CHRONOBIOLOGY INTERNATIONAL
Vol. 20, No. 5, pp. 809–821, 2003

Effects of Light and Temperature on the Circadian System Controlling Sperm Release in Moth *Spodoptera littoralis*

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ABSTRACT

Reproductive physiology of male moths is regulated by a peripheral circadian system, which controls the timing of sperm release from the testis into the upper vas deferens (UVD) and timing of sperm transfer from the UVD to the seminal vesicles. We investigated various effects of light and temperature on sperm release and transfer rhythms in the moth *Spodoptera littoralis*. We report that both rhythms persist for up to 1 week in constant darkness without significant dampening and are also temperature compensated in the range from 20°C to 30°C. However, the duration of sperm retention in the UVD is temperature-dependent; consequently, temperature exerts a masking effect on the rhythm of sperm transfer. Experimental manipulations of light and temperature regime demonstrated that light dominates over temperature in entraining the timing of sperm release and transfer. Nevertheless, temperature plays a critical role in the absence of light Zeitgeber. Sperm release and transfer are arrhythmic in constant light (LL); however, both rhythms are restored by temperature cycles.

Key Words: Insects; Sperm release rhythm; Peripheral clock; Temperature compensation; Light and temperature zeitgeber; Circadian rhythm.

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DOI: 10.1081/CBI-120024217
0742-0528 (Print); 1525-6073 (Online)
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INTRODUCTION

Circadian systems of animals are composed of central brain pacemakers and multiple oscillators located in peripheral organs. Peripheral oscillators were first identified based on physiological rhythms that persisted in isolated organs (Giebultowicz, 1999). More recently rhythmic expression of clock genes have been found in most cells of animals, suggesting the existence of abundant local oscillators (Schibler and Sassone-Corsi, 2002). In the hierarchically organized circadian systems of mammals, peripheral oscillators are light-insensitive and synchronized by the central pacemaker (Pando et al., 2002), or by non-photic signals (Stokkan et al., 2001). In contrast to mammals, peripheral oscillators of insects and zebra fish are directly entrainable by light–dark (LD) cycles (Giebultowicz et al., 1989; 2000; Plautz et al., 1997; Whitmore et al., 2000). Thus, light entrainment, which is the fundamental property of circadian systems (Saunders, 2002) is shared by central and peripheral oscillators in insects and fishes. Most peripheral oscillators have not been tested with regard to other fundamental circadian properties, namely, various roles of temperature in entrainment and temperature compensation of the free-running period, with the exception of circadian rhythm in cockroach cuticle growth (Weber, 1995; Wiedenmann et al., 1986). In this paper, we examine multiple effects of temperature on the peripheral circadian system, which controls rhythms associated with sperm migration in the reproductive system of the moth, *Spodoptera littorallis.*

Circadian rhythms are critically involved in the reproductive physiology of Lepidoptera; in all species so far investigated, two overt circadian rhythms are associated with the descent of sperm in the male reproductive tract (Bebas et al., 2001; Giebultowicz and Brooks, 1998; Giebultowicz et al., 1988; LaChance et al., 1977; Riemann et al., 1974; Seth et al., 2002). The first rhythm is in the daily release of sperm bundles from the testis into the upper vas deferens (UVD). This rhythm is the result of time-restricted penetration of sperm bundles through the epithelial barrier separating the testis from the UVD (Giebultowicz et al., 1997). The second rhythmic event is the transfer of sperm from the UVD into the seminal vesicles. This is triggered by the increased myogenic contractions of the UVD wall at a certain time in the LD cycle (Giebultowicz et al., 1996). The phases of both sperm release and sperm transfer rhythms are similar in relation to the LD cycles in different species of moths; sperm release occurs in the evening and sperm transfer in the morning. Sperm migration rhythms are not correlated with species-specific mating rhythms (Giebultowicz and Zdarek, 1996); rather, they seem to coordinate the maturation of sperm as it interacts with materials secreted by the UVD epithelium (Bebas et al., 2002a,b). It appears that the circadian system aids effective release and maturation of daily sperm batches, allowing accumulation of fertile sperm in the male storage organs. In constant light (LL), all rhythms associated with sperm descent are disrupted; consequently, sperm release is dramatically reduced and males are essentially sterile (Bebas and Cymborowski, 1999; Giebultowicz et al., 1990; Riemann and Ruud, 1974). This demonstrates the critical role of the circadian system for male fecundity and fertility.

There is evidence that the rhythms of sperm release and transfer are generated by a local circadian system. These rhythms persist in isolated testis-vas deferens complexes and can be phase-shifted by light applied to such complexes in vitro; this has been demonstrated in the two moth species, *Lymantria dispar* (Giebultowicz et al., 1989) and *S. littoralis* (Bebas et al., 2001). In addition, the clock gene *period* is expressed

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rhythmically in cells at the testis base that are involved in sperm release, and in the UVD wall (Gvakharia et al., 2000), verifying the existence of peripheral oscillator.

The effects of temperature on the peripheral circadian system in the moth reproductive tract have not been investigated. It is not known whether the free-running periods of sperm release and transfer cycles are temperature-compensated. Likewise, the relative strength of temperature and light in entraining sperm migration rhythms has not been determined in any species. Finally, it is not clear whether high/low temperature cycles can restore the rhythm of sperm release disrupted by LL. In this paper, we address the above questions in the cotton leafworm, *S. littoralis*, a model species in which several hundreds of sperm bundles are released daily from the testes in a robust circadian rhythm (Bebas et al., 2001). We report here several effects of temperature on the regulation of sperm release from the testes, and sperm transfer to the seminal vesicles.

MATERIAL AND METHODS

The colony of the cotton leafworm, *Spodoptera littoralis* was reared on an artificial diet (PREMIX) as described before (Bebas et al., 2001); adults were fed with 10% honey diluted in water. Insects were reared in cycles of 12h of bright light and 12h of dark (LD, 12 : 12) at 25°C. Lights were switched off at Zeitgeber time (ZT) 12. The newly emerged male adults were selected daily, kept for 1 day in LD at 25°C and then were transferred to specific light and temperature regimes, as indicated in the results section. The pattern of sperm release was determined during the fourth day in the new regime. To study phase shifting by light pulses or heat pulses, adults were exposed to a 4h pulse of bright light or elevated temperature (27°C or 30°C) at circadian time (CT) 13 or at CT 19 during the first night after transfer to DD. Males were kept for three more days in DD and on the fourth day the patterns of sperm release were determined. To study temperature effects on the free-running period, newly enclosed males were placed for 1 day in LD at 20°C, 22.5°C, 25°C, 27.5°C, or 30°C, followed by DD in the same temperature for 4 days.

To determine the patterns of sperm release and transfer, 8–10 males were dissected every 2h and the UVDs checked for the presence of clones of nucleated spermatozoa (sperm bundles). At designated times, males were chilled and dissected in cold saline formulated for moths (Weevers, 1966). The testis-UVD-SV complexes were transferred individually into wells of a culture plate containing saline. The paired UVDs were cut open, their content allowed to disperse, and then examined under a dissecting microscope. Each male releases several hundred nucleated sperm bundles into the UVD (Bebas et al., 2001). Usually, all of the bundles are transferred to the seminal vesicles before the next cycle of sperm release begins. Sometimes, however, a few bundles are trapped in the UVD. To avoid counting such remaining bundles as freshly released ones, we scored only males that contained more than 10 sperm bundles in the UVD as positive. Since the males were scored for the presence of sperm at 2h intervals, the phases of sperm release and transfer could not be precisely calculated. The hour at which 50% or more males had sperm bundles present in the UVD was taken as the best estimate of the time of sperm release. Similarly, the hour at which transfer of sperm bundles from the UVD to the SV was accomplished in 50% or more males was taken as the best estimate of the time of sperm transfer.

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RESULTS

Effects of DD and Different Photoperiods on Sperm Release and Transfer Rhythms

Males of *Spodoptera littoralis*, kept in LD 12 : 12, displayed robust rhythms of sperm release from the testis into the UVD and sperm transfer from the UVD into the SV [Fig. 1(A)]. Upper vas deferenses were void of sperm during the day; then, between 2 and 4h after lights-off (ZT 14–16), sperm was released into the UVD in all examined males and remained there throughout the night. After lights-on, between ZT 0 and 4, sperm was transferred from the UVD to the SV in all males; consequently, the UVDs were empty until the onset of sperm release on the following day. Both sperm release and transfer rhythms persisted in males that were kept in DD for up to 7 days, which is close to the lifespan of an adult moth. Rhythms in DD were less precise than in LD, but there was essentially no change in the phase of either rhythm between days 4 and 7 [Fig.1(B) and (C)]. This indicates that the free-running period of both rhythms is very close to 24h.

To determine the effects of photoperiod on the pattern of sperm release and transfer, males were kept in photoperiodic cycles with different light/dark ratios [Fig. 2(A)–(C)]. Shortening of the scotophase from 12 to 8h advanced sperm release onset by approximately 3h relative to the L : D transition, so that it coincided with the lights-off at ZT 12. Lengthening of the scotophase from 12 to 16h did not change the phase of sperm release; in both LD,

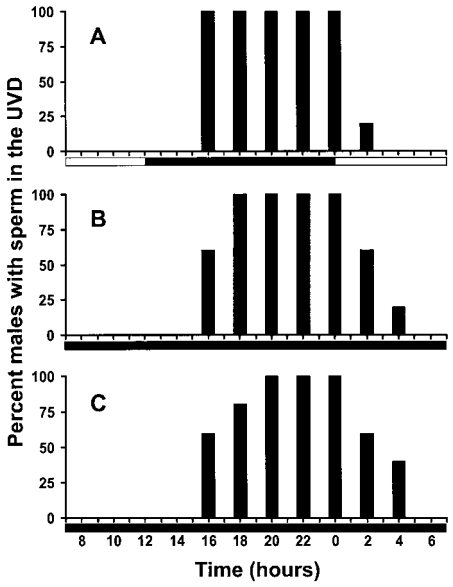

Figure 1. Patterns of sperm release into the UVD and transfer out of the UVD in LD, 12 : 12 (A), after three days in DD (B) and seven days in DD (C). Upper vas deferenses from 8–10 males were dissected every 2h around the clock and examined for sperm presence. Each bar represents the percent of males with 10 or more eupyrene sperm bundles in the UVD. Each experiment was repeated at least twice with similar results.

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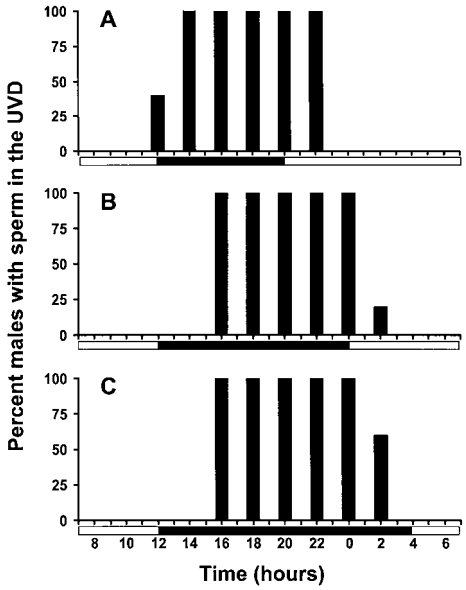

Figure 2. Patterns of sperm release and transfer in males kept in LD, 16 : 8 (A); LD, 12 : 12 (B); and LD, 8 : 16 (C). Each bar represents the percent of males with eupyrene sperm bundles in the UVD ($n = 8$–10 per time point).

12 : 12 and 8 : 16 sperm release occurred at ZT 16. In all photoperiods, sperm release was separated from sperm transfer by 10–11h. Consequently, sperm transfer fell either in the dark or light, depending on the length of the photophase. These experiments show that neither of the two rhythms is phase-locked to lights-on or lights-off. Rather, the timing of sperm release and sperm transfer remained at a stable phase angle relative to each other.

Effects of Different Constant Temperatures on Sperm Descent in DD

To determine whether the free-running rhythms of sperm release and transfer are temperature-compensated, we placed groups of males in DD at constant temperatures ranging from 20°C to 30°C. The rhythms of sperm release and transfer were evaluated after 4 days in the new environment (Fig. 3). The phase of the sperm release rhythm was very similar in males kept in temperatures from 20°C to 27.5°C. From this we infer that the free-running period of the sperm release rhythm remained close to 24h in this range of temperatures. In the constant temperature of 30°C, the phase of sperm release was advanced by approximately 4h on the fourth day in DD (Fig. 3), and by 2h on the second day (data not shown). This suggests that the free-running period of sperm release rhythm has shortened to approximately 23h. Thus, the rhythm of sperm release is temperature compensated from 20°C to 30°C.

While the rhythm of sperm release had the same phase in the range of temperatures from 20°C to 27.5°C, the rhythm of sperm transfer showed delays at 20–22.5°C and an advance at 27.5–30°C on the fourth day at a given temperature (Fig. 3). Similar timing of

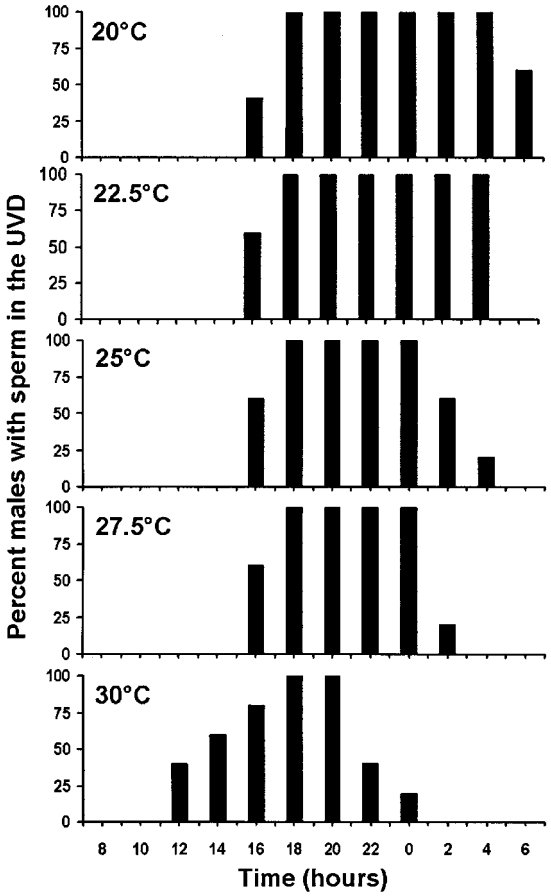

Figure 3. Effect of different constant temperatures (shown in respective panels) on the free-running patterns of sperm release in DD. Each bar represents the percent of males with eupyrene sperm bundles in the UVD ($n = 8$–10 per time point).

sperm transfer was recorded on the second and third day at these temperatures (data not shown) indicating that the free-running period of sperm transfer is also temperature-compensated. However, the time of sperm retention in the UVD was strongly temperature-dependent; sperm remained in the UVD for approximately 6h longer at 20°C than at 30°C. This suggests that some processes associated with sperm maturation occurring in the UVD may be directly dependent on temperature and may shift the phase of sperm transfer as a result of masking.

Effects of Temperature Cycles on the Rhythms of Sperm Release

To determine whether temperature cycles have modifying effects on the sperm release and transfer rhythms, males held in LD, 12 : 12 at 25°C were moved to temperature cycles

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of 12 : 12, 28/22°C, such that the high temperature coincided with either light or dark phase. The patterns of sperm release were evaluated on the fourth day in the new conditions [Fig. 4(A) and (B)]. When the dark phase coincided with the high temperature, the pattern of sperm release was similar to males kept in LD at 25°C [compare Figs. 1(A) and 4(A)]. However, when the dark phase coincided with low temperature, the onset of sperm release was advanced by 4–5h, while the time of sperm transfer remained unchanged [Fig. 4(B)]. Consequently, the time of sperm presence in the UVD has lengthened by approximately 4h relative to males kept in reversed temperature cycles. These data are consistent with the results of a previous experiment (Fig. 3), which showed that the length of sperm retention in the UVD is temperature-dependent.

Entrainment of Sperm Release and Transfer by Light and Temperature Pulses

One of the principles governing circadian oscillations that emerged from studying phase response curves is that entraining signals applied at different point of the circadian cycle have differential effects on the phase of the rhythm (Johnson, 1992). For example, light pulses applied in the first half of the night delay the phase of the free-running rhythm, while light pulses applied in the second half of the night advance it. To probe whether this rule applies to the sperm release and transfer rhythms, we exposed DD males to a single 4h light pulse at either CT 13 or CT 19 and evaluated the pattern of sperm release on the third day after the pulse relative to control (Fig. 5). A light pulse at CT 13 caused a 2h delay in both sperm release and transfer. The same pulse applied at CT 19 caused a phase advance of approximately 4h in both rhythms.

We also studied the effects of temperature pulses on the pattern of sperm release and transfer (Fig. 5). Heat pulse (30°C) applied at CT 13 did not change the timing of sperm

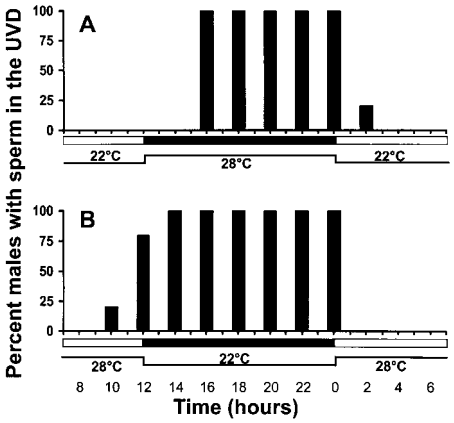

Figure 4. Effect of light and temperature in entraining the rhythms of sperm release and transfer. Males were exposed to cycles of 12 : 12 L/D and 22/28°C with the high temperature coinciding with the dark phase (A), or with the light phase (B). Each bar represents the percent of males with eupyrene sperm bundles in the UVD ($n = 6$–8). Each experiment was repeated at least twice with similar results.

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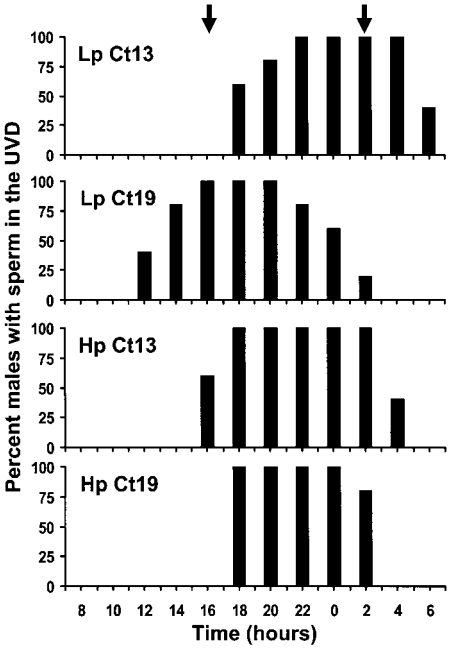

Figure 5. Effects of light pulse (Lp, 4h of bright light) or heat pulse (Hp, 4h of 30°C) applied at circadian time (CT) 13 or 19 on the phase of sperm release and transfer rhythms. All insects were kept for three days in DD at 25°C before the pattern of sperm release was evaluated. Arrows show time of sperm release and transfer in control DD males. Each bar represents the percent of males with eupyrene sperm bundles in the UVD ($n = 8$–10 per time point).

release and transfer compared to DD control. However, heat pulse at CT 19 delayed sperm release by approximately 2h. Taken together, these data show that both light and temperature serve as Zeitgebers for the rhythms associated with sperm release.

The Restoration of Sperm Release and Transfer Rhythms in Constant Light by Temperature Cycles

The rhythms of sperm release and transfer are abolished by LL in *Spodoptera* (Bebas et al., 2001). We observed that after three days in LL at 25°C, both rhythms were severely damped [Fig. 6(A)]. To determine whether the cycles of high/low temperature could entrain sperm release in LL, we kept males in LL and 12 : 12 28/22°C temperature cycles. After three days in these conditions, males displayed robust rhythms of sperm release and transfer [Fig. 6(B)]. Sperm release started 4h before the decline of temperature and the sperm transfer occurred 6h into the low temperature phase. This experiment demonstrates that in the absence of entrainment by LD cycles, temperature cycles alone can synchronize sperm release and transfer.

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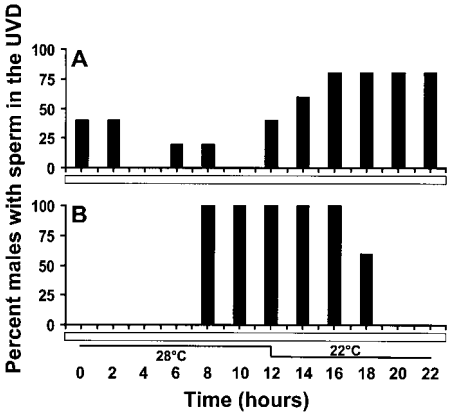

Figure 6. Patterns of sperm release and transfer in males kept for 3 days in LL at 25°C (A), or in LL at 12 : 12, 28/22°C cycles (B). Each bar represents percent of males with eupyrene sperm bundles in the UVD ($n = 8$–10 per time point).

DISCUSSION

We report several important features of the peripheral circadian system controlling two distinct but interrelated outputs: rhythmic sperm release from the testis into the UVD and sperm transfer from the VD into the seminal vesicles. It has been previously reported that both rhythms persist in DD for three days. Here we show that both rhythms continue for at least seven days with a period close to 24h and without significant dampening. The timing of sperm transfer, which in LD, 12 : 12 coincides with lights-on, is more protracted in DD. This is reminiscent of *Drosophila* eclosion rhythms, which show a sharp lights-on response in LD and more diffused response in DD (Helfrich-Forster, 1998). Monitoring sperm rhythms in different photoperiods revealed that sperm transfer is not locked to lights-on. Rather it seems to occur at a fixed number of hours after sperm release, irrespective of the photophase/scotophase length in LD cycle.

Peripheral rhythms of sperm release and transfer have been known for many years, yet they have not been rigorously tested with regard to temperature compensation of the free-running period. In this paper we report that the free-running periods of both rhythms show good temperature compensation from 20°C to 30°C. However, insects kept in temperatures lower or higher than 25°C showed delays or acceleration, respectively, in the timing of sperm transfer out of the UVD; this has been also shown in the gypsy moth held in LD cycles (Giebultowicz et al., 1988). Thus, temperature can modify the phase relationship between the time of sperm release and sperm transfer. The net effect is that sperm bundles are retained longer in the UVD at lower temperatures suggesting that temperature-dependent maturation processes may occur in the UVD lumen. Overnight retention of sperm in the UVD of *Spodoptera* has been correlated with the night-time secretion of glycoproteins from the UVD epithelium (Bebas et al., 2002) and concurrent acidification of the UVD lumen by the action of V-ATPase (Bebas et al., 2002). We hypothesize that these physiological processes may be directly dependent on temperature and their completion may affect the timing of sperm transfer within circadian gate. Further support for the direct effect of

temperature on the time of sperm retention in the UVD comes from the experiment in which cycles of 22/28°C were superimposed on LD cycles. The time of sperm retention was increased by 4h when low temperature coincided with the scotophase.

One of the fundamental properties of circadian rhythms is the change in phase of free-running oscillations in response to light or temperature pulses. Phase response curves performed in many organisms consistently demonstrated that pulses applied in early subjective night result in phase delays, while pulses applied in late subjective night advance the phase of circadian rhythms (Johnson, 1992). We show here that this principle applies to the rhythms of sperm release and transfer, although the sensitivity of this peripheral system does not match the sensitivity of central oscillators reported for other insects. Some behavioral rhythms in insects are shifted by pulses shorter than 1h (Pittendrigh, 1981). In contrast, significant phase shifts of sperm release and transfer rhythms were achieved with 4h pulses of bright light, while 2h pulses were not effective (Syrova, unpublished).

Circadian oscillations are entrained by both light and temperature, with light being the stronger zeitgeber in most insect rhythms. However, temperature has been reported as a predominant Zeitgeber in several cases (Rensing and Ruoff, 2002), for example, in rhythmic pre-ecdysis behavior in *Manduca sexta* (Truman, 1984), and the rhythm of adult eclosion in tse tse flies (Zdarek and Denlinger, 1995). Here we present two lines of evidence that temperature plays a much weaker role in the entrainment of sperm release rhythms than does light. First, superimposing a "conflicting" low/high temperature cycle on the LD cycle (cryophase with light) did not change the phase of sperm release or transfer compared to moths held in LD at constant 25°C. Second, the 4h temperature pulses of 5°C difference did not produce phase shifts of similar magnitude as 4h pulses of light applied at the same phase. Although we did not test the effects of temperature systematically at different phases of the oscillation, our limited survey clearly shows that the peripheral circadian system controlling sperm release is relatively insensitive to temperature and is entrained predominantly by light. Additional experiments showed that this system re-entrains promptly to changes in LD regime; when LD was advanced or delayed by 8h, both sperm release and transfer were re-entrained to the new LD within two days (B. Rush and J. Giebultowicz, unpublished). A similar course of re-entrainment was demonstrated before in another moth, *L. dispar*, both in vivo (Giebultowicz et al., 1988) and in vitro, when an LD shift was applied to isolated testis-VD complexes (Giebultowicz et al., 1989).

Constant light disrupts rhythms of sperm release and transfer in *Spodoptera littorallis* (Bebas et al., 2001); this dramatically reduces the number of descending sperm bundles and results in male infertility (Bebas and Cymborowski, 1999). We show here that temperature cycles restore robust rhythm of sperm release and transfer in LL-held animals. Not only is rhythmicity restored, but the amount of released bundles also increases to levels observed in LD (Syrova, unpublished) demonstrating that physiological outputs are rescued along with the clock mechanism. The restoration of activity rhythms by thermoperiod have been reported in *Drosophila melanogaster* held under LL (Tomioka et al., 1998). It is known that LL prevents the accumulation of the timeless protein in *Drosophila* (Price et al., 1995), which leads to disruption of the molecular clock feedback loop (Stanewsky, 2002). The mechanism by which temperature acts on the LL-disturbed clock is not understood. Interestingly, light and temperature appear to act via divergent input pathways in entraining the clock in the fungus *Neurospora* (Merrow et al., 1999).

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Our results provide important insights into the control of multiple output rhythms generated by a peripheral circadian system. The phase angles between sperm release and sperm transfer remained relatively constant in free-running conditions and in a range of photoperiods, but they were significantly affected by temperature. We hypothesize that the completion of certain biochemical steps involved in sperm maturation in the UVD may provide modulatory input to the oscillator controlling sperm transfer. However, it is also possible that maturation affects the timing of sperm via a pathway independent of the oscillator. The sperm release rhythm is probably generated by different clock-bearing cells than the sperm transfer rhythm. In situ hybridization and immunocytochemistry showed that the clock gene period is expressed in barrier cells involved in sperm release, and in the UVD wall involved in sperm transfer and secretory rhythms (Bebas, unpublished; Gvakharia et al., 2000). The mechanism by which the circadian system in the male reproductive tract coordinates multiple rhythms requires further investigation.

ACKNOWLEDGMENTS

We thank Brandy Rush for reading the paper. This work was supported by the USDA grant to JMG and GAAVCR grant #A5007205 to IS.

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Submitted April 27, 2003
Returned for revision May 8, 2003
Accepted May 24, 2003

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