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Adaptive Differences in Circadian Clock Gene Expression Patterns and Photoperiodic Diapause Induction in *Nasonia vitripennis*

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ABSTRACT: Day length (photoperiod) and temperature oscillate daily and seasonally and are important cues for season-dependent behavior. Larval diapause of the parasitoid *Nasonia vitripennis* is maternally induced following a certain number of days (switch point) of a given critical photoperiod (CPP). Both the switch point and the CPP follow a latitudinal cline in European *N. vitripennis* populations. We previously showed that allelic frequencies of the clock gene *period* correlate with this diapause induction cline. Here we report that circadian expression of four clock genes—*period* (*per*), *cryptochrome-2* (*cry-2*), *clock* (*clk*), and *cycle* (*cyc*)—oscillates as a function of photoperiod and latitude of origin in wasps from populations from the extremes of the cline. Expression amplitudes are lower in northern wasps, indicating a weaker, more plastic clock. Northern wasps also have a later onset of activity and longer free-running rhythms under constant conditions. RNA interference of *per* caused speeding up of the circadian clock, changed the expression of other clock genes, and delayed diapause in both southern and northern wasps. These results point toward adaptive latitudinal clock gene expression differences and to a key role of *per* in the timing of photoperiodic diapause induction of *N. vitripennis*.

Keywords: parasitoid wasp, photoperiodism, circadian clock, seasonal adaptation, latitudinal effect, RNA interference (RNAi).

Introduction

All organisms possess an internal circadian clock that runs with a period close to 24 h and modulates a variety of rhythmic behaviors, including rest, activity, mating, and feeding (Saunders et al. 2002). The clock consists of a set of transcription factors that either activate or inhibit target genes but that can also regulate their own expression through feedback loops. This constitutes an internal oscillation of gene expression that is tuned every day (entrained) by the prevailing oscillation of light-dark (LD) cycles and in turn regulates daily responses. In the fruit fly *Drosophila melanogaster*, the genes *period* (*per*) and *timeless* (*tim*) are negative regulators that also inhibit their own expression, whereas *clock* (*clk*) and *cycle* (*cyc*) are positive regulators that activate the expression of *per* and *tim* (reviewed by Peschel and Helfrich-Förster 2011). The gene *cryptochrome-1* (*cry-1*) represents the photoreceptor that transduces the light information into the core mechanism and induces the daily light-dependent degradation of *tim* every day. Not all insect species, however, possess orthologues of *cry-1* and *tim*. In such species, *cryptochrome-2* (*cry-2*) has been identified to replace *tim*, as in the mosquito *Anopheles gambiae* and some hymenoptera insects (Zhu et al. 2005; Rubin et al. 2006; Bertossa et al. 2014), but a substitute for the role of the transducing photoreceptor *cry-1* has not yet been found.

It has been hypothesized that adaptive evolution to seasonal changes in temperate climates involves the genetic mechanism of circadian timekeeping, since both involve timing of day and night length (Bünning 1960). As the duration of the circadian light period (photoperiod) can function as a reliable cue for upcoming seasonal environmental change, behaviors such as migration in birds, hibernation in mammals, and diapause in insects are triggered by photoperiodic changes (reviewed in Bradshaw and Holzapfel 2010). Two prominent models have been proposed regarding the regulation of photoperiodic responses by the internal clock (reviewed by Koštál 2011). The external coincidence model assumes the presence of one photosensitive internal oscillator, of which the phase is set by the outside light cycle. When the photosensitive phase of this cycle starts to fall in the dark, owing to the shortening of day length, a photoperiodic response is triggered (Bünning 1960). The other model is the internal coincidence model that assumes the presence of two circadian oscillators, of which the phase synchrony is determined by photoperiod. Changes in

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day length modify the phase synchrony, allowing the sensing of seasonal LD changes (Pittendrigh 1972). Despite extensive research into the role of the circadian clock in seasonal responses (Ikeno et al. 2010, 2011*a*, 2011*b*, 2011*c*; Meuti et al. 2015), its role in photoperiodism is still unresolved (Emerson et al. 2009). In particular, it is unclear whether the clock as a biological system (modular pleiotropy) or merely some of its genes (genetic pleiotropy) are involved in the seasonal photoperiodic response. This is an important evolutionary issue, as it is essential to know whether evolutionary constraints apply to the clock as a whole or to individual clock genes if we are to understand seasonal photoperiodic adaptation. Moreover, the degree to which the genetic architecture of the circadian and seasonal clock overlap determines how efficient natural selection can lead to adaptation and whether selection on one type of rhythm may result in correlated responses in the other. It is, therefore, important to investigate both the individual and the concerted effects of clock gene variation and possible associated patterns of selection in species that exhibit adaptive seasonal photoperiodic behaviors.

Insect diapause induction is a photoperiodic response, governed by both the number (counter) and the length (timer) of consecutive daylight periods (Saunders 2013). It has evolved in many insects as a form of developmental or reproductive arrest (dormancy) that allows them to survive unfavorable environmental conditions, such as low temperatures in winter. Shortening of day length is the most reliable cue to indicate oncoming winter conditions, although other cues may also be used, such as decreasing temperature and food supply (Saunders 2013). It is, however, not well understood how the photoperiodic changes are detected and processed to induce proper seasonal behavior. In any case, the mechanism must involve a means to both time and count photoperiods, store and process the information, and trigger the downstream diapause response (Denlinger 2002; Koštál 2011).

The jewel wasp *Nasonia vitripennis* has a strong seasonal response for maternal induction of diapause, a physiological state of dormancy in which development is arrested at the fourth larval instar. The sensitive phase is in the adult *Nasonia* female that senses the photoperiodic change and processes this information to start producing diapause offspring. This mechanism includes a timer to measure the duration of the light period and a counter to count the number of such LD cycles. The photoperiod at which 50% of the females induce larval diapause after a given number of LD days is called the critical photoperiod (CPP; timer), whereas the number of days at a given photoperiod that are required for inducing larval diapause is called the switch point (counter; Saunders 2010, 2013). *Nasonia* has thus a mechanism for the timing and counting of the LD cycles to trigger the photoperiodic response (reviewed by Saunders 2013), but the molecular basis of this mechanism remains unknown. Saunders (1974) used Nanda-Hamner resonance experiments (Nanda and Hamner 1958) in *N. vitripennis* to explore the role of the circadian clock in diapause induction with an internal coincidence model. In such experiments, animals are expected to show short-day responses when the total period of the LD cycle equals a multiple of 24 h and long-day responses when total period of the LD cycle differs from 24 h. However, the interpretation of these experiments assumes a circadian basis because the short-day response occurs at LD periodicities of 24, 48, and 72 h and does not consider other factors, such as light sensitivity or noncircadian features (Emerson et al. 2009). At present, it is not clear whether a positive Nanda-Hamner response is proof for the involvement of a sustained circadian oscillator in photoperiodic time measurement. It is therefore necessary to find more evidence for a connection between the *Nasonia* circadian clock and its photoperiodic response.

Indicative of adaptive evolution of *Nasonia* diapause induction is the finding of Paolucci et al. (2013, 2016), who reported natural clinal variation in photoperiodic diapause in Europe; populations at northern latitudes show an earlier switch point (counter), require longer CPPs (timer), and produce higher proportions of diapausing individuals than southern populations (Paolucci et al. 2013). Interestingly, this response correlated positively with allelic variation of the circadian clock gene *per* (Paolucci et al. 2016). Studies that investigated the geographical variation in the circadian response of *N. vitripennis* reported differences between (Bertossa et al. 2013) and within (Dalla Benetta 2018) *Nasonia* populations in activity timing and free-running rhythms. Particularly interesting is the fact that southern wasps exhibit an earlier phase of activity and a faster circadian rhythm than northern wasps (Dalla Benetta 2018). Bertossa et al. (2014) showed that *per* and *cry-2* mRNA levels oscillate with a 24-h cycle depending on applied LD conditions. Mukai and Goto (2016) provided evidence that *per* is essential for a proper photoperiodic response in *Nasonia*. These results and the observed clinal correlation of *per* gene haplotype frequencies and photoperiodic diapause induction (Paolucci et al. 2016) suggest the involvement of at least this circadian clock gene in photoperiodic time measurement in *N. vitripennis* and as a candidate regulator of seasonal diapause induction.

Here we investigate if and in what way *per* and other clock genes are involved in photoperiodic-dependent changes in circadian rhythm and diapause induction. For the clock genes *period* (*per*), *cryptochrome-2* (*cry-2*), *clock* (*clk*), and *cycle* (*cyc*), circadian expression is analyzed as a function of photoperiod and latitude of origin. Subsequently, we investigate the functional involvement of *per* in the circadian rhythm and diapause of *N. vitripennis* by knocking down its expression via RNA interference (RNAi). By analyzing how lowered expression of *per* changes expression of other clock genes and how this affects locomotor activity and photoperiodic diapause response, we consider the potential overlap in genetic architecture of these two types of rhythms in *N. vitripennis*.

Material and Methods

Experimental Lines and Rearing Conditions

For this study, isogenic lines were established from strains collected from the field in 2009 (for details, see Paolucci et al. 2013). The southern strains originate from Corsica, France (42°22′40.80″N), and the northern strains originate from Oulu, Finland (65°3′40.16″N). Isogenic lines were established by crossing a female wasp with one of her sons, followed by seven or eight generations of brother-sister crossings. This yielded an estimated homozygosity of >99%. Note that *Nasonia*, being a haplodiploid species, does not suffer strongly from inbreeding (Thornhill 1993). The southern and northern strains are homozygous for the *per*-S and *per*-N1 alleles of Paolucci et al. (2016), respectively. Lines were maintained on *Calliphora* spp. pupae as hosts in mass culture vials under diapause-preventing conditions (i.e., long photoperiod of 18L:06D, light intensity of 60 lm/ft^2, and temperature of 20° ± 1°C).

Wasp Culturing and Entrainment

To study clock gene expression under different LD conditions, mated females were allowed to oviposit under standard conditions. Offspring developed under the same conditions (16L:08D and 20°C) until the yellow pupal stage, when the host puparia were opened and five females were isolated and stored in cotton-plugged 60 × 10-mm polystyrene tubes until emergence 7–8 days later. Five biological replicates of five wasps for each time point were prepared and incubated at 20°C either under long-day 16L:08D or short-day 08L:16D conditions. Replicates were collected every 3 h throughout a 24-h period for a total of 40 samples for each tested group (southern under long-day conditions, southern under short-day conditions, northern under long-day conditions, and northern under short-day conditions; fig. A1; figs. A1–A5 are available online). To instantly kill wasps, the tubes were submerged in liquid nitrogen and stored immediately at −80°C. For the nighttime sampling points, the procedure was performed in darkness. Virgin females in each replicate were provided with fresh hosts every other day. Parasitized hosts were transferred to a new vial and cultured at 25°C, and offspring diapause was scored for each biological replicate to determine the physiological state of the wasp.

RNA Extraction, cDNA Conversion, and Real-Time Polymerase Chain Reaction (qPCR)

RNA extraction was performed from the heads of the collected wasps (five biological replicates of five wasps for each time point). Total RNA was extracted from each pool of five wasp heads with Trizol reagent (Invitrogen) according to the manufacturer's instructions. Each sample was subjected to a DNase treatment to eliminate any DNA contamination, and approximately 1 μg of total RNA was reverse transcribed with oligo-dT and hexamer primers at a 1:6 ratio with the RevertAid H Minus First Strand cDNA Synthesis Kit (Fermentas). The cDNA was then diluted 50× before being used for qPCR. qPCR was performed with SYBR Green (Quanta Biosciences) and ROX as the internal passive reference. Four microliters of diluted cDNA was used for each reaction of 20 μL total containing primer at the final concentration of 0.2 μM and 10 μL of SYBR Green/ROX buffer solution. Three technical replicates for each reaction were performed to correct for experimental errors. For normalization of the data, *elongation factor 1α* (*ef1α*) and *adenylate kinase 3* (*ak3*) were used as reference genes after confirmation that their expression level is constant throughout the day (fig. A3). Expression levels of reference genes did not differ between southern and northern lines or between LD conditions (fig. A3). Reactions were run on an Applied Biosystems 7300 Real-Time PCR System with the following qPCR profile: 3 min of activation phase at 95°C followed by 35 cycles of 15 s at 95°C, 30 s at 56°C, and 30 s at 72°C. The primers are listed in table A1 (tables A1, A2 are available online).

Expression Data Analysis and Statistics

Expression levels relative to those of the reference genes *adenylate kinase 3* and *elongation factor 1α* were calculated by normalizing the expression data with LinRegPCR (Ramakers et al. 2003; Ruijter et al. 2009). Raw fluorescence data generated by 7300 System SDS software (Applied Biosystems) were baseline corrected using LinRegPCR. Next, a window of linearity was set and PCR efficiencies per sample were calculated. N_0 values were calculated from PCR efficiency per amplicon, the C_q value per sample, the chosen fluorescence threshold to determine the C_q, and the starting concentration per sample (Ramakers et al. 2003). Relative levels were determined by dividing N_0 values of the gene of interest by the average N_0 of the two reference genes.

Circadian rhythmicity in expression was measured for each gene, and a sinusoid curve was fit to the data with CircWave (by R. Hut, available at http://www.euclock.org). CircWave employs a forward linear harmonic regression to calculate the profile of the wave with a 24-h period. This program produces a Fourier curve that describes the data better by maximizing the number of harmonics, using *F*-testing for each added harmonic. The significance level was set at .05.

Day and night average expression levels of four groups (southern wasps under long-photoperiod conditions, southern wasps under short-photoperiod conditions, northern wasps under long-photoperiod conditions, and northern wasps under short-photoperiod conditions) were compared for each gene independently with a two-way ANOVA and Tukey HDR for the multiple-comparisons test in R statistical software (R Development Core Team 2012). Data have been deposited in the

Dryad Digital Repository (https://dx.doi.org/10.5061/dryad.bt3m1p2; Dalla Benetta et al. 2019). Code for statistics is provided in a zip file, available online.[1]

Synthesis and Injection of Double-Strand RNAs (dsRNAs)

Total RNA was extracted from the heads of wasps collected between zeitgeber time (ZT) 21–24 (ZT 0 corresponds to the time when the light is turned on) and used to synthesize cDNA as described above. PCR primer pairs NV_per_dsRNA_0708 and NV_per_dsRNA_1213 (table A2) were used to amplify two fragments of *per*. These fragments were then used as A template to generate two dsRNAs. Primer set dsRNA_A spans exons 7 and 8, and dsRNA_B spans exons 12 and 13 (more details are provided in fig. A2). At both ends of these PCR fragments, a T7 polymerase-binding site was added (primers are shown in table A2). The fragments were transcribed in both directions using the Megascript RNAi Kit (Ambion). In brief, sense and antisense RNA fragments were synthesized in separate transcription reactions. After incubation for 6 h at 37°C, the two reactions were mixed and heated at 75°C for 5 min, followed by being cooled down slowly (overnight). Exonuclease digestion removed DNA, and single-strand RNA and dsRNA was subsequently purified according to the kit protocol. Finally, the dsRNA was precipitated with ethanol and redissolved in water and stored at −20°C.

Injection of dsRNAs is used for RNAi for knocking down the expression of the clock gene *period* (*per*). Female pupae of the southern and northern lines were injected in the abdomen following the procedure of Lynch and Desplan (2006), with 4 μg/μL *per* dsRNA_A (RNAi_A) or dsRNA_B (RNAi_B) mixed with red dye. Injections were performed with Femtotips II needles (Eppendorf) under continuous injection flow. Pupae were injected at the posterior end next to the ovipositor until the abdomen turned clearly pink. Slides with injected wasp pupae were incubated in an agar/phosphate-buffered saline petri dish at 25°C at the experimental photoperiods, either 08L : 16D for subsequent use in the diapause and locomotor activity experiments or 16L : 08D for a second locomotor activity experiment. Control pupae were injected with red dye mixed with water at a 1 : 4 ratio.

RNAi Efficiency

To assess the efficiency of the RNAi reaction, control and RNAi females were kept under short-photoperiod conditions (08L : 16D) at 20°C in groups of five and provided with hosts. Three days after eclosion, three biological replicates of five wasps were collected every 4 h throughout the light phase (ZT 0, ZT 4, ZT 8). They were put into liquid nitrogen to kill them instantly and stored immediately at −80°C.

RNA was extracted from pooled head samples as described above, and cDNA conversion was performed as per the manufacturer's instructions. The cDNA was diluted 50× prior to use for qPCR. Three technical replicates for each reaction served to control for pipetting variation. Reactions were run on an Applied Biosystems 7300 with the following qPCR profile: 3 min of activation phase at 95°C followed by 35 cycles of 15 s at 95°C, 30 s at 56°C, and 30 s at 72°C. Table A1 lists the primers used.

Expression data were first analyzed with LinRegPCR (Ramakers et al. 2003; Ruijter et al. 2009) as described above. After confirmation that their relative expression level is constant between treatments (fig. A4), *ef1α* and *ak3* were used as reference genes. A generalized linear mixed model (GLMM) in which expression represents the response variable and the treatments (Control_ZT0/4/8, RNAiA_ZT0/4/8, and RNAB_ZT0/4/8) represent the independent variables was used to analyze expression levels with R statistical software (ver. 3.4.1). A quasi-Poisson distribution for the GLMM corrected for overdispersion, and *F*-tests were used to compare differences in gene expression between treatments (control vs. the two RNAi treatments) and among time points. Post hoc Tukey analyses were performed with the multcomp package glhd for effects of RNAi treatments for each gene independently within lines.

Locomotor Activity

Locomotor activity was measured for adult injected females from southern and northern lines entrained to 4 days of 08L : 16D or 16L : 8D and released either in constant darkness (DD) or constant light (LL) conditions. Temperature was kept constant at 20°C. To quantify animal movement over time, individuals were placed in small tubes (diameter, 5 mm; height, 70 mm) filled for a quarter with sugar-water gel medium. They were continuously monitored for movement by infrared beams in Trikinetics *Drosophila* activity monitors. The detector records how many times per minute each individual interrupts an infrared light beam that passes through the glass tube. The monitors were placed in separate light boxes in temperature-controlled environmental chambers with 50% humidity. The light source in the box consisted of white light with a maximum light intensity of about 200 lm/ft^2 (3.15 W/m^2). Data were collected and analyzed with DAM system 2.1.3 software.

The raw locomotor activity data were first visualized with the program ActogramJ (Schmid et al. 2011; http://actogramj.neurofly.de). Double-plot actograms obtained with this software represent activity levels. Average activity was calculated under LD conditions according to Schlichting and Helfrich-Förster (2015) to find the onset, the peak, and the offset of activity. To determine the onset and offset of activity of the

1. Code that appears in *The American Naturalist* is provided as a convenience to readers. It has not necessarily been tested as part of peer review.

average day, data per wasp have to be plotted as bar diagrams with each bar representing the cumulative activity within 20 min. The first bar time when the activity starts to rise consequently represents the onset, whereas the offset is the bar time when activity reaches the level that is stable during the night phase (Schlichting and Helfrich-Förster 2015). To determine the timing of the peaks, data are smoothed by a moving average of 30 min. Through this process, randomly occurring spikes are reduced and the real maximum of the activity can be determined. The average phase of the onset, peak, and offset, represented in ZT of 30–45 wasps per treatment (southern control wasps, southern RNAi-treated wasps, northern control wasps, and northern RNAi-treated wasps), was compared between strains and treatments (controls and RNAi-treated wasps). Statistical analysis on timing of activity was performed using one-way ANOVA with Tukey's multiple-comparisons test. Only wasps that survived the entire experimental period were analyzed.

The free-running period (τ), representing the rhythm of the endogenous clock in absence of external stimuli, was determined under constant darkness and constant light with periodogram analysis, which incorporates χ^2 analysis (Sokolove and Bushell 1978). As only rhythmic individuals were analyzed, the sample sizes ranged from 17 to 44 individuals under DD conditions and from 10 to 26 individuals under LL conditions; τ values were compared between strains and treatments with one-way ANOVA and Tukey's multiple-comparisons test in R. Data have been deposited in the Dryad Digital Repository (https://dx.doi.org/10.5061/dryad.bt3m1p2; Dalla Benetta et al. 2019).

Diapause Induction

Injected wasps were tested for diapause response under 08L : 16D conditions at 20°C to study *per* knockdown effects under diapause-inducing conditions. Following Paolucci et al. (2013), 50 adult females postinjection were kept in cotton-plugged 60 × 10-mm (height × diameter) polystyrene tubes with two hosts in a light box with a controlled LD regime and constant temperature. Females were exposed to the treatment for their entire life, and the two hosts were replaced every other day. Parasitized hosts were transferred to a new vial and cultured at 25°C and constant light to ensure standardized conditions for development of offspring for all individuals in all treatments. Females produce normal developing offspring at the beginning of their life and switch to producing diapausing larvae after exposure to a certain number of LD cycles. As diapause in *Nasonia* occurs at the fourth instar larval stage, it can easily be scored by opening the hosts after 14 days. The diapause status is calculated as described in Paolucci et al. (2013). For each female, the number of diapausing broods was scored every other day, and the proportion of diapausing broods per day and per treatment group was used to determine the diapause response curve. We never found mixed broods containing developing and diapausing offspring. The switch point represents the average day at which each wasp started to produce diapause offspring.

Survival tests were used to compare diapause response curves between strains (survival package in R; Therneau and Lumley 2013) followed by pairwise comparisons with the log-rank test (survminer package in R; https://CRAN.R-project.org/package=survminer). *P* values were corrected with the Benjamini-Hochberg procedure (Benjamini and Hochberg 1995). All statistical tests were performed with R statistical software (ver. 3.4.1).

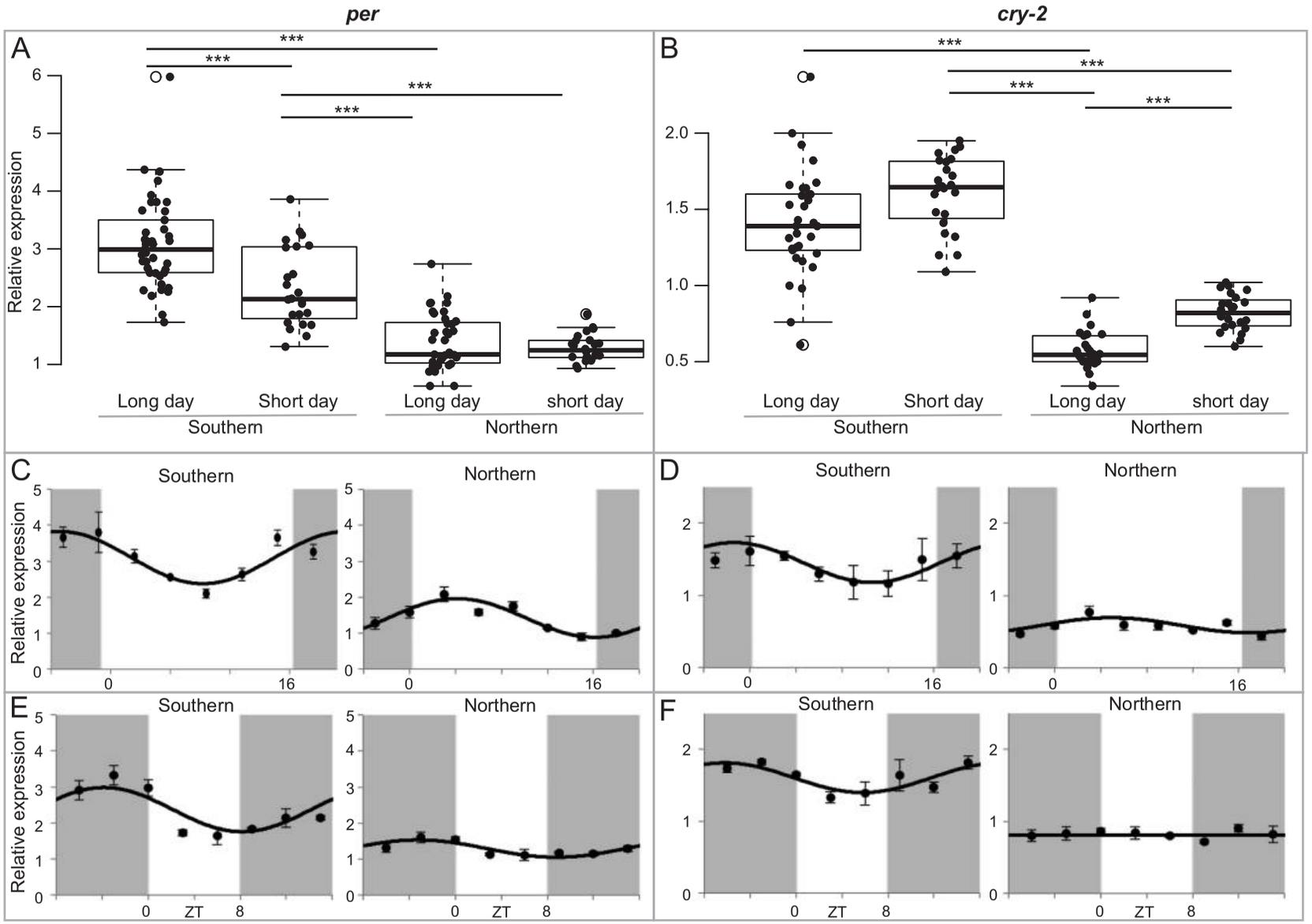
Results

Expression of period *and* cryptochrome-2

The expression levels of *per* and *cry-2* were significantly higher in southern than northern wasps under both long-photoperiod (16L : 08D) and short-photoperiod (08L : 16D) conditions (two-way ANOVA, effect of treatment: for *per*, $F_{3,120} = 88.62$, $P < .001$; for *cry-2*, $F_{1,99} = 117.362$, $P < .001$; figs. 1*A*, 1*B*, A5*A*, A5*B*). The largest differences occurred for *per* during the dark phase (fig. A5*A*) and for *cry-2* throughout the light and the dark phase (fig. A5*B*). In southern wasps, *per* expression level was lower under short-photoperiod conditions throughout the day (figs. 1*A*, A4*A*), whereas *cry-2* expression was similar between both LD regimes (figs. 1*B*, A5*B*). Interestingly, in southern wasps *per* and *cry-2* expression profiles had the same phase in both LD cycles, with the peak of expression during the end of the dark phase (around ZT 21–ZT 23) and a progressive decline during the light phase (fig. 1*C*, 1*E*). In contrast, northern wasps exhibited a shift in *per* expression phase; under long-photoperiod conditions *per* peaked in the light phase around ZT 3 (fig. 1*C*), but under short-photoperiod conditions *per* peaked during the night around ZT 21. Under short-photoperiod conditions *per* expression oscillated more weakly than under long-photoperiod conditions (fig. 1*E*), but the average expression level did not differ from that for the long photoperiod (figs. 1*A*, A5*A*). In northern wasps *cry-2* exhibited a weaker circadian oscillation under long-photoperiod conditions compared with the southern wasps, with the peak of expression during the light phase around ZT 3 (fig. 1*D*) but with no significant oscillation under short-day conditions. The constant expression under short-day conditions (fig. 1*F*) was at a higher level than under long-day conditions throughout the day and night (figs. 1*B*, A5*B*).

Expression of cycle *and* clock

The expression levels of *cyc* and *clk* were higher in southern than in northern wasps under both photoperiod conditions (two-way ANOVA, effect of treatment: for *cyc*,



$F_{1,126} = 37.843$, $P < .001$; for *clk*, $F_{1,92} = 68.89$, $P < .001$; fig. 2*A*, 2*B*). In southern wasps, *cyc* displayed the same expression level and profile under both photoperiod conditions (fig. 2*A*) with the peak of expression at the end of the light phase, in antiphase to *per* and *cry-2* (fig. 2*C*, 2*E*). Under long-day conditions the peak occurred around ZT 14 (fig. 2*C*), and under short-day conditions the peak occurred around ZT 11 (fig. 2*E*). Interestingly, in the northern line under long-photoperiod conditions, *cyc* peaked in the middle of the light phase around ZT 9 (fig. 2*C*), in phase with *per*, whereas under short-photoperiod conditions it peaked at the beginning of the dark phase (ZT 9), in antiphase to *per* (fig. 2*E*). Moreover, the amplitude of the oscillation was much weaker compared with the long photoperiod and to the southern line's expression profile, due to a decrease in the expression level during the light phase (fig. A5*C*). The gene *clk* was expressed differently between lines and photoperiods. In southern wasps no significant oscillation was evident under both photoperiod conditions (fig. 2*D*, 2*F*), and overall expression levels did not differ between photoperiods (figs. 2*B*, A5*D*). In contrast, northern *clk* expression displayed a clear circadian oscillation with a peak around ZT 13 during the light phase under long-day conditions (fig. 2*D*). Similar to the southern wasps, *clk* did not oscillate under short-photoperiod conditions (fig. 2*E*) but was expressed at a much lower level than under long-photoperiod conditions throughout the day and night (figs. 2*B*, A5*D*).

Effect of per *RNAi on Clock Gene Expression*

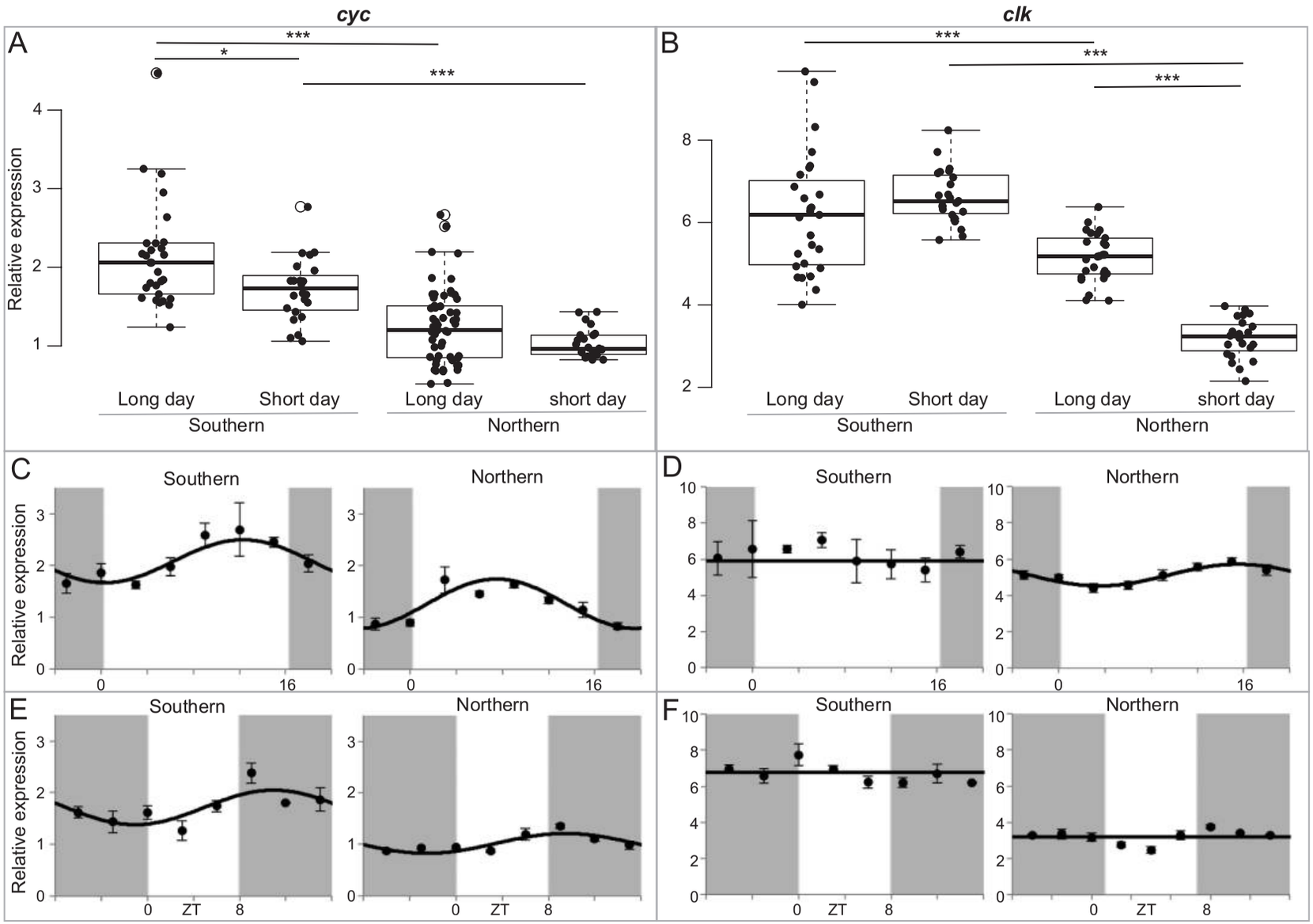
To evaluate whether *per* RNAi was efficient, the level of *per* mRNA was analyzed 3 days after eclosion at three time points during the light phase under 08L:16D conditions (ZT 0, 4 and 8). In control wasps, the expression of *per* was at the highest point at ZT 0 and at the lowest level at ZT 8 (fig. 3*A*, 3*B*), in line with the wild-type *Nasonia vitripennis* results (fig. 1*E*). The relative expression level of *per* in the dsRNA-injected wasps was lower at all three time points in both the southern and northern lines, compared with their respective controls (GLMM: for southern wasps, $F_{2,12} = 61.56$, $P < .001$; for northern wasps, $F_{2,10} = 327.5$, $P > .001$; fig. 3*A*, 3*B*; table S1; tables S1–S6 are available online), indicating an efficient *per* knockdown and a disruption of cyclical expression with stable lower *per* expression.

Expression of *cry-2*, *clk*, and *cyc* was also measured in control and *per*-RNAi-treated wasps at the same three time points in the light phase (ZT 0, 4, and 8; fig. 3*C*–3*H*; table S2–S4). In untreated southern wasps, *cry-2* expression decreased during the light phase (figs. 3*C*, 1*F*; table S2), whereas *per*-RNAi-treated wasps displayed a lower and constant *cry-2* expression at all three time points (GLMM: $F_{2,12} = 5.92$, $P < .001$; fig. 3*C*; table S4). Both *clk* and *cyc* had lower expression during all ZTs (GLMM: for *clk*, $F_{2,12} = 24.74$, $P < .001$; for *cyc*, $F_{2,12} = 16.74$, $P < .001$; fig. 3*E*, 3*G*; tables S3, S4). Moreover, the oscillation of *cyc*, whose expression increased during the light phase (figs. 2*E*, 3*G*), was disrupted in RNAi-treated wasps (fig. 3*G*; table S4). Similarly, in northern wasps *cry-2* expression was lower in *per*-RNAi-treated individuals than in control individuals (GLMM: $F_{2,12} = 5.72$, $P < .001$; fig. 3*D*; table S2). Also, the overall expression levels of *clk* and *cyc* were lower in RNAi-treated northern wasps (GLMM: for *clk*, $F_{2,12} = 4.66$, $P < .001$; for *cyc*, $F_{2,12} = 8.77$, $P < .001$; fig. 3*F*, 3*H*; tables S3, S4), with a disruption of *cyc* oscillation as in the southern wasps (fig. 3*H*; table S4). Thus, RNAi of *per* alters the phase and the expression of the whole circadian system.

Effect of per *RNAi on Daily and Seasonal Rhythms*

To assess the function of *per* in circadian and seasonal rhythms, we monitored locomotor activity and diapause response after *per* RNAi. In the locomotor activity assays, we exposed the wasps to a LD regime of either 08L:16D or 16L:08D for 4 days followed by either DD or LL for 10 days. Both southern and northern wasps displayed a unimodal activity pattern (fig. 4*A*, 4*B*) with an earlier activity in the southern line than in the northern one. Average daily activity was not affected by RNAi in the southern wasps in both LD regimes but was advanced in the northern wasps ($P < .001$, ANOVA with Tukey's multiple-comparisons test). Under 08L:16D conditions northern RNAi-treated wasps started activity about 3.5 h into the dark phase, a 4-h shift compared with control wasps. Peak of activity and offset of activity did not, however, differ between control and RNAi treatments,

Figure 1: Expression of *period* (*per*) and *cryptochrome-2* (*cry-2*) mRNA. *A*, *B*, Boxplots depicting the median (thick horizontal line within the box), 25th and 75th percentiles (box margins), and 1.5 interquartile range (thin horizontal line) of expression levels of the clock genes *per* (*A*) and *cry-2* (*B*) under long-day and short-day conditions for southern and northern lines. Asterisks represent significant differences between lines (two-way ANOVA, $^{***}P < .001$). *C* shows relative mRNA expression of *per* over 24 h under long-day conditions for southern (*left*) and northern (*right*) lines, and *E* shows *per* relative mRNA for short-day conditions for southern (*left*) and northern (*right*) lines. *D* shows *cry-2* relative mRNA under long-day conditions and *F* shows *cry-2* relative mRNA under short-day conditions for southern (*left*) and northern (*right*) lines. Each circle represents the average relative expression of three to five biological replicates per time point. The black lines represent the best sine wave fit to the experimental data over the 24-h period according to CircWave analysis. Zeitgeber time (ZT) is given in hours on the *X*-axis, where ZT = 0 represents light on. The gray area represents the night phase, and the white area represents the light phase.



although an increase in activity level in the dark phase was evident for the northern RNAi-treated wasps (fig. 4*A*; table S5). Similar behavior was reported under 16L : 08D conditions; after *per* RNAi, northern wasps displayed a strong advance of peak activity of more than 5 h ($P < .001$, one-way ANOVA with Tukey's multiple-comparisons test), whereas onset and offset of activity remained the same (fig. 4*B*; table S5).

The free-running rhythms under DD and LL conditions were compared between lines and treatments. Under DD conditions, the southern line showed a shorter free-running rhythm ($\tau = 24.67 \pm 0.10$ h) compared with the northern one ($\tau = 26.57 \pm 0.12$ h; $P < .001$, one-way ANOVA with Tukey's multiple-comparisons test; fig. 5*A*, 5*B*). After *per* RNAi, a significant shortening of τ of about 1 h was observed ($P < .001$, one-way ANOVA with Tukey's multiple-comparisons test): 23.80 ± 0.06 and 25.25 ± 0.17 h for the southern and northern lines, respectively (fig. 5*A*, 5*B*). The rhythmicity level was not clearly affected under DD conditions in the southern wasps, whereas one of the RNAi treatments (dsRNA_A) in the northern line led to an increase in the number of arrhythmic wasps (table S6). Under LL conditions, the rhythms are shorter than under DD conditions for both lines ($P < .001$, one-way ANOVA with Tukey's multiple-comparisons test; fig. 5*C*, 5*D*). The southern line has a τ of 22.32 ± 0.16 h and a high level of arhythmicity (83%); northern wasps have a τ of 23.24 ± 0.32 h and 84% of arhythmicity (fig. 5*C*, 5*D*; table S6). Interestingly, *per* RNAi in southern wasps led to an even shorter τ of 21.10 ± 0.15 h ($P < .001$, one-way ANOVA with Tukey's multiple-comparisons test) and an increase in the number of rhythmic individuals by 20% (fig. 5*C*; table S6). In contrast, *per* RNAi increased the free-running rhythm in the northern line by about 2 h, with a τ of 25.01 ± 0.44 h ($P < .001$, one-way ANOVA with Tukey's multiple-comparisons test; fig. 5*D*). Again, the number of rhythmic wasps increased in treatment RNAi_A by 10%, but in RNAi_B it was unaltered (table S6).

The diapause response under 08L : 16D conditions was also assessed in RNAi-treated wasps and controls for southern and northern lines. Although all wasps reached the switch point, southern wasps started to produce diapause offspring much later than northern ones (fig. 6). For both lines, RNAi-treated females had a later switch point and a delayed diapause response curve (for the southern line, $\chi^2 = 15.7$, $P < .0001$; for the northern line, $\chi^2 = 16.2$, $P < .0001$; log-rank test for multiple comparison). The average switch point of control wasps was day 8 for the south and day 4 for the north, in agreement with earlier observations of Paolucci et al. (2013). After *per* knockdown, southern wasps delayed the switch point by 2 days to day 10, and northern ones delayed it by 4 days to day 8 (fig. 6).

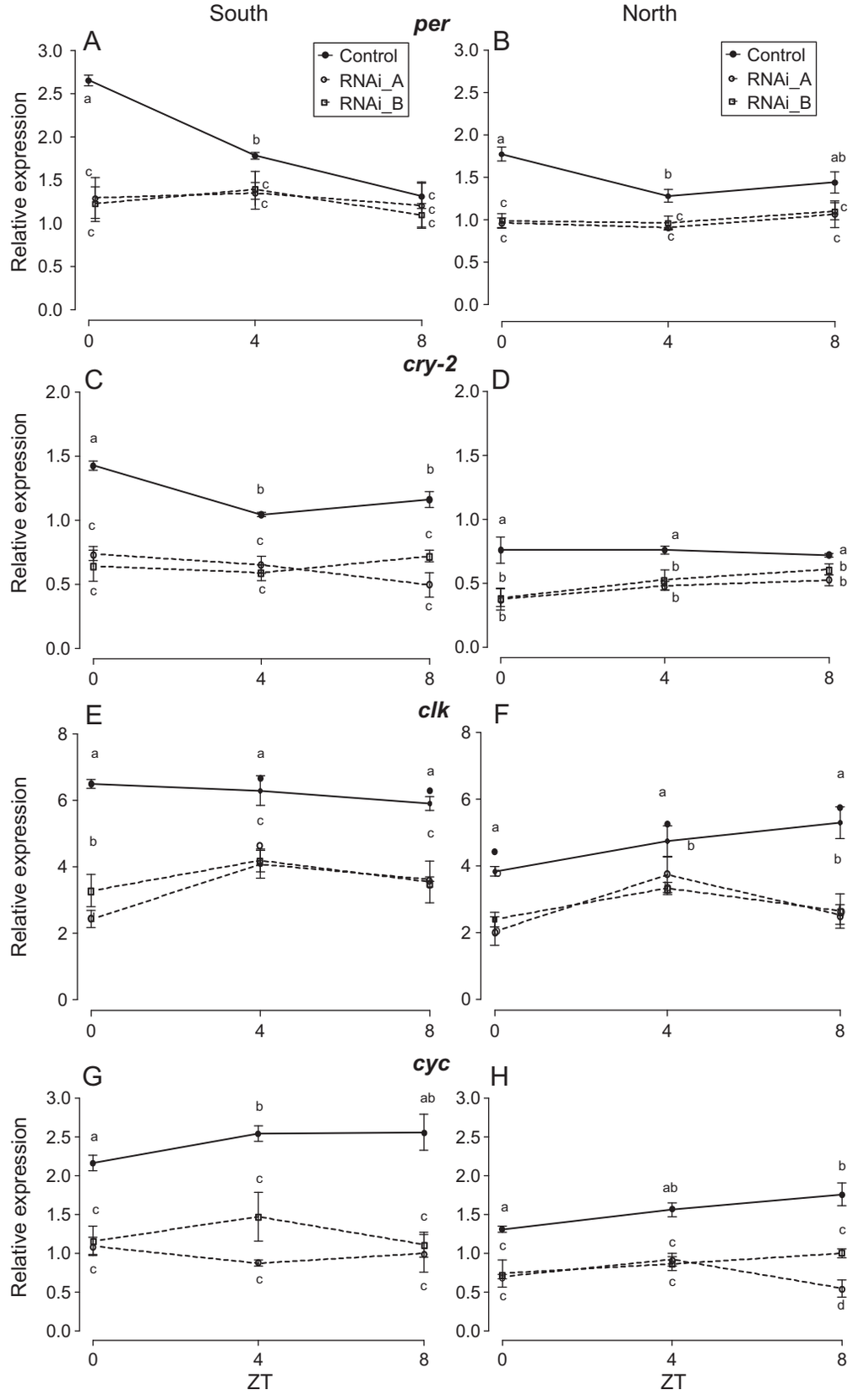
Discussion

We investigated variation in clock gene expression as function of photoperiod and latitude of origin as well as the role of the *period* (*per*) gene in regulation of circadian rhythms and photoperiodic response in the parasitoid wasp *Nasonia vitripennis*. Clock gene expression was clearly affected by both photoperiod and latitude of origin. Knockdown of *per* by RNAi altered daily rhythms under constant conditions (DD and LL), changed the timing of locomotor activity, affected the expression of other clock genes, and delayed the switch point for photoperiodic diapause response.

Clock Gene Expression Depends on Photoperiod and Latitude of Origin

The circadian clock of *N. vitripennis* includes the mammalian type *cry-2*, which is part of the core feedback loop (Yuan et al. 2007; Bertossa et al. 2014). The genes *per* and *cry-2* represent the negative elements of the *Nasonia* circadian clock and suppress their own transcription by inhibiting the positive elements *cycle* (*cyc*) and *clock* (*clk*; Hardin 2004; Stanewsky 2003). Geographical variation in photoperiodic seasonal responses in *N. vitripennis* have been associated with allelic differences of *per* (Paolucci et al. 2013, 2016). Moreover, geographical variation in circadian activity rhythms has been observed for *N. vitripennis* (Dalla Benetta 2018). To evaluate whether differential clock regulation can explain the geographical variation in seasonal and circadian responses in *N. vitripennis*, we measured expression patterns of candidate clock genes in wasps of different geographical origin under different photoperiodic conditions. We observed differences in amplitude, phase, and overall levels of expression between southern and northern wasps for all four tested genes. Moreover, gene expression was strongly affected by photoperiod in the

Figure 2: Expression of *cycle* (*cyc*) and *clock* (*clk*) mRNA. *A*, *B*, Boxplots depicting the median (thick horizontal line within the box), 25th and 75th percentiles (box margins), and 1.5 times the interquartile range (thin horizontal line) of the relative expression level of clock genes *cyc* (*A*) and *clk* (*B*) under long-day and short-day conditions for southern and northern lines. Asterisks represent significant differences between lines (two-way ANOVA, $^{***}P < .001$). *C* shows relative mRNA expression of *cyc* over 24 h under long-day conditions and *E* shows *cyc* relative mRNA under short-day conditions for southern (*left*) and northern (*right*) lines. *D* shows *clk* relative mRNA under long-day conditions and *F* shows *clk* relative mRNA under short-day conditions for southern (*left*) and northern (*right*) lines. Each circle represents the average relative expression of three to five biological replicates per time point. The black lines represent the best sine wave fit to the experimental data over the 24-h period according to CircWave analysis. Zeitgeber time (ZT) is given in hours on the *X*-axis, where ZT = 0 represents light on. The gray area represents the night phase, and the white area represents the light phase.



northern wasps, whereas only slight effects were observed in the southern wasps.

Toward high latitude, daily and annual variation in solar radiation is more extreme, especially in terms of light intensity and photoperiod. It has been argued that the light sensitivity of the circadian clock needs to be adapted to these fluctuations (Pittendrigh and Takamura 1989; Pittendrigh et al. 1991). One way of achieving this would be a lower amplitude of clock gene expression oscillations (Pittendrigh and Takamura 1989; Pittendrigh et al. 1991). Weak clocks can, more than strong clocks, easily synchronize to changes in LD cycles and more readily phase shift to light pulses (Vitaterna et al. 2006; van der Leest et al. 2009; Abraham et al. 2010). Consequently, weak circadian clocks are efficiently ticking under LD cycles and can serve as time reference for photoperiodism. Therefore, a weaker clock in the north could facilitate individuals to adapt to a more variable light environment. The observed lower amplitude caused by an overall weaker expression of the clock genes in the northern wasps, especially under short-day conditions, makes the oscillation pattern less robust, leading to a more plastic (flexible) clock in the north. These results indicate that transcriptional regulation of clock genes plays a role in daily and seasonal rhythms and suggest that adaptation to latitudinal differences in photoperiod is accomplished through selection on modulating the expression of several clock genes. It should be noted, however, that according to Hardin (2004) changes in transcript phase do not necessarily alter protein cycling in the negative feedback dynamics and that the adaptive effect of clock gene expression must be accompanied by posttranscriptional regulation.

RNAi of per *Affects Both Daily and Seasonal Rhythms*

The role of *per* in the circadian clock mechanism of *N. vitripennis* was assessed via RNAi. Interestingly, knockdown of *per* expression increased the speed of the clock (shorter τ) in both southern and northern lines under DD conditions and advanced the activity phase in the northern wasps under 16L:08D and 08L:16D conditions. These results confirm a functional role of *per* in the core mechanism of the *N. vitripennis* circadian clock. Since expression levels of *per* are higher in the south (with a faster clock) than in the north (with a slower clock), PER dosage, as argued above, may be important for setting the pace of the internal oscillator. Moreover, southern wasps are more active during the first part of the day, whereas northern ones are mostly active in the late afternoon. These data are in line with the timing of *per* expression peaks at the end of the night in the southern wasps and much later (during the light phase) in the northern ones and indicate that *per* expression is involved in setting both the pace and the phase of the circadian clock.

Under LL conditions, RNAi-treated northern wasps increased the duration of the free-running rhythm, whereas southern ones decreased it, indicating a different effect of *per* (and of the light) between the south and the north in the regulation of DD and LL rhythms. This could reflect that circadian oscillators are differently affected by light in the southern and northern wasps. If these differences indeed reflected the presence of two different neuronal oscillators with different phases, further research should identify neurons in the brain with different circadian expression between southern and northern wasps. The data also suggested a role of *per* in the as yet unknown *Nasonia* light input pathway, as the number of wasps exhibiting circadian rhythmicity under LL conditions was higher among the RNAi-treated wasps. It would also be interesting to test whether *per* functions in the light perception, since *Nasonia* does not have *tim*; whether *per* alleles in *Nasonia* differ in light sensitivity, as was reported for *tim* alleles in *Drosophila* (Sandrelli et al. 2007; Tauber et al. 2007) and for *per* in mammals (Akiyama et al. 2017); and whether the light signal is differently filtered into the clock of southern and northern wasps.

Knockdown of *per* also decreased the circadian expression levels of three other clock genes tested, *cry-2*, *clk*, and *cyc*. Although a decrease of a negative element is expected to result in an increase in gene expression of the positive elements (*cyc* and *clk*), similar results were reported in *Drosophila melanogaster* (Bae et al. 1998; Lee et al. 1998). This indicates that *per* is necessary for concerted transcription of the other clock genes and that the disruption of *per* expression affects the expression of other clock genes in a complex manner. The effects of *per* RNAi also perpetuated to the behavioral level. Although all wasps were able to induce diapause in their offspring after *per* knockdown, the timing of the photoperiodic response was delayed in both southern and northern lines. This indicates that *per* knockdown is not affecting the physiology of diapause itself but the onset of it, that is, the timer component of the photoperiodic calendar. At first sight it seems counterintuitive that low levels of *per* expression in northern lines is associated with an early switch point and a delay of switch point after RNAi. We think that the lower expression levels are important for the robustness of the clock, whereas the actual timing of diapause induction requires a threshold level of *per* expression, most likely fine-tuned with

Figure 3: Clock gene expression of control and RNA interference (RNAi)–treated wasps. Shown is clock gene expression for *per* (*A*, *B*), *cry-2* (*C*, *D*), *clk* (*E*, *F*), and *cyc* (*G*, *H*) for southern (*left*) and northern (*right*) lines. Controls are represented as closed circles with a continuous line, RNAi-treated wasps injected with dsRNA_A are represented as open circles with a dashed line, and wasps injected with dsRNA_B are represented as open squares with a dashed line. Zeitgeber time (ZT) is given in hours on the *X*-axis, where ZT = 0 represents light on. Letters indicate significant differences between ZTs and between treatments ($P < .05$, GLMM and post hoc Tukey analyses).

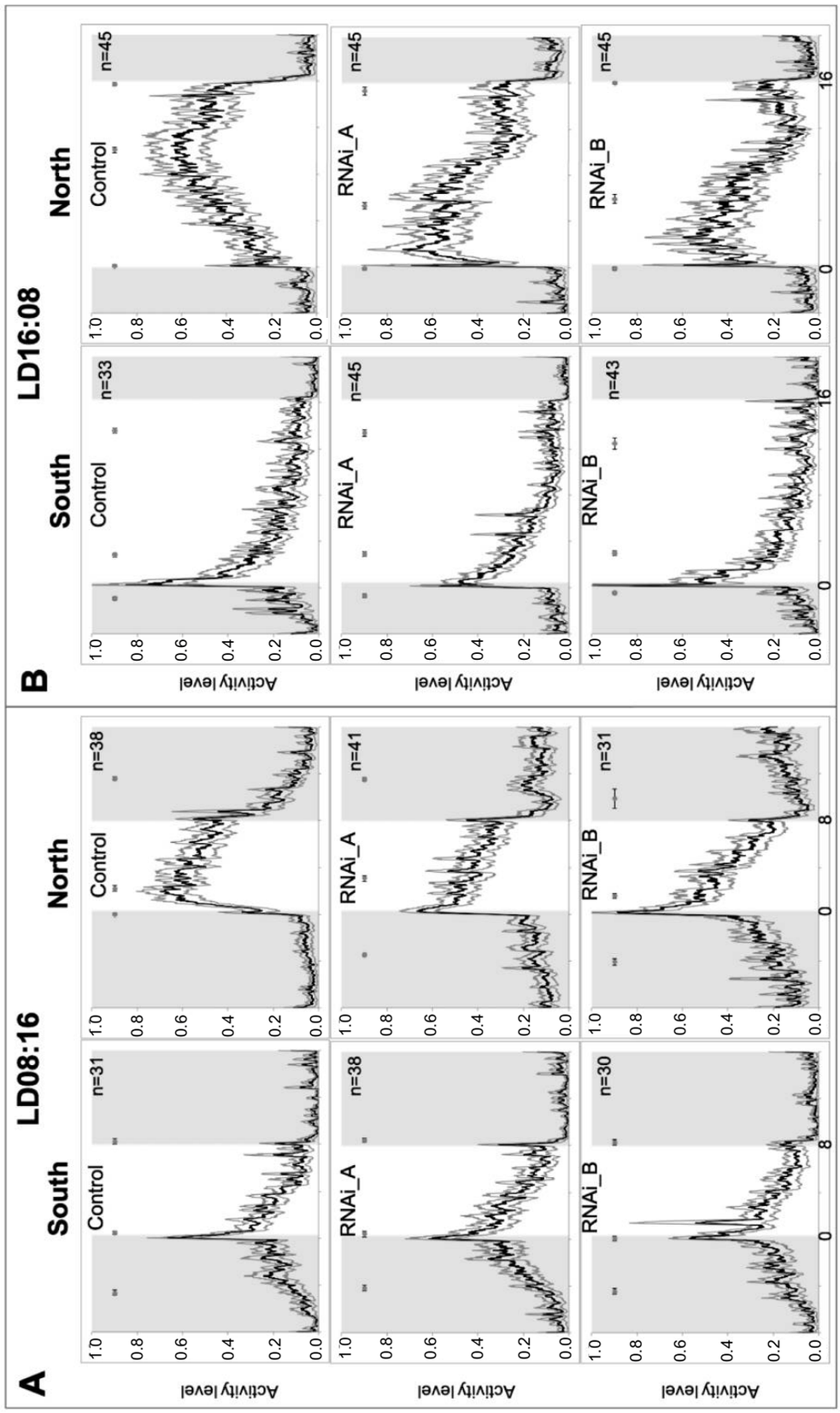


Figure 4: Locomotor activity of control and RNA interference (RNAi)–treated wasps. The locomotor activity profiles of southern and northern wasps are shown as the average of bin crosses per minute of 30–45 individuals each over 24-h periods at 08*L*:16*D* (*A*) and at 16*L*:08*D* (*B*). The night phase is indicated by gray shading, and the day phase is indicated by white. Zeitgeber time (ZT) is given in hours on the *X*-axis, where ZT = 0 represents light on. Circles at the top of each graph indicate the onset, the peak, and the offset of activity with standard errors; *n* refers to the number of individual wasps used.

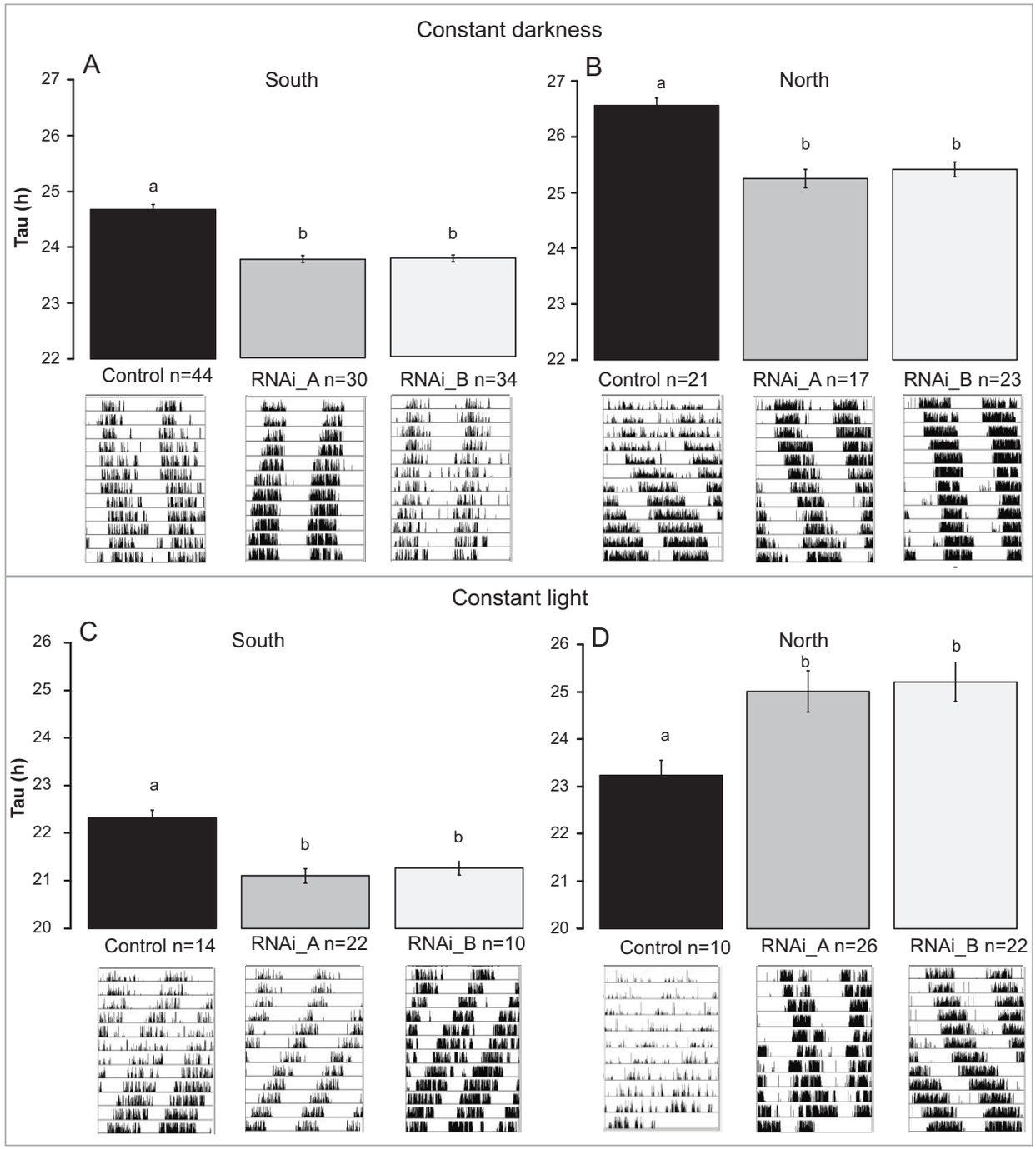


Figure 5: Free-running rhythms under constant conditions of control and RNA interference (RNAi)–treated wasps. *A*, *B*, Southern (*A*) and northern (*B*) free-running rhythms in constant darkness in control and RNAi-treated wasps injected with either dsRNA_A or dsRNA_B. *C*, *D*, Southern (*C*) and northern (*D*) free-running rhythms in constant light in control and RNAi-treated wasps injected with either dsRNA_A or dsRNA_B. Actograms below each graph bar represent activity level under constant conditions, in which black bars indicate activity. Different letters indicate significant differences ($P < .001$, one-way ANOVA with Tukey's multiple-comparisons test); *n* refers to the number of individual wasps used.

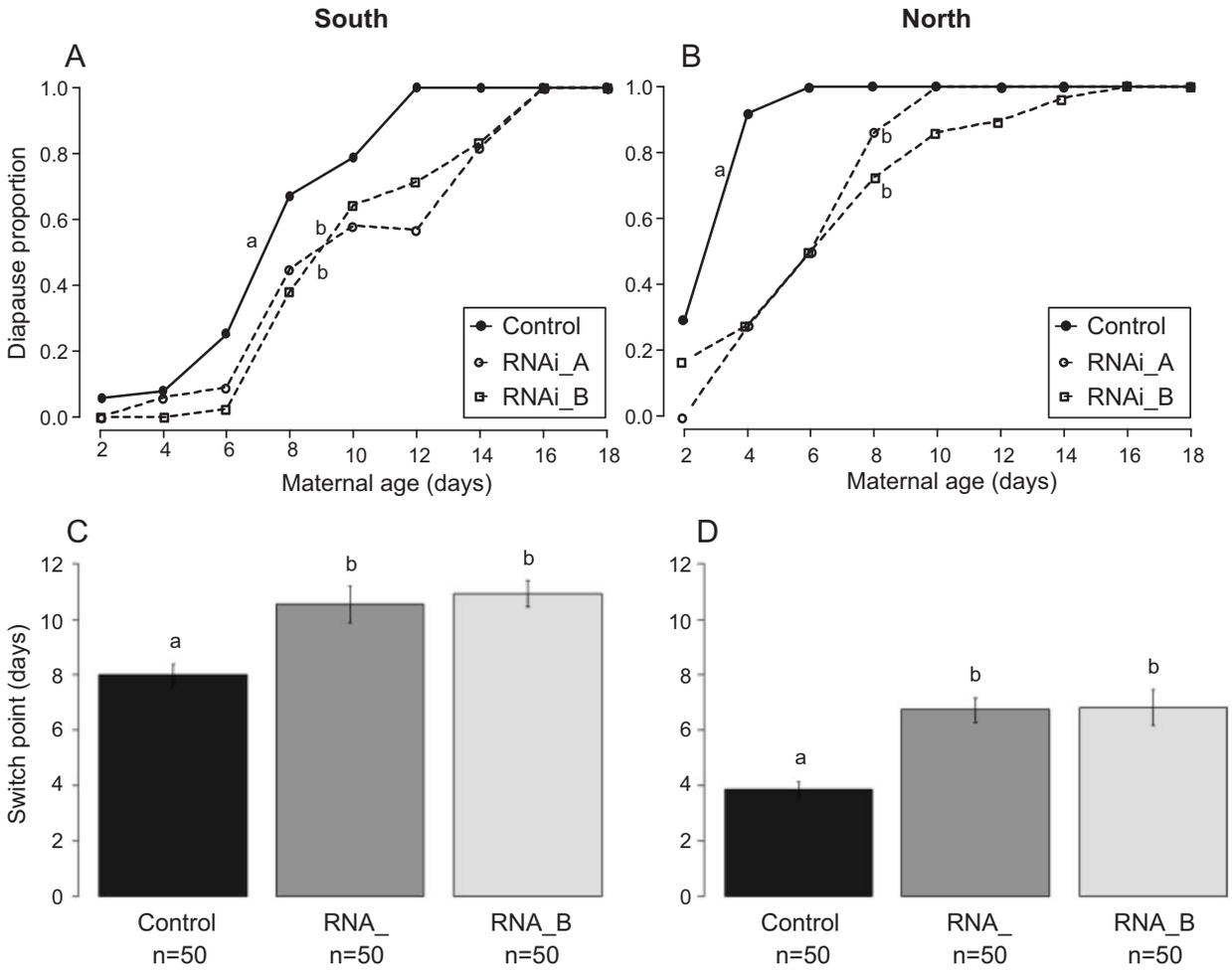


Figure 6: Diapause response of control and RNA interference (RNAi)–treated wasps. *A*, *B*, Diapause response of females under 08L:16D conditions in southern (*A*) and northern (*B*) wasps for control and RNAi-treated groups. *C*, *D*, Southern (*C*) and northern (*D*) switch point for diapause induction in control wasps, RNAi_A-treated wasps, and RNAi_B-treated wasps, calculated as the day on which wasps switch from producing developing offspring to diapause offspring. Different letters indicate statistical differences ($P < .001$, pairwise comparison using long-rank test); *n* refers to number of individual wasps used.

other clock and physiological traits. No or too low expression prevents a proper function of the timer and counter. In addition, it may not be the relative expression level of *per* but its oscillation profile that is disrupted after RNAi. Altering the phase of the internal oscillator could potentially alter the timer mechanism that relies on the phase of the circadian clock for proper timing (Koštál 2011).

Role of per *in Daily and Seasonal Responses*

There is substantial evidence to support a role of the circadian clock in photoperiodism. In 1989, Saunders et al. (1989) showed that *per* null mutations (*per*^{0}) in *D. melanogaster* did not affect its diapause incidence, which suggests that the circadian clock is not involved in photoperiodism. However, *per*^{0} flies showed a shift of the CPP (i.e., the photoperiod at which 50% of the population shows a diapause response), indicating that the timing mechanism was altered in *per*^{0} flies. Additionally, more recent studies indicate that the circadian clock gene *timeless* determines general diapause incidence (Tauber et al. 2007). Clock genes are also known to be involved in the photoperiodic response of other insects, such as *timeless* in the fly *Chymomyza costata* (Pavelka et al. 2003) and *period* in the cricket *Modicogryllus siamensis* (Sakamoto et al. 2009). Furthermore, *period*, *cycle*, mammalian type *cryptochrome*, and *clock* are important for diapause in the bean bug *Riptortus pedestris* (Ikeno et al. 2010, 2011*a*, 2011*b*, 2011*c*) and in the mosquito *Culex pipiens* (Meuti et al. 2015). Moreover, Shiga and Numata (2001) demonstrated the importance of the circadian clock neurons in

photoperiodic discrimination in the blow fly *Protophormia terraenovae* by neuron ablation experiments. Our results are in line with a role of clock genes in regulating photoperiodic diapause. RNAi of *per* affected expression of four essential *Nasonia* clock genes that correlated with changes in circadian rhythm as well as diapause induction without altering the physiology of diapause but affecting only the timing mechanism. Although these results indicate a shared genetic architecture for circadian and seasonal rhythm, they cannot distinguish between a modular versus a genetic pleiotropic function (Emerson et al. 2009) of the circadian clock in photoperiodism and diapause induction. Potentially, *per* could have a pleiotropic role by being (i) part of separate genetic pathways for circadian and seasonal rhythms or (ii) part of the circadian clock that regulates circadian and seasonal rhythms (modular pleiotropy). Importantly, after knockdown of *per* expression the expression of other clock genes was also affected. This means that any changes in the regulation of these clock genes likely also affects the expression of other genes. Hence, adaptations to the photoperiodic conditions at different latitudes may be accomplished by concerted changes in the circadian and seasonal clock. Our study has been instrumental for this: wasps of northern latitude differ in both the timing of diapause and the daily activity patterns.

In summary, our study provides clear evidence for geographical variation in clock gene regulation. Allelic differences in *per* between the north and south is associated with a weaker clock in the north that facilitates individuals to adapt to a more variable light environment. Additionally, our results indicate that natural selection acted on the sensitivity of the clock to environmental changes, suggesting that seasonal adaptation is accomplished through altering clock gene expression.

Acknowledgments

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