.

Holding pupae in constant darkness reduced the quantity of eupyrene sperm transferred by adults but not to the degree observed in males exposed to constant light (Table 3). When males were held in the 24-h light incubators, only 5% of those exposed to light transferred a full complement of sperm. In contrast, 19% of the males from containers within the grocery bags transferred a full complement of sperm. Further, of all males that transferred a full complement of sperm, males in 24-h light transferred only a moderate quantity of eupyrene sperm, whereas 1

Table 3. Sperm transfer and percentage of eggs that were embryonated for gypsy moths with a full complement of eupyrene sperm (abundant) when females were paired with males held as pupae in incubators with a photoperiod of 16:8 h (L:D), 24-h light, or 24-h darkness and constant or cyclic temperature..

Light		Temperature			♀♀ with sperm	♀♀ with eupyrene sperm•	
Periodicity	L:D	Periodicity**	°C	No.	(%)	(%)	% embryonated eggs (mean ± SEM)
Cyclic	16:8	Constant	25†	99	96	86	91 ± 2.2
Cyclic	16:8	Constant	28	20	85	50	98 ± 0.7
Cyclic	16:8	Constant	30	30	3	0	
Cyclic	16:8	Cyclic	30:25	20	95	65	96 ± 1.4
Constant	24:0	Constant	25	118	75	5	98 ± 0.8
Constant	24:0	Cyclic	28:22	41	93	90	97 ± 0.9
Constant	0:24	Constant	25†	78	95	79	95 ± 1.8
Constant	0:24	Constant	25	115	85	19	96 ± 2.5
Constant	0:24	Cyclic	28:22	41	98	95	92 ± 3.2

•These data are for ♀ ♀ with abundant eupyrene sperm only.
**For cyclic temperature, males were held for 16 h at higher temperature and 8 at lower temperature.
†These pupae were held in the rearing room.

out of 5 of the males in 24-h darkness transferred a high quantity. These differences were highly significant ($G = 16.8$; df = 3; $P < 0.001$). For males held in 24-darkness, those from the rearing room transferred a full complement of sperm about 4X more often than those from the 24-h-light incubators.

Finally, holding pupae in incubators with a cyclic temperature of 28°C for 16 h and 22°C for 8 h, nearly overcame the sterilizing effect of constant light or darkness (Table 3). There was no significant difference in sperm transfer by males of these two groups ($P = 0.75$, Fisher's Exact Test, n = 82). Although these males had a full complement of sperm as often as control males ($G = 2.2$, df = 1; $P = 0.14$), spermathecae from females receiving a full complement of sperm from control males contained a higher ranking more often than females mated with males held under constant light or darkness ($G = 7.4$; df = 1; $P < 0.01$). Forty-two percent of the females that received a full complement of sperm from control males were classified as having the highest sperm class compared with only 22% for females that received a full complement of sperm from males held in constant light or darkness and cyclic temperature of 28:22°C.

For females with a full complement of sperm, there was no difference in percentage of eggs that became embryonated ($F = 0.59$; df = 10, 222; $P = 0.82$) or in the mean egg-mass weight [$F = 1.43$; df = 10; 222; $P = 0.17$; egg mass = 912 ± 15.6 mg (mean ± SEM)] regardless of holding conditions of male pupae.

The data presented led to the following conclusions. Male pupae of the gypsy moth were sterilized at 30°C. When pupae were held in constant temperature, both constant light and darkness reduced sperm transfer, but when males were held under constant light, more were nearly sterile than when held in darkness. Light intensity (80 < lux < 3960) was not a contributing factor to sterility. Cyclic temperature overcame the sterilizing effect of constant light or darkness.

Heat-induced sterility has been observed in several species of Lepidoptera. Norris (1933) observed reduced fertility in *Ephestia kühniella* Zeller at 27°C and complete sterility at 30°C. As with males of the gypsy moth, males of *E. kühniella* successfully paired with females. A temperature of 32°C during the pupal stage sterilized males of the sugarcane gray borer, *Eucosma schistaceana* Snellen, (Cheng 1972) and silkworm, *Bombyx mori* L. (Sugai and Kiguchi 1967). Mating was reduced in both species. In the silkworm, prepupae were more sensitive than pupae to heat-induced sterility (Sugai and Ashoush 1968). Morphologically, the eupyrene and apyrene spermatozoa from these males appeared normal, but males transferred fewer motile sperm. Proverbs and Newton (1962) studied heat-induced sterility as a means of sterilizing the codling moth, *Carpocapsa pomonella* (L.), for the sterile male technique. They found it to be unsuitable because temperature that sterilized males also caused high mortality.

The sterilizing effect of constant light has been reported for several species of Lepidoptera, including the gypsy moth (Lum and Flaherty 1970, Riemann and Ruud 1974, Hagan and Brady 1981, Giebultowicz et al. 1990). In the gypsy moth, a dual rhythmic release of sperm from the testes and movement within the male reproductive system is initiated by cyclic photoperiod which becomes arrhythmic in constant light (Giebultowicz et al. 1988) or constant darkness (Giebultowicz and Joy 1992). Eupyrene sperm bundles transferred by males held as pupae under constant light fail to disassociate normally in spermatophores placed within the bursa copulatrix of the female (Giebultowicz et al. 1990). Females mated with

these males contain little or no eupyrene sperm in their spermathecae (Proshold and Bernon 1994).

Rhythmic release of sperm can be initiated by cyclic temperature in constant light or darkness as evidence by the high fertility of males held under the conditions in the present study. Although I did not observe any correlation between sperm transfer and light intensity, I did observe that males from some incubators transferred more sperm than males from other incubators. Apparently, subtle differences between incubators were able to set the biological pacemaker and initiated sperm release and movement within the reproductive system of some males.

In the standard rearing procedure for gypsy moths, males and females are placed in 4-L cartons as pupae and held at constant 25°C (Bernon[3]). Insects in these cartons remain in darkness until after reproduction. Likely, some of these males transfer a reduced quantity of sperm. The younger the male pupae when caged, the poorer would be their expected sperm transfer. Holding pupae in containers with clear lids so that they are exposed to a cyclic photoperiod or programming the rearing rooms for oscillating temperature would insure abundant sperm transfer for colony maintenance and mass production.

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