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RESEARCH ARTICLE

Functional circadian clock genes are essential for the overwintering diapause of the Northern house mosquito, *Culex pipiens*

Megan E. Meuti[1,*], Mary Stone[1], Tomoko Ikeno[1,2] and David L. Denlinger[1]

ABSTRACT

The short day lengths of late summer are used to program the overwintering adult diapause (dormancy) of the Northern house mosquito, *Culex pipiens*. Here, we investigated the role of clock genes in initiating this diapause and asked whether the circadian cycling of clock gene expression persists during diapause. We provide evidence that the major circadian clock genes continue to cycle throughout diapause and after diapause has been terminated. RNA interference (RNAi) was used to knock down the core circadian clock genes and to then assess the impact of the various clock genes on the ability of females to enter diapause. RNAi directed against negative circadian regulators (*period*, *timeless* and *cryptochrome2*) caused females that were reared under diapause-inducing, short day conditions to avert diapause. In contrast, knocking down the circadian-associated gene *pigment dispersing factor* caused females that were reared under diapause-averting, long day conditions to enter a diapause-like state. Our results implicate the circadian clock in the initiation of diapause in *C. pipiens*.

KEY WORDS: Photoperiodism, *period*, *timeless*, *cryptochrome 2*, *pigment dispersing factor*, RNA interference

INTRODUCTION

Among insects, diapause is a prominent seasonal response allowing survival during extended periods of unfavorable environmental conditions (Tauber et al., 1986; Danks, 1987; Denlinger, 2002). Temperate insects, confronted by low temperatures and limited access to food in winter, typically enter diapause in response to the short day lengths of late summer and early autumn. By utilizing this stable token stimulus, rather than changes in temperature or rainfall, which are highly variable, insects are able to anticipate the seasonal change well in advance of its arrival and thus prepare for diapause entry by accumulating fat reserves, seeking protected shelters and initiating other physiological changes that increase their resistance to low temperatures, desiccation and pathogen attacks (Denlinger, 2002). Although much is known about the photoperiodic cues, endocrine signals and physiological changes that regulate and accompany diapause, we do not fully understand how insects measure day length, store photoperiodic information or translate this information into downstream hormonal responses (Denlinger, 2002; Koštál, 2011).

Insects possess sophisticated circadian clocks, similar to clocks in mammals and other animals (Young and Kay, 2001). From the extensive body of work done on *Drosophila melanogaster*, we know that the core circadian clock consists of a self-regulatory negative feedback loop (Hardin, 2005). The positive elements of the circadian clock, the proteins CLOCK (CLK) and CYCLE (CYC), bind to E-box promoter regions of other circadian clock genes, namely *period* (*per*) and *timeless* (*tim*), and act as positive transcriptional regulators, increasing the mRNA levels of their targets. PER and TIM proteins form a heterodimer in the cytosol and translocate back into the nucleus, where they inhibit the action of CLK and CYC, thereby suppressing their own transcription. The clock is informed by light through the action of another circadian protein, CRYPTOCHROME1 (CRY1), which binds to TIM in the presence of light and degrades it. Light input into the circadian clock may also be achieved through a CRY1-independent pathway, possibly through opsins, as noted in honey bees, a species that lacks CRY1. However, among insects that possess CRY1, this protein likely serves as the dominant circadian photoreceptor. *per* and *tim* mRNA abundance shows daily oscillations in *Drosophila* and other insects, with peak expression occurring shortly after the transition from photophase (lights on) to scotophase (lights off).

In contrast to *Drosophila*, where *Clk* and *cry1* transcripts cycle with peak expression occurring early in the photophase, and where *cyc* is constitutively expressed (Hardin, 2005), *Clk* is constitutively expressed while *cyc* transcripts oscillate with peak expression occurring early in the photophase in the honey bee (Rubin et al., 2006), sand fly (Meireles-Filho et al., 2006) and monarch butterfly (Reppert, 2006). In the mosquitoes *Culex quinquefasciatus* and *Aedes aegypti*, Gentile et al. (Gentile et al., 2009) reported a peak of *cyc* expression early in photophase and the expected peak of *per* and *tim* at the onset of the of scotophase, but transcripts encoding *Clk* and *cry1* remained unchanged throughout the day–night cycle.

In addition to these core circadian components, Zhu et al. (Zhu et al., 2005) discovered that the monarch butterfly, *Danaus plexippus*, possesses another circadian protein, CRYPTOCHROME2 (CRY2), which is highly homologous to mammalian CRY and is absent in *Drosophilia*. Yuan et al. (Yuan et al., 2007) demonstrated that CRY2 proteins isolated from the monarch butterfly, the malaria mosquito, the honey bee and the flour beetle are light insensitive, and instead repress CLK:CYC-mediated transcription of *per* in *Drosophila* Schneider 2 cells. Since its initial discovery, *cry2* has been reported in numerous insects (except the higher Diptera), and its transcripts consistently cycle and reach peak expression early in scotophase (Yuan et al., 2007; Gentile et al., 2009). It is currently unknown whether CRY2 interacts with PER and TIM proteins *in vivo* in any insect, but it is likely that CRY2 serves as a more potent negative regulator of the circadian clock than either PER or TIM (Zhu et al., 2008).

Circadian clocks and the capacity for photoperiodic diapause are widely distributed among insects, and circadian clocks are known to

[1]The Ohio State University, Department of Entomology, 318 W. 12th Avenue, Room 400 Aronoff Laboratory, Columbus, OH 43210, USA. [2]Michigan State University, Psychology Department, East Lansing, MI 48824, USA.

*Author for correspondence (meuti.1@osu.edu)

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List of symbols and abbreviations

Clk	*Clock*
cry1	*cryptochrome1*
cry2	*cryptochrome2*, also known as mammalian-like *cryptochrome*
cyc	*cycle*
dsRNA	double-stranded RNA
JH	juvenile hormone
pdf	*pigment dispersing factor*
per	*period*
RNAi	RNA interference
tim	*timeless*
ZT	Zeitgeber time
β-gal	*β-galactosidase*

provide insects with important information on the time of day; thus, it seems likely that these same clocks would be used to measure day length and initiate photoperiodic responses. This hypothesis, first proposed by the German botanist Erwin Bünning (Bünning, 1936), has been well supported in plants (Putterill et al., 2010). Yet the role of circadian clocks in the insect diapause response has been less clear. This is partially because *D. melanogaster*, which has proven an excellent model for insect molecular biology, has a rather weak diapause response (Schmidt et al., 2005) and is thus not an ideal organism for probing questions about the role of circadian clocks in diapause. The involvement of circadian clocks in diapause is further complicated by experiments showing that clock genes exert many and varied pleiotropic effects on the whole organism; for example, Rund et al. (Rund et al., 2011) found that 4.5% of the genome of the malaria mosquito *Anopheles gambiae* oscillates daily and a similar study in *A. aegypti* demonstrated that genes for growth, development, immune response and detoxification/pesticide resistance are rhythmically expressed under light:dark conditions (Ptitsyn et al., 2011). Therefore, it remains unclear whether individual clock genes and/or the circadian clock as a functional unit are involved in the measurement of day length, the release of the hormonal signals that trigger the diapause response, the manifestation of individual phenotypes associated with diapause, or some combination of all of these (Emerson et al., 2009).

In spite of the pleitotropic nature of clock genes and the circadian clock, there is mounting evidence that the circadian clock is involved in the photoperiodic programming of diapause in several species. For example, classic studies using night interruption experiments, as well as Nanda–Hamner experiments, where a short photoperiod is followed by extended periods of darkness, supported what Pittendrigh (Pittendrigh, 1966) referred to as the external coincidence model. In this model, a circadian oscillator is entrained by lights off and programs a photo-inducible phase that, if illuminated, causes the insect to avert diapause. Such classic experiments have demonstrated the circadian basis of photoperiodic diapause initiation in several insect species (see reviews by Saunders, 2002; Saunders and Bertossa, 2011; Goto, 2013; Meuti and Denlinger, 2013), including a northern fruit fly, *D. montana* (Kauranen et al., 2013), and the mosquito *Aedes atropalpus* (Beach and Craig, 1977).

Additionally, neuroanatomical studies have implicated clock genes in insect photoperiodic responses. A subset of the core circadian neurons also express *pigment dispersing factor* (*pdf*), a circadian clock-associated gene that maintains synchronous cycling among individual circadian clock neurons (Shafer and Yao, 2014). These PDF-positive neurons innervate regions of the brain involved in the hormonal regulation of insect diapause in the blow fly *Protophormia terraenovae*, and ablation of these PDF-positive neurons interferes with photoperiodic diapause induction (Hamanaka et al., 2005; Shiga and Numata, 2009). Such studies suggest a physical connection between the circadian and photoperiodic clocks, as well as the potential for PDF to coordinate both daily and seasonal responses.

Some of the most convincing evidence that the circadian clock is involved in diapause initiation includes recent molecular studies on the clock genes themselves. Ikeno et al. (Ikeno et al., 2010; Ikeno et al., 2011a; Ikeno et al., 2011b; Ikeno et al., 2013) used RNA interference (RNAi) to knock down core circadian clock genes in the bean bug *Riptortus pedestris* and assessed whether the bugs are able to enter an adult, reproductive diapause. When the negative circadian regulators *per* or *cry2* are knocked down, *R. pedestris* displays a non-diapause phenotype, developing egg follicles (females) and accessory glands (males), even under short day, diapause-inducing conditions (Ikeno et al., 2010; Ikeno et al., 2011a; Ikeno et al., 2011b). In contrast, when the positive circadian regulators *Clk* or *cyc* are knocked down, the bugs display a diapause phenotype and are unable to develop their reproductive organs even under long day, diapause-averting conditions. The effects of knocking down *Clk* and *cyc* are upstream of the hormonal cues that govern diapause, as application of juvenile hormone (JH) induces ovarian development in *cyc* double-stranded RNA (dsRNA)-treated females (Ikeno et al., 2010). This work suggests that the circadian clock as a functional unit, rather than individual genes, regulates diapause initiation in *R. pedestris*.

The Northern house mosquito, *Culex pipiens* L., is a major vector of West Nile virus, as well as St Louis encephalitis and filariasis. Females of *C. pipiens* enter an overwintering, adult, reproductive diapause in response to the short day lengths experienced by fourth instar larvae and pupae in late summer and early autumn. Diapause is characterized by a number of traits including lack of host-seeking behavior, arrested egg follicle development and extensive fat hypertrophy (Spielman and Wong, 1973; Bowen et al., 1988; Mitchell and Briegel, 1989; Robich and Denlinger, 2005). As with most insects, the molecular mechanism by which *C. pipiens* measures day length is unknown. In this study, we first compared the daily expression profiles of several circadian clock genes (*per*, *tim*, *Clk*, *cyc*, *cry1* and *cry2*) in the brains of non-diapausing and diapausing adult females 1 week after adult eclosion, as well as in females at various times throughout diapause and after diapause was terminated. The circadian clock stops cycling during hibernation in European hamsters (Revel et al., 2007), but whether this is also true for insect diapause remains unclear and was tested here. Next, we used RNAi to knock down expression of several core circadian clock genes (*per*, *tim*, *cry2*, *cyc* and *pdf*) to assess the impact of this manipulation on egg follicle length and lipid content, two prominent markers of photoperiodic diapause in *C. pipiens* (Sim and Denlinger, 2008).

RESULTS

Identification of *C. pipiens* clock gene sequences

Sequences of the circadian clock genes *per*, *tim*, *Clk*, *cyc*, *cry1*, *cry2* and *pdf* in *C. pipiens* were obtained by searching the published genome of the closely related species *C. quinquefasciatus* (Arensburger et al., 2010), as well as databases for the yellow fever mosquito, *A. aegypti*, and the malaria mosquito *A. gambiae*. Primers were designed based on consensus regions in these three species, and, after initial PCR amplification of gene fragments from *C. pipiens* cDNA, 5′ and 3′ RACE were used to extend the length of these gene sequences. Using these methods, we obtained full-length gene sequences of *tim* (2998 nt; KM355979) and *pdf* (700 nt;

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Table 1. Comparison of the percentage amino acid identity of circadian clock genes identified in *Culex pipiens* with previously published sequences in the Southern house mosquito (*C. quinquefasciatus*), the yellow fever mosquito (*Aedes aegypti*), the malaria mosquito (*Anopheles gambiae*) and the fruit fly (*Drosophila melanogaster*)

Gene	Species	Amino acid identity
period in *C. pipiens* (KM355980)	*C. quinquefasciatus* (XP_001849299.1)	94%
	A. aegypti (XP_001658976.1)	72
	A. gambiae (XP_321212.4)	77
	D. melanogaster (CAA27285.1)	51
timeless in *C. pipiens* (KM355979)	*C. quinquefasciatus* (XP_001848611.1)	85%
	A. aegypti (AAY40757.1)	68
	A. gambiae (XP_001689006.1)	76
	D. melanogaster (AAB94890.1)	63
Clock in *C. pipiens* (KM355975)	*C. quinquefasciatus* (XP_001843414.1)	99%
	A. aegypti (XP_001662706.1)	93
	A. gambiae (XP_315720.4)	92
	D. melanogaster (AAD10630.1)	58
cycle in *C. pipiens* (KM355981)	*C. quinquefasciatus* (XP_001865023.1)	95%
	A. aegypti (AEX32872.1)	78
	A. gambiae (XP_556301.3)	95
	D. melanogaster (AAC39124.1)	75
cryptochrome1 in *C. pipiens* (KM355976)	*C. quinquefasciatus* (XP_001851403.1)*	97%
	A. aegypti (XP_001648498.1)*	79
	A. gambiae (XP_321104.4)	76
	D. melanogaster (NP_732407.1)	60
cryptochrome2 in *C. pipiens* (KM355977)	*C. quinquefasciatus* (XP_001869456.1)	91%
	A. aegypti (XP_001655778.1)‡	88
	A. gambiae (ABB29887.1)	85
	D. melanogaster	Absent
pigment dispersing factor in *C. pipiens* (KM355978)	*C. quinquefasciatus* (XP_001846575.1)§	100%
	A. aegypti (XP_001653971.1)§	53
	A. gambiae (XP_315791.3)	57
	D. melanogaster (AAC98309.1)	37

The GenBank accession number for each peptide is listed in parentheses.
*Peptides are listed as 'DNA photolyase' in GenBank but are identified as 'CRY1' in VectorBase. ‡This peptide is listed as 'DNA photolyase' in GenBank but no identity is given in VectorBase. §Peptides are listed as 'hypothetical proteins' in GenBank.

KM355978), including the 5′ and 3′ UTR. Large fragments of *per* (2741 nt, including the 5′ UTR; KM355980), *Clk* (1037 nt; KM355975), *cyc* (1182 nt; KM355981), *cry1* (548 nt; KM355976) and *cry2* (1825 nt; KM355977) were also obtained.

Sequences of the circadian clock genes were translated using ExPASy (Swiss Institute of Bioinformatics), and the resulting amino acid sequences were BLASTed against the genomes of *C. quinquefasciatus*, *A. aegypti*, *A. gambiae* and *D. melanogaster*. The percentage amino acid identity of each clock gene in *C. pipiens* is compared with those of the reference species in Table 1. These results show that the clock genes in *C. pipiens* are highly conserved between this species and *C. quinquefasciatus* (85–100% amino acid identity), and are highly homologous among other mosquito species (68–95% amino acid identity) as well as *D. melanogaster* (37–75% amino acid identity). The high identity among clock gene sequences in mosquitoes is also consistent with what has been reported for *per* and *tim* genes in *Aedes albopictus* (Summa et al., 2012). Furthermore, we identified two 'conserved hypothetical proteins' that are likely *pdf* in *C. quinquefasciatus* (XP_001846575.1) and *A. aegypti* (XP_001653971.1), and one peptide that is listed as DNA photolyase in GenBank but is more likely *cry2* in *A. aegypti* (XP_001655778.1).

Temporal expression patterns of clock gene mRNA

To assess the temporal expression patterns of clock genes in non-diapausing and diapausing mosquitoes, brains were collected from *C. pipiens* females at 8 h intervals over a 24 h period starting with lights on (Zeitgeber 0, ZT0). Quantitative real-time PCR was used to measure clock gene expression in 1 week old non-diapausing females that were reared under long day conditions (16 h:8 h light:dark, L:D at 18°C), and in diapausing females that were held for 1 week, 1 month or 3 months in short day, diapause-inducing conditions (8 h:16 h L:D at 18°C), as well as in females that had been in diapause for 3 months and were then exposed to long day lengths (16 h:8 h L:D) at 25°C for 2 weeks to break diapause (post-diapause females). Quantitative real-time PCR revealed that the clock genes *per*, *tim*, *cyc* and *cry2* oscillated in brains of *C. pipiens* with the expected expression profiles (peaks in early scotophase) in 1 week old non-diapausing females and continued to oscillate throughout the diapause program and after diapause termination (Fig. 1A,B,D,F). In contrast, *Clk* and *cry1* mRNA did not oscillate in the brains of either non-diapause or post-diapause females or in any of the diapausing stages (Fig. 1C,E; one-way ANOVA, $P>0.25$).

Diapausing females were exposed to short days (8 h:16 h L:D); thus, we anticipated that if core circadian clock genes were cycling during diapause, peak *per*, *tim* and *cry2* expression would occur at ZT8, i.e. at onset of scotophase (lights off). In contrast, non-diapausing mosquitoes and post-diapause *C. pipiens* were exposed to a 16 h:8 h L:D photoperiod, and hence we anticipated that peak *per*, *tim* and *cry2* expression would occur at ZT16, the time of lights off under these long-day conditions.

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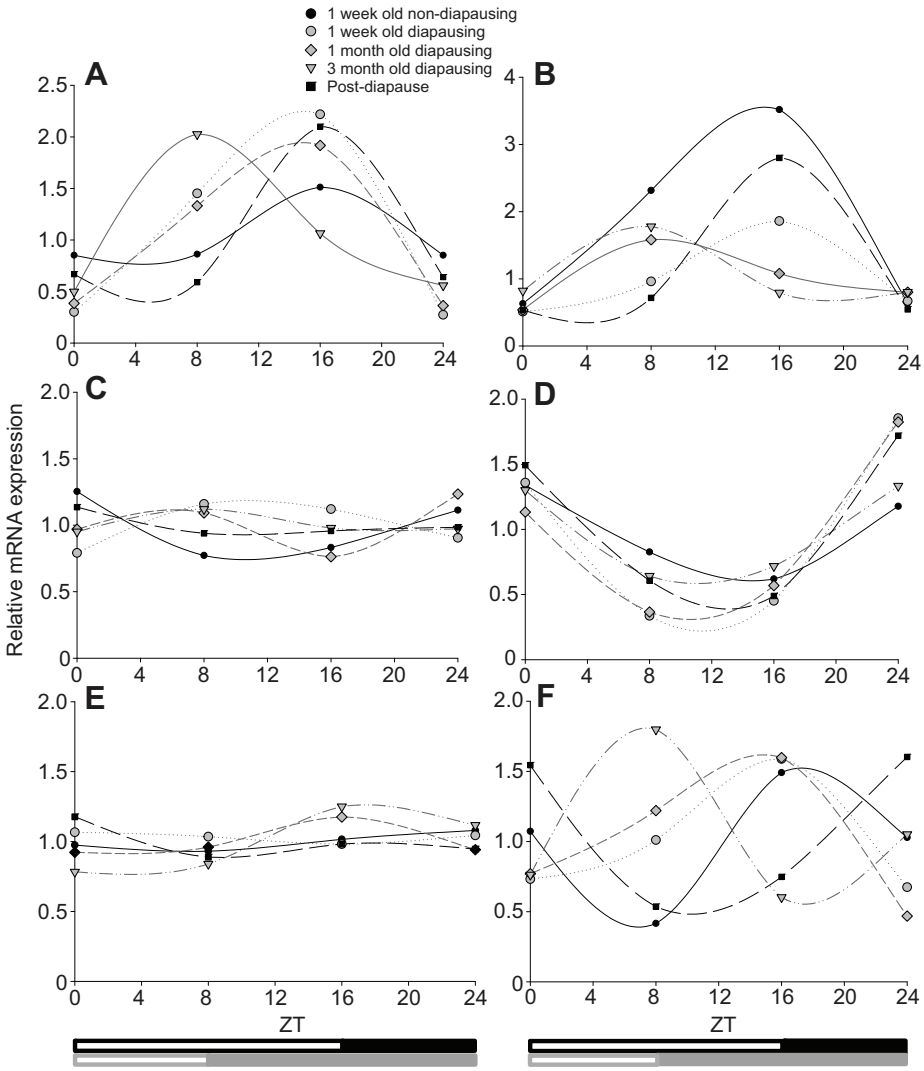

Fig. 1. Clock gene expression in non-diapausing and diapausing female *Culex pipiens*. (A) *period* (*per*), (B) *timeless* (*tim*), (C) *Clock* (*Clk*), (D) *cycle* (*cyc*), (E) *cryptochrome1* (*cry1*) and (F) *cryptochrome2* (*cry2*) relative mRNA expression measured by quantitative real-time PCR. Each point represents the average relative mRNA expression of 3–4 samples containing 10–20 female brains. One week old non-diapausing and post-diapause mosquitoes were exposed to 16 h of light and 8 h of darkness (16 h:8 h L:D), which is represented by the black open and filled bars below the *x*-axis (ZT, Zeitgeber time). One week old, 1 month old and 3 month old diapausing mosquitoes were exposed to 8 h:16 h L:D, which is shown by the gray open and filled bars below the *x*-axis. All mosquitoes were held at 18°C, except for post-diapause females, which were held at 25°C. Spline curves were fitted to the data using SigmaPlot. Standard error bars have been removed to enhance the clarity of the figure but are provided in supplementary material Figs 1–6.

In 1 week old non-diapausing females and in post-diapause females, *per* expression peaked at ZT16, as expected (one-way ANOVA, $P<0.05$). Interestingly, *per* mRNA in 1 week and 1 month old diapausing female brains showed an altered expression profile, with peak expression occurring not at the expected time of lights off (ZT8), but at ZT16 (Fig. 1A; one-way ANOVA, $P<0.001$; Holm–Sidak comparison between ZT8 and ZT16, $P<0.05$). Yet, *per* expression in 3 month old diapausing females occurred at ZT8, as expected (one-way ANOVA, $P<0.001$).

Similarly, peak *tim* mRNA expression in 1 week old non-diapausing female brains also occurred at lights off (ZT16; one-way ANOVA, $P<0.001$), but *tim* cycling was not significant in post-diapause female brains (one-way ANOVA, $P=0.216$). Like *per*, *tim* expression in 1 week old diapausing female brains also showed peak expression later in scotophase, at ZT16 (Fig. 1B; one-way ANOVA, $P<0.05$). Though *tim* did not show significant cycling in 1 month old female brains (one-way ANOVA, $P=0.099$), significant cycling was evident in 3 month old diapausing female brains, with peak expression occurring at ZT8, as expected (one-way ANOVA, $P<0.01$).

cry2 mRNA expression peaked at ZT16 in 1 week old non-diapausing female brains (one-way ANOVA, $P<0.001$). Like *per* and *tim*, peak *cry2* expression in 1 week old diapausing female brains occurred at ZT16 rather than the expected time of ZT8 (Fig. 1F; one-way ANOVA, $P<0.01$). One month old diapausing females did not show significant *cry2* cycling (one-way ANOVA on ranks, $P=0.128$), but later in diapause *cry2* displayed the expected circadian profile with peak expression occurring at ZT8 in 3 month old diapausing female brains (one-way ANOVA on ranks, $P<0.05$). Surprisingly, after diapause termination the expression profile of *cry2* reversed, with peak expression occurring at lights on (ZT0 and ZT24) and minimum expression occurring at ZT8 (one-way ANOVA, $P<0.01$).

In contrast to *per*, *tim* and *cry2*, *cyc* mRNA was expected to reach peak expression at the transition from scotophase to photophase (lights on) and be minimally expressed at lights off. As expected, peak *cyc* expression did occur at ZT0 and ZT24 in all cases (Fig. 1D; one-way ANOVA for all developmental time points, $P<0.03$). No statistical difference was noted between *cyc* expression at ZT8 and ZT16 for any of the developmental stages, indicating no difference in the timing of minimum *cyc* expression between long day- and short day-reared mosquitoes.

Effects of circadian clock gene RNAi on diapause initiation

We used RNAi to knock down core circadian clock genes and assessed the diapause phenotype of females that were reared under diapause-inducing, short days (8 h:16 h L:D at 18°C) and diapause-averting, long days (16 h:8 h L:D at 18°C). On the day of adult

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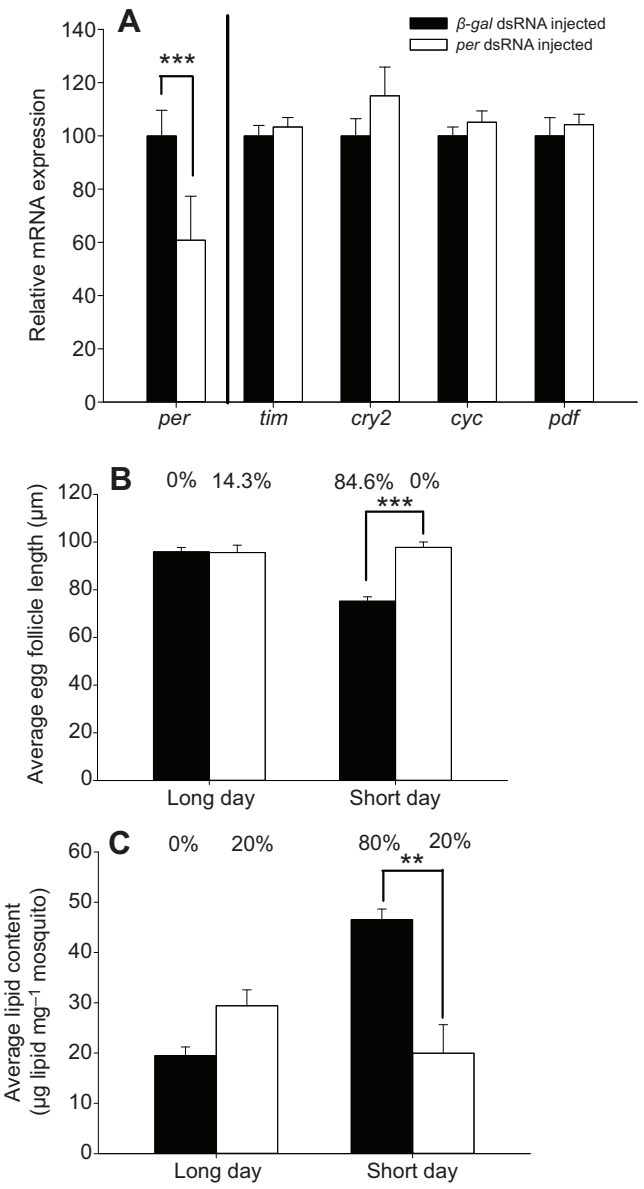

Fig. 2. Effects of *per* dsRNA injection on clock gene mRNA expression, ovarian development and lipid content. (A) Confirmation of *per* mRNA knockdown and the effects of *per* dsRNA on non-target circadian clock gene expression. Each bar represents the whole-body relative mRNA expression from mosquitoes that were collected at lights off 2 days after dsRNA injection. One week after dsRNA injection, the effects of *per* dsRNA on (B) average egg follicle length of 15 females and (C) average lipid content in 5 females that were reared under long (16 h:8 h L:D) or short day conditions (8 h:16 h L:D) at 18°C were measured. Effects of *per* dsRNA were compared with those observed in *β-galactosidase* (*β-gal*) dsRNA-injected (control) mosquitoes. Females were considered to be in diapause (percentage shown above bars) if the average egg follicle length was <80 μm and their lipid content was >30 μg mg^{-1} mosquito. Bars represent s.e.m.; ***P*<0.01, ****P*<0.001 (Student's *t*-test).

emergence, female mosquitoes were injected with dsRNA specific to *per*, *tim*, *cry2*, *cyc*, *pdf* or *β-galactosidase* (*β-gal*; positive control). Knock down of target circadian clock gene mRNA expression was assessed using quantitative real-time PCR 2 days after dsRNA injection. Because the functions of many of the circadian clock genes are interrelated, we also measured the effect of dsRNA injection on non-target circadian clock genes. The diapause phenotype was evaluated by monitoring both ovarian development and lipid accumulation. Diapausing females of *C. pipiens* have small egg follicles (50–80 μm in length) and a high lipid content (>30 μg lipid mg^{-1} fresh mass) while non-diapausing females have significantly larger egg follicles (>90 μm in length) and a lower lipid content (<20 μg lipid mg^{-1} fresh mass). We assessed the diapause status of dsRNA-injected female mosquitoes 1 week after dsRNA injection by measuring the average length of their egg follicles and whole-body lipid content.

RNAi against *per* significantly reduced *per* mRNA expression (Student's *t*-test, $P<0.001$) but did not affect expression of the other clock genes we measured (Fig. 2A). Knocking down *per* did not affect egg follicle development or lipid content of mosquitoes reared under long day conditions, but *per* dsRNA-injected females that were reared under short day conditions had significantly larger egg follicles and significantly less lipid than *β-gal* dsRNA-injected controls (Fig. 2B,C; Student's *t*-test, $P<0.01$). This indicates that *per* dsRNA prevented females of *C. pipiens* from expressing the diapause phenotype under short day, diapause-inducing conditions.

RNAi against *tim* (Fig. 3A) and *cry2* (Fig. 4A) significantly reduced *tim* and *cry2* mRNA, respectively (Student's *t*-test, $P<0.001$), but both *tim* and *cry2* dsRNA also caused a significant reduction in *per* mRNA expression (Student's *t*-test, $P<0.05$). Knock down of both *tim* and *cry2* did not affect egg follicle development or lipid content under long day conditions (Fig. 3B,C, Fig. 4B,C), but *tim* and *cry2* dsRNA-injected females had significantly larger egg follicles than *β-gal* dsRNA-injected controls under short day conditions (Fig. 3B, Fig. 4B; Student's *t*-test, $P<0.01$). These egg follicle data indicate that *tim* and *cry2* dsRNA-injected mosquitoes averted diapause. Yet, the lipid results suggest that short day-reared *tim* and *cry2* dsRNA-injected mosquitoes retained the lipid characteristics of diapause: *tim* dsRNA-injected females showed a significant increase in lipid content relative to *β-gal* dsRNA-injected controls under short day conditions (Fig. 3C; Student's *t*-test, $P<0.05$), while *cry2* dsRNA females had the same amount of lipid as diapausing *β-gal* dsRNA-injected controls (Fig. 4C).

RNAi against *cyc* significantly reduced *cyc* mRNA expression (Student's *t*-test, $P<0.01$), but did not affect expression of any of the other circadian clock genes (Fig. 5A). *cyc* dsRNA did not affect egg follicle development or lipid content in either long day- or short day-reared females (Fig. 5B,C).

pdf dsRNA strongly suppressed *pdf* mRNA expression (Student's *t*-test, $P<0.001$) but did not affect expression of non-target circadian clock genes (Fig. 6A). While knocking down *pdf* expression did not affect either egg follicle development or lipid accumulation under short day conditions, long day-reared *pdf* dsRNA-injected females had a significantly smaller egg follicle length and higher lipid content relative to *β-gal* dsRNA-injected controls (Fig. 6B,C; Student's *t*-test, $P<0.01$). Thus, knocking down *pdf* caused female *C. pipiens* to enter a diapause-like state under long day, diapause-averting conditions.

DISCUSSION

Although clock genes have been implicated in the initiation of photoperiodic diapause in several insect species, few studies have examined the ontogeny of clock gene expression during insect diapause. The role of the circadian clock during mammalian hibernation has not been extensively investigated either, but Revel et al. (Revel et al., 2007) reported that circadian clock genes do not cycle in hibernating European hamsters. In contrast, we found that the clock genes *per*, *tim*, *cyc* and *cry2* continue to show robust daily oscillations during diapause in the brains of *C. pipiens*. These

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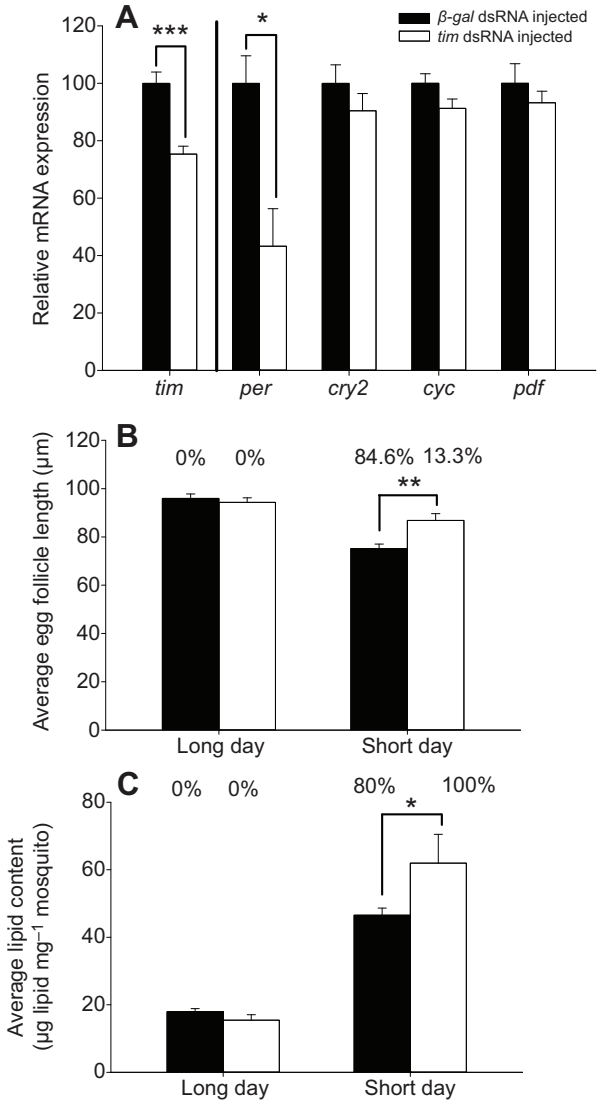

Fig. 3. Effects of *tim* dsRNA injection on clock gene mRNA expression, ovarian development and lipid content. (A) Confirmation of *tim* mRNA knockdown and the effects of *tim* dsRNA on non-target circadian clock gene expression. Each bar represents the whole-body relative mRNA expression from mosquitoes that were collected at lights off 2 days after dsRNA injection. One week after dsRNA injection, the effects of *tim* dsRNA on (B) average egg follicle length of 15 females and (C) average lipid content in 5 females that were reared under long (16 h:8 h L:D) or short day conditions (8 h:16 h L:D) at 18°C were measured. Effects of *tim* dsRNA were compared with those observed in *β-gal* dsRNA-injected (control) mosquitoes. Females were considered to be in diapause (percentage shown above bars) if the average egg follicle length was <80 μm and their lipid content was >30 μg mg^{-1} mosquito. Bars represent s.e.m.; **P*<0.05, ***P*<0.01, ****P*<0.001 (Student's *t*-test).

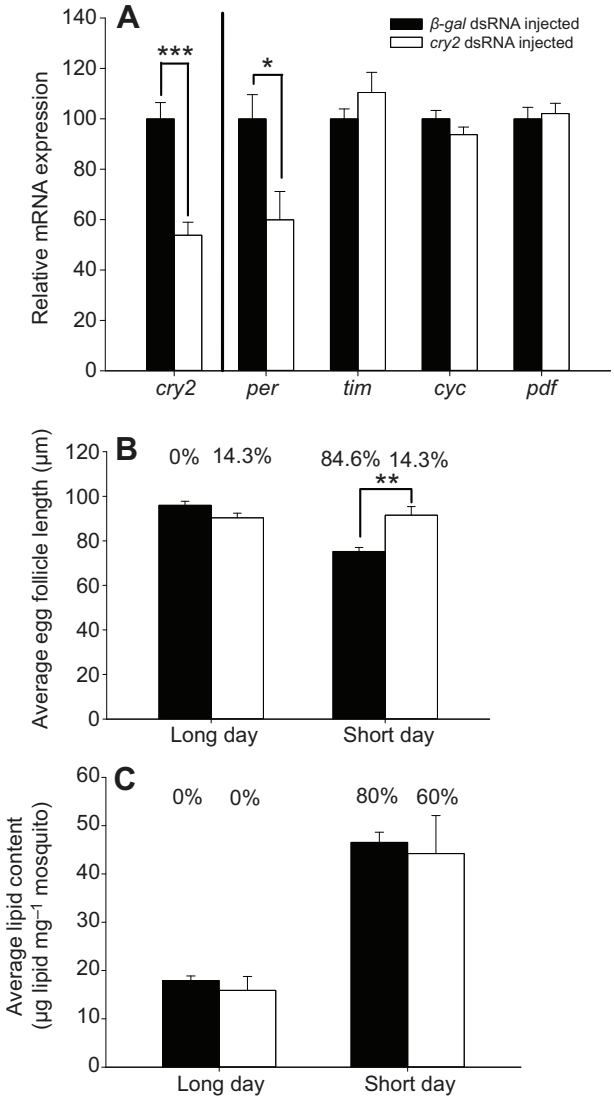

Fig. 4. Effects of *cry2* dsRNA injection on clock gene mRNA expression, ovarian development and lipid content. (A) Confirmation of *cry2* mRNA knockdown and the effects of *cry2* dsRNA on non-target circadian clock gene expression. Each bar represents the whole-body relative mRNA expression from mosquitoes that were collected at lights off 2 days after dsRNA injection. One week after dsRNA injection, the effects of *cry2* dsRNA on (B) average egg follicle length of 15 females and (C) average lipid content in 5 females that were reared under long (16 h:8 h L:D) or short day conditions (8 h:16 h L:D) at 18°C were measured. Effects of *cry2* dsRNA were compared with those observed in *β-gal* dsRNA-injected (control) mosquitoes. Females were considered to be in diapause (percentage shown above bars) if the average egg follicle length was <80 μm and their lipid content was >30 μg mg^{-1} mosquito. Bars represent s.e.m.; **P*<0.05, ***P*<0.01, ****P*<0.001 (Student's *t*-test).

observations are consistent with previous experiments demonstrating that exposure to long day lengths promotes diapause termination in female *C. pipiens* (Tate and Vincent, 1936; Sanburg and Larsen, 1973), a response that would thus require a functional time-keeping mechanism to distinguish the seasonal transition to long day lengths. Diapausing adult mosquitoes also remain somewhat active (Denlinger and Armbruster, 2014), thus further underscoring the need for a clock that continues to function during diapause. Our observations of *per*, *tim*, *cyc* and *cry2* oscillations throughout the diapause program support the circadian basis for diapause initiation and maintenance in *C. pipiens* and suggest that the rhythmic oscillations of these transcripts may be involved in continually measuring night length throughout diapause.

In contrast, *Clk* and *cry1* did not show significant changes in daily expression in the brains of mosquitoes at any of the stages we examined, a result that is consistent with the pattern of *Clk* and *cry1* expression in the heads of the Southern house mosquito, *C. quinquefasciatus* (Gentile et al., 2009). Although we did not measure the expression profiles of any of the clock genes in constant darkness, the expression that Gentile et al. report for non-diapausing

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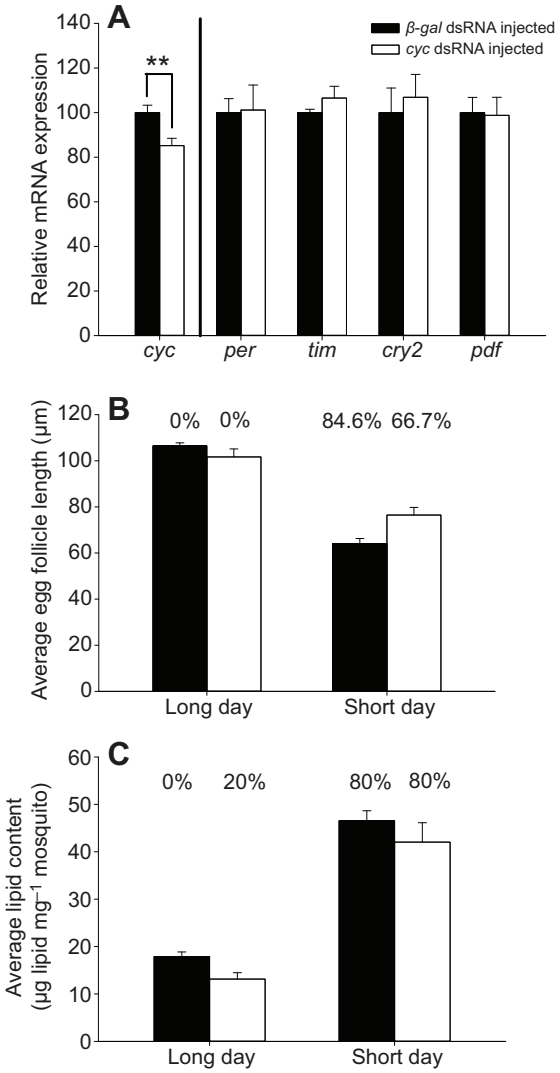

Fig. 5. Effects of *cyc* dsRNA injection on clock gene mRNA expression, ovarian development and lipid content. (A) Confirmation of *cyc* mRNA knockdown and the effects of *cyc* dsRNA on non-target circadian clock gene expression. Each bar represents the whole-body relative mRNA expression from mosquitoes that were collected at lights off 2 days after dsRNA injection. One week after dsRNA injection, the effects of *cyc* dsRNA on (B) average egg follicle length of 15 females and (C) average lipid content in 5 females that were reared under long (16 h:8 h L:D) or short day conditions (8 h:16 h L:D) at 18°C were measured. Effects of *cyc* dsRNA were compared with those observed in *β-gal* dsRNA-injected (control) mosquitoes. Females were considered to be in diapause (percentage shown above bars) if the average egg follicle length was <80 μm and their lipid content was >30 μg mg^{-1} mosquito. Bars represent s.e.m.; ***P*<0.01 (Student's *t*-test).

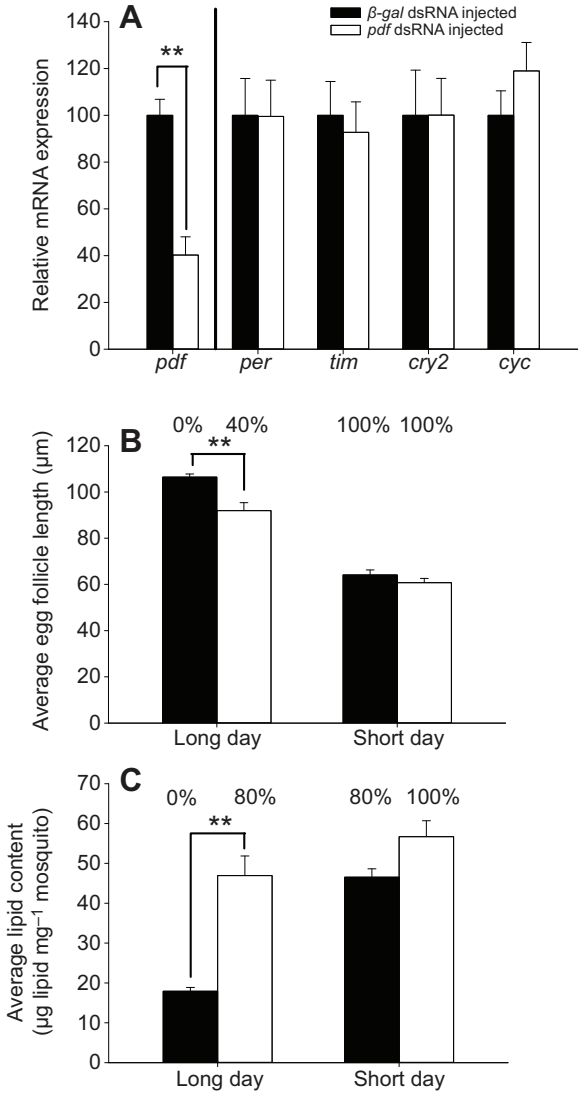

Fig. 6. Effects of *pigment dispersing factor* (*pdf*) dsRNA injection on clock gene mRNA expression, ovarian development and lipid content. (A) Confirmation of *pdf* mRNA knockdown and the effects of *pdf* dsRNA on non-target circadian clock gene expression. Each bar represents the whole-body relative mRNA expression from mosquitoes that were collected at lights off 2 days after dsRNA injection. One week after dsRNA injection, the effects of *pdf* dsRNA on (B) average egg follicle length of 15 females and (C) average lipid content in 5 females that were reared under long (16 h:8 h L:D) or short day conditions (8 h:16 h L:D) at 18°C were measured. Effects of *pdf* dsRNA were compared with those observed in *β-gal* dsRNA-injected (control) mosquitoes. Females were considered to be in diapause (percentage shown above bars) if the average egg follicle length was <80 μm and their lipid content was >30 μg mg^{-1} mosquito. Bars represent s.e.m.; **P*<0.05, ***P*<0.01 (Student's *t*-test).

C. quinquefasciatus in L:D conditions was unchanged in the absence of light. Furthermore, the expression of clock genes in non-diapausing *C. pipiens* corresponded to those reported in *C. quinquefasciatus* (Gentile et al., 2009). Therefore, we anticipate that the clock gene oscillations we observed are circadian in nature and would persist in constant darkness.

Expression profiles of *per*, *tim* and *cry2* were altered early in diapause, such that peak expression occurred later than expected in scotophase (ZT16), but 3 month old diapausing *C. pipiens* showed the expected circadian profile of all three genes, with peak expression occurring at ZT8. Bowen (Bowen, 1992) examined the circadian feeding behavior of diapausing and non-diapausing *C. pipiens* and found that while non-diapausing females fed on sugar only in the early scotophase, recently emerged diapausing females continued to gorge on sugar throughout the night. This is likely because early in diapause, females of *C. pipiens* need to accumulate additional lipid reserves to survive the winter. This trend persisted for at least 2 weeks in diapausing *C. pipiens*, but 33 day old females stopped actively feeding on sugar sources. While we are not certain why *per*, *tim* and *cry2* peaked later at night in 1 week and 1 month

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old diapausing *C. pipiens*, we suspect that the altered expression profile of these genes may reflect the shift in feeding activity observed by Bowen (Bowen, 1992) early in diapause. As these clock genes show the expected circadian profile later in diapause, with peak expression occurring at lights off, we predict that this too would drive late diapausing *C. pipiens* to limit locomotor and feeding activity to the early night. Clock genes are clearly involved in regulating mosquito feeding behavior (Das and Dimopoulos, 2008): RNAi directed against *tim*, *Clk* and *cry1* in *A. gambiae* caused a significant increase in the propensity of females to blood feed. It is thus likely that the different expression profiles of *per*, *tim* and *cry2* we observed in early and late diapausing female *C. pipiens* could similarly influence feeding behavior and other circadian activities.

A surprising result from our study is that *cry2* showed a reversed expression profile in post-diapause *C. pipiens*, with peak expression occurring at lights on rather than at lights off. A complete reversal of clock gene expression has not been documented, and we are uncertain why this occurred. However, Gentile et al. (Gentile et al., 2009) observed that while *cry2* expression peaked at lights off in *C. quinquefasciatus*, *cry2* expression peaked at both lights on and lights off in *A. aegypti*. This dual peak in *cry2* expression in *A. aegypti* likely explains their crepuscular circadian activity; these mosquitoes are active at both dawn and dusk while *C. quinquefasciatus* are active primarily at dusk. The daily activity of post-diapause *C. pipiens* mosquitoes has not been investigated, and therefore it is possible that these mosquitoes may shift their circadian behavior and perhaps become more active at dawn or earlier in the day. An alternative possibility is that *cry2* may be somehow involved in diapause termination. Further experiments on both the circadian activity and the role of *cry2* in *C. pipiens* following diapause are needed to resolve this issue.

Consistent with the results of Ikeno et al. (Ikeno et al., 2010; Ikeno et al., 2011a; Ikeno et al., 2011b) for *R. pedestris*, RNAi against the negative circadian regulators *per* and *cry2*, as well as *tim* in our *C. pipiens* experiments, stimulated ovarian development in short day-reared *C. pipiens*, suggesting that knock down of these transcripts prevents diapause induction in *C. pipiens*. However, as both *tim* and *cry2* dsRNA non-specifically suppressed *per* mRNA expression, it is unclear whether knock down of *tim* and *cry2* mRNA prompted egg follicle development directly in short day-reared *C. pipiens* or whether this phenotype was the result of knocking down *per*. We are uncertain why *tim* and *cry2* dsRNA also suppressed *per* mRNA expression as the dsRNA we designed did not BLAST to *per* transcripts in *C. quinquefasciatus* nor did it show significant sequence identity with the *per* cDNA sequences we obtained from *C. pipiens*. Possibly, *tim* and *cry2* transcripts or proteins also promote *per* mRNA transcription in *C. pipiens*. Indeed, Suri et al. (Suri et al., 1999) found that TIM acts post-transcriptionally to increase levels of *per* mRNA in *D. melanogaster*, and this may also be true for other insects. Positive regulation of *per* by *cry2* has not been reported in any insect, but should be investigated. Regardless of the method by which *tim* and *cry2* transcripts or their proteins affect *per* mRNA levels in *C. pipiens*, our results highlight the importance of measuring non-target effects in RNAi experiments. Such non-target effects of RNAi have been documented previously (Ma et al., 2006) and are likely more widespread than generally realized.

While it is difficult to determine whether *tim* or *cry2* RNAi, or the non-target effect of *per* mRNA knock down is responsible for the increased egg follicle size in short day-reared *tim* and *cry2* dsRNA-injected mosquitoes, the lipid results are more clear. Only *per* dsRNA prevented short day-reared mosquitoes from accumulating high lipid reserves, which suggests that *per* may play a more important role in diapause initiation in *C. pipiens* than either *tim* or *cry2*. It is surprising, though, that *tim* and *cry2* dsRNA-injected females that were reared under short-day conditions had non-diapause-like egg follicles, yet had diapause-like lipid stores as typically these two aspects of the diapause phenotype are in accord. The incongruence between the egg follicle and lipid results in *tim* and *cry2* dsRNA-injected females suggests that all of these genes are important for diapause initiation, but that distinct features of the diapause phenotype may be invoked by different gene pathways. A similar mechanism has been observed in *R. pedestris*, where egg follicle development is governed by the corpora allata but lipid accumulation and cuticle rigidity are controlled by an independent pathway (Morita et al., 1999).

We had limited success in knocking down *cyc* mRNA expression using RNAi; 3 µg of *cyc* dsRNA only reduced *cyc* mRNA to 85% of the level in *β-gal* dsRNA-injected controls. Although this was a statistically significant reduction in mRNA expression, it is unclear whether this level of knock down was biologically significant, especially as there was no effect on egg follicle length or lipid content. A higher dose of dsRNA (6 µg) was ineffective in further reducing *cyc* mRNA expression or in altering the diapause phenotype (data not shown).

Suppressing *pdf* mRNA in *C. pipiens* caused an effect similar to knocking down *cyc* or *Clk* in *R. pedestris*: namely, dsRNA-treated insects failed to develop their reproductive organs even under long day, diapause-averting conditions (Ikeno et al., 2010; Ikeno et al., 2011b; Ikeno et al., 2013). Additionally, RNAi against *pdf* caused long day-reared *C. pipiens* to accumulate greater lipid stores, further suggesting that *pdf* dsRNA-injected females entered a diapause-like state under long day, diapause-averting conditions.

In a recent study, Ikeno et al. (Ikeno et al., 2014) investigated the role of *pdf* on diapause initiation in *R. pedestris*. Surprisingly, *pdf* mRNA did not affect diapause initiation in bean bugs as knocking down *pdf* with RNAi did not affect the reproductive status of long day- or short day-reared bugs. However, ablation of PDF-positive neurons stimulated ovarian development in bugs that were reared under short day, diapause-inducing conditions. Shiga and Numata (Shiga and Numata, 2009) also ablated PDF-positive neurons in the blow fly *P. terraenovea* and demonstrated that flies were no longer able to measure photoperiod: 48% of long day-reared and 55% of short day-reared flies entered an adult reproductive diapause. Therefore, there is mounting evidence that PDF and/or its transcript is involved in diapause initiation in several insect species, but apparently PDF plays different roles. For example, our results suggest that *pdf* mRNA or its protein is involved in averting diapause in *C. pipiens*, as suppressing *pdf* mRNA causes long day-reared *C. pipiens* to enter a diapause-like state. In contrast, PDF neurons appear to promote diapause entry in *R. pedestris*, while PDF neurons appear to be involved in interpreting day length in *P. terraenovea*.

How *pdf* mRNA, protein or other components of PDF-positive neurons are mediating their effects on adult reproductive diapause requires further investigation. Adult reproductive diapause in *C. pipiens* is characterized by a shut-down in production of insulin-like peptide 1 (Sim and Denlinger, 2013) and the failure of the corpora allata to produce JH (Denlinger, 2002). Recently, Kang et al. (Kang et al., 2014) examined the role of allatotropin, the neuropeptide hormone that stimulates the corpora allata to produce JH, and demonstrated that knocking down *allatotropin* mRNA caused long day-reared *C. pipiens* to enter a diapause-like state with undeveloped

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egg follicles and high lipid content. As knocking down *pdf* mRNA produced results identical to those found by Kang et al., our results present the exciting possibility that *pdf* mRNA or protein may control the release of insulin-like peptide or allatotropin in *C. pipiens*, and thereby regulate diapause. Characterizing the molecular interactions between *pdf*, insulin signaling and allatotropin, as well as establishing neuronal connections between circadian clock cells, including PDF-positive neurons, and regions of the *C. pipiens* brain that produce insulin-like peptides and allatotropin, are essential for understanding how this important disease vector is able to use its circadian clock to interpret day length and translate this information into the hormonal cues that initiate diapause.

MATERIALS AND METHODS

Insect rearing

The laboratory colony of *C. pipiens* (Buckeye strain) was reared at 25°C at 75% relative humidity (RH) under a 16 h: 8 h L:D photoperiod as previously described (Robich and Denlinger, 2005). Adult mosquitoes fed *ad libitum* on a 10% sucrose solution, and females were fed chicken blood using an artificial membrane system. Two hundred first instar larvae were placed into rearing containers, and upon reaching their second instar, were either placed into diapause-averting, long day conditions (16 h:8 h L:D at 18°C and 75% RH) or diapause-inducing, short day conditions (8 h:16 h L:D at 18°C at 75% RH). Diapause was broken by placing 3 month old diapausing adult females into a 16 h:8 h L:D photoperiod at 25°C for 2 weeks (post-diapause mosquitoes).

Clock gene cDNA cloning and sequencing

Total RNA was isolated from whole bodies of non-diapausing females of *C. pipiens* using Trizol (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's recommendations. cDNA was synthesized from 3 µg total RNA using the SuperScript III First Strand Synthesis System (Life Technologies). Initial fragments of clock genes were obtained using primers designed from published clock gene sequences in the Southern house mosquito, *C. quinquefasciatus*, as well as other mosquitoes (*A. aegypti* and *A. gambiae*) and Diptera. After obtaining short fragments of the clock genes from *C. pipiens*, 5′ and 3′ RACE reactions were performed according to the protocols described in Scotto-Lavino et al. (Scotto-Lavino et al., 2007a; Scotto-Lavino et al., 2007b). Gene fragments were cloned into plasmids using the TOPO TA Cloning Kit (Life Technologies) and transfected into competent *E. coli* cells. Plasmids were purified from *E. coli* using the QIAprep Miniprep kit (Qiagen, Valencia, CA, USA), and were sequenced at The Ohio State University Plant Microbe Genomics Facility (Columbus, OH, USA).

Measurement of brain transcript levels of circadian clock genes using qRT-PCR

To compare transcript levels of the circadian clock genes in brains of non-diapausing and diapausing mosquitoes, *C. pipiens* were collected every 8 h over a 24 h period at various developmental times (1 week non-diapausing, 1 week diapausing, 1 month diapausing, 3 months diapausing and post-diapause). Female mosquitoes were frozen, decapitated, and their frozen brains were dissected in ethanol on dry ice. Total RNA was isolated from 3–4 groups of 10–20 brains at each time point using Trizol (Invitrogen, CA, USA), with a slightly modified protocol because of low sample volume. Briefly, brains were homogenized in 500 µl Trizol before adding 100 µl chloroform and centrifuging. The aqueous phase was extracted and 30 µl of 3 mol l^{-1} sodium acetate and 2 µl Glycoblue (Life Technologies) were added to facilitate RNA precipitation in 250 µl isopropanol and to allow easier visualization of the RNA pellet following subsequent centrifugation. All other steps were according to the manufacturer's protocol. RNA quality and quantity were assessed using a Nanodrop spectrophotometer (Nanodrop Products, Wilmington, DE, USA).

For cDNA synthesis, 3 µl total RNA was added to a 20 µl reaction of the TaqMan Reverse Transcription Reagents kit (Life Technologies) according to the manufacturer's protocol, and cDNA was diluted 5-fold. Quantitative real-time PCR was performed with an iQ5 real-time PCR detection system (Bio-Rad, Hercules, CA, USA), using the method described by Larionov et al. (Larionov et al., 2005). Briefly, standard curves were generated for each transcript using 10-fold serial dilutions of purified PCR product; standard curves were run on each plate. All reactions were performed in triplicate in a total volume of 20 µl containing 10 µl SYBR green PCR Master Mix (Bio-Rad), 900 nmol each primer and 2 µl sample cDNA or diluted PCR product. Primer sequences for *per*, *tim*, *Clk*, *cyc*, *cry1*, *cry2* and the control gene *Ribosomal protein 49* (*Rp49*) were the same as those used by Gentile et al. (Gentile et al., 2009) to measure clock gene expression in the Southern house mosquito, *C. quinquefasciatus*. The forward and reverse qPCR primers for *pdf* were (5′→ 3′) CAT CGC AAT CGC AAT GCC ATC C and GCC GAT TGG TTC GCT AGC CAT, respectively.

Background-subtracted fluorescence data were exported from Bio-Rad iQ5 software. Relative log concentration (RLC) was computed as in Larionov et al. (Larionov et al., 2005) using standard curves. The RLC for technical replicates were averaged. Normalized RLC (NRLC) were computed by subtracting the RLC of the internal control gene, *Rp49*, from the RLC of the gene of interest. Normalized mRNA levels were computed by 10^{NRLC}, and were centered about 1 by dividing the average of normalized mRNA level for each combination of gene of interest and developmental time, producing relative mRNA levels. Statistically significant differences in expression were assessed across a 24 h time period and among the NRLCs of the samples using a one-way ANOVA where a *P*-value less than 0.05 was considered as evidence of significant clock gene cycling.

dsRNA preparation of circadian clock genes and injection into adult females

dsRNA for various *C. pipiens* circadian clock genes and for *β-galactosidase* were prepared using the T7 RiboMAX Express RNAi System (Promega, Madison, WI, USA), according to the manufacturer's instructions. Forward and reverse primers, without the T7 promoter, used for each gene are listed in supplementary material Table S1. Each PCR-derived fragment was sequenced and BLASTed against the *C. quinquefasciatus* genome database (http://cpipiens.vectorbase.org/Tools/BLAST/) to validate the identity of the PCR product and to confirm that the dsRNA would not likely knock down non-target genes.

On the day of adult emergence, dsRNA specific to one of the clock genes or to *β-gal* (positive control) was injected into the thorax of cold-anesthetized females of *C. pipiens* using a microinjector (Tritech Research, Los Angeles, CA, USA). Experiments with *β-gal*, *per*, *tim* and *cry2* used 3 µg dsRNA with an injection volume of 0.75 µl; experiments with *pdf* used 6 µg dsRNA in a 1 µl injection volume. RNAi experiments on *cyc* were done by injecting females with either 3 µg dsRNA in an injection volume of 0.75 µl (RNA knockdown confirmation and egg follicle length data) or 6 µg dsRNA in a 1 µl injection volume (lipid content data).

Confirming RNAi efficiency using qRT-PCR

Two days after dsRNA injection, total RNA was isolated from 10 whole-body dsRNA-treated females that were collected at lights off (ZT8 for short day-reared mosquitoes; ZT16 for long day-reared mosquitoes) using Trizol (Invitrogen). cDNA synthesis and qRT-PCR were performed as described above, except that the normalized mRNA level of individual clock genes in the *β-gal* dsRNA-injected control was normalized to 100%, and the transcript level in clock gene dsRNA-treated individuals was calculated relative to this value. Significant transcript knockdown was assessed using Student's *t*-test to compare the endogenous level of clock gene expression in clock gene dsRNA-treated and *β-gal* dsRNA-injected (control) mosquitoes.

Assessing the diapause status of dsRNA-treated mosquitoes

The diapause status of dsRNA-treated females was assessed 1 week after injection by measuring the lengths of primary egg follicles and by measuring whole-body lipid content. To measure egg follicle length, ovaries were dissected in a drop of 0.9% NaCl solution, and follicles were separated using a needle and examined at 200-fold magnification (Zeiss Axioskop, Thornwood, NY, USA). Ten follicles from 15–17 females were measured and the average egg follicle length for each female was calculated. This

allowed us to assess the diapause status of individual females; a female was considered to be in diapause if the average egg follicle length was between 50 and 80 µm. Additionally, the average egg follicle length and standard error among females within each treatment group (dsRNA-injected and rearing condition) were calculated as this allowed us to assess the statistical differences between the sizes of the egg follicles between clock gene and *β-gal* dsRNA (control) treatments using Student's *t*-test.

Lipid content from five female mosquitoes per dsRNA treatment and rearing condition were measured as described elsewhere (Van Handel, 1985) with slight modifications that enabled rapid measurement of lipid content in multiple samples using a plate reader (Meuti et al., 2015). Females were individually assessed as being in a diapause state if they had a lipid content >30 µg mg^{-1} fresh mass. Additionally, the average lipid content among treatment groups was calculated and again Student's *t*-test was used to assess statistical differences in lipid content between females that had been treated with clock gene or *β-gal* (control) dsRNA.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
M.E.M. and D.L.D. designed the research and wrote the paper. M.S. obtained preliminary sequences of *pdf* and designed the RNAi primers and performed preliminary knock down experiments of *pdf*. T.I. designed the RNAi primers for *per* and *cyc* and performed preliminary knock down experiments on these genes. All of the other research was performed and analyzed by M.E.M. All authors read and approved the final manuscript.

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Supplementary material
Supplementary material available online at
http://jeb.biologists.org/lookup/suppl/doi:10.1242/jeb.113233/-/DC1

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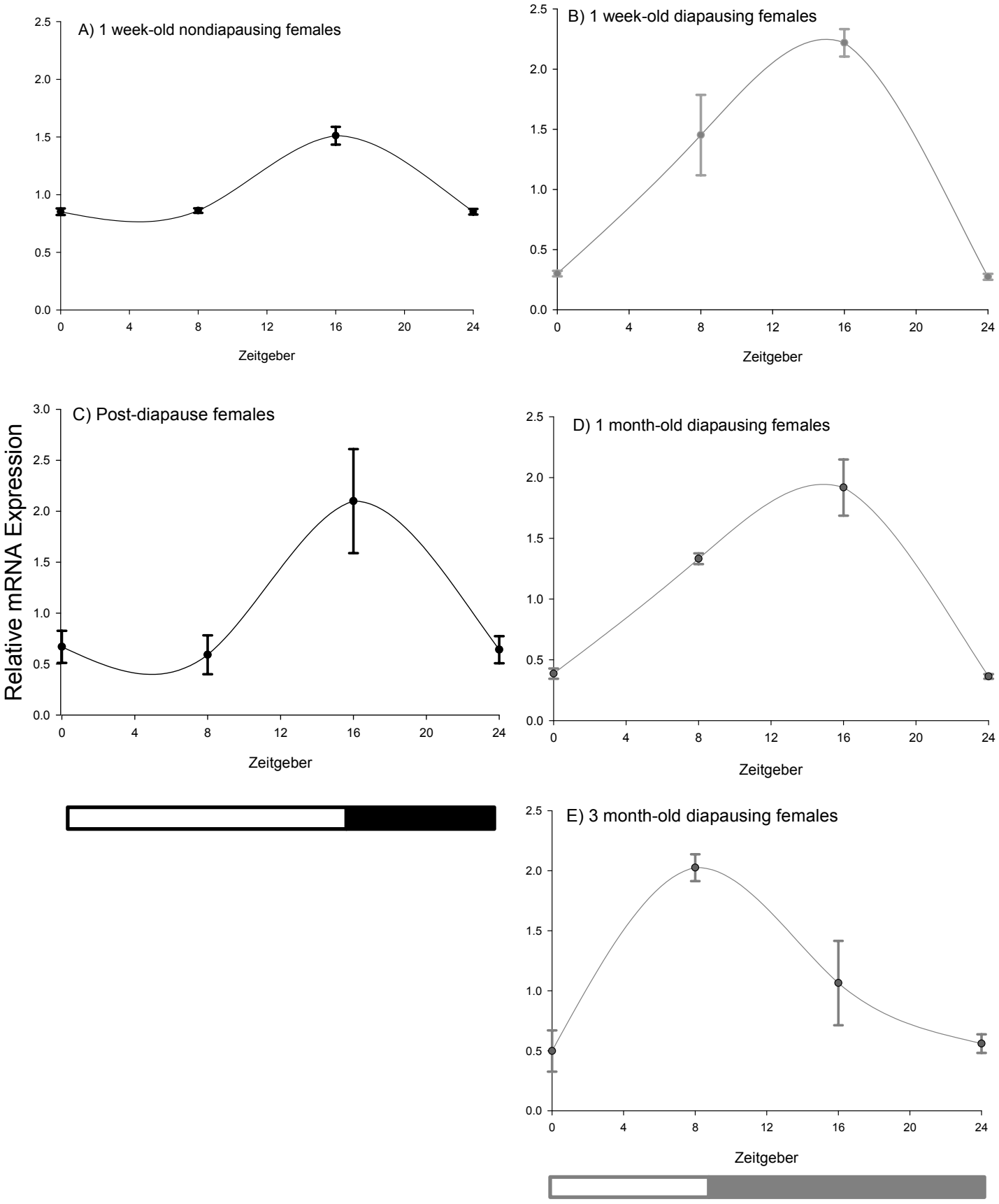

Fig. S1. Expression of the clock gene *period* in non-diapausing, diapausing and post-diapause females is shown with standard error bars. The data are an expanded version of those shown in Fig. 1, without error bars.

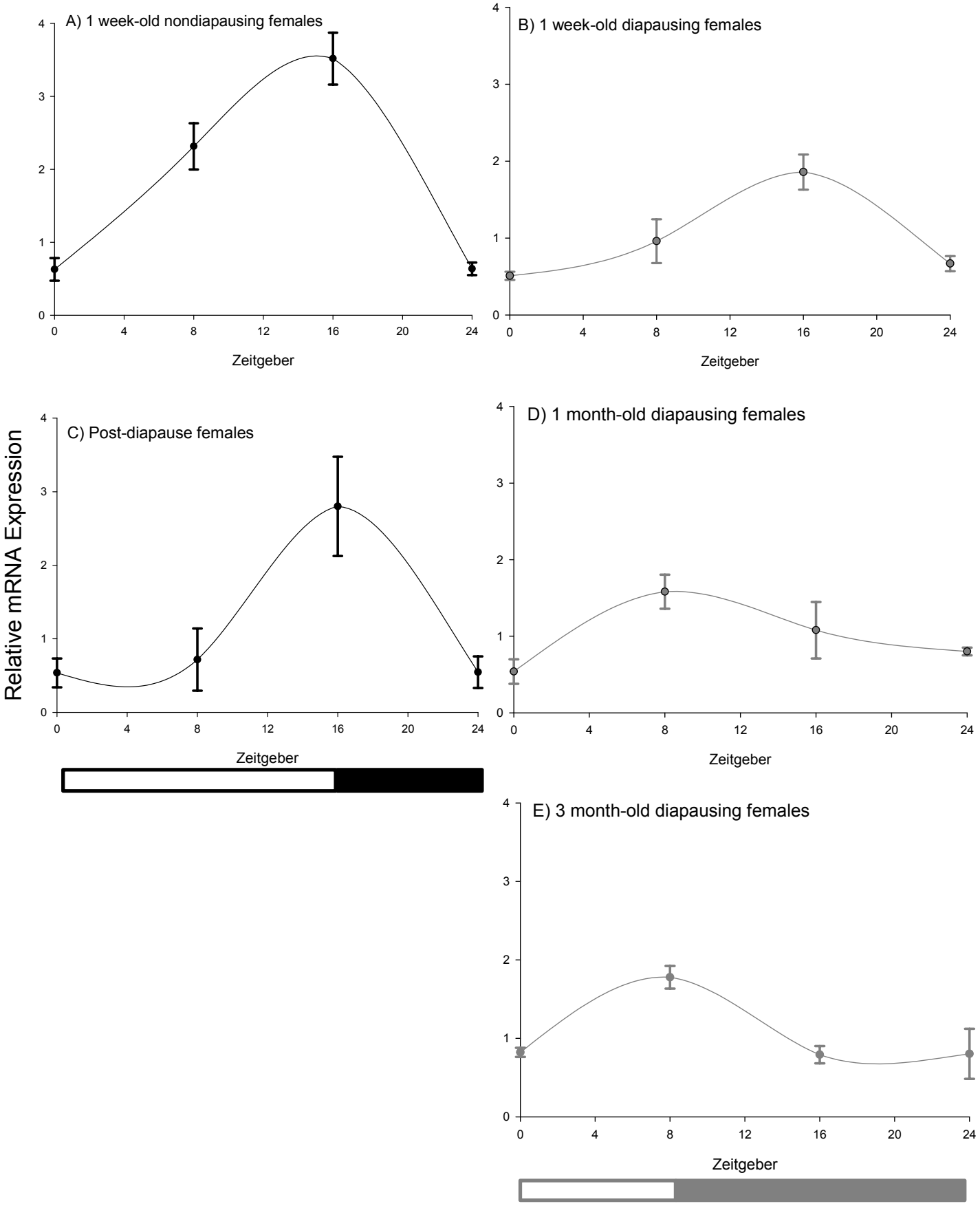

Fig. S2. Expression of the clock gene *timeless* in non-diapausing, diapausing and post-diapause females is shown with standard error bars. The data are an expanded version of those shown in Fig. 1, without error bars.

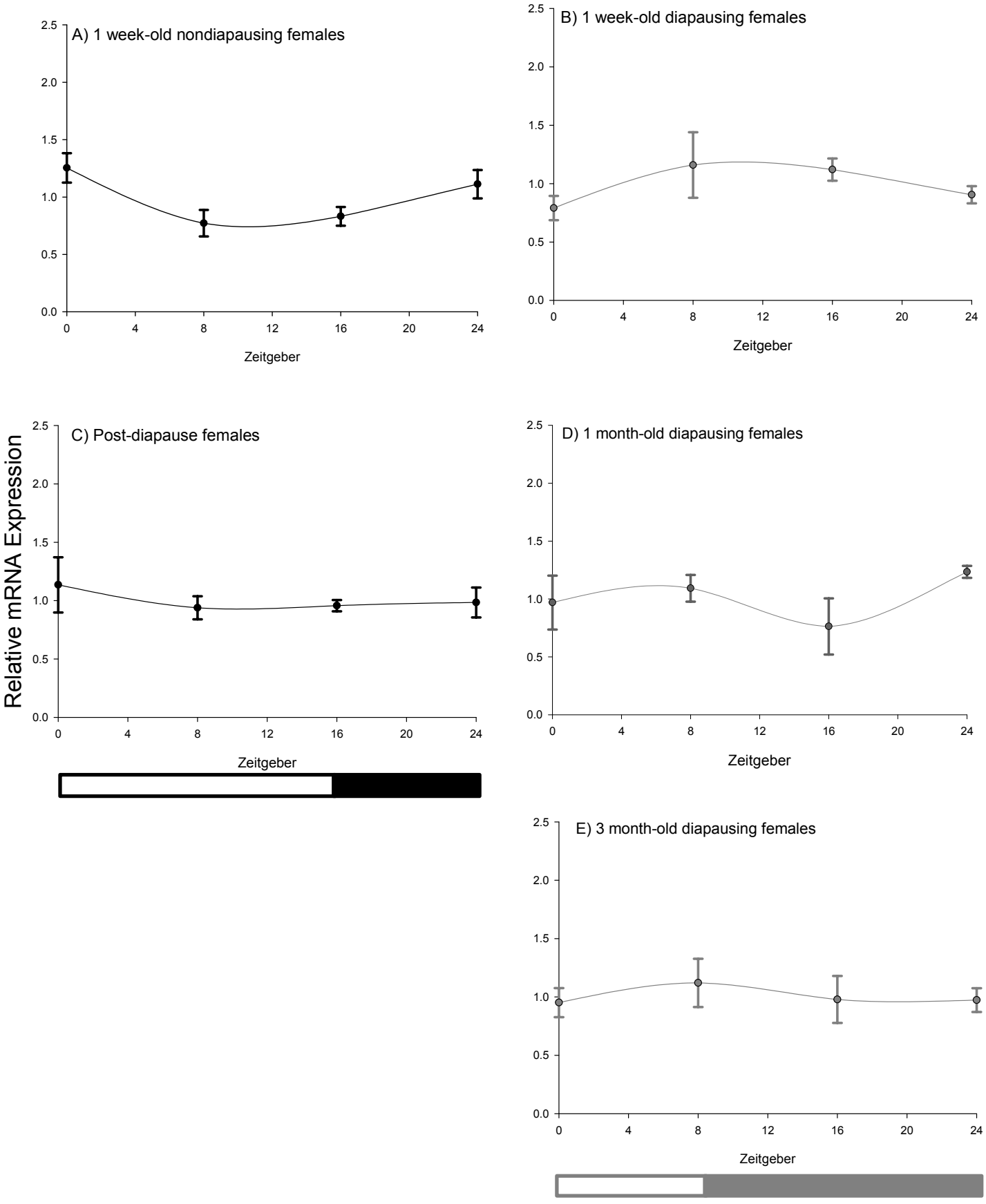

Fig. S3. Expression of the clock gene *Clock* in non-diapausing, diapausing and post-diapause females is shown with standard error bars. The data are an expanded version of those shown in Fig. 1, without error bars.

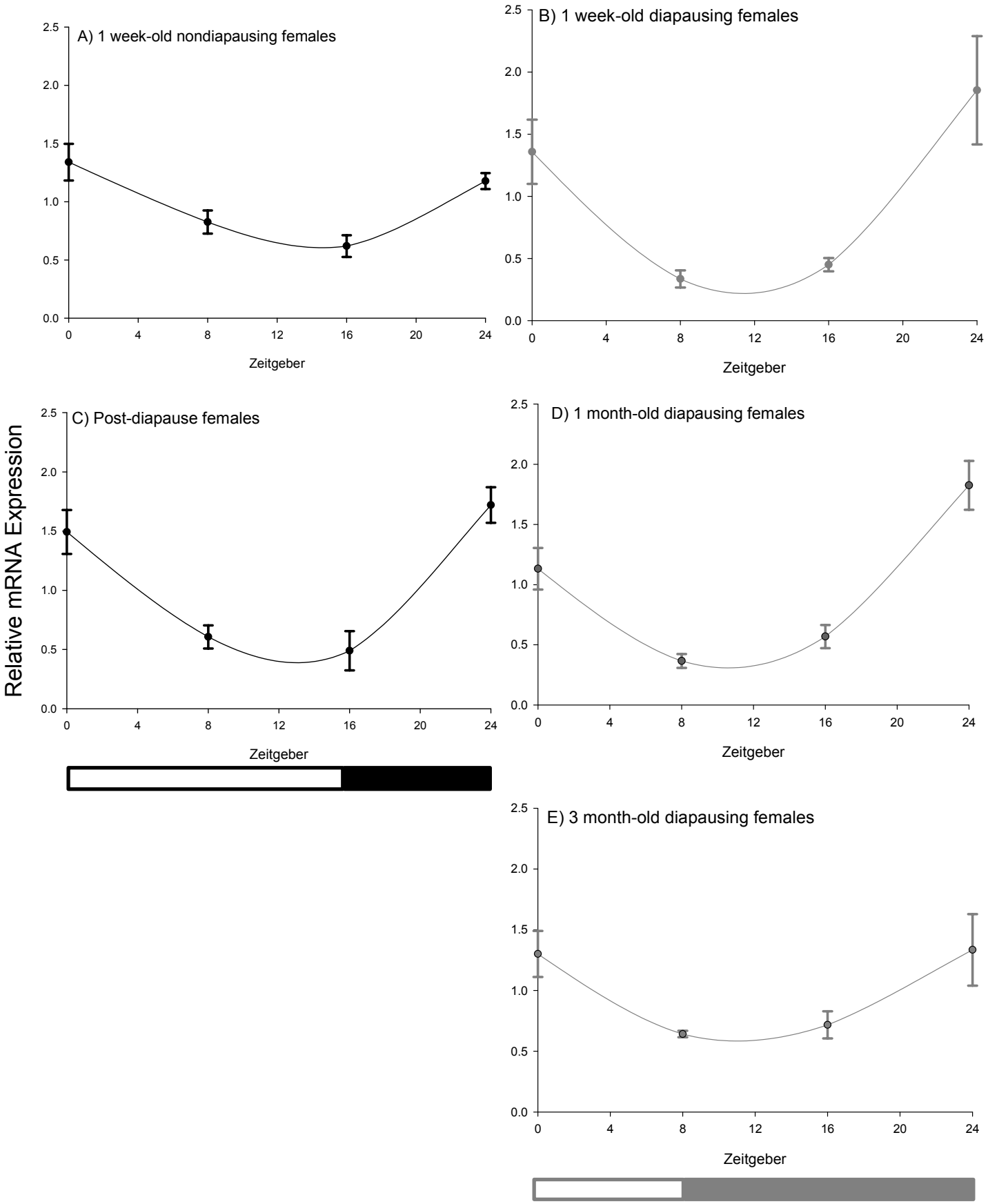

Fig. S4. Expression of the clock gene *cycle* in non-diapausing, diapausing and post-diapause females is shown with standard error bars. The data are an expanded version of those shown in Fig. 1, without error bars.

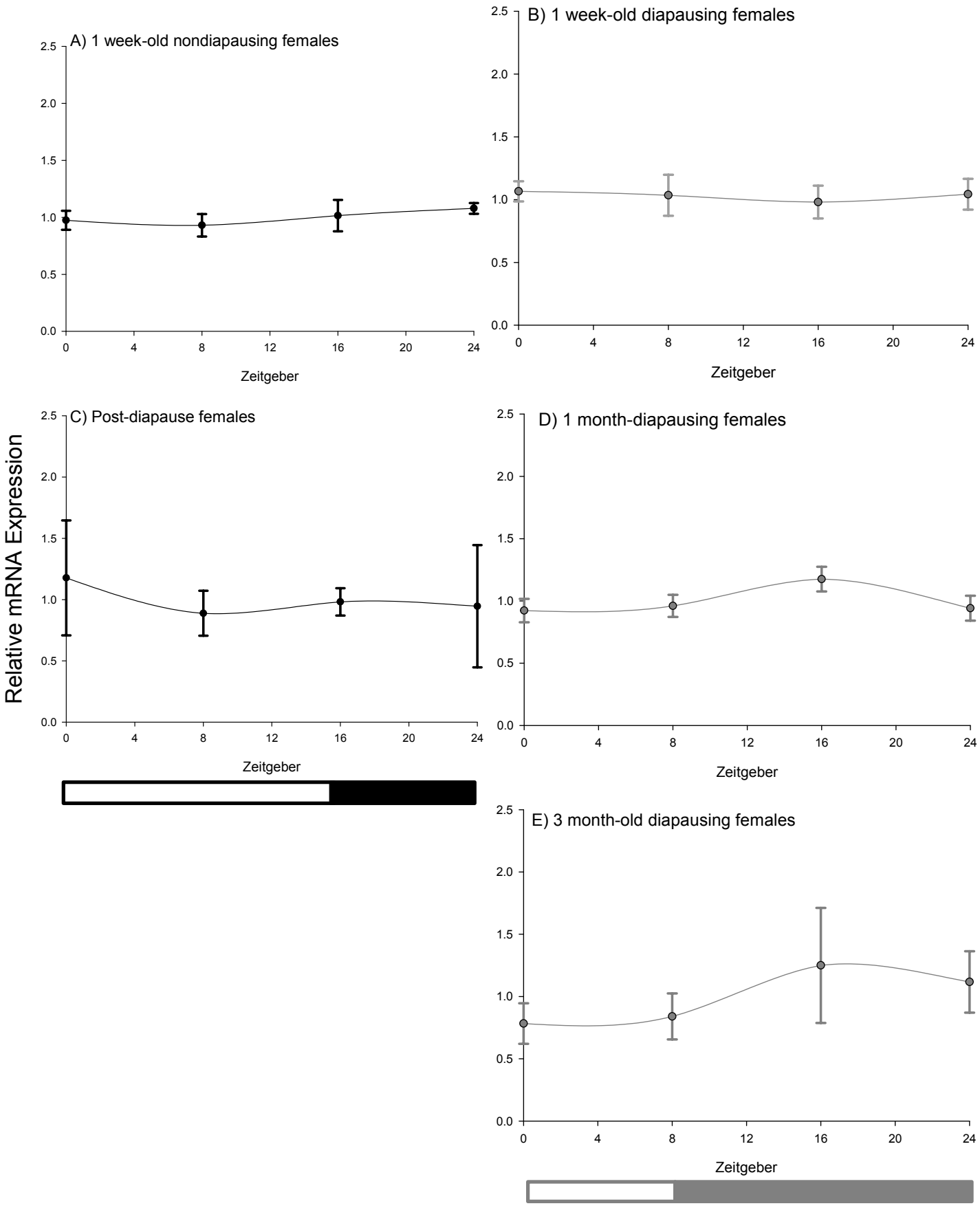

Fig. S5. Expression of the clock gene *cryptochrome1* in non-diapausing, diapausing and post-diapause females is shown with standard error bars. The data are an expanded version of those shown in Fig. 1, without error bars.

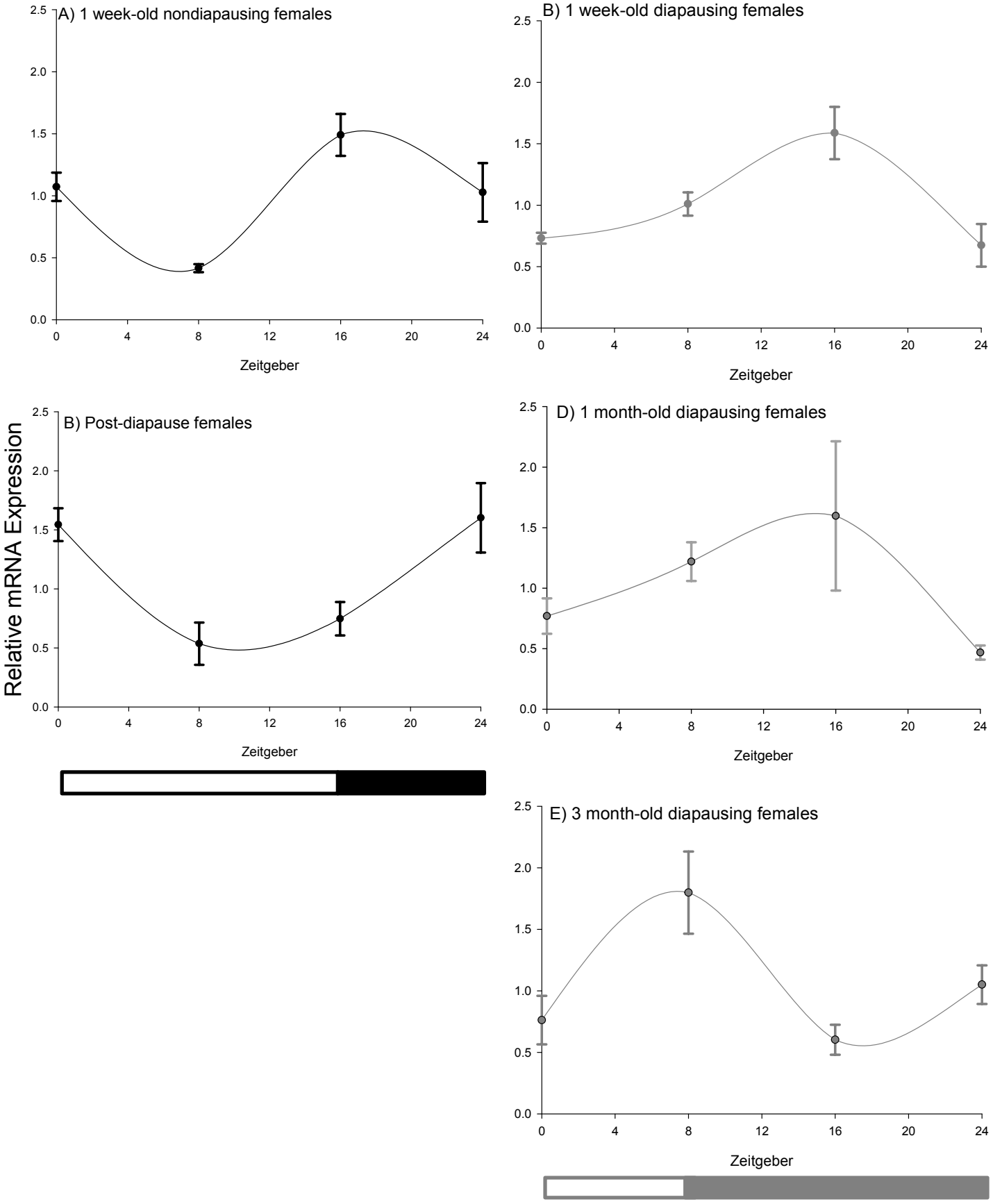

Fig. S6. Expression of the clock gene *cryptochrome2* in non-diapausing, diapausing and post-diapause females is shown with standard error bars. The data are an expanded version of those shown in Fig. 1, without error bars.

Supplementary Table 1: Primers used for dsRNA synthesis, without the T7 promoter.

Sequence name	Gene amplified	Sequence (5' → 3')
Cp.RNAi.per.Fw	*period*	CGG ACG TGT TTG CAT TGC ACT T
Cp.RNAi.per.Rev	*period*	AAT TTC CCG CACTGT CAC CAC T
Cp.RNAi.tim.Fw	*timeless*	AGC CCG CTA ACG GCT GAC G
Cp.RNAi.tim.Rev	*timeless*	GCA ACC AAA CAA CCA GCT TCC C
Cp.RNAi.*cry2*.Fw	*cryptochrome2*	CCG TAC CTC CGG TTC GGA ATG
Cp.RNAi.*Cry2*.Rev	*cryptochrome2*	ACC GGC AAA TAC CGC CG
Cp.RNAi.Cyc.Fw	*cycle*	ATG AAC ACG TAC ATC ACG GAG C
Cp.RNAi.Cyc.Rev	*cycle*	TTC GGG TGC AGA ATG TCG AA
Cp.RNAi.pdf.Fw	*pdf*	TGC TTC TAC CCG AGC TAC CAG A
Cp.RNAi.pdf.Rev	*pdf*	AGC AGT GGT ATC AAC GCA GAG T
Cp.RNAi.Bgal.Fw	*β-galactosidase*	TGT GGA ATT GTG AGC GGA TA
Cp.RNAi.Bgal.Rev	*β-galactosidase*	TAA AAC GAC GGC CAG TGA AT