EVOLUTIONARY AND PHYSIOLOGICAL GENETICS OF BIOLOGICAL TIMING

by

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There are two fundamental environmental rhythms that organisms in nature encounter: (1) the daily rhythm of light and dark that is due to the rotation of the earth about its axis and (2) the yearly seasonal rhythm due to the angle of the earth's rotation relative to the plane of its orbit around the sun. All eukaryotes have an endogenous circadian (daily) clock that allows for the timing of biological events within the context of the daily light-dark cycle. A wide diversity of plants and animals in temperate regions use photoperiodic (daylength) cues to time life history events, such as reproduction and diapause (insect dormancy) within the context of the yearly seasonal cycles. This dissertation focuses on the relationship between the circadian clock, photoperiodic time measurement and diapause.

Chapter I serves as an introduction to biological timing and briefly summarizes the chapters that follow. Chapter II outlines why Drosophila melanogaster, the workhorse of
modern insect genetics, is not an appropriate system for the study of photoperiodism. Chapter III defines the Nanda-Hamner response, the circadian phenotype used in this dissertation, and proposes that the NH response is due to a rhythmic level of circadian disorganization in response to environmental cycle length. Chapters IV and V deal primarily with the long-held proposition that the circadian clock forms the causal basis of photoperiodic time measurement. I show that variation in the circadian clock does not covary with photoperiodic phenotypes among natural populations of *Wyeomyia smithii*, and thus these two processes are evolutionarily independent. Chapter VI describes the first forward genetic screen for candidate genes involved in photoperiodism and diapause termination in any animal. Chapter VII is a discussion of the complexity involved in studies of the genetics of photoperiodism and diapause and how historical inertia of scientific hypothesis acts to confound, rather than clarify, the relationship between genotypes and phenotypes. Chapter VIII is a concluding discussion of the implications of the work presented.

This dissertation includes both previously published and co-authored material.
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CHAPTER I
INTRODUCTION

Only model organisms, living in the relative comfort of a laboratory, live in a world of endless summer; all organisms in nature encounter rhythmic, seasonal environments. There are two great rhythms that organisms encounter in nature: the daily rhythm of light and dark caused by the rotation of the earth about its axis and the seasonal rhythms of climate caused by the angle of the earth’s rotational axis relative to the plane of its orbit. All eukaryotes and some prokaryotes have endogenous circadian (~ 24 h) rhythms that control organismal and cellular events within the context of the daily light:dark cycle (Edmunds 1988; Johnson et al. 1996). A wide array of temperate plants and animals use the length of day (photoperiod) as an environmental cue that controls the timing of life history events (e.g. reproduction, migration, dormancy) within the context of the changing seasons (Bradshaw and Holzapfel 2007a; Dunlap et al. 2004). Life-history theory predicts that evolutionary fitness is maximized when organisms are able to predict and prepare for predictably rhythmic environmental changes (Roff 2002). Indeed, empirical results have shown that fitness is maximized when major biological events occur at the proper time within the daily (Chapter III, Emerson et al. 2008a; Sharma 2003) and seasonal (Bradshaw et al. 2004) cycles.

Fitness in seasonal environments is the product of an organism’s ability to exploit the favorable season, avoid or mitigate the effects of the unfavorable season and to make a
timely transition between the two lifestyles (Bradshaw et al. 2004). In temperate regions many insects overwinter in a state of hibernal dormancy (diapause). Temperate insects develop and reproduce throughout much of the summer and enter diapause early enough to minimize the exigencies of winter (Cohen 1970; Taylor 1980). Photoperiod is a highly reliable predictor of changing seasons and is used by many temperate organisms as the environmental cue for the transition between the active development of summer and the dormancy or migration of the winter (Anonymous 1960; Aschoff 1965; Bradshaw and Holzapfel 2007a; Bünning 1964; Dunlap et al. 2004; Menaker 1971; Saunders 2002; Withrow 1959). This dissertation focuses on the evolutionary and physiological genetics of photoperiodic control of seasonal timing in insects.

The Relationship Between the Circadian Clock and Photoperiodic Time Measurement

Ever since it was first proposed by Bünning (1936), the causal role of the circadian clock in photoperiodic time measurement has remained a tantalizing, yet elusive hypothesis (Saunders 2002; Saunders et al. 2004). It is an appealing hypothesis in that, if true, would mean that a single physiological time-keeping mechanism was involved in controlling rhythmic behavior on both daily and seasonal time scales (See Figure I.1).

Over the last decade, the molecular mechanism of the circadian clock in several organisms has been worked out in considerable detail (Sehgal 2004), owing in large part to work done with the fruit fly Drosophila melanogaster. In brief, the circadian clock in D. melanogaster is generated by several negative and positive feedback loops (Hardin 2005). The expression of two negative transcriptional regulators TIMELESS (TIM) and PERIOD
(PI:R) is mediated by the positive transcription factors CYCLE (CYC) and CLOCK (CLK)
(see Figure VI.2 for a simplified schematic representation of the molecular components of
the Drosophila clock).

Figure I.1. Schematic representation of the various approaches to studying the
photoperiodic control of diapause. (A) Bünning’s hypothesis states that the circadian clock
forms the basis of photoperiodism. This dissertation uses physiological experiments to
directly test for the involvement of the circadian clock in photoperiodism. (B) This
dissertation also takes an approach to studying the genetic basis of photoperiodism that is
unbiased by any assumption of the circadian involvement in photoperiodism.

Considering the influence and appeal of Bünning’s hypothesis and the increased
understanding of the molecular basis of the circadian clock over the last several years, it is
not surprising that the first attempts to dissect the genetic basis of photoperiodic time
measurement in insects used candidate genes involved in the circadian clock, most notably
period and timeless (Dolezel et al. 2007; Dolezel et al. 2005; Dumortier 1994; Goto and Denlinger 2002; Goto et al. 2006; Hodkova et al. 2003; Lankinen and Forsman 2006; Mathias et al. 2005, 2007; Pavelka et al. 2003; Saunders 1990a; Stehlík et al. 2008; Syrova et al. 2003; Tauber et al. 2007). In all of these studies, it is implicitly assumed that if variation in a circadian clock gene (at the functional DNA sequence level, or in transcriptional levels) leads to modification of the circadian clock as well as variation in photoperiodic response, then that result means that the circadian clock is involved in photoperiodic time measurement. As outlined in Chapter VI, this is not a valid assumption and has led to misunderstandings about the genetic basis of photoperiodism. For instance, showing that a particular gene involved in the circadian clock is involved in photoperiodic time measurement does not implicate the circadian clock in photoperiodism, as the gene may be acting pleiotropically in both processes.

In this dissertation I examine the relationship between the circadian clock and photoperiodic time measurement using physiological experiments to bypass the complex issues outlined above that arise when addressing this question using molecular genetic techniques. I then take a genomic, forward genetic approach to search for candidate genes involved in photoperiodic time measurement that is not biased by an assumption of circadian involvement (Figure I.1).

**Brief Outline of this Dissertation**

Herein, I present a series of studies designed to dissect the evolutionary and physiological genetics of photoperiodic time measurement.
Chapter II is a response to a question that is asked of many researchers who study genetics in non-model insects, “Why not work with *Drosophila*?” This question arises because of the power of the genetic resources available to researchers within the *Drosophila* community. The number of researchers using *Drosophila* to address diverse questions has lead to a wealth of tools for genetics and genomics, both at the descriptive and functional levels. One strain of *Drosophila melanogaster*, Canton-S, had been shown to be photoperiodic under very stringent laboratory conditions (Saunders and Gilbert 1990; Saunders et al. 1989), but the generality of photoperiodism in natural populations was yet to be determined, with the exception of weak photoperiodic response in European populations at a single moderately low temperature (Tauber et al. 2007). To determine the environmental cue controlling diapause in natural populations of *D. melanogaster* from North America, I reared flies under a variety of thermal and photoperiodic environments and found that in *D. melanogaster* temperature, and not photoperiod, determined ovarian dormancy. This is consistent with the tropical ancestry of *D. melanogaster*, as temperature plays a larger role in the timing of life history events in tropical regions that lack strong photoperiodic cues. This chapter shows that, although *D. melanogaster* has contributed a great deal to our understanding of evolutionary and molecular genetics of circadian clocks, it is not an appropriate model in which to study photoperiodism. The work presented in Chapter II was done in collaboration with P.S. Schmidt, A.M. Uyemura, and K.L. McDaniel.

Chapters III, IV and V outline a series of physiological experiments that directly address Büning’s (1936) long-held and highly influential proposition that the circadian clock forms the basis of photoperiodic time measurement. One of the main experiments historically used to implicate the circadian clock in photoperiodism is the Nanda-Hamner
(NH) Protocol, where organisms are raised in conditions of short-days with varying long-nights in separate experiments generally creating total light:dark cycle lengths ranging from 24 – 72 h. A rhythmic photoperiodic response to such a protocol was seen as evidence for the involvement of the circadian clock in photoperiodism. This interpretation of the rhythmic response to NH protocols was questioned when it was shown that there was a lack of correlation between variation in NH response and variation in photoperiodic time measurement among populations of *W. smithii* (Bradshaw et al. 2006). It is difficult to argue that two physiological processes are causally related when they evolve independently among populations.

Chapter III lays out an hypothesis for the cause of a rhythmic response to NH experiments that does not implicate the circadian clock in photoperiodism. The resonance principle states that fitness is maximized in organisms that exist in environmental cycles with periods similar to their endogenous circadian periods (Saunders 2002; Yan et al. 1998). In this chapter, I test the resonance principle in two populations of *W. smithii* and show that fitness is maximized in environmental light:dark cycles that are concordant with the endogenous circadian rhythm. This result suggests that the misinterpretation of daylength leading to the rhythmic response to NH experiments may be the result of circadian disorganization and reduced fitness under non-resonant conditions, rather than due to the involvement of the circadian clock in photoperiodic response. This chapter stresses the fact that, though it has been misleading in its historical role, NH response is an “expression of basic circadian rhythmicity, but PPTM [photoperiodic time measurement] is a separate mechanism” (Saunders 2002, p. 481, emphasis Saunders’). The work presented in Chapter
III was the result of a collaboration with W.E. Bradshaw and C.M. Holzapfel and has been previously published (Emerson et al. 2008a).

Photoperiodic time measurement consists of two main components: (1) a timer that measures the length of day, and (2) a counter that accumulates information from the timer and elicits a downstream response when enough signal has been received. Chapters IV and V focus on the relationship between the circadian clock (NH response) and the photoperiodic counter among populations of *W. smithii*. Chapter IV determines, by using extended-night environments, that the photoperiodic counter accumulates information about environmental light:dark cycles rather than endogenous circadian cycles. The work presented in Chapter IV was a collaboration with A.D. Letaw, W.E. Bradshaw, and C.M. Holzapfel and has been previously published (Emerson et al. 2008b). Chapter V shows that two circadian phenotypes (NH response in photoperiodic response and development time) do not covary with the photoperiodic timer or counter among populations of *W. smithii*. Chapter V clearly shows that variation in the circadian clock is not correlated with the evolution of either component of photoperiodic time measurement in *W. smithii*. The work presented in Chapter V was a collaboration with S.J. Dake, W.E. Bradshaw, and C.M. Holzapfel and has been previously published (Emerson et al. 2009b).

Chapter VI is a discussion of some of the complex issues that arise when trying to dissect the relationship between genotype and phenotype, with a focus on the genetic basis of diapause and photoperiodism in insects. The study of the genetic basis of photoperiodism, and its possible relationship with the circadian clock, has long been plagued by historical inertia and the failure to understand various levels of pleiotropy. Historical inertia generated by Bünning's (1936) hypothesis has led to a focus on circadian genes as
candidate loci for photoperiodism. This focus has led to a greater understanding of the circadian clock and its genetic basis, but the genetic mechanisms underlying the evolution of photoperiodic time measurement remain elusive (Bradshaw and Holzapfel 2007b). Also the field has been weighed down by the assumption that once a gene has been annotated to be involved in a particular function, any independent phenotypic effect of that gene must then necessarily be a result of that entire function. The chapter concludes by emphasizing the use of genome-wide forward genetic screens, such as Quantitative Trait Locus (QTL) mapping and gene expression analysis, to understand complex traits. The work presented in Chapter VI was a collaboration with W.E. Bradshaw and C.M. Holzapfel and has been previously published (Emerson et al. 2009a).

Chapter VII describes a microarray-based screen for candidate genes involved in photoperiodic time measurement and the earliest stages of diapause termination in *W. smithii*. The experiments described are novel in that they are the first to look at how the physiological response to daylength, rather than daylength per se, affects the expression of genes. This is critical for the determination of genes involved in photoperiodic response. A novel experimental design, incorporating evolved differences in photoperiodic response among populations of *W. smithii* under a single experimental treatment, allow for the use of genome level screens to find genes whose expression is changed in a manner consistent with involvement in the earliest stages of the photoperiodic termination of diapause. I describe several novel candidate genes for their involvement in photoperiodic time measurement.

Finally, Chapter VIII is a short discussion of how the work presented herein has contributed to the study of biological timing.
CHAPTER II
ENVIRONMENTAL CONTROL OF OVARIAN DORMANCY IN NATURAL POPULATIONS OF DROSOPHILA MELANOGASTER

The work described in this chapter was performed in collaboration with Paul Schmidt (University of Pennsylvania); two undergraduate research assistants, Alison Uyemura and Keely McDaniel; and my principal dissertation advisors William Bradshaw and Christina Holzapfel. The data outlined in this chapter used material generously supplied by Paul Schmidt and Jadwiga Giebultowicz, includes data generated by Alison Uyemura, Keely McDaniel and myself, and was written primarily by myself with considerable assistance from William Bradshaw and Christina Holzapfel.

Introduction
All organisms live in seasonal environments and exhibit life histories that permit the reduction or mitigation of the effects of unfavorable seasons. Many temperate insects enter a state of dormancy characterized by the cessation of development and an increase in stress tolerance. In temperate regions, photoperiodic (daylength) cues are a highly reliable predictor of the seasons and are used by many temperate arthropods to time the onset of dormancy in concert with the onset of winter (Danks 1987; Leather et al. 1993; Tauber et al. 1986). Photoperiodic cues are much weaker at lower latitudes, where arthropods tend to use other cues, such as temperature or humidity, to time seasonal dormancy (Denlinger 1986).
Drosophila melanogaster originated in tropical Africa and has been introduced into temperate North America over the last 400 years (David and Capy 1988). In adult females, the combined effects of short days and low temperatures induce ovarian dormancy (Saunders and Gilbert 1990; Tatar et al. 2001; Williams et al. 2006; Williams and Sokolowski 1993). However, the separate effects of day length and temperature have only been determined in a single long-established laboratory line, Canton-S (isolated ~1930, Bridges and Brehme 1944). When temperature and daylength are varied independently, longer days and warmer temperatures inhibit and shorter days and cooler temperatures promote ovarian dormancy in Canton-S females (Saunders and Gilbert 1990). To date, the effect of photoperiod in natural populations has been evaluated only in European populations and then only at a single temperature (Tauber et al. 2007). Herein, we examine the relative roles of photoperiod and temperature in determining ovarian dormancy in natural populations that reflect extreme latitudinal ranges of D. melanogaster from eastern North America.

Methods

Fly Stocks

We sampled twelve isofemale lines on strawberries from Watch Me Grow Farms in Ft. Pierce, FL (27° 28' N, 80° 21' W) in April, 2007 and ten isofemale lines from apples at Rocky Ridge Orchard in Bowdoinham, ME (44° 02' N, 69° 52' W) in October 2006, representing nearly 20° of latitudinal difference. Isofemale lines were established in the field and subsequently maintained in the laboratory under Light:Dark = L:D = 12:12 at 22-24°C prior to the experiments. All experiments with these lines were completed within 19 months of the collection of the lines.
Throughout the experiments described below, flies were reared in 4.2×10 cm (diameter×height) vials containing 4g of Formula 4-24 Instant Drosophila Medium (Carolina Biological Supply), 150 mg of Fleishman's active dry yeast, and 10 mL of nanopure water per vial. Flies were transferred to fresh vials weekly or more often as needed.

Experimental Chambers

Experimental chambers measured 53×49×18 cm (width×depth×height) with bright white interiors. Light in the chambers was provided by white-light, nine-LED strips that yield a full spectrum of visible light (Sylvania LED/UC/W/9W) creating only 0.2W of heat. LED strip lights were used as a light source because, unlike traditional incubator lights, LEDs produce negligible heat. Heat production by lights is a concern when testing for day length by temperature interaction where more total heat is produced by the lights during longer days and may falsely enhance the apparent effect of day length on development. Each chamber was cooled by air blown through light-baffled ducts. Two blocks of eight chambers were housed in each of two low temperature incubators but response to long and short days was always assessed within the same incubator. The insides of the incubators were maintained in darkness and were opened only when all experimental chambers were in the light phase of the L:D cycle.

To confirm that the flies were responsive to experimental lighting, we placed a vial of flies in the darkest corner of the chamber and noted universal, positive phototaxis within 10 s, both with all LEDs exposed and with eight of the nine LEDs covered with opaque electrical tape. As a further positive control for the efficacy of the LEDs to elicit a photoperiodic response, we exposed 90+ diapausing larvae of the mosquito *Wyeomyia smithii*
for 30 days to long and short days at 25°C with nine or only one LED exposed. Long days elicited 92 and 94% development and short days elicited 0 and 1% development in response to nine or one LED, respectively. Hence either all nine or a single LED was sufficient to elicit a full photoperiodic response in a mosquito known to be sensitive to day length (Bradshaw and Lounibos 1977).

**Scoring Ovarian Development**

We collected flies under stock rearing conditions, sorted them into cohorts with five males and 20 females each and placed them on experimental conditions within three hours of adult eclosion. Since flies may be sensitive to small differences in temperature and incubator thermometers are unreliable indicators of temperatures actually experienced by flies, temperature data were recorded for the duration of the experiment in each treatment by three replicate 15 x 5 mm (diameter x height) data loggers (Watchdog Data Loggers – 100 series, Spectrum Technologies) held within fly vials. At the end of the experimental period, we dissected all the females in each cohort and scored them for ovarian development. For all experiments, we defined reproductive dormancy following King (1970): Dormancy was defined as the presence of only stage 1-7 ovarioles in both ovaries; development was defined as at least one stage 8 ovariole in either ovary (Schmidt and Conde 2006; Schmidt et al. 2005a; Schmidt et al. 2005b).

**Experimental Protocol**

Replicated isofemale lines from Maine and Florida were used to determine the effects of temperature, photoperiod and latitude (population) on ovarian dormancy in disjunct natural populations of *D. melanogaster*. Each cohort was placed into one of 8 environmental treatments made up of either long days (Light: Dark = L:D = 18:06) or short days (L:D =
10:14) at 10, 11, 12 or 14°C (Figure II.1). We chose these temperatures to span those used in previous studies of dormancy in *D. melanogaster* (Tatar et al. 2001; Tauber et al. 2007; Williams et al. 2006). Two to three replicate cohorts for each isofemale line were initiated for each of the eight treatments. Flies were kept in each treatment for 25 (11, 12, and 14°C treatments) or 28 (10°C treatment) days, at which point the animals were frozen, dissected, and scored for ovarian development. We scored an average of 41 ± 10 (mean ± SD) females for reproductive dormancy in each of the 22 lines in each of the eight temperature x photoperiod treatments. A total of 8,551 flies were scored for this experiment.

Figure II.1 Experimental treatments used to study the induction of dormancy in *D. melanogaster*. Representative thermal profiles for each of the four thermal treatments under both long and short daylengths. Profiles are the average of 2–3 independent data-loggers (Watchdog 100, Spectrum Technologies, Inc.) in the fly vials themselves for each of the eight light and temperature combinations in the study. The inset shows the actual mean ± standard deviation temperature for each of the nominal temperature treatments.
Data Analysis

The percentage of females expressing reproductive dormancy (arcsin – square root transformed) was modeled as a function of population (Pop), photoperiod (PPD), temperature (Temp) and isofemale line nested within population (Line (Pop)) and all of the associated interaction terms. The mean square for Line (Pop) was used as the mean square error term for all F-tests that included only the other effects, as the lines were the independent measurements under these conditions. The residual mean square (representing variation among replicate cohorts within lines) served as the mean square error term for all effects including Line (Pop). All statistical analyses were performed using the R program for statistical computing (R Development Core Team 2007).

Results

Temperature was the primary determinant of dormancy in natural lines of *D. melanogaster* from both Florida and Maine (Figure II.2A), alone explaining 67% of the total variation (Table II.1). Tukey’s HSD test showed that the percentage of ovarian dormancy was significantly different in all pair-wise comparisons of temperature treatments (all adjusted P-values < 0.05). Note that in the 10°C treatment, cohorts had three more days to develop than at higher temperatures so the differences between the 10°C and the other treatments are, if anything, underestimates. Population of origin (Figure II.2B), photoperiod (Figure II.2D) or their individual or combined interaction with temperature (Table II.1) had no significant effect and, together, explained only 2% of the variation in dormancy. There was a significant effect of isofemale line (nested within population) and isofemale line by temperature interaction (Figure II.2C), but no significant effect of line by photoperiod,
temperature by photoperiod or their three-way interaction (Table II.1). Hence, there was
greater variation in response to temperature within than between populations and
photoperiod had no significant effect either between populations or among lines within
populations.

Figure II.2  Percentage of individuals expressing ovarian dormancy in natural populations
of _D. melanogaster_ from Maine and Florida in response to temperature and day length. (A)
Dormancy across all lines, populations and day lengths. (B) Dormancy in the Florida and
Maine populations across all temperatures and day lengths. (C) Dormancy in the 22 lines
from Florida and Maine across all day lengths. (D) Dormancy on short and long days across
all populations and temperatures. Error bars represent mean ± two standard errors on a
percentage scale, backtransformed from data analyzed (Table 1) after arc-sin square root
transformation.
Discussion

In natural populations of *D. melanogaster* from highly disjunct populations in eastern North America, temperature is a far more important determinant of ovarian dormancy than is day length or population of origin (Fig. 2A, B, D). The control of dormancy in natural populations of *D. melanogaster* in temperate eastern North America resembles that of tropical insects where temperature and not photoperiod is the major factor initiating dormancy (Denlinger 1986). *Drosophila melanogaster* in eastern North America therefore retains the more tropical control of dormancy, even in a high-latitude population (Maine).

Table II.1 ANOVA table for the effects of experimental treatments on the incidence of ovarian dormancy within and between natural populations of *D. melanogaster* from Maine and Florida (Temp, temperature; PPD, photoperiod; Pop, population; Line, isofemale line. Significant effects have bold P-values.

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>Df</th>
<th>SS</th>
<th>MS</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temp</td>
<td>3</td>
<td>46.824</td>
<td>15.608</td>
<td>70.062</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>PPD</td>
<td>1</td>
<td>0.114</td>
<td>0.114</td>
<td>0.511</td>
<td>0.483</td>
</tr>
<tr>
<td>Pop</td>
<td>1</td>
<td>0.423</td>
<td>0.423</td>
<td>1.898</td>
<td>0.183</td>
</tr>
<tr>
<td>Temp x PPD</td>
<td>3</td>
<td>0.164</td>
<td>0.055</td>
<td>0.246</td>
<td>0.863</td>
</tr>
<tr>
<td>Temp x Pop</td>
<td>3</td>
<td>0.883</td>
<td>0.294</td>
<td>1.321</td>
<td>0.295</td>
</tr>
<tr>
<td>PPD x Pop</td>
<td>1</td>
<td>0.013</td>
<td>0.013</td>
<td>0.056</td>
<td>0.815</td>
</tr>
<tr>
<td>Temp x Pop x PPD</td>
<td>3</td>
<td>0.098</td>
<td>0.033</td>
<td>0.147</td>
<td>0.931</td>
</tr>
<tr>
<td>Line (Pop)</td>
<td>20</td>
<td>4.455</td>
<td>0.223</td>
<td>6.170</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Temp x Line (Pop)</td>
<td>60</td>
<td>3.858</td>
<td>0.067</td>
<td>1.842</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>PPD x Line (Pop)</td>
<td>20</td>
<td>0.677</td>
<td>0.034</td>
<td>0.937</td>
<td>0.540</td>
</tr>
<tr>
<td>Temp x PPD x Line (Pop)</td>
<td>60</td>
<td>1.225</td>
<td>0.021</td>
<td>0.585</td>
<td>0.993</td>
</tr>
<tr>
<td>Cohorts = Residuals</td>
<td>327</td>
<td>11.806</td>
<td>0.036</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1 Error mean square based on lines within populations
2 Variation within populations; isofemale lines nested within populations; error mean square based on cohorts within lines

In the Canton-S laboratory line of *D. melanogaster*, day length has an effect on ovarian dormancy only over a narrow range of temperatures (Saunders and Gilbert 1990) relative to other species of temperate drosophilids that exhibit a robust photoperiodic response over a
wider range of warmer temperatures (Kimura 1984, 1988; Lankinen 1986; Riihimaa and Kimura 1988). We have shown that even in natural populations of North American D. melanogaster, a decrease in temperature from 12 to 10°C results in an increase in ovarian dormancy from 20 to 80%. Hence, a 2°C change in temperature experienced by eastern North American populations elicits approximately the same response in the incidence of diapause as a 10 h change in day length among European populations (Tauber et al. 2007).

In order to express dormancy reliably, flies must be transferred from warmer or room temperature to diapause-inducing low temperatures within a few hours of adult eclosion (Saunders and Gilbert 1990; Tauber et al. 2007; Williams et al. 2006). After a transition from dormancy-inducing temperatures to room temperature under short-day conditions, ovarian development is observed within 8-12 h in Canton-S flies (Richard et al. 1998), showing that transient warm temperatures rapidly override the effect of day length even in the most clearly photoperiodic line of D. melanogaster. It is therefore unlikely that photoperiod plays a substantive ecological role in the regulation of ovarian dormancy in D. melanogaster in nature where temperatures are not constant but are highly variable.

In eastern North American populations of D. melanogaster, selection in stressful laboratory environments results in increased incidence of ovarian dormancy, increased cold resistance, and increased lipid content, but also a tradeoff of reduced fecundity early in adult life (Schmidt and Conde 2006). In a rural orchards, the genetic propensity to enter ovarian dormancy is high in the spring and declines during the summer and early fall (Schmidt and Conde 2006). When lines of D. melanogaster are selected for early and late reproduction and then grown in outdoor cages in eastern Australia, the late selected lines achieve higher fecundity in the spring and, hence, higher overwintering fitness than early selected lines.
(Hoffmann et al. 2003). These experiments indicate that ovarian dormancy represents a genetically variable phenotypic plasticity that is under seasonal selection in nature. We conclude that in *Drosophila melanogaster*, low temperatures herald the imminent approach of winter and are the primary environmental factors that induce a syndrome of traits apparently co-adapted for overwintering: ovarian dormancy, high lipid stores and greater tolerance of environmental stress, especially cold.

**Bridge**

In natural populations from eastern North America, *Drosophila melanogaster* uses thermal, not photoperiodic, cues to time the transition from active development to reproductive dormancy. Although *D. melanogaster* has been a highly active and rewarding model system for the study of biological timing at the circadian (daily) level, its weak (Tauber et al. 2007) or total lack of photoperiodic response (results presented herein) shows that it is not an appropriate organism in which to study photoperiodic time measurement (Chapter VI, Emerson et al. 2009a). There is a wealth of other insects that show clean, reliable, strong photoperiodic responses that are more appropriate for studies of insect photoperiodism (Danilevskii 1965; Danks 1987; Tauber et al. 1986). The remainder of this dissertation will focus on one such insect, the pitcher-plant mosquito, *Wyeomyia smithii*.

In the following chapter, I show that the long-held, though poorly supported, hypothesis that circadian clocks are adaptive in natural populations, is true. A side effect of the work presented in chapter III is its support for an alternative hypothesis for the presence of a rhythmic response to Nanda-Hamner (NH) experiments, which have traditionally been
seen as support for a causal relationship between the circadian clock and photoperiodic time measurement (Saunders 2002).
CHAPTER III

CONCORDANCE OF THE CIRCADIAN CLOCK WITH THE ENVIRONMENT IS NECESSARY TO MAXIMIZE FITNESS IN NATURAL POPULATIONS

The work described in this chapter was performed in collaboration with William Bradshaw and Christina Holzapfel. The experiments were designed by all three participants; the data were collected, analyzed, and written by myself with considerable assistance from William Bradshaw and Christina Holzapfel. This chapter includes previously published, co-authored material (Emerson, K. J., W. E. Bradshaw, and C. M. Holzapfel. 2008. Concordance of the circadian clock with the environment is necessary to maximize fitness in natural populations. Evolution 62:979-983).

Introduction

Circadian clocks with a period of about a day are ubiquitous among eukaryotes and also occur in Cyanobacteria (Edmunds 1988; Johnson et al. 1996). The pervasiveness of circadian clocks across a diverse spectrum of organisms and the requirement of a functional circadian clock for the temporal coordination of both overt behavior and internal organization of cellular biochemistry is often used as evidence that circadian rhythmicity serves an adaptive function (Aschoff 1964; Bünning 1960; Hastings et al. 1991; Pittendrigh 1961, 1993; Sharma 2003; Yan et al. 1998). Indeed, mutant strains of the cyanobacterium *Synechococcus* are most competitive in light:dark (L:D) environments whose period (light plus
dark) approximates that of their circadian free-running period (Yan et al. 1998). In the drosophilid *Drosophila melanogaster* (Klarsfeld and Rouyer 1998; Pittendrigh and Minis 1972), the blowfly *Phormia terranovae*, (von Saint Paul and Aschoff 1978), and the golden hamster *Mesocricetus auratus* (Hurd and Ralph 1998), adult longevity is enhanced in environmental cycles that are the same length as the endogenous circadian period. Relative to wild type, null mutants of genes involved in the circadian clock (clock genes) show reduced carbon fixation, vegetative growth and survivorship in *Arabidopsis* (Dodd et al. 2005), reduced adult longevity in *Drosophila* (Hendricks et al. 2003) and reduced reproductive success in both male and female *Drosophila* (Beaver et al. 2002; Beaver et al. 2003).

In these cases, though, it is not clear what effects the mutations are having on reproductive physiology independently of their effects on circadian organization. Also, in studying the effect of a genetic mutant on fitness, one must maintain a standardized genetic background for both the experimental and control lines, making it hard to generalize the effects of those mutations in natural populations where dominance and epistasis play such large roles (Wolf et al. 2000). Thus it is important to test the effects of phenotypes on fitness using populations of animals segregating naturally occurring levels of genetic variation. With this in mind, we study the effects of concordance of the circadian clock with the environment in natural populations of the pitcher-plant mosquito.

Circadian clocks can be rendered dysfunctional either through genetic means (Hall 1999; Sehgal 2004) or by imposing external light:dark (L:D) cycles whose period \( T = L + D \) varies substantially from the period of oscillation \( \tau \) of an organism's internal circadian clock (Pittendrigh 1965, 1966). The circadian clock resonates with the external L:D cycle when \( T = 24 + n \tau \), but resonance fails when \( T = 24 + \tau (n + 0.5) \), where \( n \) is an integer.
Periods of endogenous circadian rhythms in insects generally range from $\tau \sim 19-26$ h (Länkinen and Forsman 2006; Saunders 2002). The endogenous circadian rhythm of the flesh fly, *Sarcophaga argyrostoma*, is about 24 h (Saunders 1976). When *S. argyrostoma* are exposed to resonant T cycles of 24 or 48 h, there is a strong pupal eclosion rhythm, but when they are exposed to non-resonant T cycles of 36 or 60 h the rhythm is very weak (Saunders 1978a), reflecting disorganization of the circadian clock much as if the flies were subjected to perpetual jet lag.

The pitcher-plant mosquito, *Wyeomyia smithii*, lays its eggs and completes its preadult development entirely within the water-filled leaves of the purple pitcher plant in eastern North America. Throughout their range, they enter a larval dormancy (diapause) that is initiated, maintained, and terminated by day length (Bradshaw and Lounibos 1977). Under a 24 h L:D cycle, short days initiate and maintain diapause whereas long days avert or terminate diapause. Under longer L:D cycles with a fixed short day and increasing night lengths, *W. smithii* exhibit a rhythmic response, alternating between peaks of development with valleys of diapause (Fig. 1a). In *Drosophila melanogaster* (Saunders 1990b), *D. auraria* (Pittendrigh et al. 1991; Pittendrigh and Takamura 1993), *Calliphora vicina* (Saunders 1997), and *Sarcophaga argyrostoma* (Saunders 1973, 1978a), the peak-to-peak or valley-to-valley interval equals the period of adult eclosion or locomotor rhythms and represents the period of the underlying circadian rhythm (Pittendrigh 1981; vaz Nunes and Saunders 1999). In *W. smithii* (Figure III.1A), these experiments repeatedly show resonant short-day responses when $T$ equals 24, 46 or 68 h and non-resonant long-day responses when $T$ equals 35 or 56 h, indicating a circadian period ($\tau$) of $\tau \sim 21$ h in *W. smithii*. 
Herein, we test whether a composite measure of fitness, the net, per-capita expectation of future offspring, depends on resonance of T with the circadian clock in natural populations of *W. smithii*. We break down fitness into its components: pupal survivorship, fecundity and embryonic viability and, because of its frequent use as a surrogate for fitness, we also consider adult longevity. We test the specific a priori hypothesis that mosquitoes exposed to a non-resonant cycle (peak B in Figure III.1A) achieve lower fitness than mosquitoes exposed to resonant cycles (valleys A and C in Figure III.1A). A comparison of fitness between two different resonant cycles (valleys A and C in Figure III.1A) controls for the possibility that a decline in fitness in non-resonant cycles might be due to mosquitoes experiencing extended nights. In addition, we determine fitness in response to long days in a resonant cycle (L:D = 18:6). A comparison of fitness between the 24 h short-day resonant cycle (valley A in Figure III.1A) and L:D = 18:6 controls for the possibility that a decline in fitness in the non-resonant cycle might be due to a response to day length, per se.

**Methods**

Animals from two populations from northern Florida, USA (30°N and 31°N, populations WI and CR from previous studies) were used in this experiment. Both populations were maintained as large, outbred populations with N > 1,000 in the laboratory for 2 (WI) or > 10 (CR) generations to reduce field and maternal effects while maintaining the naturally occurring genetic diversity of populations. Assuming genetic drift, after 10 generations of size of N = 200, heterozygosity of these populations would be reduced by
less than four percent, and thus the populations represent naturally occurring genetic variation.

Before starting the experiment larvae were reared under short-day conditions in order to synchronize all individuals into diapause. Diapausing larvae from each population were then reared (Bradshaw et al. 2003a) on a L:D = 18:6 cycle to promote continuous development without diapause. On the day of pupation, 6 - 10 replicate cohorts were either maintained on L:D = 18:6 (long days) or transferred to L:D = 10:14 (valley A in Figure III.1A), L:D = 10:25 (peak B in Figure III.1A), or L:D = 10:36 (valley C in Figure III.1A) at 23±0.5°C and 80% RH in light-tight experimental chambers held within a climate-controlled room. Cohort size was determined by the number of larvae actually pupating on a single day and ranged from 50-85 pupae in each cohort. The pupae, resulting adults, and their embryonating eggs were maintained under these same conditions until the eggs hatched. Fitness was then equated with the per-capita expectation of future offspring = (number of first instar larvae eventually hatching from a cohort) ÷ (number of pupae in the original cohort).

Pupal survivorship was measured as the number of pupae initiating the cohort that survived to adult eclosion; fecundity was calculated as the mean number of eggs per eclosing female; embryonic viability was measured as the percentage of eggs laid that successfully hatched; and adult longevity was measured as the time between median adult eclosion and median adult death of each cohort.

Differences among light regimens in both fitness, its components, and adult longevity were tested with a one-way ANOVA; if there was a significant effect of light treatment, orthogonal contrasts with 1 degree of freedom each were used to test the specific
a priori hypotheses (Sokal and Rohlf 1995). Prior to analysis of the data, all assumptions of ANOVA were tested and the appropriateness of ANOVA was confirmed (Sokal and Rohlf 1995). Data from both populations were pooled to increase power in the final analysis as the two separate populations showed the same qualitative results.

**Results**

Fitness varied among L:D treatments (Figure III.1B) ($F_{3,24} = 5.12, P < 0.001$). Fitness achieved under L:D = 10:25 was 55% lower than the three other L:D regimens ($F_{1,24} = 14.62, P < 0.001$), while fitness under the latter three regimens did not differ from each other ($P > 0.40$). Fitness under the longer L:D = 10:36 cycle did not differ from the two 24 h cycles ($F_{1,24} = 0.63, P = 0.43$), showing that the reduction in fitness in the L:D = 10:25 cycle is not due to a longer exotic L:D cycle, per se. Finally, in 24-h regimens, fitness did not differ ($F_{1,24} = 0.13, P = 0.72$) between long days (L:D = 10:14) and short days (L:D = 18:06), showing that fitness was not due to variation in day length.

Pupal survivorship ($F_{3,24} = 0.33, P = 0.80$), embryonic viability ($F_{3,24} = 1.43, P = 0.26$), and adult longevity ($F_{3,24} = 0.25, P = 0.85$) did not vary among L:D treatments (Figure III.2). By contrast, per capita female fecundity varied among L:D treatments ($F_{3,24} = 5.12, P < 0.001$). Fecundity under L:D = 10:25 was 58% lower than the three other L:D regimens ($F_{1,24} = 15.36, P < 0.001$), while fecundity under the latter three regimens did not differ from each other ($P > 0.09$). In a similar pattern to that of our composite measure of fitness, fecundity under the longer L:D = 10:36 cycle did not differ from the two 24-h cycles ($F_{1,24} = 3.11, P = 0.09$) nor did it differ ($F_{1,24} = 0.47, P = 0.49$) between short days (L:D = 10:14) and long days (L:D = 18:06).
Discussion

We show for the first time in natural populations of animals that concordance of the circadian clock with the cycling environment is necessary to maximize fitness. We show that the fitness reduction in non-resonating L:D environments is mainly due to a reduction in

![Graph showing rhythmic response and fitness](image)

Figure III.1. Resonance experiments, and fitness in Nanda-Hamner environments. (A) Rhythmic response of *Wyeomyia smithii* from the Gulf Coast of North America (30-31°N) to light:dark (L:D) cycles ranging from L:D = 10:14 - 10:62 in separate experiments (Bradshaw et al. 2003a). The data are pooled from the two populations used in this study. Note that all of these regimens consist of diapause-maintaining short days and long nights so that the rhythmic long-day response represents rhythmic transitions from resonant cycles producing short-day response “valleys” to non-resonant long-day response “peaks”. The arrows indicate three of the four experimental regimens: A, L:D = 10:14; B, L:D = 10:25; C, L:D = 10:36. (B) Fitness (per-capita expectation of future offspring) in response to the L:D cycles indicated in Figure III.1A and to a long-day L:D = 18:6 cycle (L). Error bars represent two standard errors. **P<0.001 when comparing L:D = 10:25 with the other three cycles, which did not differ from each other.
fecundity, whereas pupal survivorship, embryonic viability, and adult longevity are not significantly affected by such environments.

Figure III.2 Components of fitness in response to Nanda-Hamner environments: (a) pupal survivorship; (b) average fecundity per female; (c) embryonic viability, and (d) adult longevity, in the four different light treatments (Light: Dark) used in this study. Error bars represent two standard errors. ***P<0.001 when comparing L:D = 10:25 with the other three cycles, which did not differ from each other.

Much of the recent data on the fitness consequences of circadian organization in animals has only been in reference to a single component or correlate of fitness, in most cases longevity (Hurd and Ralph 1998; Klarsfeld and Rouyer 1998; Kumar et al. 2005; Pittendrigh and Minis 1972). Studying the effect of circadian disorganization on longevity
alone may be misleading in that longevity is often not positively correlated with composite measures of fitness (Bell 1984; Partridge and Harvey 1985; Reznick 1992; Roff 1992; Sheeba et al. 2000; Stearns 1992; Zwaan 1999). For instance, Sheeba et al. (2000) compared adult lifespan and fecundity among several lines of *Drosophila melanogaster* under constant light (LL), 12L: 12D, and constant darkness (DD) and found that a reduction in longevity in adult flies in LL is at least partly a function of an increase in reproductive output early in life. In *W. smithii*, we found that adult longevity did not vary among treatments, despite significant differences in fitness. This result underscores the unreliability of using a single fitness correlate as a surrogate for an appropriate composite index of fitness.

Using loss-of-function mutants in central circadian clock genes *period, timeless, cycle* and *clock* (*per*\(^0\), *tim*\(^0\), *cycl*\(^0\), and *Clk*\(^{ab}\)) in *Drosophila melanogaster*, Beaver et al. (2002) showed that single matings using mutant males produced ~40% fewer progeny, and those progeny had low survivorship to adulthood. This reduction in fitness was associated with the reduction in the amount of sperm released from the testes to the seminal vesicles in null mutant males (Beaver et al. 2002). Mutations in *per* and *tim* may also have clock-independent effects on the production of mature oocytes and viable progeny in females of *D. melanogaster* (Beaver et al. 2003). The mutant lines from these studies come from different genetic backgrounds and are compared to Canton-S flies as a control, making generalizations from these comparisons difficult. In addition, using mutant, inbred lines, allows for pleiotropic and epistatic effects to be fixed within the line, potentially affecting the resulting phenotype. We overcome this problem by using natural populations of mosquitoes that are segregating naturally occurring alleles among large numbers of individuals.
Several studies on the fitness effects of having an environment with the same periodicity as the circadian clock involve the use of short- and long- period mutant lines (Dodd et al. 2005; Yan et al. 1998). These studies use L:D treatments that have the same total period length as the mutant lines and show that fitness is optimized under the environment that matches the organism's free-running period. Here we avoid having to induce mutations by using two classes of extended night environments, one which is resonant with the underlying circadian rhythm and one that is non-resonant as shown in experiments with natural populations of W. smithii (Figure III.1a). We have controlled for the novelty of the experimental treatments by using resonant cycles with integral (1 and 2) multiples of the circadian period. We also control for the effect of genetic background by averaging across many individuals from populations with naturally segregating genetic variation; and, the error term in our analyses incorporates variation between as well as within populations. We have shown that the loss of fitness in the non-resonant cycle is not due either to the extended nights of that cycle or to an effect of day length. The loss of fitness in W. smithii in environmental cycles that are not integral multiples of the circadian clock's period is primarily due to reduced female fecundity (Fig. 2). We observed no effect of cycle length on pupal survivorship, indicating that the switch itself from a resonant (L:D = 18:06) to a non-resonant (L:D = 10:25) was not the cause of reduced fitness. Finally, we observed no effect of cycle length on adult longevity and, had we measured longevity alone, we would not have observed the effect of cycle length on fitness. We conclude that concordance of the period of the circadian clock with the environment is necessary to maximize fitness and confirm the long-held proposition that circadian clocks are adaptive in natural populations.
Bridge

In this chapter, I show that circadian organization, resulting from the concordance of the period of the external environment with that of the endogenous circadian clock, is necessary in order to maximize fitness in two natural populations of *W. smithii*. This is the first example of its kind in animals that has used a composite measure of fitness, rather than a fitness component or correlate. The data presented herein support the hypothesis that a rhythmic responses to NH experiments (eg. Figure III.1) is the result of circadian disorganization under conditions where the period of the environmental rhythm does not match that of the circadian clock. NH response, then, is a direct circadian phenotype (ie. a circadian response to environmental cycle length), and is an example of Saunders’ third scenario for the relationship between NH response and photoperiodic time measurement, namely that “Nanda-Hamner periodicity is an expression of circadian rhythmicity, but PPTM [photoperiodic time measurement] is a separate mechanism” (Saunders 2002, p. 481, emphasis Saunders’).

Photoperiodic time measurement is composed of two separate mechanisms: (1) the photoperiodic timer that measures the length of day, and (2) the photoperiodic counter that accumulates information (number of inductive events) from the timer and elicits a downstream response when some threshold is reached. NH response and the photoperiodic timer have evolved independently among populations of *W. smithii*. The next two chapters explicitly test for a relationship between the circadian clock and the photoperiodic counter in *W. smithii*. Chapter IV determines whether the photoperiodic counter accumulates information about the number of inductive events in a circadian (24h) or environmental (light: dark cycle) fashion.
CHAPTER IV
EXTRINSIC LIGHT: DARK CYCLES, RATHER THAN ENDOGENOUS CIRCADIAN CYCLES, AFFECT THE PHOTOPERIODIC COUNTER IN THE PITCHER-PLANT MOSQUITO, *WYEOMYIA SMITHII*

The work described in this chapter was performed in collaboration with Alathea Letaw, William Bradshaw and Christina Holzapfel. The experiments were designed by all participants; the data were collected and analyzed by Alathea Letaw and myself, and written by myself with considerable assistance from William Bradshaw and Christina Holzapfel. This chapter includes previously published, co-authored material (Emerson, K. J., A.D. Letaw, W. E. Bradshaw, and C. M. Holzapfel. 2008. Extrinsic light:dark cycles, rather than endogenous circadian cycles, affect the photoperiodic counter in the pitcher-plant mosquito, *Wyeomyia smithii*. Journal of Comparative Physiology A 194: 611 – 615).

Introduction

Photoperiod, or the length of the day, is a highly reliable predictor of seasonal change in the temperate regions and a wide diversity of organisms including plants, fish, birds, mammals, insects, and other arthropods use photoperiod to cue the appropriate timing of life-history events with respect to variation in the seasons (Anonymous 1960; Aschoff 1965; Bradshaw and Holzapfel 2007a; Bünning 1964; Menaker 1971; Withrow 1959).

Photoperiodic time measurement in arthropods is generally considered to be the result of
two separate, though related, components: (1) a photoperiodic timer that distinguishes between long and short days (or nights), and (2) a photoperiodic counter that accumulates information from the timer and then triggers downstream processes when some threshold of information has been reached (Saunders 2002; vaz Nunes and Saunders 1999). Herein, we are concerned with the role of light in the accumulation of inductive ('long-day') cycles by the photoperiodic counter used to terminate diapause and how that role changes with latitude and altitude.

Models of the physiological mechanism of the photoperiodic counter fall into two categories: (1) models that require the repeated input of light for the counter to accumulate inductive cycles and, (2) models that require light only to set internal circadian oscillators that are then able to accumulate inductive cycles in the absence of light (vaz Nunes and Saunders 1999). The first category includes two classes of models that do or do not rely on circadian rhythmicity. First, in the "hourglass" model, the circadian clock plays no role and the photoperiodic counter measures an inductive cycle if there is a period of light followed by a period of darkness (Bradshaw et al. 2003b; Lees 1973; Veerman 2001; Veerman and Veenendaal 2003). Second, in the external coincidence model, light plays a dual role: light both entrains a circadian oscillation and triggers the photoperiodic counter if light occurs during the appropriate "inducible" phase of the oscillation (Bunning 1936; Pittendrigh and Minis 1964; Saunders 2002). In both of these models, light is required for the photoperiodic counter to accumulate inductive cycles.

The second category is represented by the internal coincidence model. In this model, dawn and dusk each entrain separate circadian oscillators that trigger the counter to accumulate inductive cycles if the appropriate phases of the two rhythms overlap in time
(Danilevsky et al. 1970; Pittendrigh 1972; Saunders 1978b). Light is required only to set the phases of the dawn and dusk oscillators and repeated inductive events are accumulated even during extended darkness when the appropriate phases of the dawn-set and dusk-set endogenous rhythms overlap.

The first category can be distinguished from the second by using a long-day followed by nights of varying duration. For example, a long day of 18 h followed by a dark period of 6, 30 or 54 h would count as a single cycle under either the hourglass or external coincidence model but would count as 1, 2 or 3 cycles, respectively, under the internal coincidence model. This comparison has been used to determine the role of light in single populations (Veerman and vaz Nunes 1987) but, to our knowledge, no study has considered how the role of light in the photoperiodic counter varies among populations representing climatic extremes due to latitude and altitude within the species’ range. It is important to use geographic variation in the photoperiodic counter in these studies to show that the results are not specific to single populations, but rather generally applicable to the species as a whole.

Herein, we discriminate between single and repeated measurements by the photoperiodic counter in extended night environments using southern, northern, lowland and mountain populations of the pitcher-plant mosquito, Wyeomyia smithii (Coq). We use 24, 48 and 72 h cycles to distinguish between an hourglass/external coincidence and an internal coincidence mechanism for the photoperiodic counter among geographically disparate populations of W. smithii.

We test two predictions: First, if the mechanistic basis of the photoperiodic counter is due to internal coincidence, then multiple inductive events will be recorded by the
photoperiodic counter in extended night environments. Thus the number of light:dark (L:D) cycles to induce 50% development ($LDC_{50}$) among diapausing larvae should be greater for an L:D = 18:06 than either an L:D = 18:30 or an L:D = 18:54 cycle; alternatively, if the mechanistic basis is due to an hourglass or to external coincidence then $LDC_{50}$ should not differ among cycles of varying duration. Second, if the basis of the photoperiodic counter is due to an internal coincidence mechanism, then the ratio between $LDC_{50}$ in response to an L:D = 18:06 cycle and $LDC_{50}$ in response to an L:D = 18:30 or to an L:D = 18:54 cycle should equal 2.0 or 3.0, respectively, due to the counter measuring two inductive events in the 18:30 treatment and three events in the 18:54 treatment; alternatively, if the basis of the photoperiodic counter is due to an hourglass or to external coincidence, then these ratios should both equal 1.0.

Materials and Methods

*Wyeomyia smithii* lays its eggs and completes all of its pre-adult development within the water-filled leaves of the purple pitcher-plant, *Sarracenia purpurea*, and its range closely follows that of its host plant in North America. Throughout its range, short days induce and maintain diapause while long days avert or terminate diapause (Bradshaw and Lounibos 1977). The phenotypic output of the photoperiodic timer is represented by the critical photoperiod, the number of hours of light per day that initiates 50% diapause or 50% development. Critical photoperiod in *W. smithii* increases with latitude and altitude of population origin and has evolved independently of the circadian clock (Bradshaw et al. 2006; Bradshaw et al. 2003a, b). The phenotypic output of the photoperiodic counter is represented by the depth of diapause, the number of long days necessary to terminate
diapause in 50% of a cohort. Depth of diapause also increases with latitude and altitude but depth of diapause has heretofore been evaluated only under 24-h light:dark cycles (Bradshaw and Lounibos 1977).

Mosquitoes were collected as diapausing larvae from geographic extremes of latitude and altitude (Table IV.1) and passed through two to ten generations in the laboratory with effective population sizes $N_e = \frac{4N_MN_F}{N_M + N_F} > 200$ in order to reduce field and maternal effects and to maintain genetic variability ($N_M$ and $N_F$ equal the number of males and females, respectively). With $N_e > 200$ each generation, the cumulative inbreeding after 10 generations would be $F_{10} < 1 - \left(1 - \frac{1}{2 \times 200}\right)^{10} = 2.5\%$ (Hartl and Clark 1989), and thus the samples used represent naturally occurring levels of genetic variation. Each of the four geographic regions (southern, lowland, mountain, and northern) was represented by two independent populations.

At the start of the experiment, larvae were synchronized into diapause by being raised from eggs under short-day conditions for at least 30 days. Diapausing larvae were then placed into one of three experimental long-day cycles, L:D = 18:06, 18:30, or 18:54 for a pre-determined number of cycles in light-tight environmental chambers maintained in a controlled-environment room at 21°C ± 0.5°C. After the pre-determined time under the experimental conditions larvae were transferred to short-day conditions (L:D = 10:14) for at least 14 days to allow for the expression of any development that might have been induced by the experimental treatments. Samples of at least 105 larvae were used for each combination of population, experimental cycle and number of cycles, totaling over 43,000
Table IV.1. Summary of LDC<sub>50</sub> for experimental populations. Source of experimental populations, stage of diapause (III or IV larval instar), LDC<sub>50</sub> ± SE (the number of long-day L:D cycles, followed by short day L:D cycles, that terminates diapause in 50% of a population) and observed ratios for LDC<sub>50</sub> 18:06/18:30 and for LDC<sub>50</sub> 18:06/18:54 with expected ratios of 2.0 and 3.0, respectively, for an internal coincidence model.

<table>
<thead>
<tr>
<th>Region</th>
<th>Loc</th>
<th>Latitude (°N)</th>
<th>Altitude (m)</th>
<th>Stage of Diapause</th>
<th>LDC&lt;sub&gt;50&lt;/sub&gt; 18:06</th>
<th>LDC&lt;sub&gt;50&lt;/sub&gt; 18:30</th>
<th>LDC&lt;sub&gt;50&lt;/sub&gt; 18:54</th>
<th>Expected Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Southern</td>
<td>WI</td>
<td>30.1</td>
<td>10</td>
<td>IV</td>
<td>4.38±0.30</td>
<td>5.54±0.12</td>
<td>2.34±0.13</td>
<td>0.79</td>
</tr>
<tr>
<td></td>
<td>GR</td>
<td>30.8</td>
<td>67</td>
<td>IV</td>
<td>3.56±0.32</td>
<td>4.51±1.22</td>
<td>2.92±0.28</td>
<td>0.79</td>
</tr>
<tr>
<td>Lowland</td>
<td>GS</td>
<td>34.2</td>
<td>20</td>
<td>IV</td>
<td>5.37±0.39</td>
<td>3.27±0.27</td>
<td>3.15±0.05</td>
<td>1.64</td>
</tr>
<tr>
<td></td>
<td>SH</td>
<td>35.0</td>
<td>107</td>
<td>IV</td>
<td>7.25±0.21</td>
<td>6.66±0.52</td>
<td>6.94±0.83</td>
<td>1.09</td>
</tr>
<tr>
<td>Mountain</td>
<td>DB</td>
<td>35.0</td>
<td>900</td>
<td>III</td>
<td>5.20±0.88</td>
<td>7.33±0.51</td>
<td>7.18±1.42</td>
<td>0.71</td>
</tr>
<tr>
<td></td>
<td>HS</td>
<td>35.1</td>
<td>1190</td>
<td>III</td>
<td>9.89±0.26</td>
<td>11.77±2.31</td>
<td>10.81±1.06</td>
<td>0.84</td>
</tr>
<tr>
<td>Northern</td>
<td>RY</td>
<td>45.8</td>
<td>295</td>
<td>III</td>
<td>6.72±0.21</td>
<td>11.36±0.63</td>
<td>11.23±0.38</td>
<td>0.59</td>
</tr>
<tr>
<td></td>
<td>KC</td>
<td>46.2</td>
<td>365</td>
<td>III</td>
<td>9.63±0.33</td>
<td>13.92±0.50</td>
<td>14.08±0.84</td>
<td>0.60</td>
</tr>
</tbody>
</table>

1 Locality code referred to in previous publications from this lab.
larvae used in this study. This experiment was performed in two blocks over the course of one year. The first block was used to define the broader range of response and the second block was used to fill in or extend the results of the first block. To ensure consistency, there was at least one treatment in the second block that duplicated a treatment in the first block.

Logistic regression of long-day response (pupation) as a function of number of experimental cycles was performed using the DRC package of the statistical computing package R (R Development Core Team 2007; Ritz and Streibig 2005). A three-parameter logistic regression was fit to each set of data, assuming a lower bound of 0% long-day response and an upper bound of 100%. LDC$_{50}$ was computed as the 50% intercept along with its standard error for each curve. To test the first prediction, LDC$_{50}$ was calculated after pooling populations within regions; to test the second prediction, LDC$_{50}$ was calculated for each population.

**Results**

All three L:D treatments used in this study were interpreted as long days by the mosquitoes. Long-day response was > 96% in cohorts of larvae maintained for 15 (southern, lowland) or 25 (northern, mountain) cycles at L:D = 18:06, 18:30 or 18:54.

Within regions, long-day response increased as a sigmoid function of the number of long-day cycles experienced by an experimental cohort (Figure IV.1). LDC$_{50}$ was higher for northern and higher altitude populations, which diapause as third instars (9.93 ± 2.31, mean ± SE), than for southern and low altitude populations, which diapause as fourth instars (4.66...
± 0.82) (F_{1,23} = 30.73, P < 0.001). These results confirm previous studies in *W. smithii* (Bradshaw and Lounibos 1972, 1977).

Figure IV.1. Developmental response curves for the four geographic regions in three different long-day extended-night Light:Dark regimens (18:06, 18:30 & 18:54). Animals were exposed to the specified number of long-day L:D cycles and then transferred to short days for at least 14 days before scoring pupation (long-day response).
Table IV.2. Statistical tests of equality of LDC₅₀ for different L:D cycle lengths. LDC₅₀ by geographic region (Table 1) under different light:dark regimens (L:D) and tests for inequality of LDC₅₀ for L:D = 18:06 > L:D = 18:30 and L:D = 18:06 > L:D = 18:54, which are the predictions based on an internal coincidence model. Bold t-values indicate that the relationship between the two LDC₅₀ values is opposite of the direction expected under an internal coincidence model.

<table>
<thead>
<tr>
<th>Region</th>
<th>L:D</th>
<th>LDC₅₀ ± SE</th>
<th>18:06 &gt; 18:30</th>
<th>18:06 &gt; 18:54</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>t</td>
<td>d.f.</td>
</tr>
<tr>
<td>Southern</td>
<td>18:06</td>
<td>3.83 ± 0.20</td>
<td>-2.80</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td>18:30</td>
<td>4.63 ± 0.20</td>
<td>1.17</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>18:54</td>
<td>2.57 ± 0.14</td>
<td>-1.80</td>
<td>45</td>
</tr>
<tr>
<td>Lowland</td>
<td>18:06</td>
<td>6.03 ± 0.35</td>
<td>-1.80</td>
<td>45</td>
</tr>
<tr>
<td></td>
<td>18:30</td>
<td>5.34 ± 0.47</td>
<td>1.17</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>18:54</td>
<td>5.00 ± 0.54</td>
<td>-1.80</td>
<td>45</td>
</tr>
<tr>
<td>Mountain</td>
<td>18:06</td>
<td>8.82 ± 0.50</td>
<td>-15.0</td>
<td>51</td>
</tr>
<tr>
<td></td>
<td>18:30</td>
<td>10.07 ± 0.50</td>
<td>-15.0</td>
<td>51</td>
</tr>
<tr>
<td></td>
<td>18:54</td>
<td>9.01 ± 0.45</td>
<td>-15.0</td>
<td>51</td>
</tr>
<tr>
<td>Northern</td>
<td>18:06</td>
<td>7.99 ± 0.22</td>
<td>-15.0</td>
<td>51</td>
</tr>
<tr>
<td></td>
<td>18:30</td>
<td>13.57 ± 0.31</td>
<td>-15.0</td>
<td>51</td>
</tr>
<tr>
<td></td>
<td>18:54</td>
<td>12.41 ± 0.25</td>
<td>-15.0</td>
<td>51</td>
</tr>
</tbody>
</table>

For each region, LDC₅₀ under the L:D = 18:06 regimen was not greater than under the L:D = 18:30 regimen (Table 2) and LDC₅₀ under the L:D = 18:06 regimen was not greater than under the L:D = 18:54 regimen, except in the southern region (Table IV.2). Hence, with the exception of one of the two comparisons (L:D = 18:06 vs. 18:54) in the southern region, our data support the predictions of an hourglass/external coincidence timer within each region.

To determine whether the L:D = 18:06 vs. 18:54 comparison in the southern region reflected a consistent or inconsistent response of the two replicate populations, we made this comparison in each of the two populations individually (Table IV.1, lines 1-2). LDC₅₀
for one population, CR, was not significantly greater for an L:D = 18:06 than an L:D = 18:54 cycle (t = 1.51, df = 19, P_{1-tail} = 0.074), supporting predictions for an hourglass/external coincidence timer. LDC_{so} for WI was indeed higher for an L:D = 18:06 than for an L:D = 18:54 cycle (t = 6.24, df = 18, P_{1-tail} < 0.001), supporting predictions for an internal coincidence model. Hence, the apparent exception of the southern region is due to the response of a single population to only one of four tests within that region. The results are therefore generally consistent with predictions for an hourglass/external coincidence counter mechanism within each region.

There was no evidence that, across all eight populations, the counter accumulated two inductive cycles in the L:D 18:30 treatment as the ratio, LDC_{so} (18:06) + LDC_{so} (18:30), was significantly less than 2.0 (t = 9.33, df = 15, P_{1-tail} < 0.001) and was not significantly different from 1.0 (t = 0.90, df = 15, P_{2-tail} = 0.397). A similar result was found in the L:D 18:54 treatment where the ratio, LDC_{so} (18:06) + LDC_{so} (18:54) was significantly less than 3.0 (t = 9.32, df = 15, P_{1-tail} < 0.001) and was not significantly different from 1 (t = 0.57, df = 15, P_{2-tail} = 0.578). These results uniformly support predictions for an hourglass/external coincidence counter across all populations as the basis for keeping track of the number of long days.

Discussion

Our results conform to the predictions of an hourglass/external coincidence model for the mechanistic basis of the photoperiodic counter in *W. smithii*. Both the observed inequalities and observed ratios of LDC_{so} between L:D 18:06 and either L:D 18:30 or L:D 18:54 across a broad range of latitudes and altitudes support this category of models and
rejects the internal coincidence model. These results do not distinguish between an
hourglass model or an external coincidence model as the basis for the photoperiodic counter
in *W. smithii*; but, these results do mean that light is necessary to accumulate photoperiodic
information and that the photoperiodic counter of *W. smithii* counts external light:dark
cycles and not internal circadian cycles. Despite variation among populations in the stage
and depth of diapause (Table IV.1), the basic importance of light for the physiological
mechanism underlying the photoperiodic counter prevails throughout the geographic range
of *W. smithii*, including southern, northern, lowland and mountain populations.

In the northern hemisphere, as one proceeds northwards or increases in elevation,
winter arrives progressively earlier in the year and becomes progressively harsher. Critical
photoperiod, a measure of the photoperiodic timer, increases with latitude and altitude
among a wide variety of arthropods (Danks 1987, Table 24; Saunders 2002, Table 10.1;
Taylor and Spalding 1986), including *W. smithii* (Bradshaw and Lounibos 1977). Similarly in
*W. smithii*, LDCso, a measure of the photoperiodic counter, increases with latitude from
southern to lowland to northern localities and increases with altitude from lowland to
mountain localities under either 24-h (Danks 1987, Table 25). Hence, as populations
encounter earlier, harsher winters, they become more prone to enter and remain in diapause.
The positive correlation among populations between critical photoperiod and depth or
intensity of diapause (LCDso) is also reflected by their positive genetic correlation within
populations and forms part of the evolutionarily flexible diapause syndrome in *W. smithii*
(Campbell and Bradshaw 1992).
Bridge

Herein, I have shown that the photoperiodic counter in *W. smithii* accumulates information about the number of inductive Light:Dark cycles rather than the number of inductive circadian cycles. This is consistent with the hypothesis that the circadian clock is not causally involved in the evolution of the photoperiodic counter among populations.

Chapter V explicitly tests this by comparing two circadian phenotypes (NH response in long-day response and development time) with both the photoperiodic timer (critical photoperiod) and the photoperiodic counter (depth of diapause) among populations of *W. smithii*. 
CHAPTER V

EVOLUTION OF PHOTOPERIODIC TIME MEASUREMENT IS INDEPENDENT OF THE CIRCADIAN CLOCK IN THE PITCHER-PLANT MOSQUITO, WYEOMYIA SMITHII

The work described in this chapter was performed in collaboration with Sabrina Dake, William Bradshaw and Christina Holzapfel. The experiments were designed by all participants; the data were collected and analyzed by Sabrina Dake and myself; the chapter was written by myself with considerable assistance from William Bradshaw and Christina Holzapfel. This chapter includes previously published, co-authored material (Emerson, K. J., S.J. Dake, W. E. Bradshaw, and C. M. Holzapfel. 2009. Evolution of photoperiodic time measurement is independent of the circadian clock in the pitcher-plant mosquito, Wyeomyia smithii. Journal of Comparative Physiology A 195: 385 – 391).

Introduction

There are two great rhythms of light and temperature in the biosphere: the daily rhythm caused by the rotation of the earth about its axis and the yearly seasonal rhythm caused by the rotation of the earth about the sun. All eukaryotes (Edmunds 1988) and some prokaryotes (Johnson et al. 1996) possess an endogenous circadian (daily) clock that has been shown to be adaptive in 24-h environments (Emerson et al. 2008a; Sharma 2003; Yan et al. 1998). A wide diversity of plants, annelids, arthropods, echinoderms, fish, birds and
mammals use daylength (photoperiodism) to anticipate and prepare for the changing seasons (Anonymous 1960; Bradshaw and Holzapfel 2007a; Büning 1964; Menaker 1971; Withrow 1959), and this photoperiodic response has been shown to be adaptive in year-long seasonal environments (Bradshaw et al. 2004).

Photoperiodism consists of two separate, though related, components. The photoperiodic timer measures the length of day (or night), and the photoperiodic counter accumulates information from the timer and elicits the downstream photoperiodic response after a threshold number of inductive days has been counted (reviewed in Saunders 2002, Ch 12). Over seventy years ago, Büning (1936), hypothesized that the circadian clock formed the basis (Grundlage) of photoperiodic response and since then, the main focus of studies of the mechanistic basis of photoperiodism has been on this relationship (Danks 2005; Saunders 2002). Büning's (1936) model posits that there is a rhythmic, circadian sensitivity to light that persists in constant darkness. If light interacts with the rhythm when it is in its sensitive phase, induction of the photoperiodic response follows, i.e., Büning's (1936) model assumed a causal connection between the circadian clock and photoperiodic response.

The main experimental support for Büning’s hypothesis of the causal relationship between the circadian clock and photoperiodism are results from Nanda-Hamner experiments (NH) in which organisms are exposed for the duration of the experiment to light for a fixed number of hours (usually a short-day ~ 10-12 h) followed by long nights of varying duration in separate experiments with separate individuals, creating cycle lengths ($T = \text{Light} + \text{Dark}$) of typically 24 to 72 h total duration (Blaney and Hamner 1957; Bradshaw et al. 2003a; Nanda and Hamner 1958). For instance, some individuals are exposed to L:D (Light: Dark) = 10:14 ($T = 24$) for the duration of their life, while others are exposed to L:D
= 10:26 (T = 36), or L:D = 10:62 (T = 72). Historically, the idea was that if, during the long dark phase, there were a circadian-based sensitivity to light, then the animals should exhibit a rhythmic long-day response to increasing duration of the T cycle, (e.g., Bradshaw et al. 2003a; Pittendrigh 1981; Saunders 1968; Saunders 1974).

Recently, however, it has been argued that rhythmic responses to NH experiments in and of themselves do not support Büning’s hypothesis of a causal circadian basis of photoperiodism (Bradshaw et al. 2006; Danks 2005; Veerman 2001). When all experiments are run under the same environmental conditions using populations that have been reared through at least two laboratory generations to minimize maternal (field) effects, then persistent phenotypic variation among populations represents evolved (genetic) differences among them. If the evolutionary modification of two physiological processes is causally (genetically) connected through common genes (pleiotropy), then there should be a strong correlation between the two processes among evolutionary lineages: If the evolution of photoperiodic response is due to evolution of the circadian clock, then the formal properties of both processes should be correlated among populations within a single species. Among populations of the pitcher-plant mosquito, Wyeomyia smithii, critical photoperiod (an overt expression of the photoperiodic timer) is closely correlated with latitude and altitude of population origin (R² repeatedly > 0.92) but is not correlated with either the period or amplitude of response to NH with a short day and variable night lengths (Bradshaw and Holzapfel 2001; Bradshaw et al. 2006; Bradshaw et al. 2003a). Bradshaw et al. (2006) therefore concluded that W. smithii provides an example “that Nanda-Hamner periodicity is an expression of basic circadian rhythmicity, but PPTM [photoperiodic time measurement] is a separate mechanism” (one of three alternate proposals by Saunders 2002, page 481;
emphasis Saunders'). We follow Bradshaw et al. (2006) and use NH response as a direct assay of circadian rhythmicity (i.e. a circadian phenotype analogous to eclosion rhythms) and argue that in order to understand the relationship between the circadian clock and the evolutionary modification of photoperiodic time measurement, a comparative approach testing for associations between circadian and photoperiodic phenotypes among populations, is required.

Herein, we determine whether there is an evolutionary association of critical photoperiod (an overt expression of the photoperiodic timer) and depth of diapause (an overt expression of the photoperiodic counter) with the circadian clock (as measured by a rhythmic response of development time to a long-day NH). A long-day NH consists of a diapause-terminating long day (18h) followed by night lengths of 6-54 hours to create T-cycles from 24-72 h using different animals in the different L:D = 18:D regimens. For the termination of diapause, \textit{W. smithii} counts an L:D = 18:D cycle as a single long day, regardless of night length (Emerson et al. 2008b). The question then remains whether there is a rhythmic expression in development time of diapausing larvae exposed to T cycles from 24-72 h and whether the degree of rhythmicity covaries with critical photoperiod or depth of diapause over the geographic, evolutionary trajectory of \textit{W. smithii}.

**Methods**

The pitcher-plant mosquito, \textit{Wyeomyia smithii} (Coq), completes its pre-adult development within the water-filled leaves of the purple pitcher-plant, \textit{Sarracenia purpurea}, and its range closely follows that of its host plant in North America. Throughout its range, \textit{W. smithii} undergoes an hibernal larval diapause whose onset, maintenance and termination
are regulated by photoperiod (Bradshaw and Lounibos 1977). Ancestral, southern populations enter diapause in the fourth instar; derived northern (and southern mountain) populations enter diapause in the third instar (Armbruster et al. 1998; Bradshaw and Lounibos 1977). The critical photoperiod for the onset, maintenance and termination of diapause increases linearly with both altitude and latitude (Bradshaw and Lounibos 1977). Critical photoperiod and depth of diapause form part of a diapause syndrome (Campbell and Bradshaw 1992) where southern, diapause-averse populations enter a shallower diapause at a later instar under shorter days later in the fall; northern, diapause-prone populations enter a deeper diapause at an earlier instar under longer days earlier in the fall (Table 1).

Eight populations of *W. smithii* were collected from four geographic regions, with two populations at least 80 km apart within each region, representing extremes in latitude from 30 to 46°N and altitude from 20 to 1000m (Table V1). All eight populations used in this study are the same as those reported in (Emerson et al. 2008b): two from Florida (Southern), two from the coastal plain and piedmont of North Carolina (Lowland), two from the mountains of North Carolina (Mountain), one from northern Maine and one from northern Wisconsin (Northern) (populations WI, CR, GS, SH, DB, HS, KC and RY, respectively from Emerson et al. 2008b). After factoring out maternal effects by rearing the populations in the laboratory as detailed in (Emerson et al. 2008b), experiments were carried out as a single block to ensure constant temperature, light and humidity conditions across all treatments. Experimental larvae were reared in short days (Light:Dark = 8:16) at 21 ± 0.5°C for at least thirty days to ensure that all animals were synchronized in diapause before the experiment began. Diapausing larvae from each population were exposed to Light:Dark
Table V.1. Circadian and photoperiodic phenotypes across the geographic range of *W. smithii*. Geographic origin, stage of diapause, critical photoperiod, depth of diapause, and both short-day (L = 10 hr) and long-day (L = 18 hr) Nanda-Hammer (NH) responses of the eight populations (two from each region) used in this study.

<table>
<thead>
<tr>
<th>Clade</th>
<th>Region</th>
<th>Latitude</th>
<th>Altitude</th>
<th>Stage of Diapause</th>
<th>Critical Photoperiod</th>
<th>Depth of Diapause</th>
<th>Short-day NH Response</th>
<th>Long-day NH Response</th>
</tr>
</thead>
<tbody>
<tr>
<td>South</td>
<td>Southern</td>
<td>30-31°N</td>
<td>&lt; 100 m</td>
<td>IV</td>
<td>12.3 ± 0.1</td>
<td>4.0 ± 0.4</td>
<td>Strong rhythm</td>
<td>Linear</td>
</tr>
<tr>
<td></td>
<td>Lowland</td>
<td>34-35°N</td>
<td>&lt; 110 m</td>
<td>IV</td>
<td>12.9 ± 0.2</td>
<td>6.3 ± 0.9</td>
<td>Strong rhythm</td>
<td>Rhythmic</td>
</tr>
<tr>
<td>North</td>
<td>Mountain</td>
<td>35°N</td>
<td>≥ 200 m</td>
<td>III</td>
<td>13.9 ± 0.0</td>
<td>7.5 ± 2.3</td>
<td>Arrhythmic</td>
<td>Rhythmic</td>
</tr>
<tr>
<td></td>
<td>Northern</td>
<td>46°N</td>
<td>295-365 m</td>
<td>III</td>
<td>15.2 ± 0.2</td>
<td>8.2 ± 1.5</td>
<td>Weak Rhythm</td>
<td>Linear</td>
</tr>
</tbody>
</table>

1 Taxonomic clade (Armbruster et al. 1998; Bradshaw and Lounibos 1977)
2 General geographic region of origin: Southern - Gulf Coast of FL; Lowland - coastal and piedmont NC; Mountain - southern Appalachian Mountains in NC; Northern, ME and WI.
3 Stage of diapause, III or IV instar (Bradshaw and Lounibos 1977)
4 Mean ± SE (hours) critical photoperiod, a measure of the photoperiodic timer, of populations within the region (Bradshaw et al. 2003a). Note that in *W. smithii*, critical photoperiod is the same for both the initiation and termination of diapause in unchilled animals (Bradshaw and Lounibos 1972).
5 Mean ± SE (days) depth of diapause, a measure of the photoperiodic counter, within each region using the same populations as in this study. Data are from Emerson et al. (2008b, Table 1, LDCso 18:06)
6 NH response with a light period of 10 h (Bradshaw et al. 2003a)
7 NH response with a light period of 18 h (Fig. 1)
cycles with 18 hours of light and, in separate experiments with separate individuals, from 6-
54 hours of darkness in 2-hour increments. The experiment involved 25 treatments \( \times 8 \)
populations \( \times 105 \) larvae treatment \(^1\) population \(^1\), totaling 21,000 larvae in a single block.
Experiments were carried out until all larvae developed (< 70 days for the longest L:D
cycles). All experiments were run in light-tight experimental chambers with an air-cooled 4
W cool-white fluorescent lamp at 21\(^\circ\) ± 0.5\(^\circ\)C.

Development time was measured as the time from the start of the experiment with
diapausing larvae until pupation. Development time is therefore a composite index
including both the time required for larvae to terminate diapause using the photoperiodic
counter and the time required for larvae to complete post-diapause development culminating
in pupation. Experimental animals were cleaned and fed and all pupae were counted and
removed every 2 – 4 days during the light phase of the L:D cycles.

We tested whether there is a significant rhythm in the response to a long day T-
experiment using a likelihood-based statistical approach. We independently fit two classes of
models of the relationship between development time (DT) and cycle length (T) to the data
from the four separate geographic regions: (1) a linear model of the form
\[ DT = a + bT, \]
(2) a rhythmic model of the form
\[ DT = a + bT + c\cos\left(2\pi \frac{T - d}{e}\right), \]
where \( a, b, c, d, e \) are parameters fit by maximum likelihood: \( a \), intercept; \( b \), slope of the linear function; \( c \), amplitude of the rhythmic function, \( d \), lag between the internal rhythm and the external L:D
cycle; \( e \), the period of the internal rhythm.

Within each geographic region, Akiaké's Information Criterion (AIC), a measure of
the relative correctness-of-fit for a given model, was used to determine whether a linear or a
rhythmic model was better supported by the data (Burnham and Anderson 2004). This measure gives an estimate of the "information lost" when using the model rather than the data itself and can be thought of as a measure of distance between the model and the data. Hence, smaller values for AIC indicate a better fit of the model to the observed data. We determine whether the relationship between development time and T is better explained by a linear or rhythmic function by choosing the model with the smallest AIC value.

We use log-likelihood ratios as a measure of the strength of our test between the two models. The log-likelihood ratio is calculated as $\text{Log}(L) = \log(\text{likelihood of non-linear model} / \text{likelihood of linear model})$. Large positive values of $\text{Log}(L) > 5$ indicate confidence in the non-linear model being a better fit than the linear model; whereas large negative values of $\text{Log}(L) < -5$ indicate confidence in linear models being a better fit than the non-linear model. All analyses use the statistical computing program R (R Development Core Team 2007).

**Results**

Percent development was greater than 95% in all treatments used in this study, showing that all the treatments were interpreted as long (diapause-terminating) days by the mosquitoes, confirming previous results that showed that *W. smithii* measures the length of day (Emerson et al. 2008b) rather than the length of the dark period.

Development time in all four regions increased with increasing duration of the L:D cycle (Figure V.1). There was a rhythmic response in development time as a function of cycle length in the lowland and mountain regions, whereas the relationship had no significant rhythm in southern and northern regions (Figure V.1, Table V.2). The magnitude of the log-
Figure V.1 Long-day Nanda Hamner Response. Developmental time (mean number of days ± 2 SE) as a response to long days (18 h) followed by 6-54 h of darkness in 25 separate experiments for eight populations of *W. smithii* from four geographic regions; lines plot the best fitting model (Table 2).
likelihood ratios, a quantitative measure of support for one model over the other, ranged from 9 to 62, suggesting that, at a minimum, the better supported model was more than $10^9$ times more likely to have produced the observed data. The period of oscillations for the rhythmic regions were slightly less than 24 hr (Lowland: 23.90 ± 0.78 SE, Mountain: 22.29 ± 0.56; $t = 1.68$, $P = 0.342$), and fell within the range of 19 – 26 hr reported for insect circadian rhythms (Lankinen and Forsman 2006; Saunders 2002).

Table V.2. Support statistics for the linear and rhythmic models fit to development time for each geographic region.

<table>
<thead>
<tr>
<th>Geographic Region</th>
<th>Log(L) Linear¹</th>
<th>Log(L) Rhythmic¹</th>
<th>Log(L) Ratio²</th>
<th>AIC Linear¹</th>
<th>AIC Rhythmic¹</th>
<th>Better fitting model</th>
</tr>
</thead>
<tbody>
<tr>
<td>Southern</td>
<td>-161.78</td>
<td>-199.15</td>
<td>-37.37</td>
<td>749.03</td>
<td>927.14</td>
<td>Linear</td>
</tr>
<tr>
<td>Lowland</td>
<td>-169.67</td>
<td>-160.65</td>
<td>9.02</td>
<td>785.36</td>
<td>749.84</td>
<td>Rhythmic</td>
</tr>
<tr>
<td>Mountain</td>
<td>-380.47</td>
<td>-332.09</td>
<td>48.38</td>
<td>1756.11</td>
<td>1539.31</td>
<td>Rhythmic</td>
</tr>
<tr>
<td>Northern</td>
<td>-321.59</td>
<td>-383.75</td>
<td>-62.16</td>
<td>1484.98</td>
<td>1777.24</td>
<td>Linear</td>
</tr>
</tbody>
</table>

¹Log-likelihood of the given model
²Estimate of the strength of support for the better fitting model: large negative values < support the linear model over the rhythmic model; large positive values > support the rhythmic model over the linear model.
³Akiake's information criterion, a measure of the relative correctness of fit to the linear and to the rhythmic models. Boldface indicates the better fitting model for each geographic region.

Discussion

Critical photoperiod in *W. smithii* is closely correlated with latitude and altitude ($R^2$ repeatedly > 0.92, Bradshaw and Holzapfel 2001). To be rhythmic a function must have both a period and an amplitude of oscillation. The period of the rhythmic response to short-day NH is not correlated with latitude, altitude or critical photoperiod (Bradshaw et al. 2003a). The amplitude of the rhythmic response to short-day NH is correlated only with
altitude and is not significantly correlated with critical photoperiod over *W. smithii*’s range (Bradshaw et al. 2006). In the present study using long-day NH experiments, a linear model best describes development times in regions representing the extremes in both critical photoperiod and depth of diapause (Figure V.1 and Table V.2, southern and northern); a rhythmic model best describes development times in regions representing intermediate critical photoperiods and depths of diapause (Fig. 1 and Table 2, lowland and mountain). Hence, there is no consistent geographic association of critical photoperiod or depth of diapause with the rhythmic response to long-day NH.

*Wyeomyia smithii* is the single temperate species of an otherwise neotropical genus (Lane 1953; Stone et al. 1959). *Wyeomyia smithii*’s geographic distribution, natural history, morphology, reproductive biology, physiology and allozyme variation all support an evolutionary history that originates with the invasion of North America from the tropics along the Gulf of Mexico and was subsequently followed by dispersal to higher latitudes and altitudes (Armbruster et al. 1998; Bradshaw and Lounibos 1977). There is a strong association (Table 1) of evolutionary history with both critical photoperiod and depth of diapause. This association is not true for the rhythmic response to long-day NH, which is more similar among regions between than within evolutionary groups. Hence, there is no consistent evolutionary association between critical photoperiod (a measure of the photoperiodic timer) or depth of diapause (a measure of the photoperiodic counter) with the circadian-based, rhythmic response to long-day NH.

Saunders et al. (2004) propose that the presence of a robust photoperiodic response curve despite the absence of a response to Nanda-Hamner experiments may be due to rapid damping of the circadian oscillator during long dark periods. This proposition might have
applied to the mountain populations that show a flat, arrhythmic developmental response to short-day NH (Table 1). Our present results provide an unintended test of Saunders’ proposal. The same mountain populations that are arrhythmic in response to short-day NH show a clear rhythmic response to long-day NH that persists for at least 54h in darkness (Fig. 1). Hence, there is a robust, non-damping circadian oscillation in the mountain populations and the lack of a rhythmic response to the short-day NH is not due to damping of the circadian oscillator.

All populations of *W. smithii* exhibit a rhythmic response to short-day NH, to long-day NH or to both, but neither the period nor the amplitude of this rhythmic response is associated with the photoperiodic timer or counter. We therefore conclude that the circadian rhythm expressed through NH experiments is not a causal factor in the evolution of photoperiodic response over the eco-climatic gradient of North America by *W. smithii*.

The hypothesis of a causal connection between circadian rhythms and photoperiodism has been a tantalizing concept for over 70 years. Such a relationship would mean that one or more key genes are mediating both processes, i.e., their functional connection is due to pleiotropy. Pleiotropy is bi-directional: selection on one trait generates a correlated response in the other trait, leading to potential tradeoffs between fitness-related traits (Roff 1992; Rose 1991). If the circadian clock were related to photoperiodic time measurement through pleiotropy, the evolutionary modification of one trait would have a modifying effect on the other. The circadian clock orchestrates the daily temporal coordination of hundreds of genes in *Drosophila* (Claridge-Chang et al. 2001; McDonald and Rosbash 2001); and, by one estimate, nearly all genes in mouse adipose tissue have a circadian component to their expression (Ptitsyn et al. 2007). Proper entrainment of the
The daily circadian clock and the seasonal photoperiodic timer serve two, separate adaptive functions and are affected by different suites of environmental inputs (Danks 2005). This is not to say that a particular gene involved in the circadian clock mechanism may not also be involved in photoperiodic time measurement, independently of its role in the circadian clock (Bradshaw and Holzapfel 2007b; Mathias et al. 2005; Stehlik et al. 2008; Tauber et al. 2007); rather, the evolutionary modification of photoperiodic response is independent of the evolution of the circadian clock as a system. This independent evolution should be expected over geographic gradients that vary in the amplitude of seasonal day length, in the mean and amplitude of daily temperature, in the length of the
growing season, in the duration and severity of winter and in ecological contexts that are not the same between times of day and between times of year.

**Bridge**

In this chapter I have shown that the circadian clock, represented by two distinct NH response phenotypes, has evolved independently of both the photoperiodic timer and counter among populations of *W. smithii*. For this reason, future studies concerning the mechanistic basis of photoperiodic time measurement should not assume an association between the two processes. Forward genetic approaches, unbiased by assumptions about circadian involvement, will be the most fruitful approach to understanding the genetic basis of photoperiodism.

In the following chapter, I outline how the historical inertia generated by Bünnings's (1936) influential hypothesis, that the circadian clock formed the causal basis of photoperiodism, has confounded rather than clarified the mechanistic basis of photoperiodism.
CHAPTER VI
COMPLICATIONS OF COMPLEXITY: INTEGRATING ENVIRONMENTAL,
GENETIC AND HORMONAL CONTROL OF INSECT DIAPAUSE

The work described in this chapter was written in collaboration with William Bradshaw and Christina Holzapfel. This chapter includes previously published, co-authored material (Emerson, K. J., W. E. Bradshaw, and C. M. Holzapfel. 2009. Complications of complexity: Integrating environmental, genetic and hormonal control of insect diapause. Trends in Genetics 25(5):193-242).

Genes and Modules

Mapping genotype to phenotype, understanding how genes interact with other genes, and how genes can affect multiple phenotypes (pleiotropy) have been long-standing goals of developmental and evolutionary biology. It has been recognized for many years that organisms tend to be modular insofar that they are composed of developmental or physiological processes that are tightly integrated into units operating to a certain degree independently of other processes. This basic concept of modularity dates back at least to Herbert Simon (Simon 1962) who argued that genes can be integrated into functionally autonomous groups. Modular pleiotropy can then be defined as the effect of a module on multiple phenotypes, being distinct from gene pleiotropy, which defines the typical effect of individual genes on multiple phenotypes. Therein lies the complication of complexity
because the fact that a gene constitutes a component part of a functional module does not preclude that gene from having a separate and important ancillary function outside of the module. Modularity has been extensively considered in developmental pathways, especially those involved in cell signaling such as Hedgehog (Cohen 2003), transforming growth factor (TGFβ) (Kitésin et al. 2007), Wnt (Logan and Nusse 2004) and Notch (Bray 2006) in which investigators generally recognize the distinction between modular and gene pleiotropy; however this distinction is less well appreciated in physiological genetics. Herein, we examine genetic control of a physiological trait, using insect dormancy (diapause) as an illustrative example, and show how not recognizing the difference between modular and gene pleiotropy, especially when bound by long-standing historical expectations, has confounded rather than clarified the genetic basis of this important phenotype.

Physiological processes represent the connection between the genome and the external world. Approximately two-thirds of the world’s land mass lies within temperate and polar regions of the earth. Within these regions, the majority of insects escape the exigencies of winter through dormancy or migration, which is initiated by a physiological response to day length called photoperiodism (Danilevskii 1965; Danks 1987; Masaki 1984; Saunders 2002; Tauber et al. 1986). Seasonality, in turn, is a reflection of latitude and altitude (Critchfield 1974). In response to recent rapid climate change, both plants and animals have expanded their ranges northwards and have altered the pattern of their seasonal activities (Hughes 2000; Parmesan and Yohe 2003; Peñuelas and Filella 2001; Root et al. 2003; Walther et al. 2002; Warren 2006). Perhaps not surprisingly, the first evidence for an evolutionary response (genetic change) to recent climate warming over a geographic gradient involved a genetic shift towards shorter more southern day lengths controlling diapause
(Bradshaw and Holzapfel 2001; Bradshaw and Holzapfel 2008), thereby making this phenotype one of the most powerful diagnostic measures of the global threat of a warming world. Hence, examining the genetic basis for environmental control of diapause is particularly timely because of the societal implications of biotic response to rapid climate change and because of the opportunity to observe evolutionary change in contemporary time.

Understanding the genetic foundations of insect diapause and other complex adaptations will largely be gained by using forward genetic approaches (proceeding from phenotype to genotype), by matching the appropriate organism with the appropriate phenotype, and by probing the genetic basis of natural phenotypic variation over geographical gradients.

The Structure of the Photoperiodism–Hormone–Diapause Axis

Many insects have evolved a strategy called diapause – a stage of developmental arrest – that enables them to survive harsh seasonal conditions, such as extremes in temperature or drought. Diapause can occur at any stage of the life cycle, from embryonic to adult reproductive dormancy. A wide variety of arthropods living at temperate and polar latitudes use the length of day (photoperiodism) to initiate a cascade of hormonal events that culminate in diapause (Figure VI.1). This photoperiodic response incorporates the input of light, a timer that assesses the length of day or night, a counter that accumulates the number of long- or short-day signals received, and an output signal to the hormonal control of diapause (Saunders 2002). Each of these processes from the input of light to the output signal, and the genes controlling them, interact to elicit photoperiodic response. Therefore
Figure VI.1. Schematic illustration of genetic events leading to diapause. Each pentagram represents a module of functionally related genes. The photoperiodism (blue) – hormonal event (green) – diapause (red) axis shows sequential events that are established and accepted. The role of the circadian clock (purple) in affecting diapause remains uncertain and is the primary subject of this figure. (a) Modular pleiotropy. A mutation of a clock gene (★) modifies the function of the circadian clock and this modified clock function then affects the expression of diapause. The circadian clock might potentially exert its control through photoperiodism or hormonal control of diapause, or might act on diapause directly, as indicated by the broken lines through unknown connections (black square). (b) Gene pleiotropy. A mutation of a clock gene (★) might or might not affect clock function but does affect the expression of diapause, independently of its role in the circadian clock. This single gene might exert its control through photoperiodism or hormonal control of diapause, or might act on diapause directly, as indicated by the broken lines through unknown connections (black square). Distinctions between gene and modular pleiotropy and knowledge of where in pathways mutations have their effects is crucial for understanding the genetic basis of phenotypes.

Photoperiodism can be considered as a module, which we define as a group of functionally related genes that interact with each other to control integrated processes. Generally, genes within a module interact with each other more than they do with genes in other modules. Nonetheless, individual genes within a module can have important ancillary effects that are independent of the role they play within a given module.
When the photoperiodism module produces an output signal that ultimately results in diapause, the hormones involved in diapause must be synthesized, secreted and degraded at the appropriate rates and times. Hence, the hormonal control of diapause can be considered as a second module between photoperiodism and diapause itself (Figure 1). Finally, output from the endocrine system elicits a syndrome of diapause traits that includes the cessation of active development, reproductive dormancy, the accumulation and sequestration of energy reserves, increased stress resistance, reduced metabolic rate or a combination of these traits. Diapause is then the final module in the photoperiodism-hormone-diapause axis.

The distinction between an individual gene and a module is important in complex traits because, regardless of its exact definition, gene actions are often perceived as part of functionally integrated processes (modules). Understanding the modular structure of processes has greatly advanced our knowledge of how a genotype becomes a phenotype (Wagner et al. 2007). However, undue focus on modularity leads to the logical temptation to conclude that the effects of a single mutant gene in the lab or a single allele segregating in natural populations must have its effect through its principal module and that the alteration of the entire module determines the causal change in the phenotype of interest. This conclusion is tantamount to assuming that a gene cannot have an effect outside of its principal module; however, effects of single genes on multiple phenotypes (pleiotropy) are very common (Mackay 2001). Likewise, modules themselves can have pleiotropic effects (Schlosser and Wagner 2004). It is the failure to distinguish between gene and modular pleiotropy that complicates interpretation of genetic mutations in laboratory lines or interpretation of allelic variation segregating in natural populations.
The Role of the Circadian Clock in Diapause

The circadian clock controls daily timing at molecular, cellular, physiological and behavioral levels in organisms from Cyanobacteria to mammals (Dunlap et al. 2004). The circadian clock might be considered as a fourth module potentially affecting diapause (Figure VI.1). Since Bünning's influential hypothesis (Bünning 1936) that circadian rhythms formed the basis (Grundlage) of photoperiodism, investigators have sought physiological parallelisms between circadian behavior and photoperiodic response. With a deeper understanding of the molecular basis of circadian rhythmicity in Drosophila melanogaster, circadian rhythm genes quickly became candidate loci for photoperiodism. However a distinction needs to be made between Bünning's Grundlage that the circadian clock forms a causal, necessary basis of photoperiodism and the concept that the circadian clock can influence the photoperiodic response. Given the pervasive effects of the circadian clock on cellular biochemistry (Keegan et al. 2007), it would be surprising to find any physiological function that is not affected in some way by the circadian clock. Mutation or allelic variation in genes involved in circadian rhythmicity could affect diapause by modifying clock function so that the circadian clock as a module, would alter photoperiodism, hormonal control or diapause itself (Figure VI.1a). Alternatively, mutation or allelic variation in a clock gene could affect diapause directly as an individual gene, independently of its role in the circadian clock (Figure VI.1b). There are two known clock genes with a role in photoperiodic Diptera that illustrate this distinction.

The genes period (per) and timeless (tim) code for the proteins PERIOD and TIMELESS that form an integral part of the negative regulatory loop of the circadian clock of D. melanogaster (Figure VI.2). If the circadian clock, as a module, has a causal role in
photoperiodism, laboratory mutants or naturally segregating alleles of these loci should alter both circadian function and photoperiodic response.

Figure VI.2. Simplified schematic of the period–timeless feedback loop in the Drosophila circadian clock. (a) The CLOCK–CYCLE (CYC–CLK) heterodimer is a transcription factor that promotes the transcription of period and timeless (per and tim) mRNA. (b) per and tim mRNAs are transported to the cytoplasm in the early evening where they are translated into their respective proteins, PERIOD (PER) and TIMELESS (TIM). After a delay of a few hours, PER and TIM are translocated back into the nucleus (probably as a dimer) in the late evening. (c) Once in the nucleus, PER–TIM might dissociate, and PER physically disrupts the transcription factor CLK–CYC, leading to a fall in per and tim levels. (d) Nuclear TIM and, later, PER are then degraded, and this degradation enables the derepression of per and tim promoters via CLK–CYC, so the molecular cycle starts again with per and tim transcription late in the day. (e) In the presence of light, the protein CRYPTOCHROME (CRY) binds TIM, preventing its dimerization with PER and leading to its phosphorylation and degradation. This cycle continues in constant darkness but is amplified in light–dark cycles, when the blue light receptor CRY is activated. CRY physically interacts with TIM and the F-box protein JETLAG, and TIM is phosphorylated and degraded (Peschel et al. 2009). When the PER–TIM heterodimer is accumulating in the early evening, light retards the accumulation of the PER–TIM dimer and sets the clock back. When PER–TIM is degrading late in the night, light accelerates this breakdown and sets the clock ahead.
Clock genes and diapause

The wild-type Canton-S strain of D. melanogaster (isolated c.1930 near Canton, Ohio, USA (Bridges and Brehme 1944)) is photoperiodic for delayed ovarian maturation (diapause) over a restricted range of moderately low temperatures in the laboratory (Saunders and Gilbert 1990; Saunders et al. 1989). Initially, three mutants were discovered that altered clock function: \textit{pel} \textsuperscript{1}, \textit{per}' and \textit{pel}\textsuperscript{Oater}, \textit{pe:l'}], which eliminate, shorten or lengthen the period of circadian rhythmic activity or eclosion, respectively (Konopka and Benzer 1971; Smith and Konopka 1981). Saunders and colleagues (Saunders 1990a; Saunders et al. 1989) determined the photoperiodic response curves for these mutants and an additional mutant, \textit{per}, a double deletion of the \textit{per} locus (Figure VI.3). All four mutants, including the arrhythmic \textit{per} \textsuperscript{01} and \textit{per} \textsuperscript{-} mutants, exhibited robust photoperiodic response curves, leading them to conclude that the period locus was not causally involved in photoperiodic time measurement and that the crucial genes were to be found elsewhere in the genome (Saunders et al. 1989). These results mean that neither \textit{per} nor a functional circadian clock module is necessary for photoperiodic response.

The results from Saunders' experiments with \textit{per} mutants led multiple laboratories, including our own (Mathias et al. 2005, 2007), to investigate genes that function in the pathway between the input of light and the feedback loop involving \textit{PER} (Figure VI.2). Both circadian rhythmicity and photoperiodism are triggered by light and it is the interaction of light, TIM and CRYPTOCHROME (CRY) proteins that sets the circadian clock. In the presence of light CRY binds TIM whereupon TIM is phosphorylated and degraded (Peschel et al. 2009). In \textit{per} null mutants, \textit{tim} is transcribed continuously and is translated into the TIM protein. In the presence of light, TIM still binds to CRY and is ultimately degraded; in
Figure VI.3. Photoperiodic response of D. melanogaster and period mutants. (a) Photoperiodic response of wild-type Canton-S (redrawn, with permission, from Saunders et al. 1989). Light gray circles represent replicate groups; solid black circles represent group means. (b) Photoperiodic response of Canton-S wild type compared with various per mutants.

Explanation of terms: per\#, two replicates of a short period line; per\$, a long period line; per\^, an arrhythmic line; per, double overlapping deletion line lacking the per gene altogether.

Data points represent means of groups at that day length (Saunders 1990a) (adapted, with permission, from Sage Publications). We fit the lines by logistic regression (R Development Core Team 2007), omitting day lengths of 0 and 24 h because all lines were not represented at these day lengths. Critical photoperiods and their standard errors were determined from logistic regression. After applying sequential Bonferroni (Rice 1989) to account for a posteriori multiple comparisons, we found no significant differences in critical photoperiods among lines, either using all possible comparisons (P ≥ 0.40) or comparing each mutant line against only wild-type Canton-S (P ≥ 0.43). These comparisons mean that there are no significant differences in left-right position of the sigmoid curves.

In the absence of light, TIM continues to accumulate. Hence, even in the absence of a functional circadian clock, the TIM protein continues to cycle in proportion to day length (Myers et al. 1996; Zeng et al. 1996). TIM could then function as an interval timer and provide the downstream cue for hormonal control of diapause. In the only quantitative trait locus (QTL) map for photoperiodism in animals (the mosquito, Wyeomyia smithii), tim does not fall under any of the 6–9 QTL identified so far for photoperiodic response, although ltm or a closely linked gene interacts epistatically with six other markers on the third
chromosome that affect the evolution of photoperiodism (Mathias et al. 2007). The evolution of photoperiodic response in North American *W. smithii* is not correlated with either the period or amplitude of the circadian clock (Bradshaw et al. 2006; Bradshaw et al. 2003a; Emerson et al. 2009b). These results are consistent with *tim* acting through gene and not modular pleiotropy to influence, but not to provide the basis for the evolution of photoperiodic time measurement in *W. smithii*.

*Timeless* is also involved in diapause of the Japanese drosophilid *Chymomyza costata*. *C. costata* enters a larval diapause that is mediated by photoperiod at 18°C (Riihimaa and Kimura 1988). A non-diapausing mutant, *npd* (for non-photoperiodic diapause), was isolated from a recently collected population south of Sapporo. The *npd* mutant is arrhythmic for adult eclosion and the *npd* larvae lack daily and circadian oscillations of *per* and *tim* transcription (Koštál and Shimada 2001; Pavelka et al. 2003). Ultimately, *npd* was traced to the *tim* locus (Stehlik et al. 2008). Compared with wild-type, the *npd* mutant has an 1855 base-pair deletion in the 5'UTR and promoter regions, which removed the start of transcription, and all the regulatory motifs found in the wild-type flies. The *npd* mutant also included 37 nonsynonymous substitutions in five of the 11 exons, including the PER interaction domains, five amino acid substitutions in the cytoplasmic localization domain, and one in the stop codon. Clearly, *tim* function in *C. costata* is necessary for the expression of circadian behavior and for diapause, but, it is not clear whether the effect of *tim* on diapause is exerted through the circadian clock (modular pleiotropy) or independently (gene pleiotropy) through independent interaction with photoperiodism, with the hormonal control of diapause or with diapause itself.
European populations of *D. melanogaster* are photoperiodic for the regulation of ovarian diapause (Taubet al. 2007). Within European populations, *tim* is polymorphic for transcribing an ancestral short form of *tim*, *s-tim*, or both a long and short form, *ls-tim* (Rosato et al. 1997). In both Italian and Dutch populations, there is a linear decline in the incidence of diapause with increasing day length and, at each daylength, the incidence of diapause is greater in *ls-tim* than *s-tim* flies (Taubet al. 2007). However, the photoperiodic response curves of both Italian and Dutch populations are linear and parallel. Hence, there is no significant *tim* genotype by day length interaction. In transgenic flies with a common genetic background, the derived L-TIM protein binds less tightly to CRY than the ancestral S-TIM protein, thereby inhibiting the breakdown of TIM by light and rendering the circadian clock less sensitive to light (Sandrelli et al. 2007). The *l-tim* transgenics exhibit a higher incidence of diapause than the *s-tim* transgenics and again there is no genotype by day length (short vs. long days) interaction. In the natural populations and the transgenics, the lack of a genotype by day length interaction means that *tim* and day length have additive and independent effects on the incidence of diapause (Figure VI.4). The effect of *tim* genotype on sensitivity of the circadian clock (Sandrelli et al. 2007) then means that *tim* presents a clear case of gene but not modular pleiotropy, either through hormonal control or by affecting diapause directly.

A consideration of *period* and *timeless* illustrate the risk in assuming that, because a gene plays a major role in a given module, the effect of that gene must be due to modular and not gene pleiotropy. Neither mutants of *per* nor natural allelic variation in *tim* provide a clear demonstration that the circadian clock as a functional module is responsible for or contributes to diapause expression in adult flies.
Figure VI.4. Photoperiodic response of *D. melanogaster* from Salice, Italy. Analysis of covariance (ANCOVA) shows no significant day length by timeless genotype interaction: the regression lines for *ls-tim* and *s-tim* flies are parallel. The parallel lines indicate that an increase or decrease in day length by 2 h has the same effect on diapause in both the *ls-tim* and *s-tim* lines; or, the effect of a timeless allele on diapause is the same regardless of photoperiod. Hence, the two factors, day length and timeless allele, are acting additively and independently. Figure adapted, with permission, from Tauber et al. (2007).

**Diapause and Hormonal Control of Ovarian Maturation**

Operationally, adult diapause in *D. melanogaster* is scored by ovarian development, which ceases or at least slows significantly at the pre-vitellogenic state (before yolk-protein uptake by the oocyte) in diapausing individuals over a narrow range of intermediate temperatures (Saunders et al. 1989). Warmer temperatures invariably avert diapause; colder temperatures halt development at any stage. Furthermore, if maintained long enough at the diapause-inducing temperature (12°C), ovarian maturation resumes after a delay of several weeks, even in the long-established, photoperiodic Canton-S line (Saunders et al. 1989). Clearly, if one wants to study the effect of latitude or genetic variation on diapause, one selects the constant temperature at which a chosen effect on the incidence of diapause is greatest. For good measure, assays of diapause are run on short days, although day length has not been
shown to affect the incidence of diapause in any natural population of *D. melanogaster* from North America. Not surprisingly, separate studies of diapause in populations of *D. melanogaster* have each been carried out at a constant moderately low temperature between 11 and 13°C on a short day (10L:14D) with controls at a warm temperature on a long day.

Diapause in *D. melanogaster* is characterized by delayed vitellogenesis but also involves increased stress resistance and lipid sequestration (Schmidt and Conde 2006). The incidence of diapause increases with latitude in eastern North America (Schmidt et al. 2005a; Williams et al. 2006) and in eastern Australia (Mitrovski and Hoffmann 2001). Diapause in *D. melanogaster* therefore represents a syndrome of traits expressed in a formerly tropical insect that seems to improve fitness in progressively more temperate seasonal environments.

A composite picture of hormonal control of ovarian maturation and diapause in Diptera is emerging from studies on ovarian diapause in *D. melanogaster* and mosquitoes (Figure 5). Diapause is mediated by the insulin-signaling pathway (Figure VI.5). Insulin-like proteins (Ilp) are secreted by the dorsal medial neurosecretory cell and travel down the recurrent nerve to the ring gland. This gland consists of three endocrine organs fused into one structure surrounding the oesophagus: the prothoracic gland, which synthesizes and secretes ecdysteroids (steroid hormones), the corpora allata, which synthesize juvenile hormone (JH, a sesquiterpenoid lipid-like molecule), and the corpora cardiaca. The corpora cardiaca are neurohaemal organs that release hormones into the haemolymph. In combination, the presence or absence of JH from the corpora allata and ecdysteroid from the ring gland and the ovaries control ovarian diapause (Garofalo 2002; Nijhout 1994; Richard et al. 2001; Riddiford 2008; Wu and Brown 2006).
Figure VI.5. Endocrine control of ovarian maturation in flies (Diptera). (a) Major hormonal events. Upon appropriate environmental input, neurosecretory cells in the dorsal midbrain (NSC) secrete insulin-like peptides (Ilp) that stimulate the corpora allata (CA) in the ring gland to secrete juvenile hormone (JH). JH acts early in ovarian maturation to stimulate yolk protein synthesis in the ovaries and fat body. In response to JH, the ovaries secrete ecdysteroid (Ecd) that stimulates uptake of yolk protein. Ecd from the ovaries stimulates sustained yolk protein synthesis later in ovarian maturation. (b) The PI3K–FOXO cascade in the insulin signaling pathway. Ilps are bound by the insulin receptor (InR) and the insulin receptor substrate (CHICO) that, along with PI3K, phosphorylate phosphatidylinositol (4-5)-bisphosphate [PtdIns(4,5)P2; PIP2] to phosphatidylinositol (3,4,5)-trisphosphate [PtdIns(3,4,5)P3; PIP3]. This, in turn, acts as a phosphate source to phosphorylate serine–threonine kinase (AKT/PAKT). PAKT then acts as a phosphate source to phosphorylate the forkhead transcription factor (FOXO), leading to its degradation. FOXO enters the nucleus where it regulates transcription. Figure compiled from several references (Claeys et al. 2002; Edgar 2006; Partridge et al. 2005; Richard et al. 2001; Wu and Brown 2006).
In *D. melanogaster*, the insulin signaling pathway is involved in aging (Broughton et al. 2005; Claeyss et al. 2002; Partridge et al. 2005; Tatar et al. 2003) and manipulations of the phosphatidylinositol 3 kinase (PI3K) – Forkhead box O (FOXO) cascade in *D. melanogaster* and mosquitoes result in phenotypes similar to diapause (Figure VI.5). Insulin-like peptides from the dorsal median neurosecretory cells in the brain promote yolk uptake and ecdysteroid production by the ovaries (Brown et al. 2008), lipid mobilization, increased fecundity, and shorter lifespan (Broughton et al. 2005). The insulin receptors are necessary for ovarian development; knockouts are rescued by JH in a dose-dependent manner (Sim and Denlinger 2008; Tu et al. 2005; Williams et al. 2006). The insulin receptor substrate (CHICO) is necessary for normal levels of JH synthesis (Tu et al. 2005) and PI3K is necessary for ovarian development (Tu et al. 2005; Williams et al. 2006). FOXO controls lipid sequestration in diapausing adults and insulin-dependent signaling in the fat body, in addition to oxidative stress resistance as well as extended lifespan (Hwangbo et al. 2004; Sim and Denlinger 2008). Finally, allelic variation in PI3K is associated with latitudinal increase in the incidence of diapause in North American *D. melanogaster* between Georgia and Ontario (Williams et al. 2006).

Together, these results and observations indicate that the insulin-like peptides, JH and ecdysteroids acting through the PI3K–AKT–FOXO cascade provide the pivotal hormonal connection between the environment and ovarian diapause in adult Diptera. The hormonal control of diapause thereby provides the integrated module that, through its pleiotropic effects, influences the diverse traits associated with diapause and longevity, not only in mutant flies in the laboratory, but also in the geographically variable expression of diapause in natural populations.
**Couch Potato, Linkage and Co-adaptation**

*Couch potato* (*cpo*) is so called because hypomorphic adults are lethargic, recover slowly from anesthesia, are reluctant to fly, and exhibit only weak positive phototaxis or negative geotaxis (Bellen et al. 1992). In eastern North America, the latitudinal cline in diapause is associated with increased stress and cold resistance and increased lipid storage (Schmidt and Conde 2006; Schmidt et al. 2005a). Increasing diapause along the latitudinal cline is also associated with an increasing frequency in the derived substitution of lysine for ancestral isoleucine in residue 462 in the 3’ end of exon 5 at this locus. *cpo* is expressed in the ring gland and has a series of upstream ecdysone response elements, suggesting that the effects of *cpo* on diapause in *D. melanogaster* is mediated by ecdysteroids (Schmidt et al. 2008). Therefore, *cpo* could provide the link between the insulin signaling pathway and the downstream hormones involved directly in the regulation of vitellogenesis.

Both *cpo* and *Pi3K* affect the incidence of diapause and are involved in latitudinal clines in propensity to diapause. These genes and the gene encoding the insulin-like receptor, InR, are located on the right arm of the third chromosome within the boundaries of a known inversion, *In(3R)Payne*. The frequency of *In(3R)Payne* declines with increasing latitude in both North America and Australia and shows strong linkage disequilibrium between marker loci within the inversion (Kennington et al. 2006). This linkage disequilibrium indicates that diapause-promoting alleles might be positively linked (coupled), forming a co-adapted gene complex that restricts recombination between diapause-prone, winter alleles and diapause-averse summer alleles.
Concluding Remarks

Our current understanding of the genetics of diapause illustrates both how historical inertia and the failure to distinguish between gene and modular pleiotropy has impeded progress in understanding the relationship between circadian rhythmicity, photoperiodism and diapause. The historical inertia created by Bünning’s hypothesis that circadian rhythms constitute the basis of photoperiodic control of diapause led to a focus on circadian clock genes as candidate loci for photoperiodism. Until recently (Tauber et al. 2007), it has been assumed that mutations or allelic variants in clock genes that modify both overt rhythmicity and diapause have done so by modifying the effect of the entire circadian clock on photoperiodism, and not by having direct effects of single clock genes on diapause, independently of their role in circadian rhythmicity (i.e., the failure to distinguish between modular and gene pleiotropy). Awareness of this distinction is important because, with the emergence of new molecular tools and an exploding genomics data base, today’s association, correlation, presumptive connection or logically plausible role, like Bünning’s hypothesis, has a tendency to acquire a momentum of its own and become tomorrow’s accepted assumption or established dogma.

Nonetheless, many years of careful research at physiological, evolutionary and genetic levels have resulted in a solid working framework for understanding reproductive diapause in insects. There is general agreement that input of light is interpreted by a timing mechanism that assesses the length of day or night, a counter that accumulates the number of long or short days received, and an output signal. The output signal, which is not yet known, initiates a cascade of hormonal events that ultimately results in diapause itself. The hormonal events are initiated by the release of insulin-like peptides from the brain and
involve sequential secretion of juvenile hormone by the corpora allata in the ring gland and ecdysteroid by the ovaries to promote yolk protein synthesis in the ovaries and fat body. Hence, the insulin signaling pathway is involved not only in aging but also in regulating diapause, both of which involve increased stress resistance, lipid sequestration and postponed senescence in adult Diptera.

On an evolutionary scale, diapause as a phenotype has provided unexpected opportunities to assess the impact of global warming on natural populations, on agriculture and on vector borne disease. Contemporary evolution is proceeding faster than was conceived a generation ago (Grant and Grant 2006; Hendry et al. 2007). As the length of the growing season increases at temperate latitudes, there already have been genetic shifts towards more southern phenotypes in photoperiodic response and the timing of diapause which has been observed over as short a time span as five years in mosquitoes and moths (Bradshaw and Holzapfel 2001; Gomi et al. 2007). The frequencies of In(3R)Payne – the summer inversion – have increased over a latitudinal gradient during the last 20 years in Australian populations of *D. melanogaster* (Anderson et al. 2005), suggesting that evolution of diapause in response to recent rapid climate change has involved not only photoperiodism but also downstream events associated with *couch potato* and at least one of the genes (*Pi3K*) of the insulin signaling pathway.

**Future Directions**

Reverse genetic approaches require an a priori assumption of which genes are important to consider. The use of circadian clock genes as candidate loci has shed little light on the genetic basis of photoperiodism whereas QTL mapping has identified 6-9 QTL contributing
to the evolution of photoperiodism in a mosquito (Mathias et al. 2007). Even in a genetic system as well understood as the circadian clock in *Drosophila*, a new negative feedback loop involving *clockwork orange* was discovered through the use of an expression microarray (Kadener et al. 2007; Lim et al. 2007). Along with others (Bradshaw and Holzapfel 2007b; Stinchcombe and Hoekstra 2008; Tauber and Kyriacou 2008), we therefore propose that forward genetic approaches such as fine-scale mapping, expression arrays, and bulked segregant analysis are fruitful avenues for gene discovery, even in systems that are considered to be well understood.

Reverse genetic approaches typically use induced mutations in cloned genes that are introduced into cells or the germ line and then observed for the phenotypic consequences. This procedure works well for discovering genes of major effect in model organisms but is less likely to identify genes with allelic variation undergoing selection in natural populations. A forward genetic approach starting with latitudinal variation in diapause ultimately led to discovery of the role of *couch potato* in the regulation of diapause in *D. melanogaster* (Schmidt et al. 2005a; Schmidt et al. 2008). We therefore propose that more advantage be taken of natural geographic variation to reveal the genes responsible for adaptive evolution in the real world.

We are in agreement with others (Riddiford 2008; Saunders et al. 1989) that choice of the appropriate insect for the phenotype under consideration is essential. Although making a huge contribution to genetics, development, and evolution, the weak photoperiodic response of *Drosophila melanogaster* unfortunately makes it a lesser organism for the study of photoperiodism despite heroic efforts to do so (Saunders and Gilbert 1990; Saunders et al. 1989; Tauber et al. 2007). There is a wealth of other insects with clean,
precise and robust photoperiodic responses (Danilevskii 1965; Danks 1987; Tauber et al. 1986) that would produce more definitive results. The increasing accessibility of molecular tools for non-model organisms makes the choice of the appropriate insect especially imperative.

Finally, we propose that when experiments are designed and data are interpreted, special care be taken to determine whether the effects of a gene are realized through modification of the module to which it contributes, or are realized through effects of that single gene functioning independently of its contribution to modular function. One approach would be to show whether or not the effects of multiple, independent mutations or single gene knockouts that each eliminated modular function also each had an identical effect on the phenotype. If not, the gene in question would be having an ancillary effect on the phenotype. Complexity in connecting genotypes and phenotypes presents complications that are tedious to unravel. However, experimental conclusions will only be as robust as our willingness to apply rigorous standards in interpreting how genetic variation determines phenotypic variation.

Bridge

This chapter has outlined several of the complexities that arise when studying the genetic basis of physiological traits. The chapter ends with a discussion of the need to use forward genetic approaches if one hopes to understand the basis of complex physiological traits in natural, non-model systems.

Chapter VI presents the first forward genetic screen for candidate genes involved in the physiological response to daylength in any animal. I use a newly developed cDNA
expression microarray and a novel experimental design to find genes whose transcription is regulated as a function of the physiological response to daylength, rather than daylength \textit{per se}. A new list of candidate genes for the involvement in photoperiodism and diapause termination is described.
CHAPTER VII
CANDIDATE GENES INVOLVED IN DEVELOPMENT FOLLOWING DIAPAUSE TERMINATION IN THE PITCHER-PLANT MOSQUITO, *WYEOMYIA SMITHII*

Introduction

A diversity of arthropods mitigate the negative effects of harsh seasons in a state of hibernal dormancy (diapause) that is characterized by the cessation of development, increased lipid stores and increased stress tolerance (Danilevskii 1965; Leather et al. 1993; Tauber et al. 1986). As daylength is a highly reliable predictor of the time of year, arthropods from temperate and polar regions use photoperiod (the length of day) as a cue for the timing of diapause.

The photoperiodic control of diapause is a complex physiological process that involves the photoperiodic timer that measures the length of the day, the photoperiodic counter that accumulates information from the timer to elicit downstream events in the endocrine system that then triggers the go/no-go switch that determines the developmental fate of the organism (active development vs. diapause) (Emerson et al. 2009a; Saunders 2002). It is difficult to study the genetic basis of physiological processes along the photoperiodism-diapause developmental axis (Emerson et al. 2009a) due to the fact that the phenotype measured (diapause) is well downstream of the process that determines it (photoperiodism). For studies of photoperiodism, for instance, great care must be taken when designing experiments to isolate the appropriate time at which the photoperiodic timer
and counter are acting, well before the target phenotype, termination of diapause, is observable.

Herein, I describe a novel experimental design that allows for the study of changes in gene expression due to differential physiological responses to a single light:dark environment. I take advantage of the power of the pitcher-plant mosquito, Wyeomyia smithii (Coq), as a model for the study of photoperiodism. Its clean, robust photoperiodic response, along with a wealth of data concerning the physiology of its photoperiodic termination of diapause (Bradshaw et al. 2003a; Emerson et al. 2008b), allow for the isolation of a particular time in development during which the photoperiodic signals are being interpreted. This design is intended to capture the gene expression differences at the inception of diapause termination so as to reduce the signal of downstream developmental processes.

Methods

The Experimental System: Wyeomyia smithii

The pitcher-plant mosquito, W. smithii, completes its pre-adult development exclusively within the water-filled leaves of its host-plant, the purple pitcher-plant, Sarracenia purpurea. Throughout its range in eastern North America, W. smithii undergoes an hibernal larval diapause that is initiated and maintained by short photoperiods, and terminated or averted by long photoperiods (Bradshaw and Lounibos 1977). Critical photoperiod (the daylength that elicits 50% developmental response within a population) and depth of diapause (the number of long days required to elicit 50% development within a population)

increase linearly with both latitude and altitude (Bradshaw and Lounibos 1977; Emerson et al. 2008b).

Experimental Approach and Design

To determine genes involved in photoperiodism or the first stages of diapause termination, a cDNA expression microarray was used to detect differences in expression between two populations at a fixed daylength that one population interprets as a long day and the other as a short day (Fig VII.1). Two populations from the northern clade of *W. smithii* were used: a population from Western Ontario, Canada (DR: 46°09.370'N, 77°37.582'W, 154m elevation), and a population from the mountains of North Carolina, USA (DB: 35°01.871'N, 83°03.850'W, 900m elevation). This allowed for the greatest difference in critical photoperiod, while maintaining a constant stage of diapause (III instar).

On Day 0 of the experiment, mosquitoes were transferred from short days (Light:Dark = L:D = 10:14) to the experimental L:D cycle (L:D = 14.6:9.4). The experimental L:D cycle is interpreted as a long day by < 2% of animals from population DR and by > 98% of animals from population DB (Bradshaw et al. 2003a, unpublished results). Transitions between L:D cycles were arranged so as to maintain a constant circadian time of dawn (Zt=0). RNA samples (six replicates of 50 whole larvae per day per population) were collected at Zt10 (10 h after lights-on) on days 0 and 6. Since dawn was synchronized, no long-day cues are seen by either population by Zt10 on day 0, and these samples act as a control for evolved differences in gene expression between the two populations. By Zt10 on day 6, more than 50% of individuals within population DB have interpreted enough long-day cues to have terminated diapause (Emerson et al. 2008b) whereas no individuals from population DR have seen any long-day cues. Differences between the day 6 samples represent variation due
to both population and photoperiodic response. Linear models were used to test for transcripts that display a significant interaction between day and population.

Figure VII.1. Schematic representation of microarray experimental design. Two populations (northern population DR and southern population DB) with different critical photoperiods (CPP), were be transferred from short days (L:D = 10:14) to the experimental L:D = 14.6:9.4 environment on day 0. All RNA samples were taken at a single time of day (Zt=10) on days 0 and 6. On day zero, none of the animals had seen any long days. On day 6, only animals from population DB had seen long days, since the critical photoperiod for population DR is greater than the experimental daylength. Note that after 6 long days, >50% of animals in population DB had terminated diapause (Emerson et al. 2009b).

cDNA Library Construction

To allow for the greatest representation of variation in the cDNA library, all RNA used was collected from a hybrid mosquito population representing the nuclear and mitochondrial genetic diversity of eight populations from throughout the range of *W. smithii*. Total RNA was extracted (Trizol Reagent, Invitrogen) from three replicate samples of 30 III and IV instar larval heads sampled at 4 times of day (Zt1, 7, 13, 19) under both
short-day (L:D = 10:14) and long-day (L:D = 18:06) conditions. These samples (all with a 260/280 ratio of 2.0± 0.1) were all pooled into a total RNA pool. One µg from this pooled RNA preparation was used to create a cDNA library using the Clontech SMART cDNA Library Construction Kit (BD Biosciences; Protocol #PT3000-1), following the manufacturer's instructions. Sfi-I digested and purified cDNAs were directionally ligated and packaged into the Lambda TrplEx2 vector using Gigapack III Gold Packaging Extract (Stratagene). The library was amplified according the Kit's Instructions (BD Biosciences; Protocol #PT3000-1).

The phage library was converted to a plasmid library and transformed into competent Escherichia coli (BM25.8) (Clontech protocol PT3003-1). Aliquots of the library were diluted with LB broth, spread on LB agar plus carbenicillin (50 µg/ml) plates, and incubated at 37°C overnight. Isolated colonies were handpicked at random into 96-well plates containing 150 µL LB plus carbenicillin (50 µg/ml). After overnight incubation, these plates were used as templates for Polymerase Chain Reaction (PCR) amplification using the LD-amplimer primer pair from Clontech (forward: 5′ TCCGAGATCTGGACGAGC 3′; reverse: 5′TAATACGACTCACTATAGGG 3′) with a 5′ amine modification (IDT; /5AmMC12/). Sixteen reactions from each 96-well PCR plate were examined using 1.0% agarose gel electrophoresis to check product sizes. Insert sizes were between 0.25 kb and 3 kb, and a vast majority of these were single products.

All of the original cultures were transferred to Nunc 384-well plates with 25% glycerol and stored at −80°C. PCR reactions were precipitated (2.5 volumes 95% EtOH, 1/10 volume 3M NaOAc), re-suspended in nanopure water and transferred to 384-well plates. Once transferred, the plates were dried under vacuum and each reaction was re-suspended in 10 µL
3X SSC. The cDNA library was assessed for redundancy by sequencing 1000 random clones. Approximately 40% of the 1000 sequences were unique suggesting that the library has a redundancy of ~60%. As the redundancy was low for a cDNA library, no normalization was performed.

Printing and Probing of Microarrays

The *W. smithii* microarrays were printed on silylated aldehyde (CEL-1) glass slides at the Genomics Facility of the University of Oregon. The slides were printed, allowed to dry overnight, and then UV cross-linked at 6000 J. On the day of hybridization, slides were post-processed by immersing slides in a wash buffer (5X SSC, 0.1% SDS, 0.1 mg/mL BSA) at 42°C for 60 minutes, and then washing the slides three times in 0.1X SSC. The slides were then immersed in the blocking solution (2X SSC, 0.05% SDS, 2.5 mg/mL NaBH₄). After 3 washes in 0.1X SSC, slides were dried by centrifugation at 800 rpm for 5 minutes.

For each array, 15 μg of each of two RNA samples (one experiment, one control) was reverse transcribed to cDNA and labeled with Cy3 and Cy5 dUTP fluorescent dyes (PerkinElmer) using the Superscript cDNA Labeling System (Invitrogen) according to the manufacturer's protocol, but reducing the reagents by half. Unincorporated dyes were removed from the samples using a Qiagen PCR Purification Kit following the manufacturer's protocol. Samples were then dried completely under vacuum and re-suspended in 42 μL of hybridization buffer containing 50% Formamide, 3X SSC, 1% SDS, 5X Denhardt’s solution (Sigma), and 0.8 mg/mL poly(A) (Sigma). Samples were then boiled at 100°C for 2 minutes, centrifuged, and added to the arrays. The arrays were covered by Lifter slips (Erie Scientific) and allowed to hybridize overnight in Corning microarray
chambers submerged in a 42°C water bath. All samples were hybridized against a pooled reference sample containing 15μg of RNA from the reference sample.

Following overnight hybridization, arrays were washed in 3 solutions containing: 1) 1X SSC and 0.015% SDS; 2) 0.2X SSC; and 3) 0.05X SSC. The microarrays were then dried by centrifugation at 800 rpm for 5 minutes. Microarrays were scanned using a GenePix 4000B scanner and software (Axon GenePix 6.0) and normalized to obtain a 635/532 nm ratio (of medians within arrays) between 0.90 and 1.1.

Array Analysis

All data were analyzed using the LIMMA package for the statistical computing package R (R Development Core Team 2007; Smyth 2005) which implements empirical Bayes methods for analyzing microarray data (Smyth 2004). All spots showing significant interactions between day and population and showing differential expression in the long-day response treatment and the three short-day response treatments were sequenced from the cDNA clone library, and homology was assigned by blastx search of the *Drosophila melanogaster*, *Aedes aegypti*, and *Anopheles gambiae* databases at NCBI. Gene names were assigned the names from the highest scoring blast hit from *D. melanogaster*, preceded by Ws. Where there was no significant blast results, genes were named WsUnknown followed by a number. Data for spots that represented the same transcript were pooled.

Results and Discussion

Thirty transcripts have differential expression due to a physiological response to daylength, with 5 transcripts being significantly down-regulated, and 25 up-regulated, in the long-day response treatment (Figure VI.2), after correction for multiple testing using
Benjamini-Hochberg False Discovery Rate criterion (Table VI.1). Ten of the genes are orphan genes in *W. smithii*, having no significantly similar sequences found in *D. melanogaster*, *A. aegypti*, or *A. gambiae*. Expression patterns of two down-regulated genes (*Ws*Sodh-1 and *Ws*Obo243B) and two up-regulated genes (*Ws*13043 and *Ws*Cpr65Az) were confirmed by quantitative real-time PCR.

*Diapause Associated Gene*

Sorbitol dehydrogenase (*Sodh*) is a two gene system (*Sodh-1* and *Sodh-2*) composed of two paralogous genes that appear in tandem in insect genomes (Luque et al. 1998) and have a highly conserved sequence (>90% within both *W. smithii* and *D. melanogaster*). In *W. smithii*, both copies of *Sodh* are highly expressed in larval diapause, and are down-regulated in animals that have terminated diapause (Figure VI.2). *Sodh* is expressed late in the embryonic diapause of *Bombyx mori* and permits the conversion of sorbitol into glycogen, which is used as an energy source for the termination of diapause (Denlinger 2002; Niimi et al. 1993a; Niimi et al. 1993b). During adult reproductive diapause in the bean bug *Pyrrhocoris apterus*, the expression level of *Sodh* is high, with levels decreasing until they reached trace levels with the termination of diapause (by either thermal or photoperiodic cues) and the resumption of development (Koštál et al. 2008). The generality of the reduction of *Sodh* expression with the termination of diapause across insect orders and stages of diapause...
Figure VII.2. Gene expression that is regulated by differential physiological response to daylength. Blue cells show down-regulated transcripts, yellow cells represent up-regulated transcripts. The four different treatments from Figure VII.1 are represented along the bottom of the figure. Note that all of the genes presented here have a different pattern of expression in the single long-day response treatment (DB day 6) relative to the other treatments.
suggests that it is a general property of insect diapause. In fact, Koštál et al. (2008) suggest using it as a molecular marker of diapause.

**Cuticle Proteins**

Seven cuticle proteins are up-regulated in the long-day response treatment. Most arthropod cuticle proteins share a conserved Rebbers-Riddiford (RR) consensus sequence, which can be further partitioned into one of three types. RR-1 and RR-2 domains correspond to proteins found mainly in “soft” and “hard” cuticle respectively, while RR-3 domains tend to be found in proteins are found mainly in post-ecdysial cuticle (Andersen 2000; Karouzou et al. 2007; Rebers and Riddiford 1988). Five of the cuticle proteins found to be up-regulated in the long-day response treatment include an RR-1 domain (Karouzou et al. 2007; Rebers and Riddiford 1988), and two had no discernable RR domain. This is consistent with the resumption of development following diapause termination as the mosquitoes are preparing for a molt from III to IV instar larvae.

It is worth speculating about the possibility that cuticle proteins serve a function in photoperiodic response independent of their structural role in the generation of new cuticle. The earliest reported photoperiodic response in animals was the transition between parthenogenetic (long-day) and sexual (short-day) forms of the pea aphid, *Acrthosiphon pisum* (Markovich 1924; Moran 1992). Using an EST based microarray, Le Trionnaire et al. (2007) show the differential regulation of 19 cuticle proteins in response to different day lengths. This result was unexpected in that the photoperiodic response in aphids is not diapause, but rather the mode of reproduction. The authors speculate that there may be some modification of cuticle structure associated with photoperiodic response in aphids, but there
Table VII.1. List of genes shown in Figure VII.2. Gene name, and symbol are derived from the *Drosophila* ortholog shown by its FlyBase ID. Adjusted p-values are of the interaction term between day and population from the linear model analysis of the gene expression data.

<table>
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<th>Gene Symbol</th>
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<th>Adjusted P value</th>
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**Genes with lower expression in long-day response treatment**

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<th>Gene Name</th>
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is no experimental evidence to confirm this speculation. It may be that cuticle proteins serve an as yet unknown pleiotropic role in cuticle structure and photoperiodic response.

Alternatively, when one considers the extent to which insect genomes are made up of members of the cuticle protein family, with > 1% of all genes in Anopheles gambiae annotated as cuticle proteins (Cornman et al. 2008), it may not be surprising to find a large number of cuticle proteins emerge from both studies, particularly if they play an as yet unidentified functional role outside of their structural roles in cuticle.

Other Transcripts

One transcript significantly up-regulated in the long-day response treatment, Ws13043, is located in a genomic region that is involved in complex interactions regulating critical photoperiod, stage of diapause and ecdysone-regulated development in W. smithii (Mathias et al. 2007). Using an F2G hybrid line (generated as in Mathias et al. 2007 and maintained under panmictic optimal laboratory conditions for 26 generations) and a previously designed genotyping protocol (Mathias et al. 2007), the strong association between northern and southern alleles of Ws13043 and critical photoperiod is maintained ($\chi^2 = 58.62$, d.f. = 1, $P < 0.001$).

The rest of the transcripts emerging for this microarray based screen do not have any known annotation that relates to diapause, development, or interaction with light. More functional studies are required to determine the role these transcripts play in the photoperiodically controlled termination of diapause. Several of these genes (WsPebIII, WsOha23B, WsLep65A, WsCprA, WsSep) show no association with critical photoperiod in an F2G hybrid line (data not shown), indicating that though they are differentially expressed in
response to daylength, they may not be involved in the evolutionary modification of photoperiodic time measurement among populations of *W. smithii*.

Many complexities arise when studying physiological processes such as photoperiodic time measurement and diapause (Emerson et al. 2009a). For instance, though we have found quite a number of genes that are differentially expressed due to a physiological response to daylength, it is unclear whether these genes are acting on the photoperiodic time measurement system, diapause, active development or any combination of these processes. More work is required to further parse out the roles that these genes are having on the photoperiodically controlled termination of diapause as well as where along the photoperiodism-diapause axis they have their effect.

In this chapter, I report the first genomic screen for candidate genes involved in the photoperiodic control of diapause termination in an insect. This is only the second screen (see Le Trionnaire et al. 2007) for genes involved in photoperiodic time measurement in animals that is not biased by the assumption that the circadian clock is involved in photoperiodism. The list of genes presented in Table VII.1 is the first list of candidate genes underlying the evolutionary modification of photoperiodic time measurement.
CHAPTER VIII
CONCLUSIONS

Understanding the physiological and genetic basis of the photoperiodic control of diapause in insects has been a major focus of many researchers for decades. The control of insect diapause in natural populations is one method that has been suggested for the management of insect pests (Chippendale 1982). All of the examples, to date, of adaptation to recent, rapid climate change over the last few decades consist of the genetic modification of the timing of seasonal phenotypes, including photoperiodic diapause in insects (Bradshaw and Holzapfel 2001; Bradshaw and Holzapfel 2008). Clearly, diapause and its control are ecologically and evolutionarily important phenotypes, and a deeper understanding of them is critical for our increased understanding of organisms living in seasonal environments.

The focus of this dissertation has been the physiological and genetic basis of photoperiodic time measurement. The dissertation began with a study that shows the importance of the proper choice of organism in biological studies. *Drosophila melanogaster*, though being the laboratory work-horse of geneticists for over 100 years, is not the proper organism in which to study the genetic basis of photoperiodism as natural populations have at best a very weak (Tauber et al. 2007) and at worst a non-existent (Chapter II) photoperiodic response.

One major theme that surfaced throughout the work presented herein is that the historical inertia generated by Bünning’s (1936) hypothesis that the circadian clock formed
the causal basis of photoperiodism has had a profound effect on the last seven decades of chronobiological research. Herein, I show that there is no evidence supporting this hypothesis in *W. smithii*, and in fact, there is strong evidence that these two physiological processes, the circadian clock and photoperiodic time measurement, that control the timing of biological events on daily and seasonal scales, are evolutionarily independent (Emerson et al. 2009b; Emerson et al. 2008b). Furthermore, the work presented herein offers an alternative hypothesis for the generation of a pattern of experimental data (rhythmic response to Nanda-Hamner experiments) that historically has been used as evidence for the role of the circadian clock in photoperiodism (Emerson et al. 2008a), namely that the rhythm is an overt expression of the circadian rhythm, independent of any role the clock may have in photoperiodism.

Lastly, this dissertation discusses the complications that arise when trying to understand how a genotype becomes a phenotype. Insect diapause and photoperiodism are used as a case in point. Care must be taken when studying photoperiodic control of diapause to determine where allelic variation or genetic mutation is playing its role within the photoperiodism-diapause developmental axis in order not to have negative effects on future work (Emerson et al. 2009a). This discussion is followed by the first genomic screen for candidate genes involved in the physiological response to daylength that was not biased by *a priori* assumptions concerning the role of the circadian clock. Several genes were highlighted for their involvement in the photoperiodism-diapause axis (Chapter VII).

The study of biological timing, both at the daily and seasonal scales, has historically only been possible within a few model organisms (Sehgal 2004). The recent advent of molecular tools available for non-model organisms, such as expression microarrays (Chapter...
VII) and next-generation sequencing (Mardis 2008) yield great promise for the field over the next several years. Researchers are no longer limited to the few model organisms to address fundamental questions in the evolution of biological timing. Researchers are now able to ask the fundamental questions in the appropriate organisms, exhibiting clear robust phenotypes without being limited by the tools available. The next decade of chronobiological research is indeed very exciting and I look forward to being a part of it.
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