MOLECULAR LINKAGE BETWEEN CIRCADIAN AND PHOTOPERIODIC
CLOCKS IN THE FLESH FLY, *Sarcophaga bullata*

DISSERTATION

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ABSTRACT

A non-diapausing (nd) strain of the flesh fly, *Sarcophaga bullata*, loses the normal daily adult eclosion rhythm as well as the normal photoperiodic diapause response. The inheritance mode of diapause was investigated by crossing this mutant strain with two other rhythmic diapausing strains having different diapause capacities. The crossing results consistently indicated that diapause incidence is inherited in a simple Mendelian pattern, thus a single gene or a small gene cluster controls the seasonal response of diapause in the nd strain. The essential circadian clock genes, *period*, *timeless*, *cycle* and *cryptochrome* were originally targeted as candidates of the single malfunctioning gene in the nd strain. As the initial step to study the genetic differences among strains, the full length cDNA sequences of the *cryptochrome* and the *cycle* genes in *S. bullata* were obtained. The cDNA of the *cryptochrome* gene is 1629 base pairs long, which encodes a protein of 542 amino acids. The *cycle* gene cDNA is 1517 base pairs long, and its product consists of 410 amino acids. Some functional domains are highly conserved. However, no significant differences at the cDNA level were discovered in the four circadian clock genes between the nd strain and the wild-type flies, suggesting that none of these clock genes is the major cause of circadian rhythm loss in the nd strain.
Meanwhile, various mutations on the *period* gene were discovered using another strain of *S. bullata*. Among these mutations, *per<sup>d</sup>* interferes with light detection when determining time of dawn eclosion, while *per<sup>m2</sup>* shifts the daily eclosion time window significantly earlier. These mutations help detailed investigation of functional motifs on the *period* gene. More interestingly, deletion and insertion mutations at the C-terminal region, as well as their correlations with diapause capacities, were discovered. This region on the *period* gene is involved in photoperiodism in *S. bullata*, suggesting that circadian and photoperiodic clocks are related through a shared molecular component.
Dedicated to my family
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CHAPTER 1

INTRODUCTION

1.1 Photoperiodic clocks: seasonal time measurement

1.1.1 Overview

Photoperiodism refers to the functional or behavioral responses of an organism to changes of duration in seasonal cycles of light and darkness in an entire year. It is hypothesized that in insects, some photoperiodic clock system measures the yearly cycle of daylength and determines correspondingly the responses such as seasonal growth, reproduction and migration and most importantly, diapause (reviewed by Danks, 2003).

* D. melanogaster * has only a very limited seasonal response, which makes it less than ideal as a model system to investigate molecular mechanisms of photoperiodic clocks as in circadian studies. One has to turn to other insects that have a more dramatic photoperiodic response. As a result, molecular components and mechanisms of photoperiodism are currently largely unknown and photoperiodism has been studied only
with some theoretical approaches. For example, Veerman (2001) suggested an hour glass model, in which insects measure a single light-dark interval and this is used to determine their seasonal activities.

1.1.2 Diapause: a photoperiodic response

Diapause is a seasonal developmental arrest in insects. During the diapause stage, development and reproduction of insects is slowed or stopped. It is an adaptive developmental response to unfavorable conditions, such as cold, drought or lack of food, in the temperate region.

In a single insect species, diapause occurs at a particular developmental stage. Embryonic diapausing insects such as the silkworm, *Bombyx mori* enter diapause as eggs (Wu et al., 1996). This type of diapause is usually induced and controlled by maternal effects. Some other species such as the blow fly, *Calliphora vicina*, enter a larval diapause (McWatters and Saunders, 1996). Other insects like the cabbage butterfly, *Pteris melete*, enter diapause as pupae (Xue et al., 1997). A tropical beetle, *Callosobruchus maculates*, enters a reproductive diapause during which adult females do not lay eggs (Zannou et al., 2003).

Diapause occurrence is programmed by the insects and is greatly influenced by many external factors. Among all the environmental factors that are responsible for diapause determination, the yearly periodic change of photoperiod is the most powerful
one (Christiansen-Weniger and Hardie, 1999). Another factor that affects diapause, temperature (Christiansen-Weniger and Hardie, 1999), also follows a similar changing pattern on a seasonal basis. Therefore, it has been suggested that some central photoperiodic clock system integrates the daylength and temperature information and controls the diapause response based on these seasonal cues. Saunders and Sutton (1969) have discovered that the photoperiodic clock, which itself is phase set by the light and dark cycle, has a long-term time measuring ability and have demonstrated that the clock is directly involved in diapause of the parasitic wasp, *Nasonia vitripennis*.

Internally, diapause induction and termination is under endocrinological control. Insect hormones play different roles in diapause determination in different species. A neuropeptide hormone, diapause hormone, was discovered in the female silkworm, *Bombyx mori*, inducing embryonic diapause in the offspring (Yamashita, 1996). Other hormones also show diapause-related functions. For example, ecdysteroids are important in induction of diapause in gypsy moth, *Lymantria dispar* (Lee et al., 1997), and juvenile hormone prevents the release of PTTH and ecdysteroids, thus maintaining larval diapause in the bamboo borer, *Omphisa fuscidentalis* (Singtripop et al., 2000). Furthermore, some exogenous messenger molecules were discovered affecting the seasonal diapause. For example, dopamine is a key factor for the induction of diapause in a drosophilid fly, *Chymomyza costata* (Kostal et al., 1998).

Diapause is accompanied by dramatic physiological changes. During diapause, insects reduce their metabolism and reserve energy by protein and lipid storage (Danks,
Diapausing insects also produce some molecules associated with water proofing and cold hardiness, such as glycerol (Han and Bauce, 1995). In addition, insects exhibit diapause specific gene expression patterns. Transcription of many functional genes is shut down in dormancy (Flannagan et al., 1998). On the other hand, expression of some other genes is elevated in different stages of diapause. For example, proteins related to immune responses such as hemolin (Lee et al., 2002) and sarcotoxin II (Rinehart et al., 2003) and heat shock proteins related to stress such as hsp 70 (Rinehart et al., 2003) are all upregulated during diapause. All these events generate products that help insects survive harsh environments.

1.1.3 Genetics: determination of diapause

Diapause has a genetic basis (e.g. Hayes et al., 1987; Roff and Bradford, 2000; Waldbauer and Sternburg, 1973). The fact that much geographic and latitudinal variation in photoperiodism has been observed suggests that there is a great amount of genetic polymorphism for this trait within populations. Various adaptive clines are formed under different local selection pressure (Riihimaa et al., 1996).

Although the heritable characteristic of diapause has been confirmed, conclusions about the mode of inheritance are somewhat contradictory, possibly due to species differences. Fortunately, the availability of different phenotypes provides the possibility of studying the genetic mode by reciprocal crossing. Based on the fact that diapause is characterized by diverse inherent traits in different species, is induced and regulated by
various seasonal environmental changes, and is coordinated by distinct endocrinological factors in different insect species (Danks, 1987; Lee et al., 1997; Yamashita, 1996), it has been suggested that a polygenic system is involved in diapause determination (Wipking and Kurtz, 2000), a view that is partially supported by results obtained in the rice stem maggot, *Chlorops oryzae* (Takeda, 1998). Diapause-related genes are sex linked in some species, such as the European corn borer, *Ostrinia nubilalis* (Reed et al., 1981). Inheritance can be biased towards either parent depending on the species. For instance, the diapause incidence is mainly determined maternally in the blow fly, *Calliphora vicina* (McWatters and Saunders, 1996, 1997).

More evidence, however, suggests that diapause can be determined by only a small subset of diapause-related genes. Several experiments show that inheritance of diapause does not fit an additive model and that the capacity for diapause is genetically transmitted in a manner of incomplete dominance (Henrich and Denlinger, 1983; Kim et al., 1995). In several cases the number of major diapause-controlling genes may be as few as one, as suggested by the simple Mendelian inheritance pattern exhibited by progeny crosses. For example, in crosses of the spider mite, *Tetranychus pueraricola* (Suwa and Gotoh, 2006) and the linden bug, *Pyrrhocoris apterus* (Dolezel et al., 2005), the segregation rate in hybrids was close to 3:1, implying a major role for one gene in diapause induction. Besides the low number of genes involved, the incomplete or complete dominant effects may also be caused by gene integration, based on the fact that multiple diapause-related genes tend to be closely linked in *Culex* mosquitoes (Mori et al., 2007).
1.2 Circadian clocks: daily gene network

1.2.1 Overview

To synchronize life activities to the daily light-dark cycle, almost all organisms possess internal clocks (Ouyang et al., 1998). The clocks are oscillators that maintain free-running rhythms with a period of roughly 24 hours and generate daily rhythms endogenously in cells. This clock system is called the “circadian clock”. The circadian clock, in many cases, is autonomous, and does not rely on constant environmental input (Saunders, 2002). Circadian rhythms are exhibited by various organisms and the ways in which circadian clocks are built are highly conserved (reviews by Young and Kay, 2001).

Membrane electrical activity in the special pacemaker neurons located in the brain is essential for the circadian oscillator to work properly (Nitabach et al., 2005). Brain transplantation experiments further proved that the pacemaker is located in the brain and the humoral factors controlling circadian activity rhythms are released from the brain (Handler and Konopka, 1979).

Independent from the central oscillator in the brain, there are some self-sustained peripheral clocks that control local rhythms. The persistence of molecular rhythms in a greatly variety of cells or peripheral tissues in vitro was discovered in insects, for example, in the Malpighian tubules (Giebultowicz et al., 2000). These peripheral clocks
adjust time in the local tissues and thereby contribute to rhythmicity of the whole organism under the governing of the central clock.

1.2.2 Drosophila: a system for circadian clock mechanism discoveries

With recent progress in molecular biology, many genetic components of the clock were discovered, and the scheme of the clock mechanism has been determined in many different organisms. The fruit fly, Drosophila melanogaster, has been used extensively for circadian rhythm studies.

It was discovered that in D. melanogaster, two interlocked transcriptional feedback loops involving many clock genes generate molecular oscillations that control the daily rhythm. Circadian clock genes involved in the loops include: the two central clock genes whose oscillation provide basic circadian information, the period (per) gene (Konopka and Benzer, 1971) and the timeless (tim) gene (Sehgal, 1995); other genes that serve as transcriptional factors or protein kinases thus regulate the expression patterns of per and tim, such as dClock (Clk) and dBmal1 (Cyc) (Allada et al., 1998; Darlington et al., 1998; Rutila et al., 1998), doubletime (dbt) (Price et al., 1998), vrille (vri) (Blau and Young, 1999), shaggy (sgg) (Martinek et al., 2001), as well as jetlag (jet) (Koh et al., 2006).

Both the per and tim genes are transcriptionally activated by the heterodimers of two circadian clock proteins, CLK and CYC (Allada et al., 1998; Rutila et al., 1998). The
mRNA of per and tim is maintained at a high level throughout the day. Translation at around midnight results in the peak level of TIM protein (Marrus et al., 1996). PER is then stabilized by forming heterodimers with TIM to initiate the nuclear entry of the PER-TIM dimer (Kloss et al., 1998; Martinek et al., 2001). After that, this complex inhibits the function of the CLK-CYC dimer (Lee et al., 1999). After the PER-TIM complex enters the nucleus, TIM protein starts to be degraded (Marrus et al., 1996), which leads to PER degradation by DBT dependent phosphorylation (Rothenfluh et al., 2000). As soon as the fly detects light, another protein named JET transmits light signals to TIM (Koh et al., 2006) and TIM is degraded via the protein kinase SGG (Naidoo et al., 1999) to entrain to the light-dark cycle. This scheme is depicted in Figure 1.

In Drosophila, the blue light photoreceptor cryptochrome (CRY) is involved in input of the light signal (Ceriani et al., 1999). On the other hand, an insect neuropeptide, PDF (Pigment-dispersing factor), is likely to be a principal component carrying circadian information originating from the central oscillator as output effectors (Helfrich-Förster, 1995).

1.2.3 Eclosion time: a circadian response

Many behavioral activities show circadian rhythmicity in insects, such as flight, walking, feeding, mating and so on. Among these activities, adult eclosion rhythms have been most thoroughly investigated. Eclosion is initiated and directed by a neuroendocrine cascade under clock regulation. Truman (1973) showed that circadian eclosion rhythms
in the silkmoth are controlled by brain factors, given the fact that surgical removal of brains from pupae caused an arrhythmic eclosion pattern. It has already been demonstrated that the eclosion pattern is phased by the circadian clock (Pittendrigh and Skopik, 1970). For instance, the emergence of adult flies from their puparia occurs in an 8-10 hours time window determined by the circadian clock (Qiu and Hardin, 1996). Those individuals that have reached a certain developmental stage by this phase emerge during the first available time window, whereas those that have not wait until the next one (Saunders, 2002).

1.3 Controversy: relationships between circadian and photoperiodic clocks

Seasonal clocks are similar to the circadian clock system in that they both rely mainly on input information from daylength. It has been suggested that photoperiodic responses like diapause have a circadian basis (Saunders, 2001). It was hypothesized that the two clock systems are closely related by assuming some circadian clock components belong to the functional components of photoperiodism (Saunders, 2002; Veerman, 2001).

Unfortunately, debates exist and many insist that photoperiodic clocks are independent from circadian clocks due to the fact that photoperiodism is affected by a large number of other inputs such as temperature, food, moisture, density and mating. Some evidence suggests that the two clock systems are basically irrelevant. For example, in the non-diapause mutant of linden bug, *Pyrrhocoris apterus*, the *per* gene is not causally involved in diapause (Dolezel et al., 2005).
However, many researchers believe the existence of a close relationship between circadian and seasonal clock systems, based on the thought that information obtained by the circadian clock can at least be used as a daily timing reference in photoperiodism. Many facts suggest that the two clock systems are related. For example, in the onion fly, *Delia antique*, circadian eclosion rhythm between nondiapause and diapause pupae are found to be totally different (Watari, 2002). Another conclusive study discovered that the two core circadian clock genes, *per* and *tim*, are involved in photoperiodism in *C. costata*. In the *npd*- mutants of the flies that lost their ability to enter larval diapause, an arrhythmic daily expression pattern of *per* and very low expression levels of *tim* were recorded (Kostal and Shimada, 2001; Pavelka et al., 2003).

Therefore, current knowledge on the relationships between the circadian and photoperiodic clocks seems contradictive. The seasonal molecular network and the seasonal model based on daily input are urgently required to understand mechanisms of photoperiodism and to determine the ultimate answer on whether circadian components are involved in seasonal clocks.

1.4 Flesh fly: an ideal subject to test the relationships

The flesh fly, *Sarcophaga bullata*, is ideal to serve as the test case of whether there is a close relationship between the two clock systems because manifestations of both systems can be observed in this species. The flesh fly normally exhibits a circadian
rhythmic pattern of adult eclosion with most adults emerging during the first 10 hours after light-on (Goto et al., 2006).

The flesh fly also enters a pupal diapause, which is a cessation of early pupal development. When the embryos and young larvae are exposed to a daylength shorter than 13.5 hours as well as low temperature during the crucial period of diapause determination (Gnagey and Denlinger, 1984), early pupae temporally stopped their development at a stage before the antenna discs become visible (Denlinger, 1971). During this stage, the brain cells are arrested in G0/G1 phase of the cell cycle (Tammariello and Denlinger, 1998). The diapause programming is controlled by genetic information and is greatly influenced by maternal effects at the same time (Rockey et al., 1989; Webb and Denlinger, 1998).

Another reason to use *S. bullata* to test the possible relationships is because more than one strain of *S. bullata* has been obtained, among which alteration of diapause capacities and abnormal patterns of daily adult eclosion can be observed at the same time. First, the concurrence of lacking both circadian and photoperiodic responses in a non-diapausing strain of *S. bullata* has been confirmed. Secondly, reduced diapause incidence and altered daily eclosion time have been observed in another colony of *S. bullata*. Searching for malfunctions on circadian clock genes and looking at their effects on photoperiodic diapause can be performed on this species. Should any correlations between circadian clocks and photoperiodic responses be determined, the hypothesis that the circadian clock components are involved in photoperiodic determination of pupal
diapause can be tested. The main objectivity of the current study is to discover possible linkage on the molecular level between the circadian and photoperiodic clock systems.
CHAPTER 2

A NON-DIAPAUSING STRAIN

2.1 Direct questions

A long established variant of the flesh fly, *S. bullata*, fails to enter diapause even under strong diapause-inducing conditions. It also displays an arrhythmic daily adult eclosion pattern. These results suggest that a functional circadian clock is necessary for the expression of photoperiodism in this species. This strain of flies could prove useful for discovering the linkage between circadian clock gene and photoperiodic induction of diapause at the molecular level.

2.2 Materials and methods

2.2.1 Insects

Two colonies of *S. bullata* Parker were maintained as described (Denlinger, 1972). In the present study, an old wild-type (wt) strain and a nondiapausing (nd) variant were
used. The old wt strain has been maintained at the Ohio State University and used in the previous studies (e.g., Henrich and Denlinger, 1982). The nd variant was spontaneously derived from the original wt strain when they were maintained by Dr. John H. Werren at the University of Rochester as hosts for the parasitoid wasp, *Nasonia vitripennis*. Stocks of both colonies were reared throughout development in long-day photoperiod conditions (LD 15:9 h) at 25°C.

2.2.2 Diapause incidences

To provide diapause-inducing conditions and to eliminate a possible maternal effect (Denlinger 1971; Henrich and Denlinger 1982), flies of the parental generation were transferred at adult eclosion from a long-day photoperiod to LD 12:12 h at 25°C for adult life and LD 12:12 h at a low temperature for the larvae and pupae that the adults produced. The low temperatures used to induce diapause were 20°C, 17°C, 15°C and 14°C. Diapause incidence for the two strains was recorded at all four low temperatures. The diapause incidence was determined 35 days after larviposition by removing the caps of the puparia to observe the developmental status of the pupae, using criteria previously described (Fraenkel and Hsiao 1968).

2.2.3 Eclosion rhythm

Daily adult eclosion time was measured under LD 15:9 h at 25°C. A fraction collector was used and the eclosion time was monitored by the falling ball method similar
to that described by Saunders (1978). For the wt strain and the nd variant, the balls were collected at intervals of 32 and 30 minutes, respectively.

The degree of rhythmicity in eclosion was measured by the parameter ‘R’ (Winfree, 1970). The theoretical range of R is from 0 when all eclosions occur within the gate to 200, which represents a completely uniform pattern of eclosion. Values of R greater than 90 show statistically uniform eclosion, while values less than 60 are considered to represent a rhythmic pattern (Saunders, 1978).

2.3 Results

2.3.1 Diapause incidence

Diapause in the wt colony was quite high, and decreased at higher rearing temperatures (Table 1). By contrast, no diapause was observed in the nd variant, even at 14°C, the threshold for survival of most flies.

2.3.2 Eclosion rhythm

The adult eclosion patterns, shown in Figure 2, demonstrated that most wt individuals eclosed early in the photophase. The R-value of 8.8 indicates a clear rhythmic diel pattern. By contrast, the nd variant showed a much more uniform eclosion pattern, and the R-value of 103.8 indicates arrhythmicity.
2.4 Discussion

Abnormalities in both daily rhythmicity and seasonal responses coexist in the same strain of *S. bullata*. This strongly suggests that the two clock systems are somehow linked.

It has been reported that the expression levels of two circadian clock genes, *period* and *timeless*, are elevated in this nd strain (Goto et al., 2006). It implies that the aberrant characteristics observed in the nd strain are related to the circadian clock system. Besides malfunctions of these two genes themselves, an upstream component of the clock or a defect in one of the autoregulatory feedback loops are all possibly responsible for the loss of circadian rhythm and diapause induction. In this sense, analysis of the molecular background of the nd strain would provide basic background knowledge for the future probing of potential relationships between circadian and seasonal clocks.
CHAPTER 3

MENDELIAN INHERITANCE OF DIAPAUSE

3.1 Direct questions

The nondiapausing (nd) strain (See Chapter 2) of *S. bullata* fails to enter pupal diapause. Discovery of the reasons for the aberrant responses would be helpful in understanding mechanisms of photoperiodic regulation. As the first step, genetic methods were used to check the inheritance mode of the nd phenotype. Interestingly, the nd phenotype is inherited in a Mendelian manner, suggesting that only one gene is responsible for loss of diapause in the nd strain.

3.2 Materials and methods

3.2.1 Insects

Three colonies of *S. bullata* Parker were maintained as described (Denlinger 1972). In addition to the nd strain (Chapter 2), a novel strain exhibiting low diapause
incidence (ld) was also reared; this strain was derived from a colony of wild-type flies described in Chapter 2, presumably due to the inadvertent artificial selection when rearing the flies. In addition, a new wild-type (wt) colony with a high diapause incidence recently collected in Alabama (30°N latitude), was used. Stocks of all these colonies were reared throughout development in long-day photoperiod conditions (LD 15:9 h) at 25°C.

3.2.2 Reciprocal crossing

Reciprocal crosses between the nd strain and each of the two diapausmg strains were performed. To start the crossing, the sexes were separated on the first day of adult eclosion, after which 20 males flies of one strain and 20 female flies of the other strain were combined for each cross. Hybrids of the first (F1) and second generation resulting from self-crossing of F1 flies (F2), as well as progeny resulting from backcrossing with the nd strain (BcN) and the diapauing strain (BcD), were obtained from these crosses.

3.2.3 Diapause incidences

To provide diapause inducing conditions and to eliminate a possible maternal effect (Denlinger 1971; Henrich and Denlinger 1982), flies of each individual cross were transferred at adult eclosion from a long-day photoperiod to LD 12:12 h at 25°C for adult life and LD 12:12 h at a low temperature for the larvae and pupae that the adults produced. The low temperatures used to induce diapause in the ld and the wt strains were 20°C and 23°C, respectively.
The diapause incidence was determined 35 days after larviposition by removing the caps of the puparia to observe the developmental status of the pupae, using criteria previously described (Fraenkel and Hsiao 1968). After determining the diapause incidences of F1, F2, BcN, BcD and each parental strain, an exact test was performed to determine whether there was a specific coherent incidence ratio within each crossing line (Martin and Austin 1996).

3.3 Results

The recorded diapause incidences are shown in Table 2. The two parallel sets of crossings yielded a similar pattern. First, the diapause incidences were nearly the same in both directions of crossing, suggesting that genetically based differences in diapause induction between the strains does not involve sex linkage. Secondly, the non-diapausing phenotype was an almost completely dominant trait as evidenced by the lack of diapause (nd × ld) or extremely low diapause incidence (nd × wt) in the F1 generation. Thirdly, the diapause incidence in the F2 generation was roughly a quarter of that in the diapausing strain, implying a 3:1 Mendelian segregation ratio. Finally, the back crossing results showing no diapausing flies in the backcross BcN and a diapause incidence reduced by half in BcD, further indicated that inheritance of diapause operates in a simple Mendelian manner. Collectively, these results are consistent with a Mendelian inheritance model in which the non-diapause phenotype in the nd strain is a single autosomal dominant trait.
According to the hypothesized Mendelian inheritance model, the diapause incidences in these crossing results should exhibit (1) no sex biases and (2) simple numeric relationships. The lack of sex biases was tested by comparing the diapause incidences between the two directions in the F1 and F2 hybrids, as well as among the four directions in the backcrosses. Because no significant differences were found (p>0.05, exact test), cumulative diapause incidences of F1, F2 and backcrosses were calculated. As shown in Figure 3, the relationships among recorded diapause incidences also supported this model, which was constructed by compiling all the observed data in a “least-error” way. The ratio of cumulative diapause incidences in the nondiapausing parent (N), BcN, F1, F2, BcD and the diapausing parent (D) was close to 0:0:0:1:2:4 (p>0.05, exact test), as predicted by the model. In both crosses, the non-diapausing characteestic in the nd strain was traced to one gene locus, based on the fact that diapause induction in flesh flies is inherited in a Mendelian manner. Therefore, it is concluded that a single gene, or a cluster of genes arranged closely on the same chromosome, completely controls pupal diapause in S. bullata.

3.4 Discussion

Diapause is a seasonal response programmed by the central photoperiodic clock (Saunders and Sutton 1969), using integrated information of photoperiod and temperature (Christiansen-Weniger and Hardie 1999). Environmental input, however, is not required by all individuals and for all species (Lees 1955), suggesting the existence of inherent factors that control diapause in some cases. Studying the inheritance mode of these
factors provides indirect ways to investigate the processes of diapause induction such as the number and localization of diapause determinants, and eventually, to understand the mechanisms of photoperiodic clocks.

In some species such as the parasitic wasp, *Nasonia vitripennis*, genetic factors affecting the diapause response are mainly cytoplasmic, as implicated by the purely maternal role of diapause determination (Saunders 1965). However, in most cases, these determinants are directly derived from genomic information. Moreover, diapause can be monogenetically controlled in some species. For example, mutations on a single photoreceptor gene are sufficient to affect the photoperiodic response of diapause in the spider mite, *Tetranychus urticae* (Veerman 1980). Finding these single diapause-controlling genes and studying their influence on diapause is a promising way to decipher the mechanisms of unknown genes involved in the photoperiodic clock system.

The diapause-controlling genes are not necessarily homologous in various species, as suggested by different modes of inheritance discovered so far. Even between very closely related species, the identities of diapause-controlling genes may differ. For instance, a diapause-controlling gene is on an autosome in *Drosophila littoralis* but on the X-chromosome in *Drosophila lummei*, thus suggesting a role for two different genes given that gene translocation is unlikely to occur between these two interbreeding species (Lumme and Oikarinen 1977; Lumme and Keranen 1978). It is reasonable to hypothesize that different genes play essential roles at different levels of the complex photoperiodic machinery. Thus, we can expect that a number of different genes are
involved in the diapause response, but that a mutation in any one of these genes may result in a loss of the diapause phenotype.

It has been suggested that a small number of genes play essential roles in pupal diapause determination in *S. bullata*, with the conclusion derived from crossing two lines selected for a high and a low diapause incidence, respectively (Henrich and Denlinger 1983). Our current study involved crosses from a recently collected wild type strain of *S. bullata* and two laboratory lines, one that exhibited no diapause and another line that exhibited a low diapause incidence. In the present study, we discovered the existence of only one single genetic unit that completely controls diapause in this species. An abnormality on a single autosomal gene, most likely due to a mutation, deprives the flies of the capacity to enter pupal diapause, despite the presence of strong diapause-inducing conditions. This abnormal non-diapausin (nd) allele is dominant to the wild type allele. In other words, a single gene or closely-linked gene cluster, when altered, completely shuts down the photoperiodic induction of diapause. The locus is so essential for the proper functioning of the photoperiodic clock that almost all individual flies are prevented from entering diapause as long as the nd allele is present, even under a heterozygous condition. This gene is thus a major “diapause-controlling” gene that regulates entry into diapause.

No noticeable morphological or developmental aberrances are exhibited by the nd strain of *S. bullata*, but we suspect that this gene is involved in the circadian clock system. In addition to displaying a diminished photoperiodic response (*i.e.* diapause), the nd flies
also lost the circadian rhythmicity of adult eclosion (Goto et al. 2006). The flies emerge throughout the day, in contrast to wild-type flies that show an eclosion peak at dawn (Saunders 2002). Therefore, this diapause-controlling gene is likely to be responsible for the loss of circadian timing as well. The involvement of this single diapause-controlling gene in circadian clock function is supported by the significant transcriptional elevation and reduced daily oscillation of two major circadian clock genes, *period* and *timeless* (Goto et al. 2006).

A link has been proposed between the circadian and photoperiodic systems, mainly based on a demonstrated correlation between transcription of circadian clock genes and photoperiodic inputs (Goto and Denlinger 2002; Syrova et al. 2003), although seemingly contradictory results reporting independence of diapause and circadian clock components have been obtained in some species (Dolezel et al. 2005; Lankinen and Forsman 2006; Saunders et al. 1989). Additional evidence suggesting that molecular components are shared between these two time measuring systems is obtained by experiments in other species such as the drosophilid fly, *Chymomyza costata* (Kostal and Shimada 2001; Pavelka et al. 2003) and *Drosophila melanogaster* (Tauber et al. 2007).

In this sense, central circadian clock genes including *period* (Kenopka and Benzer 1971), *timeless* (Sehgal et al. 1994), *cycle* (Rutila et al. 1998) as well as the circadian photoreceptor *cryptochrome* (Stanewsky et al. 1998) are all candidate genes that could potentially be responsible for loss of the diapause response. A survey for functional mutations in these candidate genes in the nd strain may suggest the basis for this
nondiapausing phenotype, but it is also quite possible that the gene represents a novel photoperiodic clock gene or an upstream or downstream gene that is essential for the proper functioning of clock input or output mechanisms.
MOLECULAR CHARACTERIZATION OF CIRCADIAN CLOCK GENES

4.1 Direct questions

It is hypothesized that mutations on at least one of the circadian clock genes in the nd variant (See Chapter 2) of *S. bullata* deprived it of its daily eclosion rhythm. As the first step of testing the hypothesis, the full length sequences of the major circadian clock genes need to be obtained.

In this study, the entire cDNA sequences of two genes involved in circadian clock network, *cry* and *cyc*, were determined using RACE techniques. In addition to the other two circadian clock genes, *per* and *tim* previously sequenced (data not shown), a molecular profile of the essential circadian clock genes of *S. bullata* was constructed. The sequences of these genes were compared between the nd variant and the wild-type strain (See Chapter 3) to see whether there are any mutations responsible for the arrhythmic eclosion of the nd flies.
4.2 Materials and methods

4.2.1 Insects

The colony of the wild-type S. bullata Parker was maintained as described (Denlinger, 1972). Flies were reared throughout development in long-day photoperiod conditions (LD 15:9 h) at 25°C.

4.2.2 RNA extraction

RNA was extracted from heads of male adult flies 8 days after eclosion, 3 hours after light on. Fly heads were homogenized in TRIzol reagent (GIBCO BRL). RNA was purified according to TRIzol standard protocols (GIBCO BRL). The total RNA was resuspended in molecular grade water (GeneMate) and stored at −20°C. The RNA quality was tested using formaldehyde RNA denaturation 1% agarose gel electrophoresis. Total RNA was quantified using a BioSpec-mini DNA/RNA/Protein Analyzer (SHIMADZU).

4.2.3 Synthesis of first strand cDNA

Using purified total RNA, the first strand cDNA was synthesized. A SMART RACE cDNA amplification kit (BD Biosciences Clontech) was used according to the
supplier’s instructions. The SuperScript III Reverse Transcriptase (Invitrogen) was used in constructing the first strand cDNA by heating at 50°C for 1 hour.

4.2.4 RACE (rapid amplification of cDNA ends)

Partial sequences of the cryptochrome and the cycle genes in *S. bullata* were obtained from the GenBank database (245 bp for *cry*, GenBank accession number AB08023; 346 bp for *cyc*, GenBank accession number AB080234). Gene specific primers were designed subsequently based on the partial sequence information. All primers used in amplification are listed in Table 3.

3’ and 5’ RACE PCR for the two genes was conducted using a SMART RACE cDNA amplification kit (BD Biosciences Clontech) to obtain the entire sequences of *cry* and *cyc*. The reaction system included cDNA derived from 50 ng total RNA as the template and 1.25 U of BD Advantage 2 DNA polymerase (BD Biosciences Clontech) in a total volume of 25 µl. The final concentration was 1×BD Advantage 2 PCR buffer, 0.2 mM of dNTP, 0.2 µM of the gene specific primer and 1.0 µM of the universal primers mix provided by BD Biosciences Clontech. Amplification was achieved with the following thermal cycling program: 94°C for 1 min; 8 cycles of 94°C for 30s, 70°C for 30s, 72°C for 3 min; 27 cycles of 94°C for 30s, 68°C for 30s, 72°C for 3 min; and 72°C for 7 min.
4.2.5 Subcloning and sequencing

For each RACE PCR reaction, a single dominant amplified band was selected. Amplicons were specifically cut out from a 1% agarose gel after electrophoresis and were purified using Ultra-free DA tubes (Amicon). A TOPO TA cloning kit (Invitrogen) was used to subclone the amplicons into a pCR2.1-TOPO vector which was used to transform One-Shot Top10 competent cells according to the manufacturer’s standard protocol (Invitrogen). Plasmid DNA was purified using a QIAPrep Spin Miniprep kit (Qiagen).

The purified plasmid DNA was sequenced at the Plant-Microbe Genomics Facility of the Ohio State University using a 3730 DNA Analyzer (Applied Biosystems) with BigDye terminator cycle sequencing chemistry. After new sequence information was obtained, more gene specific primers were designed in order to finish the whole amplicon sequencing (Table 3). The full length sequences of cry and cyc cDNA were determined by combination of the 3’ RACE and 5’ RACE results. The BLAST program was used to search the GenBank sequence repository for sequence identities.

4.2.6 Phylogenetic studies

After comparing the putative amino acid sequences of S. bullata with other species, fast minimum evolution trees (Kimura, 1980) were constructed. In this system, formulae were adopted to estimate evolutionary distances. The evolutionary distances are positively correlated to the number of nucleotide substitutions between homologous sites.
4.3 Results

4.3.1 RNA

On average, 17.8 µg of total RNA was extracted from each adult fly head. Different samples of RNA extracted have OD260 / OD280 values varying from 1.8 to 1.9, which eliminated the possibilities of contamination. After electrophoresis in a formaldehyde agarose gel, three distinct bands were displayed that correspond to 28S, 18S and 5S rRNA. This indicated the integrity of the RNA extracted.

4.3.2 RACE

3’ RACE PCR the primer Cry-F amplified a band of approximately 0.5 kb. 5’ RACE PCR using the primer Cry-R amplified a band of approximately 1.7 kb. The sequencing results were combined to obtain the full length cry cDNA sequence as well as deduced amino acid sequence (Figure 4).

3’ RACE PCR the primer Cyc-F amplified a band of approximately 1.2 kb. 5’RACE PCR using the primer Cyc-R amplified a band of approximately 0.5 kb. The sequencing results were combined to obtain the full length cyc cDNA sequence as well as deduced amino acid sequence (Figure 5).
4.3.3 Sequence of cryptochrome

The cDNA of cry in S. bullata is 2011 base pairs long. Nucleotide sequence shows high similarity to cry in many other insect species. The nucleotide identities to S. crassipalpis and D. melanogaster are 90% and 69%, respectively. The sequence contains a start codon and a stop codon. The 5’ UTR region is 195 base pairs long, while the 3’ UTR region is 187 base pairs long and contains multiple polyadenylation signals slightly upstream of the poly(A) tail (Figure 4).

The encoding region of cry cDNA is 1629 base pairs long, encoding a CRY protein consisting of 542 amino acids residues. The calculated molecular weight of the protein is 62 kDa.

4.3.4 Sequence of cycle

The cDNA of cyc in S. bullata is 1517 base pairs long. Nucleotide sequence shows high similarity to cyc in many other insect species. The nucleotide identities to S. crassipalpis and D. melanogaster are 95% and 70%, respectively. The sequence contains a start codon and a stop codon. The 5’ UTR region is 186 base pairs long, while the 3’ UTR region is 98 base pairs long and contains multiple polyadenylation signals slightly upstream of the poly(A) tail (Figure 5).
The encoding region of cyc cDNA is 1233 base pairs long, encoding a CYC protein consisting of 410 amino acids residues. The calculated molecular weight of the protein is 47 kDa.

4.3.5 Phylogenetic analysis

The putative amino acid sequences of the two circadian clock genes reported in the current project are closely related to the well studied CRY and CYC genes in D. melanogaster. Considerable similarities were also discovered when compared with many other insect species. This indicates that the circadian clock components of a variety of organisms originated from common ancestral genes. After the number and type of amino acid substitutions were compared, phylogenetic trees were constructed according to the gene sequence information, as shown in Figure 6 and Figure 7.

4.4 Discussion

In the present study, full length cDNA and amino acid sequences of the structural homologues of both D. melanogaster’s cry and cyc genes were obtained from a closely related dipteran species, S. bullata. Combined with the already known full length sequences of per and tim genes (unpublished data), a molecular profile of essential circadian clock components has been constructed.
Excluding alignment gaps, 71% of the CRY amino acids are identical between *D. melanogaster* and *S. bullata* (Table 4). In comparison, 82% CYC amino acid identity was discovered between the two species, indicating that CYC as a central clock component is more conserved than the light transducer CRY (Table 5). Generally, the two genes show high nucleotide and deduced amino acid sequence similarities to their homologues obtained from other insects (data not shown), suggesting a common working mechanism of circadian clocks originating very anciently.

In *D. melanogaster*, the central part of the CRY protein is a flavin adenine dinucleotide (FAD) binding domain important for blue light reception (Emery et al., 2000; Stanewsky et al., 1998). A DNA photolyase homology domain functioning in light detection and transduction is present close to the amino-terminal end, while a carboxyl-terminal region is essential for regulating stability of CRY and its interaction with TIM (Emery et al., 1998). In *D. melanogaster*, the CYC protein contains a basic-helix-loop-helix (bHLH) motif that binds to an E-box in the promoter regions of two other circadian clock genes, *per* and *tim* (Hao et al., 1997). In addition, two PER-ARNT-SIM (PAS) domains were discovered mediating dimerization of CYC with its heterodimeric partner dCLOCK as well as interacting with PAS regions of the PER protein (Huang et al., 1993; Rutila et al., 1998). SMART analysis (Schultz et al., 2000) revealed that these domains were also present in the CRY and CYC protein of *S. bullata* (Figure 4 and Figure 5). Therefore, it was concluded that CRY is a typical blue light receptor synchronizing the circadian clock to the light-dark cycle and CYC is a bHLH-PAS type transcription factor that participates in the temporal regulation of circadian clock in *S. bullata*.
These functional domains on the two circadian related proteins show especially high conservation between *D. melanogaster* and *S. bullata*. PAS and bHLH domains in CYC, FAD binding and the C-terminal domain in CRY are highly conserved because of their importance in correct functioning of the circadian clock system (Table 4 and Table 5). On the other hand, slightly reduced conservation of the photolyase like domain of CRY was observed (Table 5), implying possible diverse light intense and wavelengths used as circadian clues by various organisms.

Two *cry* mutations have been isolated in *D. melanogaster*: *cry*\(^b\) exhibits no circadian oscillation of *per* and *tim* gene products (Stanewsky et al., 1998); *cry*\(^m\) elongates the free-running period to 25.1 ~ 26.6 hours as light intensity decreases (Emery et al., 1998). The sites to which these mutations have been mapped are also conserved in *S. bullata* (Figure 4).

The cDNA sequences of four circadian clock genes, *per*, *tim*, *cry* and *cyc* were compared between the nd variant and the wt strain (data not shown). Surprisingly, no consistent mutations were discovered in any of the four genes. This suggests that any mutations in the nd flies, if present, exist on promoter region of a circadian clock gene or another unchecked clock gene. Finding the mutation resulting in the aberrant circadian phenotypes in the nd flies would possibly lead to discovery of a new circadian clock gene or a new role in photoperiodism of a known gene.
CHAPTER 5

PER MUTATIONS CAUSING EARLY ECLOSION

5.1 Direct questions

A wild-type strain of Sarcophaga bullata has been maintained for a long time (See Chapter 2). Recent pilot studies indicated that various mutations of a central circadian clock gene, period, exist in this strain (data not shown). Moreover, flies of this strain exhibit an altered circadian eclosion pattern when compared with previous generations monitored years ago as well as newly-field collected, wild-type flies.

Is the change of circadian eclosion time caused by per mutations? Possible correlations between specific mutations and alteration of circadian phenotypes would be important for understanding gene function and to study the molecular mechanisms regulating how the circadian clock genes function to control daily insect activities. In this study, flies of S. bullata were separated based on their adult eclosion time in the hope of isolating colonies homozygous on the per locus so that effects of specific mutations can be characterized.
5.2 Materials and methods

5.2.1 Insects and variant selection

Colonies of *S. bullata* Parker were maintained in long-day photoperiod conditions (LD 15:9 h) at 25°C as described (Denlinger 1972).

The new wild-type (wt) colony described in Chapter 3 was used. The original strain used for selection was the old wild-type strain described in Chapter 2. A ‘single pair’ method was used for selection to increase the possibility of obtaining homozygous colonies. In brief, one male and one female fly that emerged during the same time window were isolated from the original colony on the first day of adult eclosion, and the offsprings produced by this single pair constituted the selected colony. Five consecutive generations of single pair selection using the same time window were performed on the same line to obtain the resulting colony. The time windows used for selection were: (1) during scotophase; (2) 4–5 hours after light-on; (3) 9 hours after light-on and before light-off.

5.2.2 RNA extraction

Total RNA was extracted from the head of a single male adult fly from each colony, 8 days after eclosion, 3 hours after light on. The fly head was homogenized in
TRIzol reagent (GIBCO BRL). RNA was purified according to TRIzol standard protocols (GIBCO BRL). The total RNA was resuspended in molecular grade water (GeneMate) and stored at −20°C. The RNA quality was tested using formaldehyde RNA denaturation with 1% agarose gel electrophoresis.

5.2.3 RT-PCR

With purified total RNA, the SuperScript III Reverse Transcriptase (Invitrogen) was used to synthesize the first strand cDNA according to the supplier’s instructions.

The full length sequence of the period gene in *S. bullata* was obtained from the GenBank database (GenBank accession number AB080236). Gene specific primers were designed subsequently at the 5’ and 3’ UTR region. The primers used in amplification were Per-F and Per-R (Table 3).

The PCR reaction system included cDNA derived from 10 ng total RNA as the template and 1.25 U of BD Advantage 2 DNA polymerase (BD Biosciences Clontech) in a total volume of 25 µl. The final concentration was 1×BD Advantage 2 PCR buffer, 0.2 mM of dNTP, 0.2 µM of each primer. Amplification was achieved with the following thermal cycling program: 94°C for 1 min; 35 cycles of 94°C for 30s, 58°C for 30s, 68°C for 4 min; 68°C for 10 min.
5.2.4 Subcloning and sequencing

In each RT-PCR reaction, a band of roughly 3 kb was selected. The amplicon was specifically cut out from a 1% agarose gel after electrophoresis and was purified using Ultra-free DA tubes (Amicon). A TOPO TA cloning kit (Invitrogen) was used to subclone the amplicon into a pCR2.1-TOPO vector which was used to transform One-Shot Top10 competent cells according to the manufacturer’s standard protocol (Invitrogen). Plasmid DNA was purified using a QIAprep Spin Miniprep kit (Qiagen).

The purified plasmid DNA was sequenced at the Plant-Microbe Genomics Facility of the Ohio State University using a 3730 DNA Analyzer (Applied Biosystems) with BigDye terminator cycle sequencing chemistry. The entire encoding sequence of per cDNA from each colony was determined by combination of different sequencing results.

5.2.5 Homozygosity test on the per locus

For each selected line, as well as the wt strain, the sequence of the per encoding region was determined. The sequences obtained from 3 randomly selected individuals in the same colony were compared. If sequence differences at the nucleotide level between any two individuals were less than 0.05%, which was within the error range caused by errors in cDNA synthesis, DNA amplification and sequencing, the colony was considered homozygous.
5.2.6 Eclosion time

Daily adult eclosion time of each homozygous colony was measured under LD 15:9 h at 25°C. A fraction collector was used and the eclosion time was monitored by the ‘falling ball’ method similar to that described by Saunders (1978). The balls were collected at intervals of 32 minutes.

5.3 Results

5.3.1 Circadian mutants of per

Intra-colony sequence comparisons revealed that the wt strain is homozygous at the per locus. In addition, seven homozygous colonies were isolated from the single pair selection based on circadian phenotypes.

Comparison of the per encoding region as well as deduced protein sequence between each isolated line and the wt strain demonstrated that four homozygous colonies selected in the first time window are actually per mutants. Besides gene polymorphism among colonies, two mutations consistently exist in these isolated strains: a single amino acid deletion (per$^{d}$) and a double point mutation (per$m^2$). Deletion of 3 consecutive nucleotides, ATT, near the N-terminus results in loss of Ile (109) in the putative protein sequence. Furthermore, change of nucleotides ACG to GAT causes point mutations on two neighboring amino acids: Asn (750) and Ala (751) are substituted with Arg and Ser,
respectively. Both $per^d$ and $per^{m2}$ are present in three of the isolated strains consequently named dm-1, dm-2 and dm-3. The other mutant strain, d-1, bears only $per^d$ but not $per^{m2}$. No consistent per sequence variations were discovered in the other selected strains compared with the sequence of the wt strain. The strain selected using the second time window is named wt-1 and the other two strains isolated during the third time window are named wt-2 and wt-3, respectively.

5.3.2 Eclosion time

All the isolated strains, including the per mutants, exhibited solid eclosion rhythmicity, as shown in Figure 8 and Figure 9. The circadian eclosion rhythm is supported by Winfree’s R values lower than 60 (Winfree, 1970). Evidently, the mutations on the per gene are not severe enough to ruin completely the functions of PER. Moreover, consistent diel eclosion patterns persisted over many days of examination and over generations, suggesting that the period for adult eclosion is roughly 24 hours.

However, different timing patterns were observed in the per mutants and the wt strains when determining the daily time window for emergence (Figure 8 and Figure 9). The strains with both mutations on the per gene emerged much earlier than strains with the wild-type per gene (Mann-Whitney test, p<0.01). Timing of dm-1, dm-2 and dm-3 strains were roughly 3 hours earlier than that of the wt-1 strain and approximately 5 hours earlier than those of the wt-2 and wt-3 strains (Table 6). No differences were discovered
among the three dm strains (Mann-Whitney test, p>0.05), suggesting that the two mutations only were sufficient to change daily eclosion time.

The daily eclosion time of all 7 isolated strains is summarized in Figure 10. The time window of eclosion was shifted so much in the dm strains that most of the mutant individuals emerged during the scotophase, which is different from many wild-type dipteran insects that have eclosion peaks in early photophase (Brett, 1955; Saunders, 2002). The d-l strain generally emerged during a late time window and was more similar to the wt strains, with approximately 10% of the individuals emerging before light-on (Figure 10; Table 6).

5.4 Discussion

In the present study, two new mutations were reported in the circadian clock gene per in the flesh fly, S. bullata. Specifically, per^d^ is a deletion of a single amino acid due to three missing nucleotides on per mRNA; per^m^ is a double point mutation of two consecutive amino acids resulting from triple nucleotide substitutions.

Several per mutations have been discovered in D. melanogaster. These mutations result in phenotypes of either loss of circadian rhythmicity such as per^0^ or lengthened or shortened circadian rhythms as flies of per^l^, per^s^ and per^t^ exhibit (Baylies et al., 1992; Hamblen et al., 1998). Phenotypic changes in per mutants have been used in identification of new circadian clock genes and to study essential functional domains of
the PER protein (Konopka and Benzer, 1971; Rutila et al., 1996). The per mutations discovered in *S. bullata*, on the contrary, result in no loss of rhythm. This implies that these mutations do not occur at the active sites of important functional domain such as PAS. The main effect of the two mutations is the shifting of daily eclosion time towards the scotophase. This type of shift to an earlier time window has been observed in the mutant *per* in *D. melanogaster* (Qiu and Hardin, 1996).

In *S. bullata*, adult emergence during the night occurs very rarely, in contrast to the large number of eclosions as soon as light is received (Figure 2; Figure 9). A light detection system seemingly exists to determine eclosion time in addition to the central clock system.

Fortunately, a mutant strain with only one mutation *per* was isolated. Therefore, results of each mutation can be studied. The population eclosion time is not shifted earlier in the d-1 strain, indicating that this time window shift is mainly the result of *per* in *D. melanogaster*. In addition, although not as many as in the dm strains, some d-1 flies do emerge during the scotophase. This leads to the conclusion that *per* is related to light detection when eclosion time is determined.

The sites at which the two mutations are mapped are not within known functional domains of PER (Figure 11), which explains the intact diel eclosion rhythm. The positions of the mutations may represent locations of unknown motifs, such as functional domains responsible for daily time determination. Investigations on the detailed
mechanisms of early time shift and night eclosion would be helpful to understand how
the circadian behavioral rhythm is determined by the clock genes in a few central
pacemaker neurons.
CHAPTER 6

CP REGION OF PER INVOLVED IN PHOTOPERIODISM

6.1 Direct questions

A wild-type strain of *Sarcophaga bullata* has been maintained for a long time (See Chapter 2). Recent pilot studies indicated that various mutations of a central circadian clock gene, *period*, exist in this strain (data not shown). Moreover, a gradually lowered diapause incidence was observed in this strain when compared with previous generations monitored years ago, as well as in newly field-collected, wild-type flies.

Is the change of diapause incidence caused by *per* mutations? Possible correlations between specific *per* mutations and alteration of diapause induction would help construct a relationship between a circadian clock gene and photoperiodism in insects. In this study, flies of *S. bullata* were separated based on their photoperiodic responses in hope of isolating colonies homozygous on the *per* locus so that effects of specific mutations can be clarified.
6.2 Materials and methods

6.2.1 Insects and variant selection

Stocks of *S. bullata* Parker were maintained in long-day photoperiod conditions (LD 15:9 h) at 25°C as described (Denlinger 1972). To induce diapause, flies were reared under LD 12:12 h at 25°C for adults of the parental generation and at 23°C for larvae and pupae.

The new wild-type (wt) colony described in Chapter 3 was used. The original strain used for selection was the old wild-type strain described in Chapter 2. A ‘single pair’ method was used for selection to increase the possibility of obtaining homozygous colonies. In brief, under diapause inducing conditions, one male and one female fly of the same photoperiodic phenotype were isolated from the original colony on the first day of adult eclosion and reared in LD 15:9 h at 25°C. The offspring produced by this single pair were reared in a long-day photoperiod before further selections were performed on their progeny to avoid possible maternal effects (Denlinger 1971). Three consecutive single pair selections were performed on the same line to obtain a resulting colony.

The photoperiodic phenotypes used for selection were: adults emerging within 35 days after larviposition; with no antennal discs visible 35 days after larviposition (*i.e.* diapausing), at 23°C. Diapausing flies used for further selection were transferred to LD 15:9 h at 25°C. In order to perform selection on the offspring of these diapause flies, 5 µL
hexane (CQ Concepts) was topical applied on the head of each pupa after removing the caps of the puparia to terminate diapause (Denlinger et al., 1980).

6.2.2 RNA extraction

Total RNA was extracted from the head of a single male adult fly from each colony, 8 days after eclosion, 3 hours after light on. The fly head was homogenized in TRIzol reagent (GIBCO BRL). RNA was purified according to TRIzol standard protocols (GIBCO BRL). The total RNA was resuspended in molecular grade water (GeneMate) and stored at −20°C. The RNA quality was tested using formaldehyde RNA denaturation 1% agarose gel electrophoresis.

6.2.3 RT-PCR

With purified total RNA, the SuperScript III Reverse Transcriptase (Invitrogen) was used to synthesize the first strand cDNA according to the supplier’s instructions. The full length sequence of the period gene in S. bullata was obtained from the GenBank database (GenBank accession number AB080236). Gene specific primers were designed subsequently at the 5’ and 3’ UTR region. The primers used in amplification were Per-F and Per-R (Table 3).

The PCR reaction system included cDNA derived from 10 ng total RNA as the template and 1.25 U of BD Advantage 2 DNA polymerase (BD Biosciences Clontech) in
a total volume of 25 µl. The final concentration was 1×BD Advantage 2 PCR buffer, 0.2 mM of dNTP, 0.2 µM of each primer. Amplification was achieved with the following thermal cycling program: 94°C for 1 min; 35 cycles of 94°C for 30s, 58°C for 30s, 68°C for 4 min; 68°C for 10 min.

6.2.4 Subcloning and sequencing

In each RT-PCR reaction, a band of roughly 3 kb was selected. The amplicon was specifically cut out from a 1% agarose gel after electrophoresis and was purified using Ultra-free DA tubes (Amicon). A TOPO TA cloning kit (Invitrogen) was used to subclone the amplicon into a pCR2.1-TOPO vector which was used to transform One-Shot Top10 competent cells according to the manufacturer’s standard protocol (Invitrogen). Plasmid DNA was purified using a QIAprep Spin Miniprep kit (Qiagen).

The purified plasmid DNA was sequenced at the Plant-Microbe Genomics Facility of the Ohio State University using a 3730 DNA Analyzer (Applied Biosystems) with BigDye terminator cycle sequencing chemistry. The entire encoding sequence of per cDNA from each colony was determined by combination of different sequencing results.

6.2.5 Homozygosity test on the per locus

For each selected line, the sequence of the per encoding region was determined. The sequences obtained from 3 randomly selected individuals in the same colony were
compared. If the sequence differences on the nucleotide level between any two individuals were less than 0.05%, which was within the error range caused by errors in cDNA synthesis, DNA amplification and sequencing, the colony was considered homozygous.

6.2.6 Diapause incidences

Under diapause inducing conditions, the diapause incidence for each homozygous colony was determined 35 days after larviposition, using criteria previously described (Fraenkel and Hsiao 1968).

6.3 Results

6.3.1 Photoperiodic mutants of *per*

Intra-colony sequence comparisons revealed that the wt strain is homozygous at the *per* locus. In addition, five homozygous colonies were isolated from single pair selection based on their diapause responses.

Comparison of the *per* encoding region as well as deduced protein sequence between each isolated line and the wt strain demonstrated that two out of the five homozygous colonies isolated are actually *per* mutants. Both mutations involve changes of a large piece of the amino acid chain near the C-terminus.
One mutant strain isolated using the non-diapausuing phenotype bears an insertion of 9 amino acids in the PER protein, and a deletion of 33 amino acids occurs in PER of the other mutant strain isolated using the diapausuing phenotype (Figure 12). The two mutant strains are correspondingly named insert (ins) and deletion (del), respectively.

6.3.2 Diapause incidences

The diapause incidences of the ins and del strains as well as the wt flies under LD 12:12 h at 23°C were recorded in Table 7.

Compared with the wt flies, the ins strain exhibited a dramatically diminished diapause response (Student’s t-test, p<0.01). By contrast, the diapause incidence of the del strain was elevated significantly (Student’s t-test, p<0.01).

6.4 Discussion

In the present study, two new mutations were reported in the circadian clock gene per in the flesh fly, S. bullata. Correlations were discovered between the mutations and the seasonal response of pupal diapause.

Interestingly, the insertion and deletion were mapped to the same position in PER in these two mutant lines of S. bullata. This suggests that the region where the mutations
 occur is a hot spot for variation. Based on the fact that length of the region is correlated with photoperiodic diapause responses, it is named a carboxyl-terminal photoperiodic (CP) region (Figure 11).

A TG (threonine-glycine) repeat is normally a characteristic of PER proteins in insects such as D. melanogaster. It has been proposed that the number of TG repeating units is used to compensate for temperature differences in photoperiodic clock systems (Peixoto, 1998). This hypothesis is based on the observation that northern and southern strains of D. melanogaster show different lengths of TG repeats. However, only two repeating units (i.e. TGTG) were found in S. bullata (data not shown) as well as in its close relative, S. crassipalpis (Goto and Denlinger, 2002), which makes length variations unlikely to occur, thus suggesting a potential functional substitution for the TG repeat in flesh flies.

The CP region is a good candidate for the functional counterpart of the TG repeat in S. bullata. Similar to the TG repeat, the CP region acts by ‘adjusting’ length of a certain part of the PER protein. Compared with changing amino acid constitutions, variations by length allow more variation and are more resistant to harmful or nonsense mutations. The similar strategies adopted by different insects are possibly the result of evolutionary convergence. It has been indicated that the TG repeat is not included in the adaptive clock variability in D. littoralis (Lankinen and Forsman, 2006). It is likely that another domain similar to the CP region with different lengths exist in the PER protein in
different populations. Testing this hypothesis would help propose a general mechanism of photoperiodic regulation by circadian clock genes in dipteran insects.

The hypothetical function of the CP region in photoperiodism is supported by the different diapause capacities observed in different CP length variants. Although diapause incidences seem higher in short CP variants, it is risky to draw a conclusion of negative correlations between the CP length and diapause tendencies based on observations of merely 3 strains. Isolation of more CP length mutants would be useful for further testing the relationship between structure of the CP region and the photoperiodic response. In addition, searching for other CP variances of *S. bullata* from different latitudinal areas, such as examining the PER sequences of some tropical subspecies, would be a promising way of understanding functions of the CP region in photoperiodic control of diapause.

Establishing a causal relationship between the circadian and photoperiodic clocks has been a challenge. In this study, it was demonstrated in *S. bullata* that they are related through a small region on the product of a circadian clock gene, *per*. As illustrated in Figure 11, different functions are performed by different domains of the same circadian protein. This may be the reasons why much evidence of independence of photoperiodic phenotypes from circadian clock genes has been reported (e.g. Larkin and Riihimaa, 1992), even if a circadian clock gene is indeed involved in photoperiodism. It should not be denied that the functional roles of circadian clock genes in photoperiodism can be performed by various members of the circadian clock family in different species, such as *tim* (Kostal and Shimada, 2001; Pavelka et al., 2003). The involvement of different
circadian clock genes in photoperiodism reflects the diversity of mechanisms in photoperiodic regulation of insects, which is a direct result of long-time evolution of the circadian clock system.
<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Diapause incidence (%)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>wt</td>
<td>nd</td>
</tr>
<tr>
<td>20</td>
<td>61.9 (215)</td>
<td>0.0 (477)</td>
</tr>
<tr>
<td>17</td>
<td>74.6 (134)</td>
<td>0.0 (480)</td>
</tr>
<tr>
<td>15</td>
<td>82.8 (239)</td>
<td>0.0 (431)</td>
</tr>
<tr>
<td>14</td>
<td>99.2 (125)</td>
<td>0.0 (435)</td>
</tr>
</tbody>
</table>

Table 1: Diapause incidence of a wild-type and a nondiapausing (nd) variant of *S. bullata* under LD 12:12 h at various temperatures. The number of individuals is shown in parentheses.
<table>
<thead>
<tr>
<th>Strain / hybrid</th>
<th>nd × ld</th>
<th>nd × wt</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Diapause incidence (%)</td>
<td>Total number</td>
</tr>
<tr>
<td>P D</td>
<td>28.4</td>
<td>229</td>
</tr>
<tr>
<td>N</td>
<td>0.0</td>
<td>262</td>
</tr>
<tr>
<td>F1 N × D</td>
<td>0.0</td>
<td>277</td>
</tr>
<tr>
<td>D × N</td>
<td>0.0</td>
<td>272</td>
</tr>
<tr>
<td>F2 (N×D) × (N×D)</td>
<td>8.7</td>
<td>321</td>
</tr>
<tr>
<td>(D×N) × (D×N)</td>
<td>3.6</td>
<td>252</td>
</tr>
<tr>
<td>BcD D × (N×D)</td>
<td>14.8</td>
<td>54</td>
</tr>
<tr>
<td>(N×D) × D</td>
<td>8.6</td>
<td>35</td>
</tr>
<tr>
<td>D × (D×N)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>(D×N) × D</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>BcN N × (N×D)</td>
<td>0.0</td>
<td>174</td>
</tr>
<tr>
<td>(N×D) × N</td>
<td>0.0</td>
<td>272</td>
</tr>
<tr>
<td>N × (D×N)</td>
<td>0.0</td>
<td>33</td>
</tr>
<tr>
<td>(D×N) × N</td>
<td>0.0</td>
<td>13</td>
</tr>
</tbody>
</table>

Table 2: Diapause incidences of progeny from reciprocal crossing between the nd strain (N) and the two diapausing strains (D) of *S. bullata*, designated low diapause (ld) and wild type (wt). The strain of the female parent is designated on the left. Parentheses indicate hybrids resulting from each specific crossing.
### Oligonucleotides

<table>
<thead>
<tr>
<th></th>
<th>Sequence (5’→3’)</th>
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<tr>
<td><strong>For RACE:</strong></td>
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<tr>
<td>Cyc-F</td>
<td>GCA ACA TCT GCG CAG TAT ACG TGG TG</td>
</tr>
<tr>
<td>Cyc-R</td>
<td>GGC TTA CAA TCT CCA CCG CTG TAG GC</td>
</tr>
<tr>
<td>Cry-F</td>
<td>GTG CCT GAA TTA GCG AAC GTA CCC AAG</td>
</tr>
<tr>
<td>Cry-R</td>
<td>GAG TTC GCA TGG CAA TCG TGT TAC G</td>
</tr>
<tr>
<td><strong>For sequencing:</strong></td>
<td></td>
</tr>
<tr>
<td>Cyc-Fs</td>
<td>GCA CTC GGT TGA TGG TAA A</td>
</tr>
<tr>
<td>Cry-Fs</td>
<td>CAA TCC GTT GGC TAT AAT C</td>
</tr>
<tr>
<td>Cry-Rs</td>
<td>CTC CGG AGT AGG TGA CTC</td>
</tr>
<tr>
<td>Per-Fs</td>
<td>CTA AGC TAT CAG AAG ATT CTC</td>
</tr>
<tr>
<td>Per-Rs</td>
<td>GCA AGT GCT TGA CAC CTT CTA GAG ACC</td>
</tr>
<tr>
<td><strong>For PCR amplification:</strong></td>
<td></td>
</tr>
<tr>
<td>Per-F</td>
<td>CCT CAG CAA CAC CAT ATT GAA AAG ACC AT</td>
</tr>
<tr>
<td>Per-R</td>
<td>GTT TTC CGC TTT CTT TAT GAC TCG ATG CT</td>
</tr>
</tbody>
</table>

Table 3: Sequences of primers.
<table>
<thead>
<tr>
<th>Domain</th>
<th>identities</th>
<th>positives</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>71</td>
<td>84</td>
</tr>
<tr>
<td>DNA photolyase</td>
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<tr>
<td>FAD binding</td>
<td>79</td>
<td>89</td>
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<tr>
<td>C-terminal</td>
<td>88</td>
<td>94</td>
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</table>

Table 4: Percentage (%) of amino acid similarities between some functional domains of CRY in *S. bullata* and *D. melanogaster*.

<table>
<thead>
<tr>
<th>Domain</th>
<th>identities</th>
<th>positives</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
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<td>91</td>
</tr>
<tr>
<td>bHLH</td>
<td>94</td>
<td>96</td>
</tr>
<tr>
<td>PAS-1</td>
<td>85</td>
<td>95</td>
</tr>
<tr>
<td>PAS-2</td>
<td>84</td>
<td>94</td>
</tr>
</tbody>
</table>

Table 5: Percentage (%) of amino acid similarities between some functional domains of CYC in *S. bullata* and *D. melanogaster*. 
<table>
<thead>
<tr>
<th>Strain</th>
<th>Number of individuals</th>
<th>Eclosion peak (hours after light-off)</th>
<th>Average eclosion time (hours after light-off)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt</td>
<td>168</td>
<td>13</td>
<td>13.2</td>
</tr>
<tr>
<td>wt-1</td>
<td>96</td>
<td>11</td>
<td>12.2</td>
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<tr>
<td>wt-2</td>
<td>106</td>
<td>15</td>
<td>14.1</td>
</tr>
<tr>
<td>wt-3</td>
<td>86</td>
<td>14</td>
<td>14.1</td>
</tr>
<tr>
<td>d-1</td>
<td>81</td>
<td>13</td>
<td>13.3</td>
</tr>
<tr>
<td>dm-1</td>
<td>68</td>
<td>8</td>
<td>8.8</td>
</tr>
<tr>
<td>dm-2</td>
<td>122</td>
<td>8</td>
<td>9.9</td>
</tr>
<tr>
<td>dm-3</td>
<td>66</td>
<td>8</td>
<td>9.2</td>
</tr>
</tbody>
</table>

Table 6: Daily eclosion time of homozygous *per* variants of *S. bullata*. The highest eclosion peak is roughly estimated. The eclosion time on average is calculated in each strain.
<table>
<thead>
<tr>
<th>Strain</th>
<th>Diapause incidence (%)</th>
<th>Number of individuals</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt</td>
<td>78.1</td>
<td>811</td>
</tr>
<tr>
<td>ins</td>
<td>4.0</td>
<td>173</td>
</tr>
<tr>
<td>del</td>
<td>93.0</td>
<td>128</td>
</tr>
</tbody>
</table>

Table 7: Diapause incidence of a wild-type strain and two homozygous *per* variant of *S. bullata* under LD 12:12 h at 23°C. Compared with the wt flies, the two mutations strains exhibit significantly elevated and decreased diapause incidences, respectively (Student’s t-test).
Figure 1: Model of the molecular basis of circadian rhythms in *D. melanogaster*. Stars represent repression. Nuclear PER/TIM dimer preferentially binds the CLK/CYC dimer which serves as the transcriptional activator of *per* and *tim* transcription. Light degrades TIM through the light receptor CRY thus destabilising PER in both the cytoplasm and the nucleus. Adapted from Helfrich-Förster, 2002.
Figure 2: Distribution of adult eclosion in wild-type (upper panel, N = 459) and a nondiapausing (nd) variant (lower panel, N = 265) of *S. bullata* at 25°C under LD 15:9 h. Shaded area indicates the scotophase. Winfree’s R-values are calculated.
Figure 3: Comparisons of diapause incidences of *S. bullata* with theoretically predicted values by a Mendelian inheritance model in the two crosses: nd × ld (A) and nd × wt (B). The four arrows (from left to right) point out the theoretically predicted diapause incidences of N, F2, BeD, D, respectively. BeN and F1 share the same diapause incidence values with N. Vertical lines represent observed diapause incidences.
Figure 4: Nucleotide and putative protein sequence of the cry gene of S. bullata. Nucleotide numbering of the cDNA is on the left side; amino acid numbering is on the right side. The predicted stop codon of translation is marked with an asterisk. Functional motifs are marked: the box indicates DNA photolyase homology domain; FAD binding domain is shaded; The C-terminal domain is underlined. Some sites to which mutations have been mapped are indicated with arrows.
Figure 5: Nucleotide and putative protein sequence of the cyc gene of *S. bullata*. Nucleotide numbering of the cDNA is on the left side; amino acid numbering is on the right side. The predicted stop codon of translation is marked with an asterisk. Functional motifs are marked: bHLH domain is shaded; PAS domains are indicated with boxes.
Figure 6: Fast minimum evolution trees constructed based on amino acid sequence information of CRY protein. Isoforms of CRY homologues are indicated with names of the species. The length of each branch is proportional to the scale of amino acid substitution. The maximum difference included is 50% to study phylogeny of the major groups in insects. The evolutionary status of *S. bullata* is labeled.
Figure 7: Fast minimum evolution trees constructed based on amino acid sequence information of CYC protein. Isoforms of CYC homologues are indicated with names of the species. The length of each branch is proportional to the scale of amino acid substitution. The maximum difference included is 40% to study phylogeny of the major groups in insects. The evolutionary status of *S. bullata* is labeled.
Figure 8: Daily eclosion time of three selected strains of *per* mutants of *S. bullata* at 25°C under LD 15:9 h. The vertical scale is from 0 to 40%. Shaded area indicates the scotophase.
Figure 9: Daily eclosion time of three selected strains of *S. bullata* without mutations at the *per* locus at 25°C under LD 15:9 h. The vertical scale is from 0 to 40%. Shaded area indicates the scotophase.
Figure 10: Summary of daily eclosion time of isolated homozygous colonies of *S. bullata*. Bars represent the time windows in which 95% flies emerge. Vertical lines in the bars point out the median time of eclosion. The large box indicates time of a day. Shaded area indicates the scotophase.
Figure 11: Structure of the PER protein of *S. bullata*. Black boxes represent important functional domains: PAS, CLD and NLS. The shaded box represents the newly discovered C-terminal photoperiodic (CP) region. Positions of the mutations *per*<sup>d</sup> (single deletion) and *per*<sup>m2</sup> (double mutation) are indicated with triangles.

Figure 12: Putative amino acid sequences of the CP region in PER of *S. bullata* showing length variations. Sequences of two photoperiodic mutants are compared with that of the wild-type flies. The blank box indicates the segment deleted in the del strain. The shaded box indicates the segment inserted in the ins strain.
BIBLIOGRAPHY


Helfrich-Förster C., 1995. The period clock gene is expressed in central nervous system neurons which also produce a neuropeptide that reveals the projections of circadian pacemaker cells within the brain of Drosophila melanogaster. Proc. Natl. Acad. Sci. USA 92, 612-616.


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