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Research article

Circadian rhythm of glycoprotein secretion in the vas deferens of the moth, *Spodoptera littoralis*

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Abstract

Background: Reproductive systems of male moths contain circadian clocks, which time the release of sperm bundles from the testis to the upper vas deferens (UVD) and their subsequent transfer from the UVD to the seminal vesicles. Sperm bundles are released from the testis in the evening and are retained in the vas deferens lumen overnight before being transferred to the seminal vesicles. The biological significance of periodic sperm retention in the UVD lumen is not understood. In this study we asked whether there are circadian rhythms in the UVD that are correlated with sperm retention.

Results: We investigated the carbohydrate-rich material present in the UVD wall and lumen during the daily cycle of sperm release using the periodic acid-Shiff reaction (PAS). Males raised in 16:8 light-dark cycles (LD) showed a clear rhythm in the levels of PAS-positive granules in the apical portion of the UVD epithelium. The peak of granule accumulation occurred in the middle of the night and coincided with the maximum presence of sperm bundles in the UVD lumen. These rhythms persisted in constant darkness (DD), indicating that they have circadian nature. They were abolished, however, in constant light (LL) resulting in random patterns of PAS-positive material in the UVD wall. Gel-separation of the UVD homogenates from LD moths followed by detection of carbohydrates on blots revealed daily rhythms in the abundance of specific glycoproteins in the wall and lumen of the UVD.

Conclusion: Secretory activity of the vas deferens epithelium is regulated by the circadian clock. Daily rhythms in accumulation and secretion of several glycoproteins are co-ordinated with periodic retention of sperm in the vas deferens lumen.

Background

Many life functions in organisms ranging from prokaryotes to humans display daily rhythms that are controlled by internal circadian clocks. The mechanisms of circadian clocks involve rhythmic expression of several "clock" genes, including the *period* (*per*) gene. Clock genes interact in a transcription – translation feedback loops with circa 24 hour oscillation period. The core clock mechanism is conserved in animals from insects to mammals [1]. Circadian clocks located in specific brain regions control behavioral rhythms, such as rest/activity cycles [2]. Recent studies show that clock genes are also expressed in most

organs of insects and vertebrates [3–5]. Rhythmic expression of clock genes in peripheral organs isolated *in vitro* suggests that these organs contain oscillators that coordinate specific physiological processes [6]. While clock mechanism is well-understood, little is known about output rhythms generated by most peripheral clocks and their biological significance to the organism as a whole.

A remarkable peripheral clock with a clear function in reproductive physiology was identified in insects. This circadian clock controls the rhythm of sperm release from the testis in several species of moths belonging to different orders [7–12]. In all species examined, there are two rhythmic steps entrained by light-dark cycles (LD). The first step, the release of sperm from the testis into the upper vas deferens (UVD), occurs in the evening. The second step, the transfer of sperm from the UVD to the seminal vesicles, takes place in the morning. Both rhythms persist in constant darkness (DD) but are abolished in constant light (LL). The number of sperm bundles released from the testis under LL conditions is dramatically reduced and females mated with LL males lay mostly unfertilized eggs [13,14]. Taken together, these results suggest that circadian rhythms in the insect reproductive system are essential for the optimal production of fertile sperm. The circadian system is also important for reproductive fitness in *Drosophila melanogaster*. Mutations that disrupt circadian clock lower fecundity and fertility in male flies [15].

The circadian clock, which control rhythms of sperm release is located in the reproductive system itself. The autonomy of this circadian system was first inferred based on the continuation of the sperm release rhythm in isolated abdomens of moth *Anagasta kuehniella* [16]. Subsequent studies demonstrated persistence of sperm release rhythms in reproductive tracts of *Lymantria dispar* and *Spodoptera littoralis* isolated in vitro [12,17]. Furthermore, the phase of sperm release rhythm could be shifted by light in vitro [17]. These results lead to the conclusion that testis-vas deferens complexes of moths contain a self-sustained photoreceptive circadian clock. This was supported by the finding that the clock gene *per* is rhythmically expressed in the vas deferens of the codling moth, *Cydia pomonella* [18].

In all species of moths studied so far, sperm bundles that have been released from the testis are retained in the lumen of the UVD overnight, before being transferred to the seminal vesicles. What is the significance of periodic retention of sperm in this part of the seminal tract? Previous ultrastructural and histochemical studies of the UVD in two moth species suggested that the UVD epithelial cells produce secretory granules which are released into the UVD lumen [19–21]. There was a temporal correlation between the release of the electron-dense secretory granules and the appearance of sperm in the UVD lumen, suggesting that these materials may be involved in sperm maturation. In most animals, spermatozoa leaving the testes are immature and do not have fertilizing capacity. An extra-testicular maturation of sperm occurs in the vas deferens and other segments of the seminal ducts, leading to sperm mobility and ability to bind and penetrate the egg [22]. The surface of each sperm cell is modified by "maturation antigens" secreted by specialized epithelial cells of the reproductive tract [23]. These components have been studied mostly in mammals and to a lesser degree in *Drosophila*; several of them have been identified as glycoproteins [22,24].

Taken together, the above data led us to a hypothesis that the overnight retention of moth sperm in the UVD may be associated with clock-controlled secretion of glycoproteins from the UVD epithelium. To test this hypothesis, we investigated temporal changes in carbohydrate-rich material in the UVD epithelium and conducted biochemical analysis of glycoproteins extracted from the UVD wall and lumen in the cotton leafworm, *S. littoralis*. This moth displays a robust two-step rhythm of sperm release that persists with high amplitude in constant darkness, and is disrupted in constant light [12]. We demonstrate circadian rhythms in the levels of secretory granules in the UVD of *S. littoralis* and show that several glycoproteins are released into the UVD lumen in a rhythmic fashion.

Results

Circadian rhythm in the levels of carbohydrate-rich materials in the UVD

The initial part of the reproductive tract of S. littoralis consists of testes that are separated from paired UVD by the epithelial barrier. We have established previously that the daily period of sperm release starts at approximately Zeitgeber time 12 (ZT 12), which, by convention, corresponds to lights-off point in the LD cycle. A section of testis-UVD fixed at ZT12 and processed for PAS reactions is shown in Figure 1a. Elongated sperm bundles stained dark red can be seen exiting into the UVD through the epithelial barrier. Columnar epithelial cells with elongated nuclei form the UVD wall. The UVD lumen is filled with pinkish, PAS-positive material indicating the presence of carbohydrates. Higher magnification of the border between the UVD lumen and the epithelium demonstrates the presence of PAS-positive granules in the apical portions of the UVD epithelium and suggest that this carbohydrate-rich material is released into the lumen (Fig. 1b).

Comparison of UVDs fixed at different times of day in the LD cycle revealed daily changes in the level of PAS-positive granules (Fig. 2a). In the morning, at ZT4, there was no PAS-positive reaction in the cytoplasm. Four hours later, at ZT8, a thin layer of PAS-positive material was detected in the apical cytoplasm closest to the lumen. At the

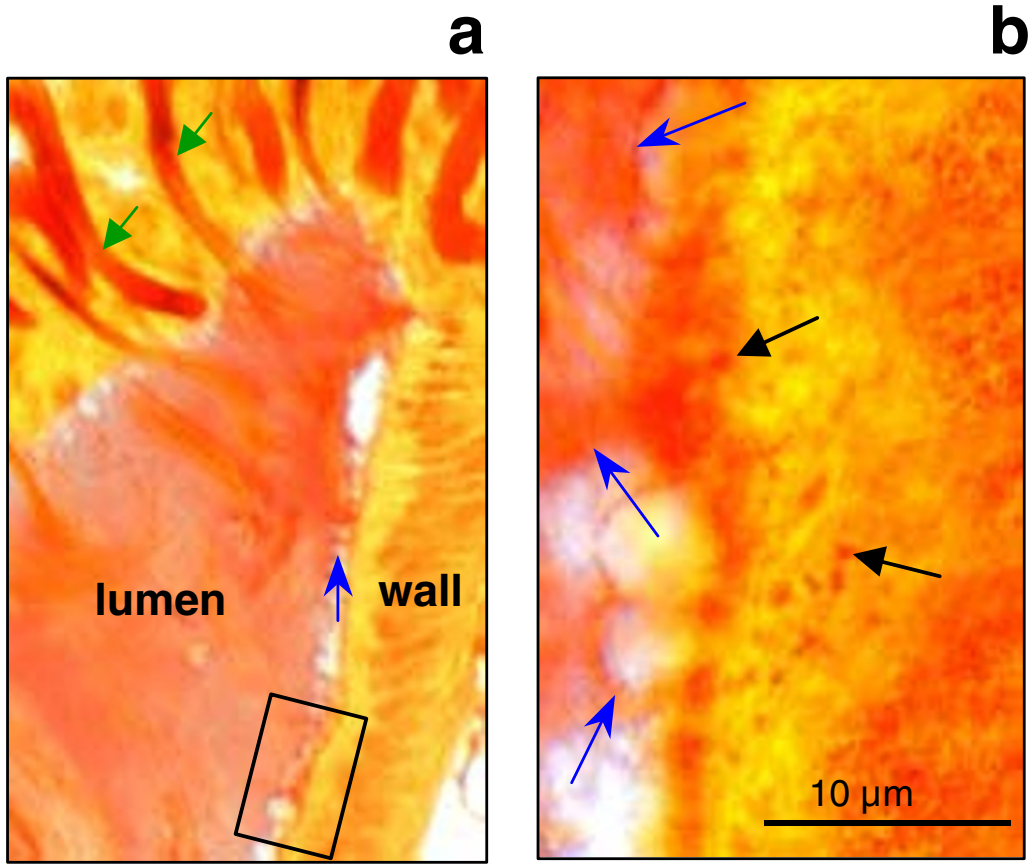

Figure 1
Carbohydrates in the reproductive system of *S. littoralis*.a. Section of the UVD showing sperm bundles (green arrows) penetrating through the epithelial barrier into the UVD lumen. Pinkish, PAS-positive material is present among sperm in the lumen (blue arrow) and in the apical region of the UVD epithelium. **b**. Higher magnification of the apical region (delineated by black rectangle in a). Distinct secretory granules (black arrows) are visible in the cytoplasm and strands of PAS-positive material (blue arrows) are visible in the lumen.

lights-off (ZT12), distinct dark granules appeared in the apical cytoplasm between the cell membrane and the nucleus. Less distinct and visually larger granules were present in this region throughout the night, at ZT 16 and 20. Four hours after lights-on (ZT 24), PAS-positive granules were no longer present in the cytoplasm. A similar rhythm in the levels of PAS-positive material was observed in males that were held in DD for 3 days (Fig 2b). In contrast, no rhythm was detected in the UVD of males held for 3 days in LL (Fig. 2c). In these conditions, intermediate levels of PAS-positive material were observed in the apical region at all time points. In some preparations, uneven staining was observed along the epithelium suggesting lack of co-ordination in the secretory activity between adjacent cells.

The PAS reaction used in this study detects both glycogen and sugar components of glycoproteins. To determine whether PAS-positive material contained glycogen, we treated several UVD sections from LD males with diastase prior to the PAS reaction. These preparations showed PAS staining indistinguishable from untreated controls (data not shown). The fact that the treatment with diastase did not weaken PAS reaction indicates that carbohydrates present in the UVD do not include discernible amounts of glycogen. The failure of diastase to remove staining and the oscillations in glycoprotein abundance (see below) are consistent with the majority of PAS signals coming from glycoproteins.

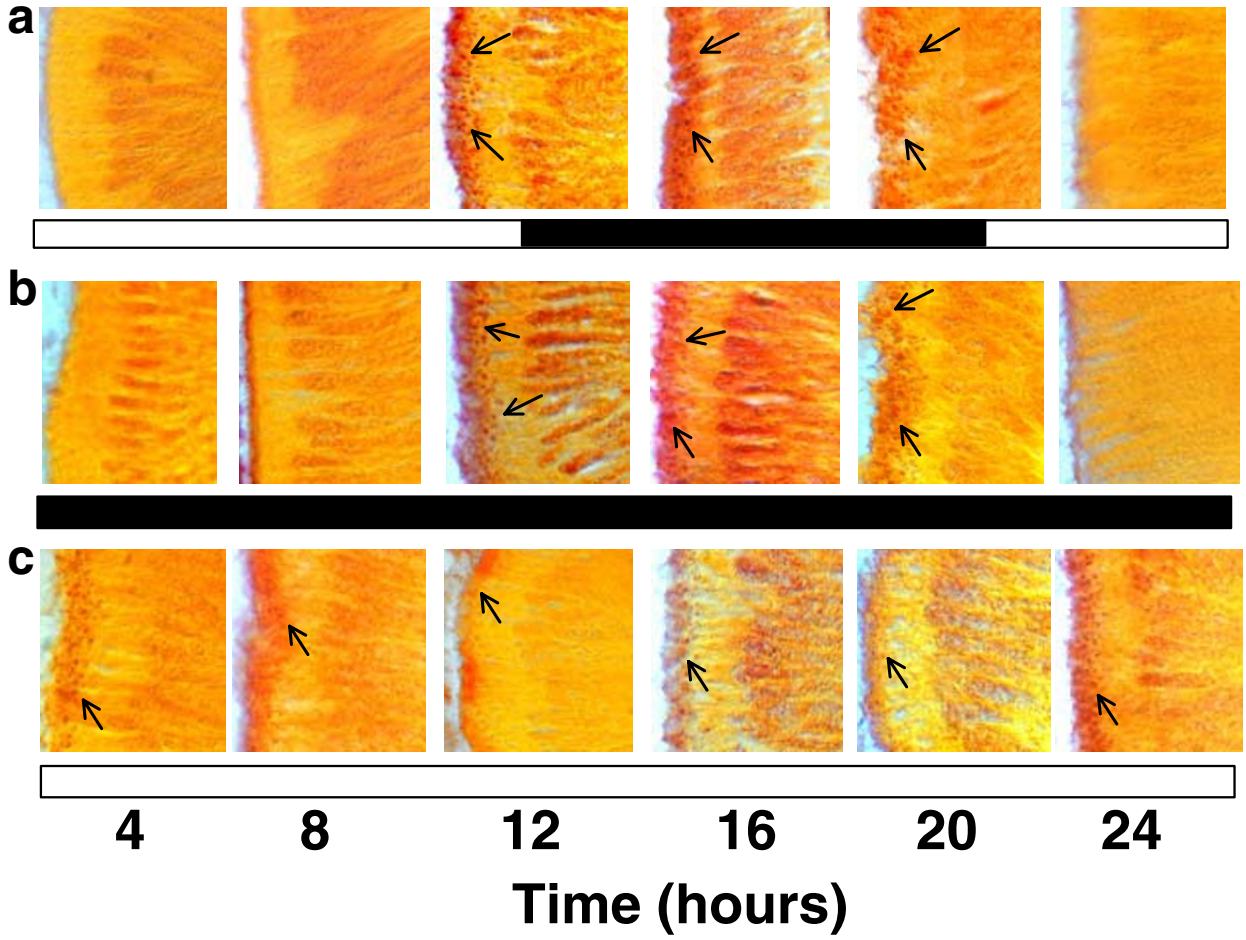

Figure 2
Circadian rhythm of secretory activity in the UVD of *S. littoralis* Longitudinal sections of the UVD showing time-dependent accumulation of PAS positive granules in apical cytoplasm of epithelial cells in LD (**a**), DD (**b**), and LL (**c**). The levels of PAS-positive granules (arrows) show daily oscillations in LD and DD conditions with high accumulation occurring at night. In LL, intermediate levels of PAS-positive material are present at all times. The experiment was performed on 8–10 males per time point in each photo regime with similar results. White and black horizontal bars indicate periods of light and darkness, respectively.

Quantitative changes in the number and size of PAS-positive granules in the UVD

Measurements of the cytoplasmic area containing dark, PAS-positive granules at different times of day confirmed a strong circadian rhythm in the accumulation of granules in the apical cytoplasm of the UVD epithelium in LD males (Fig 3a). Dark granules were not detected in the middle of the day, at ZT 4. Later in the day, at ZT8 and 12, dark granules occupied on average 6 % of cytoplasm. During the night, the percentage of cytoplasm occupied by dark granules increased to above 10% and then dramatically decreased 4 hours after lights-on (ZT24). A similar rhythmic changes in the percentage of the cytoplasmic area occupied by PAS-positive granules was observed in males reared in DD (Fig 3b), except that the accumulation of granules was delayed by a few hours relative to LD males as may be expected in a free-running conditions. In males reared under LL, there were no statistically significant differences in the amount of granules in the UVD apical cytoplasm as a function of time. At all time points, 4–6% of cytoplasm contained PAS-positive material (Fig. 3c). These quantitative data agree well with microscopic observations described in the previous section (Fig 2a,2b,2c).

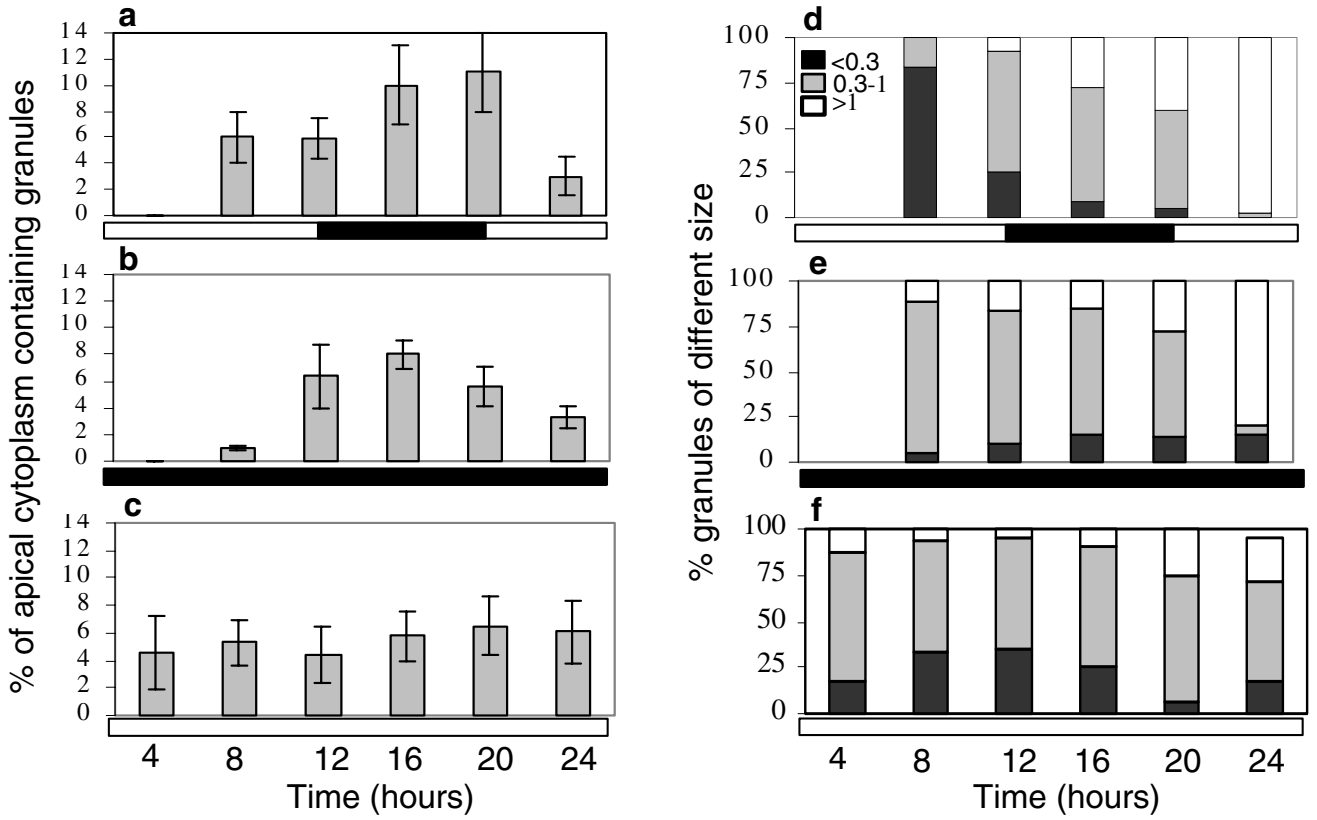

Figure 3
Circadian rhythm in abundance and size of PAS-positive granules (**a-c**) Average (± SD) per cent of PAS-positive granules in the apical cytoplasm of the UVD epithelium in LD (**a**), DD (**b**), and LL (**c**). (**d-f**) Changes in the size of dark granules as a function of time. Stacked bars represent the proportion of small (black), medium (grey) and large (white) granules at given time point. See text for further explanation. Data shown are averaged from the UVD sections representing 8–10 males per time point. White and black horizontal bars indicate periods of light and darkness, respectively.

To determine whether the size of dark, PAS-positive granules changed with time, we arbitrarily divided dark granules into 3 category sizes and calculated the proportion of each size at sequential time points. In LD males, an increase in the size of dark granules was observed as a function of time (Fig 3d). When PAS-positive material first appeared at ZT8, it was predominantly in the form of small granules, less than 0.3 μm^2. At later time points, these small granules were gradually replaced by medium granules between 0.3 and 1.0 μm^2. In the morning, when PAS-positive material decreased to a low level, apical cytoplasm contained only sparse large granules above 1.0 μm^2 in size. A similar increase in the size of granules was observed in DD males (Fig. 3e), except that medium rather then small granules predominated at ZT8. It should be noted that only 2 males out of 8 examined for this time point had PAS-positive material and therefore were included in the calculations. No rhythmicity in the size of granules was detected in males kept in LL (Fig. 3f).

Rhythmic changes of glycoprotein content in the UVD wall and lumen

Rhythmicity in the abundance of carbohydrate-rich granules in the UVD epithelium suggested that the epithelial cells may rhythmically produce and secrete glycoproteins. To begin testing this hypothesis, we extracted proteins from UVD walls, collected at different times of LD cycle, and separated them on SDS-PAGE. Proteins were electro-

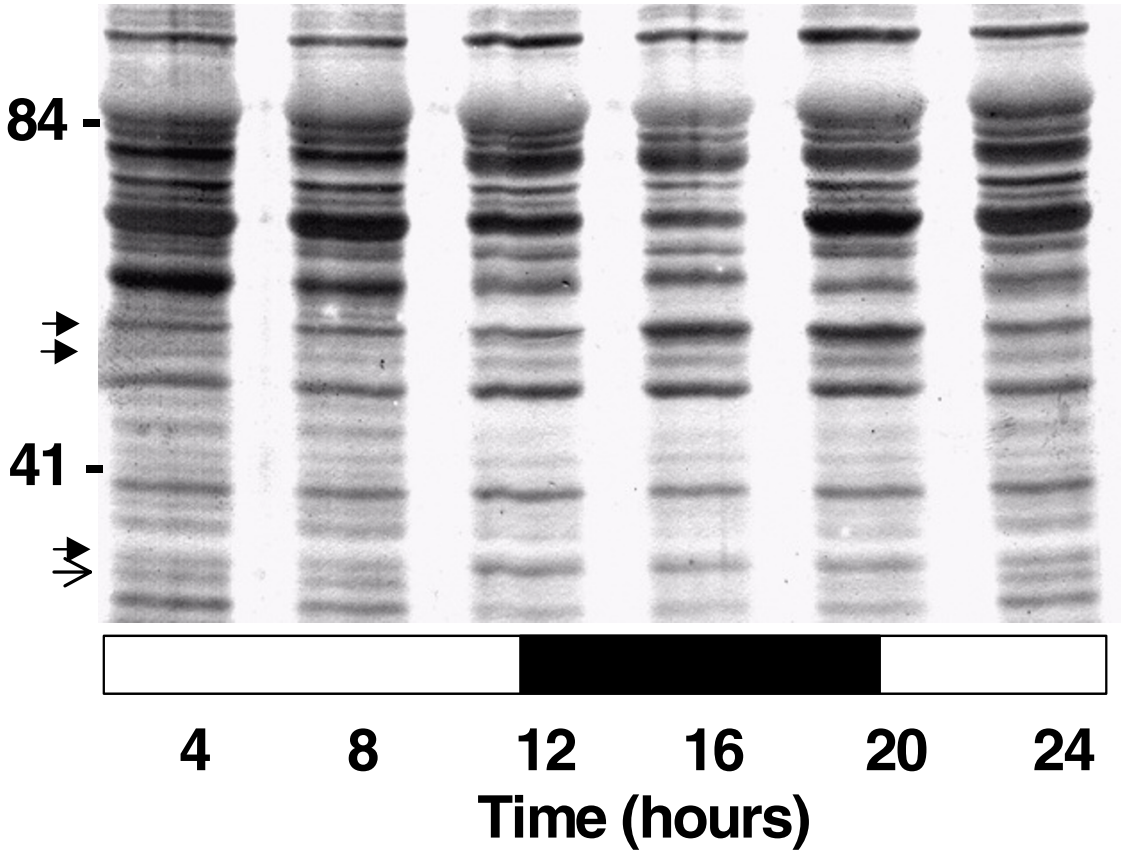

Figure 4
Temporal pattern of glycoproteins in the UVD wall. Glycoproteins in the UVD wall homogenates separated on SDS-PAGE, electroblotted, and probed with BCHZ. Arrows indicate glycoproteins that displayed daily rhythms in abundance in LD conditions. The experiment was repeated 3 times with similar results. White and black horizontal bars indicate periods of light and darkness, respectively.

transferred to the PVD membrane, and the blot was treated with biocytin hydrazide (BCHZ) to label glycoproteins (see Materials and Methods for details). Several distinct glycoprotein bands were detected in the homogenates of the UVD wall at all times of day (Fig 4). Of those, at least four glycoproteins displayed a daily rhythm in abundance. Two adjacent glycoprotein bands with a molecular weight above 41 kDa were more abundant at ZT16 and ZT 20 than at other times. One glycoprotein with the molecular weight below 41 kDa increased in abundance at ZT12 and remained at high levels through ZT24. An adjacent protein (Fig. 4, open arrow) appeared to oscillate with a reverse phase, showing low levels at night.

To determine whether there are daily changes in glycoprotein abundance in the UVD lumen, we blotted and BCHZ labelled gel-separated proteins from the seminal fluid in the UVD lumen. Many glycoproteins were detected in the seminal fluid and several of them showed robust oscillations in LD moths (Fig. 5a). Each glycoprotein showed characteristic phases of accumulation and decline, however, the majority of proteins were upregulated during the night. Interestingly, the glycoprotein with molecular weight just below 84 kDa showed a rhythm in abundance with a reverse phase, with lowest levels detected at ZT 16 and 20 (Fig. 5a, open arrow). Most of the rhythmic changes in the glycoprotein levels were abolished in moths held in LL such that intermediate levels of these proteins were present in blots from different time points, or changes in protein levels were random with respect to time of day (Fig. 5b).

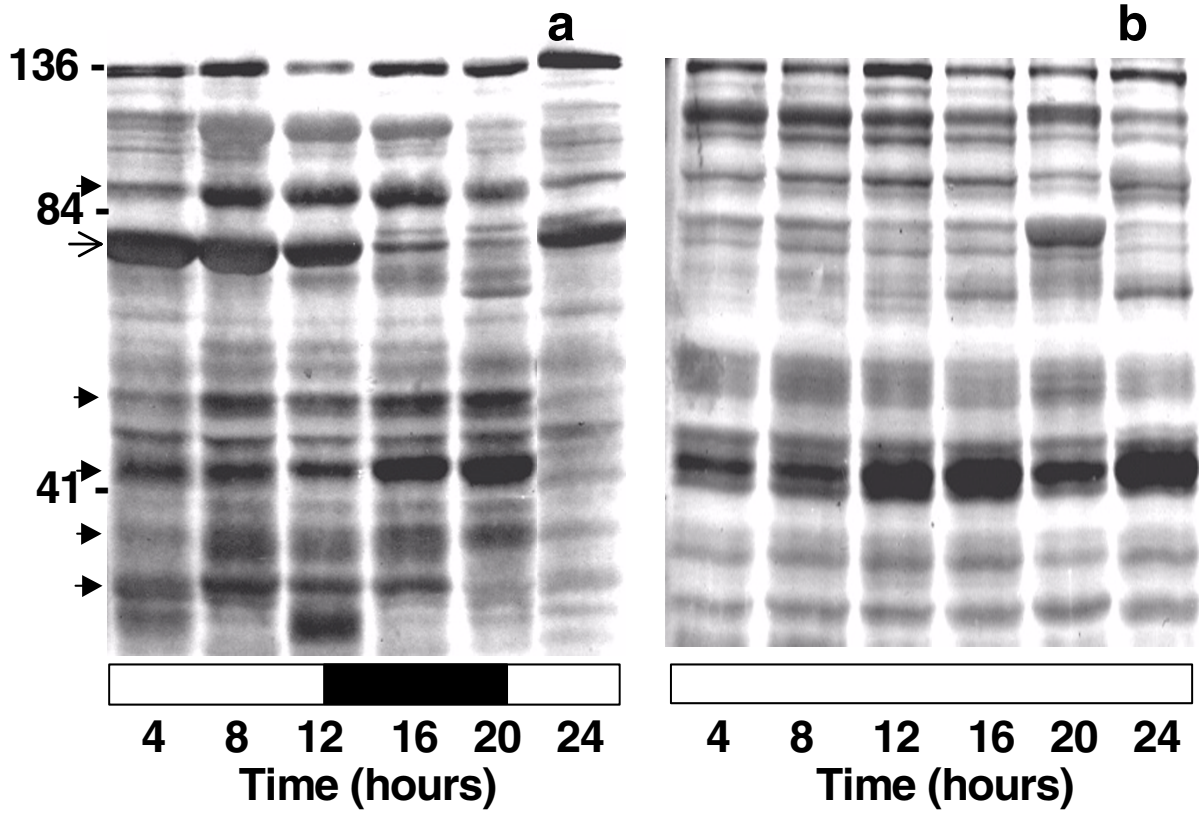

Figure 5
Temporal pattern of glycoproteins in the UVD lumen. Glycoproteins from the seminal fluid of LD (**a**) and LL (**b**) males were separated on SDS-PAGE, electro-blotted, and probed with BCHZ. In LD, several glycoproteins show distinct rhythms in their levels (arrows). In LL, there were no consistent rhythms in the levels of glycoproteins. The experiment was repeated 3 times with similar results. White and black horizontal bars indicate periods of light and darkness, respectively.

Discussion

Our histochemical data showing rhythms in the levels of carbohydrate-rich material combined with rhythmic glycoprotein release provide strong evidence for circadian rhythms in the secretory activity of the UVD epithelial cells in *S. littoralis*. In LD, carbohydrate-rich material is present in the apical portion of the UVD cells during the night and absent during the day. A similar pattern persists in DD demonstrating that the rhythm is circadian in nature and not driven by light-dark cycles. In constant light, moderate levels of secretory granules were detected in the UVD at all times of day. Our initial qualitative assessments of the daily pattern in the levels of secretory granules were fully confirmed with imaging software measurements of darkly stained granules in the apical cytoplasm. No dark granules were detected in the cytoplasm in mid-day; the proportion of dark cytoplasm increased at the end of the day and during the night. Such rhythmic changes were observed in both LD and DD but not in LL. Quantitative analysis also revealed that the size of the granules changes with time. Small-sized granules, which predominate in the early night, are later replaced by granules of larger-size. There are several possible interpretations of this phenomenon. First, smaller granules may be combined into larger ones before their content is secreted to the lumen, or granules may enlarge in the process of maturation [25–27]. Second, granules of different sizes may be independently produced and contain different secretory materials. Finally, large granules may represent multivesicular bodies that are formed during endocytosis

[28]. Although our present investigation cannot distinguish between these possibilities, it demonstrates the existence of complex rhythms in the cellular activities associated with the formation and modification of secretory granules.

Our data strongly suggest that periods of intense secretion occur in the UVD epithelial cells. In most epithelial cells, the secretory materials are released via exocytosis following fusion of secretory vesicles with the cell membrane. However, apocrine secretion is also known in insects during which membrane-bound proteins are released from the cells. Both types of secretion may co-exist in one tissue. For example, different digestive enzymes in *Tenebrio molitor* are delivered to the gut lumen by either exocytosis or apocrine release [29]. The fact that PAS-positive material in the UVD lumen has amorphous rather than punctuate appearance suggests that exocytosis is the main route for the delivery of the secretory material to the seminal fluid. Previous ultrastructural studies showed the presence of a microvillar border in the UVD epithelium of two moth species; *A. kuehniella* [19] and *L. dispar* [20]. This, together with our current results on *S. littoralis*, strongly supports the secretory function of the UVD in moths.

In our attempt to characterise the nature of PAS-positive material secreted from the UVD, we excluded glycogen by showing that PAS staining persists after treatment with the glycogen-digesting enzyme, diastase. We demonstrated instead that several of the glycoproteins present in the homogenates of the UVD wall increase in abundance in a temporal pattern consistent with the accumulation of PAS-positive material in the epithelial wall. In addition, we found daily rhythms in the levels of several glycoproteins in the UVD lumen. Side-by side separation of epithelial and luminal proteins did not reveal rhythmic proteins with corresponding molecular weights. It is possible that secreted proteins undergo modifications such as cleavage of the signal peptide or changes in carbohydrate moieties. Such modifications of sperm duct glycoproteins were reported in mammals [30]. Future sequencing of rhythmically secreted proteins will help to establish relations between glycoproteins in the UVD wall and lumen in S. littoralis.

We hypothesize that glycoproteins released from the UVD epithelium may be involved in post-testicular sperm maturation. Sperm maturation (capacitation) which is important for the acquisition of fertilizing ability, have been investigated mostly in mammals. Spermatozoa released from seminiferous tubules of testes acquire fertilizing ability during interaction with the epididymal epithelium and seminal fluid [30]. There is evidence that glycoconjugates play many roles in these processes. Enzymes present in the upper part of the mammalian seminal tract modify sperm surface glycoproteins by adding or cleaving sugar residues [31,32]. Also, new glycoproteins from the epididymal fluid are added to the sperm surface during epididymal transit [33]. Several lines of evidence suggest that common sperm-maturation processes may occur between mammals and insects. First, homologs of mammalian sperm surface glycosidases have been identified in Drosophila [34,35]. Second, ultrastructural studies in moths demonstrated changes in the spermatozoa membrane during their passage through the UVD [21,36]. These data, together with our demonstration that glycoproteins are released from the UVD of *S. littoralis*, suggest that sperm maturation may involve conserved processes from insects to mammals.

The novel aspect of our study is the demonstration that the accumulation and release of several glycoproteins occurs in a circadian rhythmic fashion in *S. littoralis*. It is likely that the secretory activity of the UVD epithelium is controlled by a local circadian clock. Previous research showed that the reproductive system of *S. littoralis* contain a circadian clock which can drive rhythmic release of sperm in vitro [12]. We also determined by RT-PCR that the clock gene *per* is expressed in the reproductive system of *S. littoralis* and PER protein shows rhythms in nuclear localization in all UVD epithelial cells (Bebas and Gvakharia, unpublished). Therefore, it is likely that each cell of the UVD possess a clock mechanism, which may be driving rhythmic production and secretion of glycoproteins. Rhythmic secretion of specific neuromodulators has been reported in the central clock of mammals, the suprachiasmatic nucleus (SCN) [37,38]. Recent microarray analysis of circadianly regulated genes in the SCN showed rhythmic expression of genes encoding components of the secretory pathway [39]. Thus, it appears that the circadian clock may regulate secretory activities in different organisms and different cell types, from epithelial cells to neurons.

What is the functional significance of the circadian clock in the reproductive system? Our hypothesis is that the clock provides temporal coordination of processes associated with sperm maturation. In addition to rhythmic secretory activity reported here, we recently demonstrated a pH rhythm in the UVD lumen, which is generated by rhythmic changes in the levels of the proton pump, vacuolar H+ATPase [40]. A functional link between the UVD rhythms and fertility is inferred from the fact that in LL both the rhythms are disrupted and the fertility is impaired. [13,14]. A similar link may also exist in flies. Recently we obtained direct evidence that the circadian clock plays an important role in reproductive fitness of *D. melanogaster*. The loss of clock function in flies with mutated clock genes results in approximately a 40% decline in fertility compared to wild-type flies [15]. Thus, involvement

of the circadian clock in the reproductive physiology may be conserved across species.

Methods

Animals

S. littoralis, were reared at 25 ± 0.5°C, at 70% relative humidity on artificial diet [41], adults were fed with 20% honey solution in water. Sexed pupae and newly emerged males were kept in cycles of 16 h light and 8 hours darkness (LD, 16:8) in thermostats equipped with a fluorescent lamp providing illumination of approximately 0.7 Wm^{-2}. Lights were switched off at Zeitgeber time (ZT) 12, and switched on at ZT 20. In specific experiments, males were transferred into constant light (LL), or constant darkness (DD) one day prior to adult eclosion. All males used in this study were two-day old adults.

Histochemistry

Tissues for histological investigations were collected every 4 hours, for one day, beginning at ZT4 on the second day after eclosion. The testis-vas deferens complexes were dissected in buffered saline from 8–10 males per time point, fixed in Bouin's solution [42] for 12 hr, dehydrated in increasing ethanol series, embedded in paraffin and cut into 6 μm thick sections. Following the removal of paraffin and re-hydration, sections were subjected to periodic acid-Schiff (PAS) reaction which detects carbohydrates in tissues, and Feulgen reaction to visualize cell nuclei, according to published protocol [42]. The orange-G cytoplasmic stain was used as a counter-stain and then specimens were dehydrated and mounted in DPX medium. Because the PAS reaction detects carbohydrates conjugated with proteins as well as glycogen, we checked whether the latter is present in the UVD by digesting tissues sections with diastase (Fisher Scientific). For this control experiment, tissue sections were hydrated, treated with 0.05, 0.1 and 0.5% solution of diastase in water at 37°C for 15 min, and then stained with PAS and Feulgen reactions as described above.

Protein electrophoresis and blotting

Patterns of glycoproteins in the UVD wall and lumen were determined in tissues dissected every 4 h for 24 h, at times corresponding to histochemical examination of glycoproteins in the UVD. UVDs were cut out from the reproductive system and collected in cold Grace's medium (Gibco BRL). UVD walls were gently cut open to release the lumen content, then the walls were removed, washed several times in Graces medium, collected in an Eppendorf tube and sonicated. The lumen content was transferred to a new Eppendorf tube and centrifuged at 1500 × g to separate sperm from the seminal fluid. Total soluble proteins were extracted from the wall and seminal fluid samples as described previously [43]. Equal amounts of total protein were loaded onto 10% SDS-PAGE gels, separated, and transferred to PVDF membrane. Blots were probed for glycoprotein conjugates using biocytin hydrazide (BCHZ) as described [44]. Blotted glycoproteins were chemically oxidised with periodate and then labeled with BCHZ. The latter was detected with avidin-conjugated alkaline phosphatase (both reagents from Pierce). The signal was visualized with a color reaction involving NBT/BCPI (Roche Molecular Biochemicals) as alkaline phosphatase substrates.

Quantitative analysis of secretory granules

For the quantitative assessment of the levels of darkly stained, PAS-positive granules, PAS-stained sections of testis-vas-deferens were compared using image analysis software. The images were analysed using a Nikon Eclipse E-600 microscope, with a Panasonic GP-KR222E camera, equipped with Lucia G version 4.21 software (Laboratory Imaging Ltd.). The analysed areas were chosen to encompass the apical portions of 4–5 UVD epithelial cells (ca. 1/3 of the cells' length, between the nuclei and the brush border). A rectangle of 100 × 150 pixels was measured in each preparation (measurement frame). Threshold values (in a range from 0 to 255) were set by investigators separately for red, blue and green channels to select areas corresponding to pinkish-brown PAS-positive granules. To quantify the levels of secretory granules, the Lucia software measured cytoplasmic regions within the threshold values and then calculated % of the apical cytoplasm containing granules. To determine the size of granules at different times of day, the software calculated the area of each individual granule, as well as their number in the measurement frame. Three size classes of the granules were delineated: small – below 0.3 μm^2; medium – between 0.3 and 1 μm^2; and large – above 1 μm^2. The data were analysed statistically using Statistica software (StatSoft).

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