AN ABSTRACT OF THE THESIS OF

Laura M. Beaver for the degree of Master of Science in Environmental Sciences presented on December 10, 2002. Title: Involvement of Circadian Clock Genes in Reproduction of *Drosophila melanogaster*

Redacted for privacy

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Daily (circadian) rhythms exist at molecular, physiological, and behavioral levels and coordinate many life functions. This coordination is believed to contribute to an organism's fitness, however, such contributions have not been convincingly demonstrated in any animal. The most significant measure of fitness is the reproductive output of the individual and species. In this thesis I examine the consequences of loss of clock function on reproductive fitness in *Drosophila melanogaster*. I demonstrated that single mating among couples with mutated *period* (per^0), *timeless* (tim^0), *cycle* (cyc^0), and *Clock* (Clk^{Jrk}) genes resulted in approximately 40% fewer progeny compared to wild-type flies. Male and female contribution to this phenotype was demonstrated by a decrease in reproductive capacity among per^0 and tim^0 flies mated with wild-type flies of the opposite sex. The important role of clock genes for reproductive fitness was confirmed by reversal of the low fertility phenotype in flies with rescued *per* or *tim* function. These results prompted an investigation to determine the relative contribution of each sex to the fertility phenotype. Males lacking a functional clock showed a significant decline in the quantity of sperm released from the testes to seminal vesicles (SV), suggesting that this peripheral oscillator is involved in sperm maturation. We found that clock genes are rhythmically expressed in these tissues and the cycling of *per* and *tim* expression continued in vitro, hence the testes and SV complex contained an autonomous circadian

clock. In contrast to males, PER and TIM were constantly present in the cytoplasm of follicular cells in fly ovaries. Ovarian expression of *per* and *tim* is not disrupted by constant light and females lacking *per* and *tim* produced nearly 50% fewer mature oocytes then wild-type flies. These results suggest that *per* and *tim* are acting in a non-circadian pathway in the ovaries. Taken together, this data demonstrates that circadian clock genes significantly contribute to the fitness of *Drosophila melanogaster* by affecting the fecundity of both sexes.

Involvement of Circadian Clock Genes in Reproduction of *Drosophila melanogaster*

by

Laura M. Beaver

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CONTRIBUTION OF AUTHORS

Dr. Jaga Giebultowicz was involved in the design, analysis, and writing of each manuscript. She additional performed the real time expression studies in chapter 2 in collaboration with Dr. Ralf Stanewsky's lab. Dr. Barbara Gvakaharia and David Hege performed the in situ hybridizations studies in chapters 2 and 3. Trevor Vollintine participated in ICC and the quantification of sperm experiments in chapter 2. Brandy Rush participated in ICC and in the quantification of mature oocytes experiments in chapter 2.

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Involvement of Circadian Clock Genes in Reproduction of *Drosophila melanogaster*

CHAPTER 1

Introduction and Background

General Overview of Thesis

Body functions are synchronized with the daily oscillations of light and temperature caused by the rise and fall of the sun. The internal biological mechanism that maintains this synchrony is called the circadian clock (latin *circa* for "about" and *dies* for "day"). Circadian clocks which generate circadian rhythms persist even in the absence of external cues, thus demonstrating that there is an endogenous timing mechanism.

Circadian clocks allow the body to anticipate daily changes in the environment such that different physiological and behavioral processes in an organism can occur at the most appropriate time of day. An example of this is the control of the eclosion rhythm by the circadian clock in many insects (Brady, 1974; Saunders, 1982). In several insect species including *Drosophila melanogaster,* adult eclosion from the pupal case is timed for the early morning when suitable environmental conditions of moisture and temperature exist. These conditions are most appropriate for eclosion because they provide the optimal physical environment for wing spreading and sclerotization of the cuticle (Truman, 1992).

Circadian clocks have been found in organisms ranging from cyanobacteria to humans but their direct contribution to an organism's fitness has not been convincingly demonstrated in any animal. In *D. melanogaster,* mutations in the circadian clock gene *period* demonstrated that the circadian clock had pleitropic effects on developmental timing and longevity (Hall, 1995). The most significant measure of fitness is the reproductive output (fertility and fecundity) and survival of the individual and species (King and Stansfield, 1985). The effect of the circadian system on reproductive fitness has not been directly investigated in any species.

D. melanogaster is an excellent model species for gaining insights into the circadian clocks role in fitness since the core circadian clock mechanism is known and mutations in key circadian clock genes have been generated. I initiated reproductive studies in *D. melanogaster* to directly investigate the possible links between circadian clock genes and fitness. The specific aims of this thesis are: 1) Determine whether circadian clock genes play a significant role in fertility. 2) Perform a detailed spatial and temporal examination of circadian clock genes in the reproductive system. 3) Examine the relative contribution of circadian clock genes to fecundity in male and female reproductive systems.

Molecular Basis of Circadian Clock in *Drosophila melanogaster*

The genetic basis of the circadian clock was initially demonstrated by R. Konopka in 1971. He discovered the first circadian clock gene, *period* (*per*), by demonstrating that mutations at this locus altered the rhythms of eclosion and locomotor activity (Konopka and Benzer, 1971). Extensive studies investigating the molecular mechanism that controls circadian rhythms ensued in several different organisms leading to discoveries of additional circadian clock genes. These studies revealed that the core molecular mechanism that controls circadian clocks is highly conserved among animals while the details vary between species (Young and Kay, 2001). Below I will discuss the circadian clock mechanism as it is now known in *Drosophila.*

The core circadian clock mechanism of *D. melanogaster* is comprised of two interacting transcriptional feedback loops (Fig. 1.1). The first loop consists of the rhythmic transcription and translation of clock genes *period* (*per*) and *timeless* (*tim*) followed by accumulation and nuclear translocation of their proteins, PER and TIM in the form of a hetrodimer (Hardin et al., 1992; Sehgal et al., 1995). These proteins indirectly inhibit activity of their own genes and may activate or inhibit activity of other genes. TIM is degraded in response to light; and this light-sensitivity contributes to synchronization of the internal clock mechanism to the external cycles of light and dark (Hunter-Ensor et al., 1996; Myers et al., 1996; Zeng et al., 1996). The gene *cryptochrome* (*cry*), encodes a photoreceptor protein CRY, which mediates this degradation of TIM (Emery et al., 2000; Ivanchenko et al., 2001; Stanewsky et al., 1998). Together, the proteins TIM and CRY are involved in a mechanism which resets the clock on a daily basis. Interestingly, the same mechanism that causes the synchronization of circadian clocks in light/dark cycles

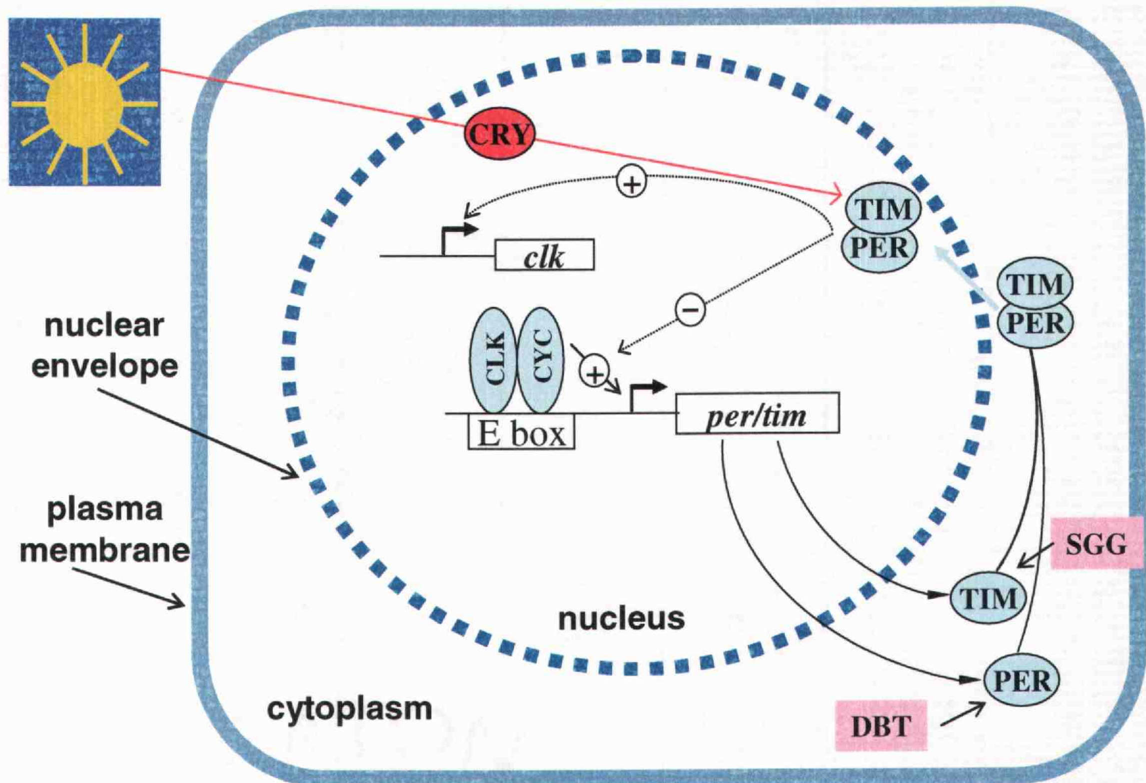

Figure 1.1 Molecular mechanism of the circadian clock in *Drosophila melanogaster*. Abbreviations in italic represent genes, capitals represent proteins. The four core clock proteins PERIOD (PER), TIMELESS (TIM), CLOCK (CLK), and CYCLE (CYC) are shown in blue circles. The photoreceptor protein CRYPTOCHROME (CRY) is illustrated in red and the proteins that participate in the circadian clock via phosphorylation, SGG (SGG) and DOUBLETIME (DBT), are shown in pink.

(LD) results in a disruption of circadian clocks in constant light (LL). Under LL conditions, no TIM is available to dimerize with PER due to the constant degradation of the former (Price et al., 1998). Consequently the circadian clocks in the fly are disrupted in the presence of constant light, and this disruption results in the loss of all circadian rhythms.

The second circadian loop involves the rhythmic transcription of the gene *dClock* (*dClk*) (Allada et al., 1998). Transcription of *dClk* is positively regulated by the protein PER and occurs in the opposite phase with regard to *per* and *tim* transcription (Bae et al., 1998). The protein dCLOCK (dCLK) forms a heterodimer with the protein product of a constitutively expressed circadian clock gene *cycle* (*cyc*) (Rutila et al., 1998b). Together, dCLK-CYC heterodimers activate the transcription of *per* and *tim*. Accumulating PER-TIM dimers enter the nucleus and interfere with dCLK-CYC, thus completing the clock cycle in an approximate time span of 24 hours (Darlington et al., 1998; Lee et al., 1999). There are two additional genes that are known to contribute to the circadian clock; *shaggy* (*sgg*) and *doubletime* (*dbt*) participate in the circadian loop through their role in phosphorylation of TIM and PER respectively reviewed in (Stanewsky, 2002). Recently, DBT was shown to physically associate with the PER-TIM complex and facilitate nuclear translocation of this complex (Kloss et al., 2001).

Production and Fertilization of Gametes in *Drosophila melanogaster*

Spermatogenesis. The male reproductive tract in *Drosophila melanogaster* consists of the testes, seminal vesicles, accessory glands, ejaculatory duct, and the ejaculatory bulb. These organs are organized as shown in Figure 1.2. Spermatogenesis, the production of sperm, primarily takes place in the testes although the seminal vesicles may play a role in sperm maturation (Fuller, 1993).

Sperm production begins at the apical tip of a testis with the mitotic division of a stem cell located within the germarium (Fig. 1.3). This division results in a new stem cell and a primary spermatogonial cell. The primary spermatogonial cell is enveloped by two somatic cyst cells. The primary spermatogonial cell and two cyst cells are known as a cyst. After the cyst is formed and exits the germarium, the primary spermatogonial cell undergoes four mitotic divisions in which cytokinesis is incomplete. These divisions results in a cyst containing 16 spermatocytes that remain connected by cytoplasmic bridges.

The cyst then enters into a growth phase in which the cyst grows 25 times in volume (Fuller, 1993). This growth is due to a massive amount of transcription and translation of genes that are required to trigger cellular differentiation. Once growth is completed and all necessary genes have been expressed the spermatocytes within the cyst enter meiosis. These incomplete meiotic divisions results in a cyst containing 64 haploid spermatids that are joined by cytoplasmic bridges. Spermatids undergo the process of elongation and differentiation; the acrosome is formed, nuclei become compact and elongated, mitochondria fuse into two mitochondrial

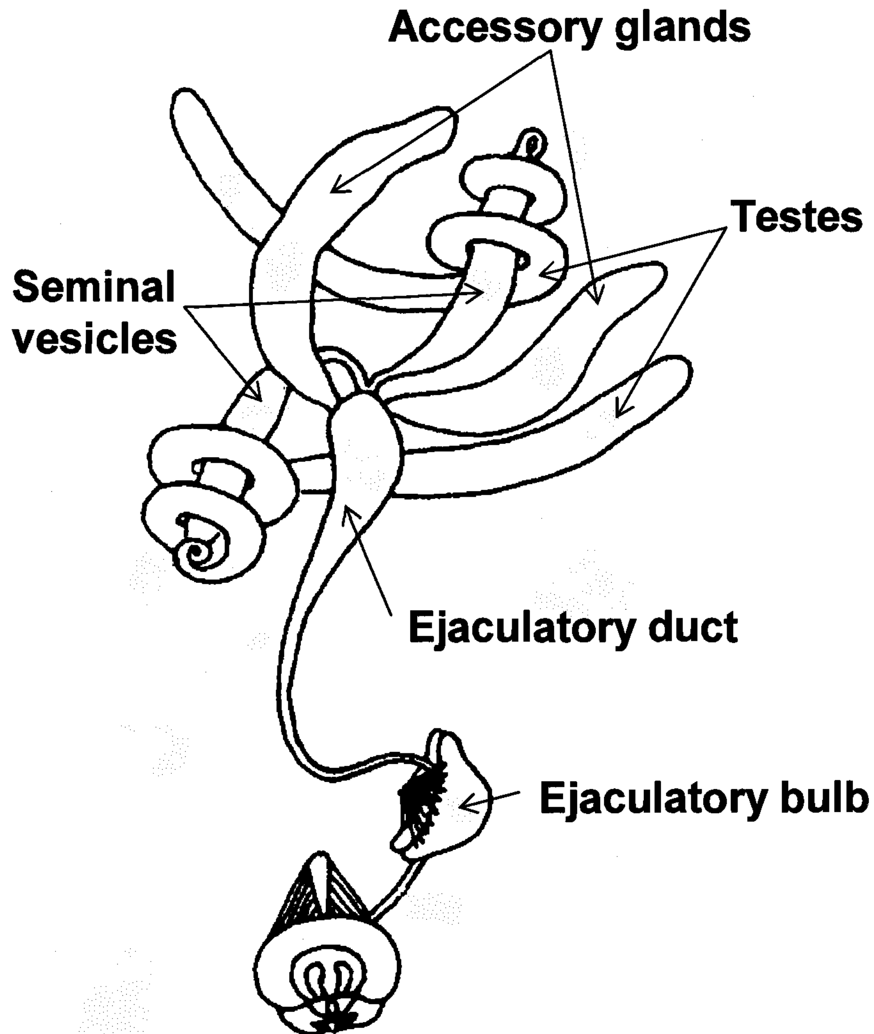

Figure 1.2 Organization of the male reproductive tract in *Drosophila melanogaster*. The male reproductive tract primarily consists of the testes, seminal vesicles, accessory glands, ejaculatory duct, and the ejaculatory bulb.

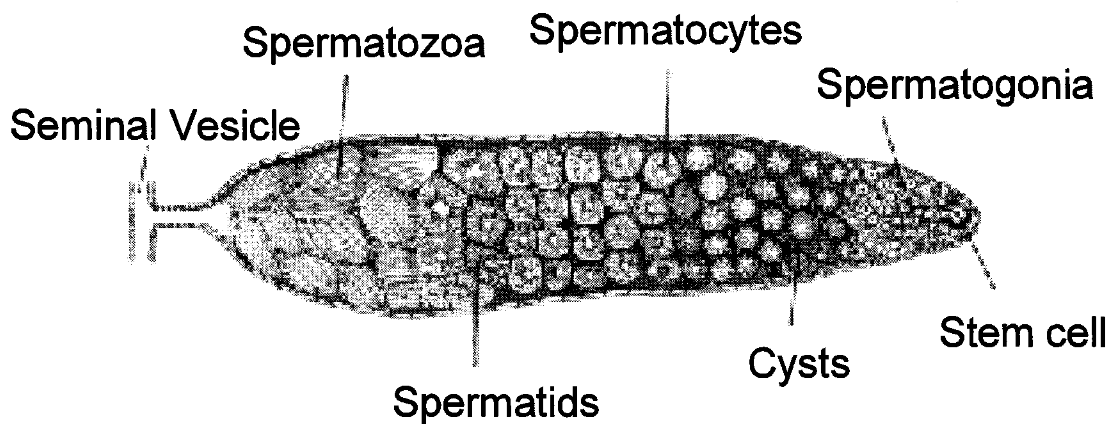

Figure 1.3 Anatomy of spermatogenesis. This diagram illustrates the general process of spermatogenesis in an insect testis. Spermatogenesis in *Drosophila melanogaster* begins with the division of a stem cell at the apical tip of the testis and continues until 64 individual spermatozoa are released into the seminal vesicle.

spermatids purge the majority of their cytoplasm causing the disruption of the derivatives, and the axoneme elongates into flagellum-like tail. At this stage the cytoplasmic bridges. In this process the spermatids become individualized and separated from each other but remain confined within the cyst (sperm bundle). At the end of the differentiation process sperm bundles undergo coiling and the somatic cyst cells become embedded in the terminal epithelium of the testis. Here the 64 individual spermatozoa are released into the base of the testis and subsequently enter the seminal vesicle where they are stored until copulation takes place. The sperm is thought to undergo further maturation processes in the seminal vesicles but the details of this are not full understood in *Drosophila.* At some undetermined point in the testes base or in the seminal vesicles the sperms flagellated tails become active (Fuller, 1993).

Oogenesis. The female reproductive tract in insects consists primarily of the ovaries, lateral oviducts, common oviduct, spermatheca, accessory glands, and the genital chamber; these organs are shown in Figure 1.4. Oogenesis, the production of mature oocytes (eggs), is a complicated developmental process during which oocytes are produced in an "assembly line" within an ovariole (Fig. 1.5B). In *Drosophila melanogaster* there are on average 16 ovarioles in each ovary that are held together by a connective tissue known as the peritoneal sheath (Fig. 1.5A). The ovaries are also intertwined with trachea that provides as vehicle for gas exchange. Several additional organs participate in oogenesis including vitellogenin-producing fat body and the brain, which controls oocyte production via hormonal cues.

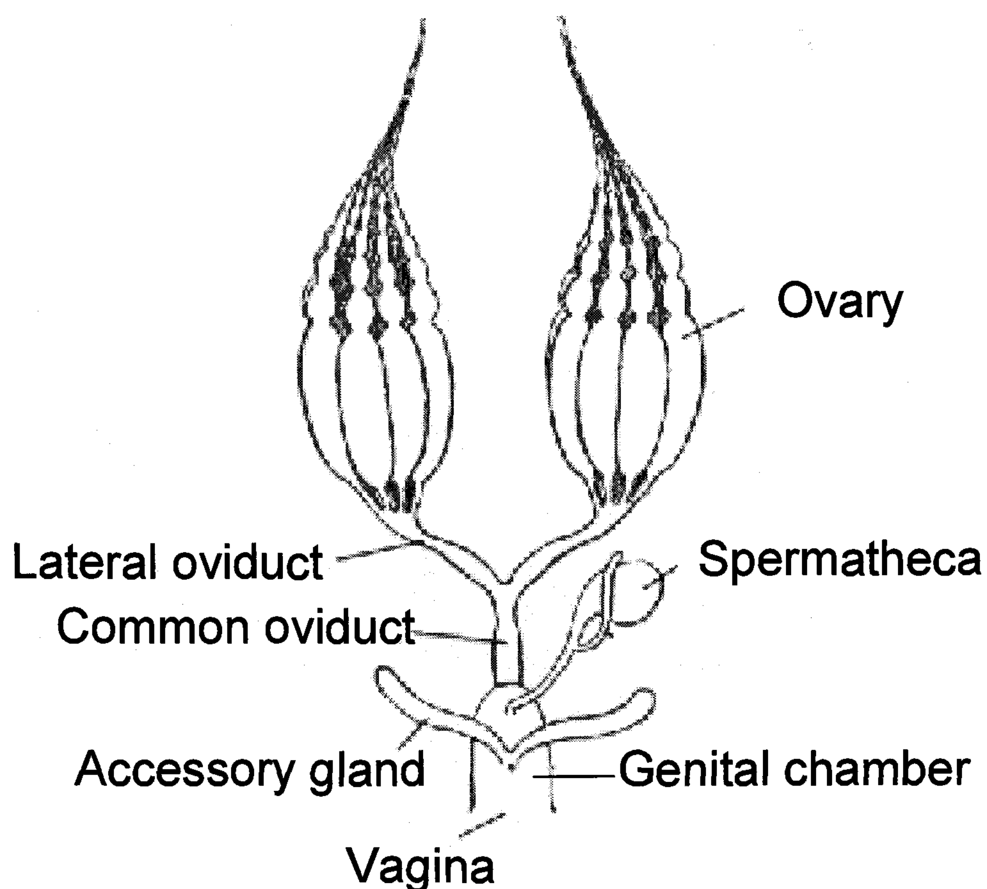

Figure 1.4 Female reproductive system. This diagram illustrates the general organization of the female reproductive tract in insects. The female reproductive system primarily consists of the ovaries, lateral oviducts, common oviduct, spermatheca, accessory glands, and the genital chamber.

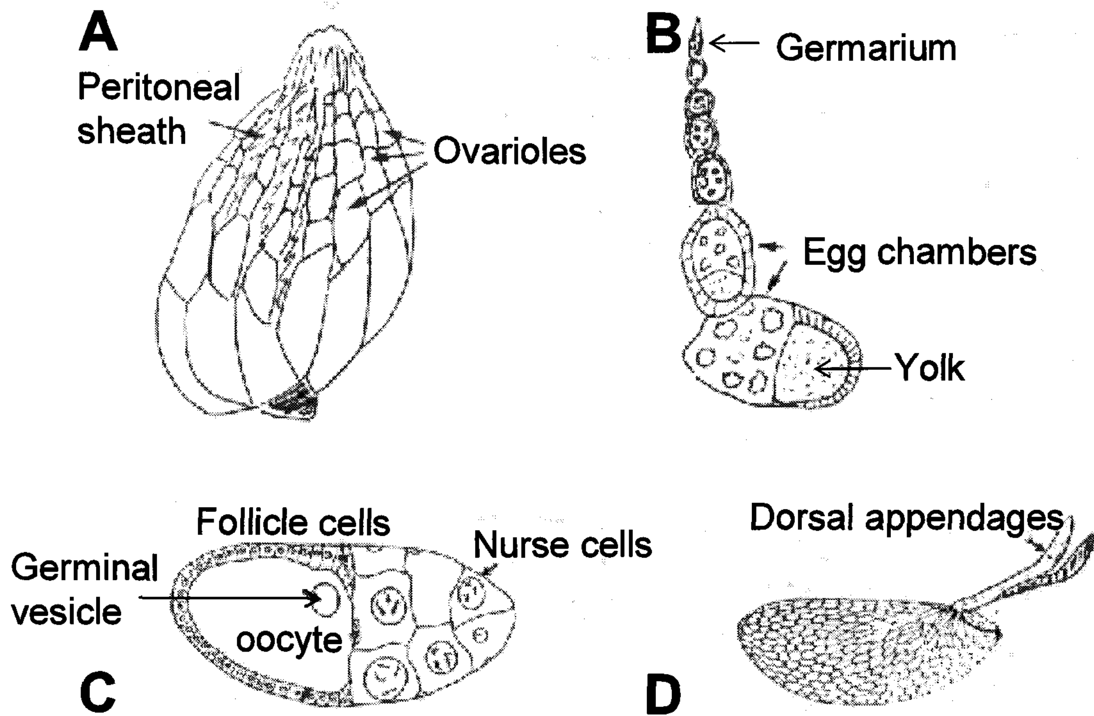

Figure 1.5 Anatomy of oogenesis in *Drosophila melanogaster*. The illustration shows an ovary containing 16 ovarioles (A), a single ovariole (B), a vitellogenic egg chamber (C), and a mature oocyte (D).

The formation of a mature oocytes (mature egg chambers) begins in the germarium of an ovariole when a germ cell undergoes a meiotic division (Spradling, 1993). This division results in the formation of a haploid cell known as the cystoblast. While still in the germarium the cystoblast undergoes 4 mitotic divisions yielding a 16 cell cystocyte. The cystocyte is composed of 15 cells that will differentiate into nurse cells and one cell that becomes the oocyte. These 16 cells are interconnected by cytoplasmic bridges due to incomplete cytokinesis during mitosis. As the cystocyte exits the germarium it is enveloped by somatic follicular epithelium (follicle cells). At this point in oogenesis the egg chamber enters a growth phase known as previtellogenesis.

During previtellogenesis, the oocyte differentiates from the nurse cells. The chromosomes within the nucleus of the oocyte, the germinal vesicle, become condensed into karyosomes. In contrast to the oocyte, the nurse cells begin producing large quantities of mRNA, protein, ribosmes and mitochondria. These products are eventually passed to the oocyte via polarized transport through the cytoplasmic bridges. Follicle cells undergo 4-5 divisions during this time in order to maintain a monolayer of cells around the periphery of the egg chamber. Follicle cells also produce yolk proteins (YPs) during this phase of oogenesis. Previtellogenesis takes approximately 2 days from the time the egg chamber leaves the germarium until it is ready to enter the next stage, vitellogenesis.

Vitellogenesis is dominated by the uptake of YPs, vitellogenins, by the oocyte. These YPs will provide the embryo with a rich source of nutrients for growth (Spradling, 1993). Vitellogenesis begins with the migration of the majority of the follicle cells over the oocyte leaving only a few associated with the nurse cells (Fig. 1.5C). These follicle

cells then release the YPs that they produced to the oocyte. The follicle cells also regulate the uptake of vitellogenins produced by the fat body by controlling their passage to the oocyte via intercellular spaces (patency). The YPs are taken up by the oocyte via receptor-mediated endocytosis. Juvenile hormone and ecdysone regulate the entry of egg chambers into vitellogenesis.

After completion of yolk uptake, the egg chamber enters the last phase of oogenesis known as post vitellogenesis or choriogenesis (Spradling, 1993). The oocyte gains a shell and becomes competent for fertilization during this phase. Choriogenesis begins when the nurse cells complete the dumping of their contents and begin to degenerate. During this phase follicle cells produce a waterproof vitellin membrane, secrete the egg shell (chorion), and finally degenerate. Several additional distinct features are formed during choriogenesis including the dorsal appendages (provides oxygen for the developing embryo), a micropyle (allows the sperm to enter and fertilize the mature oocyte), and a larval exit door (Fig 1.5D).

Fertilization of gametes. In order for fertilization to occur a male and female fly (possessing mature sperm / oocytes respectively) must first meet. The male must then successfully woe the female by song, smell, and touch, and then copulation may take place. During copulation the male passes sperm and seminal fluid to the female which is then stored in the female's seminal receptacles and spermatheca. After the female has successfully copulated with a male she allows a mature oocyte to pass from her ovaries through her lateral and common oviducts. The stored sperm then has the opportunity to fertilize the egg. If fertilization takes place one sperm will successfully enter the

micropyle of the mature oocyte. Fertilization is then followed by oviposition of the egg by the female on a suitable substrate for the developing embryo and larva to grow.

Involvement of Circadian Clocks in Reproduction

The four core clock genes, *per, tim, dClk,* and *cyc* are essential for circadian rhythms in eclosion and locomotor activity. A null mutation in any of these genes results in a dysfunctional clock that yields behaviorally arrhythmic flies (Williams and Sehgal, 2001). Outside of the circadian clocks role in rhythmic activity, the clock has also been linked to other rhythmic events that occur throughout the day in many insect species, such as rhythms in the levels of hormones and metabolites in the hemolymph (Giebultowicz, 2001; Saunders, 1982) .

Several circadian rhythms have been described in insects that fall into the category of reproductive behaviors. Daily rhythms in pheromone release and spermatophore production have been described in moths and crickets respectively (Loher, 1974; Raina and Menn, 1987); a daily rhythm in oviposition was also described in *Drosophila* (Sheeba et al., 2001). Circadian clock genes have been directly linked to a mating rhythm in *Drosophila* (Sakai and Ishida, 2001). Additionally the circadian clock gene *period* was shown to play a role in the male courtship song. Mutations that effect the free-running period of behavioral rhythms per^{S} and per^{L} also changed the ultradian rhythm which controls the frequency of wing beating that generates the courtship song (Kyriacou et al., 1990b).

The most robust physiological circadian rhythms in reproduction are the rhythms associated with release and maturation of sperm in moths. Release of sperm bundles

from the testis to the vas deferens (VD) is restricted to a certain time of day (Bebas et al., 2001; Giebultowicz et al., 1988). The lumen of the VD shows a daily rhythm in pH while VD epithelial cells display rhythms in levels of apical targeting of vacuolar H+ ATPase and secretion of glycoproteins (Bebas et al., 2002a; Bebas et al., 2002b). These rhythmic cellular activities in the VD epithelium are thought to aid the maturation of sperm released from the testis. Disruption of this rhythm by constant light renders males sterile due to a severe decline in the number of sperm bundles released and incorrect processing of sperm in the VD (Giebultowicz et al., 1990). This decline in sperm quantity and quality in LL gave us indirect evidence that the circadian clock may play a significant role in fitness of insects.

Organization of the Circadian System in *Drosophila*

Molecular characterization of the circadian clock mechanism in different animals was followed by the discovery that circadian clock genes are widely expressed in many different tissues. Rhythmic expression of clock genes in *Drosophila* was detected in the "Lateral Neurons" (LNs) of the brain which are considered the central clock and regulate activity rhythms in the fly (Siwicki et al., 1988). In addition, many peripheral organs isolated *in vitro* maintain oscillations of clock genes that are directly entrainable by light-dark (LD) cycles (Hege et al., 1997; Plautz et al., 1997a). This knowledge raised the possibility that independent circadian oscillators may exist in each tissue that rhythmically express circadian clock genes (Hall, 1995; Tei et al., 1997; Whitmore et al., 1998).

The expression of *per*, *tim* and the proteins that they encode were shown to occur with the same phase in the brain and different peripheral organs (Giebultowicz and Hege, 1997; Giebultowicz et al., 2000). Studies on the peripheral clock in Malpighian (renal) tubules demonstrated substantial autonomy of this oscillator. First, it was demonstrated that the central clock does not mediate the re-setting of the tubule oscillator in response to a shift in the LD regime. Second, tubules transplanted into a host entrained to a reversed LD cycle continued to cycle out of phase with the host despite sharing a common hormonal milieu, thus demonstrating substantial autonomy of the timing mechanism in the Malpighian tubule oscillator (Giebultowicz and Hege, 1997; Giebultowicz et al., 2000). Additionally, a circadian clock in the fly antenna was shown to be independent of the central clock. The circadian clock in the antenna mediates the sensitivity of chemoreceptors to food-related odors (Krishnan et al., 1999; Krishnan et al., 2001). These studies gave us a good understanding of the organization of the circadian system and the scope of its expression in the fly. The wide range of clock expressing tissues and the independence of each oscillator suggested that the circadian clock performed an important function in each clock expressing tissue. Yet, the concrete evidence for the physiological role of most peripheral clocks is absent.

Studies that looked at the range of clock gene expression in the body revealed that clock genes were expressed in the reproductive system of the fly but the spatial and temporal patterns were not examined in detail (Kaneko and Hall, 2000; Liu et al., 1988; Saez and Young, 1988) . It had also not been determined whether the gonads contained an oscillator mechanism or whether the genes were acting in a non-circadian pathway. The presence of clock genes in the gonads and the indirect evidence suggesting the

importance of circadian rhythms in moth reproduction prompted my study of the functional significance of circadian clock genes in male and female reproductive systems.

CHAPTER 2

Loss of circadian clock function decreases reproductive fitness in males of *Drosophila melanogaster*

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Abstract

Circadian coordination of life functions is believed to contribute to an organism's fitness, however, such contributions have not been convincingly demonstrated in any animal. The most significant measure of fitness is the reproductive output of the individual and species. Here we examined the consequences of loss of clock function on reproductive fitness in *Drosophila melanogaster* with mutated *period* (per^0), *timeless* (tim^0), *cycle* (cyc^0), and *Clock* (Clk^{Jrk}) genes. Single mating among couples with clock-deficient phenotypes resulted in approximately 40% fewer progeny compared to wild-type flies, due to a decreased number of eggs laid and a greater rate of unfertilized eggs. Male contribution to this phenotype, was demonstrated by a decrease in reproductive capacity among per^0 and tim^0 males mated with wild-type females. The important role of clock genes for reproductive fitness was confirmed by reversal of the low fertility phenotype in flies with rescued *per* or *tim* function. Males lacking a functional clock showed a significant decline in the quantity of sperm released from the testes to seminal vesicles and these tissues displayed rhythmic and autonomous expression of clock genes. By combining molecular and physiological approaches, we identified circadian clock in the reproductive system and defined its role in the sperm release that promotes reproductive fitness in *D. melanogaster*.

Introduction

Many life functions, from cellular activities to behavior, display daily (circadian) rhythms. These rhythms are generated by cell-autonomous circadian clocks and involve several genes encoding transcriptional regulators, which are substantially conserved among animals from fruit flies to humans (Dunlap, 1999). The core clock mechanism in

Drosophila melanogaster involves rhythmic transcription of the *period* (*per*) and *timeless* (*tim*) genes followed by nuclear accumulation of their proteins, PER and TIM. Another gene, *Clock* (*Clk*), shows mRNA oscillations that are out of phase with the oscillations of *per* and *tim* mRNAs. Protein encoded by *Clk*, together with protein encoded by the gene *cycle* (*cyc*), activate transcription of *per* and *tim*. These four genes are essential for the function of the brain clock and a null mutation in any of them renders the fly behaviorally arrhythmic (Williams and Sehgal, 2001).

While the importance of circadian clocks in controlling behavior is well documented, their functional significance for physiology is less understood. Clock genes are rhythmically and autonomously expressed in peripheral tissues of *D. melanogaster*, (Giebultowicz et al., 2000; Krishnan et al., 2001; Plautz et al., 1997a), zebrafish (Whitmore et al., 1998), and mammals (Yamazaki et al., 2000), suggesting that peripheral clocks may coordinate physiological processes ultimately affecting fitness. Few studies have compared physiological parameters between individuals with a normal or disrupted circadian clock in any species. A study in cyanobacteria demonstrated that colonies in which free-running circadian periods are in resonance with environmental LD cycles out-compete colonies in which internal and external periodicities are out of synchrony (Ouyang et al., 1998). In *D. melanogaster*, mutations in the *per* gene and photoperiodic conditions affect developmental time and longevity (Klarsfeld and Rouyer, 1998; Kyriacou et al., 1990a; Sheeba et al., 2000)

The role of the circadian clock in fitness, defined as a measure of reproductive success, has not been addressed in animals with a genetically disrupted pacemaker. However, physiological data indicated an important role of circadian timing in

reproduction of moths. In these insects, a circadian clock controls rhythms associated with the release and post-testicular maturation of sperm (Bebas et al., 2001; Giebultowicz et al., 1989). Disruption of circadian rhythms by rearing male moths in constant light reduces sperm release and male fertility (Giebultowicz et al., 1990). These experiments provided indirect evidence that the circadian system is important for reproduction.

We tested genetically the links between the circadian system and reproductive fitness in *D. melanogaster*, in which both spermatogenesis and reproductive behavior are relatively well understood (Fuller, 1993; Hall, 1994). We report here that flies with loss-of-function mutations in the *per*, *tim*, *Clk*, or *cyc* genes produce significantly fewer progeny than wild-type flies. Clock mutant males contribute to this phenotype by releasing smaller quantities of sperm than their wild-type counterparts. Both fertility and sperm release are restored to wild-type levels in flies with rescued clock function. Finally, we demonstrate that clock genes are rhythmically and autonomously expressed in testes and seminal vesicles (SV) of male flies, suggesting that these tissues harbor a circadian system important for optimal sperm output and fertility.

Material and Methods

Fly rearing and strains. Flies were raised on cornmeal-yeast medium in 12 h light: 12 h dark cycles (LD) at 25 °C. By convention, the time of lights-on is denoted as Zeitgeber Time (ZT) 0 and time of lights-off as ZT 12. Wild-type flies used were Canton-S. The following loss-of-function alleles of the clock genes *per*, *tim*, *Clk*, and *cyc* were applied: per^{01} (Konopka and Benzer, 1971), per^{04} (Hamblen-Coyle et al., 1989), tim^{01}, (Sehgal et

al., 1994), *Clk*^{Jrk} (Allada et al., 1998)), and *cyc*^{01}, (Rutila et al., 1998b). Additionally, a novel timeless null-allele, *tim*^{03} was analyzed (Stempfl et al., 2001).

Rescue of *per* function in a *per*^{01} genetic background was performed by crossing *per*^{01} virgins to transgenic males carrying a P-element containing a 13.2-kb genomic per DNA fragment. This transgene restores behavioral rhythmicity when crossed to *per*^{01} flies (Citri et al., 1987). Similarly, *tim* function was rescued by introducing a transgene expressing the tim cDNA under the control of the tim promoter into the background of tim null-alleles. The transgene used restores behavioral rhythmicity in *tim*^{01} and *tim*^{03} mutant flies (Rutila et al., 1998a; Stempfl et al., 2001). For the rescue, *tim*^{01} males carrying two copies of the transgene were crossed to *tim*^{03} females. The resulting transheterozygous *tim*^{01}/*tim*^{03} F1 males, carrying one copy of the rescue transgene, were analyzed for their reproduction phenotype. *tim*^{01}/*tim*^{03} flies were used as a control to exclude the possibility of rescue by complementation.

To elucidate expression patterns of per and tim in the reproductive tract, the following reporter strains were used: 1. "BG" flies carrying *per-lacZ* fusion gene (encoding two-thirds of PER fused to ßgal) in a *per*$^{+}$ genetic background (Stanewsky et al., 1997a); 2. doubly transgenic flies carrying *tim-gal4* combined with UAS-*gfp* (Kaneko and Hall, 2000); 3. transgenic flies in which *luciferase* (*luc*) cDNA was fused to upstream flanking material of either *per* (BG-*luc*) or *tim* (*tim*-luc) gene (Stanewsky et al., 1997b).

Mating and fertility assessments. Males and females of the appropriate strain were collected one day before eclosion and isolated in individual vials with 1ml of diet. Flies

of appropriate age, sex, and genotype were paired in mating wheels (Hall, 1979) approximately 3 h after lights-on (Sakai and Ishida, 2001) and their copulation observed. After single mating was completed, individual females were transferred to 50 mm Petri dishes with 2ml of diet where they oviposited for 4 days. Flies that were allowed to mate ad libitum were aspirated directly into Petri dishes, where both sexes remained together for 4 days. Flies were then removed, and larvae allowed to hatch for 24 hours. The number of larvae, dark un-hatched embryos and white unfertilized eggs were counted. All data were analyzed for statistical significance using ANOVA and Fischer's least significant difference procedure (LSD intervals) at 95% confidence intervals.

Quantification of sperm. To determine the quantity of sperm released from testes into SVs, individual spermatozoa were counted in SVs dissected from 1- and 2-days old males at 12 h intervals. Individual SVs were placed in a drop of distilled water and ruptured to facilitate dispersion of spermatozoa. After water evaporated dispersed spermatozoa were mounted in Vectashield supplemented with DAPI to stain sperm nuclei. Digital images were acquired using a SPOT CCD camera and software (Diagnostic Instruments) and printed. Thin and elongated sperm nuclei contained in individual SVs were counted, and data were averaged for each strain. Data were analyzed using ANOVA and Fischer's LSD intervals at 95% confidence intervals.

Assessments of clock gene expression. Reproductive systems (RSs) from doubly transgenic *tim-gal4*, UAS-*gfp* flies were dissected in phosphate-buffered saline and observed live under Zeiss Axiovert microscope. RSs from flies carrying *per-lacZ* were

fixed and stained with X-gal as described (Hege et al., 1997). For whole-mount immunocytochemistry and in situ hybridizations RSs were fixed in 4% Paraformaldehyde for 15 min. Immunodetection of PER and TIM were performed on RSs dissected and fixed at ZT 8 and ZT 20. Anti-TIM (diluted 1:4,000) or anti-PER (diluted 1:15,000) primary antisera were used, followed by application of anti-rat or anti-rabbit (for TIM and PER detection, respectively) secondary antibodies conjugated to Alexa Fluor 594 (Molecular Probes) diluted 1:1,000. In situ hybridizations were performed as described (Gvakharia et al., 2000). Dig-labelled *per* and *Clk* antisense RNA probes were hybridized to whole RSs dissected and fixed at ZT 4 and ZT16. Tissues were whole-mounted in Vectashield-DAPI medium (Vector Laboratories) to allow localization of clock proteins and mRNAs relative to cell nuclei.

To determine *luciferase*-reported activity of *per* and *tim* genes in vitro, testes-SV complexes were dissected and cultured individually in wells of 96-well plates (Giebultowicz et al., 2000). The bioluminescence from each well was measured once per hour in a Packard Topcount Multiplate Scintillation Counter (Stanewsky et al., 1997b); the resulting counts were analyzed for phase, period and amplitude (Plautz et al., 1997b).

Results

Mutations in clock genes decrease reproductive capacity. To investigate the effects of loss-of-clock-function on reproductive capacity, we compared offspring produced by wild-type and clock mutant flies after matings between males and females of a given genotype. Null mutations in clock genes, per^{01}, tim^{01}, Clk^{jrk} and cyc^{01}, invariably led to a significant decline in average progeny as compared to wild-type flies (Fig. 2.1). Flies

carrying alternative null genotypes, *per*^{04} and *tim*^{03}, as well as *tim*^{01}/*tim*^{03} mutations also showed statistically significant decreases in progeny compared to wild-type flies (Table 2.1). The decline in progeny was caused by a lower number of eggs laid per couple, and a higher than normal production of unfertilized eggs; these two factors contributed to a varying degree to the lower fertility (Table 2.1). This phenotype was rescued to wild-type in *per*^{01} males and females carrying a *per*$^{+}$ construct and in *tim*^{01}/*tim*^{03} flies rescued with a *tim*$^{+}$ construct. Thus, the reduction in progeny observed in flies with a disrupted circadian clock maps to the *per* and *tim* loci.

The significant declines in fertility observed with singly mated flies are not as readily detected in routine laboratory cultures where many males and females are housed in one vial for several days, such that flies can potentially mate multiple times. We investigated whether leaving pairs of mutant flies together for 4 days, thus allowing them to mate ad libitum, would affect their fertility. *per*^{01} couples mated *ad libitum* produced an average of 57.6 larvae (n=15), a 20.5% increase over single mating. *tim*^{01} couples mated *ad libitum* produced on average 55.6 larvae (n=7), a 31.3% increase over singly mated flies. These increases were statistically significant ($p<0.05$) in both mutant strains. In contrast, *ad libitum* conditions (n=23) did not significantly increase the average number of progeny over single matings (n=16) in wild-type flies. These data suggest that clock mutants may compensate for their lower reproductive fitness via re-mating.

Crosses between *per*^{01} or *tim*^{01} mutant flies of either sex with wild-type flies of another sex indicated that both sexes contribute to the low fertility phenotype. In this paper, we examined in detail the male contribution to the low fertility phenotype (the results describing female contribution will be reported elsewhere). Single matings of

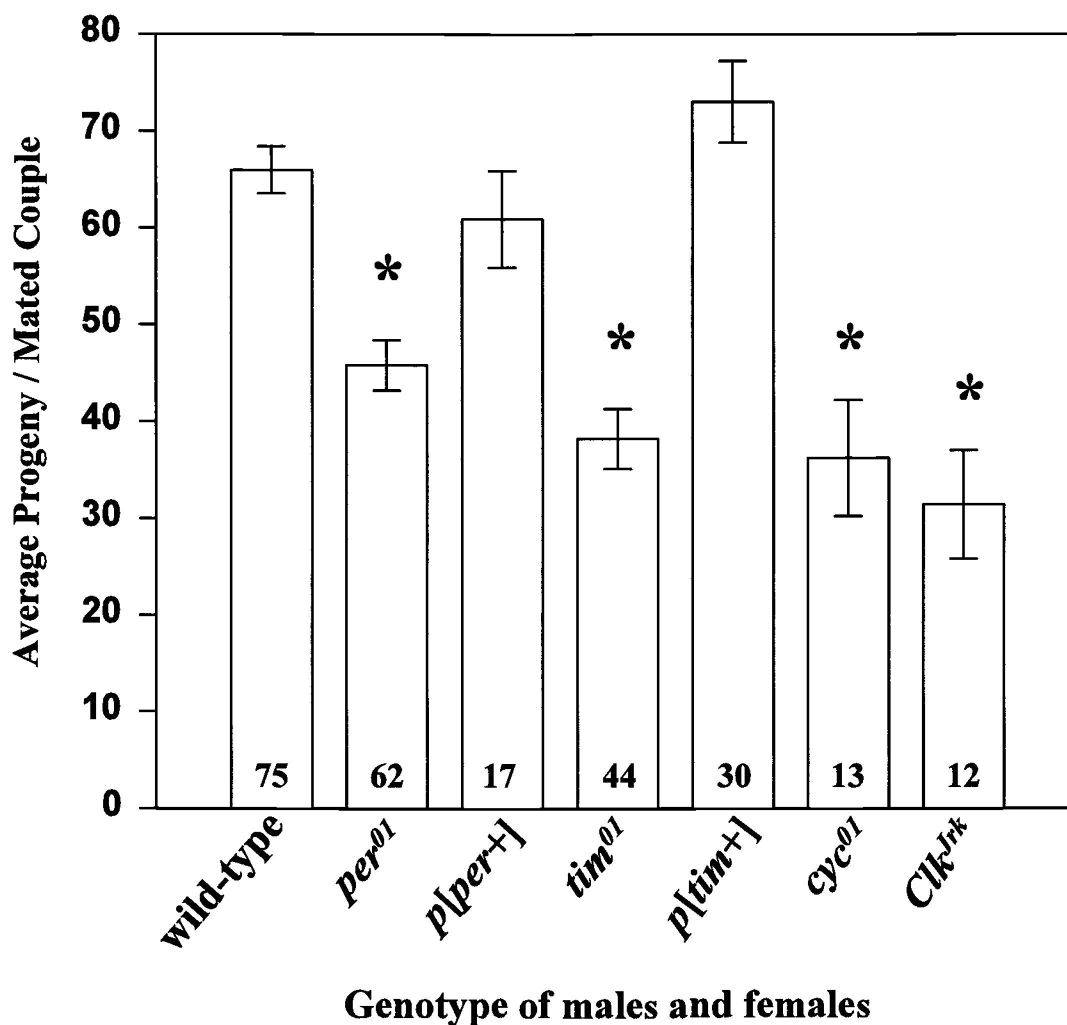


Figure 2.1 Mutations in four clock genes dramatically reduce fertility in *D. melanogaster*. Bars represent average number (±SEM) of progeny produced by couples of a given genotype (numbers of couples tested are shown in bars). Asterisks denote strains that produce significantly less progeny then wild-type flies as calculated by ANOVA and Fishers Least Significant Difference (LSD) intervals ($p < 0.05$). Males and females were 4 day-old when mated.

Table 2.1 Effects of clock genes on reproductive output in *D. melanogaster*

Strain[a]	n[b]	Eggs Laid	% Unfertilized[c]	Progeny[d]
wild-type	75	71.2	6.2	65.7
per^{01}	62	**49.7**	10.3	**45.7**
per^{04}	7	71	**40.2**	**42.3**
per^{01}; $p[per^{+}]$	17	65.7	4.6	60.9
tim^{01}	44	**50.9**	**24.9**	**38.2**
tim^{03}	14	67.9	**25**	**43.6**
tim^{01}/tim^{03}	7	64.2	7.8	**50.6**
tim^{01}/tim^{03}; $p[tim^{+}]$	30	89	4.2	73.1
Clk^{Jrk}	12	**40.5**	9.5	**36.2**
cyc^{01}	13	**40.2**	12.4	**31.4**

Numbers in bold are significantly different from wild-type ($p < 0.05$).
[a]Males and females were of the same strain and were 4-days-old when mated
[b]Number of pairs tested
[c]Ratio of unfertilized eggs / total eggs laid. Statistical analysis showed non-normal distribution of this parameter. The Kruskal-Wallis test confirmed statistically significant differences between the medians ($p < 0.05$).
[d]Average offspring /mated pair.

per^{01}, *tim*^{01},or *tim*^{01}/*tim*^{03} males with wild-type females resulted in a significant reduction in progeny over wild-type pairs (Fig. 2.2). The average number of progeny was raised to normal levels in females mated with *per*^{01} mutants carrying a *per*$^{+}$ construct or with *tim*^{01}/*tim*^{03} heterozygotes carrying a *tim*$^{+}$ construct (Fig. 2.2). The decline in progeny produced by *per*^{01}, *tim*^{01}, and *tim*^{01}/ *tim*^{03} males was caused by a significantly lower number of eggs laid per couple (76.2, 67.5, and 63.4, respectively, compared with 95.5 in wild-type) and by an increased percentage of unfertilized eggs (data not shown). Both parameters reverted to wild-type levels in flies with rescued clock function (Fig. 2.2).

Mutations in clock genes decreases male fecundity. A factor affecting oviposition rate in *D. melanogaster* is the amount of sperm transferred to a female during copulation. To analyze the causes of lower fertility in clock mutant males, we compared the quantity of spermatozoa released from testes and stored in SVs in wild-type and clock-deficient males. The number of spermatozoa released during the first two days after adult emergence was significantly lower in males expressing either of two null mutations of the *per* or *tim* gene (Fig 2.3A). The buildup of sperm was most impeded at the last time point; therefore, we chose this time to assess rescue of the mutant phenotype by *per*$^{+}$ or *tim*$^{+}$ transgenes. *per*^{01} and *tim*^{01}/*tim*^{03} males, each carrying the appropriate construct accumulated a significantly higher quantity of sperm in the SV than their respective clock-deficient genetic variants (Fig. 2.3B). ($p>0.05$) The mean number of spermatozoa accumulated in *tim*-rescued males was similar as in wild-type males ($p<0.05$) while in *per*-rescued males it was lower than in wild type.

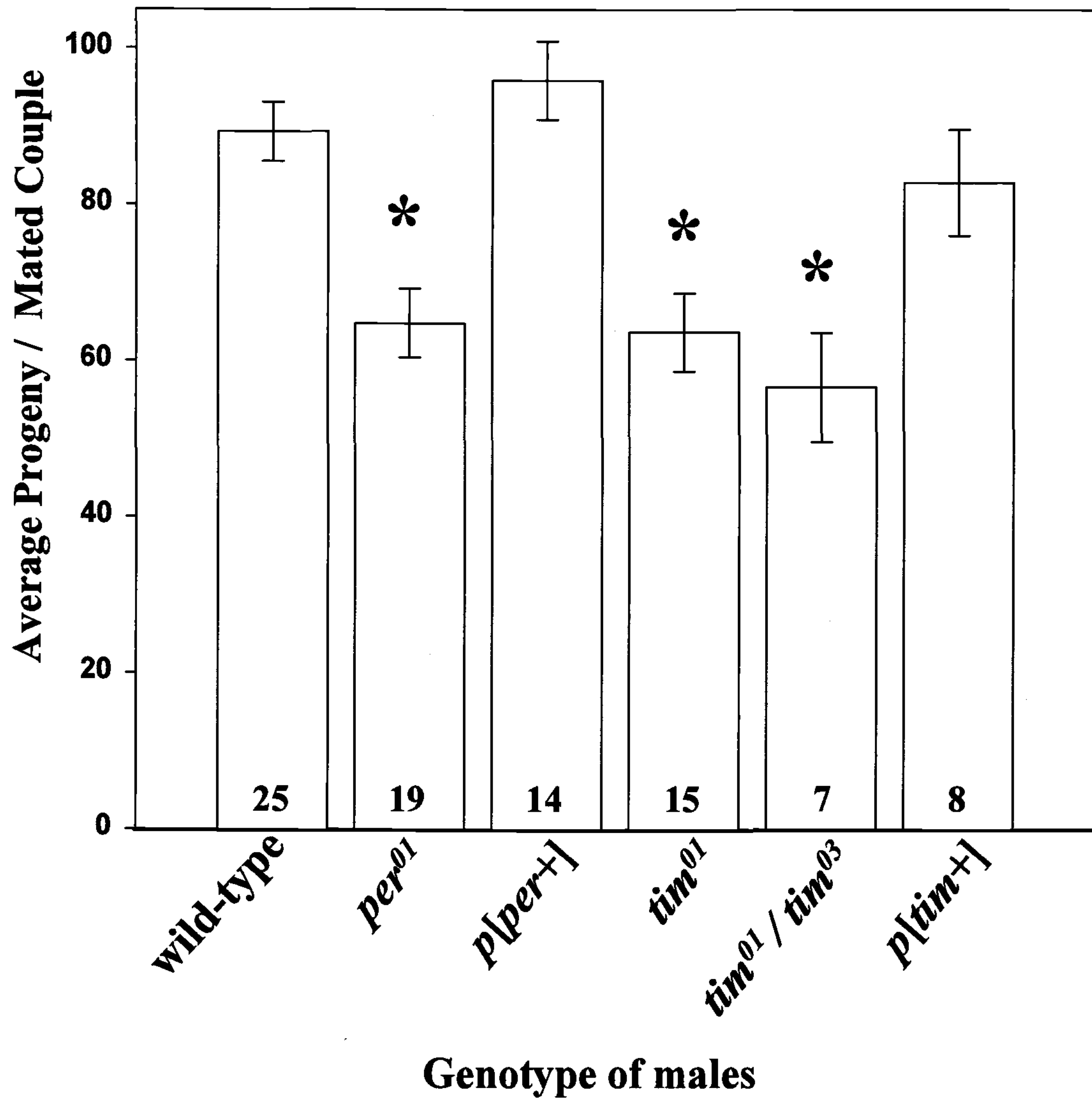


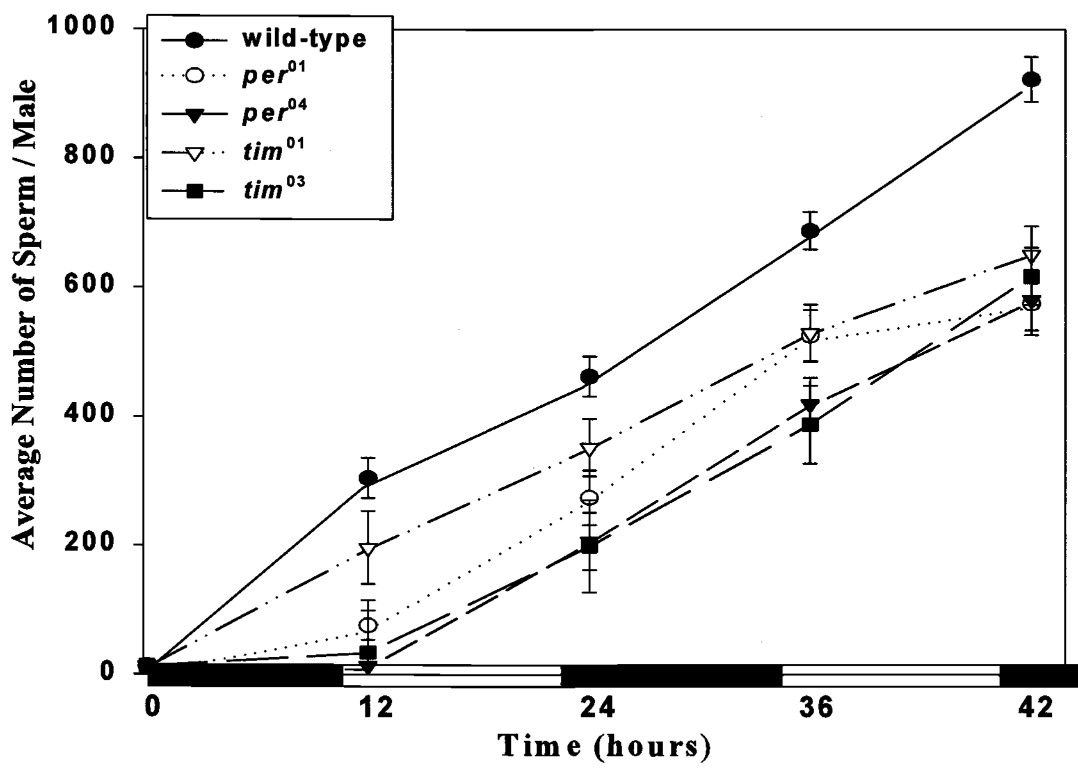
Figure 2.2 Effects of *per* and *tim* mutations on male fertility. 2-day-old males of the indicated genotypes were mated with 5-day-old wild-type females. Bars represent the average number (±SEM) of progeny produced by couples of given genotype (n shown in bars). Asterisks denote genotypes that produced significantly fewer progeny compared with wild-type, as calculated by ANOVA and LSD intervals ($p < 0.05$).

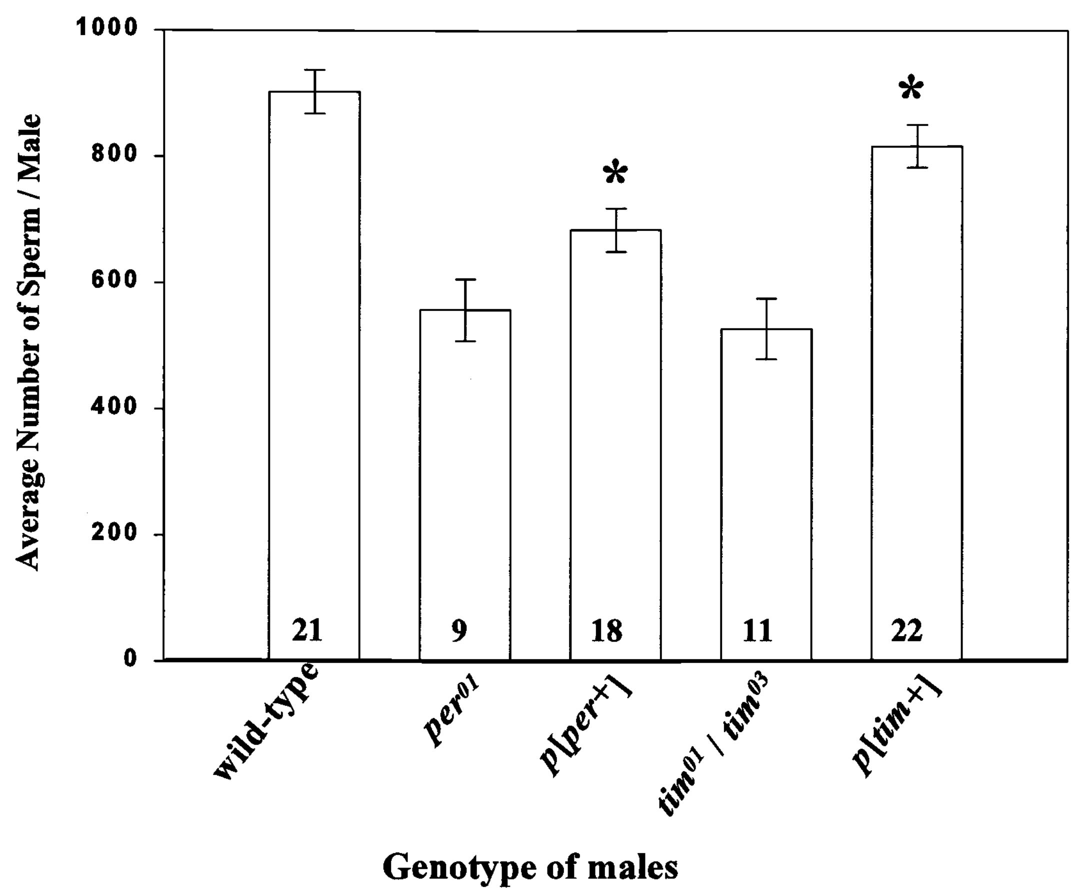
Expression of clock genes in the male reproductive system. To determine whether the low sperm phenotype is correlated with clock function in the reproductive system, we studied the activity of clock genes in these tissues. Spatial expression of *per* and *tim* was evaluated in flies which carry a *per-lacZ* reporter construct or express GFP under control of the *tim* promoter. Both reporters exhibited strong activity in the lower testes and SVs, weak activity in the ejaculatory duct and the upper testes, and no activity in the paragonial (accessory) glands (Fig. 2.4A). Immunocytochemical analysis showed rhythmic expression of PER and TIM proteins limited to the lower testes and the SVs. PER and TIM were not detected at ZT 8, while at ZT 20 both proteins were ubiquitously expressed in the nuclei of the epithelial cells forming the lower testes and the SV (Fig. 2.4B). PER and TIM proteins were absent in per^{01}, per^{04}, and tim^{01} flies. However, similar to wild-type distribution of both proteins in the SV and lower testes was observed in the reproductive system of per^{01} mutants rescued with a per^{+} construct and tim^{01} mutants transformed with a tim^{+} construct. Nuclear localization of PER and TIM was evident in those flies at ZT 20 (data not shown).

Because the functional clock of the fruit fly involves out of phase cycling of *per* and *Clk* mRNAs (Williams and Sehgal, 2001), we did in situ hybridization of the male RS with antisense probes for both genes. Both *per* and *Clk* mRNA were detected in the lower testes-SV epithelium. The level of *per* mRNA was low at ZT 4 and high at ZT 16 (Fig. 2.4C), while *Clk* mRNA showed cycling in the opposite phase with high levels at ZT 4 and low levels at ZT 16 (Fig. 2.4D). Taken together, these results demonstrate cycling of clock components that is similar to patterns observed in fly brains and is consistent with the existence of a circadian clock in the male RS.

Figure 2.3 Effects of *per* and *tim* mutations on male fecundity. (A) Time course of sperm accumulation in SVs of young virgin males. Each point represents the average number (± SEM) of spermatozoa from 5-30 SVs dissected at 2 h after lights-off and lights-on. Clock mutants accumulated significantly fewer sperm at all time points tested as calculated by ANOVA and LSD intervals ($p < 0.05$). The arrow indicates approximate time of adult emergence. Black and white bars indicate times when lights were on and off, respectively. (B) Reversal of low sperm phenotype in males with rescued clock function. Bars represent average number (± SEM) of spermatozoa from the of SVs of given genotype (n shown in bars) dissected at 42 h. Asterisks denote *per*-rescue and *tim*-rescue flies that accumulated significantly more sperm than did their corresponding clock-deficient males.

Figure 2.3





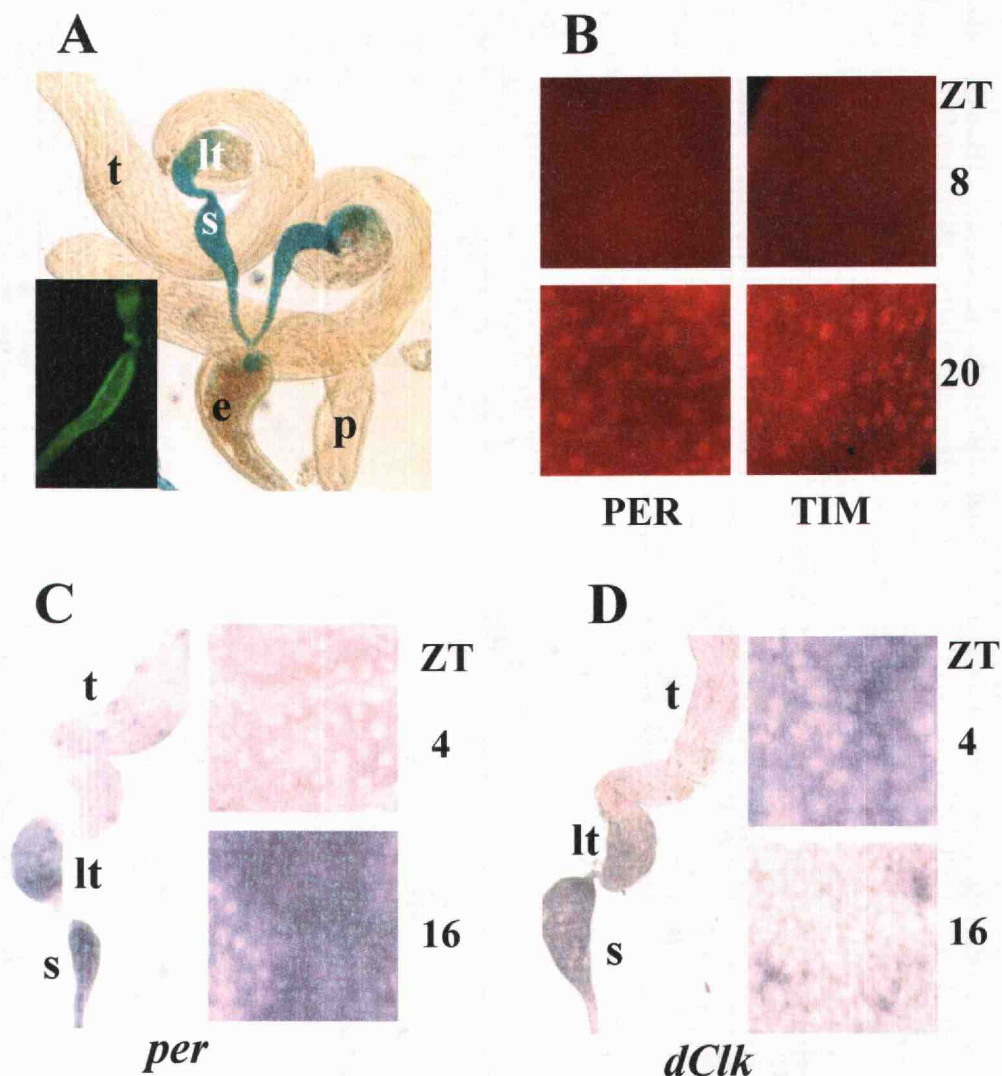


Figure 2.4 Spatial and temporal expression of clock genes in the reproductive system of *D. melanogaster*. (A) X-gal staining demonstrating strong *per-lacZ* expression in the SV (s) and the lower testes (lt), weak signal in the ejaculatory duct (e) and in the upper testes (t), and no signal in the paragonial glands (p). Inset shows expression of *tim*-driven GFP reporter in the lower testes-SV. (B) Immunofluorescence detecting PER and TIM proteins in the nuclei of SV epithelial cells at ZT 20 but not at ZT 8. (C) In situ hybridization with antisense probes detecting *per* mRNA in the SV and lower testis. Higher magnification of SV showing that epithelial cells are *per*-negative at ZT 4 and *per*-positive at ZT 16. (D) In situ hybridization at ZT 4 with antisense *Clk* mRNA show co-localization with *per* in the SV and lower testis. Higher magnification of SV showing that *Clk* is expressed in epithelial cells at ZT 4 but not at ZT16. Control hybridization with respective sense probes yielded no signal. Images represent the prevailing pattern observed among at least 5 specimens used to assess staining with each reagent at a given time point.

To elucidate the autonomy of the testes-SV circadian system, we used BG-*luc* (reporting *per*) or *tim-luc* transgenic flies. Testes-SV complexes were dissected from these flies and individually cultured in vitro in LD cycles followed by DD and return to LD. Isolated organs showed clear, high-amplitude cycling of BG-*luc* and *tim-luc* activity during the initial LD cycles with peak expression during the night (Fig. 2.5). Quantitative analysis of the data revealed that 59% of testes-SVs from BG-*luc* flies (n=49) and 76 % of same organs from *tim-luc* flies (n=41) were rhythmic in vitro in the circadian range. Upon transfer to DD, cycling continued in 36 % of both BG-*luc* and *tim*-luc organs with reduced amplitude. When the free-running cultures were returned to LD cycles, the amplitude increased for both constructs, demonstrating direct light-responsiveness of the testes-SV circadian system (Fig. 2.5). When both constructs were crossed into genetic backgrounds carrying a loss-of-function mutation for the respective clock gene (BG-*luc* into per^{01} and *tim-luc* into tim^{01}), circadian oscillations were eliminated (data not shown), indicating that wild-type alleles of the two clock genes are needed to support reporter-gene cycling.

Discussion

Our results identify a new circadian clock in the reproductive systems of male flies with known clock components detected in the lower testes and SV. Three lines of evidence confirm earlier suggestion (Hall, 1995) that a clock mechanism is located in these tissues. First, *per* and *Clk* mRNA appear to cycle out of phase such that *per* mRNA levels are high in the early night while *Clk* mRNA levels are high in the morning. This pattern is consistent with the current model of brain clock function

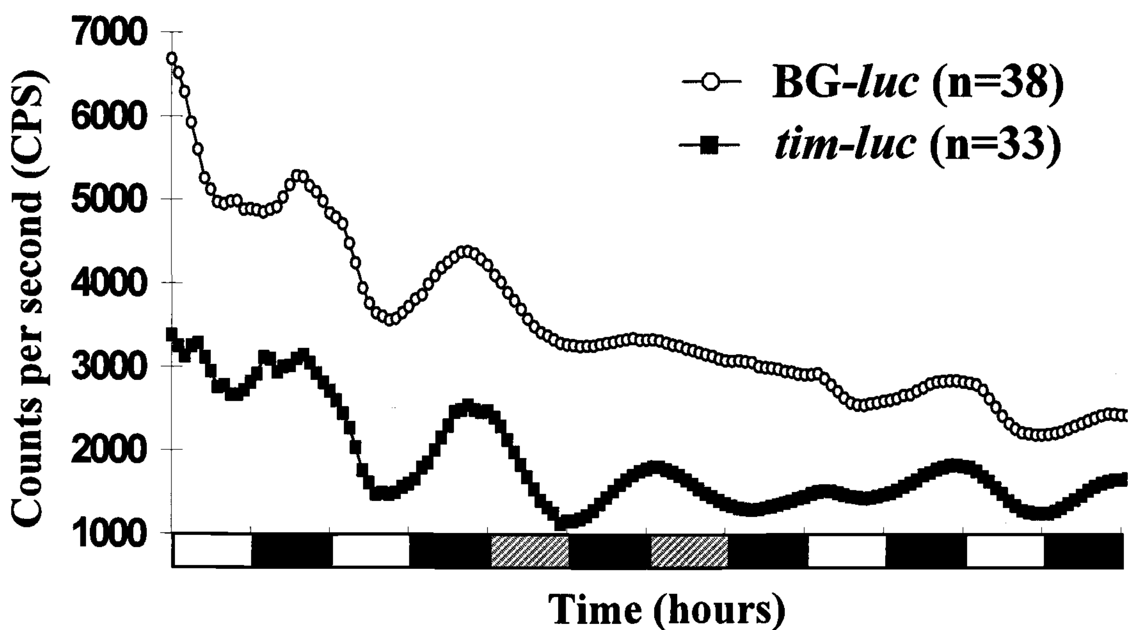


Figure 2.5 Real-time expression of BG-*luc* (*per-luc*) and *tim-luc* reporter genes in testes-SVs from individual flies. Plots show average bioluminescence from BG-*luc* and *tim-luc* expressing tissues, which were determined rhythmic in LD and DD by quantitative analysis. The average LD oscillation period of 23.2 h for BG-*luc* was not significantly different from the average period of 23.7 h for *tim-luc*. Black and white bars indicate 12 h periods where lights were on and off respectively, shaded bars indicate subjective day, where the lights would be on in LD.

(Williams and Sehgal, 2001) and is demonstrated here for the first time for any peripheral clock in flies. Second, PER and TIM proteins were detected in the SV cell nuclei late at night, as previously reported for other clock-containing cells (Williams and Sehgal, 2001). Third, cultures of testes-SVs taken from flies transformed with *per-luc* or *tim-luc* showed that reporter-gene activity in the RS free-runs in constant darkness and is autonomously light sensitive. Thus, the testes-SV complex appears to contain a bona fide circadian clock that is self-sustained and brain-independent. Such clocks were previously reported in the fly excretory system (Giebultowicz et al., 2000) and antennae (Krishnan et al., 2001).

Our study suggests a link between the molecular clockworks in the reproductive tissues and normal sperm output in *D. melanogaster.* We showed that null mutations in the *per* or *tim* genes lead to a significant decline in the number of spermatozoa released from the testes into the SV. In flies with rescued *per* and *tim* function this low sperm phenotype is reversed and PER and TIM expression is restored in the testes-SV complex. The significance of the RS clock for male fecundity has been shown previously in moths. The release of sperm from moth testes follows a robust circadian rhythm, which persists in the RSs isolated in vitro (Bebas et al., 2001; Giebultowicz et al., 1989). Disruption of the moth circadian system by constant light leads to a dramatic reduction in the amount of released sperm (Bebas et al., 2001; Giebultowicz et al., 1990). Thus physiological disruption of the circadian mechanism in moths and genetic disruption of clock genes in flies both cause a decline in male fecundity. These data suggest that sperm release from testes may also be rhythmic in flies, however, our experiments monitoring sperm accumulation in SVs at every 12h (Fig. 2.3A) and in more frequent intervals

(Giebultowicz, unpublished), did not uncover a rhythmic component in this process. Although the principles of sperm release are shared in moths and flies (Giebultowicz et al., 1997; Tokuyasu et al., 1972), fly spermatozoa are extremely long (up to 2 mm) and their slow translocation into the SV via a narrow testis neck (see Fig 2.4A) may mask a putative release rhythm in the lower testes. The expression of clock genes in the sperm release zone in both moths (Gvakharia et al., 2000) and flies further implies that the circadian clock may aid sperm release in both groups of insects. Clock genes are also expressed in testes of zebrafish, mouse, rat, and humans (Giebultowicz, 2001), suggesting a possibility that circadian clocks may be involved in fecundity across animal phyla.

Our finding that clock-mutant males have fewer sperm available for mating provides an explanation for the low fertility phenotype of such males. Males lacking *per* or *tim* function sire 30-50% fewer larvae, because females inseminated by them deposit fewer eggs than those inseminated by wild-type males (Fig. 2.2). The rate of oogenesis and oviposition in *D. melanogaster* is dependent on transfer of sperm during mating (Heifetz et al., 2001; Xue and Noll, 2000). Clock mutant males, having about 40 % less sperm stored in the SV than wild-type males, are likely to transfer proportionally fewer spermatozoa to females; this may adversely affect egg output. Proteins produced by male accessory glands also stimulate egg production in mated females (Wolfner, 1997); however, clock genes are not expressed in accessory glands, therefore their contribution to mating should not be affected directly by mutations in those genes.

The second factor contributing to low offspring in females inseminated by clock-mutant males is a higher incidence of unfertilized egg deposition. The lower number of stored sperm may underlay the female phenotype; however, it is also possible that the

loss of circadian clock function adversely affects quality or viability of sperm. It is known that disruption of the circadian system in moths leads to production of defective sperm that fail to fertilize eggs (Giebultowicz et al., 1990). It has been proposed that the circadian system orchestrate the physiological processes involved in post-testicular maturation of sperm in moths (Bebas et al., 2001; Giebultowicz, 2001). Rhythmic expression of clock genes in the sperm-storing SVs suggests that clock-controlled processes may also aid sperm maturation in flies. Although we surmise that lowered reproductive potential of clock mutant males is attributable to the loss of clock function in the RS, the lack of clock function in other organs may adversely affect the physiological and metabolic state of the fly (Krishnan et al., 2001; Sarov-Blat et al., 2000). This could lead to diminished resources available for gamete production.

In conclusion, we demonstrated a novel low fertility phenotype in clock-deficient mutants of *D. melanogaster*, which correlates with decreased sperm release. Our results provide direct evidence that genes essential for the operation of the circadian clock are important for reproductive fitness. Although these genes may also have clock-unrelated functions, the low-fertility phenotype consistently observed in flies defective in any of the four clock genes tested (*per*, *tim*, *Clk* and *cyc*) suggests that they aid reproduction via their respective roles in clock mechanism. Working at the molecular and physiological level, we identified an autonomous clock in the reproductive system and its role at the end of the clock-output pathway. In order to dissect molecular components of this pathway, we initiated microarray screening of ESTs for testis specific genes differentially expressed during day and night (Andrews et al., 2000).

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CHAPTER 3

Novel non-circadian role of the circadian clock genes *period* and *timeless* in oogenesis of *Drosophila melanogaster*

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Summary

Circadian clocks are ubiquitous in the nervous system and peripheral tissues of complex animals. While clock genes in the brain are essential for behavioral rhythms, the physiological roles of clock genes in the periphery are not well understood. Constitutive expression of the clock gene *period* was reported in the ovaries of *Drosophila melanogaster*, however its molecular interactions and functional significance remained unknown. Here we demonstrate a fitness-related phenotype of circadian clock genes *period* (*per*) and *timeless* (*tim*) that is directly linked to their expression in the ovaries. Virgin females lacking either *per* or *tim* show nearly a 50% decline in the production of mature oocytes. Several lines of evidence demonstrate that *per* and *tim* are involved in a novel non-circadian pathway in the ovary. PER and TIM are constantly expressed in the follicle cells enveloping young oocytes. The levels of these proteins are affected neither by light nor by the lack of clock-positive elements *dClock* (*dClk*) and *cycle* (*cyc*). Despite genetic evidence that PER and TIM interact in the ovaries, they do not translocate to the nucleus. Taken together, our data demonstrate a novel function of PER and TIM that is related to oogenesis.

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Daily rhythms in life functions are generated by molecular circadian clocks which are substantially conserved among animals from fruit flies to humans (Young and Kay, 2001). The core clock mechanism is based on the daily oscillations of auto-regulatory proteins that repress their own genes by interfering with their transcriptional activators (Stanewsky, 2002). The two most intensely studied clock genes in *Drosophila*

melanogaster are *period* (*per*) and *timeless* (*tim*). Oscillations in the levels of *per* and *tim* mRNA lead to rhythmic translation of PER and TIM proteins which form dimers and accumulate in the cell nuclei. PER-TIM dimers indirectly suppress activity of their own genes by interacting with their transcriptional activators encoded by *Clock* (*Clk*) and *cycle* (*cyc*). These four clock genes are essential for the function of the brain clock and a loss of function mutation in any of them renders the fly behaviorally arrhythmic. Clock genes are also expressed rhythmically in many peripheral tissues, where they seem to drive local oscillators (Giebultowicz, 2001); however, very little is known about the roles of these genes in physiology and fitness. We previously established that circadian clock genes are involved in reproductive success of the fly. Flies with loss-of-function mutations in the *per*, *tim*, *dClk*, or *cyc* genes produce significantly less progeny than wild-type flies. This low fertility phenotype correlates with decreased sperm production in clock mutant males. The circadian oscillator that we identified in the testes-seminal vesicles (testes-SV) complex appears to be relevant to this phenotype (Beaver et al., 2002).

Our interest in the role of the circadian clock in reproduction prompted us to focus on the expression of clock genes in the ovary. In contrast to other peripheral tissues, *per* mRNA levels in the ovary remain high at all times of day (Hardin, 1994). This, together with cytoplasmic localization of PER (Liu et al., 1988; Saez and Young, 1988), suggests that *per* has a non-circadian function in this tissue; however, *tim*, which is a clock partner of *per*, was detected in ovaries of flies carrying tim-Gal4/ UAS-GFP reporter (Kaneko and Hall, 2000). We investigated the expression and interactions of *per*, *tim*, and other clock genes in the ovaries to resolve their molecular context. To understand the

functional significance of clock genes in the ovary, we studied phenotypic consequences of the loss of function mutations in these genes. Crosses between clock mutant females and wild-type males illustrates that females strongly contribute to the low fertility phenotype. Females lacking functional *per* (*per*^{0}) or *tim* (*tim*^{0}) that were mated with wild-type males produced on average 45% fewer progeny then wild-type females (Fig. 3.1). This low fertility phenotype was reversed to wild-type in *per*^{01} females rescued with a *per*$^{+}$ construct and in *tim*^{01}/*tim*^{03} flies rescued with a *tim*$^{+}$ construct. Thus, the reduction in progeny maps to the *per* and *tim* loci, which demonstrates that these two clock genes confer an adaptive advantage on the female fly.

To investigate the cause of low fertility, we examined the time course of oogenesis in *per*^{01} and *tim*^{01} virgin females (Fig. 3.2A) and revealed that they produce significantly fewer mature oocytes than wild type females. Since this phenotype was most pronounced on day 5, we used 5-day old females to examine oogenesis in additional mutants as well as in flies that contained a rescue construct for *per* or *tim* (Fig. 3.2B). Independent mutations *per*^{04} and *tim*^{03} as well as *tim*^{01}/ *tim*^{03} transheterozygotes all displayed significantly lower fecundity than wild-type flies. In contrast, females with rescued *per* or *tim* function produced wild-type levels of mature oocytes. These results demonstrate that both *per* and *tim* genes are involved in the process of oogenesis in flies. The lack of either gene causes a similar reduction in the number of mature oocytes, leading to lower fertility in mutant females.

Egg production in flies depends on protein intake (Spradling, 1993). To determine whether elevation of dietary protein would result in a less severe phenotype in *per*^{01} and *tim*^{01} females, we examined the production of mature oocytes on diet containing

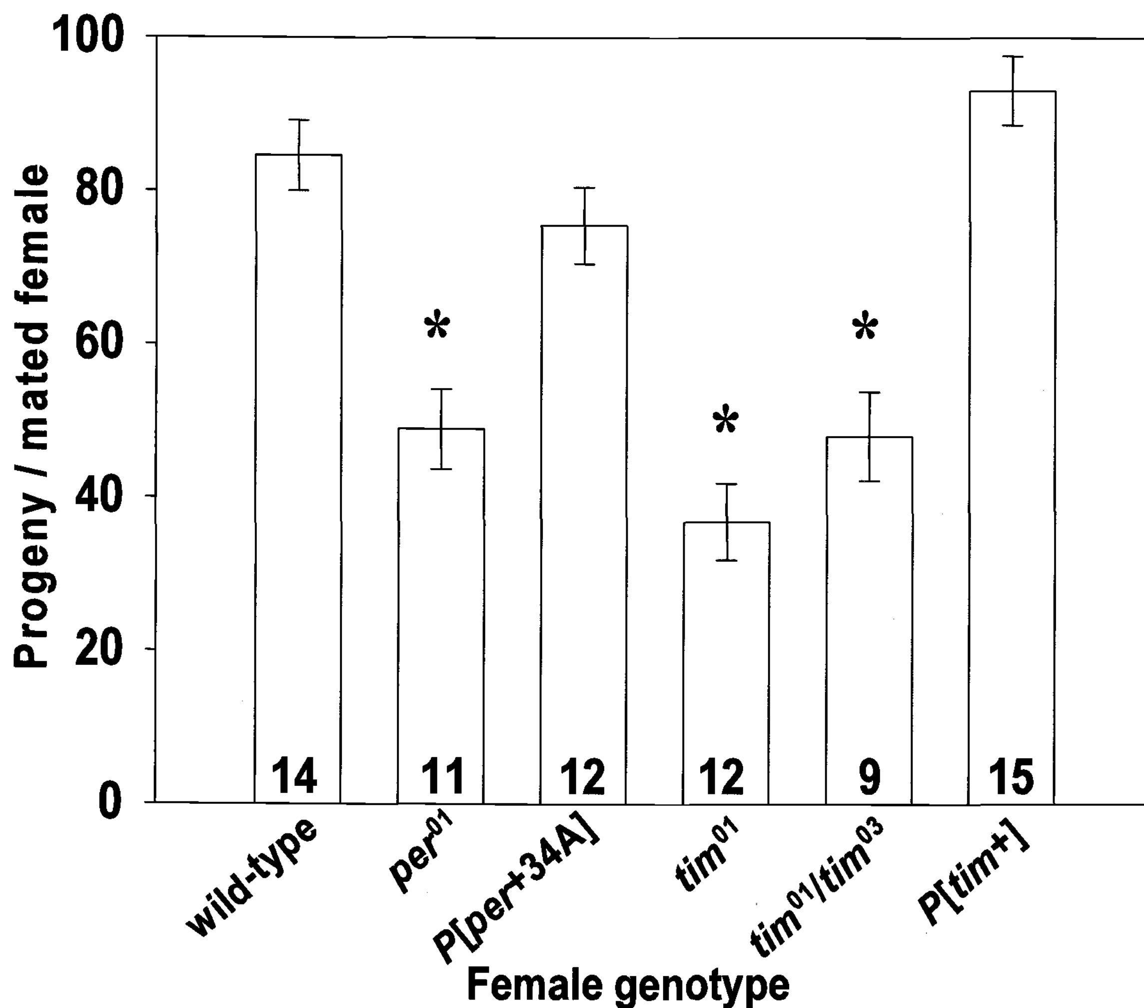
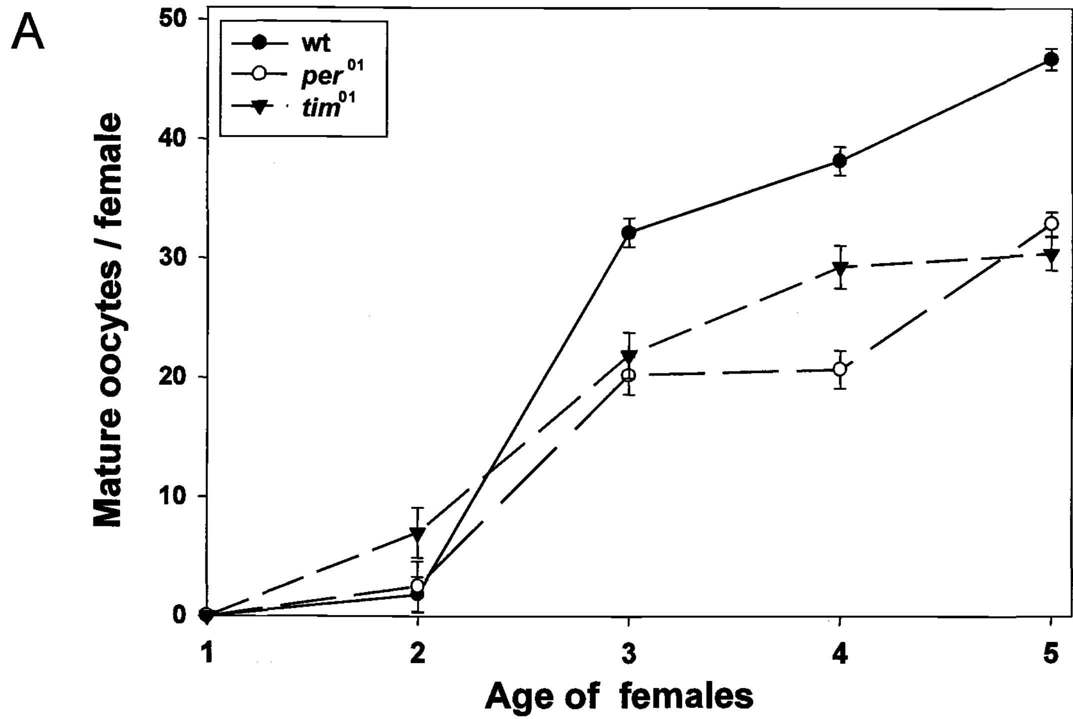
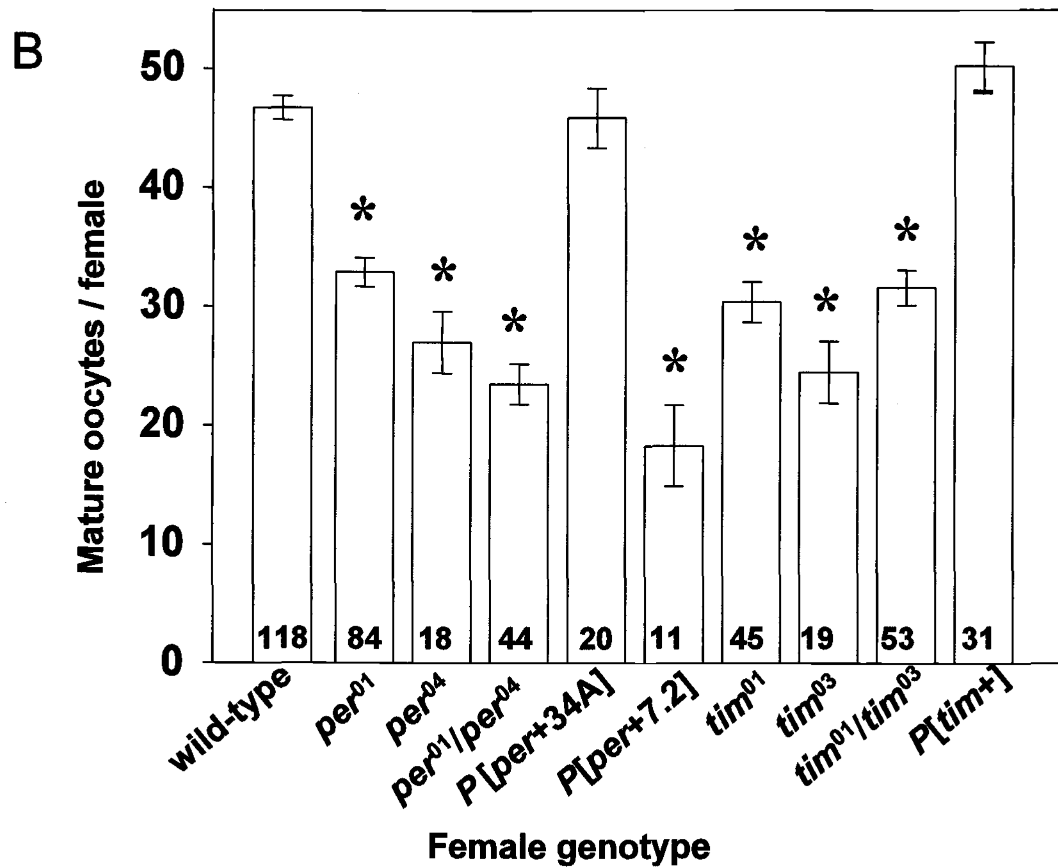


Fig. 3.1 Effects of *per* and *tim* mutations on female fertility. 2-day-old females of the indicated strain were mated once with 5-day-old wild-type males and allow to oviposit for 5 days. Bars represent the average number (± SEM) of progeny produced by couples of given phenotype (*n* shown in bars). Stars mark genotypes that produced significantly fewer progeny compared to wild-type, as calculated by ANOVA and LSD intervals ($p<0.05$, see Materials and Methods).

Fig. 3.2 Effects of *per* and *tim* mutations on female fecundity. (A) Time course of production of mature oocytes in virgin females. Each point represents the average number (± SEM) of mature oocytes from at least 20 females dissected at 2 h after lights-on for five consecutive days post eclosion). per^{01} and tim^{01} females produced significantly fewer mature oocytes on days 3-5 than the wild-type females. (B) Low fecundity is evident in 5-day-old virgin females with different null mutations in *per* and *tim* and in *P*[*per*+7.2] females in which per is rescued only in LN's. This phenotype is reversed in females with rescued clock function throughout the body. Genotypes that produced significantly fewer mature oocytes compared with wild-type are marked by a star (*).

Figure 3.2





increasing amounts of yeast (Fig. 3.3A). Augmenting the diet's yeast content increased the average number of mature oocytes in all strains examined, however *per*^{01} and *tim*^{01} females never achieved wild-type levels of fecundity. In fact, the difference in fecundity levels became more pronounced on double yeast diet; therefore, we conducted subsequent fecundity experiments on adult females reared at this protein concentration. We demonstrated that mutant flies showed no decline in resources for egg production. Western blotting showed that yolk proteins are produced at the same levels in *per*^{01}, *tim*^{01}, and wild-type females (Fig. 3.3B). Additionally, weights of wild-type and mutant females raised at similar densities were not significantly different from each other. The average weights for wild-type, *per*^{01}, and *tim*^{01} females were 1.15, 1.20, and 1.19 mg respectively (30-39 individuals were tested for each strain).

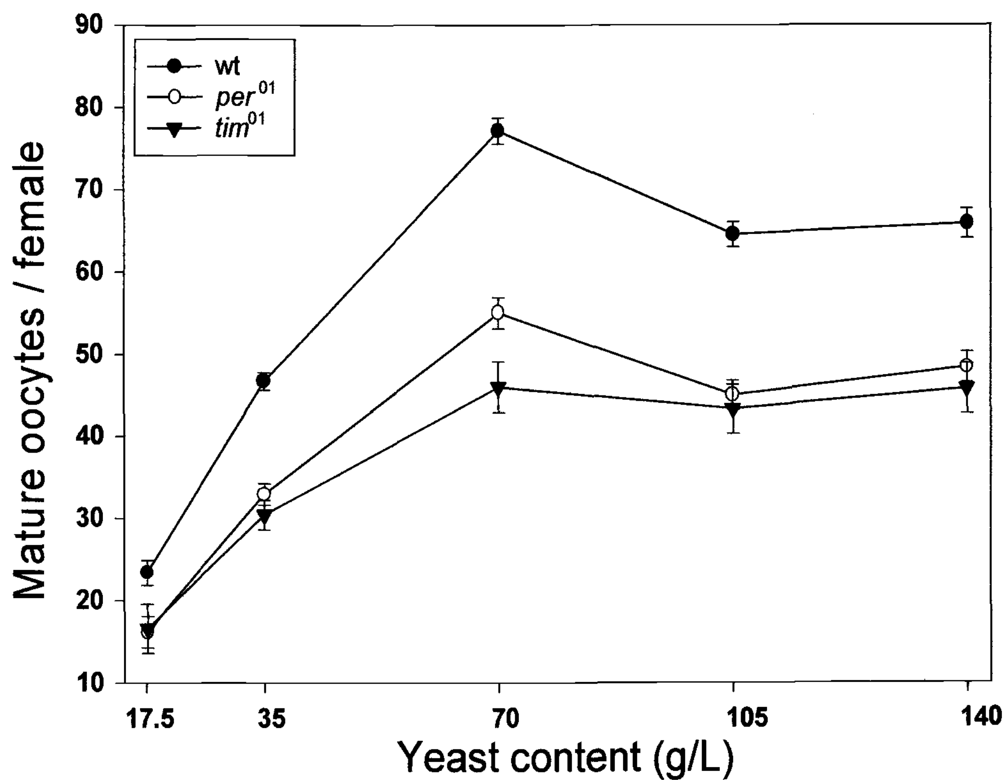
Oogenesis in flies is a complicated developmental process during which mature oocytes are produced in ovarioles (Spradling, 1993). The anterior end of each ovariole contains the germarium where germ and somatic stem cells divide and form egg chambers (follicles). Each follicle contains an oocyte with 15 interconnected nurse cells and is enveloped by follicular epithelium (follicle cells). In the maturation process follicles proceed through three developmental phases: previtellogenic growth, vitellogenesis (when yolk proteins, vitellogenins, accumulate in the oocytes), and post-vitellogenesis during which oocytes gain a shell and become competent for fertilization (Spradling, 1993).

Several organs participate in oogenesis; namely the ovaries, vitellogenin-producing fat body, and the brain which controls oocyte production via hormonal cues (Bownes, 1989). *per* and *tim* are expressed in each of these tissues thus, it became

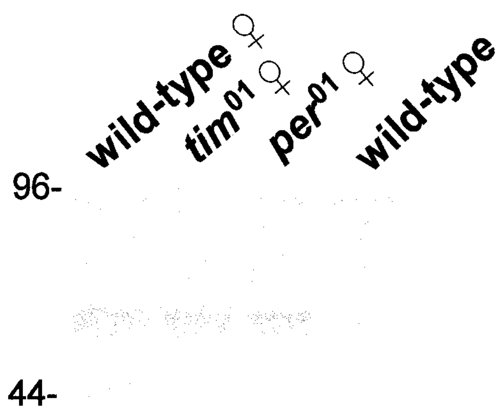
Fig. 3.3 Nutrient utilization in wild-type and *per*^{01} and *tim*^{01} females. (A) Production of mature oocytes in virgin females reared on diet containing varying levels of protein. Each point represents the average number (± SEM) of mature oocytes from at least 15 5-day-old females dissected at 2 h after lights on. *per*^{01} and *tim*^{01} females produced significantly fewer mature oocytes than wild type females on all diets. (B) Western Blot detecting yolk proteins level in wild-type, *per*^{01} and *tim*^{01} females. No difference was observed in the levels of yolk protein among females of different strains. As expected, male abdomens contained no yolk proteins. Protein molecular weight markers in kDa are indicated on the left. Each lane is marked with the sex and strained used.

Figure 3.3

A



B



important to determine which organs contribute to the fecundity phenotype. To elucidate whether the low fecundity phenotype is dependent on the central clock in the brain we tested *per*^{0} females that contain a *per*7.2 rescue construct, which rescues *per* expression only in the lateral neurons (Frisch et al., 1994). This construct failed to elevate the production of mature oocytes despite the fact that it recovers rhythmic locomotor activity (Fig. 3.2B). This suggests that the lower fecundity phenotype is not dependent on the function of *per* in the central clock, but rather on its expression in peripheral tissues. To elucidate whether low fecundity of females lacking clock genes is linked to their ovarian expression, we examined in detail the spatial and temporal distribution of *per* and *tim* in this organ.

In situ hybridization with antisense *per* and *tim* probes demonstrated that both genes are strongly expressed in the germaria. mRNA levels dissipate as egg chambers are formed and mRNA is no longer detected in late previtellogenic follicles (Fig. 3.4A). Both *per* and *tim* mRNA levels were prominent in ovaries dissected at ZT 4 and ZT 16 showing lack of temporal regulation, in contrast to oscillatory expression of these genes in circadian clock tissues. We then used immunocytochemistry (ICC) to investigate spatial and temporal profiles of PER and TIM proteins. Ovaries dissected at 6 h intervals throughout a light/dark (LD) cycle revealed that expression of PER and TIM is constant in somatic follicle cells of young egg chambers (Fig. 3.4B). Both proteins are first detected in mid-previtellogenic egg chambers when *per* and *tim* mRNA decline to a very low level. Thus, similar to the circadian mechanism, the accumulation of PER and TIM is delayed by several hours relative to the high levels of their respective mRNA. The levels of both proteins reach a maximum intensity in late previtellogenic oocytes and then

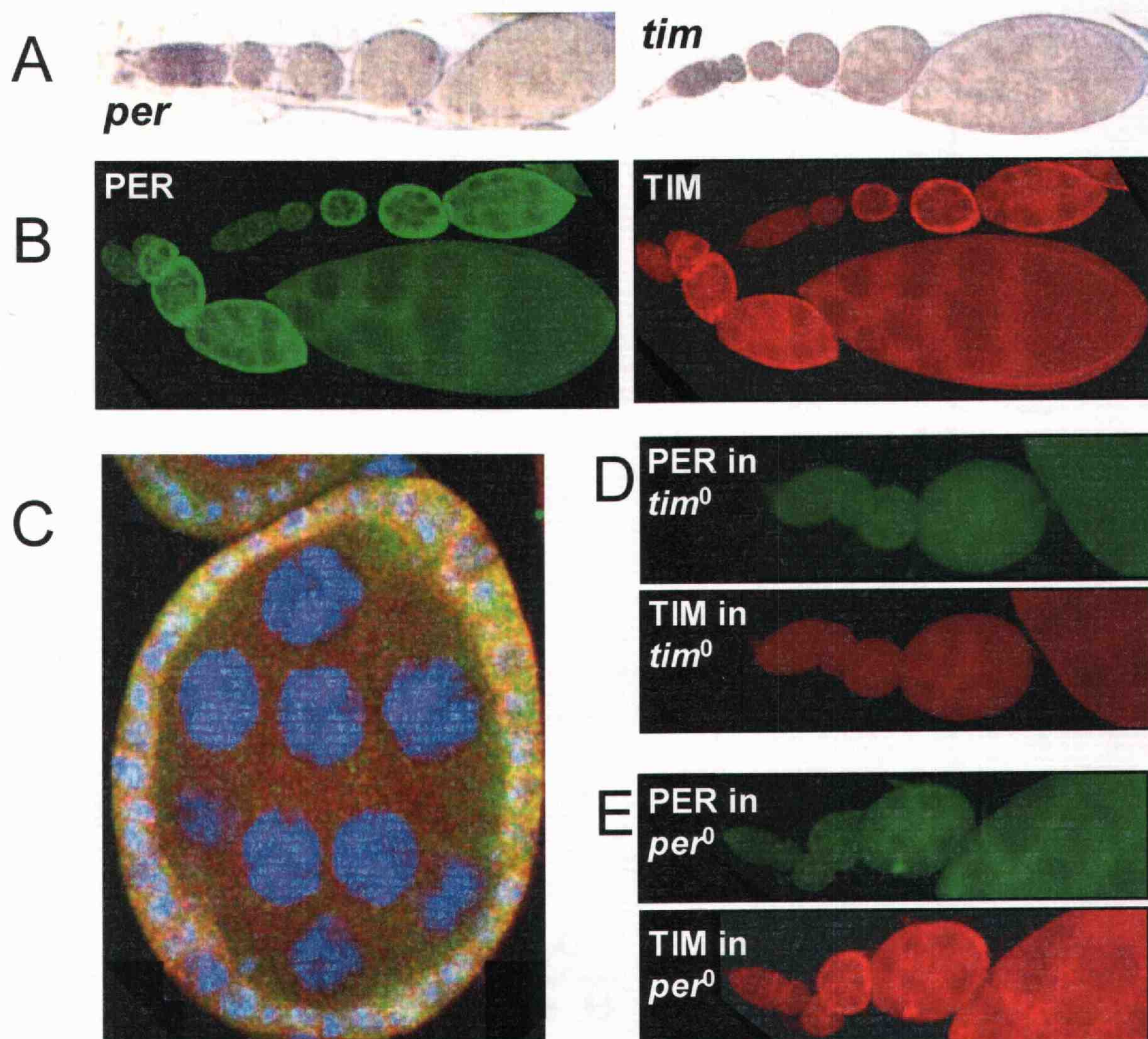


Fig. 3.4 Expression of *per* and *tim* in the ovaries. (A) *in situ* hybribization with antisense probes detected *per* and *tim* mRNA in the germarium and previtellogenic egg chambers of wild-type (wt) females. (B) Immunofluorescence detected PER (green) and TIM (red) proteins in follicle cells enveloping previtellogenic and early vitellogenic oocytes in wt females. (C) Co-localization of PER and TIM (yellow) in the cytoplasm of follicle cells. Small nuclei of follicle cells and large nuclei of nurse cells are stained blue by DAPI. (D) PER expression is not detected in the ovaries of tim^{01} flies. (E) TIM is present in per^{01} ovaries.

decline in vitellogenic oocytes (Fig. 3.4B). An overlay of confocal images showed that PER and TIM are excluded from DAPI-stained cell nuclei at all time points examined (Fig. 3.4C). Thus we confirmed the cytoplasmic location of PER and revealed that the lack of nuclear translocation of PER is not caused by the absence of its dimerization partner TIM in the cytoplasm of the same follicle cells.

Since PER and TIM co-localize in the cytoplasm but do not enter the nucleus, it was of interest to test whether these proteins interact with each other. In the circadian feedback loop, stability of PER and TIM depends on their dimerization; PER and TIM proteins interact in the ovary as illustrated by the absence of PER in tim^0 ovaries and the presence of TIM in per^0 females (Fig. 3.4D & 3.4E). Since this data is aligned with expression patterns found in previous circadian clock studies we suggest that TIM stabilizes PER expression in the cytoplasm of ovarian tissue in a similar manner as was observed in the circadian clock mechanism (Myers et al., 1996; Price et al., 1998).

One of the canonical features of the fly circadian clock is degradation of TIM in response to light (Hunter-Ensor et al., 1996; Myers et al., 1996; Zeng et al., 1996). The presence of TIM in oocytes throughout the light phase of the LD cycle suggested that TIM may not be sensitive to light in this tissue. To verify this observation, we inspected the levels of TIM and PER in tissues dissected from females kept in constant light (LL) for 3 days. High and persistent levels of TIM were observed in follicle cells after three days in LL while TIM was absent from control, clock-carrying cells in Malpighian tubules and other peripheral tissues (Fig. 3.5). Prominent expression of PER was also detected in the follicle cells of LL females, but not in the Malpighian tubules (data not shown).

We utilized this divergence in TIM and PER response to light to study the relative contribution of ovarian and non-ovarian PER/TIM to the low fecundity phenotype. If circadian oscillators in other tissues, such as fat body, are important for fecundity then production of mature oocytes would be lower in wild-type females reared in LL as compared to wild-type females in LD. If, however, ovarian expression of PER and TIM is the only significant contributor to fecundity then LL females should produce a similar number of oocytes as LD females. We found no significant differences in the average number of mature oocytes produced by LL or LD wild-type females, while *tim*^{01} females produced 47% fewer oocytes (Fig. 3.6A). This suggests that the fecundity phenotype is entirely dependent on ovarian expression of PER and TIM.

To verify that functional circadian clocks are irrelevant for the normal level of oocyte maturation, we used flies with over-expressed PER protein, a condition that was previously shown to disrupt circadian clocks and behavioral output rhythms (Yang and Sehgal, 2001). By crossing *tim*(UAS)-Gal4 females with UAS-*per* males we achieved a constant high expression of PER in all tissues containing TIM (data not shown). Fecundity in *tim*(UAS)Gal4/UAS-*per* females was as high as in the females from one parental line and even higher than the other parental line (Fig. 3.6B). Thus, disruption of circadian clocks in the brain and body by either depletion or continuous expression of PER does not affect the production of mature oocytes. Taken together this data demonstrates that the lower fecundity phenotype is entirely based upon *per* and *tim* in the ovaries and suggest that they are acting in a non-circadian mechanism.

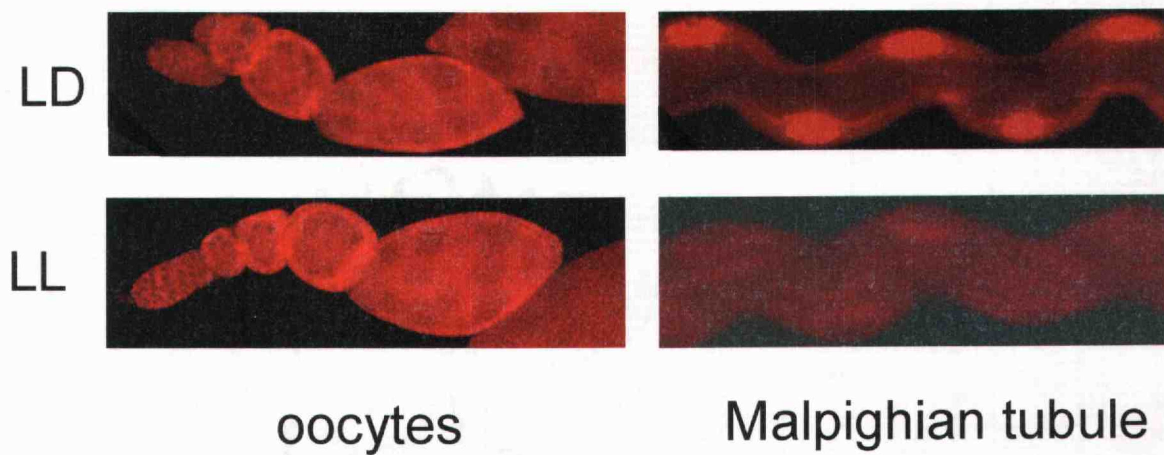
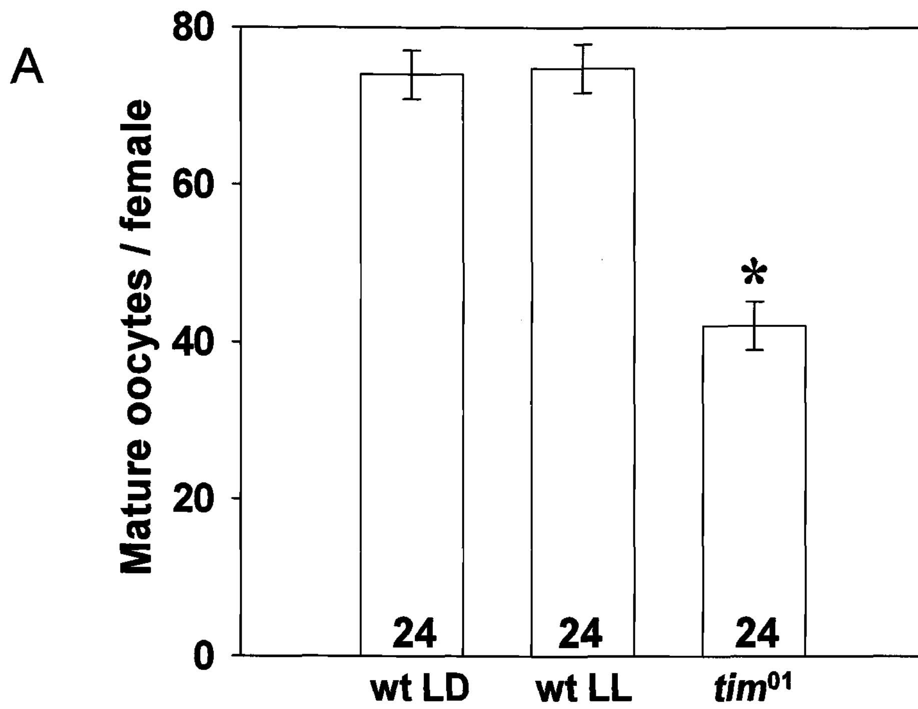
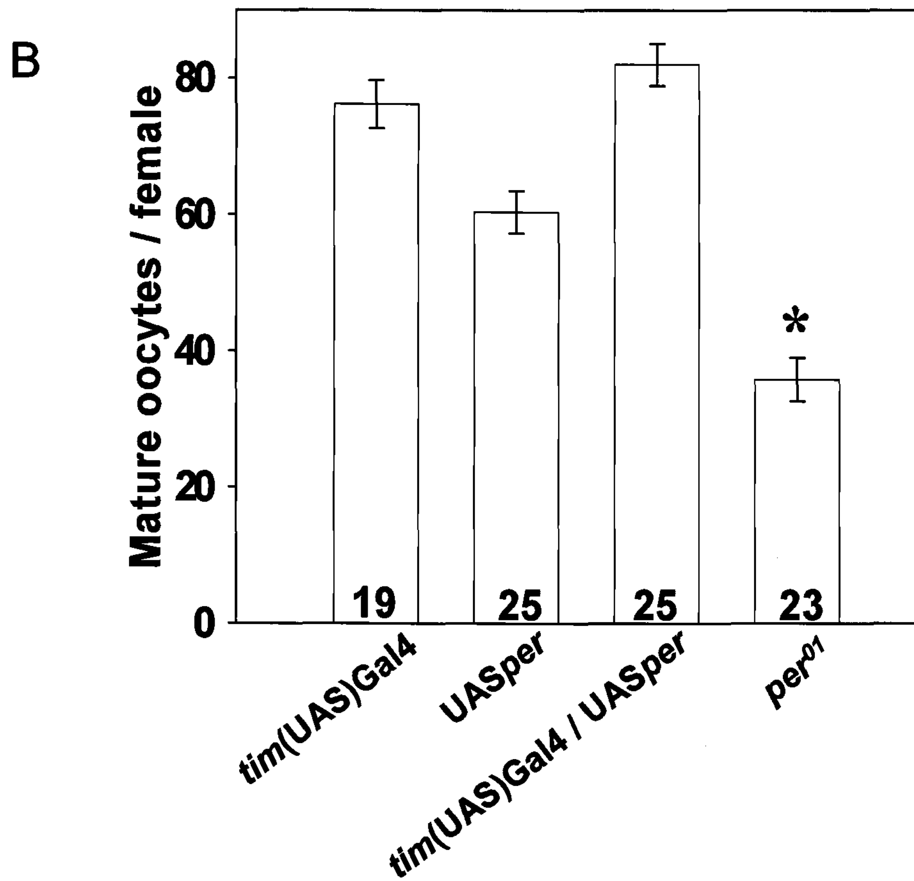


Fig. 3.5 Effects of constant light on TIM expression in wt females kept in LL or LD for 3 days and dissected at ZT 20. Immunofluorescence showed high levels of TIM in ovaries but lack of TIM in the Malpighian tubules of LL females.

Fig. 3.6 Disruption of circadian clocks does not affect female fecundity. (A) No difference was observed in the average number of mature oocytes produced by wt females kept in LL or LD, while *tim*^{01} females reared in LD produced significantly fewer mature oocytes. (B) Flies carrying transgene were crossed to to cause continuous expression of *per* in all *tim* expressing cells. Production of mature oocytes in the *tim*(UAS)-*gal4*/ UAS-*per* females with continuous expression of *per* was no lower than the parental lines but was significantly higher than *per*^{01} females. Bars represent the average number (± SEM) of mature oocytes produced by 5-day-old females (*n* shown in bars).

Fig. 3.6





The activation of *per* and *tim* in the circadian feedback loop depends on two transcription factors, dCLOCK (CLK) and CYCLE (CYC) which bind to the *per* and *tim* promoters (Darlington et al., 1998). Consequently, flies with mutated *cyc* or *dClk* express low levels of PER and TIM in the head due to low *per* and *tim* transcription (Allada et al., 1998; Rutila et al., 1998b). To investigate whether *dClk* and *cyc* are necessary for PER and TIM expression in the ovary, we tested the levels of these proteins in ovaries of $dClk^{jrk}$ and cyc^{0} flies by ICC. Malpighian tubules, which harbor a peripheral oscillator, were used as a control tissue. Both, PER and TIM were depleted from Malpighian tubules of $dClk^{jrk}$ and cyc^{0} flies, but remained at high levels in ovaries (Fig. 3.7). These results clearly show that CLK and CYC do not affect transcription of *per* and *tim* in the ovary.
In summary, we present several lines of evidence that the essential components of the negative circadian feedback loop, *per* and *tim,* behave in a non-circadian manner in the ovary. First, *per* and *tim* are not stimulated by transcriptional activators encoded by *dClk* and *cyc*. Second, despite genetic evidence of PER and TIM interaction they remain confined to the cytoplasm in the follicular cells of young egg chambers. Given this localization, they must perform functions different than transcriptional regulation for which they are known in the circadian mechanism.

A third observation inconsistent with the clock function of *tim* is that TIM is not degraded by light in the ovary. This is the first demonstration of a novel role for TIM that is otherwise known as a light-sensitive element of the clock. Degradation of TIM in central and peripheral clocks is mediated by the *cryptochrome* (*cry*) gene encoding a photoreceptor CRY (Emery et al., 2000; Ivanchenko et al., 2001; Stanewsky et al., 1998). It would be of interest to determine the presence and subcellular localization of CRY in

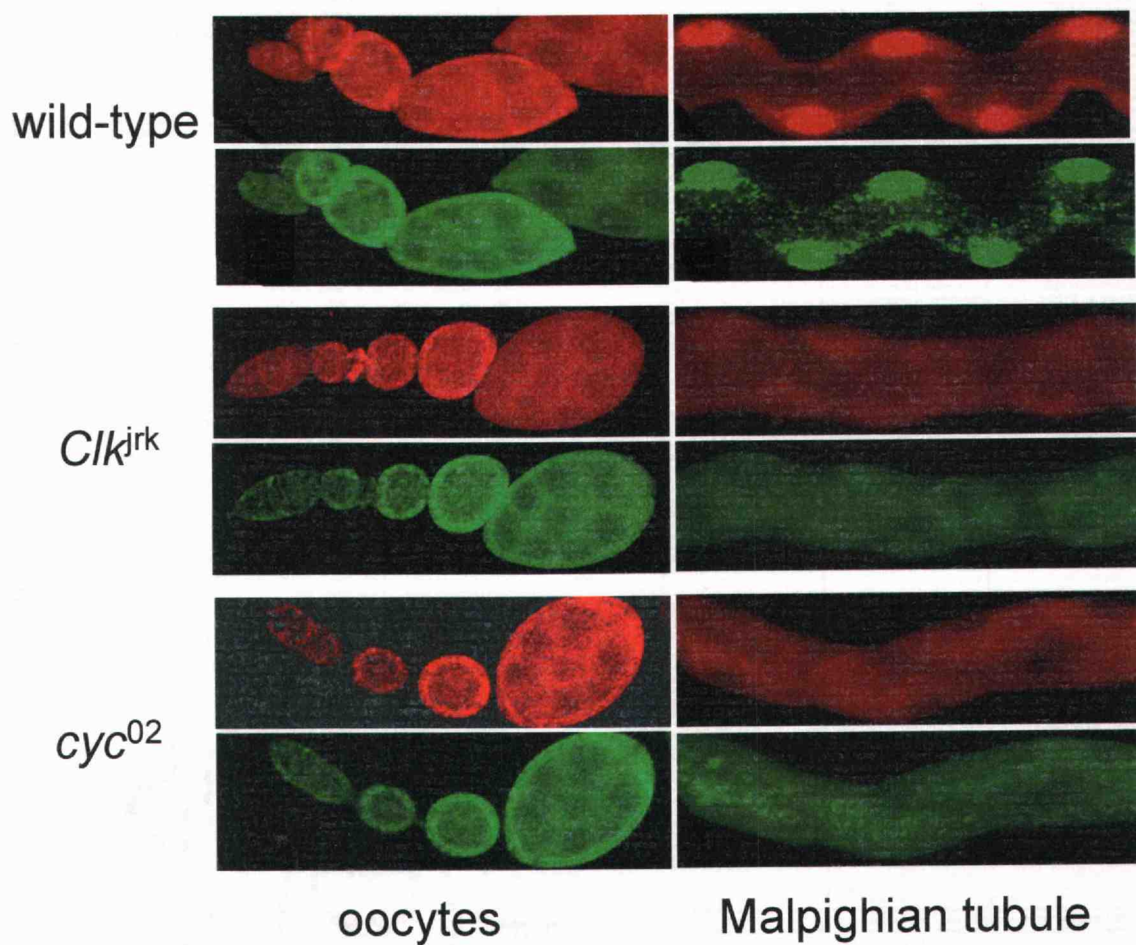


Fig. 3.7 Effects of *dClk* and *cyc* on expression of PER and TIM. Immunofluorescence detected high levels of TIM (red) and PER (green) in ovaries and Malpighian tubules of wild-type females. Both proteins were present in the ovaries of Clk^{Jrk}, and cyc^{02} females while they were absent from the Malpighian tubules. All females were reared in LD and dissected at ZT 20.

the ovaries. Previous studies suggested that *per* is involved in sensing the critical day-length for the induction of ovarian diapause in flies (Saunders, 1990). The light-insensitivity of ovarian TIM and PER demonstrated here excludes ovaries as a candidate tissue contributing to this function.

Despite non-canonical performance of *per* and *tim* in the ovary, two aspects of their behavior are reminiscent of the circadian mechanism. First, there is a several hour delay between high levels of *per* and *tim* mRNA and PER and TIM proteins. The rise in protein levels in previtellogenic egg chambers coincides with the decline of their mRNA. Thus, temporal separation of mRNA and protein synthesis, which is a hallmark of the fly clock (Williams and Sehgal, 2001), is present as a developmental delay in the ovaries. Second, our data suggests an interaction between PER and TIM in follicle cells. Based on the expression results in the *per* and *tim* mutant flies, we infer that TIM stabilizes PER, probably by forming dimers, as they do in clock cells.

Our results generate an interesting question of how the function of PER is controlled at the level of subcellular localization in clock cells versus the ovary. Some answers regarding the lack of nuclear translocation of PER in the ovary may be gleaned from the regulation of other PAS proteins in fly development. For example, nuclear translocation of a bHLH-PAS protein Tango (Tgo) depends on its dimerization with Single-minded or Trachealess; Tgo remains cytoplasmic in cells that do not express either protein (Crews and Fan, 1999; Ward et al., 1998). Translocation of the PER-TIM complex to the nucleus in clock cells depends on their association with a kinase encoded by *doubletime* (*dbt*) (Kloss et al., 2001). It would be of interest to determine if DBT protein is co-expressed with PER and TIM in the follicular cells.

The PAS domain is a multifunctional interaction domain found in proteins from bacteria to humans. In addition to their prominent role in circadian clocks, PAS proteins are involved in development and in sensing the external and internal environment of cells (Gu et al., 2000). We demonstrate here that *per* and *tim* play a developmental role in oogenesis because lack of those genes result in a reduced production of mature oocytes. Preliminary evidence suggests that there is a delay in time and number of oocytes that enter vitellogenesis in per^{01} and tim^{01} females. Vitellogenesis is an important checkpoint in the progression of oogenesis. If yolk is produced at low levels, egg chamber progress into vitellogenesis is retarded (Spradling, 1993). Our data demonstrates that per^{01} and tim^{01} females are not deficient in YP levels (Figure 3B); therefore, the delay seen in per^{01} and tim^{01} females is not due to lack of resources. Rather, we suggest that PER and TIM may be involved in "sensing" the level of nutrients and accordingly regulating the entry of oocytes into the vitellogenic phase.

Several studies have demonstrated non-circadian phenotypes associated with mutations in core clock genes (Andretic et al., 1999; Gotter et al., 2000; Kyriacou et al., 1990a; Kyriacou et al., 1990b; Shaw et al., 2002). However, in no case were these pleiotropic effects linked to the expression of clock genes in specific patterns and locations of the body. Here we link an important developmental phenotype to non-circadian expression of PER and TIM in follicle cells of the ovary. Expression of clock genes in ovaries of the zebrafish and mouse (M. Menaker, personal communication) suggest these genes may have a conserved role in the regulation of oogenesis throughout the animal kingdom.

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Material and Methods

Fly rearing and strains. Flies were raised on cornmeal-yeast-agar-molasses medium. Basic diet contained 35 g of yeast per 1 liter of diet; for specific experiments the amount of yeast varied as noted in the results. Flies were kept in 12 h light: 12 h dark cycles (LD) at 25 °C; by convention, the time of lights-on is denoted as Zeitgeber Time (ZT) 0 and time of lights-off as ZT 12. Wild-type flies used were Canton-S. The following loss-of-function alleles of the clock genes *per*, *tim*, *Clk*, and *cyc* were used: per^{01} (Konopka and Benzer, 1971), per^{04} (Hamblen-Coyle et al., 1989), tim^{01} (Sehgal et al., 1994), tim^{03} (Stempfl et al., 2001), Clk^{Jrk} (Allada et al., 1998), and cyc^{01}, (Rutila et al., 1998b). Additionally, the following two transgenic lines were used *tim*(UAS)-*gal4* (Blau and Young, 1999)and UAS-*per* (Blanchardon et al., 2001). Partial rescue of *per* function only in the lateral neurons was obtained in a per^{01} genetic background in the *per*7.2 line (Frisch et al., 1994). Complete rescue of *per* function in a per^{01} genetic background was performed by crossing per^{01} virgins to transgenic males carrying a *P*-element containing a 13.2-kb genomic *per* DNA fragment. This transgene restores behavioral rhythmicity in per^{01} background (Citri et al., 1987). Similarly, *tim* function was rescued by introducing a transgene expressing the *tim* cDNA under the control of the *tim* promoter into the

background of *tim* null-alleles. The transgene used restores behavioral rhythmicity in tim^{01} and tim^{03} mutant flies (Rutila et al., 1998a; Stempfl et al., 2001). For the rescue, tim^{01} males carrying two copies of the transgene were crossed to tim^{03} females. The resulting transheterozygous tim^{01}/tim^{03} F1 females, carrying one copy of the rescue transgene, were analyzed for their reproduction phenotype. We used per^{01} $/per^{04}$ and tim^{01}/tim^{03} transheterozygotes as a control to exclude the possibility of rescue by complementation.

Mating and fertility assessments. Males and females of the appropriate strain were collected one day before eclosion and isolated in individual vials with 1ml of diet. Flies of appropriate age, sex, and genotype were paired in mating wheels (Hall, 1979) approximately 3 h after lights-on (Sakai and Ishida, 2001) and their copulation observed. After single mating was completed, individual females were transferred to 50 mm Petri dishes with 2ml of diet and allowed to oviposit for 4 days. Females were then removed, and larvae allowed hatching for 24 hours. The number of larvae, dark un-hatched embryos, and white unfertilized eggs were counted. All data were analyzed for statistical significance using ANOVA and Fischer's least significant difference procedure (LSD intervals) at 95% confidence intervals.

Quantification of mature oocytes. To determine the quantity of mature oocytes produced, females of the appropriate strain were collected one day before eclosion and isolated in individual vials containing 1 ml of diet. Virgin females were dissected at the indicated age between ZT0 and ZT4 and both ovaries were placed in PBS with a trace of

the dye Trypan Blue (Sigma) to assist in the visualization of oocytes. Chorionated oocytes with fully formed dorsal filaments contained within the ovary plus eggs that were laid on the diet were counted as mature oocytes. The number of mature oocytes was averaged for each strain and the data were analyzed using ANOVA and Fischer's LSD intervals at 95% confidence intervals.

Assessments of protein and gene expression. For Western blots, fly abdomens were homogenized in lysis buffer and centrifuged for 10 min at 12,000 g. Protein concentration in the supernatant was measured using Bio-Rad protein assay (Bio-Rad Laboratories), and an equal amount of total protein was loaded onto 12% polyacrylamide-SDS gel. Polypeptides resolved by electrophoresis were electroblotted onto the PDV membrane (Milipore). Blots were incubated with a primary polyclonal antibody detecting the three yolk proteins of *Drosophila* (Butterworth et al., 1991). Blots were incubated for 1 h in primary antibody diluted 1:1000 in blocking buffer containing 1% bovine serum albumin (BSA) in 0.05 M sodium phosphate-buffered saline (PBS), washed twice in PBS, and incubated for 30 min in a 1:1000 dilution of alkaline phosphatase-conjugated anti-rabbit secondary antibody (Southern Biotechnology Inc). Blots were then washed twice in PBS and color reaction was developed according to the manufacturer's instructions (Roche). Pre-stained protein molecular weight standards (Bio-Rad Laboratories) were used for estimates of molecular weights of the blotted proteins.

Tissues for immunocytochemistry were fixed at room temperature in 4% Paraformaldehyde for 25 min (ovaries) or 15 min (Malpighian tubules). Anti-TIM (diluted 1:4,000) and anti-PER (diluted 1:15,000) primary antisera were used, followed by

application of anti-rat Alexa Fluor 594 and anti-rabbit $F(ab)_2$ Alexa Fluor 488 (Molecular Probes) secondary antibodies diluted 1:1,000 (for TIM and PER detection, respectively). Tissues were mounted in Vectashield-DAPI medium (Vector Laboratories) to allow localization of clock proteins relative to cell nuclei. Digital images were acquired using a SPOT CCD camera and software (Diagnostic Instruments) and processed in Adobe Photoshop 6.0. To assess temporal pattern of PER and TIM expression, immunodetection was performed on ovaries dissected and fixed at 6 hour intervals at ZT2, ZT8, ZT14 and ZT 20. Approximately 20 images for each time point and strain were randomized and scored subjectively but blindly for PER and TIM intensity on a scale from 0 to 3 by three independent investigators. The averaged scores show no significant changes in the level of PER and TIM at investigated time points.

For in situ hybridizations ovaries were fixed in 4% Paraformaldehyde for 15 min and hybridizations were performed as described (Gvakharia et al., 2000). Dig-labelled *per* and *tim* antisense RNA probes were hybridized to ovaries dissected and fixed at ZT 4 and ZT16. Tissues were whole-mounted in Vectashield-DAPI medium (Vector Laboratories). Digital images were acquired using a SPOT CCD camera and software (Diagnostic Instruments).

CHAPTER 4

Conclusions and Future Directions

Circadian clocks have been found in organisms ranging from cyanobacteria to humans and the molecular timekeeping mechanism is highly conserved in animals (Young and Kay, 2001). These mechanisms generate many circadian rhythms at the molecular, physiological, and behavioral levels. Temporal coordination of life functions is believed to contribute to an organism's fitness; however, such contributions had not been convincingly demonstrated in any animal. Since the most significant measures of fitness are fertility and fecundity, it is important to focus on the role of circadian clocks in controlling these parameters. The overall goal of my research was to directly investigate the links between circadian clock genes and fitness in order to establish whether these genes confer an adaptive significance in *Drosophila melanogaster.* To this end, I demonstrated that circadian clock genes play a significant role in fertility via their respective functions in the production of gametes in both sexes of *Drosophila.*

The detailed spatial and temporal examination of circadian clock genes in the testes-SV complex revealed that a *bona fide* circadian clock exists in this tissue (Chapter 2). I demonstrated that males with loss of clock function produced fewer progeny than wild-type males when mated to wild-type females. I also showed that *per* and *tim* null males released significantly less sperm from the testes to the SV than wild-type. Given these results, I suggest that this local circadian clock may play an important role in sperm

maturation and or release, although this was not directly demonstrated here. To determine whether the circadian clock in the testes-SV complex is necessary and sufficient for wild-type fertility in males; future research should involve the disruption of the circadian clock in this tissue. Specific disruption of this clock could be achieved through constant expression of circadian clock genes in this tissue which can be accomplish through the yeast Gal4/UAS system (Brand and Perrimon, 1993; Yang and Sehgal, 2001). This experiment could determine if the testes-SV clock is directly responsible for the decline in fertility described here.

There is little knowledge of what output pathways are generated by peripheral circadian clocks and how these pathways are regulated. It would therefore be of great interest to determine if any specific output pathways are under circadian clock control in the testes-SV of male flies. Some candidate output rhythms can be gleaned from research performed on moths in this laboratory. A circadian rhythm in the trans-membrane proton pump, V-ATPase leads to rhythmic acidification of the VD lumen in *Spodoptera littoralis* (Bebas et al., 2002a). Further, a rhythm in secretion of glycoproteins was found in the epithelial cells of the VD in the same moth (Bebas et al., 2002b). These rhythms are believed to aid sperm maturation since their disruption by constant light leads to male sterility. Molecular mechanisms of these rhythms and their possible control could be directly studied via the genetic tools that are available in *Drosophila.*

While studying the effects of circadian clock genes on fertility I also discovered that mated females with loss of clock function in *per* and *tim* produced significantly fewer progeny then wild-type females (Chapter 3). To further understand this phenotype, I investigated oogenesis and showed that *per* and *tim* null females produced nearly 50%

fewer mature oocytes than wild-type. These results suggested that these genes are expressed in the female reproductive system. Spatial examination of *per* and *tim* in the ovaries revealed that their mRNA are expressed in the germaria while the proteins PER and TIM are expressed in follicle cells of previtellogenic and vitellogenic oocytes. Temporal examination of these genes revealed that they are involved in a non-circadian pathway in oogenesis, although the specific mechanism by which this occurs remains unknown. It is intriguing that PER and TIM seem to interact and still remain restricted to the cytoplasm in contrast to their nuclear translocation in clock cells. A detailed molecular study should be completed to determine what circadian components are present and absent in the follicle cells. Knowledge of this may allow us to further dissect what genes are necessary and sufficient for nuclear translocation of the PER-TIM heterodimer. The presence or absence of the protein DBT, which is known to bind to the PER-TIM heterodimer and facilitate translocation of this complex, would be of particular interest (Kloss et al., 1998; Kloss et al., 2001).

In the course of my research I established that TIM which is present in follicle cells is not sensitive to light. Studies in this tissue may give us insights into what genes are essential for resetting the clock via TIM's rapid degradation in the presence of light. The presence or absence of the gene *cry* that is in the TIM degradation pathway and the localization of the CRY protein in the follicle cells would be acutely relevant to understanding this pathway (Emery et al., 1998; Emery et al., 2000).

Using ovaries as a model system, we can also investigate what other genes regulate *per* and *tim* expression since the known regulators *dClk* and *cyc* are not playing this role in the ovary (Darlington et al., 1998). The non-circadian nature of *per* and *tim*

expression in the ovary provides us with an excellent opportunity to gain insights into the circadian clock mechanism.

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