Involvement of clock genes in seasonal, circadian and ultradian rhythms of Nasonia vitripennis

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Chapter 3

Circadian clock gene expression in *Nasonia vitripennis* depends on photoperiod and latitude of origin

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Abstract

Light and temperature are important environmental factors that show daily and seasonal oscillations. They are cues for season-dependent behaviours, including timing of migration, hibernation and reproduction. *Nasonia vitripennis* is a parasitic wasp with maternal induction of larval diapause, a form of dormancy, as a response to cues for an approaching winter. Populations of *N. vitripennis* collected along a latitudinal gradient in Europe show a cline in photoperiodic diapause induction. Allelic frequencies of the circadian clock gene *period* are correlated with this cline, suggesting involvement of the circadian clock in diapause regulation. We compared expression levels of the clock genes *period (per)*, *chryptochrome-2* (*cry-2), clock* (*clk)* and *cycle* (*cyc)* in wasps from a southern (Corsica, France) and a northern (Oulu, Finland) population, to further evaluate this correlation. For all genes, circadian oscillations depending on both photoperiod and latitude of origin were observed, with less influence of photoperiod for wasps from southern than northern origin. These results provide further evidence for a role of clock genes in responses of insects to seasonal changes.

Introduction

Migration, hibernation and reproduction are behaviours of which timing is often adapted to annual changes in the environment as a result of natural selection to optimize survival and reproduction. Photoperiod (hours of light per day) is used as an environmental cue for the time of year. It often correlates to environmental conditions like temperature and food availability. Seasonal variation of photoperiod depends on latitude. The variation of day length is extreme at high latitudes where the day varies from constant darkness during winter to constant light during summer. In contrast, at the equator light-dark (LD) cycles are constant, although other environmental factors, such as precipitation, may vary seasonally. Many organisms have evolved a photoperiodic response for several behaviours to the latitudinal variation in photoperiod (reviewed in Hut et al., 2013).

Latitudinal variation in photoperiodic response is well known from many insects (Tauber et al., 1986; Danks, 1987; Tyukmaeva et al, 2011; Wang et al., 2012). Shortening photoperiod signals the upcoming of an unfavourable season. 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. Short photoperiod elicits a higher induction of larval diapause than long photoperiod. The photoperiod, at which 50% of the females induce larval diapause, after a precise number of LD cycle, is called the critical photoperiod (CPP; timer), while the number of CPP days that are required for inducing larval diapause is called the switch point (counter) (Saunders, 2013; Saunders, 2010). A clock mechanism is responsible for the timing and counting of the LD cycles necessary for starting the photoperiodic response (Saunders, 2013). Under long photoperiods, the switch point occurs later or not at all (Saunders, 1969). Paolucci et al., (2013) found a positive correlation between geographical origin and proportion of diapausing broods: wasps from northern regions had an earlier switch point and a longer CCP than wasps from southern regions. Short day conditions induced earlier switching than long day in all populations. QTL analysis (Paolucci et al., 2016) identified two genomic regions associated with diapause induction in *N. vitripennis*. One of these regions contains the *period* (*per*) locus, and further investigation identified three *per* haplotypes with frequencies that correlated with the earlier observed cline in photoperiodic diapause induction (Paolucci et al. 2016). These results indicate that *per* and possibly other clock genes play a role in photoperiodic diapause induction in *N. vitripennis*.

Many behavioural and physiological processes in animals, including rest-activity rhythms, mating and cell division, are regulated by circadian clocks (Sandrelli et al., 2008, Tomioka & Matsumoto, 2010). In many organisms, including insects, these clocks consist of transcriptional-translational feedback loops that regulate the expression of clock controlled genes. In *D. melanogaster* the circadian clock consists of negative elements, like *period* (*per*) and *timeless* (*tim*), and positive elements, like *clock* (*clk*), *cycle* (cyc), and the

photoreceptor *cryptochrome-1* (*cry-1*) (Konopka et al., 2007; Peschel & Helfrich-Forster, 2011). The discovery of the clock gene *cryptochrome-2* (*cry-2*) by Zhu et al. (2005) suggested that regulation of the circadian clock in the honeybee (*Apis mellifera*) (Rubin et al., 2006), monarch butterfly (*Danaus plexippus*) (Zhu et al., 2005), and the mosquito, *Anopheles gambiae* (Zhu et al., 2005) is different from *Drosophila*. It has been argued that clock genes provide the time measurement for diapause induction in insects (Ikeno et al., 2010, 2011a; Meuti et al., 2015). However, given the variation in components of the circadian clock among insect species, and that diapause apparently evolved numerous times, a similar variation in the regulation of photoperiodism is to be expected (Meuti and Denlinger, 2013).

The clinal correlation in *per* gene haplotypes and photoperiodic diapause induction suggests involvement of the circadian clock in photoperiodic time measurement in *N. vitripennis* (Paolucci et al., 2016). Furthermore, Mukai and Goto (2016) provided evidence that *per* is essential for a proper photoperiodic response in *Nasonia*. Bertossa et al., (2014) investigated the circadian oscillation of *per* and *cry-2* mRNA in a lab line of *N. vitripennis* under two different photoperiodic conditions. Both *per* and *cry-2* mRNA levels displayed a synchronized circadian oscillation under LD 18:06 and LD 12:12. Interestingly, changes in LD conditions caused a phase shift in the expression pattern. Recently Menegazzi et al., (2017) described a different neuroanatomical architecture of circadian clock neurons between southern and northern *Drosophila* species reflecting their different ability to adjust to long photoperiod. The study indicates the presence of a weaker clock in the northern species, which allows them to adapt to extreme photoperiods. Natural variation in circadian response with a faster clock and lower activity in southern than northern European *N. vitripennis* lines was also described by Paolucci (2014). Here we follow up on this study and further test the hypothesis that clock genes are responsible for photoperiodic-dependent changes in life history traits by investigating clock gene expression patterns of *Nasonia* wasps from different geographical origin under different photoperiodic conditions. For the clock genes *period (per)*, *chryptochrome-2* (*cry-2)*, *clock* (*clk)* and *cycle* (*cyc)*, circadian expression depending on photoperiod and latitude of origin was analysed in order to assess if a response to the latitudinal variation in photoperiod correlates with the adaptive behaviour of different photoperiodic diapause induction in *Nasonia* and if clock genes may play a role in photoperiodic diapause induction in *N. vitripennis*. The results contribute to the understanding of the link between photoperiodism and circadian clock, as hypothesized long ago.

Materials and methods

Experimental lines and rearing conditions

We used isogenic lines established from wasps collected from the field in 2009 (see for details Paolucci et al., 2013). The southern lines originate from Corsica, France (42°22'40.80N) and the northern lines from Oulu, Finland (65°3'40.16N). Isogenic lines were established by crossing a female wasp with one of her sons, followed by 7-8 generations of brother-sister crossing. This yields an estimated homozygosity level of 99.99%. Lines were maintained on *Calliphora spp*. pupae as hosts in mass culture vials under diapause-preventing conditions, i.e. long photoperiod of LD16:08, light intensity of 60 lum/sqf) and temperature of 20 ± 1 °C.

Wasp culturing and entrainment

In order to study clock gene expression in southern and northern lines of *N. vitripennis* under different light-dark (LD) conditions, mated females were allowed to oviposit under standard conditions. Offspring developed under the same conditions (LD16:08 and 20°C) until the yellow pupal stage, when the host puparia were opened and 5 females were stored in cotton-plugged 60 x 10 mm polystyrene tubes until emergence. Three to five biological replicates for each time point were prepared and incubated at 20°C either at long day LD16:08 or short day LD08:16 conditions. Seven to eight days later, virgin females had eclosed and were provided with hosts that were replaced every other day. Three to five biological replicates of five wasps each were collected every three hours throughout a 24h period (Fig. 3.1). To instantly kill wasps, we put the tubes into liquid nitrogen and stored them immediately at -80°C. For the night-time sampling points, the procedure was performed in darkness. 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 qPCR

RNA extraction was performed from the head of the collected wasps. Total RNA was extracted from each pool of five wasp heads with Trizol reagent (Invitrogen, USA) according to the manufacturer's instructions. Each sample was subjected to a DNase treatment to eliminate any DNA contaminations, and about 1ug of RNA was used to synthetize cDNA with RevertAid H Minus First Strand cDNA Synthesis kit (Thermo Scientific). The cDNA was then diluted 50x before being used for real time PCR (qPCR).

qPCR was performed with SYBR Green (Quanta Biosciences) and ROX as the internal passive reference, and 4ul of diluted cDNA was used for each reaction of 20ul total containing primer at the final concentration of 200nM and 10ul of SYBR Green/ROX buffer solution. Three technical replicates for each reaction were performed to correct for experimental errors. Reactions were run on an Applied Biosystems 7300 Real Time PCR System with the following qPCR profile: 3 min of activation phase at 95^{o}C, 35 cycles of 15 s at 95^{o}C, 30 s at 56^{o}C and 30 s at 72^{o}C. The primers used are listed in Table S1.

Expression data analysis and statistics

Relative expression levels were calculated by normalizing the expression data of the genes of interest with LinRegPCR (Ramakers et al. 2003, Ruijter et al. 2009). *Elongation factor 1 α* (e*f1α)* and *arginine kinase 3 (ak3*) were used as reference genes, after confirmation that their expression level is constant throughout the day, Furthermore their expression levels did not differ between southern and northern lines and between LD conditions (Fig. S1). Circadian rhythmicity was evaluated for each gene, and a sinusoidal curve was fitted to the data using Circwave (by R. Hut, available atwww.euclock.org). CircWave employs a forward linear harmonic regression to calculate the profile of the wave with a 24h period. Average expression levels between lines and photoperiods were compared with ANOVA in R statistical software (2012).

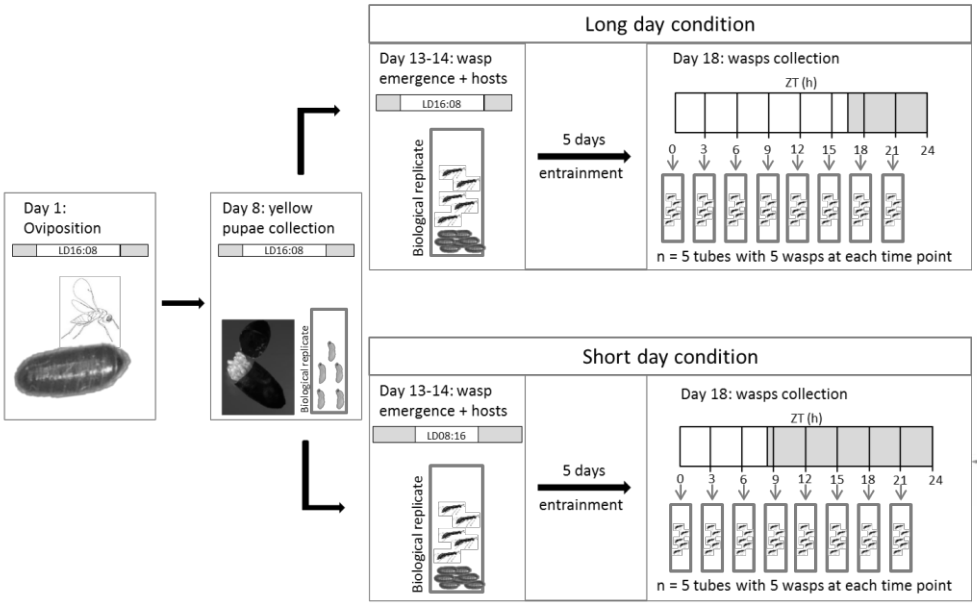

Fig. 3.1. Schematic representation of wasp sample collection

Mated females were allowed to oviposit under standard conditions in light-dark 16:08. Offspring developed under the same conditions (LD16:08) until the yellow pupal stage at day 8, when the host puparia were opened and 5 females were stored in tubes until emergence. Five biological replicates for each time point were prepared and incubated at 20oC either at long day LD16:08 or short day LD08:16 conditions. At day 13-14, virgin females had eclosed and were provided with hosts and at day 18 five biological replicates of five wasps each were collected per ZT (*Zeitgeber time*), where ZT=0 represents light on. Grey area represents the night phase and white area the light phase.

Results

Variation in clock gene expression was measured in *Nasonia vitripennis* wasps from different geographic origin and under different light-dark (LD) conditions. Expression patterns of *period* (*per)*, *cryptochrome-2* (*cry-2)*, *clock* (*clk)* and *cycle* (*cyc)* differed significantly between southern and northern lines depending on the applied photoperiod.

Period *and* cryptochrome-2 *expression differs between the southern and northern lines*

The expression level of *per* and *cry-2* was significantly higher in southern than in northern wasps under both long (LD16:08) and short (LD08:16) photoperiods ($p<0{,}001$ Fig. 3.2A, B). The strongest differences were evident for *per* during the dark phase (Fig. S2A) and for *cry-2* throughout the light and the dark phase (Fig. S2B). In the southern wasps *per* expression level was lower at the short photoperiod, throughout the day ($p<0.001$, Fig. 3.2A, Fig. S1A, S2A), whereas *cry-2* expression did not change significantly between LDs (Fig. 3.2 B, Fig. S2B, S2B). Interestingly, in the 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 21-23) and a progressive decline during the light phase (Fig. 3.3A, B). In contrast, northern wasps showed a shift in *per* expression phase, during long photoperiod *per* peaks in the light phase around ZT 3 (Fig. 3.3A), but under short photoperiod during the night around ZT 21. Under short photoperiod *per* expression showed a weaker oscillation than during the long photoperiod (Fig. 3.3B), however the average expression level under short photoperiod did not differ from the long photoperiod (Fig. 3.2A, S2A). In northern wasps *cry-2* showed a weaker circadian oscillation during long photoperiod compared to southern one, with the peak of expression during the light phase around ZT 3 (Fig. 3.3C), but no significant oscillation was observed under short days. The constant expression under short days (Fig. 3.3D) was at a significantly higher level than under long days throughout the day and the night ($p<0.001$, Fig. 3.2B, S2B).

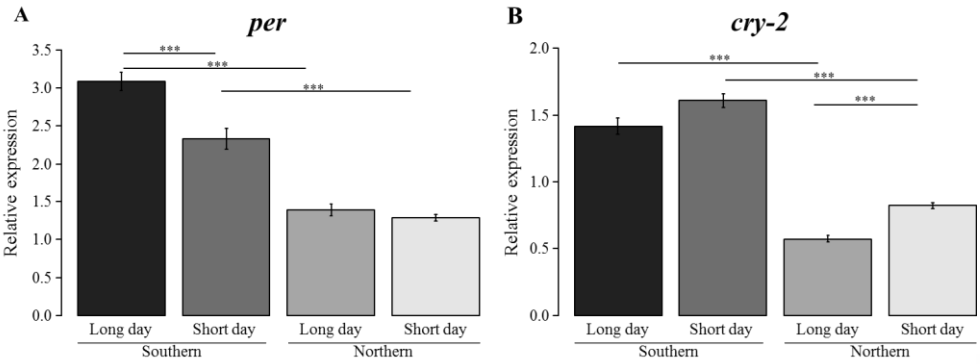

Fig. 3.2. *Period* and *cryptochrome-2* expression level.
(A) Depicts the average relative expression of clock gene *period* (*per*), and **(B)** for *crypthochrome-2* (*cry-2*) under long day and short day conditions for southern and northern lines. Asterisks represent significant differences between lines(one way ANOVA, ***p<0,001).

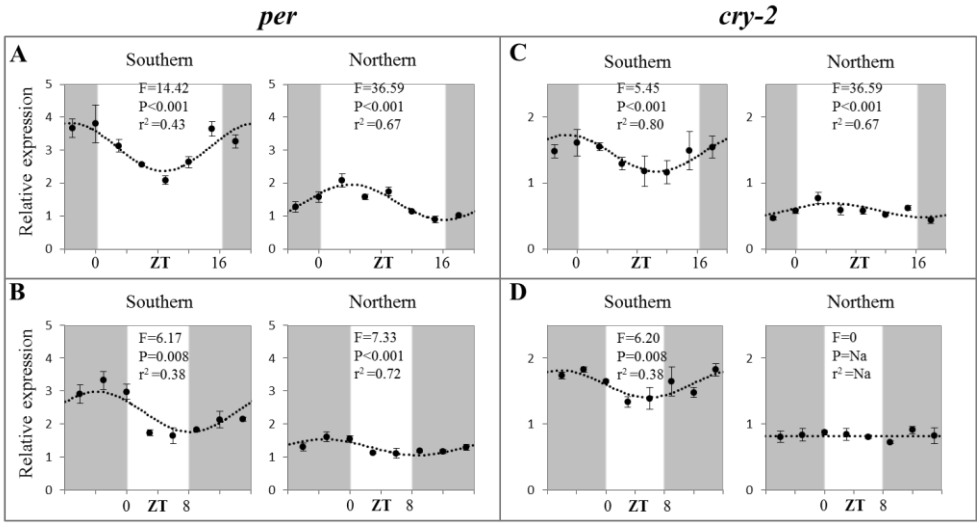

Fig. 3.3. *Period* and *cryptochrome-2* expression of southern and northern lines under long and short day conditions.
(A) represents the relative mRNA expression of *per* over 24h under long days for southern and northern lines, **(B)** represents the *per* relative mRNA, but for short days, for southern and northern lines. **(C)** represents *cry-2* relative mRNA under long days and **(D)** under short days, for southern and northern lines. Each dot represents the average relative expression of three to five biological replicates per time point. The dotted lines represent the best sine wave fit to the experimental data over the 24h period according to Circwave analysis. *Zeitgeber time* (ZT) is given in hours on the X-axis where ZT=0 represents light on. Grey area represents the night phase and white area the light phase. Na = not applicable.

Amplitude of* cycle *expression is affected by photoperiod in the northern line

The expression level of *cyc* was higher in the southern than the northern wasps under both photoperiods (Fig. 3.4). In southern wasps *cyc* displayed the same expression level and profile under both photoperiods with the peak of expression at the end of the light phase. It was in antiphase to *per* and *cry-2* (Fig. 3.5A, B), however under long days the peak occurred around ZT 14 (Fig. 3.5 A), while under short photoperiod the peak occurred around ZT 11 (Fig. 3. 5 B). Interestingly in the northern line, under long photoperiod c*yc* peaked in the middle of light phase around ZT 9 (Fig. 3.5A), with a similar phase of *per*, whereas under short photoperiod the expression phase peaked at the beginning of the dark phase (ZT 9), in antiphase to *per* (Fig. 3.5B) and the amplitude of the oscillation was much weaker compare to long photoperiod and to the southern expression profile, due to a decrease in the expression level during the light phase (Fig. S2C).

Photoperiod affects* clock *expression the northern lines

Clk was expressed differently between lines and photoperiods. The expression level of *clk* in southern wasps was much higher than in northern ones ($p<0.001$, Fig. 3.6). In southern wasps no significant oscillation was evident under both photoperiods (Fig. 3.7A, B), and expression levels did not change between photoperiod (Fig. 3.6, S2D). In contrast, northern *clk* expression showed a clear circadian oscillation with a peak of expression around ZT 13, during the light phase under long days (Fig. 3.7A). Similar to the southern wasps, *clk* showed no significant oscillation under short photoperiod (fig. 3.7 B), but was expressed at a much lower level under short than long days throughout the day and the night ($p<0.001$, Fig. 3.6, S2D).

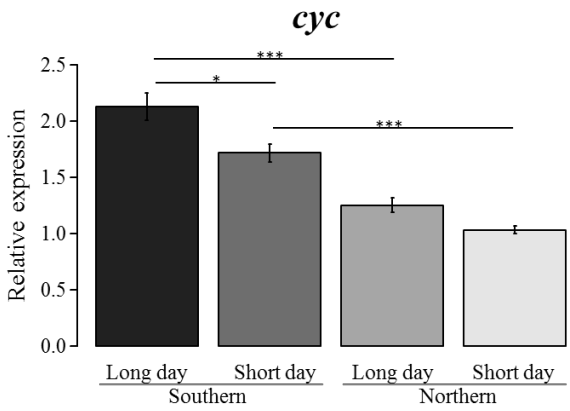

Fig. 3.4. ***Cycle*** **expression level**

The graph depicts the average relative expression of clock gene *cycle* (*cyc*) for long day and short day conditions and for southern and northern lines. Asterisks represent significant differences between lines (one way ANOVA, ***p<0,001; *p<0.05).

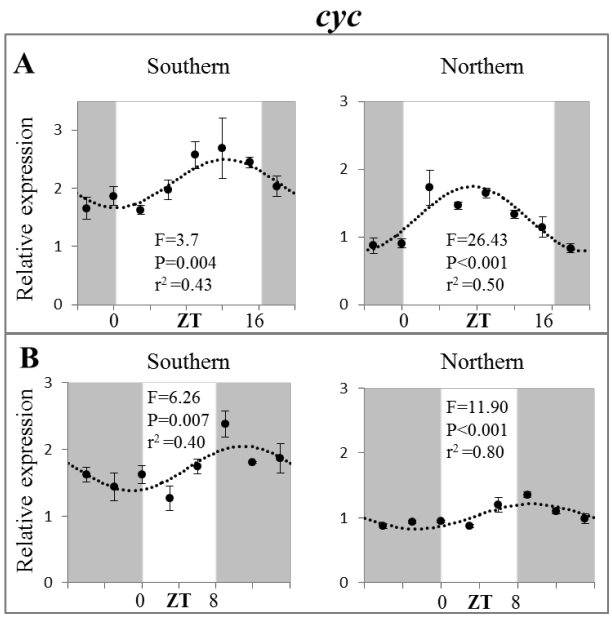

Fig. 3.5. ***Cycle*** **expression of southern and northern lines under long and short days**

(A) represents the relative mRNA expression over 24h under long days, for southern and northern (right) lines. **(B)** Similar, but for short days. Each dot represents the average relative expression of three to five biological replicates per time point. The dotted lines represent the best sine wave fit to the experimental data over the 24h period according to Circwave analysis. *Zeitgeber time* (ZT) is given in hours on the X-axis where ZT=0 represents light on. Grey area represents the night phase and white area the light phase.

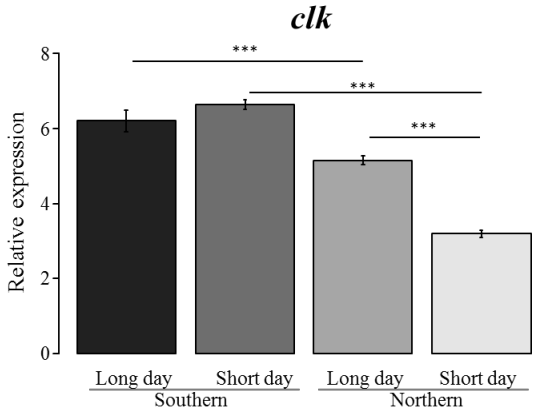

Fig. 3.6. ***Clock*** **expression level**

The graph depicts the average relative expression of clock gene *clock* (*clk*) for long day and short day conditions and for southern and northern lines. Asterisks represent significant differences between lines (one way ANOVA, ***p<0,001).

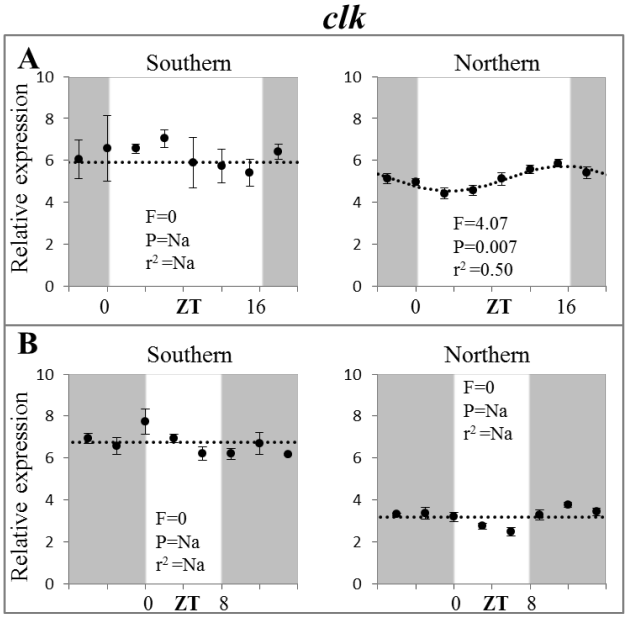

Fig. 3.7. ***Clock*** **expression of southern and northern lines under long and short days**

(A) represents the relative mRNA expression over 24h under long days, for southern and northern lines. **(B)** Similar, but for short days. Each dot represents the average relative expression of three to five biological replicates per time point. The dotted lines represent the best sine wave fit to the experimental data over the 24h period according to Circwave analysis. *Zeitgeber time* (ZT) is given in hours on the X-axis where ZT=0 represents light on. Grey area represents the night phase and white area the light phase. Na = not applicable

Photoperiodic diapause induction differs between northern and southern lines

Diapause phenotype was scored for each biological replicate used in the gene expression analyses (Fig. 3.8). Diapause is presented as percentage of diapausing offspring of the total number of offspring produced by the five females pooled in each biological replicate. Southern wasps showed no diapause response under long photoperiod and less than 25 % of the offspring went into diapause under the short days condition. In contrast, northern wasps displayed a variable percentage of diapause (15-80 %) under long photoperiod, whereas diapause response was 100% under short photoperiod.

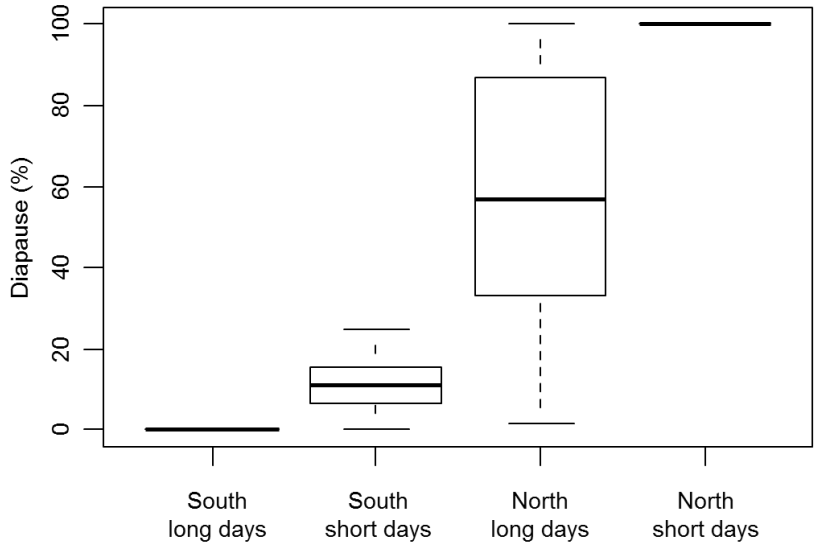

Fig. 3.8. Diapause percentage of samples used for expression analysis
Box plots show the median (thick horizontal line within the box), the 25 and 75 percentiles (box), and 1.5 times the interquartile range of the data (thin horizontal lines). Diapause percentage at day 5 after emergence (collection day) of 24 to 40 pools of 5 wasps each. Different letters indicate significant differences between conditions analysed by two way ANOVA ($p<0,0001$).

Discussion

We investigated geographical variation in clock gene expression as function of photoperiod in the parasitoid wasp *Nasonia vitripennis*. *Per* and *cry-2* represent the negative elements of the circadian clock, acting as repressor of their own transcription, beside their possible role as transcription factor for regulating other clock-controlled genes (Stanewsky, 2003). We found higher expression levels of *per* and *cry-2* in the southern than northern wasps and different amplitude and phase in expression profile. Moreover, expression levels and phase were differently affected by photoperiod in wasps of the two localities. Similar results in *per* and *cry-2* expression phase between photoperiods was also reported by Bertossa et al., (2014) in a laboratory line of *Nasonia* that originated from The Netherlands (which is intermediate in latitude to the lines used in this study), but this study provided no comparison of relative expression levels between photoperiods. Mukai and Goto (2016) found no differences between photoperiods in a Japanese line of *Nasonia*, with *per* and c*ry-2* peaking at the end of the night phase, although they showed a different expression level of the two transcripts between photoperiods, resembling our results for the southern line. Taking together these results indicate that *per* and *cry-2* expression in southern wasps is differently regulated by photoperiod than in northern wasps.

Geographical studies of clock genes, in terms of allelic variants and expression differences, are still scarce. Latitudinal clines in clock gene variation are well known from *Drosophila melanogaster* (Costa et al., 1992; Sawyer et al., 1997; Sandrelli et al., 2007; Tauber et al., 2007). The *Drosophila period* gene is characterized by a length polymorphism for a threonine-glycine (Thr-Gly) encoding repetitive stretch. Northern populations exhibit high frequencies of the longer (Thr-Gly)20 length variant compared to southern ones, in which the shorter (Thr-Gly)17 variant predominates (Costa et al., 1992). The different Thr-Gly variants have an effect on the temperature compensation of the circadian clock (Sawyer et al., 1997). A second component of the circadian clock of *D. melanogaster*, the *timeless* gene, is also polymorphic with allele frequencies following a latitudinal cline in Europe (Tauber et al., 2007). A recently derived mutation of this gene was found to influence diapause incidence, suggesting a link between the seasonal and circadian clock in flies (Tauber et al., 2007). Paolucci et al., (2013, 2016) reported a cline in *per* allele frequencies, correlated with diapause phenotype in *Nasonia*. We have now also found differential expression of the southern and northern *per* alleles providing further evidence for a role of *per* in photoperiodic diapause induction in this wasp. We concomitantly measured the proportion of diapausing offspring in our experimental lines (Fig. 3.8), which reflected the physiological state of the wasps. Southern wasps produced no diapausing broods under long days and a low number (<25%) under short days, which corresponds to little difference in *per* and *cry-2* gene expression between both LD cycles. The proportion of diapausing broods in the northern wasps varied strongly under long days,

whereas all offspring went into diapause during short days, which coincides with a phase shift in *per* and higher *cry-2* expression pattern. These results suggest that natural variation in *per* and *cry-2* expression pattern might be important for latitude-dependent diapause induction in *Nasonia*.

Cycle (*cyc*) and *clock* (*clk*) represent the positive elements of the circadian clock, activating the expression of E-Box genes like *per* and *cry-2* (Hardin, 2004; Stanewsky, 2003). *Nasonia cyc* is homologous to mammalian BMAL1, like in other hymenopterans such as the honeybee (Rubin et al., 2006) that have the BMAL1-terminal region (BCTR) domain at the C-terminal. The BCTR domain was characterized as an activation of CLK/BMAL1 heterodimer in mammalian cell cultures (Takahata et al., 2000) representing the region where CRY-2 binds to act as repressor (Sato et al., 2006). We found that *cyc* expression has a daily oscillation profile similar to mammals and other Hymenoptera (Rubin et al., 2006). However, the southern wasps had much higher expression levels than the northern ones. Photoperiod affected the amplitude of *cyc* expression profile only in northern wasps, indicating again different transcriptional regulation between lines and under different photoperiods. Similar results were recently reported for the moth *Sesamia nonagrioides* in which photoperiodic conditions affected the expression pattern and amplitude of *cyc* (Kontogiannatos et al., 2017). These authors concluded that *cyc* expression is associated with diapause because under diapause conditions the photoperiodic signal altered the mRNA accumulation. They also reported an effect of photoperiod on *per* expression that showed the same oscillation phase as *cyc*. We also found similar phase for *per* and *cyc* in northern wasps under LD16:08, however we cannot extrapolate these data to oscillations at the protein level. Hardin (2006) showed that it is possible that changes in transcript phase do not alter the protein cycling in the negative feedback dynamics. Furthermore the presence of different *per* splicing variants could lead to different post-translational regulations. Nevertheless, our results indicate that expression of both genes is influenced by photoperiod in a different way, suggesting that transcriptional regulation of these clock genes may play a role in programming diapause response, although more data about protein expression profile, post-transcriptional and post-translational regulation are needed to give a more complete model. Southern *clk* expression did not show any oscillation under both photoperiods, consistent with other hymenopterans (Rubin et al., 2006). Northern wasps, however, had a lower overall expression level of *clk* with a clear oscillation in phase only under long photoperiods. Hence, *clk* expression can be differentially regulated depending on line and photoperiod.

We found that diapause response was higher in the northern wasps in agreement with the cline reported by Paolucci et al., (2013). Expression of the four clock genes was strongly affected by photoperiod in the northern wasps whereas only slight effects were seen in the southern wasps. Overall we found a weaker expression profile of the clock genes in the northern wasps under short photoperiodic conditions, indicating the presence

of a "weaker" (more plastic) clock in the north. This could facilitate northern wasps to adapt a very variable environment. This potentially could imply a higher light sensitivity in the northern wasps in order to respond quickly to photoperiodic changes, but more data from natural variation in light sensitivity are needed to justify such a conclusion. These results indicate the potential of clock gene expression in the regulation of diapause induction as a function of photoperiod in *Nasonia vitripennis*. Differential expression of clock genes was also reported for strains of *Pyrrhocoris apterus* (Syrová et al. 2003) and for the aphid *Acyrthosipnon pisum* (Barberà et al., 2017). Additional evidence for a role of clock genes in diapause regulation comes from functional studies in the bean bug *Riptortus pedestris* and the mosquito *Culex pipiens* in which RNAi knockdown of *cyc* and *clk* induced diapause under non-diapausing conditions (Ikeno et al., 2011a; Meuti et al., 2015). Mukai and Goto (2016) also found a lack of diapause response after *per* RNAi in *Nasonia*, indicating again a functional role of clock genes in photoperiodic diapause induction in *Nasonia*. Although evidence for involvement of clock genes in diapause regulation is growing, further functional analyses and *in situ* localization studies of clock neurons are required to determine the precise role of clock genes in photoperiodism in *Nasonia vitripennis*.

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Supplementary information

Table S1. Primers used for qPCR

Gene	NCBI Ref. seq.	Forward primer	Reverse primer
per	XM_008211021.1	5'-GCCTTCATTACACGCATCTC-3'	5'-ACCATTCGCACCTGATTGAC-3'
cry-2	XM_008206206.1	5'-TCGCTTGTTTCCTCACCAG-3'	5'-GGTAACGCCGAATGTAGTCTC-3'
cyc	XM_008217573.1	5'-GATGCCAAGACGATGCTTCC-3'	5'-GCTCTTTCCTTGATCTGCGAC-3'
clk	XM_008216216.1	5'-ACTACCATATAGACGACCTTGAC-3'	5'-CCTGTATCCTCAAATGTTTGACCA-3'
ef1α	XM_008209960.1	5'-CACTTGATCTACAAATGCGGTG-3'	5'-CCTTCAGTTTGTCCAAGACC-3'
ak3	XM_016986045.1	5'-AATTCAATCGGGTTCTGCTC-3'	5'-CAGCATCTCATCTAACTTCTCTG-3'

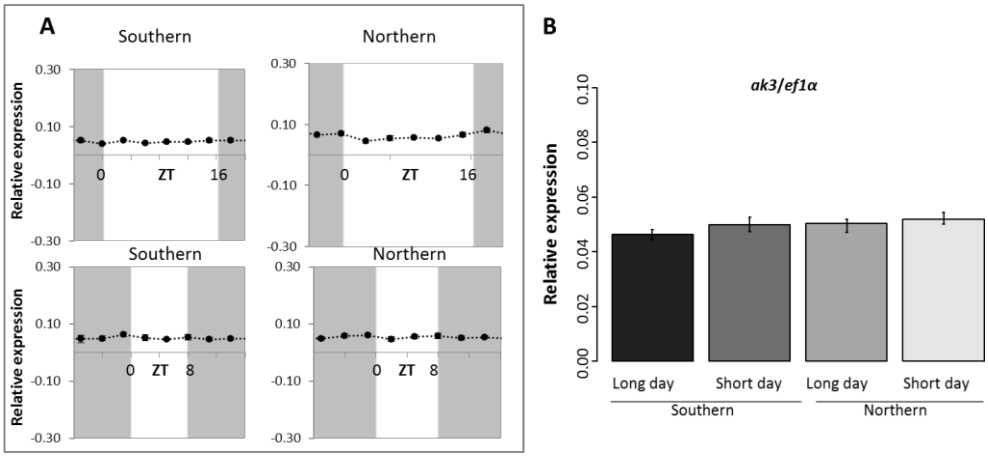

Fig. S1. Expression of the reference under long and short days in the southern and norther lines.
(A) The average relative expression of *ef1α* and *ak3* is compared among time points, under long days and short days, in the southern and northern lines. **(B)** the overall average relative expression is compared between long and short days and between southern and northern lines by two way ANOVA. *Zeitgeber time* (ZT) is given in hours on the X-axis where ZT=0 represents light on.

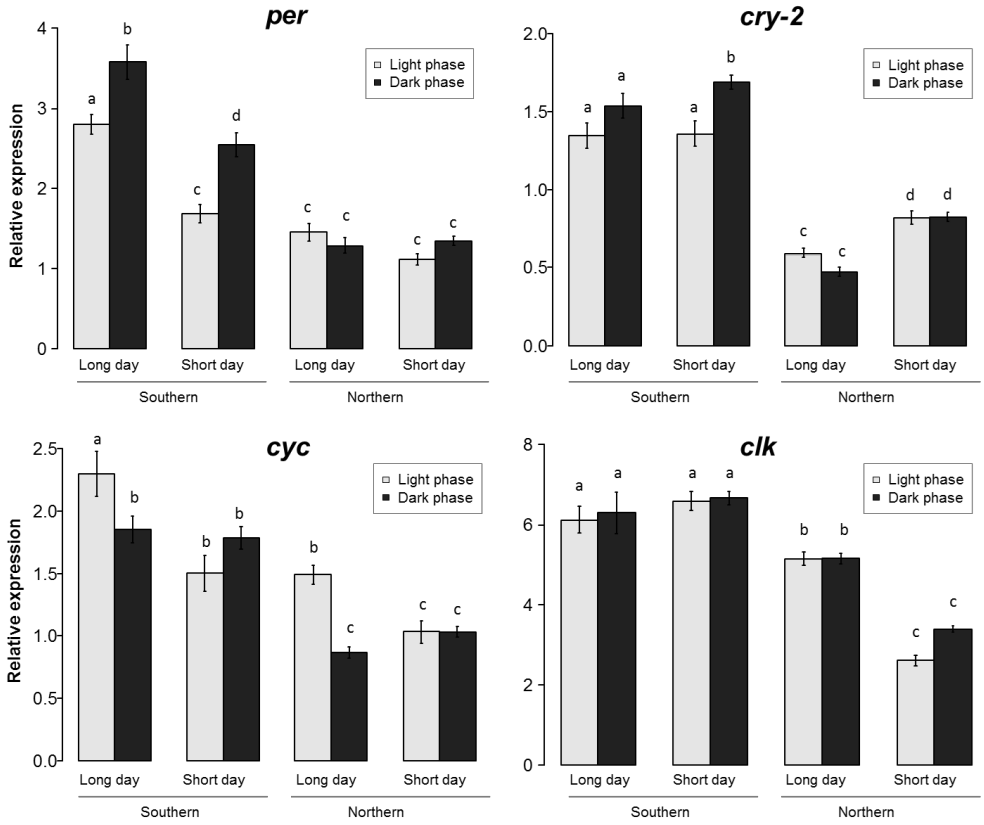

Fig. S2. Light and dark phase expression levels of southern and northern lines.
The average relative expression of clock genes *period* (*per*), *cycle* (*cyc*), *crypthochrome-2* (*cry-2*) and *clock* (*clk*) is compared between long days and short days and between light and dark phases in the southern and northern lines. Different letters represent significant differences between conditions analysed by two way ANOVA ($p<0,001$).