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

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This research has been carried out at the Groningen Institute for Evolutionary Life Sciences (GELIFES) of the University of Groningen (The Netherlands), according to the requirements of the Graduate School of Science (Faculty of Science and Engineering, University of Groningen).

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General introduction

Elena Dalla Benetta

Biological rhythms

The environment is the biotic and abiotic surroundings of an organism and a powerful driving force behind evolution. Since life first appeared on this planet it has been subjected to daily cycles of light and dark, and to seasonal cycles of climatic change, caused by the rotation of the earth around its axis and around the sun. All living organisms need to adapt their physiology, behaviour and metabolism in order to cope with this periodicity, especially those living towards high latitude where, owing to the Earth's axial tilt, daily and seasonal changes include extreme fluctuations in light and temperature. As a result, the evolution of endogenous clocks enables organisms from bacteria to plants and animals to exhibit biological rhythms to 'time' daily and annual events.

Biological rhythms can be divided into three broad classes on the basis of their periodicity: infradian, circadian and ultradian rhythms. Infradian (from the Latin infra, meaning "below", and dies, meaning "day") rhythms have periods longer than 24 hours, including seasonal or annual periods. Circadian (from the Latin circa, meaning "around", and dies, meaning "day") rhythms have an approximately 24 hours period and are found from single-celled organisms to vertebrates. Ultradian (from the Latin ultra, meaning "beyond", and dies, meaning "day") rhythms have a period shorter than 24 hours, ranging from several milliseconds to several hours. All these rhythms are necessary components of living organisms to ensure the proper timing of cellular/metabolic events, allowing the synchronization between external cues and internal functions.

Insects are particularly suited for studies on biological clocks. They are widely distributed and adapted to a wide range of climate conditions. Extensive studies have been made of insect rhythms, and these have contributed largely to our knowledge of basic characteristics of clocks that are common to all animals (Saunders et al., 2002). For example, many laboratory experiments on circadian rhythmicity in insects have been concerned with locomotor activity. Since such activity affects, or is affected by, most of the individual's physiological processes, an understanding of activity rhythms is an essential step towards understanding how living organisms adapt to their natural environment. Also photoperiodism, a response to the length of the light or dark period in a day, has been studied in many insects. It regulates many seasonal responses, such as diapause, seasonal morphs, growth rate, migration strategy, and a variety of associated physiological states. In this thesis I report further investigations into insect clocks, particularly the genetic basis of daily, seasonal and ultradian rhythms. I focus on seasonal photoperiodic diapause response, an adaptive trait that allows individuals to synchronize their life cycle with seasonal environmental cycles.

Infradian (seasonal) rhythm

Seasonality represents a selective factor influencing life-history traits of organisms. Most biotic and abiotic sources, used by organisms, are directly or indirectly related to seasonal changes. In order to cope with these seasonal changes, organisms need to synchronize growth, development, and reproduction with the seasonal presence of energy resources, mates, and favourable physical conditions. Organisms display a variety of seasonal responses such as hibernation in rodents, migration in birds and in large mammals, dormancy in plants and diapause in insects. Among different environmental cues, photoperiod (i.e. the ratio of light phase to dark phase within one light-dark cycle) is a reliable indicator that can be used to predict unfavourable conditions and sensitivity to photoperiodic signals helps organisms to adjust their life-cycle and development according to the seasons.

The system to detect and respond to photoperiodic changes includes: (i) an input pathway to measure external stimuli, such as light, (ii) a photoperiodic timer that distinguishes between short and long night (or day), (iii) a photoperiodic counter that accumulates information of successive short or long night (or day) periods, and (iv) an output pathway that transduces the photoperiodic information into signals that will lead to the proper seasonal response (Denlinger, 2002; Kostal, 2011). A photoperiodic calendar is responsible for the timing and counting of the light-dark (LD) cycles necessary for starting the photoperiodic response during the sensitive period (Saunders, 2013). When the number of photoperiodic cycles reaches a threshold, the response can be expressed, changing the activity of the output pathways (such as inducing diapause or stopping development) (Fig. 1.1). The photoperiod at which 50% of the population enters dormancy (or diapause), after a specific number of LD cycles determined by the counter, is called the critical photoperiod (CPP). The precise physiology of the timer and the counter remains however obscure, it is possible that they collaborate and are part of the same mechanism.

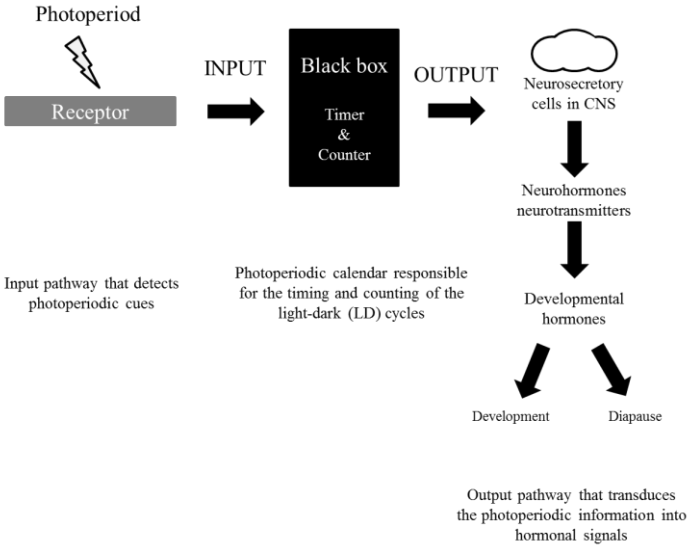

Fig.1.1. Schematic structure of the photoperiodic calendar
The environmental signals are transmitted by unknown receptors to a core mechanism composed of a photoperiodic calendar and counter. The mechanisms that underlie the function of the core complex are still unknown and for this reason they are represented as a black box. The output pathway involves neurosecretory cells and neurotransmitters that influence development via hormone production, such as the switch between active development and diapause (Kostal, 2011). CNS = central nervous system

Insects are the most diverse group of animals on the planet and occur in almost all terrestrial habitats. Their seasonal adaptation responses therefore evolved under the pressure of many different seasonal environments. For this reason insects are particularly suited to study seasonal adaptations. To survive adverse environmental conditions, many insects undergo diapause. Diapause is a state of reduced metabolism during which morphogenesis is stalled, resistance to environmental extremes is enhanced by an increase concentrations of cryoprotectants (Li et al., 2015) and behavioural activity reduced (Tauber et al., 1986). Due to its crucial role in seasonal regulation of life cycle, diapause is considered an important life-history trait that has been shaped by adaptive evolution.

Diapause typically occurs at a specific life stage and during a specific season. Insect species differ in the stage during the life cycle at which diapause occurs. For example, in the order Diptera, there are examples of photoperiodically induced embryonic diapause (e.g. the Asian tiger mosquito, *Aedes albopictus*), larval diapause (e.g. the pitcher plant mosquito, *Wyeomyia smithii*), pupal diapause (e.g. the flesh fly, *Sarcophaga bullata*), and adult reproductive diapause (e.g. the northern house mosquito, *Culex pipiens*). Furthermore, the sensitive stage and the diapause stage do not always overlap, they can occur in one and the same generation or in successive generations (Tauber et al., 1986).

When the sensitive stage occurs in one generation and the diapause stage in the next it is considered a "parental effect", i.e. the sensitive stage occurs in the female parent and the effect in the offspring. Examples of species with maternal induction of diapause are found among Sarcophagid flies, mosquitoes and parasitic Hymenoptera. In other species, such as Drosophilids, diapause occurs at the adult stage and sensitive and responsive stages overlap. The enormous variation in stage-specificity of diapause between and within a single insect order, and sometimes even within a single genus, indicates that there are multiple ways to evolve photoperiodic diapause, and that it likely has been altered several times during evolution.

Diapause is regulated by environmental cues that signal an upcoming change in the environment (for example approaching winter). Particularly in temperate and polar environments, where seasonal differences are pronounced, many animals evolved the capacity to measure and respond to changes in day length, or photoperiod. Photoperiod is not affected by changes from year to year. Hence, its predictability and persistency provide a reliable seasonal signal. Although less predictable, temperature has also a regular seasonal pattern and insects can respond to seasonal changes in temperature. In fact, in most insect species photoperiod and temperature interact to induce diapause (Hodkova & Socha 1995; Christiansen-Weniger & Hardie 1999; Saunders et al. 2002). Lower temperature increases the critical photoperiod CPP (i.e. the photoperiod at which 50% of the population enters diapause after a precise number of LD cycles), whereas high temperature decreases the length of the CPP necessary to induce the diapause response (Hodkova and Socha 1995; Christiansen-Weniger and Hardie 1999; Saunders et al., 2002). Other environmental factors can also affect diapause regulation, such as food availability, population density and humidity (Tauber et al., 1986). Insects are exposed to different biotic and abiotic factors that vary seasonally and that can interact with each other to induce diapause. In this thesis, I am investigating the role of photoperiod in the regulation of diapause, all the other environmental factors are carefully controlled and maintained constant throughout my experiments.

Circadian (daily) rhythms

In addition to the rotation around the sun, the axial rotation of the Earth has also deeply impacted the evolution of life. It causes daily variation in light and temperature. As a consequence, organisms show daily rhythms in metabolic function, physiological processes in order to be active at the "right time". The best example is the evolution of an internal timer that represents a very old biological clock, the so-called circadian clock. It can be found from bacteria to plants and animals. The daily light-dark cycles are the main forces to synchronize the endogenous clock every day to 24h, allowing adaptation of the organism to the environmental conditions. This introduction provides a short description of what is

known about the circadian clock mechanism of insects and mammals.

A timekeeping system responsible for daily rhythms involves three interacting elements: (i) an input pathway that transmits the environmental signals, such as light and temperature, (ii) the clock that represents a pacemaker characterized by clock genes, which expression oscillates with a period of 24h, and (iii) the output signals that control physiology and behaviour (such as rest-activity rhythms, courtship and mating), and development (such as hatching, pupation, or eclosion in insects) (Saunders et al., 2002) (Fig. 1.2). The rhythmic expression of clock genes within pacemaker cells and transcriptional-translational feedback loops between their products trigger the daily rhythms. Individual insects have active and inactive phases throughout the day, providing easily understandable examples of how behavioural or physiological rhythms are produced by circadian clocks and making insects a great model for chronobiology studies.

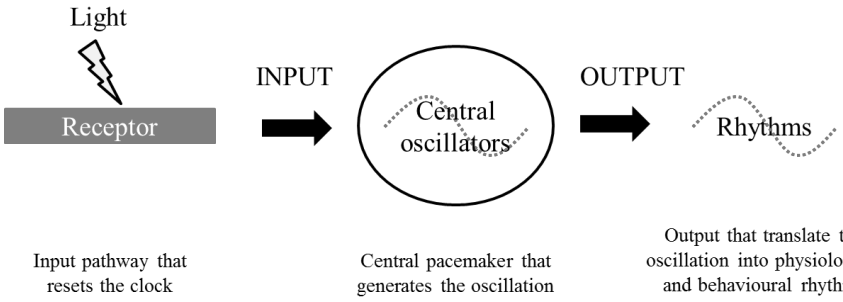

Fig. 1.2. General model for the circadian system
Light represents the environmental cue sensed by the input pathway. The light information is transduced to the pacemaker characterized by the clock genes and it generates the output rhythms, such as rest-activity, mating, and eclosion.

The molecular mechanism of circadian clock function is well known in some organismal groups, such as insects and mammals. Some differences between insects and mammals have been well characterized. The rhythm generation involves interaction and transcriptional translational feedback loops between the principal clock proteins (Table. 1.1). Transcription factors, kinases, phosphatases and other factors are indispensable for timing of expression. Despite the high degree of conservation between mammalian and dipteran circadian mechanisms, some genes appear to have different function in the two models.

In *Drosophila* CLOCK (CLK) and CYCLE (CYC) activate the transcription of other circadian genes such as *per* (*period*) and *tim* (*timeless*). The helix-loop-helix of these transcription factors (CLK and CYC) bind as heterodimers to E-Box sequences to enhance

the transcription of *per* and *tim* The transcription levels of *per* and *tim* start to increase during the second half of the day, and their mRNA reach peak expression at the end of the day (Fig. 1.3A). TIM and PER proteins accumulate in the cytoplasm only during the night because of the light sensitivity of TIM. Without TIM protection, during the light phase, PER is phosphorylated and targeted to degradation via proteasome. Consequently PER and TIM proteins accumulate with a delay of 6h with respect to their mRNA peaks. TIM and PER enter into the nucleus, in the second half of the night, where PER acts as transcriptional repressor. It inhibits CLK /CYC promoting the hyper phosphorylation of CLK and thus prevents CLK/CYC dimers from binding to promoters. Subsequently, the light causes the degradation of TIM during the next morning mediated by the photoreceptor CRY-1 (CRYPTOCHROME-1) (Emery et al., 1998). Hence TIM and PER prevent their own transcription in a periodic manner with this negative feedback loop (Peschel & Helfrich-Forster, 2011). Importantly the cycle re-starts every day owing to the light activation of CRY-1, allowing the synchronization of the rhythms to 24h. Furthermore, a second feedback loop regulates *clk* transcription; its mRNA peak reaches the maximum in the late night to early morning. CLK/CYC activates also the transcription of *vri* (*vrille*) and *pdp1ε* (*par-domain protein1-ε*) that bind the V/P-boxes in the promoter region of *clk*. Whereas VRI inhibits the transcription of *clk*, PDP1ε activates *clk*'s transcription helping thus the regulation of the positive element CLK. (Peschel & Helfrich-Forster, 2011).

Although the vertebrate clock follows the same pattern of transcriptional translational feedback loops, there are some differences in the players involved. First of all, CRY-2 and PER (instead of TIM and PER) inhibit CLK/BMAL1 transcriptional activity and they thus prevent their own transcription (Fig. 1.3B). BMAL is the mammalian orthologue of *Drosophila* CYC (Table.1). Furthermore, in mammals the activity of CRY appears independent of light and for this reason the vertebrate CRY is called CRY-2 (Froy et al., 2002; Staknis, & Weitz, 1999; Zhu et al., 2005). There is no true orthologue of *Drosophila* TIM, but rather an orthologue of *Drosophila* TIMEOUT of which the function is unknown in the fly's clock. In addition BMAL1 (represents the positive element that oscillates in anti-phase to the negative elements (PER and CRY) (Fig. 1.3B).

Several studies from Hymenoptera, such as *Apis mellifera* and *Nasonia vitripennis* indicate that different insects present different circadian models compared to *Drosophila*. These species do not display orthologues of *Drosophila cry-1* and *tim*, rather, they possess orthologues of *Drosophila timeout* (*tim2*) and mammalian-type *cry-2* (Fig. 1.3C). Moreover, analysis of the honeybee and wasp clock protein oscillations revealed an expression pattern more similar to mammals than *Drosophila* (Bertossa et al., 2014; Rubin et al., 2006). This illustrates that natural selection may have led to the evolution of different gene functions, while the same mechanisms of rhythm generation have been maintained. The main difference among Diptera and vertebrates is the role of CRY proteins. In mammals CRY-2 works in the negative feedback loop and represents the main

transcriptional repressor of the clock (Froy et al., 2002; Staknis, & Weitz, 1999; Zhu et al., 2005). In contrast, in *Drosophila* CRY-1 represents the photoreceptor working in the light input pathway of the clock (Konopka et al., 2007), but the hymenopteran clock system appears more vertebrate-like than *Drosophila*-like. More studies on non-model organisms are clearly needed for a more comprehensive picture of the evolution of biological clocks..

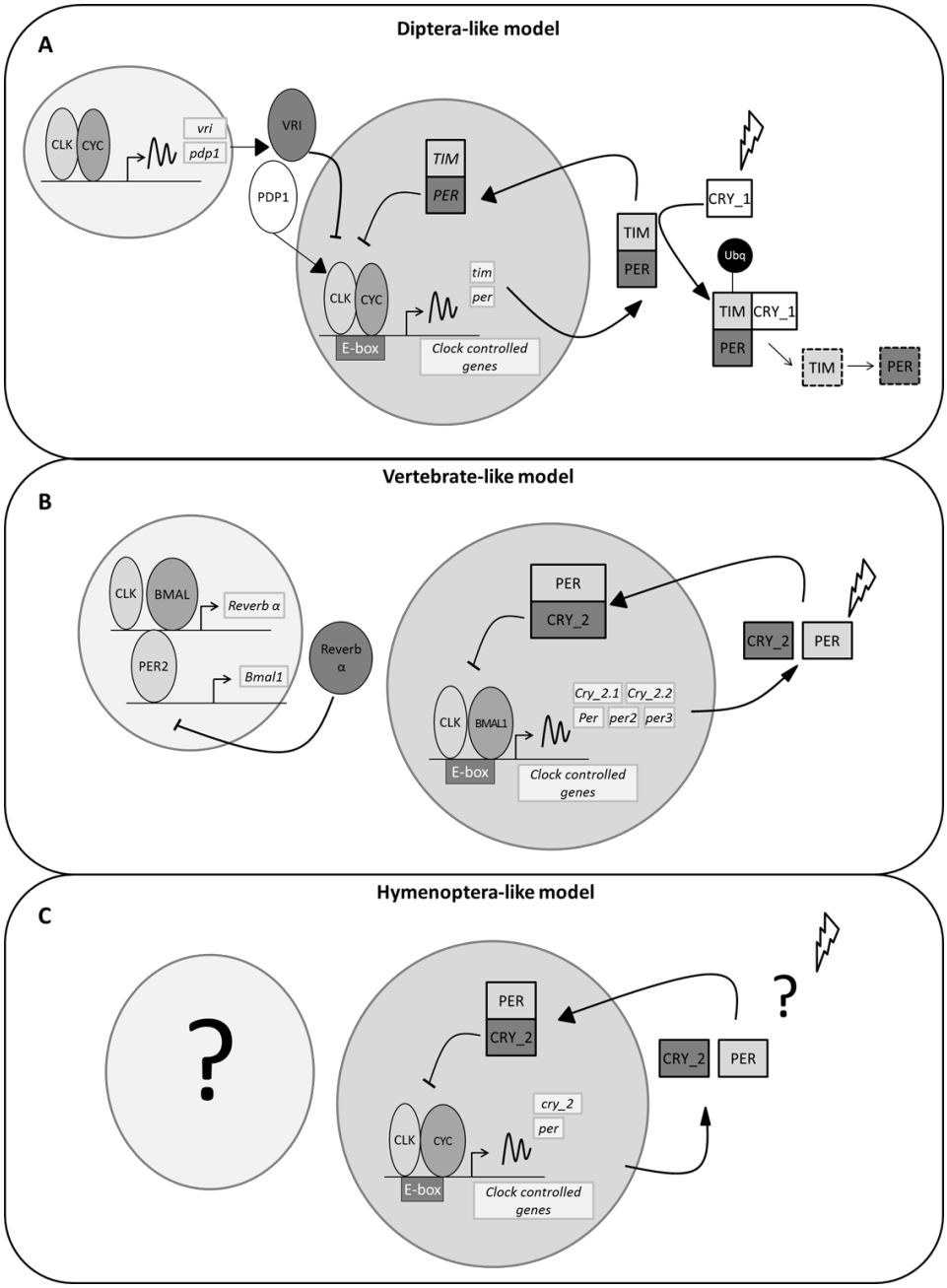

Fig. 1.3. Circadian clock models

(A) Schematic representation of the Diptera-like circadian mechanism. **(B)** The vertebrate clock system and **(C)** the Hymenoptera system (details in the text).

Table 1.1 Principal proteins involved in dipteran and vertebrate circadian clocks

Protein	Abbrev.	Function in Diptera-like model	Homologous protein in mammals	Function in vertebrate-like model
CLOCK	CLK	Transcription factor that binds E-box of target genes	CLK	Transcription factor that binds E-box of target genes
CYCLE	CYC	Transcription factor that binds E-box of target genes	BMAL1	Transcription factor that binds E-box of target genes
PERIOD	PER	Transcriptional repressor that inhibits CLK/CYC functions	PER1/2	Transcriptional repressor that inhibits CLK/CYC functions
TIMELESS	TIM	Part of negative feedback loop in conjunction with PER	No homologue found	-
CRYPTOCHROME	CRY	Blue light photoreceptor	No homologue found	-
CRYPTOCHROME-2	CRY-2	Absent	CRY-2	Part of negative feedback loop in conjunction with PER
TIMEOUT	TIM2	Unknown function in the clock	TIM2	Unknown function in the clock
VRILLE	VRI	Transcriptional repressor of *CLK*	REVERB-α is analogue but no homologue	Transcriptional repressor of *BMAL*
PAR-domain protein-1	PDP1	Transcriptional activator of *CLK*	ROR is analogue but no homologue	Transcriptional activator of *BMAL*
DOUBLETIME	DBT	Phosphorylation of PER	Casein kinase 1 (CK1-ε)	Phosphorylation of PER1/2
CASEIN KINASE 2	CK2	Phosphorylation of TIM/PER complex	-	-
PROTEIN PHOSPHATASE 2A	PP2A	De-phosphorylation of TIM/PER complex	-	-
SLIMB	SLIMB	Ubiquitin E3 ligase to target PER to proteasome	-	-
SHAGGY	SGG	Phosphorylation of TIM and CRY light-dependent	-	-
JETLAG	JET	F-box protein that mediates ubiquitination of TIM and CRY	-	-

Ultradian rhythm

A biological rhythm is called ultradian if its period is shorter than 20 hours (Halberg et al., 1965). Ultradian rhythms have been observed in physiological functions, like cellular processes, respiration, circulation, and hormonal release and sleep stages, as well as in behavioural functions, often related to feeding patterns. Specific stages of reproduction are also accompanied by short-term rhythms, e.g. courtship behaviour and breeding (Daan & Aschof, 1981). These rhythms have been recognized only since 1979 with their discovery in the soil amoeba *Acanthamoeba castellanii*. They were initially called the "Epigenetic clock" and characterized as a temperature-compensated ultradian timekeeper (Edwards & Lloyd, 1978, 1980; Lloyd et al., 1982). Due to the difficulties in studying such fast rhythms, very little is known about the genetic mechanisms underlying ultradian rhythmicity. Ultradian rhythms appear to not only differ in length (from hours to milliseconds) but also in mechanisms and functions. In general, functions of ultradian rhythms have been described in terms of energetic optimization, internal coordination or social communication, as in the case for insect courtship songs (Kyriacou & Hall, 1980).

Of particular interest are the ultradian rhythms in the range of seconds such as insect courtship songs. Male song is an important courtship display in many insect species. It differs between species and can affect mate choice and reproductive isolation (reviewed in Alt et al., 1998; Greenspan & Ferveur, 2000), as shown by females that mate more when played songs with species-typical parameters (Bennet-Clark & Ewing, 1969; von Schilcher, 1976b; Kyriacou & Hall, 1982; Ritchie et al., 1999). The rhythmic component of this behaviour might involve a timer mechanism that regulates the pace of the rhythm. It has been hypothesized that the circadian clock also plays a role in ultradian rhythms, as mutations of clock genes alter in a parallel fashion both circadian and courtship song cycles (Kyriacou and Hall, 1980). Despite the broad recognition that these cycles exist, we know virtually nothing about the underlying genetics of ultradian rhythms.

Latitudinal variation of biological rhythms

Species with a wide distribution range encounter a great diversity of climate conditions and variability in seasonal conditions among localities because annual patterns in light and temperature vary with latitude (reviewed in Hut et al., 2013). For example, the variation in day length is larger at higher latitude, where photoperiod strongly increases during summer and decreases during winter, with continuous light and continuous darkness near the poles at the peak of the season. In contrast, at the equator light-dark cycles are constant, although other environmental factors, such as precipitation, may vary seasonally. Throughout the year, the incidence of solar radiation is more perpendicular at the equator than at the poles,

causing higher temperatures around the equator. This leads to latitude-specific selection pressures in many organisms, corresponding to large variation in life cycles as a result of local adaptation. Phenotypic and genotypic clines are often the result, which can yield information on the underlying mechanisms of seasonal adaptation (Hut & Beersma, 2011).

Latitudinal variation in seasonal response

The latitudinal variation in photoperiodic response has been investigated in many insects in which shortening of the photoperiod indicates the upcoming of unfavourable season and trigger the induction of diapause. Positive correlation between latitude and the critical photoperiod CPP, necessary for triggering a diapause response, has been first described in 1965 by Danilevskii for the knot grass moth *Acronicta rumicis* (Danilevskii, 1965) and later for many other insects (Saunders, 2013), including in the maternally induced diapause of *Nasonia vitripennis* (Paolucci et al., 2013),. Latitudinal clines in CPP has an adaptive significance. At higher latitudes winter starts early in the year when days are still long, thus a long critical photoperiod allows proper winter anticipation. On the other hand, at lower latitudes, temperature interacts with photoperiod to modulate diapause. Higher temperature at lower latitudes decreases the CPP necessary to trigger the seasonal response. Populations at lower latitudes enter diapause under much shorter photoperiod compared to populations at higher latitudes. This shorter CPP at lower latitudes allows organisms to enter diapause later in the year and to fully exploit the favourable season. Photoperiodic induction of diapause includes an accumulation counter, where individuals must experience a specific number of short days to enter diapause (Saunders, 2013). The latitudinal variation in the required number of short days has a similar adaptive significance, in which faster responses are beneficial at higher latitude where seasonal changes are faster (reviewed by Hut et al., 2013). However the genetics and the mechanism behind this faster and slower latitude-dependent photoperiodic response are unknown.

Latitudinal variation in circadian response

Latitudinal variation in circadian rhythms has not been studied as extensively as photoperiodism. Daily rhythms may also associate with latitude because they are synchronized by light-dark cycles. As previously discussed, light-dark cycles vary with latitude. Therefore, such variation might reveal important selection pressures on circadian function as well. Latitudinal clines in free running rhythms have been observed in plants (*Arabidopsis thaliana*), where the period (τ) of the rhythms in constant darkness (DD) increased with latitude (Michael et al., 2003). In insects similar findings were described for the linden bug *Pyrrhocoris apterus* (Pivarciova et al., 2016) and for *Nasonia vitripennis*

(see below; Paolucci, 2014). A positive latitudinal cline in DD rhythms was reported for several *Drosophila* species. Some species, such as *D. auraria* (Pittendrigh & Takamura, 1989) and *D. ananassae* (Joshi & Gore, 1999), showed a positive correlation between latitude and length of free running rhythm, whereas *D. littoralis* and *D. subscura* show an opposite cline with shorter tau towards northern latitude (Lankinen, 1986). Other geographical differences in circadian clock properties have been described in *Drosophila* and reflect a different neuronal architecture in the expression of clock genes (Menegazzi et al., 2017). Northern *Drosophila* species (such us *D. montana* and *D. littoralis*) present unimodal evening activity compared to more southern *Drosophila* species (such as *D. melanogaster*), which exhibit a bimodal activity rhythm with morning and evening activity bouts. Interestingly the northern *Drosophila* species lack the neuropeptide pigment-dispersing factor, PDF, in one set of lateral clock neurons. PDF in these neurons controls the morning activity of the southern *Drosophila* species (Hermann et al., 2013; Kauranen et al., 2013). Northern and southern *Drosophila* species evolved thus differential clock gene expression in their pacemaker, important for their local adaptation. All together these findings indicate that selection acted also on the circadian system. Yet, little is known about natural genetic variation in clock genes and how they determine phenotypic variation.

Interaction of circadian and photoperiodic systems

Bünning (1936) first proposed that the photoperiodic response is based on circadian functions because both phenomena react to time-giving cues from environmental light-dark cycles. Although both processes are essential to organize the temporal pattern of a variety of processes, these two functions are very different but at the same time complementary. Although both need the environmental stimuli (such as light) to synchronize with the environment, the circadian pacemaker can free run in the absence of external cues (i.e. under DD or LL). The light in this case is important to reset the clock every day to 24h .On the other hand, the photoperiodic calendar needs the interaction with the environment to start the photoperiodic response. Furthermore, the circadian system is temperature compensated and able to keep a 24h period in a wide range of temperatures. In contrast, the photoperiodic system is highly sensitive to changes in temperature (Tauber & Kyriacou, 2001). Bünning's hypothesis has been supported by studies on fungi, plants, mammals and insects (Putterill, 2001; Roenneberg et al., 2010). However, among the insects, some species show clear evidence for the involvement of the circadian system in photoperiodic time measurement, such as the spider mite *Tetranychus urticae* (Goto, 2016), whereas others do not, such as the aphid *Megoura viciae* (Lees, 1973) and the fly *Drosophila ezoana* (Vaze & Helfrich-Förster, 2016). For this reason, the role of circadian rhythmicity in insect photoperiodism has remained somewhat controversial (Bradshaw & Holzapfel, 2010b).

Models of interaction of circadian and seasonal mechanisms

Nowadays, three models exist to explain the influence, or the absence, of the circadian clock on photoperiodic response (Kostal, 2011)(Fig. 1.4). First, the Independence or Hourglass model (Lees, 1973) excludes the involvement of the circadian clock in photoperiodic time-measurement. It considers the two systems completely separate and while the circadian pacemaker system controls the daily rhythms, the hypothetical hourglass-based photoperiodic system is responsible for the seasonal adaptation. An hourglass is a mechanism that follows a set time course in darkness after being initiated at lights off and needs a minimum duration of light to restart the measurement process at the beginning of the next dark phase. When dawn interferes with this process the photoperiod is sensed as a short night. Importantly, at the end of the night phase the hourglasses are turned and the whole sequence of reactions must be restarted daily by light (Fig. 1.4A).

The other two types of models try to explain the possible interaction between circadian and seasonal timing: the external- and internal-coincidence models. In the external coincidence models (Bünning, 1960) light has a dual role: entrainment of the circadian rhythm and photoperiodic induction, thus there is only one system with dual function and the daily synchronization to 24h is sufficient to secure both daily and photoperiodic responses. In this system the circadian clock is sufficient to drive daily rhythms and photoperiodic response because the photoperiodic calendar activities rely on seasonal plasticity in clock genes expression. When the photo-inducible phase of the oscillator generated by the circadian system falls in the dark, owing to the shortening of the day length, the day is sensed as short (long night). Vice versa, if this phase falls in the light, the day will be sensed as long (short night) (Fig. 1.4B). Finally, the internal coincidence model (Pittendrigh, 1972) involves the cooperation between two oscillators. They are physically separated, or partially overlapping, but in close cooperation. The light has only one role: entrainment of multiple oscillators. The internal coincidence model assumes a change in phase between the two circadian oscillators triggered by the change of photoperiod to be responsible for sensing seasonal changes (Fig. 1.4C). Functional studies aimed to disrupt circadian clock properties were also able to disrupt diapause induction (see below), indicating a role of clock genes in the regulation of diapause. However, the pleiotropic effect of single clock genes in both systems cannot be excluded, and due to the complexity of separating the two systems, the debate is still ongoing.

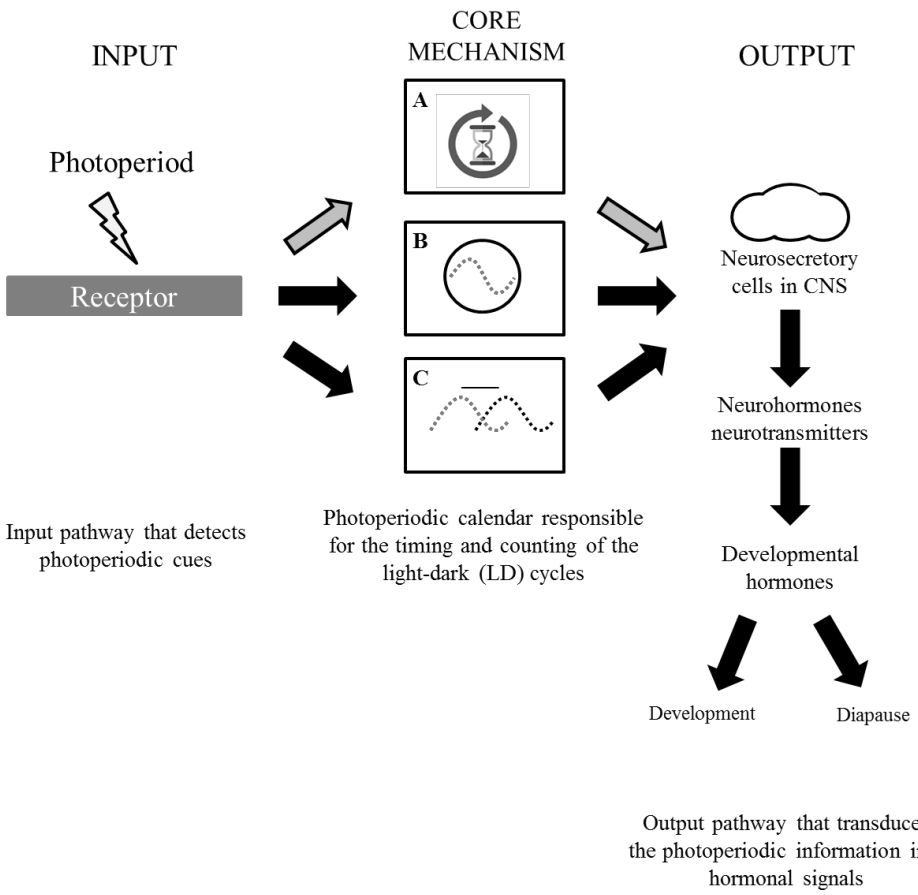

Fig. 1.4. Models of no-interaction and interaction between seasonal and circadian clock
(A) Represents the hourglass model in which the two mechanisms are physically separated and functional independent. **(B)** Assumes the presence of only one system with both functions. **(C)** Predicts the cooperation of the two systems although they can be physically separated whereby different subsets of neurons perform circadian clock and photoperiodic calendar functions.

Evidence for the role of the circadian clock in insect photoperiodism

Although the role of the circadian clock in photoperiodism is still controversial, there are some observations in support of the fact that the clock perceives time in a seasonal temporal domain, and turns it into a photoperiodic response. Most of studies are of clock mutants, geographical clines in the frequencies of clock genes, and from functional studies that knock down clock gene expression. Here, I review the most important observations for an involvement of clock genes in the regulation of diapause in insects.

Geographical variation in clock genes, associated to local adaptation, has been reported in many insects. *Period* is a very conserved clock gene among organisms (Konopka & Benzer, 1971). The first important characteristic of the *Drosophila* PER orthologue is the presence of a threonine-glycine repetitive (Thr-Gly) encoding-repeat; it is polymorphic in length and shows a robust latitudinal cline in natural *Drosophila melanogaster* populations (Costa et al., 1992; Sawyer et al., 1997; Zamorzaeva et al., 2005). Northern European populations exhibit higher frequencies of the longer $(Thr\text{-}Gly)_{20}$ length variant compared to the southern lines, and accordingly, the shorter $(Thr\text{-}Gly)_{17}$ variant predominates in the south (Costa et al. 1992). The cline might reflect an adaptive response to climatic variation due to differences in thermostability of PER variants. Northern populations indeed have very efficient temperature compensation mechanisms in which the free running period at high (25°C), as well as low (18°C) temperature is close to 24h. Contrary, in southern populations, with high frequencies of the shorter repeat $(Thr\text{-}Gly)_{17}$, the free running period changes significantly under the two temperature conditions; τ is shorter than 24h at lower temperature (Sawyer et al. 1997). Therefore, carrying the $(Thr\text{-}Gly)_{20}$ variant is more favoured in the thermally variable northern areas, and the shorter allele in the warmer southern locations. However a functional role of this variation in diapause induction of *Drosophila* is not proven. Interestingly a latitudinal cline of *per* alleles has also been found in *Nasonia vitripennis*, correlating with a latitudinal gradient in diapause induction. (Paolucci et al., 2013, 2016; more details below).

In *Drosophila* a similar cline was observed for the clock gene *timeless* (*tim*) (Tauber et al., 2007). The *tim* alleles differ in light sensitivity (due to different interaction with the CRY-1 proteins) leading to different photo-responsiveness. Moreover, the different alleles of this gene were found to influence diapause incidence (Sandrelli et al., 2007; Tauber et al., 2007). Thus, it has been hypothesized that different circadian photo-responsiveness associated to the two *tim* alleles contributes to translating the photoperiodic information (Tauber et al., 2007; Zonato et al., 2017a, 2017b). Similarly to *Drosophila*, *tim* has been suggested as a possible linker between the circadian and photoperiodic system in the fly, *Chymomyza costata* (Stehlík et al., 2008). The cycling of *tim* mRNA was lost in the heads of non-diapausing (npd) mutants and TIM protein was undetectable (Pavelka et al., 2003; Stehlík et al., 2008). Similarly, work on npd mutants showed a reduced *per* mRNA

level in the non-diapause mutant (Kostal & Shimada, 2001). Work on photosensitive larvae of the fly, *Sarcophaga crassipalpis* (Kostal et al., 2009) also suggests photoperiodic sensitivity of *per* and *tim* expression, as a phase-shift of peak of mRNA was observed under long day and short day conditions. Therefore, it appears that selection on clock gene expression plays an important role in shaping local adaptation in many insect species. However, these data raise a number of interesting questions that require further investigation. For example, whether different alleles of clock genes are involved in seasonal adaptation and how they can translate the photoperiodic information into a diapause response.

Involvement of the circadian clock in photoperiodic diapause has recently been studied with the RNA interference (RNAi) technique in the bean bug *Riptortus pedestris* (Ikeno et al., 2010, 2011a, 2011b) and the mosquito *Culex pipiens* (Meuti, 2015). Knock down of the negative circadian regulators *per* or *cry-2* (and *tim*) resulted in a non-diapause phenotype under short day, diapause-inducing conditions. In contrast, when the positive circadian regulators *clk* or *cyc* were knocked down, the insects displayed a diapause phenotype under long day, diapause-preventing conditions. These results contradicted Saunders (1989) who showed that mutation of *per* genes in *D. melanogaster* were not able to affect diapause phenotype, however *Drosophila per* mutants showed a change in the critical photoperiod necessary to trigger the diapause response (Saunders et al., 1989). Although these experiments revealed a disruption of the circadian rhythm as well as of the diapause response, the pleiotropic effect of a single clock gene in the regulation of daily and seasonal responses cannot be excluded.

The presence of clines in the frequencies of clock gene alleles associated with local adaptation, and the functional involvement of clock genes in circadian and seasonal responses, point toward a regulative role of the circadian clock in diapause induction in insects. However, the function of clock genes could also be interpreted as pleiotropic effects of a single clock gene playing an independent role in both mechanisms. Therefore, more studies are necessary to access the exact role of the clock in the regulation of photoperiodic time measurement. I will investigate the possible role of clock genes in photoperiodism and diapause induction in the wasp *Nasonia vitripennis*, making use of natural genetic variation in circadian as well as in seasonal rhythms, clock gene expression and by performing functional analysis on the *period* gene.

Nasonia as an emerging insect model for chronobiology

Nasonia is a genus of small parasitoid wasps belonging to the hymenopteran order. They are 2-3 mm in size and parasitize blowfly pupae (Calliphoridae). *Nasonia vitripennis* (jewel wasp) has become a model organism in evolutionary genetics and development because of

some unique advantages (Beukeboom & Desplan, 2003). First of all, they have short generation time of about 15 days at 25°C and are easy to culture on commercially available host pupae. Offspring can be sexed at the white or black pupal stages, in the pupal stage females are usually larger than males, they have longer wings that are visible in lateral view and an ovipositor that can be easily identified frontal view. In addition, wasp lines can be maintained in diapause for more than one year allowing the maintenance of many stocks. Another important feature is its haplodiploid reproduction, males are haploid and develop from unfertilized eggs and females are diploid and develop from fertilized eggs. This allows the expression of recessive traits in males and facilitates screens for developmental mutants and the identification of candidate genes. Moreover, the genome of *Nasonia* is sequenced (Werren et al., 2010). Consequently, there are a lot of molecular tools available, such as high-density markers for QTL analysis, RNAseq, and systemic RNAi (Lynch, 2015).

Four species of *Nasonia* are known: the cosmopolitan *N. vitripennis* and three endemic North America species *N. giraulti*, *N. longicornis* and *N. oneida*. They differ in morphology and behaviour but can produce fertile hybrids in the laboratory allowing analysis of the genetic basis of these differences. *Nasonia vitripennis* will be used in this study because of its cosmopolitan distribution. As it can be found all over the world covering a wide range of climate conditions, it is ideally suited as a model for studying photoperiodism and circadian rhythms in insects.

Timing components in *Nasonia* biology

Nasonia vitripennis is an excellent model for chronobiology studies because it is known to exhibit several biological rhythms. Its widespread distribution provides the opportunity to analyse the adaptive value of these biological rhythms and their genetic basis. Here, I describe seasonal, circadian and ultradian rhythms of *N. vitripennis* and the current knowledge of the genetics underlying these rhythms.

Seasonal rhythm

In temperate zones, *Nasonia* overwinter as diapause larvae inside the host puparium. Diapause is induced by different factors, such as day length, temperature and food deprivation, which affect the maternal generation. (Fig. 1.5B). In general when adult females experience short day conditions, or more precisely the Critical Photo Period (CPP), they initially produce normal developing larvae, but after exposure to a specific number of light-dark cycles they switch to production of diapausing larvae. The photoperiod, at which

50% of the females induce larval diapause, after a precise number of LD cycles, 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). Therefore a clock mechanism is responsible for the timing and counting of the light-dark cycles necessary for starting the photoperiodic response (Saunders, 2013). Under longer photoperiods the switch occurs later or not at all and short photoperiod elicits a higher induction of larval diapause than long photoperiod. (Saunders, 1969, 2010, 2013). Paolucci et al., (2013) described geographical variation in photoperiodic diapause response, and the existence of a positive correlation between geographical origin and proportion of diapausing broods that is underlain by genetic differences. Wasps from northern European 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 predominant *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*.

Circadian rhythm

The circadian clock of *Nasonia* resembles that of other hymenopteran species like honeybees and ants (Fig. 1.3C). They miss insect *cryptochrome-1 (cry-1)*, which is still present in Dipterans and Lepidopterans. Instead they have the mammalian type *cry-2*, that it is part of the core feedback loop (Yuan et al., 2007, Bertossa et al., 2013). In *Nasonia per* and *cry-2* represent the negative elements of the circadian clock. They inhibit their own transcription, by inhibiting *cycle* (*cyc*) and *clock* (*clk*). c*lk* and *cyc* represent the positive elements of the clock, activating the expression of E-Box genes like *per* and *cry-2* (Hardin, 2004; Stanewsky, 2003). *Nasonia cyc* is homologous to mammalian BMAL, 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/BMAL heterodimer in mammalian cell cultures (Takahata et al., 2000) representing the region where CRY-2 binds to act as repressor (Sato et al., 2006). Due to the absence of the photoreceptor *cry-1* and the other negative element *tim*, the light signalling pathway in *Nasonia* is still unknown. Different candidate genes can be involved in the light pathway including *per*, *cry-2* and opsin genes.

Circadian rhythms in activity and emergence from the host puparium have also been found in *N. vitripennis* (Bertossa et al., 2010). Emergence of males from the host occurs at the same time of the day in consecutive days, between dark and light phase,

indicating that they are able to advance the light-on signal. Locomotor activity rhythms present a unimodal diurnal pattern for both sexes of all four *Nasonia* species (Bertossa et al., 2013). In addition, *Nasonia* shows free running rhythms under constant darkness (DD) and constant light (LL) conditions (Fig. 1.5C). Geographical variation of circadian properties was also described by Paolucci (2013), with an increasing of the length of the DD rhythm towards northern latitude. The adaptive significance of latitude-dependent circadian properties is not well understood and further investigation of these geographical differences in circadian behaviours of *Nasonia* in relation to different *per* alleles is required.

Ultradian rhythm

Courtship in many animal species consists of a repertoire of specific signals delivered by the male and the female during the mating event. These signals may play a role in species recognition as well as in sexual selection within the species. Male signals are an important courtship display in many insect species. They are often species-specific and can affect mate choice and reproductive isolation (reviewed in Alt et al., 1998; Greenspan & Ferveur, 2000; Talyn & Dowse, 2004). *Nasonia* male courtship behaviour represents an example of ultradian rhythm. *Nasonia* males court females by performing series of strong movements with their head, so-called "head-nods" that are accompanied by wing vibrations, and that are interrupted by pauses, together making up a series of cycles (Fig. 1.5D) (van den Assem et al., 1980) . The rhythmic head-nods display during male courtship behaviour is important for inducing female receptivity by enabling the rhythmic release of pheromones (reviewed in van den Assem & Beukeboom, 2004). The ultradian pattern of cycle duration and head-nods number is species specific and genetically determined (van den Assem et al., 1980), but very little is known about the genetic mechanism that underlies this ultradian rhythmicity.

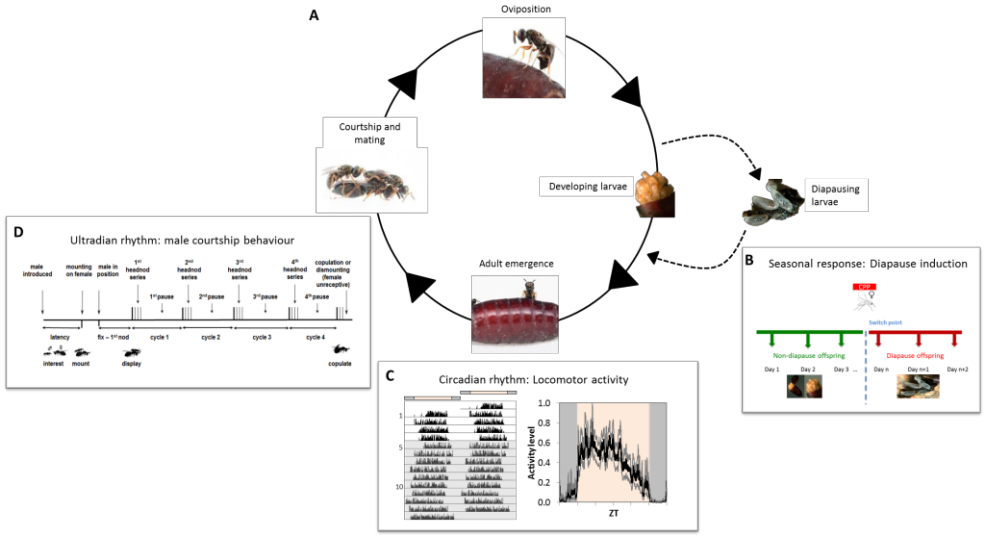

Fig. 1.5 Life cycle and biological rhythms of *Nasonia vitripennis*

(A) Schematic representation of the life cycle in *Nasonia vitripennis*. *Nasonia* is a gregarious parasitic wasp, which uses the pupae of different fly species as host. The flies are parasitized by *Nasonia* females, which drills through the host puparia wall with her ovipositor, inject venom that kills the fly, and lay eggs that develop inside the host. The wasp larvae feed on the host, pupate and emerge as adults from the host puparium. Males typically emerge 1 or 2 days earlier than females and wait for females outside the host to mate. **(B) Seasonal rhythm**. Diapause response in which a *Nasonia* female senses the photoperiod and produces normal developing offspring (green) until the switch point, after which she produces diapausing offspring (red) that stall their development at the fourth instar larval stage**. (C) Circadian rhythm.** Locomotor activity is represented by a double plotted actogram on the left in which the black bars represent the activity level. The dark phase is shown by the grey bars and the light phase by the orange bars. The grey shade represents the DD (constant darkness) conditions. On the right the average daily activity indicates the timing of locomotor activity throughout 24h. Zeitgeber Time (ZT) is given in hours on the x-axis where ZT=0 represents light on, grey shade represents the dark phase and orange shade the light phase. **(D) Ultradian rhythm.** Schematic representation of *Nasonia* courtship display. After introduction of the male to the female there is a latency period during which the partners locate each other. The male mounts the female and positions himself on her head. Following rhythmic display movements include repeated series of head noddings and pauses in cycles. Vertical lines represent separate head-nods. The interval between the first nod of two consecutive series is used as a measure of cycle duration. Figure is adapted from van den Assem & Beukeboom (2004). Wasp symbols are from Clark et al. (2010). Photos by Jitte Groothuis.

Aims of this thesis and chapter overview

This PhD research is part of the EU funded Marie Curie Initial Training Network (ITN) "INsecTIME", a consortium of academic institutes and SMEs (Small and Medium Enterprises) spanning seven EU countries, including Israel. Its aim is to study the molecular genetic basis of insect biological timing.

The focus of this thesis is the study of genetic variation in the architecture of biological rhythms in *Nasonia vitripennis*, with particular focus on the role of clock genes in photoperiodic diapause response. I address the following questions:

1. Does the circadian rhythm differ between populations of different latitude, i.e. southern and northern *Nasonia* lines, carrying different *per* alleles?
2. Are photoperiodic changes able to affect the daily expression of candidate clock genes?
3. How can geographical variation in the expression of clock genes be translated into a seasonal response to allow local adaptation?
4. Is the clock gene *period* involved in ultradian, circadian and seasonal responses in *Nasonia*? What is its function in regulating these rhythms?

I selected southern and northern populations from the extreme ends of a cline described by Paolucci et al (2013, 2016), carrying either a southern- or the northern-specific *per* allele. To further investigate the geographical variation in biological rhythms, I generated isogenic lines and used a combination of behavioural assays, gene expression analyses and functional tests.

In Chapter 2 I describe the natural variation in circadian rhythmicity in *N. vitripennis* originating from southern (Corsica, France) and northern (Oulu, Finland) populations, which represent the two extremes of the cline described by Paolucci and co-workers (2013, 2016). I compare five different isogenic lines from both localities for their timing and level of locomotor activity under long and short photoperiods. I also analyse whether their free running rhythms differ in constant darkness (DD) and constant light (LL). Significant differences are detected in the timing of onset, maximum peak and offset of activity as well as in the level of activity. I also find different free running rhythms under DD and LL between southern and northern lines.

In **Chapter 3**, I investigate the role of clock genes in photoperiodism, 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 is analysed. This allows assessing if changes in clock gene expression correlate with the adaptive behaviour of diapause induction in *Nasonia*. The results contribute to the understanding of the link between photoperiodism and circadian clock, as hypothesized long ago. Additionally, in **box 1**, I describe two splicing

variants of the clock gene *per* that could lead to different PER proteins, which in turn can play a role in detecting and translating photoperiodic information.

In order to understand the adaptive evolution of seasonal response, it is essential to establish if and how the circadian clock is involved in this process as a module or single gene. Therefore, in **chapter 4,** I investigate the functional involvement of the clock gene *per*, both in the circadian rhythm and in the photoperiodic diapause response of *N. vitripennis*. I first analyse the effect of *per* knock-down by RNA interference (RNAi) on locomotor activity behaviour under LD cycles and under constant conditions. Second, I test how per RNAi affects photoperiodic diapause response. Additionally, it is important to understand whether the expression pattern of other clock genes, *cry-2*, *clk*, and *cyc*, is also affected by *per* RNAi to determine the role of *per* in the feedback loop of the circadian clock. I also ask whether genetic variation for clock genes can influence the seasonal phenotypes by comparing southern and northern strains of *N. vitripennis* that differ in *per* alleles, locomotor activity and diapause response. These data reveal a role of *per* in the core mechanism of the circadian clock, and a role in photoperiodic time measurement in *N. vitripennis*.

In **chapter 5** I investigate the role of the clock in ultradian rhythms of male courtship behaviour. I test whether southern and northern lines of *N. vitripennis* differ in male courtship behaviour in terms of cycle duration and head-nod number by knocking down *period*. I show an involvement of this gene in timing of head-nods series and cycle time during the courtship performance. The implementation of a new genetic tool in *Nasonia*, namely CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) is described in **box 2**. This technique, that employs RNA-guided endonucleases to specifically target and degrade DNA, will allow to induce gene know-out and knock-in of any gene of interest in the future. In particular it will allow making stable lines carrying one or multiple mutations in clock genes.

In the final **chapter 6** I synthesize the results of my research, summarize the current knowledge about photoperiodism in insects and propose some directions for future research. I present a possible model for the interaction between the circadian clock and other timing-related traits (seasonal diapause and ultradian courtship).

Chapter 2

Geographical variation in circadian clock properties of *Nasonia vitripennis*

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To be submitted

Abstract

The endogenous circadian clock regulates many physiological processes of living organisms. In the parasitic wasp *Nasonia vitripennis*, natural variation in photoperiodic diapause response is correlated with allelic variation of the clock gene *period* (*per*), which in turn affects circadian clock properties. To investigate if this variation is also correlated with circadian behaviour, we compared the locomotor activity and free running rhythms of northern (Oulu, Finland) and southern (Corsica, France) lines of *N. vitripennis* that carry different *per* alleles. Southern wasps have their onset, peak and offset of activity much earlier during the 24 h period and exhibit an overall lower level of circadian locomotor activity than northern wasps. Differences were also found in the free running rhythms under constant darkness and constant light, with southern wasps having shorter tau than northern ones. We discuss the observed natural variation in properties of seasonal and circadian rhythmicity in the light of natural selection on clock genes for local adaptation.

Introduction

The daily rotation of the Earth around its axis has profound impact on daily activity patterns of organisms. Many behavioural and physiological activities like mating, feeding, and sleeping, show a distinct oscillating rhythm with a peak of activity at a certain moment during the light-dark cycle. These rhythms are driven by an endogenous circadian clock that runs with a period close to 24h (Pittendrigh, 1993). Besides daily fluctuations in environmental conditions, there are also seasonal changes in day length and temperature caused by the tilt of the Earth's axis relative to its orbit around the sun. This causes the degree of daily and seasonal changes to depend on latitude with almost constant conditions near the equator and increasing environmental variation at higher latitudes.

In insects, the circadian clock modulates a variety of rhythms, including rest and activity, eclosion, mating and feeding (Saunders et al., 2002). The photoperiodic mechanism regulates seasonal adaptations, such as diapause; an arrest of development associated with changes in metabolism, physiology and behaviour. The extent to which the circadian and seasonal systems are intertwined is still debated despite the accumulating evidence for a role of the circadian clock in photoperiodism in many species (Saunders, 2010; Kostal, 2011). Several studies have shown that seasonal responses differ geographically as result of variation in photoperiodic conditions that signal seasonal changes. However, it is still unclear whether the observed natural variation in photoperiodic response is controlled by specific circadian clock properties, such as the speed and the phase of the endogenous clock (Hut & Beersma, 2011). Investigation of geographical variation in circadian systems will therefore contribute to our understanding of the role of the internal circadian clock in photoperiodic regulation.

The parasitoid wasps *Nasonia vitripennis* shows robust photoperiodic response for the maternal induction of larval diapause, in which the development is arrested at the fourth instar larval stage (Paolucci et al., 2013; Saunders, 2013). Short photoperiod elicits a stronger diapause response than long photoperiod. The photoperiod at which 50% of females induce larval diapause, after a precise number of LD cycles, is called the critical photoperiod (CPP; timer), whereas the number of CPP days that are required for inducing larval diapause determine the switch point (counter) (Saunders, 2010, 2013; Saunders & Bertossa, 2011). A clock mechanism is responsible for the timing and counting of the light-dark cycles necessary for starting the photoperiodic response (Saunders, 2013). Under long photoperiods, the switch point occurs later or not at all (Saunders, 1969). Natural variation in switch point for photoperiodic induction of diapause and frequencies of allelic variants of the clock gene *period* follow a similar latitudinal cline. In addition, similar clinal variation was described for the circadian locomotor activity by Paolucci (2014). Free running rhythm (τ) under constant conditions increased towards higher latitude. All this suggests an involvement of the circadian clock in diapause induction in *Nasonia* (Paolucci et al., 2013,

2016).
In this study, we further investigate the timing and level of locomotor activity under long and short photoperiod in two geographically separated populations. We compare five different isogenic lines from southern populations collected in France (Corsica) and northern populations collected in Finland (Oulu), which represent the two extremes of the cline described by Paolucci and co-workers (2013, 2016). We also analyse whether free running rhythms differ in constant darkness (DD) and constant light (LL).

Materials and methods

Experimental lines

The experimental lines for this study are isogenic lines established from isofemale lines collected from the field in 2009 (for collection details see Paolucci et al., 2013). The southern lines S1, S2, S3, S4, S5 were collected in Corsica, France (42°22'40.80N) and the northern lines N1, N2, N3, N4, N5 come from Oulu, Finland (65°3'40.16N). Isogenic lines were established by crossing a virgin female wasp with a son. This cross was followed by 7-8 generations of brother-sister mating. In this way, we obtained an estimated homozygosity level of 99.99%. The lines were maintained on *Calliphora spp*. pupae as hosts in mass culture vials under diapause-preventing conditions, i.e. long photoperiod with a light-dark (LD) cycle of LD16:08 at 20°C.

Locomotor activity

To quantify animal movement over time, virgin females were placed individually in small tubes (diameter 10mm and height 70mm) that were half filled with an agar gel containing sugar. Trikinetics *Drosophila* activity monitors (www.trikinetics.com) were used for activity registration with 32 wasps per monitor. Monitors were placed in light boxes in temperature-controlled environmental chambers with 20°C temperature and 50% humidity. The light source in the box consisted of white light with a maximum light intensity of about 60 lum/sqf. A detector recorded how many times per minute each individual interrupted an infrared light beam that passes through the glass tube. Data were collected and analysed with DAM System 2.1.3 software. We tested the locomotor activity of adult virgin females from northern and southern populations exposed to LD16:08 and LD08:16. We also measured the free-running period under constant darkness (DD) and constant light (LL) conditions.

Behavioural data analysis and statistics

The raw locomotor activity data were first visualized with the program ActogramJ (Schmid, Helfrich-Forster, and Yoshii 2011; available at http://actogramj.neurofly.de). Double-plot actograms obtained with this software represent activity levels. Under LD conditions the average activity was calculated as described by Schlichting and Helfrich-Forster (2015). Every single wasp activity profile was also analyzed with Chronoshop (K. Spoelstra, Netherland Institute of Ecology, Wageningen, the Netherlands) to find the onset, the peak and the offset of activity, and compared between southern and northern wasps. To determine the onset and offset of activity of the average day data per wasp have to be plotted as bar diagrams with each bar representing the sum of activity within 20 min. The onset represents the first time bar when activity starts to rise consecutively, whereas the offset is when activity reaches the level, which is stable during the night phase. To determine the timing of the peaks, the data are smoothed by a moving average of 30. 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 *Zeitgeber time* (ZT), was compared between different lines and treatments. Statistical analysis was performed with ANOVA and a Tukey's multiple-comparisons test.

Under constant darkness and constant light, it was possible to measure the period of activity (τ) with periodogram analysis, which incorporates chi-square test (Sokolove & Bushell, 1978). A generalized linear mixed effect model (glm) was used with a quasi-poisson distribution to correct for overdispersion and *F*-tests to compare differences τ between lines. Post-hoc analyses were performed using the multicomp package. The activity level of each individual was defined as an average of the locomotor activity based on 60 minutes/bins. Average activity levels were compared between lines and photoperiods by ANOVA with a Tukey's multiple-comparisons test. All statistical analyses were performed with R statistical software (version 3.4.1, R Development Core Team 2012).

Results

Timing of circadian activity depends on latitude and photoperiod

To investigate the activity timing of southern and northern wasps, animals were exposed to a light dark (LD) regime of either 16:08 or 08:16h per 24h for 4 days. Under LD16:08, both southern and northern lines displayed a unimodal activity pattern (Fig. 2.1), but with significant regional differences in the timing of onset, peak and offset of activity (Table 2.1, Table S1). Southern lines started activity when the light was turned on, on average around ZT 0, which is about two hours earlier than northern lines (Fig. 2.1, Table 2.1, Table S1).

Subsequently, southern lines reached the maximum activity around ZT 5, which is two and half hours earlier than northern ones, that have the peak of activity at ZT 8 (Fig. 2.1, Table 2.1, Table S1). In addition, southern wasps ceased activity in the late afternoon, on average around ZT 13, while northern wasps ceased activity around the light-off signal, at ZT 16 on average, being three hours later than the southern wasps (Fig. 2.1, Table 2.1, Table S1). Thus, southern *N. vitripennis* were more active in the first half of the day while northern wasps were more active towards the end of the day.

Similar differences were found under the shorter photoperiod LD08:16 (Fig. 2.1, Table 2.1, Table S2). Southern wasps started their activity, when the light was still off around ZT 21,5, in contrast to the northern wasps that became active when the light was turned on, namely at ZT 0, two hours later than the southern ones (Fig. 2.1, Table 2.1, Table S2). The peaks of activity also differed about one and half hour, at ZT 2.5 and ZT 4 for southern and northern wasps, respectively (Fig. 2.1, Table 2.1, Table S2). Interestingly, while southern wasps ceased activity when the light was turned off at ZT 8, the northern ones prolonged activity for more than two hours into darkness, until ZT 10.5 on average (Fig. 2.1, Table 2.1, Table S2).

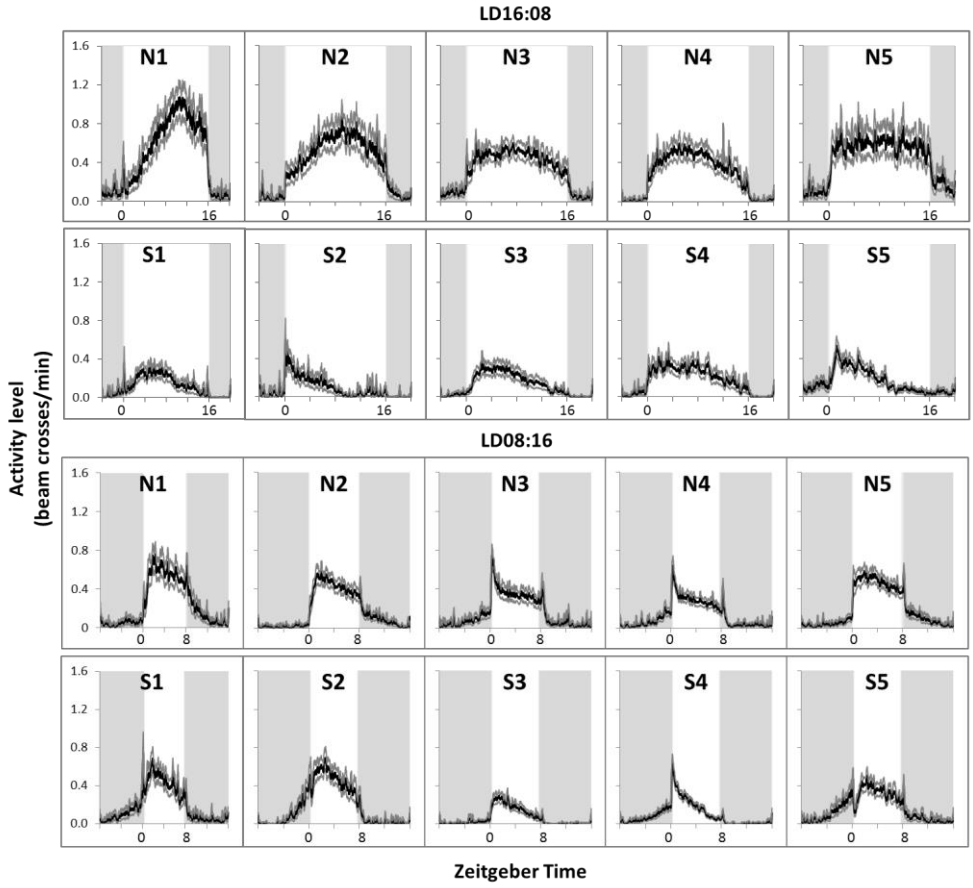

Fig.2.1 Locomotor activity patterns of northern and southern wasps under LD16:08 and LD08:16
Locomotor activity profiles of northern (N1, N2, N3, N4, N5) and southern (S1, S2, S3, S4, S5) lines are shown at long (LD16:08) and short (LD08:16) day regimes. The night phase is indicated by grey shading, the day phase in white. *Zeitgeber time* is indicated along the X-axis and ZT0 represents the time when light turn on. Activity is estimated as average of bin crosses/minute of 25-32 individuals each over 24 hours periods.

Table 2.1 Timing of onset, peak and offset of activity for northern and southern lines under long (LD16:08) and short (LD08:16) day conditions. ZT (h) is *zeitgeber time* in hours. Different letters indicate statistical differences ($p<0.05$, ANOVA with a Tukey's multiple-comparisons test).

	LD16:08			**LD08:16**		
Lines	Onset ± SE ZT (h)	Peak ± SE ZT (h)	Offset ± SE ZT (h)	Onset ± SE ZT (h)	Peak ± SE ZT (h)	Offset ± SE ZT (h)
N1	3.07 ± 0.32 (a)	10.80 ± 0.30 (a)	18.66 ± 0.56 (a)	0.05 ± 0.18 (a)	4.48 ± 0.31 (a)	11.60 ± 0.42 (ab)
N2	1.66 ± 0.35 (ab)	8.24 ± 0.38 (b)	16.19 ± 0.25 (b)	23.97 ± 0.17 (a)	4.51± 0.20 (a)	11.92 ± 0.38 (b)
N3	2.29 ± 0.42 (b)	7.16 ± 0.23 (bc)	17.85 ± 0.19 (bc)	22.67 ± 0.27 (ab)	3.15 ± 0.20 (b)	10.56 ± 0.32 (a)
N4	1.25 ± 0.17 (bc)	6.65 ± 0.27 (bcd)	14.34 ± 0.25 (c)	22.59 ± 0.27 (ab)	3.12 ± 0.16 (b)	10.77 ± 0.36 (a)
N5	1.29 ± 0.49 (bc)	6.35 ± 0.66 (cd)	14.57 ± 0.64 (bc)	22.89 ± 0.20 (ab)	2.81 ± 0.16 (b)	8.66 ± 0.37 (c)
Overall N	**2.00 ± 0.17**	**7.96 ± 0.21**	**16.05 ± 0.23**	**23.38 ± 0.18**	**3.54 ± 0.11**	**10.48 ± 0.21**
S1	1.09 ± 0.36 (ab)	6.44 ± 0.39 (cd)	12.66 ± 0.41 (c)	21.70 ± 0.24 (bc)	2.99 ± 0.22 (b)	8.65 ± 0.21 (c)
S2	0.13 ± 0.11 (c)	5.29 ± 0.22 (d)	13.05 ± 0.23 (c)	22.25 ± 0.22 (bc)	3.03 ± 0.32 (b)	8.57 ± 0.11 (c)
S3	23.78 ± 0.18 (c)	5.79 ± 0.31 (cd)	14.16 ± 0.27 (c)	21.06 ± 0.24 (c)	1.52 ± 0.15 (c)	7.53 ± 0.27 (c)
S4	23.88 ± 0.25 (c)	5.37 ± 0.39 (d)	13.07 ± 0.43 (c)	21.97 ± 0.28 (bc)	3.15 ± 0.28 (b)	9.02 ± 0.23 (c)
S5	23.70 ± 0.15 (c)	3.63 ± 0.21 (e)	13.18 ± 0.27 (c)	21.05 ± 0.24 (c)	0.89 ± 0.14 (c)	7.82 ± 0.13 (c)
Overall S	**0.12 ± 0.11**	**5.28 ± 0.16**	**13.20 ± 0.15**	**21.56 ± 0.11**	**2.29 ± 0.13**	**8.30 ± 0.10**

Free running rhythms differ between northern and southern wasps

Under constant conditions it is possible to evaluate the speed of the endogenous clock. Thus, after the entrainment period under LD, the wasps were released either in constant darkness (DD) or constant light (LL). Representative examples of double-plotted actograms, which display the activity of single individuals during the monitoring period, are shown in Fig. 2.2. Southern and northern wasps differed in free-running rhythms under constant conditions (Fig. 2.3A). Four out of five southern lines displayed a DD rhythm with τ around 24h (Fig 2.3A, Table 2.2, Table 3S). On the other hand, northern lines had rhythms well above 24h. Although there was some variation between lines, the average free-running rhythm of southern wasps was 24.3+0.1h, which differed significantly from the longer τ of 26.7+0.1h of the northern ones ($p<0.001$) (Fig. 2.3A, Table 2.2, Table S3). These data indicate that the southern clock is faster than the northern one in DD.

Under constant light, southern and northern lines displayed a shortening of the free-running period compared to DD (Fig. 2.3A, Table 2.2; Table S3). The average LL free-running rhythm of southern wasps (23.5+0.1h) was significantly shorter than the average DD tau ($p<0.001$) and also shorter than the average LL tau of the northern ones (26.1+0.2h) ($p<0.001$). Therefore, also under LL, the southern clock runs faster than the northern one. Significant differences were also found between northern LL and DD tau ($p=0.003$) for some lines but not all (Fig. 2.3A, Table 2.2; Table S3) indicating a general pattern in which LL rhythms are shorter than DD rhythms in accordance to Achoff's rule (Pittendrigh & Takamura, 1989).

We further analysed the number of rhythmic wasps under these constant conditions (DD and LL). There is not a clear effect of latitude of origin and light conditions on the number of rhythmic individuals in the tested lines (Fig. 2.3B Table 2.2).

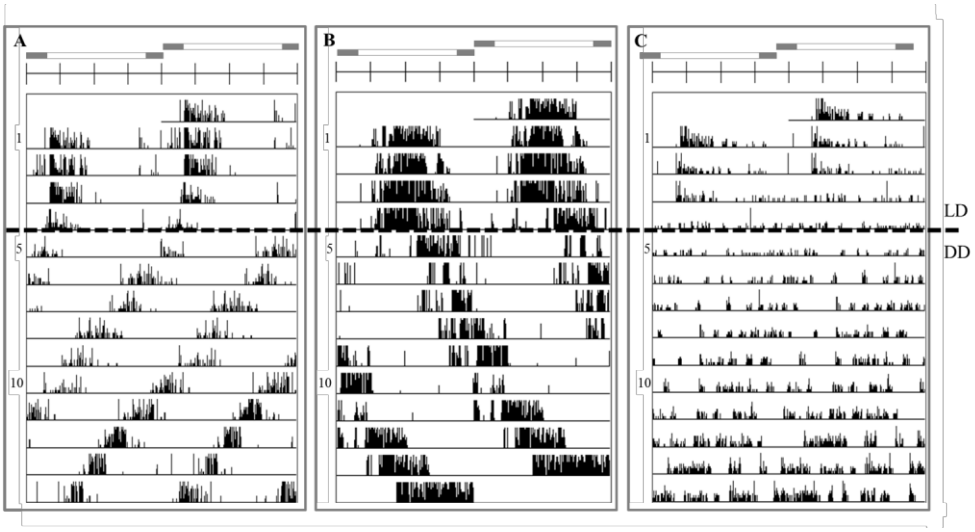

Fig.2.2 Representative double-plotted actograms for northern and southern Nasonia wasps
Animals were entrained under LD16:08 for 4-5 days followed by constant darkness (DD) for 10 days. The day phase is indicated in white and the night in grey shading. Activity is indicated by black bars. (A) a rhythmic wasp with $\tau < 24h$, (B) a rhythmic wasp with $\tau > 24h$ and (C) an arrhythmic wasp under free running condition.

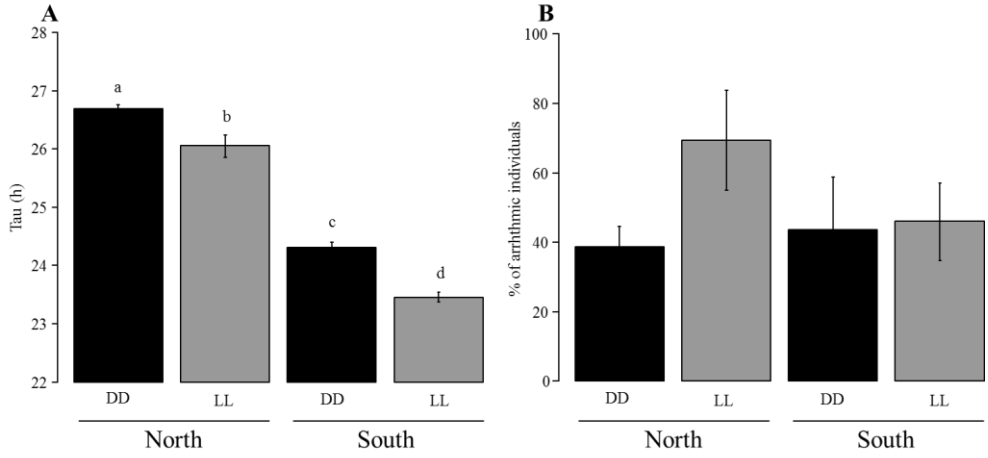

Fig.2.3 Locomotor activity under constant darkness (DD) and constant light (LL) for southern and northern lines. (A) Average free running period (τ) and **(B)** percentage of arrhythmic wasps for northern and southern lines. Different letters indicate significant differences ($p<0.05$, posthoc multiple comparison).

Table 2.2 Free running rhythms and percentage of arrhythmic animals under constant darkness (DD) and constant light (DD) conditions. Different letters indicate statistical differences between lines ($p<0.05$ posthoc multiple comparison). Asterisks indicate significant differences between DD and LL (***$p<0.001$; **$p<0.05$) .

	DD			LL	
	Tau ± SE (h)	Arrhythmic %		Tau ± SE (h)	Arrhythmic %
N1	26.93 ± 0.12 (a)	19.35		25.00 ± Na (bcd)	95.00
N2	26.67 ± 0.16 (a)	55.17		26.67 ± 0.22 (b)	31.03
N3	26.57 ± 0.17 (a)	36.67	**	25.66 ± 0.25 (c)	39.13
N4	26.47 ± 0.25 (a)	40.63		-	100.00
N5	26.72 ± 0.12 (a)	41.67	***	24.13 ± 0.26 (d)	81.82
S1	23.83 ± 0.11 (b)	27.03		23.12 ± 0.15 (bd)	50.00
S2	24.67 ± 0.17 (c)	16.98	***	23.45 ± 0.13 (bd)	20.69
S3	24.36 ± 0.35 (b)	50.98		24.09 ± 0.20 (bd)	36.00
S4	-	100.00		23.20 ± 0.52 (bd)	86.21
S5	24.32 ± 0.10 (b)	22.58	***	23.19 ± 0.14 (bd)	36.67

Circadian activity level depends on photoperiod and latitude of origin

Activity level was calculated under both LD cycles, constant darkness (DD) and constant light (LL). Generally, activity of southern wasps was much lower than that of northern ones (Fig. 2.4, Table 2.3). Under LD16:08 the night activity was very low in all lines but still with southern activity being lower than northern. Southern wasp showed an average activity level of 2.16 $\pm$ 0.23 per hour bin during the night and 11.22 $\pm$ 0.52 during the day, compared to northern wasps with 4.44 $\pm$ 0.38 and 31.19 $\pm$ 1.06 for dark and light phase respectively ($p<0.001$) (Fig. 2.4A, Table 2.3).

Under short photoperiod LD08:16, southern wasps increased their average activity level to 3.51 ± 0.29 per hour bin during night and to 18.72 ± 0.84 during light phase (Fig. 2.4B). Although this is a significant increase ($p<0.001$), not all southern lines showed differences between long and short photoperiod (Table 2.3). On the other hand, none of the northern lines showed differences in activity level between the two photoperiods (Fig. 2.4, Table 2.3). Despite the larger increase in activity level under short photoperiod of the southern lines, the northern wasps remained, on average, more active than southern ones during the light phase ($p<0.001$).

Finally, under constant conditions DD and LL, southern wasps always displayed a lower activity level (13.34 $\pm$ 0.45 per hour bin under LL and 11.49 $\pm$ 0.51 under DD) compared to northern wasps (22.34 $\pm$ 0.93 and 19.97 $\pm$ 0.90 under LL and DD respectively) ($p<0.001$) (Fig. 2.4C, table 2.3). DD activity level was not significantly different from LL activity in both southern and northern lines. All these data together indicate that activity level is physiologically different between southern and northern lines under all the tested conditions.

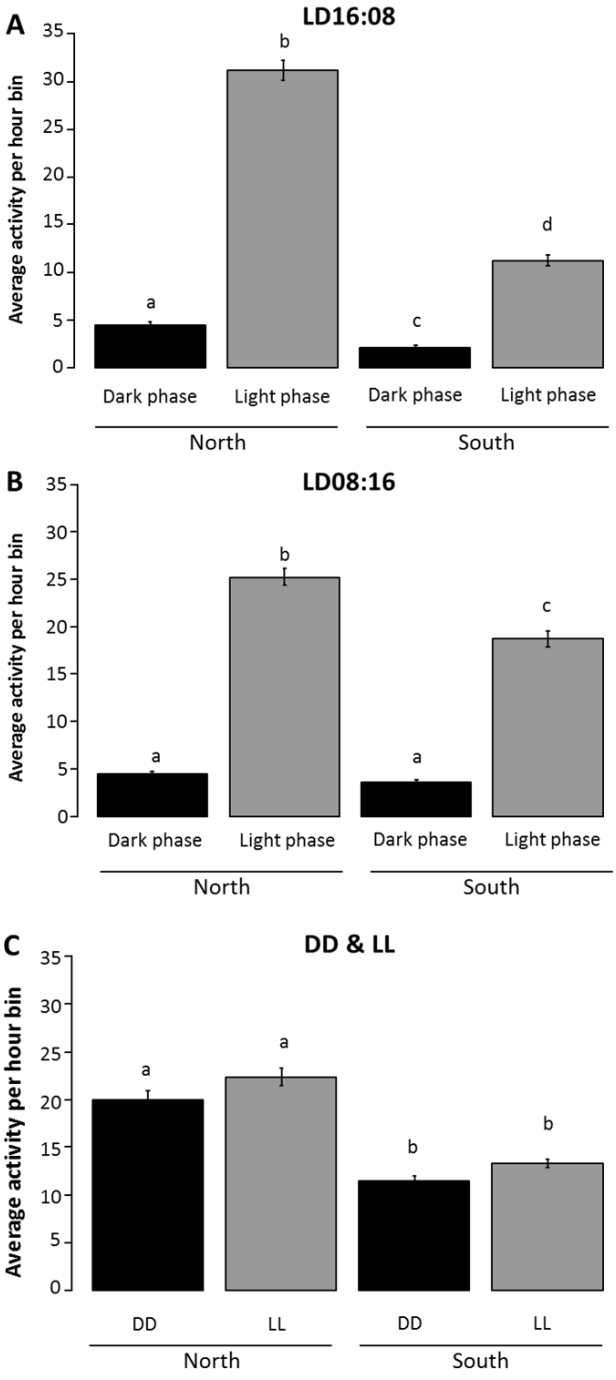

Fig. 2.4 Activity level of the southern and northern lines of *Nasonia vitripennis*

(A) Activity level of northern and southern wasps under LD16:08 during the dark and light phase (black bars and grey bars respectively). **(B)** activity level of northern and southern wasps under LD08:16 during the dark and light phase (black bars and grey bars respectively). **(C)** activity level of northern and southern wasps under constant darkness (DD, black bars) or constant light (LL, grey bars) after 4 days of entrainment under LD cycles. Different letters indicate significant differences ($p<0.001$, ANOVA with a Tukey's multiple-comparisons test).

Table 2.3 Activity level per line under LD16:08, LD08:16, DD and LL conditions. Activity is calculated as beam crosses in hour bin. Different letters indicate statistical differences between lines ($p<0.05$) and asterisks indicate statistical differences between LD16:08 and LD08:16, and between DD and LL conditions ($p<0.001$).

	LD16:08				LD08:16		DD		LL
	Light phase	**Dark phase**	**Light**	**Dark**	**Light phase**	**Dark phase**	**DD Activity**		**LL Activity**
	Activity ± SE (per h bin)	Activity ± SE (per h bin)			Activity ± SE (per h bin)	Activity ± SE (per h bin)	Activity ± SE (per h bin)		Activity ± SE (per h bin)
N1	39.21 ± 2.58 (a)	5.14 ± 0.81 (f)			30.33 ± 3.40 (a)	5.77 ± 0.81 (f)	20.24 ±1.11 (h)		28.20 ± 2.40 (h)
N2	32.75 ± 2.07 (ab)	4.09 ± 1.06 (f)			25.42 ± 1.83 (ab)	3.51 ± 0.36 (f)	15.67 ± 1.58 (hi)	***	28.22 ± 2.67 (h)
N3	27.95 ± 1.92 (b)	5.20 ± 0.78 (f)			23.23 ± 0.91 (ab)	4.84 ± 0.37 (f)	22.82 ± 1.85 (h)		21.10 ± 1.08 (h)
N4	26.27 ± 2.27 (b)	1.31 ± 0.37 (g)			19.29 ± 0.75 (be)	3.56 ± 0.46 (fg)	12.64 ± 1.99 (ih)		17.17 ± 1.08 (h)
N5	28.89 ± 2.00 (b)	6.58 ± 0.61 (f)			28.13 ± 1.91 (b)	4.68 ± 0.59 (f)	25.94 ± 2.38 (j)		17.89 ± 1.39 (hj)
S1	10.34 ± 0.94 (cd)	1.35 ± 0.27 (g)	***	***	26.48 ± 2.53 (bde)	5.33 ± 0.96 (f)	9.94 ± 0.87 (i)	***	16.34 ±0.87 (k)
S2	7.71 ± 0.64 (c)	1.61 ± 0.40 (g)	***		27.75 ± 1.21 (bde)	3.71 ± 0.61 (fg)	11.85 ± 0.83 (i)		12.67 ±1.22 (ik)
S3	11.29 ± 1.11 (cd)	1.05 ± 0.29 (g)			10.52 ± 0.51 (c)	1.03 ± 0.13 (g)	11.21 ± 1.19 (i)		13.05 ±0.9 (ik)
S4	12.04 ± 0.91 (cd)	4.85 ± 0.71 (f)			12.69 ± 0.76 (c)	2.77 ± 0.25 (f)	14.52 ± 1.45 (i)		12.09 ± 0.81 (ik)
S5	14.11 ± 1.52 (d)	1.60 ± 0.29 (g)		***	19.97 ± 1.64 (de)	5.46 ± 0.83 (f)	10.64 ± 1.26 (i)		12.87 ± 1.09 (ik)

Discussion

Nasonia female wasps from high latitudes (northern lines, 65°N) and low latitudes (southern lines, 42°N) displayed profound differences in their daily locomotor activity. Northern wasps were mainly active at the end of the day, with a prolonged evening peak at the shorter photoperiod, whereas the southern ones showed a unimodal morning activity, with an increase of activity before the light turned on during short photoperiod. This shifted activity pattern between northern and southern wasps can reflect local adaptation. In the south, temperatures are known to become high in the middle, late afternoon and shifting the activity to the coolest part of the day (the morning) might be a strategy of insects that live in a hot environment (Prabhakaran & Sheeba, 2012, 2013). In contrast, species that live at higher latitudes would have to cope with lower temperatures and longer photoperiods. In concordance to this, the northern lines have a reduced morning activity and have their activity peak in the second part of the day when temperatures are higher. Similar differences in activity patterns between northern and southern individuals have been reported for *Drosophila*, albeit at the species rather than population level (Prabhakaran and Sheeba, 2012, 2013). Moreover in *Drosophila* these behavioural differences in timing of locomotor activity correlate with variation in the neuroanatomical architecture of the circadian clock between northern and southern species (Menegazzi et al., 2017). Although no data about the neuroarchitecture of the circadian clock of *Nasonia* are available, our results on *Nasonia* are consistent with the behaviour reported from *Drosophila*.

This different timing of activity reflects the speed of the clock in constant darkness (DD): southern lines show shorter free running rhythms (τ) close to 24h (faster clock), compared to northern ones that naturally have a τ longer than 24h (slower clock). The presence of a positive latitudinal cline in DD rhythm was previously reported for *Drosophila*. Some *Drosophila* species, such as *D. auraria* (Pittendrigh & Takamura, 1989) and *D. ananassae* (Joshi & Gore, 1999), showed a positive correlation between latitude and length of free running rhythm, whereas *D. littoralis* and *D. subscura* show an opposite cline with shorter tau towards northern latitude (Lankinen, 1986). Only few studies have addressed the variability of the free running rhythms within a species. For example, in the model plant *Arabidopsis thaliana* the free running period (*tau*) under DD increases towards northern latitude, and correlates with clinal variation in seasonal flowering time regulated by photoperiodic cycles (Michael et al., 2003). In insects, similar results (*i.e.* longer *τ* towards northern latitude) were reported from the mosquito *Culex pipiens* (Shinkawa et al., 1994), the linden bug *Pyrrhocoris apterus* (Pivarciova et al., 2016) and also *N. vitripennis* by Paolucci (2014). This could possibly be explained by the fact that at higher latitudes, organisms must continue to accurately entrain to the 24-hour day, despite the sharp increase in day length during the Spring. In accordance with Aschoff's rule, pacemakers with periods longer than 24h are more efficient in tracking and interpreting the dawn and thus

photoperiodic changes (Pittendrigh & Takamura, 1989). Therefore, clocks with τ exceeding from 24 hours should enhance seasonal acuity, particularly at high latitudes. This suggests that the latitudinal differences in free running period are the result of selection on the circadian rhythm mediated through selection on traits that are genetically correlated with circadian rhythms (such as seasonal response)

Additionally, we found that the activity level of northern wasps was higher than southern ones, whereas southern strains tend to have higher activity when photoperiod is shorter. Such difference could result from variation in the sensitivity to light. For example, it could reflect different adaptation to local light intensity in nature. In summary we described natural variation on the pace, phase and level of daily rhythms between southern and northern *N. vitripennis* lines that likely are the results of different selection pressure and local adaptation.

Acknowledgements

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Supplementary information

Table S1: Statistical analysis of circadian timing between southern and northern lines under LD16:08. Indicated are p-values from ANOVA with a Tukey's multiple-comparisons test. In bold p<0.05.

	Onset LD16:08									
	N1	N2	N3	N4	N5	S1	S2	S3	S4	S5
N1		0.281	0.456	**< 0.001**	**0.006**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**
N2			0.948	0.995	0.999	0.938	**0.009**	**< 0.001**	**0.001**	**< 0.001**
N3				0.364	0.601	0.139	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**
N4					1.000	0.999	0.161	0.239	**0.040**	**0.010**
N5						0.999	0.277	0.060	0.093	**0.031**
S1							0.337	0.065	0.104	**0.030**
S2								0.997	0.999	0.988
S3									1.000	1.000
S4										0.999
S5										

	Peak LD16:08									
	N1	N2	N3	N4	N5	S1	S2	S3	S4	S5
N1		**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**
N2			0.4334	0.05	**0.01**	**0.008**	**0.009**	**< 0.001**	**0.001**	**< 0.001**
N3				0.989	0.837	0.878	**0.004**	0.138	**0.007**	**< 0.001**
N4					0.999	0.999	0.157	0.791	0.216	**< 0.001**
N5						1.000	0.569	0.988	0.653	**< 0.001**
S1							0.346	0.950	0.435	**< 0.001**
S2								0.994	1.000	**0.009**
S3									0.997	**< 0.001**
S4										**0.010**
S5										

	Offset LD16:08									
	N1	N2	N3	N4	N5	S1	S2	S3	S4	S5
N1		**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**
N2			0.999	**0.031**	0.124	**< 0.001**	**< 0.001**	**0.011**	**< 0.001**	**< 0.001**
N3				0.127	0.366	**< 0.001**	**< 0.001**	0.055	**< 0.001**	**< 0.001**
N4					0.999	0.053	0.272	0.999	0.337	0.463
N5						0.021	0.132	0.999	0.173	0.256
S1							0.998	0.143	0.998	0.986
S2								0.512	1.000	0.999
S3									0.585	0.721
S4										0.999
S5										

Table S2: Statistical analysis of circadian timing between southern and northern lines under LD 08:16. Indicated are p-values from ANOVA with a Tukey's multiple-comparisons test. In bold p<0.05.

	Onset LD08:16									
	N1	N2	N3	N4	N5	S1	S2	S3	S4	S5
N1		1.000	0.878	0.611	0.376	**< 0.001**	**0.014**	**< 0.001**	**0.003**	**< 0.001**
N2			0.891	0.60	0.342	**< 0.001**	**0.008**	**< 0.001**	**0.001**	**< 0.001**
N3				0.999	0.999	**0.030**	0.507	**< 0.001**	0.202	**< 0.001**
N4					0.999	0.081	0.751	**< 0.001**	0.382	**< 0.001**
N5						0.157	0.899	**< 0.001**	0.569	**0.001**
S1							0.964	0.765	0.999	0.897
S2								0.093	0.999	0.169
S3									0.401	0.999
S4										0.574
S5										

	Peak LD08:16									
	N1	N2	N3	N4	N5	S1	S2	S3	S4	S5
N1		1.000	**< 0.001**	**< 0.001**	**0.006**	**< 0.001**	**< 0.001**	**< 0.001**	**0.002**	**< 0.001**
N2			**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**0.009**	**< 0.001**	**0.001**	**< 0.001**
N3				1.000	0.999	1.000	1.000	**< 0.001**	0.999	**< 0.001**
N4					0.997	0.999	1.000	**< 0.001**	0.999	0.999
N5						0.999	0.999	**0.001**	0.985	**< 0.001**
S1							1.000	**< 0.001**	0.999	**< 0.001**
S2								**< 0.001**	0.999	**< 0.001**
S3									**< 0.001**	0.709
S4										**< 0.001**
S5										

	Offset LD08:16									
	N1	N2	N3	N4	N5	S1	S2	S3	S4	S5
N1		0.999	0.156	0.369	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**
N2			**0.008**	**0.04**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**
N3				0.999	**0.016**	**0.010**	**0.006**	**< 0.001**	0.171	**< 0.001**
N4					**0.006**	**0.004**	**0.002**	**< 0.001**	**0.079**	**< 0.001**
N5						1.000	1.000	0.316	0.999	0.697
S1							1.000	0.292	0.998	0.677
S2								0.413	0.994	0.799
S3									0.053	0.999
S4										0.211
S5										

Table S3: Generalized linear model analysis of free running rhythms between southern and northern lines, under constant darkness (DD) and constant light (LL). Indicated are p-values from a postdoc multiple comparison. In bold p<0.05.

	τ DD								
	N1	N2	N3	N4	N5	S1	S2	S3	S5
N1		0.997	0.954	0.841	0.997	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**
N2			1.000	0.999	1.000	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**
N3				1.000	0.999	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**
N4					0.995	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**
N5						**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**
S1							**0.002**	0.389	0.550
S2								0.921	0.861
S3									1.000
S5									

	τ LL								
	N1	N2	N3	N5	S1	S2	S3	S4	S5
N1		0.461	0.995	0.983	0.262	0.477	0.95	0.391	0.228
N2			**0.006**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**
N3				**0.040**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**
N5					0.476	0.838	1.000	0.753	0.413
S1						0.156	**0.010**	1.000	1.000
S2							0.156	0.999	0.925
S3								0.421	**0.013**
S4									1.000
S5									

Chapter 3

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

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To be submitted

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).

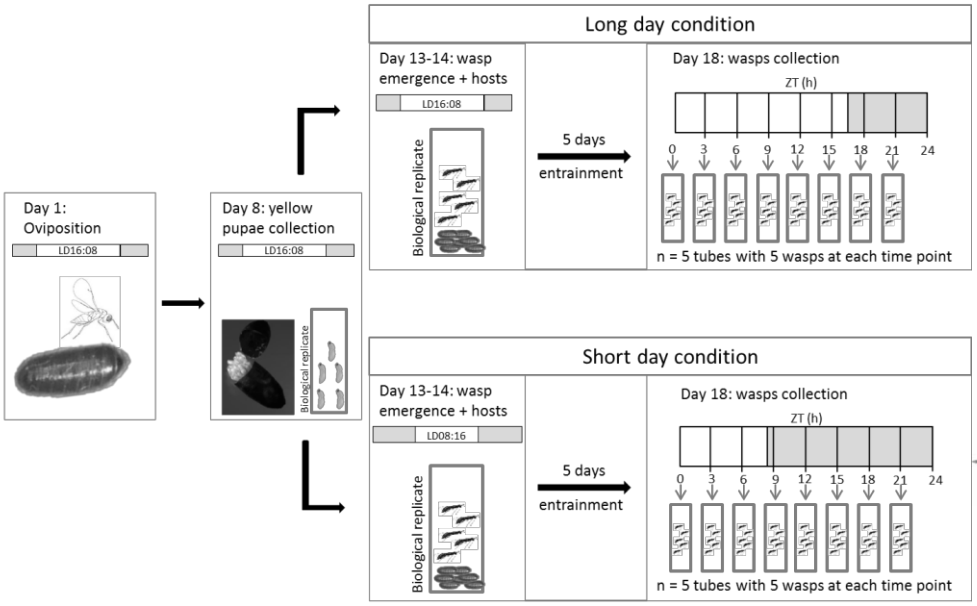

Fig. 3.1. Schematic representation of wasp sample collection

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).

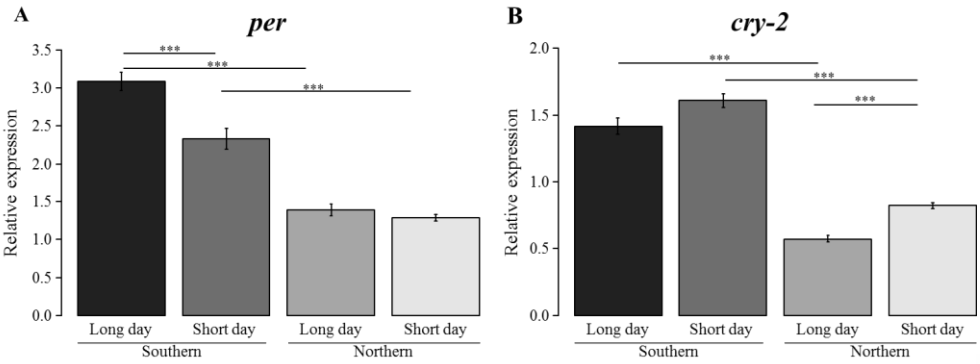

Fig. 3.2. *Period* and *cryptochrome-2* expression level.
(A) Depicts the average relative expression of clock gene *period* (*per*), and **(B)** for *crypthochrome-2* (*cry-2*) under long day and short day conditions for southern and northern lines. Asterisks represent significant differences between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

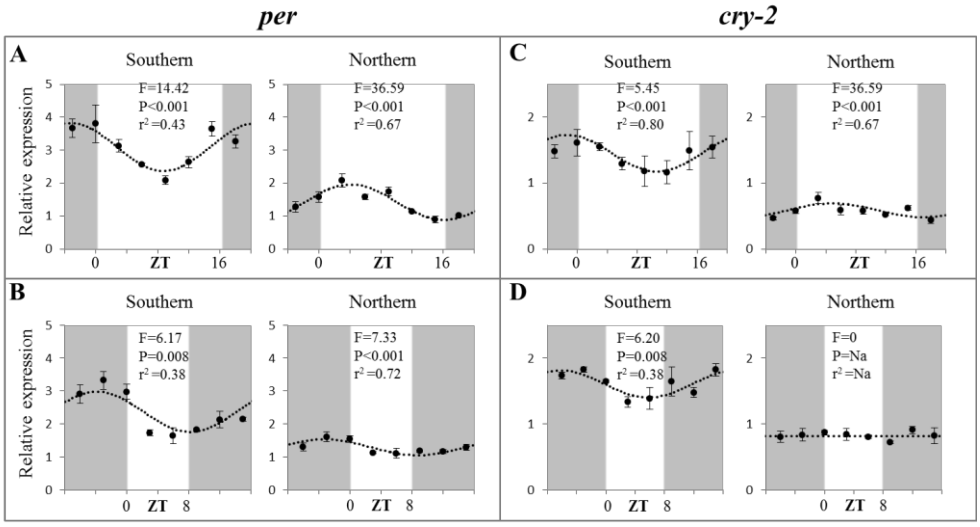

Fig. 3.3. *Period* and *cryptochrome-2* expression of southern and northern lines under long and short day conditions.
(A) represents the relative mRNA expression of *per* over 24h under long days for southern and northern lines, **(B)** represents the *per* relative mRNA, but for short days, for southern 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).

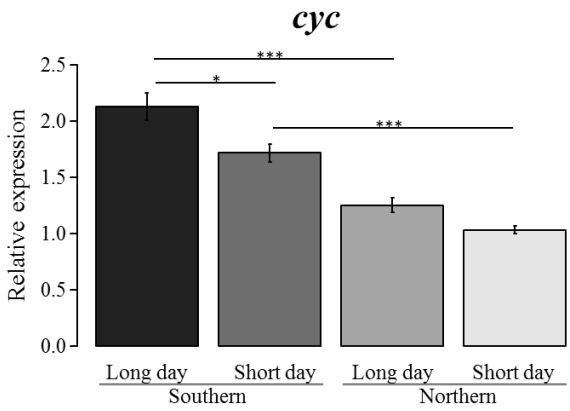

Fig. 3.4. ***Cycle*** **expression level**

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).

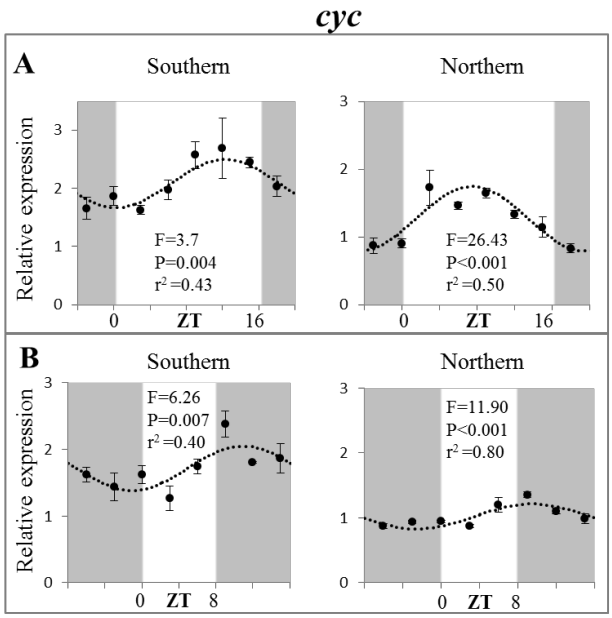

Fig. 3.5. ***Cycle*** **expression of southern and northern lines under long and short days**

(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.

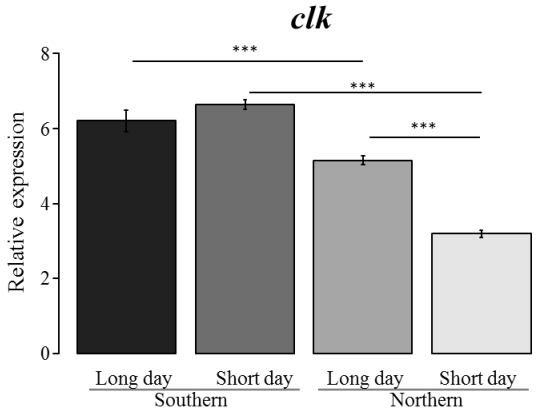

Fig. 3.6. ***Clock*** **expression level**

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).

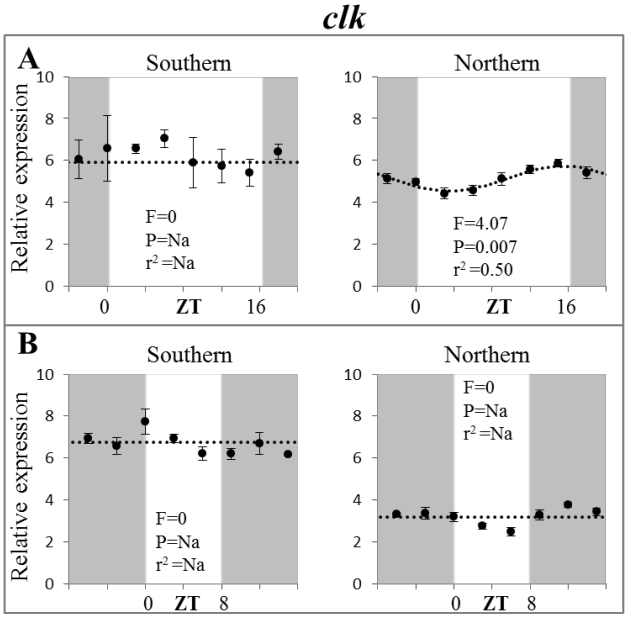

Fig. 3.7. ***Clock*** **expression of southern and northern lines under long and short days**

(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.

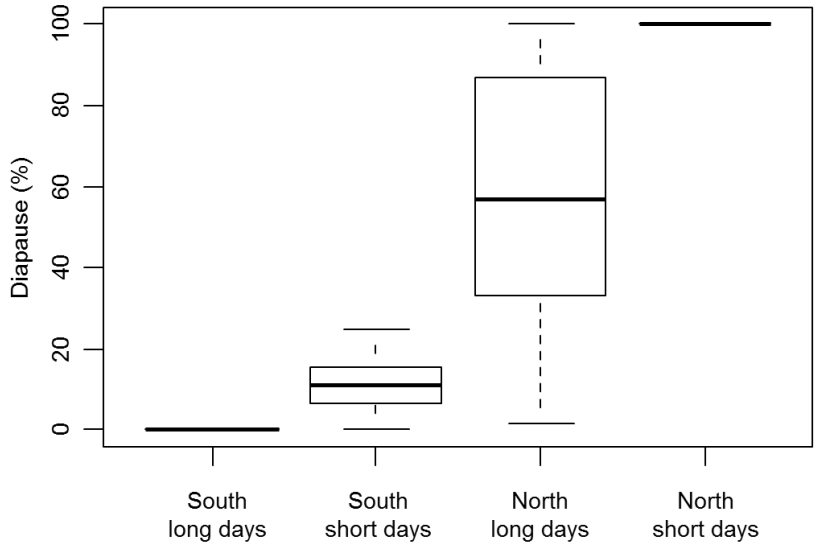

Fig. 3.8. Diapause percentage of samples used for expression analysis
Box plots show the median (thick horizontal line within the box), the 25 and 75 percentiles (box), and 1.5 times the interquartile range of the data (thin horizontal lines). Diapause percentage at day 5 after emergence (collection 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'

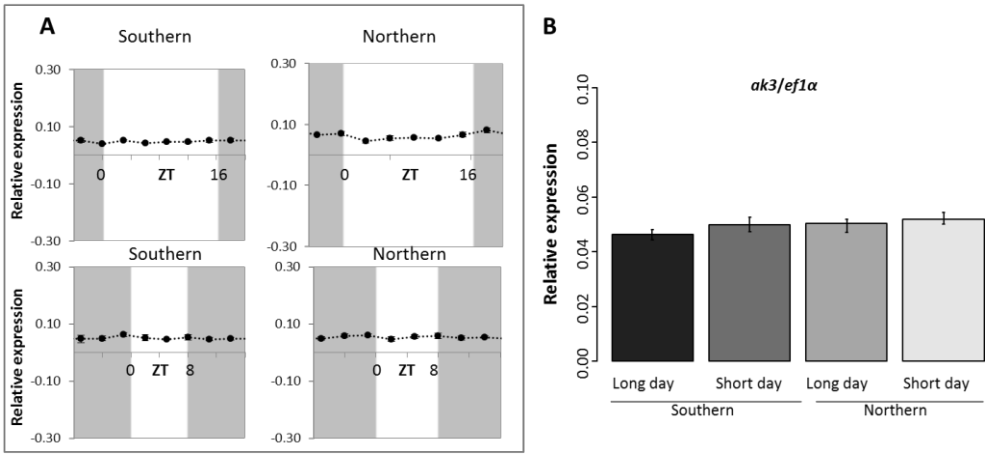

Fig. S1. Expression of the reference under long and short days in the southern and norther lines.
(A) The average relative expression of *ef1α* and *ak3* is compared among time points, under long days and short days, in the southern and northern lines. **(B)** the overall average relative expression 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.

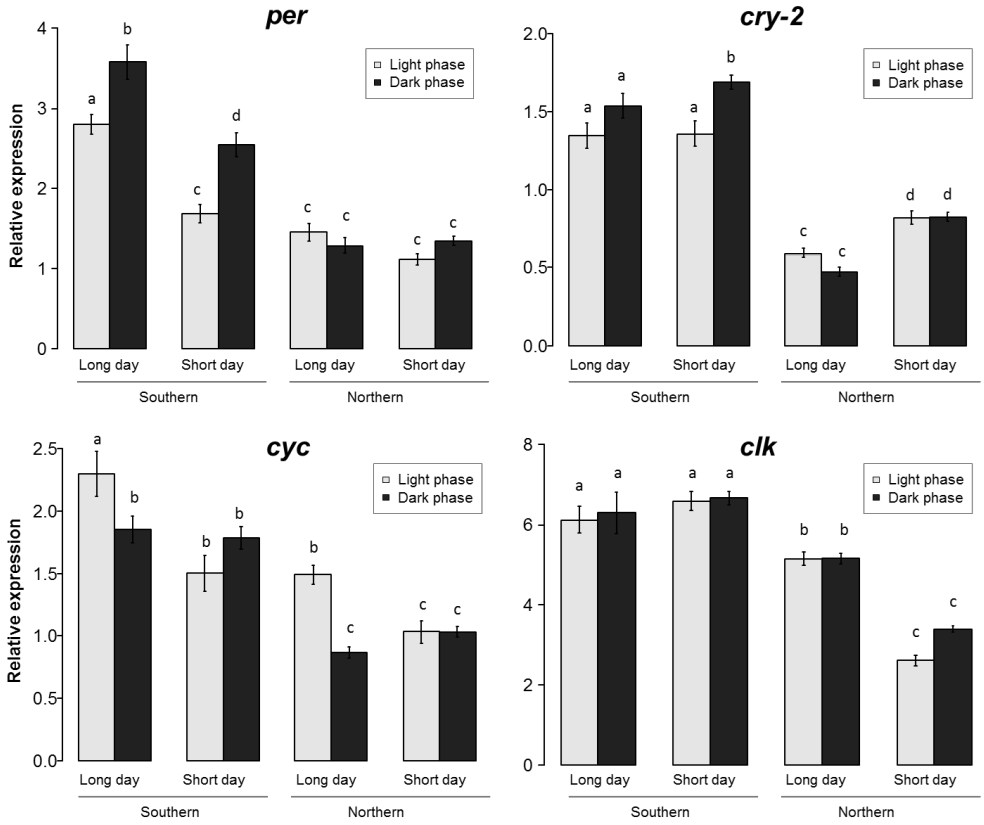

Fig. S2. Light and dark phase expression levels of southern and northern lines.
The average relative expression of clock genes *period* (*per*), *cycle* (*cyc*), *crypthochrome-2* (*cry-2*) and *clock* (*clk*) is compared between long days and short days and between light and dark phases in the southern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

BOX 1

Identification of alternative splicing of period in *Nasonia vitripennis*

Elena Dalla Benetta

Background

Circadian clocks are endogenous timing mechanisms that generate daily rhythms in various organisms, ranging from cyanobacteria to humans. Although rhythms controlled by a circadian clock are self-sustained and persist robustly with a period close to 24 hours under conditions of constant darkness and temperature, they are entrained by environmental cues (*zeitgebers*), such as light, temperature, and food intake (Rusak et al., 1993; Soriano, 1981). Transcription is considered the prime mechanism driving daily rhythms in gene expression. The control of gene expression is a complex process, mRNAs and proteins can undergo many processing and regulatory steps that influence their expression. These post-transcriptional and post-translational regulations, such as alternative splicing, phosphorylation and dephosphorization, modulate sub-cellular localization, protein interactions and protein functions, and thus are very important to shape biological rhythms (Virshup et al., 2007; Zheng & Sehgal, 2012).

The *period* (*per*) gene has been demonstrated to play a crucial role in circadian rhythm generation in many organisms, including mammals (Chen et al., 2009; Lee et al., 2011) and insects (Hardin et al., 1990; Konopka & Benzer, 1971; Sauman & Reppert, 1996). Different *per* isoforms have been described in many insects. For example, in *Drosophila melanogaster* alternative splicing of *per* is induced by low temperature and results in two different isoforms (Colot et al., 2005; Diernfellner et al., 2005; Majercak et al., 1999). The ratio of the two isoforms is crucial to phase mRNA and protein expression of *per*. Alternative splicing of *per* has also been reported from the Silk Moth *Bombyx mori*, in which the two isoforms differ in 5 amino acids (GTQEK) on the PAS A domain (Takeda et al., 2004). A similar set of PERIOD isoforms has been reported for honeybees, *Apis mellifera* and *Apis cerana*, but in this case for a deletion/insertion of eight amino acid residues near the so-called *perS* mutation site (Minamoto et al., 2012; Shimizu et al., 2001).

In *Nasonia vitripennis*, the *per* gene shows a latitudinal cline in allele frequencies that correlate with a latitudinal cline in photoperiodic diapause response (Paolucci et al., 2016). In chapter 3, expression profiles of *per* mRNA were investigated and revealed a different expression pattern between southern and northern lines and between photoperiods. Here, I address the question whether *N. vitripennis* also expresses different *per* isoforms, whether southern and northern lines show differences in the expression of those isoforms, and what function could be associated to these *per* isoforms.

Results and discussion

Different *Nasonia per* transcripts have been found in the NCBI (National Centre for Biotechnology Information) (NCBI Resource Coordinators, 2017) database. They have been aligned with the homologous *per* of *Apis mellifera* and *Apis cerana japonica*, indicating an amino acid identity of about 46%. Two of these *per Nasonia* transcripts correspond to the *alpha* and *beta* versions described from the honeybee (Shimizu et al., 2001) (Fig. 1). We confirmed the presence of the two *per* transcripts in *Nasonia* by reverse transcription (RT)-PCR, and by cloning and sequencing of the variants (Fig. 2B, C). The alternative splicing interest the last 24bp (8nt) of the intronic region between exon 9 and 10 (Fig. 2A; 3). The cDNA structure of *per* clearly showed the existence of splice consensus sites (Maniatis & Tasic, 2002; Norton, 1994), i.e. GU (GT in cDNA) at the 5' side and AG at the 3' side of the specific 24 bp sequence. This strongly suggests that the isoforms with the 24 bp sequence difference were produced by alternative splicing from an intron adjacent to this splicing site (Fig. 3).

Both *alpha* and *beta* variants were detected in both heads and bodies of southern and northern lines (Fig. 2B). Unfortunately qPCR methods failed to quantify the two mRNAs due to lack of good sequence for designing specific primers. A semi-quantitative analysis following the procedure described by Shimizu et al., (2001) also failed to give reliable information about expression levels of the two variants. Although the functional difference between PERIOD α and β is unknown, it is necessary to quantify the ratio of isoform α and isoform β in PERIOD protein or *period* mRNA forms. So far, we have not succeeded in such quantifications in spite of attempts to differentiate the ratio between period α and period β mRNAs. As shown in Fig. 2, the amount of period α mRNA was judged to be larger than that of period β mRNA. To quantify this difference more firmly, we may need an elite system to amplify each mRNA isoform accurately and quantitatively. Moreover, it would be very interesting to investigate the temporal pattern of expression of the two isoforms through the day, in southern and northern wasps. As reported in chapter 3 *per* expression profile is affected differently by photoperiod in southern and northern lines of *N. vitripennis*, it is thus necessary to investigate whether light or temperature affect the splicing efficiency in favour of one or the other forms in the two lines.

Comparative analysis of the *Nasonia* and *Drosophila per* gene confirmed the high similarity with the *Drosophila* region containing the *perS* mutation site as in *Apis* (Shimizu et al., 2001; Minamoto et al., 2012) (Fig. 4). Interestingly, in *Drosophila* this region is very important for phosphorylation regulation (Garbe et al., 2013) and mutations in this area lead to different PER phosphorylation that leads to different protein stability (Garbe et al., 2013) (Fig. 5, 6). Comparing the residuals, targeted by phosphorylation in *Drosophila*, we could predict a differential phosphorylation between the α and β isoforms present in *Nasonia* that could lead to different protein stability and subsequently different protein timing.

Therefore, it is also necessary to investigate the stability of the two isoforms at the protein level. Isoforms of the PERIOD proteins can be detected by sophisticated monoclonal antibodies that can differentiate the insertion/deletion of the 8 amino acids, so that immunocytochemical study could then clarify the phosphorylation pattern, stability and neuronal localization of the two isoforms.

Fig. 1 mRNA alignment of *period* from *Apis cereana japonica* and *Nasonia vitripennis*
Per variants alignment revealed the presence of both *alpha* and *beta per* mRNA in *Nasonia vitripennis*.

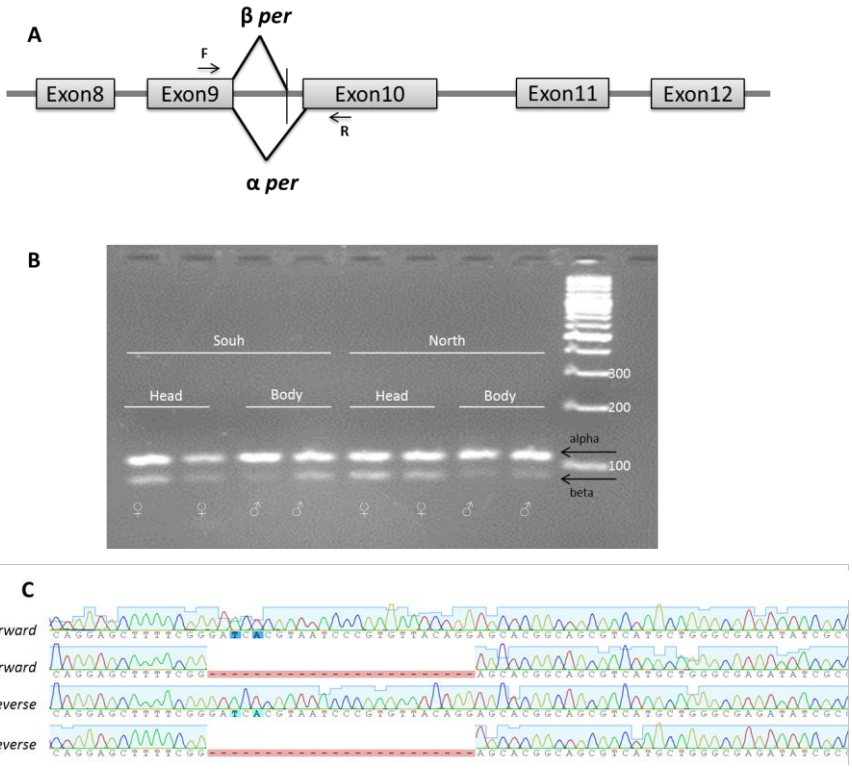

Fig. 2 Detection of *alpha* and *beta* variant of *per* in *Nasonia vitripennis*
(A) Gene structure of *per* from exon 8 to exon 12. Grey boxes indicate exons, grey lines introns and black lines the alternative splicing. Small black arrows indicate respectively the location of forward (F) and reverse (R) primers to amplify the region of the alternative splicing. **(B)** RT-PCR amplification of the splicing region detects the presence of boths *alpha* and *beta* variants. **(C)** Alignment of the sequenced α and β fragments using either the forward (F) or the reverse (R) primer.

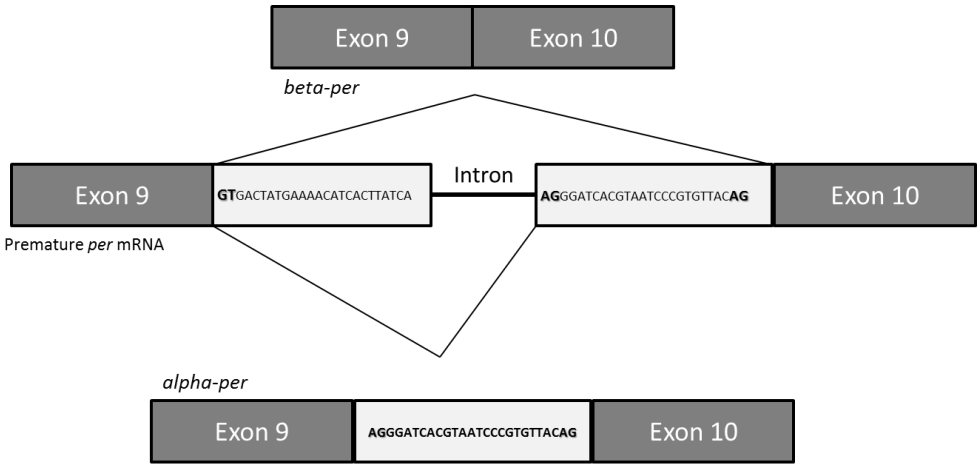

Fig. 3 Structure of the *per* splicing site

cDNA structure of the splicing site revealed the presence of the splice consensus sites **GT** at the 5' side and **AG** at the 3' side of the specific 24 bp sequence.

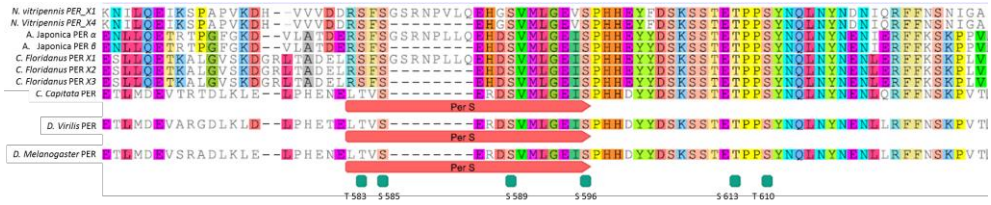

Fig. 4 Protein alignment for *per*

PER sequences alignment revealed conservation of the *per* S region of *Drosophila* (red arrows) and of the phosphorylated residues (green boxes).

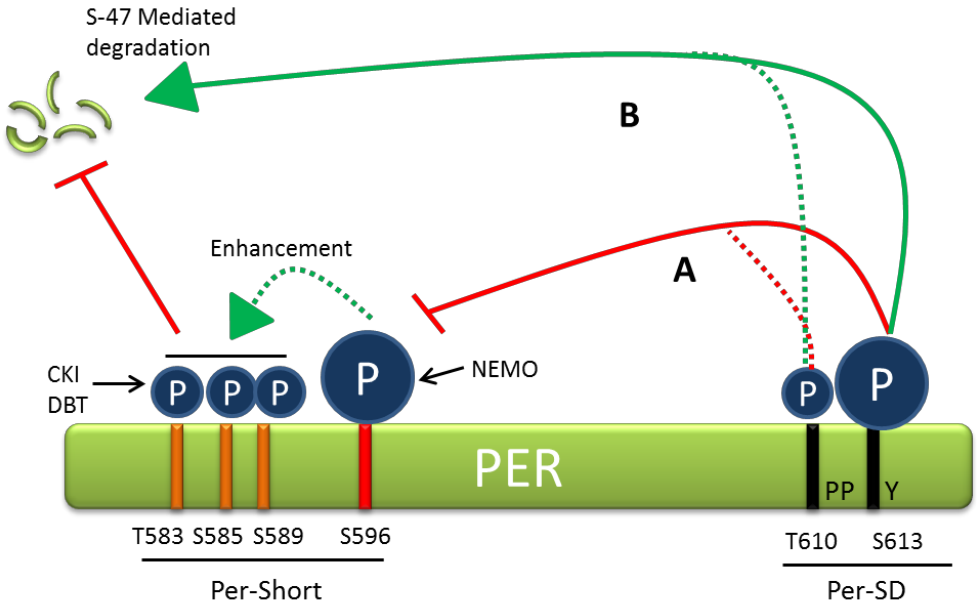

Fig. 5 Potential regulation of *Drosophia* PER via phosphorylation
In scenario (**A**), the PER-SD domain regulates phosphorylation of residues within the PER-short domain, while scenario **(B)** proposes that phosphorylation of the PER-Short and PER-SD domains control clock speed in parallel. In both cases the degradation process is regulated.

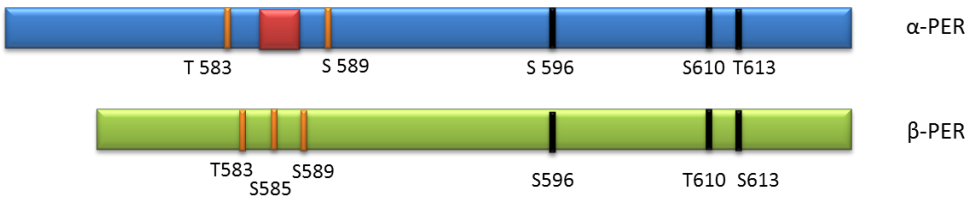

Fig. 6 Predicted phosphorylation site in *Nasonia alpha* and *beta per* variants.
The two bars represent the two PER variants, in blue the longer one (α-per) and in green the shorter one (β-per). In red is represented the acceptor splicing site that in the α sequence includes the first 24bp of the exon 10. Black bars represent the conserved predicted phosphorylation sites and orange bars the predicted phosphorylation sites that differ between the two variants.

Conclusion and open questions

To maintain approximate 24-hour cycles at the molecular level, clocks must be tightly regulated at several steps to maintain the correct period, phase and amplitude of the rhythms of thousands of proteins that generate the wide range of rhythmic biological processes. As described above, there is now abundant evidence that post-transcriptional mechanisms play an important role in shaping these rhythms (Kojima et al., 2011). However there are many open questions that need to be addressed to understand the function of different *per* isoforms. For the future it will be very important not only to specify the functions of these two *per* isoforms, but also investigate their temporal and spatial expression pattern. Especially it would be very interesting to explore functional differences in southern and northern lines of *Nasonia* in order to contribute to understand the mechanism of circadian rhythms and photoperiodism in this species.

Material and methods

Experimental line and rearing conditions

For this study we used isogenic lines established from wasps collected from the field in 2009 (see for details Paolucci et al., 2013). The lines were maintained on *Calliphora* spp. pupae as hosts in mass culture vials under diapause-preventing conditions, LD16:08, and temperature of 20 ± 1 °C.

Bioinformatic search

Protein and cDNA sequences of *Nasonia period* (*per*) were found in NCBI (NCBI Resource Coordinators, 2017), orthologous sequences of *Apis cerana Japponica*, *Drosophila*. *melanogaster* and *virilis*, *Camponotus floridanus* and *Ceratitis capitata* have been found through BLAST (Altschul et al., 1990) search using the *Nasonia per* mRNA as input sequences. Accession numbers are reported in table 1. Sequence alignments of *per* from different insect species were performed with *Geneious* (Kearse et al., 2012).

Identification of the two isoforms using reverse transcription (RT)-PCR

Primers were design with primer3 (Untergasser et al., 2012) in the region surrounding the alternative splicing sites. Amplicon size was about 100bp in order to facilitate the visualization on gel after reverse transcription (RT)-PCT.

RNA was extracted from individual bodies and heads, separately for females and males, 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). cDNA was used as template for RT-PCR using the forward primer (5'-TGTGGTTGTGGACGACAGG-3'), that maps in exon 9, and the reverse primer (5'-CTGTCGAAGTACTCGTGGTG-3'), that maps in exon 10 (Fig. 2A). The RT-PCR profile was: 3 min of activation phase at 95^{o}C, 35 cycles of 15 sec at 95^{o}C, 30 sec at 56^{o}C and 30 sec at 72^{o}C. In order to detect small size differences, PCR product was run on 3% TBE agarose gel containing ethidiumbromide. To confirm the two splicing variants, PCR products were ligated into PGEM-T vector (Promega, Madison, WI, USA) after purification using the GeneJET PCR purification kit (Fermentas, Hanover, MD, USA). Ligation reaction was used to transform competent JM-109 *E. coli* (promega, madison, WI, USA). Colony-PCR was conducted with PGEM-T primers (5'-GTAAAACGACGGCCAGT-3' and 5'-GGAAACAGCTATGACCATG-3') at 94°C for 3 min, 30 cycles of 94°C for 30s, 55°C for 30s and 72°C for 2min, with a final extension of 7 min at 72°C. Both strands were sequenced and fragments were aligned to one another and to *period* mRNA variants to inspect the splicing variation.

Table 1. Sequences used for aligment

Sequence name	Accession number	Protein ID	Gene ID
***N. vitripenis_period* x1**	XM_008211021.2	XP_008209243.1	100121302
***N. vitripennis_period* x4**	XM_008211024.2	XP_008209246.1	100121302
A.japonica_period α	AB190207.1	BAK79120.1	292787
A. japonica_period β	AB190208.1	BAB55601.1	292787
***C. floridanus _period* x1**	XM_011266355.2	XP_011264657.1	105256454
***C. floridanus _period* x2**	XM_011266356.2	XP_011264658.1	105256454
***C. floridanus _period* x3**	XM_011266359.2	XP_011264661.1	105256454
***C. capitata _period* x1**	XM_004529490.3	XP_004529547.1	101453268
***D. virilis_period* A**	XM_002056770.2	XP_002056806.1	6633594
***D. melanogaster_period* A**	NM_080317.2	NP_525056.2	31251

Acknowledgements

I would like to thank Ties Ausma for his attempts to quantify the two splicing variants, Leo W. Beukeboom and Louis van de Zande for fruitful discussion and comments.

Chapter 4

The clock gene *period* is involved in circadian and seasonal timing in *Nasonia vitripennis*

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Louis van de Zande

to be submitted

Abstract

It has been hypothesized that the endogenous circadian clock is also involved in seasonal timekeeping. In the parasitic wasp *Nasonia vitripennis*, southern (Corsica, France) and northern (Oulu, Finland) populations show allelic variation of the clock gene *period* (*per*) correlating with differences in seasonal response to photoperiodism. They also differ in circadian rhythm, southern wasps have earlier onset of activity and shorter free running rhythms in constant conditions. In this study, we further investigated the role of *per* in circadian and seasonal timing with RNA interference (RNAi). Knockdown of *per* in northern wasps led to a shorter rhythm in constant darkness (DD), an advance of the daily activity and a delayed photoperiodic diapause response. In the southern wasps *per* RNAi also induced a shorter DD rhythm and later diapause response. In constant light (LL) an increase of rhythmicity after RNAi was observed for both southern and northern wasps, suggesting an additional role for *per* in the light sensitivity pathway. Knockdown of *per* also affected the expression levels of the clock genes *cryptochrome-2* (*cry-2*), *clock* (*clk*) and *cycle* (*cyc*). These data reveal a role of *period* in the core mechanism of the circadian clock, and a role in photoperiodic time measurement in *N. vitripennis*.

Introduction

Photoperiodic diapause in insects is a prominent seasonal response allowing survival during unfavourable environmental conditions, such as low temperatures during winter. Shortening of day length is the most reliable cue to indicate an oncoming winter. A photoperiodic mechanism is responsible for detecting photoperiodic changes and storing this information in order to change the behaviour of the organism accordingly. This mechanism includes a timer to measure the duration of the light period and a counter to count the number of light-dark (LD) cycles to adequately start a photoperiodic response (such as diapause induction). It is, however not well understood how insects measure day (and night) length, store photoperiodic information and process this information to trigger the downstream diapause response (Denlinger, 2002; Kostal, 2011).

Organisms possess an internal circadian clock that synchronizes their behaviour and physiology with environmental light-dark (circadian) cycles. This clock runs with a period close to 24 hours, and modulates a variety of rhythmic processes, including rest and activity, eclosion, mating and feeding (Saunders et al., 2002). On the mechanistic level, the clock consists of several transcription factors that either activate or inhibit their own expression through feedback loops, enabling the generation of an internal oscillator that is reset every day by the light-dark cycles. For example, in *Drosophila,* the genes *period* (*per*) and *timeless* (*tim*) are negative regulators, that 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-Forster 2011). The identification of *cryptochrome-2* (*cry-2*) as a clock gene (Zhu et al., 2005) showed that regulation of the circadian clock in the honeybee *Apis mellifera* (Rubin et al., 2006), monarch butterfly *Danaus plexippus* (Zhu et al., 2005), mosquito *Anopheles gambiae* (Zhu et al., 2005) and *Nasonia vitripennis* (Bertossa et al., 2014) is different from *Drosophila*. Thus, not all clocks have the same genetic composition.

It has been hypothesized that the genetic basis of the seasonal (photoperiodic) system overlaps with the circadian mechanism of time keeping, since both involve timing of light-dark cycles (Bünning, 1960). There is experimental evidence for involvement of the circadian clock in photoperiodism (i.e. the response of an organism to seasonal changes) (reviewed in Saunders, 2013), and two prominent models were proposed to explain this involvement. One is the external coincidence model that assumes the presence of one photosensitive internal oscillator, of which the phase is set by the light cycle. When the photosensitive phase of this cycle falls into the dark, owing to the shortening of the day length, a photoperiodic response is triggered. The other model is the internal coincidence model, which assumes the presence of two circadian oscillators of which the phase-synchrony is influenced by the change of the photoperiod, making it possible to sense seasonal light-dark changes. However the role of the circadian clock in photoperiodism is

still controversial, despite recent studies that showed a functional involvement of the circadian clock gene *per* in diapause induction in the bean bug *Riptortus pedestris* (Ikeno et al., 2010; Ikeno et al., 2011a, 2011b) and the mosquito *Culex pipiens* (Meuti et al., 2015). These studies, however, did not address the possibility of a pleiotropic role of *per* in both circadian and seasonal systems (Emerson et al., 2009).

The jewel wasp *Nasonia vitripennis* shows a robust photoperiodic response, where maternal induction of larval diapause leads to developmental arrest at the fourth instar stage. Moreover, it has 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 is unknown. Paolucci et al., (2013, 2016) reported natural clinal variation in photoperiodic diapause response that correlates with allelic variation of the clock gene *per*. *Per* affects circadian clock properties, southern European *N. vitripennis* lines have a faster clock and lower activity compared to northern European lines (chapter 2). In addition, the daily expression profiles of several circadian clock genes, including *period*, is strongly dependent on photoperiod in *Nasonia* females (Chapter 3), indicating that *per* may play a role in regulating photoperiodic responses. Yet, geographical variation in circadian rhythms has not been as extensively studied as photoperiodism, making it difficult to understand how genetic variation in clock genes determines phenotypic variation.

Saunders (1974) hypothesized that *N. vitripennis* uses an internal coincidence model to detect photoperiodic changes to induce diapause, by Nanda–Hamner experiments (Nanda & Hamner, 1958). However, recent work of Vaze and Helfrich-Förster (2016) suggests that *N. vitripennis* may use a strongly damped circadian oscillator as part of an external coincidence model, to measure night length. In addition, behavioural and gene expression data, described in previous chapters, also indicate the presence of a single circadian oscillator with a morning phase in the south and an evening phase in the north, suggesting that the external coincidence model might be more applicable to explain photoperiodic diapause induction in *Nasonia*.

A role of *per* in the circadian clock of *Nasonia* has not been reported yet. Therefore, we first investigated the functional involvement of *per* in establishing the circadian rhythm of *N. vitripennis* by analysing the effect of *per* knockdown by RNA interference (RNAi) on locomotor activity behaviour under LD cycles and under constant conditions. Second, we tested how *per* RNAi affects photoperiodic diapause response. Additionally, we monitored the expression pattern of the other clock genes *cry-2*, *clk*, *cyc* after *per* RNAi, in order to carefully consider the modular role of the circadian clock in diapause regulation. Finally, we compared southern and northern strains of *N. vitripennis* that differ in *per* alleles, for locomotor activity and diapause response.

Material and methods

Experimental lines

The experimental strains for this study were isogenic lines established from isofemale lines collected from the field in 2009 (Paolucci et al., 2013). The northern wasps come from Oulu, Finland (65°3'40.16N), and the southern ones were collected in Corsica, France (42°22'40.80N). The isogenic lines were established by crossing female wasps with their son, followed by 7-8 generations of brother-sister crossing. The lines were maintained using *Calliphora* spp. pupae as hosts in mass culture vials under diapause-preventing conditions: (LD16:08) at 20°C. The same lines were used in chapter 3 for gene expression analysis. Northern line represents the N1 strains of chapter 2 and southern one the S1 strain of chapter 2.

RNA extraction, cDNA conversion

In order to obtain a sufficient amount of dsRNA for knocking down the expression of *period*, RNA extraction was performed only from the head (where the master clock is located) of wasp collected between ZT 21-24 (*Zeitgeber time*, ZT 0 corresponds to the time when the light turned on). This period corresponds to the time at which *period* is most highly expressed (chapter 3). Total RNA was extracted using Trizol reagent (Invitrogen, USA) according to the manufacturer's instructions. Each sample was submitted to a DNase treatment to eliminate any DNA contaminations, and about 1µg of RNA was used to synthetize cDNA with RevertAid H Minus First Strand cDNA Synthesis kit (Thermo scientific).

Synthesis and injection of dsRNAs

knockdown of *period* via RNAi was induced in early female pupae. Primer pairs NV_per_dsRNA_0708 and NV_per_dsRNA_1213 in table 4.1 were used for PCR to amplify two fragments of the *period* (*per*) gene. These fragments were then used as template to generate two dsRNAs. Primer set dsRNA_A is spanning exons 7 and 8, dsRNA_B is located in exons 12 and 13 (more details in Fig. S1). Both 5' and 3' of these PCR fragments, a T7 polymerase binding site was added (primers in table 1). The fragments were transcribed in both directions using the Megascript RNAi kit (Ambion, Austin, Texas, USA). Briefly, sense and antisense RNA fragments were synthetized in separate transcription reactions. After 6h incubation at 37°C, the two reactions were mixed and heated at 75°C for 5 min followed by cooling down slowly (overnight). Nuclease

digestion removed DNA and ssRNA and dsRNA was purified according to the kit protocol. Finally, the two dsRNAs were precipitated with ethanol for better purification, re-dissolved in water and stored at -20°C.

Female pupae from the southern and northern lines were injected in the abdomen following the procedure of Lynch and Desplan (2006), either with 4 µg/µl of *per* dsRNA_A (RNAi_A) or dsRNA_B (RNAi_B) mixed with red dye. Injections were performed with Femtotips II (Eppendorf, Hamburg, Germany) needles 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/PBS Petridish at 25^{o}C at the experimental photoperiods, either LD 08:16 for subsequent use in the diapause and locomotor activity experiments, or LD 16:08 for a second locomotor activity experiment. Control pupae were injected with red dye mixed with water in a 1:4 ratio.

Entrainment and sample collection for gene expression analysis

Control and RNAi females were kept under short photoperiod (LD08:16) at 20^{o}C in groups of 5 and provided with hosts. After three days post eclosion, three biological replicates of 5 wasps were collected every four hours 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^{o}C.

RNA was extracted from pooled head samples as described before, and cDNA conversion was performed as per manufacturer's instructions. The cDNA was diluted 50x prior to use for Real Time PCR (qPCR). qPCR was performed with SYBR green (Quanta Biosciences) and *rox* as the internal passive reference. 4µl of diluted cDNA was used for each reaction of 20µl total containing primer at the final concentration of 200nM and 10µl of SYBR green/ROX buffer solution. Three technical replicates for each reaction served to control for pipetting variation. Reactions were run on an ABI7300 with the following qPCR profile: 3 min of activation phase at 95^{o}C, 35 cycles of 15s at 95^{o}C, 30s at 56^{o}C and 30s at 72^{o}C. Table 4.2 lists the primers for *per*, *cry-2*, *cyc* and *clk*. *Elongation factor 1α* (*ef1α*) and *arginine kinase* (*ak*) were used as reference genes.

Expression data were first analysed with LinRegPCR (Ramakers, Ruijter, Deprez, & Moorman, 2003; Ruijter et al., 2009). *Ef1α* and *ak* were used as reference genes, after confirmation that their expression level is constant throughout the day (chapter 3) and between treatments (Fig. S2). A generalized linear mixed effect model (glm) was used to analyse expression levels with R statistical software (R version 3.4.1). A quasi-poisson distribution for the glm 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 analyses were performed with the multcomp package for effects of RNAi treatments within lines.

Locomotor activity

Locomotor activity was measured of adult injected females from southern and northern lines entrained to 4 days of LD08:16 or LD16:8 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 5mm, height 70mm) a quarter filled with sugar-water gel medium. They were continuously monitored for movement by infrared beam arrays in Trikinetics *Drosophila* activity monitors (www.trikinetics.com), each monitor allowing for the recording of 32 wasps. The detector records how many times per minute each individual interrupts an infrared light beam that passes through a 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 60 lum/ft^2 (3.15 W/m2). Data were collected and analysed with DAM System 2.1.3 software.

Table 4.1: Primers to produce dsRNA of *period* region A (named dsRNA_0708 or RNAi_A) and region B (named dsRNA_1213 (RNAi_B)

Primers name	Forward primer	Reverse primer
NV_per_dsRNA_0708 (region A)	5'-CCTTCTTCCAACCCATACGG-3'	5'-CTCAATGATCTTGGCTTCCTG-3'
NV_per_dsRNA_1213 (region B)	5'-CTGCTGTCGTTAGATGTGAG-3'	5'-GTCGCCATATCAGTTATCGG-3'
NV_per_dsRNA_1213_T7 (region A)	5'-TAATACGACTCACTATAGGGC CTTC TTCCAACCCATACGG-3'	5'-TAATACGACTCACTATAGGGCTC AATGATCTTGGCTTCCTG-3'
NV_per_dsRNA_1213_T7 (region B)	5'-TAATACGACTCACTATAGGG CTGCTGTCGTTAGATGTGAG-3'	5'-TAATACGACTCACTATAGGGGT CGCCATATCAGTTATCGG-3'

Table 4.2: Primers used for qPCR of clock genes and reference genes

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'

Diapause induction

Injected wasps were tested for diapause response under LD08:16 at 20°C to study *per* knockdown effects under diapause-inducing conditions. Following Paolucci *et al*. (2013), 50 adult females post-injection were kept in cotton-plugged h60mm × d10mm polystyrene tubes with two hosts in a light box with a controlled light-dark 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 produce diapausing larvae after exposure to a certain number of light-dark 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 indicated by the presence of larvae at this stage (Paolucci, et al., 2013). For each female, the daily production of diapausing offspring per host was measured as the proportion of diapausing brood relative to the total number of host pair per day. The diapause switch point is calculated as the day at which wasps switch from producing developing offspring to diapause offspring.

Behavioural data analysis and statistics

The raw locomotor activity data were first visualized with the program ActogramJ (Schmid et al., 2011; available at http://actogramj.neurofly.de). Double-plot actograms obtained with this software represent activity levels. Under LD conditions the average activity was calculated according to Schlichting and Helfrich-Forster (2015) to find the onset, the peak and the offset of activity, and compare them between southern and northern wasps and between treatments (control vs. RNAi). To determine the onset and offset of activity of the average day data per wasp have to be plotted as bar diagrams with each bar representing the sum of activity within 20min. The onset represents the first time bar when activity starts to rise consecutively, whereas the offset is when activity reaches the level, which is stable during the night phase. To determine the timing of the peaks, the data are smoothed by a moving average of 30min. 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 *Zeitgeber Time* (ZT), was compared between strains and treatments. Statistical analysis on timing of activity was performed by ANOVA with a Tukey's multiple-comparisons test.

The free running period (τ) was determined under constant darkness and constant light, with periodogram analysis, which incorporates X^2 analysis (Sokolove & Bushell, 1978). τ values were compared between strains and treatments with ANOVA and a Tukey's

multiple-comparisons in R.

Survival tests were used to compare diapause response curves between strains (package *survival* in R (Therneau & Lumley, 2014) followed by pairwise comparisons with Log-Rank test (package *survminer* in R) (Kassambara et al., 2017). P-values have been corrected with Benjamini-Hochberg (BH) procedure (Benjamini & Hochberg, 1995). All statistical tests were performed with R statistical software (R version 3.4.1).

Results

Efficiency of per *RNAi*

The level of *per* expression was analysed three days after eclosion under LD08:16 at ZT 0, to assess the efficiency of RNAi. In wildtype *N. vitripennis*, the expression of *per* is highest at this time point (chapter 3, Fig. 3.3B). The relative expression level of *per* in the dsRNA-injected wasps was lower in both the southern and northern lines compared to their respective controls ($p<0.001$) (Fig. 4.1A, B; Table S1), indicating an efficiency of 50 to 60 % of *per* knockdown. Two additional time points analysed during the light phase (ZT 4 and ZT 8) show that *per* expression decreased during the light phase in control wasps ($p<0.001$) whereas the RNAi-treated wasps, displayed a stable lower *per* expression (Fig. 4.1A, B; table S1).

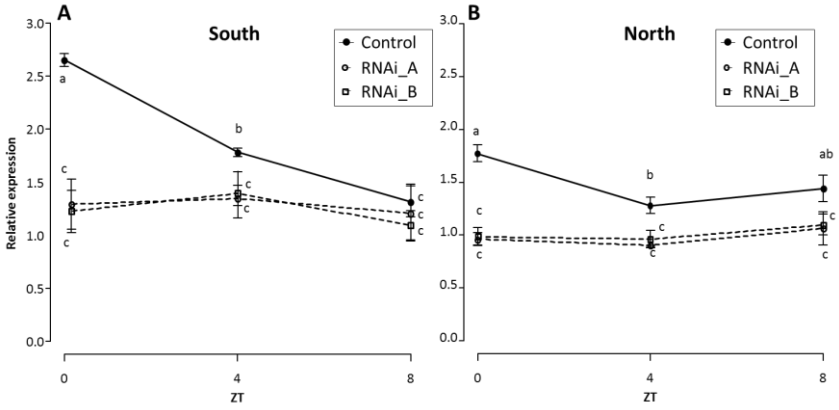

Fig. 4.1: *Period* expression in control and RNAi wasps
(A) Southern *period* mRNA expression in control and RNAi wasps injected either with dsRNA_A or with dsRNA_B. **(B)** Northern *period* mRNA expression in control and RNAi wasps respectively injected with dsRNA_A and dsRNA_B. *Zeitgeber time* (ZT) is given in hours on the X-axis where ZT=0 represents light on. Letters indicate significant differences between ZTs and between treatments ($p<0.05$).

Free running rhythms under DD and LL

In the locomotor activity assays, we exposed the wasps to a light-dark regime of either 08:16 followed by constant darkness (DD) for 10 days, or 16:08 for 4 days, followed by constant light (LL) for 10 days. The free running rhythms under DD and LL were compared between each line and treatment. Under DD, the southern line shows a shorter free running rhythm (τ=24.67 $\pm$ 0.10 h) compared to the northern one (τ=26.57 $\pm$ 0.12 h) (Fig. 4.2). After *per* RNAi a significant shortening of about one hour of τ was observed ($p<0.001$), 23.80 $\pm$ 0.06 h and 25.25 $\pm$ 0.17 h for the southern and northern lines, respectively (Fig 4.2). The rhythmicity level was not clearly affected under DD 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 S2).

Under constant light (LL) the rhythms are shorter than under DD for both lines (Fig. 4.3). The southern line has a τ of 22.32 $\pm$ 0.16 h, and a high level of arrhythmicity (83%); northern wasps have a rhythm of 23.24 $\pm$ 0.32 h and 84% of arrhythmicity (Fig. 4.3; Table S2). Interestingly, *per* RNAi in southern wasps led to an even shorter τ of 21.10 $\pm$ 0.15 h and an increase in the number of rhythmic individuals by 20% (Fig. 4.3A, Table S2). In contrast, *per* RNAi increased the free running rhythm in the northern line by about 2h, with a τ of 25.01 $\pm$ 0.44 h (Fig. 4.3B). Again, the number of rhythmic wasps increased in treatment RNAi_A by 10%, but in RNAi_B the number was unaltered (Table S2).

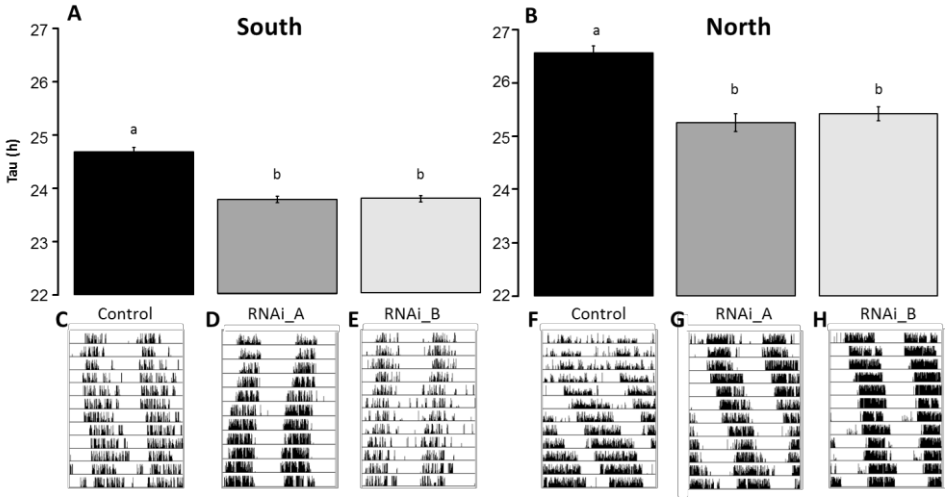

Fig. 4.2: Constant darkness (DD) rhythms of control and RNAi wasps(A) Southern free running rhythms in DD in in control and RNAi- injected wasps with either dsRNA_A or with dsRNA_B. **(B)** Northern free running rhythms in DD in in control and RNAi wasps injected either with dsRNA_A or with dsRNA_B. **(C-E)** Southern double plot actograms and **(F-H)** northern ones. Black bars indicate activity. Different letters indicate significant differences.

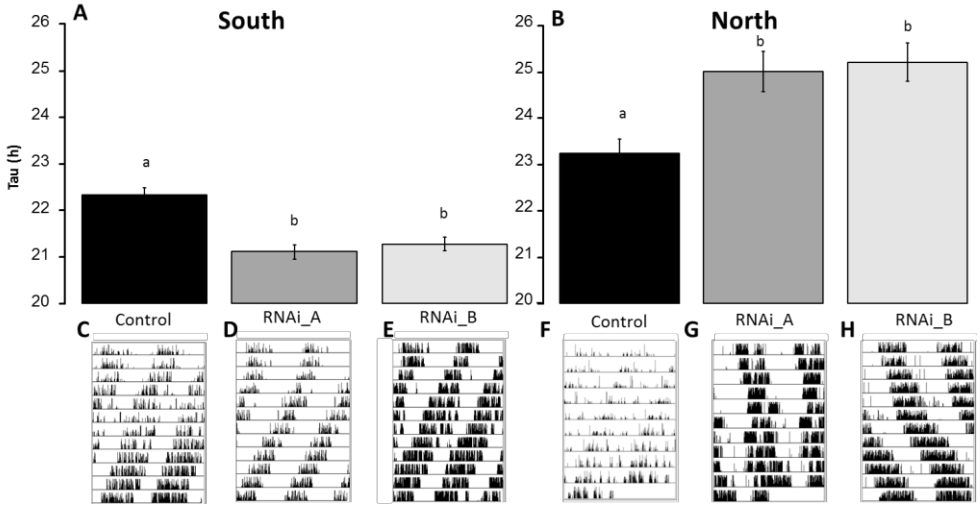

Fig. 4.3: Constant Light (LL) rhythms of control and RNAi wasps
(A) Southern free running rhythms in LL in in control and RNAi wasps injected either with dsRNA_A or with dsRNA_B. **(B)** Northern free running rhythms in LL in in control and RNAi wasps injected either with dsRNA_A or with dsRNA_B. **(C-E)** Southern double plot actograms and **(F-H)** northern ones. Black bars indicate activity. Different letters indicate significant differences.

Timing of locomotor activity

The timing of the locomotor activity was analysed under both LD conditions for 4 days. Both southern and northern wasps displayed a unimodal activity pattern (Fig. 4.4); however, significant differences were detected in the timing of onset, maximum peak and offset of activity (Table S3). Under LD 08:16 southern wasps started activity on average four hours before the light turn-on, while northern wasps became active four hours later, around the time the light was turned on (ZT 0)($p<0.001$). The peaks of activity differ by about 1.5 hour, occurring around ZT 1 for the southern wasps and around ZT 2.5 for the northern ones ($p<0.001$). Interestingly, whereas southern wasps ceased activity when the light was turned off (ZT 8), the northern ones prolonged activity for more than 3 hours into the dark phase (until ZT 11.5) ($p<0.001$) (Fig. 4.4A, D, Table S3). Average daily activity was not affected by RNAi in the southern wasps but was advanced significantly in the northern wasps ($P<0.001$). Northern RNAi wasps started activity about three-and-half hours into the dark phase, a four-hour shift compared to control wasps. Peak of activity and offset of activity, however, did not differ in timing between control and RNAi treatments although an increase of the level of activity in the dark phase for the northern RNAi-treated wasps

could be observed (Fig. 4.4A, Table S3).

Under LD16:08 both southern and northern wasps started activity around ZT 0 when the light was turned on, but the peak of activity occurred much earlier in the morning in the southern line; ZT 3 compared to ZT 10 at the end of the day in the northern line. Southern wasps ceased activity in the late afternoon at ZT 14, whereas the northern ones became inactive when the light was turned off around ZT 16 (Fig. 4.4B, Table S3). After *per* RNAi, southern wasps did not significantly change the timing of activity, whereas northern wasps displayed a strong advance of the activity peak to ZT 5.5 ($p<0.001$). Onset and offset of activity remained the same (Fig. 4.4B; Table S3).

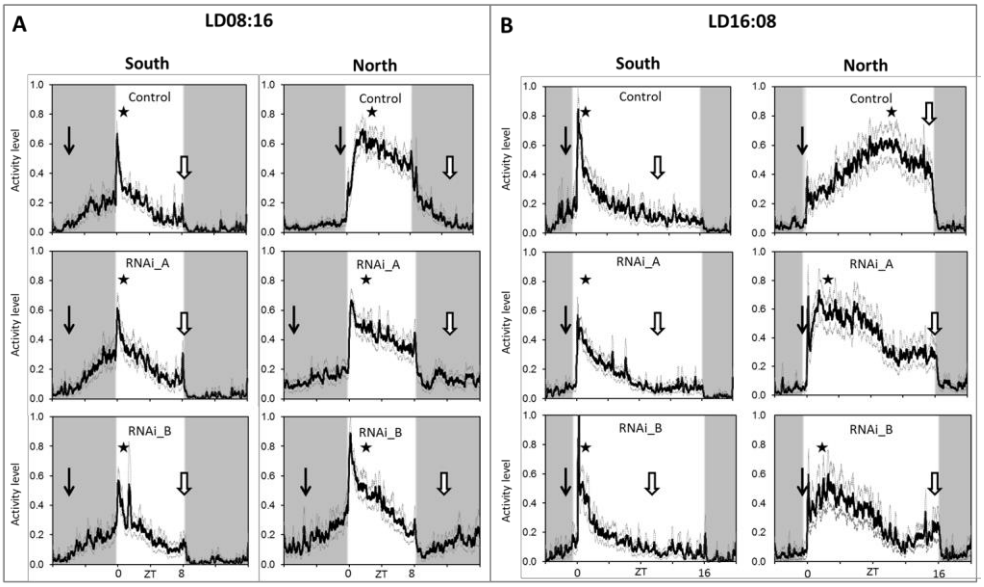

Fig. 4.4: Locomotor activity of control and RNAi wasps

Locomotor activity profile of southern) and northern wasps (control and RNAi respectively) are shown as average of bin crosses/minute of 25-32 individuals each over 24 hours periods at (A) LD08:16 and at (B) LD16:08. The night phase is indicated by grey shading, the day phase in white. *Zeitgeber time* (ZT) is given in hours on the X-axis where ZT=0 represents light on. Black arrows indicate the onset, stars the peak and white arrows the offset of activity.

Expression pattern of cry-2, clk and cyc

Cry-2, *clk* and *cyc* expression was measured in control and *per* RNAi-treated wasps during three points in the light phase (ZT0, ZT4, ZT8) (Fig. 4.5; Table S4, S5, S6). In southern wild type (chapter 3, Fig. 3.3D) and control wasps, *cry-2* decreased its expression during the light phase (Fig. 4.5A; Table S4), whereas *per*-RNAi-treated wasps displayed a lower and constant *cry-2* expression for all the three time points (Fig. 4.5A; Table S4). Both c*lk* and *cyc* had a significant lower expression during all ZTs ($p< 0,001$) (Fig. 4.5C, E; Table S5, S6). Moreover the oscillation of *cyc*, which increased during the light phase (Fig 4.5E; chapter 3, Fig. 3.4B, 3.5B), is disrupted in RNAi-treated wasps (Fig 4.5E; table S6). Similarly, in the northern wasps, *cry-2* expression is significantly lower in *per* RNAi-treated wasps compare to the control ($p<0.05$) (Fig. 4.5B; Table S4). Expression levels of *clk* and *cyc* are also significantly lower in RNAi-treated northern wasps (Fig. 4.5D, F; Table S5, S6), with a disruption of *cyc* oscillation, like in the southern wasps (Fig. 4.5F; Table S6). Thus, RNAi of *per* alters the phase and the expression of the whole circadian system.

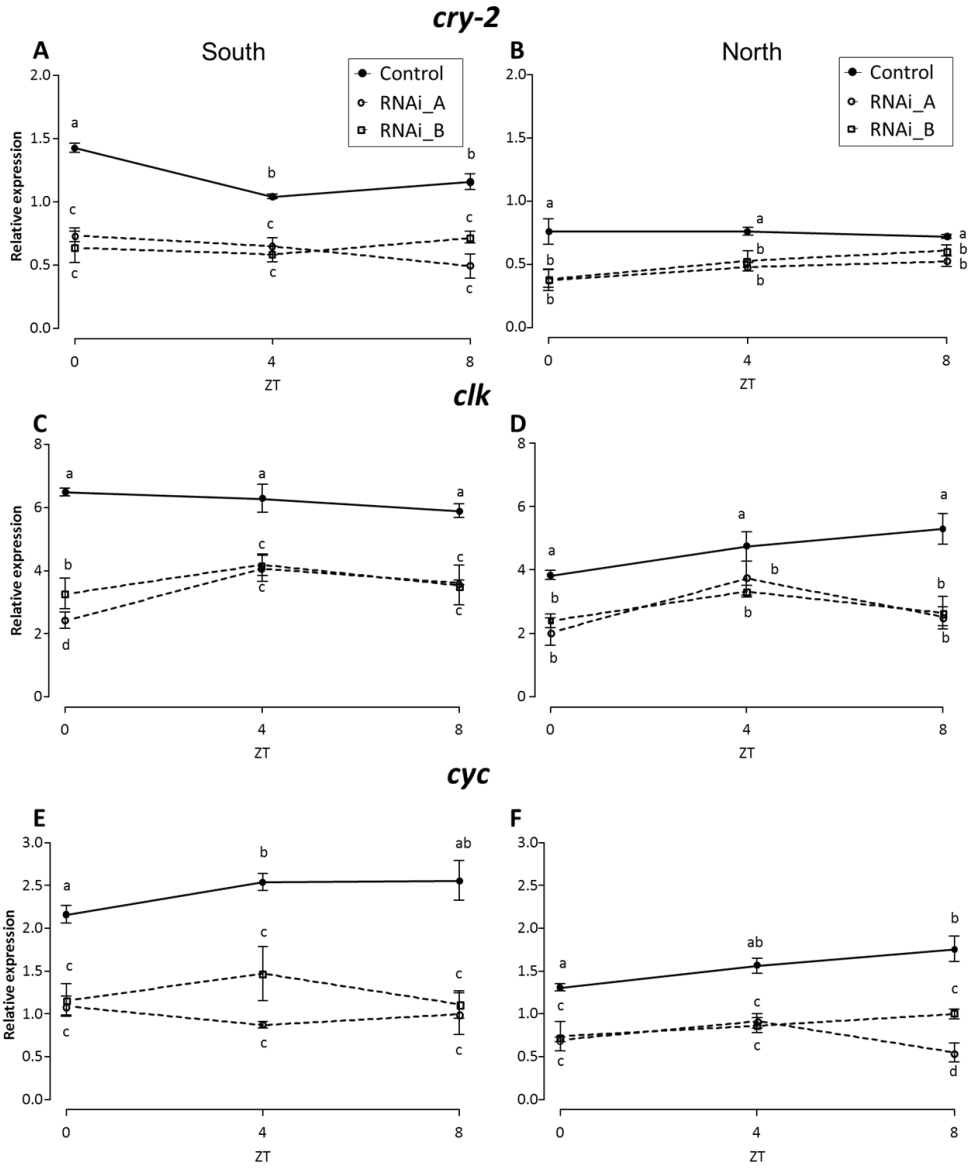

Fig. 4.5. clock gene expression in control and *per* RNAi wasps

Clock gene expression for **(A, B)** *cry-2*, **(C,D)** *clk* and **(E,F)** *cyc* respectively for southern and northern lines *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<0.005).

Diapause response

The diapause response under LD08:16 was assessed in RNAi-treated wasps and controls for southern and northern lines. Although all wasps reach the switch point, the southern wasps started to produce diapause offspring much later than the northern ones. For both lines, RNAi-treated females showed a later switch point and a delayed diapause response curve (Long-Rank test for survival multiple comparison $P<0.05$). The average switch point of controls was day 8 in the southern wasps and day 4 in the northern ones, in agreement with earlier observations (Paolucci et al, 2013). After *per* knock-down, southern wasps delayed the switch point with 2 days to day 10 and the northern ones with 4 days to day 8 (Fig. 4.6; S2). This means that both the absolute and relative delay time was twice as long for northern than for southern wasps.

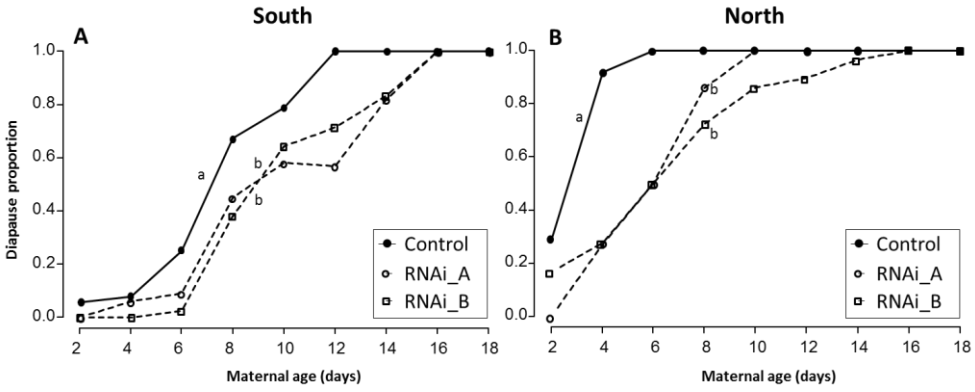

Fig. 4.6: Diapause response of control and RNAi wasps
Diapause response of females under LD08:16. **(A)** Southern wasps, control, RNAi-treated wasps and RNAi_B treated ones. **(B)** Northern wasps, control, RNAi-treated wasps and RNAi_B treated ones. Letters indicates significance differences (pairwise comparisons using Log-Rank test).

Discussion

In this study we investigated the role of the *period* gene in the regulation of circadian rhythms and photoperiodic response in *Nasonia vitripennis*. Knock down of *per* alters the daily rhythm under constant conditions (DD and LL), changes the timing of locomotor activity, and affects the expression of other clock genes. We further found that photoperiodic diapause response is delayed in RNAi-treated wasps, irrespective of their geographic origin.

DD circadian rhythms are affected by **per** *RNAi*

Wasps from southern and northern lines showed differences in circadian clock properties. Southern lines are more active during the first part of the day, whereas northern ones are active in the late afternoon. This different timing of activity reflects the speed of their clocks in constant darkness (DD). Southern lines show shorter free running rhythms (τ) close to 24 h, compared to northern ones that naturally have a τ longer than 24h. The presence of such a positive latitudinal cline in the DD rhythm for *N. vitripennis* was previously reported by Paolucci (2013) (see chapter 2) and also described for the linden bug *Pyrrhocoris apterus* (Pivarciova et al., 2016). This could mean that the latitudinal differences in free running period are the result of selection acting on traits genetically correlated with circadian rhythms (such as seasonal response), instead of acting on the circadian rhythm itself. The knockdown of *per* expression resulted in a shortening of the free running period in constant darkness, and advanced the activity phase of the northern wasps in LD16:08 and LD08:16. Moreover, it increased the speed of the clock (shorter τ) in both southern and northern lines. It is clear from these data that *per* is involved in setting the speed and the phase of the clock, in agreement with its described role in *Drosophila* (Konopka & Benzer, 1971) and mammalian systems (Chen et al., 2009; Lee et al., 2011). The effects of speeding up the clock in the northern lines are more pronounced, because after *per* RNAi the daily timing resembles that of the natural pattern in the southern lines, namely an advance in activity profile with a peak of activity in the morning rather than in the evening.

LL circadian rhythms are affected differently in southern and northern lines by **per** *RNAi*

Under LL conditions, *per* RNAi increased the duration of the free running rhythm in northern wasps, whereas it decreased in southern wasps. This indicates a different effect of *per* (and of light) in the regulation of DD and LL rhythms. This difference could reflect the

presence of different circadian oscillators in southern and northern wasps, of which the phases are set by dawn in the south and dusk in the north. As proposed by Pittendrigh and Daan (1976), in the dual oscillators model, the two oscillators show different responses to light: one is accelerated and the other decelerated by constant light. One oscillator will thus shorten and the other oscillator will lengthen its period when exposed to LL (Daan et al., 2001; Pittendrigh & Daan, 1976). This difference is also visible in wildtype *Nasonia* wasps with an intact circadian clock, but as soon as we start manipulation by knocking down *per*, this differential regulation becomes more evident. This confirms that southern and northern wasps not only differ in the speed of their clock, but also in the phase of their circadian oscillator. It is in agreement with the gene expression data reported in Chapter 3, where the phase of *per* mRNA expression differed between southern and northern lines and between photoperiodic conditions. However, it must be noted that this is only one of a number of possible interpretations. If these differences indeed reflect the presence of two different neuronal oscillators with different phases in the south and the north, further analysis should identify neurons in the brain with different circadian expression between southern and northern wasps.

Surprisingly, the fraction of wasps exhibiting circadian rhythmicity under LL was higher among the RNAi-treated wasps than the control wasps, indicating that *per* could be regulated by light, as is the case in the mammalian system where light induces *per* expression (Okamura et al., 1999). It has been shown that in mammals *per* is important for light induced resetting of the circadian clock (Albrecht et al., 2001), and recently Akiyama et al., (2017) showed geographical variation in the frequencies of *per* haplotypes in humans associated with variation in light sensitivity. In *Drosophila* a similar geographical variation was observed for the clock gene *timeless* (*tim*) (Tauber et al., 2007). Different *tim* alleles confer different light-sensitivity phenotypes (due to different interaction of TIM isoforms with the CRY-1 proteins) leading to different photo-responsiveness. Moreover, the different alleles of this gene were found to influence diapause incidence (Sandrelli et al., 2007; Tauber et al., 2007). Thus, it has been hypothesized that different circadian photo-responsiveness associated to the two *tim* alleles contributes to translating the photoperiodic information. We hypothesize a similar role of *per* in *Nasonia* as its function seems to resemble *tim* in stabilizing the main clock repressor CRY-2 (Buricova et al., unpublished), and its knockdown increased wasp rhythmicity in LL. It has been argued that, due to the variable environment in temperate zones, the light sensitivity of the circadian clock needs to be adjusted to northern latitude. One possible mechanism for this process could involve different filters of the light input into the clock between southern and northern regions. We already showed that photoperiod differently affects clock gene expression patterns in the two lines carrying two different *per* alleles (chapter 3). This points to a higher photo-responsiveness at higher latitude that, together with a weaker clock oscillation, could make northern wasp more flexible in adjusting to a variable environment. Furthermore, our data

point towards a role of *per* in the light sensitivity pathway. It would be very interesting to test whether the different *per* alleles in *Nasonia* also differ in light sensitivity as was reported for *tim* alleles in *Drosophila* (Sandrelli et al., 2007; Tauber et al., 2007), and whether the light signal is differently filtered into the clock system of southern and northern wasps.

Cry-2, clk *and* cyc *expression is affected by per RNAi*

We further analysed the effect of *per* knockdown on the expression of the clock genes *cry-2*, *clk* and *cyc*. Generally the expression levels of *cry-2* transcript in *per* RNAi wasps was altered and lower than the controls. In addition, its oscillation was disrupted in RNAi-treated wasps and the expression of *clk* and *cyc* transcripts was also lowered in southern and northern wasps. Taken together, these data indicate, as expected, that manipulating one of the genes in the feedback loop of the circadian clock, causes the whole system to go out of phase. This obviously complicates the interpretation of results, i.e. whether the phenotypic differences are due to one of the genes altered by per RNAi or to the circadian system being out of phase as a whole.

Diapause response is delayed in per *RNAi-treated wasps*

Interestingly, in the RNAi-treated wasps photoperiodic diapause response was also affected. Although all wasps were able to induce diapause in their offspring after *per* knock down, the timing of the photoperiodic response was delayed in both lines. This indicates that *per* knockdown is not affecting the physiology of diapause itself, but the onset of it, i.e. the timer component of the photoperiodic calendar. These data can be interpreted in two ways: (i) there is a pleiotropic role of a single clock gene in photoperiodism or (ii) the circadian clock, as a functional module, underpins photoperiodism. Under the first hypothesis, *per*iod could have a pleiotropic role in both circadian and seasonal response. Alternatively, the pleiotropic role can be from one of the other genes whose expression is altered by *per* RNAi. On the other hand, the role of the circadian clock as a functional module cannot be excluded, both processes (daily and seasonal) rely primarily on the input of light and it is the interaction between light and the photosensitive element (unknown in *Nasonia*) to set the circadian clock. Moreover, the different *per* alleles, associated with different circadian and seasonal responses (Paolucci et al., 2013, 2016), seem to be responsible for the different speed and phase of the southern and northern circadian clock. Changing the phase and the speed of this circadian clock, by manipulating *per* expression profile, results in an altered diapause response. Taken together, these results indicate the presence of a single oscillator whose phase differs in the southern and northern wasps.

Thus, we can conclude that both the pace and the phase of the circadian clock are important for photoperiodic measurement. Moreover, the external coincidence model seems to be the system used by *Nasonia* to detect photoperiodic changes. New studies, using the newly designed T-cycle experiments of Vaze and Helfrich-Förster (2016), in conjunction with experiments aimed to identify this oscillator at the neuronal level, may help to test this hypothesis.

This second hypothesis, in which the circadian clock as a module plays a role in photoperiodism, is in agreement with the results of knockdown experiments in the bug *Riptortus pedestris* (Bradshaw & Holzapfel, 2010; Ikeno et al., 2011a) and the mosquito *Culex pipiens* (Meuti et al., 2015).In these studies the disruption of the circadian clock by knocking down clock gene expression also induced disruption of a proper diapause response. In 1989, Saunders et al., showed that *per* null-mutations (per^0) in *D. melanogaster* did not affect the diapause incidence. Initially, these results were hard to judge, but they showed a shift of the critical photoperiod (i.e. the photoperiod at which 50% of the population shows a diapause response), indicating that the timing mechanism was altered in this per^0 flies (Saunders et al., 1989). Despite the difficulties to distinguish between pleiotropic and modular involvement of *per* and the circadian clock in photoperiodism, there are now more studies in favour of the involvement of the same robust oscillator in circadian activity control than there are against it.

Our study also added a geographical variation component to clock gene involvement in seasonal adaptation. We found that *per* knockdown affects northern wasps twice as strong as southern ones. This can also be interpreted in the context of the two-abovementioned hypotheses (e.g. pleiotropy and modular effect). Under both scenarios, *per* is important in sensing the photoperiodic changes, independently or through the circadian clock mechanism, and if it is light sensitive (directly or indirectly) as suggested from the LL data, the allelic differences between north and south might reflect the presence of a more sophisticate system in northern populations. This will allow them to detect small environmental changes, in which a small perturbation of the system generates a greater effect, allowing northern populations to respond faster and stronger to a changeable environment and allowing them to adjust their physiology and behaviour accordingly. We recently showed that northern *Nasonia* wasps have lower amplitude in circadian clock gene oscillation and that expression of *per* and the other clock genes, is also affected by photoperiodic changes (chapter 3). These data together suggest the evolution of more sensitive clocks at higher latitude that would allow northern wasps to adapt quicker to a variable environment. These clocks might represent a more sophisticated mechanism for LD measurement and/or counting that is the main cue for both daily and photoperiodic responses.

Conclusion

Knockdown of the circadian clock gene *per* affects the speed of the circadian clock and the timing of photoperiodic diapause response. Our results indicate that *per* is a core component of the circadian clock generating daily rhythms in locomotor activity and also involved in photoperiodic time measurement in *Nasonia vitripennis* without affecting the diapause physiology itself. This effect on timing suggests that natural selection acted on the timer component of the wasps rather than on the physiology of diapause. *Per* could have a pleiotropic role in both circadian and seasonal clocks (Emerson et al., 2009). However, different *per* alleles are associated with different circadian and seasonal responses (Paolucci et al., 2013, 2016), they have different expression profiles, and are differently regulated by photoperiod (chapter 3). We showed that manipulating the expression of *per* also leads to shifts in the expression of the other clock genes *cry-2*, *clk* and *cyc* causing northern wasps to behave more similar to southern ones. Taken together, these results indicate that the circadian clock acts as a functional unit, rather than that an individual gene regulates diapause initiation in *N. vitripennis*. We also suggest a role for *per* in the light sensitivity pathway, as an increased rhythmicity was found for RNAi-treated wasps, and we suggest that the differences in *per* alleles can lead to different photo-sensitive proteins, in agreement with the idea that natural selection acted on the sensitivity of the wasps. Additional functional studies, like a complete knock out of *per* and/or inducing a targeted mutation aimed to slow down the circadian clock through CRISPR/CAS9 method, could give more information about the role of *per* in regulating photoperiodic diapause response in *N. vitripennis*. Furthermore, functional studies on other candidate clock genes and/or neuropeptides as Pigment Dispersing Factor (PDF) would also give a more complete picture of the molecular mechanism underlying photoperiodic time measurement.

Acknowledgements

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Supplementary Tables and Figures

Table S1. Comparison of *per* expression between treatments (control vs the two RNAi) and among time points. P value from Posthoc analyses, P<0.05 are in bold.

***per* North**									
	ZT0 control	ZT4 control	ZT8 control	ZT0 RNAi_A	ZT4 RNAi_A	ZT8 RNAi_A	ZT0 RNAi_B	ZT4 RNAi_B	ZT8 RNAi_B
ZT0 control		**0.015**	0.085	**<0.001**			**<0.001**		
ZT4 control			0.527		**0.021**			**0.014**	
ZT8 control						**0.012**			**0.017**
ZT0 RNAi_A					0.999	1.000	1.000		
ZT4 RNAi_A						0.890		1.000	
ZT8 RNAi_A									1.000
ZT0 RNAi_B								1.000	1.000
ZT4 RNAi_B									0.965
ZT8 RNAi_B									
***per* South**									
	ZT0 control	ZT4 control	ZT8 control	ZT0 RNAi_A	ZT4 RNAi_A	ZT8 RNAi_A	ZT0 RNAi_B	ZT4 RNAi_B	ZT8 RNAi_B
ZT0-control		**<0.001**	**<0.001**	**0.006**			**<0.001**		
ZT4 control			**<0.001**		**0.035**			**0.002**	
ZT-8 control						0.966			1.000
ZT0-RNAi_A					1.000	0.947	1.000		
ZT4 RNAi_A						0.904		1.000	
ZT-8 RNAi_A									1.000
ZT0-RNAi_B								1.000	1.000
ZT4 RNAi_B									1.000
ZT-8 RNAi_B									

Table S2. Free running rhythms and arrhythmicity. Letters indicates significant differences among treatments and southern and northern lines (ANOVA with Tukey's multiple comparison p<0.001).

Treatment	DD		LL	
	Tau (τ)	Arrhythmicity	Tau (τ)	Arrhythmicity
	h	%	h	%
Control (north)	26.57 ± 0.12 (a)	65	23.24 ± 0.32 (a)	83
RNAi_A (north)	25.25 ± 0.17 (b)	73	25.01 ± 0.44 (b)	72
RNAi_B (north)	25.41 ± 0.13 (b)	60	25.21 ± 0.41 (b)	89
Control (south)	24.67 ± 0.10 (b)	43	22.32 ± 0.16 (c)	84
RNAi_A (south)	23.78 ± 0.06 (c)	38	21.10 ± 0.15 (d)	55
RNAi_B (south)	23.80 ± 0.06 (c)	35	21.26 ± 0.15 (d)	72

Table S3. Timing of locomotor activity. Letters indicates significant differences among treatments and southern and northern lines (ANOVA with Tukey's multiple comparison p<0.001).

Treatment	LD08:16			LD16:08		
	Onset	Peak	Offset	Onset	Peak	Offset
	ZT (h)	ZT (h)	ZT (h)	ZT (h)	ZT (h)	ZT (h)
Control (north)	23.98 ± 0.16 (a)	2.21 ± 0.39 (a)	11.61 ± 0.26 (a)	0.08 ± 0.10 (a)	10.06 ± 0.25 (a)	15.74 ± 0.14 (a)
RNAi_A (north)	20.53 ± 0.30 (b)	3.04 ± 0.21 (a)	11.55 ± 0.31 (a)	23.89 ± 0.12 (a)	5.35 ± 0.30 (b)	20.32 ± 0.34 (b)
RNAi_B (north)	19.92 ± 0.28 (bc)	1.56 ± 0.19 (a)	9.88 ± 0.82 (a)	23.86 ± 0.14 (a)	5.87 ± 0.37 (b)	15.91 ± 0.08 (a)
Control (south)	19.33 ± 0.16 (c)	0.45 ± 0.16 (b)	8.24 ± 0.24 (b)	23.06 ± 0.14 (b)	2.81 ± 0.15 (c)	13.58 ± 0.21 (c)
RNAi_A (south)	19.78 ± 0.18 (b)	0.44 ± 0.24 (b)	8.47 ± 0.14 (b)	23.29 ± 0.14 (b)	2.91 ± 0.21 (c)	12.47 ± 0.47 (c)
RNAi_B (south)	19.53 ± 0.23 (b)	0.05 ± 0.19 (b)	8.26 ± 0.23 (b)	23.53 ± 0.10 (b)	2.99 ± 0.20 (c)	13.40 ± 0.27 (c)

Table S4: Comparison of cry-2 expression between treatments (control vs the two RNAi) and among time points. P value from Posthoc analyses, P<0.05 are in bold.

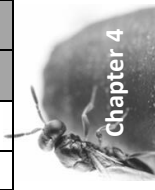
***cry-2* North**									
	ZT0 control	ZT4 control	ZT8 control	ZT0 RNAi_A	ZT4 RNAi_A	ZT8 RNAi_A	ZT0 RNAi_B	ZT4 RNAi_B	ZT8 RNAi_B
ZT0 control		1.000	1.000	**<0.001**			**<0.001**		
ZT4 control			1.000		**0.010**			**0.030**	
ZT8 control						**0.047**			**0.049**
ZT0 RNAi_A					0.964	0.919	1.000		
ZT4 RNAi_A						0.666		1.000	
ZT8 RNAi_A									1.000
ZT0 RNAi_B								0.722	0.205
ZT4 RNAi_B									0.993
ZT8 RNAi_B									
***cry-2* South**									
	ZT0 control	ZT4 control	ZT8 control	ZT0 RNAi_A	ZT4 RNAi_A	ZT8 RNAi_A	ZT0 RNAi_B	ZT4 RNAi_B	ZT8 RNAi_B
ZT0 control		**0.047**	**0.047**	**<0.001**			**<0.001**		
ZT4 control			0.984		**<0.001**			**<0.001**	
ZT8 control						**<0.001**			**<0.001**
ZT0 RNAi_A					1.000	0.125	0.974		
ZT4 RNAi_A						0.124		0.755	
ZT8 RNAi_A									0.197
ZT0 RNAi_B								0.999	0.995
ZT4 RNAi_B									0.866
ZT8 RNAi_B									

Table S5: Comparison of *clk* expression between treatments (control vs the two RNAi) and among time points. P value from Posthoc analyses, P<0.05 are in bold.

***clk* North**									
	ZT0 control	ZT4 control	ZT8 control	ZT0 RNAi_A	ZT4 RNAi_A	ZT8 RNAi_A	ZT0 RNAi_B	ZT4 RNAi_B	ZT8 RNAi_B
ZT0 control		0.681	0.197	**<0.001**			**<0.001**		
ZT4 control			0.988		**0.046**			**0.048**	
ZT8 control						**<0.001**			**<0.001**
ZT0 RNAi_A					0.234	0.963	0.996		
ZT4 RNAi_A						0.319		0.997	
ZT8 RNAi_A									1.000
ZT0 RNAi_B								0.584	0.999
ZT4 RNAi_B									0.896
ZT8 RNAi_B									
***clk* South**									
	ZT0 control	ZT4 control	ZT8 control	ZT0 RNAi_A	ZT4 RNAi_A	ZT8 RNAi_A	ZT0 RNAi_B	ZT4 RNAi_B	ZT8 RNAi_B
ZT0 control		1.000	0.990	**<0.001**			**<0.001**		
ZT4 control			0.999		**<0.015**			**<0.029**	
ZT8 control						**0.007**			**0.005**
ZT0 RNAi_A					**0.012**	0.149	0.450		
ZT4 RNAi_A						0.995		1.000	
ZT8 RNAi_A									0.639
ZT0 RNAi_B								0.635	0.999
ZT4 RNAi_B									0.948
ZT8 RNAi_B									

Table S6. Comparison of *cyc* expression between treatments (control vs the two RNAi) and among time points. P value from Posthoc analyses, P<0.05 are in bold.

***cyc* North**									
	ZT0 control	ZT4 control	ZT8 control	ZT0 RNAi_A	ZT4 RNAi_A	ZT8 RNAi_A	ZT0 RNAi_B	ZT4 RNAi_B	ZT8 RNAi_B
ZT0 control		0.836	**0.048**	**0.001**			**0.004**		
ZT4 control			0.973		**0.002**			**<0.001**	
ZT8 control						**<0.001**			**<0.001**
ZT0 RNAi_A					0.703	0.891	0.999		
ZT4 RNAi_A						0.600		0.999	
ZT8 RNAi_A									**0.012**
ZT0 RNAi_B								0.983	0.533
ZT4 RNAi_B									0.983
ZT8 RNAi_B									
***cyc* South**									
	ZT0 control	ZT4 control	ZT8 control	ZT0 RNAi_A	ZT4 RNAi_A	ZT8 RNAi_A	ZT0 RNAi_B	ZT4 RNAi_B	ZT8 RNAi_B
ZT0 control		0.936	0.049	**<0.001**			**0.002**		
ZT4 control			1.000		**0.015**			**<0.001**	
ZT8 control						**<0.001**			**<0.001**
ZT0 RNAi_A					1.967	0.999	1.000		
ZT4 RNAi_A						0.999		0.194	
ZT8 RNAi_A									0.999
ZT0 RNAi_B								0.892	1.000
ZT4 RNAi_B									0.844
ZT8 RNAi_B									



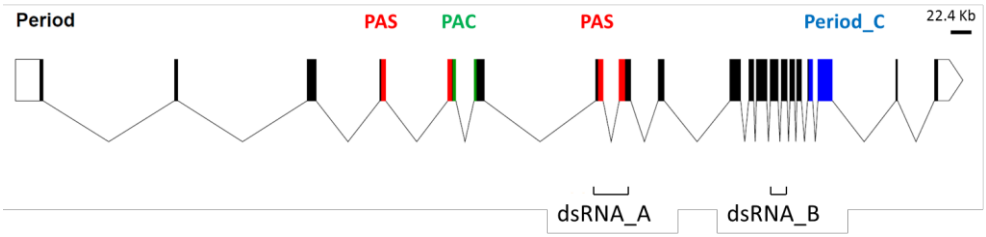


Fig S1: *period* gene structure and location of dsRNAs
Schematic representation of *period* in *Nasonia vitripennis*. Exons are indicated with boxes and intron with lines. The total length is 22.4Kb. Red boxes indicated the PAS domains, in green the PAC domain and in blue the Period_C domain. dsRNA_A and dsRNA_B indicates the region targeted by RNAi.

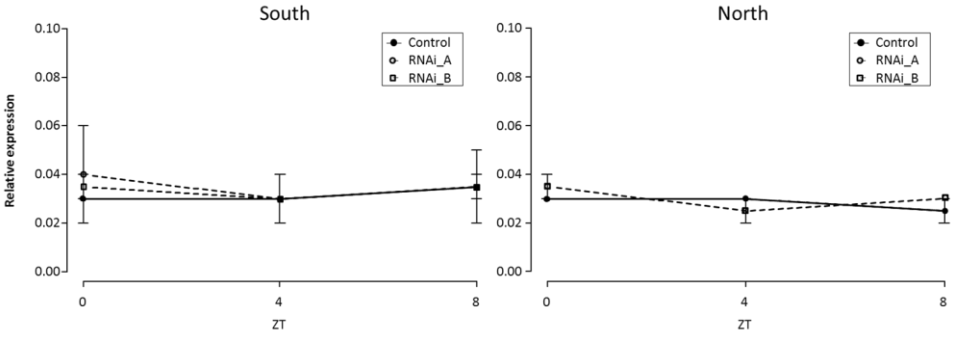


Fig. S2. Expression of the reference genes in southern and northern lines and all treatments.
A The average relative expression of EF1α and Ak3 is compared among time points, in the southern and northern lines in controls and RNAi-treated wasps by two way ANOVA**.**

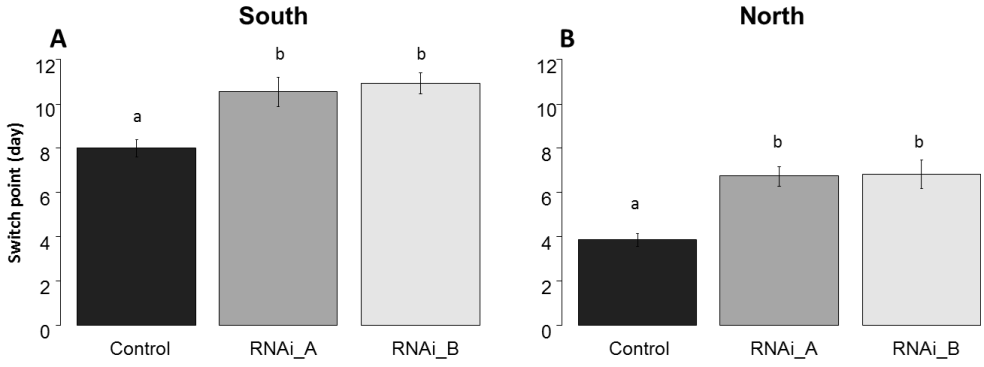


Fig S3: Switch point for diapause induction in control and RNAi-treated females
(A) southern and **(B)** northern switch point for diapause induction, in controls, RNAi_A and RNAi_B, calculated as the day at which wasps switch from producing developing offspring to diapause offspring. Different letters indicate statistical differences (Pairwise comparison using Long-Rank test, $p<0.001$).

Chapter 5

Courtship rhythm in *Nasonia vitripennis* is affected by the clock gene *period*

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to be submitted

Abstract

Males of the parasitoid wasp *Nasonia* court females by performing strong head movements ("head-nods") in repeated series within distinct cycles and accompanied by wing vibrations. The pattern of cycle duration and head-nod number is species-specific and has a genetic basis, but little is known about the genetic mechanism that underlies this ultradian rhythmicity. It has been hypothesized that the clock gene *period* (*per*), as part of the endogenous clock, plays a role in the regulation of such ultradian rhythms. *N. vitripennis* from southern and northern European populations show allelic variation of *per* that has been associated with differences in circadian rhythms. In this study, the possible involvement of *per* in regulating *Nasonia* ultradian rhythms was investigated in a southern and northern strain. *Per* knockdown by RNA interference (RNAi) resulted in a shortening of the free running rhythm in constant darkness (DD), and increased both the cycle duration as well as the number of head-nods per cycle, indicating a role of *per* in the regulation of ultradian rhythms and male courtship behaviour of *N. vitripennis*.

Introduction

Courtship in many animal species consists of a repertoire of specific male and female signals. These signals may play a role in species recognition as well as in sexual selection within species. In insects, courtship signals can be acoustic, visual, tactile or chemical (Ewing, 1983; Saarikettu et al., 2005). The production of these signals often occurs in a repeated pattern, until the signaller is accepted or rejected for mating (Thornhill & Alcock, 1983). Besides affecting mate choice within a species, courtship signals are often species-specific and serve as barrier for interspecies mating. They can also play an important role in reproductive isolation (reviewed in Alt et al., 1998; Greenspan & Ferveur, 2000; Talyn & Dowse, 2004).

Various behaviors, including locomotor activity and courtship, are controlled by an endogenous clock in insects and exhibit rhythmicity. Since Konopka and Benzer (1971) found the first circadian mutant of the clock gene *period* (*per*) in *D. melanogaster*, many circadian clock mutants have been reported from *Drosophila* species, nematodes, mice, and other species (Dunlap et al., 1999; Hall, 1995; Panda et al., 2002). These mutants frequently show behavioural differences in time-related traits owing to the altered function of the circadian clock (Kyriacou & Hall, 1980). The role of *per* was already shown in the species-specific song patterns of *D. melanogaster* (Kyriacou & Hall, 1980, 1982), and in the melon fly *Bactrocera cucurbitae* (Miyatake & Kanmiya, 2004). In both species, mutations of *per* alter, in a parallel fashion, both circadian cycles and ultradian courtship song cycles. Nevertheless, due to the difficulty of studying fast rhythms, very little is known about the genetic mechanisms underlying ultradian rhythmicity. In addition, because of the complexity of clock mechanisms, it is unclear how *per* regulates this rhythmicity.

Ethological studies have been conducted for more than half a century in *Nasonia* (Barrass, 1960a, 1960b; van den Assem & Beukeboom, 2004) and its male courtship behaviour is well characterised. It consists of a repetitive pattern of specific components that constitute an ultradian rhythm, as they are a rhythmic pattern of several stages with a duration of seconds (van den Assem & Werren, 1994). After mounting, *Nasonia* males start courtship by performing a series of strong movements with the head, the so called "head-nods", and wing vibrations, interrupted by pauses (van den Assem & Beukeboom, 2004). The first head-nod in each series is accompanied by the release of pheromones that are essential to provoke receptivity in the female (van den Assem et al., 1980; van den Assem & Werren, 1994). After a number of consecutive head-nods and pauses, the so called cycles, the female may become receptive and signals receptivity by lowering her antennae. Cycle duration and head-nod number are species-specific for the four described *Nasonia* species and are genetically determined (van den Assem & Werren, 1994; van den Assem & Beukeboom, 2004).

In the *Nasonia* circadian clock *cryptochrome-2 (cry-2)* and *per* are predicted to

regulate their own transcription by inhibition of *clock (clk)* and *cycle (cyc)* (Zhu et al., 2005; Yuan et al., 2007; Bertossa et al., 2014; chapter 4). Interestingly, the circadian clock of *Nasonia* shows different properties in natural populations along a latitudinal cline in Europe, associated with different *per* alleles (Paolucci et al., 2016). Southern wasps have a faster clock (shorter free running rhythms) compared to northern wasps (chapter 2). *per* RNA interference (RNAi) is able to change circadian clock properties by speeding up the circadian clock (chapter 4). In this study, We test the involvement of *per* in ultradian timing, by measuring the effect of *per* knockdown on male courtship behaviour of two geographically distinct strains of the wasp *Nasonia vitripennis*.

Material and methods

Experimental lines

The experimental strains used in this study were isogenic lines established from isofemale lines collected from the field in 2009 (Paolucci, et al., 2013). The northern wasps were collected in Oulu, Finland (65°3'40.16 N) and the southern lines in Corsica, France (42°22'40.80 N). The lines were maintained on *Calliphora spp*. pupae as hosts in mass culture vials with a light-dark cycle of 16 h of light and 8h of darkness (LD16:08) at 20°C.

Courtship observations

Nasonia courtship behaviour can easily be observed and quantified following the procedures described by Beukeboom and van den Assem (2001). Males and females were collected and sexed at the black pupal stage 1-2 days prior to eclosion. After eclosion, individual males were placed in 60 mm glass tubes, diameter 10mm, closed off with a plug of cotton wool, and mated females were then introduced. Mated females typically do not mate again, allowing observation of longer courtship bouts. All males were inexperienced and one-day old, since male age and previous experience may have an effect on courtship performance (Beukeboom & van den Assem, 2001). Courtship of males was recorded under a stereo binocular microscope at 10x magnification. The number of head-nods and the cycle time (time period between the first head-nod of two consecutive series) was scored for the first five cycles. A total of 40-60 pairs of RNAi-treated and untreated control males were recorded from the northern and southern strain.

Crosses between the southern and northern lines (interline crosses) were used to determine the inheritance of the courtship trait. *N. vitripennis* has a haplodiploid reproductive system in which males are haploid and develop from unfertilized eggs,

whereas females are diploid and develop from fertilized eggs. Therefore, virgin F1 females from a cross between a southern female and a northern male (reciprocal crosses were unsuccessful) were used to produce interline F2 male offspring. The courtship performance of these interline males were recorded and compared to the parental southern and northern courtship behaviour. Individuals were used only once and after mating the males were subjected to locomotor activity registration (see below).

Locomotor activity registration

To determine daily activity patterns, individuals were placed in small tubes (diameter 10 mm and length 70 mm) half filled with sugar-water gel medium and continuously monitored for movement by infrared beam arrays. Trikinetics *Drosophila* activity monitors (www.trikinetics.com) were used for the recording of 32 wasps simultaneously. The detector records the number of times per minute each individual interrupts an infrared light beam that passes through the glass tube. The monitors were placed in light boxes at 20°C in temperature-controlled environmental chambers with 50% humidity. The light-dark cycle of each light box could be controlled independently. The light source in the box consisted of white light with a maximum light intensity of about 60 lum/Ft^2 (3.15 W/m^2). Data were collected with DAM System 2.1.3 software (available at www.trikinetics.com). In order to analyse and compare the circadian behaviour of RNAi treated and control wasps, northern and southern females were simultaneously entrained to 4 days of LD16:8 and subsequently placed in constant darkness (DD).

RNA extraction, cDNA conversion

Manipulation of *period (per)* expression can be achieved via RNA interference (RNAi) by injecting double-stranded RNA (dsRNA) into wasp pupae. In order to obtain a sufficient amount of dsRNA for knocking down the expression of *per*, RNA extraction was performed only from the head (where the master clock is located) of wasps collected between ZT 21-24 (*Zeitgeber time*, ZT 0 corresponds to the time when the light turned on). This time corresponds to the peak of *period* expression (see chapter 3). Total RNA was extracted using Trizol reagent (Invitrogen, USA) according to the manufacturer's instructions. Each sample was submitted to a DNase treatment to eliminate any DNA contaminations, and about 1µg of RNA was used to synthetize cDNA with RevertAid H Minus First Strand cDNA Synthesis kit (Thermo scientific).

Synthesis and injection of dsRNAs

Knockdown of *per* was induced in early male pupae following the methods described in chapter 4. Two PCR fragments with a T7 polymerase-binding site were transcribed in both directions using the Megascript RNAi kit (Ambion, Austin, Texas, USA) following the manufacturer's protocol for dsRNA synthesis using the primers listed in the table 5.1. Sense and antisense RNA fragments were synthetized in separate transcription reactions. After 6h incubation at 37°C, the two reactions were mixed and the sample was incubated at 75°C for 5 min and subsequently cooled down slowly (overnight). Nuclease digestion was performed to remove DNA and single stranded RNA (ssRNA), and dsRNA was purified using reagents provided by the kit. Finally, RNA was precipitated with ethanol for better purification, re-dissolved in water and stored at -80°C.

Male pupae were injected in the abdomen according to the Lynch and Desplan (2006) protocol, either with 4 µg/µl of *per* dsRNA_A (RNAi_A) or dsRNA_B (RNAi_B) (Fig. S1) mixed with red dye. Injections were performed with Femtotips II (Eppendorf, Hamburg, Germany) needles under continuous injection flow. Pupae were injected at the posterior until the abdomen turned clearly pink. Slides with injected wasp pupae were incubated in a Petridish with an Agar/PBS medium at 25^{o}C and LD 16:08 for subsequent use in the courtship recording and locomotor activity experiments. Control pupae were injected with red dye mixed with water in a 1:4 ratio.

Entrainment and sample collection for gene expression analysis

DsRNA-injected and water-injected control males were kept after emergence under LD16:08 at 20^{o}C with five individuals per tube. Three biological replicates, each containing five wasps, were prepared for each treatment and three days post eclosion the wasps were collected at ZT 0. To preserve the RNA, tubes with wasps were frozen in liquid nitrogen and immediately stored at -80^{o}C.

RNA was extracted from pooled head samples and cDNA conversion was performed following the manufacturer's instruction. The cDNA was diluted 50 x before use in Real-Time quantitative PCR (RT-qPCR). The RT-qPCR was performed with SYBR green (Quanta Biosciences) and rox as the internal passive reference. 4 µl of diluted cDNA was used for each 20 µl reaction containing a final primer concentration of 200 nM and 10 µl of SYBR green/ROX buffer solution. The primers used are listed in Table 2. Three technical replicates for each reaction were performed to correct for pipetting errors. The following qPCR profile was used on the abi7300 PCR machine: 3 min of activation phase at 95^{o}C, 35 cycles of 15s at 95^{o}C, 30s at 56^{o}C and 30s at 72^{o}C. Table 5.2 lists the primers for *period (per)*, *Elongation factor 1α* (*ef1α*) and *adenylate kinase* (*ak*) genes.

Expression data were first analysed with LinRegPCR (Ramakers et al., 2003,

Ruijter et al., 2009) *Elongation factor 1α* (*ef1α*) and *adenylate kinase* 3 (*ak3*) were confirmed to have constant expression levels throughout the day (Chapter 3 and 4) and between treatments (Fig. S2) and were thus used as reference genes. A generalized linear mixed effect model (glm) was used to analyse expression levels with R statistical software. A quasi-poisson distribution for the glm corrected for over-dispersion and *F*-tests were used to compare differences in gene expression between treatments. Post-hoc analyses were performed with the multcomp package for effects of RNAi treatments within lines.

Behavioural data analysis and statistics

The raw locomotor activity data were first visualized with the program ActogramJ (Schmid et al., 2011; available at http://actogramj.neurofly.de). Double-plot actograms obtained with this software represent activity levels. Under LD conditions, the average activity was calculated as described previously by Schlichting and Helfrich-Forster (2015). We determined when wasps start to be active (onset), have the peak of activity (peak) and terminate their activity (offset) during each 24h period, and compared this activity between RNAi-treated and non-treated of both southern and northern wasps. To determine the onset and offset of activity, data were plotted as bar diagrams for each wasp, with each bar representing the sum of activity within 20 min. The onset of activity is defined as the first time bar when activity starts to rise consecutively, whereas the offset of activity is defined as the first time bar when activity reaches the level that is stable during the night phase. To determine the timing of the peaks, the data are smoothed by a moving average of 30min. Through this process, randomly occurring spikes are reduced and the real maximum of the activity can be determined. The free running period (τ) was determined under constant darkness and constant light, with periodogram analysis, which incorporates chi-square test (Sokolove & Bushell, 1978). The average phase of the onset, peak and offset, represented ZT and the τ values, were compared between strains and treatments. Statistical analysis, on timing of activity and free running rhythms, was performed with ANOVA and a Tukey's multiple-comparison test. Courtship behaviour was analysed with non-parametric Kruskal–Wallis test with a Dunn's multiple-comparison test for non-normally distributed data with Bonferroni correction for p-values. in R statistical software (version 3.4.1).

Table 5.1. Primers used for generating *period* dsRNA

Primers name	Forward primer	Reverse primer
NV_per_dsRNA_0708 Region A	5'-CCTTCTTCCAACCCATACGG-3'	5'-CTCAATGATCTTGGCTTCCTG-3'
NV_per_dsRNA_1213 Region B	5'-CTGCTGTCGTTAGATGTGAG-3'	5'-GTCGCCATATCAGTTATCGG-3'
NV_per_dsRNA_1213_T7 Region A	5'-TAATACGACTCACTATAGGG'CCT TCTTCCAACCCATACGG-3'	5'-TAATACGACTCACTATAGGG'CTC AATGATCTTGGCTTCCTG-3'
NV_per_dsRNA_1213_T7 Region B	5'-TAATACGACTCACTATAGGG'CTG CTGTCGTTAGATGTGAG-3'	5'-TAATACGACTCACTATAGGG'GTC GCCATATCAGTTATCGG-3'

Table 5.2. Primers used for period qPCR

Gene	NCBI Ref. seq.	Forward primer	Reverse primer
per	XM_008211021.1	5'-GCCTTCATTACACGCATCTC-3'	5'-ACCATTCGCACCTGATTGAC-3'
ef1 α	XM_008209960.1	5'-CACTTGATCTACAAATGCGGTG-3'	5'-CCTTCAGTTTGTCCAAGACC-3'
ak	XM_016986045.1	5'-AATTCAATCGGGTTCTGCTC-3'	5'-CAGCATCTCATCTAACTTCTCTG-3'

Results

Geographical differences in courtship behaviour

Male courtship behaviour of southern and northern lines of Nasonia vitripennis followed a general structure consistent with previous reports (Fig. 5.1) (van den Assem & Beukeboom, 2004). The duration of each cycle increased steadily throughout the consecutive cycles in all groups, starting with about 9s for the first and second cycle and reaching about 11s in the fourth cycle (Fig. 5.1; Table S1). The highest average head-nods numbers occurred in the first cycle (4.46 ± 0.20 in the southern line, 5.31 ± 0.36 in the northern wasps) followed by a lower number in the second cycle (2.95 ± 0.18 in the southern line, 4.23 ± 0.27 in the northern one), and a gradual increase in subsequent cycles (Fig. 5.1; Table S3). Cycle times did not differ between southern and northern wasps, but southern wasps had lower average head-nod numbers during all cycles ($p<0.001$; Dunn's multiple-comparisons test). This difference can be due to different nodding pace or different pause length between two consecutive cycles.

Cycle duration of the F2 interline males was similar to the parental lines. However head-nod numbers of F2 interline males resembled that of the northern (grandparental) line, and were significantly higher than those of the southern line (Fig. 5.1; Table S1) ($p<0.001$; Dunn's multiple-comparisons test). These data are consistent with a genetic basis of head-nod numbers as reported by Beukeboom & Van den Assem (2001).

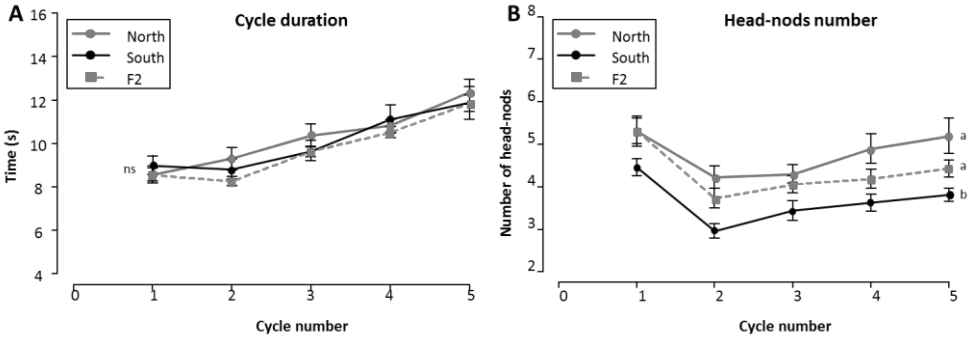

Fig. 5.1. Male courtship behaviour in southern, northern and F2 interline wasps
(A) Duration and **(B)** head-nods number (average ± standard error) of the first five cycles for southern, northern lines and F2 interline males. Different letters indicate significant differences by Kruskal–Wallis test with Dunn's multiple-comparisons ($p<0.05$).

Efficiency and effect of **period** *RNAi*

Expression of per was analysed to assess the efficiency of RNAi three days post-eclosion under LD16:08 at ZT 0. This time point represents the moment when the light is turned on and corresponds to the peak of per expression (Chapter 3). The relative expression of per in the dsRNA-injected southern and northern wasps was significantly lower compared to the controls ((both $p<0.001$; Fig. 5.2), indicating an efficiency of 50 to 60 % of per knockdown via RNAi.

To test whether the endogenous properties of the circadian clock were efficiently altered via per RNAi, as reported in chapter 4, the free running rhythms under constant darkness (DD) were compared between lines and treatments. In the behavioural assays, animals were exposed to a light dark (LD) regime of 16:08 for 4 days followed by constant darkness (DD) for 10 days. Knockdown of per efficiently shortened the free running period of both southern and northern lines by approximately one hour (both $p<0.001$;Fig. 5.3, table S2), without affecting the proportion of rhythmic wasps (table S2) nor the timing of locomotor activity (Fig. S3, Table S3), meaning that circadian clock properties where efficiently manipulated by RNAi.

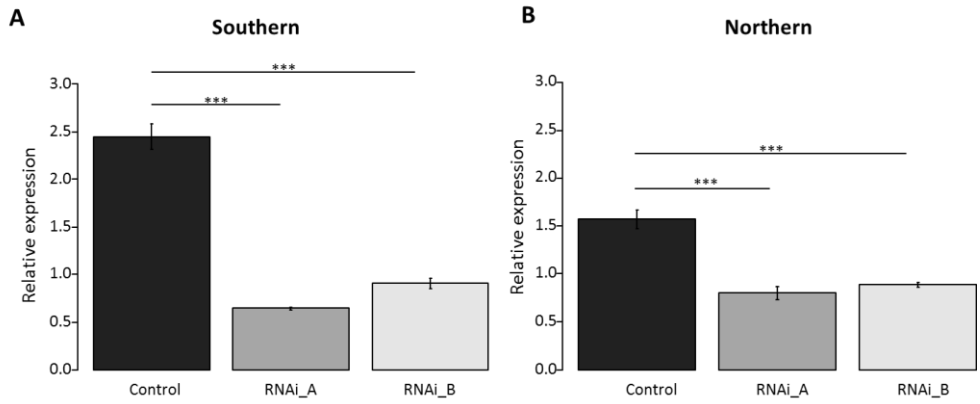

Fig. 5.2. *Period* expression in control and *per* RNAi-treated wasps
(A) Southern and (**B**) northern *per* mRNA expression in control and RNAi-injected wasps either with dsRNA_A or with dsRNA_B. Asterisks represent significant differences between treatments (***p<0.001 by two-way ANOVA).

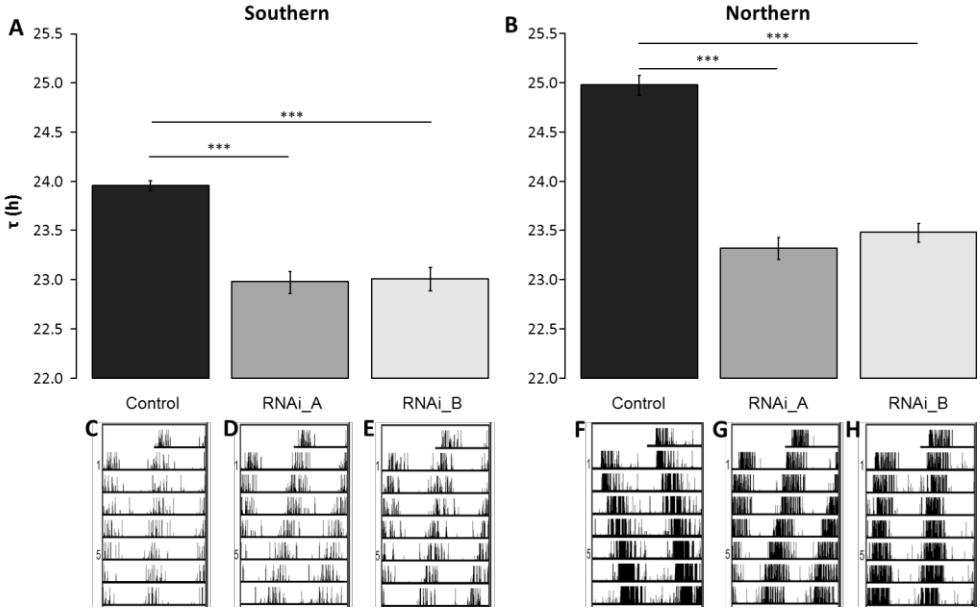

Fig. 5.3. Constant darkness (DD) rhythms of control and RNAi-treated wasps
(A) Southern and (**B**) northern free running rhythms in control and RNAi-injected wasps either with dsRNA_A or with dsRNA_B. **(C-E)** Southern and **(F-H)** northern double plot actograms for circadian rhythms. Black bars indicate activity. Asterisks indicate significant differences (***p<0.001 by two way ANOVA).

Period *RNAi affects courtship behaviour*

Southern and northern males injected with either per dsRNA_A or per dsRNA_B showed a significant increase in cycle duration (Fig. 5.4A, B), as well as in head-nod numbers per cycle (Fig. 5.4C, D) (both $p<0.05$; Dunn's multiple-comparisons test). The duration of the first cycle increased by more than 1s in both lines (from 9.22 ± 0.20s to 10.30 ± 0.38s and 10.04 ± 0.20s respectively in the northern wasps, and from 9.44 ±0.21s to 10.46 ± 0.37s and 10.93 ± 0.28s in the southern wasps for dsRNA_A and dsRNA_B respectively). The same is true for the duration of the subsequent cycles (Fig. 5.4A, B; Table S4), whereas the general pattern of steady cycle duration increase throughout consecutive cycles is maintained after per RNAi. The number of head-nods per cycle is also higher in RNAi-treated wasps in both lines throughout all cycles compared to the controls (Fig. 5.3A, B; Table. S4), whereas the pattern is not altered. Interestingly, the effect of RNAi on cycle duration and headnods- number seems slightly higher in the southern wasps, and this effect is significant for the number of head-nods (Fig. 5.3A, B; Table S4).

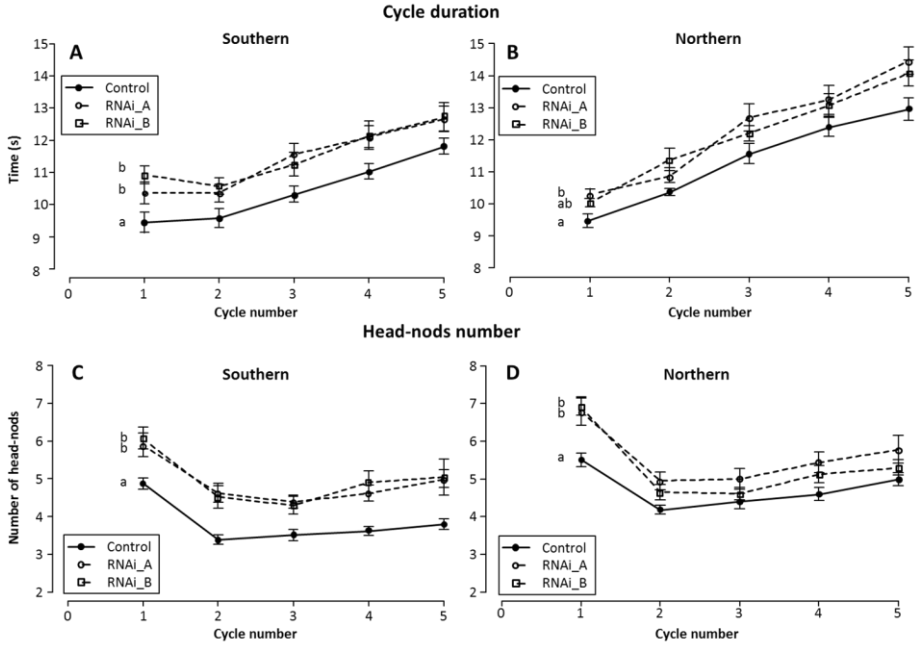

Fig. 5.4: courtship behaviour of control and RNAi-treated wasps
Duration (average ± standard error) of the first five cycles of control and RNAi-treated wasps of the (**A**) southern and (**B**) northern line. Head-nods number (average ± standard error) of the first five cycles of control and RNAi-treated wasps for (**C**) southern and (**D**) northern line. Different letters indicate significant differences by Kruskal–Wallis test with Dunn's multiple-comparisons ($p<0.05$).

Discussion

Various insect behaviours, including locomotor activity and courtship, are rhythmic and controlled by an endogenous clock. In this study we investigated the effect of knockdown of the clock gene *per* on male courtship behaviour of two geographically distinct strains of the wasp *Nasonia vitripennis*. RNA interference (RNAi) efficiently decreased per transcript in RNAi-treated wasps. We found a differential effect of *per* knockdown on the ultradian rhythm of male courtship behaviour of southern and northern wasps. As a confirmation for effective RNAi knockdown we recorded the circadian rhythm of treated and control wasps. The circadian rhythm was significantly altered after *per* knockdown, showing a shortening of the free running rhythm under constant darkness (see also chapter 4).

Our results reveal that *per* regulates, in a parallel fashion, both circadian and courtship cycles in *Nasonia*. This is the first evidence that the clock gene *per* is involved in courtship behaviour in Hymenoptera. Similar findings were previously reported for *Drosophila melanogaster* and the melon fly *Bactrocera cucurbitae*, in which mutation of the gene *per* speeds up the circadian clock and changes the periodical fluctuation of the male's courtship song (Kyriacou & Hall, 1980; Miyatake & Kanmiya 2004). Together, these studies reveal a partial conserved regulating mechanism of circadian activity and courtship behaviour between Diptera and Hymenoptera.

In Nasonia the rhythmic head-nods display during male courtship appears to be important for inducing female receptivity by enabling the rhythmic release of pheromones (reviewed in Van den Assem & Beukeboom, 2004). Similarly, in *Drosophila* the song rhythm during courtship, plays an important role in mate choice and reproductive isolation (Alt et al., 1998). Song rhythms are species-specific as *D. melanogaster* females favour males with long pulse song whereas *Drosophila montana* females prefer songs with short but frequent pulses (Ritchie et al., 1998). Interestingly, *per* female mutants do not prefer the song characteristics of the corresponding mutant male, indicating that there is no 'genetic coupling' between the male and female communication systems with respect to *per* (Greenacre et al., 1993). In contrast, if *per* in *Nasonia* is involved in setting the pace of this ultradian rhythm, it indirectly sets the pace of pheromone release and thereby might contribute to mate choice and consequently reproductive isolation between and within *Nasonia* species.

Geographical differences were observed in the number of head-nods per cycle as part of male courtship performance, in line with Diao (2017) who reported latitudinal differences in courtship traits among European populations of *N. vitripennis*. The possible adaptive significance of this variation is not known. One option is that it is merely an effect of drift and has no selective history. Male courtship is clearly essential for inducing female receptivity (van den Assem et al., 1980), but the precise behaviours may not be essential and merely serve to transmit pheromones to females. Direct selection on cycle time and

headnods numbers is therefore unlikely to occur. Instead, the observed differences in ultradian rhythms may be a correlated response to selection for different *per* alleles. The cline in *per* allele frequencies in *N. vitripennis* has been attributed to latitudinal selection for diapause response (Paolucci et al., 2013, 2016). As this study indicates a role of *per* in male courtship behaviour, genetic correlation appears the most likely explanation for the observed differences in cycle time and headnods numbers between northern and southern wasps.

We previously showed involvement of per in the core mechanism of daily and seasonal timing. The current study revealed an additional role of the clock gene *per*, and maybe of the circadian clock, in timing mechanisms of *N. vitripennis*, i.e. in the ultradian mechanism of male courtship behaviour. In terms of the genetic organisation of the clock it remains a question whether *per* regulates the ultradian courtship rhythm directly, due to pleiotropic gene function, or through altering the action of the circadian clock. In both cases, whatever transcription factor is involved in the genetic pathway of courtship behaviour, it likely is either up- or down regulated when *per* is knocked down. Clearly, the route between PER as a transcriptional regulator of downstream courtship factors is complex. It requires more sophisticated genome editing experiments to fully describe the mechanism behind its role in the regulation of ultradian rhythmicity.

Acknowledgements

This work was funded by the EU Marie Curie Initial Training Network INsecTIME. We thank all participants in the network for helpful and stimulating discussions. We thank Bas van Boekholt, Frederique Derks, Bas Verviers for help with data collection. We thank the members of the Evolutionary Genetics, Development & Behaviour Group for discussions and advice on statistical analysis.

Supplementary tables and figures

Table S1. Number of head-nods and duration of the first 5 cycles of wild type southern and southern wasps and F2 interline crosses.

	South	North	F2
Number of			
1st Head-nods	4.46 ± 0.20	5.31 ± 0.36	5.32 ± 0.30
2nd Head-nods	2.95 ± 0.18	4.23 ± 0.27	3.72 ± 0.22
2nd - 1st	-1.51 ± 0.2	-1.08 ± 0.36	-1.61 ± 0.40
3rd Head-nods	3.44 ±0.23	4.30 ± 0.22	4.06 ±0.21
4th Head-nods	3.63 ± 0.20	4.90 ± 0.37	4.19 ± 0.19
5th Head-nods	3.81 ± 0.16	5.20 ± 0.44	4.43 ± 0.26
Duration of			
1st cycle	9.15 ± 0.44	8.57 ± 0.40	8.58 ± 0.29
2nd cycle	8.83 ± 0.45	9.31 ± 0.50	8.28 ± 0.21
2nd - 1st	- 0.32 ± 0.45	-0.74 ± 0.50	-0.33 ± 0.24
3rd cycle	9.67 ± 0.48	10.36 ± 0.54	9.65 ± 0.24
4th cycle	11.13 ± 0.65	10.82 ± 0.30	10.5 ± 0.26

Table S2. Free running rhythms and arrhythmicity of southern and northern lines: controls and RNAi-treated.

Treatment	Av	St.dev	n	St.err	Arr	Rhy
N_Control	23.96	0.37	53	0.05	8.62	91.38
N_RNAi_A	22.97	0.76	47	0.11	7.84	92.16
N_RNAi_B	23.00	0.80	44	0.12	13.73	86.27
S_Control	24.98	0.79	57	0.11	20.83	79.17
S_RNAi_A	23.31	0.71	41	0.11	8.89	91.11
S_RNAi_B	23.48	0.59	37	0.10	24.00	76.00

Table S3. Timing of locomotor activity LD16:08 of southern and northern lines: controls and RNAi-treated.

LD16:08 Treatment	**Onset ± SE ZT(h)**	**Peak ± SE ZT (h)**	**Offset ± SE ZT (h)**
N_Control	23.85 ± 0.05	3.53 ± 0.30	11.81 ± 0.26
N_RNAi_A	23.75 ± 0.11	3.76 ± 0.32	11.63 ± 0.30
N_RNAi_B	23.66 ± 0.11	3.39 ± 0.23	11.61 ± 0.30
S_Control	23.32 ± 0.11	1.89 ± 0.19	9.87 ± 0.28
S_RNAi_A	23.63 ± 0.13	1.44 ± 0.17	6.99 ± 0.35
S_RNAi_B	23.69 ± 0.09	1.36 ± 0.17	6.12 ± 0.35

Table S4. Courtship of control and RNAi-treated wasps.

	South			**North**		
	Control	RNAi_A	RNAi_B	Control	RNAi_A	RNAi_B
Number of						
1st Head-nods	4.88 ± 0.15	5.9 ± 0.32	6.08 ± 0.30	5.51 ± 0.18	6.80 ± 0.36	6.91 ± 0.23
2nd Head-nods	3.38 ± 0.13	4.63 ± 0.25	4.52 ± 0.29	4.19 ± 0.12	4.95 ± 0.23	4.65 ± 0.20
2nd - 1st	-1.54 ± 0.15	-1.27 ± 0.32	-1.73 ± 0.39	-1.34 ± 0.17	-1.85 ± 0.34	-2.26 ± 0.17
3rd Head-nods	3.50 ± 0.15	4.40 ± 0.18	4.30 ± 0.24	4.39 ± 0.17	5.00 ± 0.27	4.61 ± 0.16
4th Head-nods	3.62 ± 0.11	4.62 ± 0.21	4.91 ± 0.29	4.60 ± 0.17	5.44 ± 0.27	5.13 ± 0.23
5th Head-nods	3.80 ± 0.15	5.00 ± 0.24	5.05 ± 0.48	5.00 ± 0.17	5.69 ± 0.33	5.30 ± 0.21
Duration of						
1st cycle	9.44 ± 0.21	10.46 ± 0.37	10.93±0.28	9.22 ± 0.20	10.30 ± 0.38	10.04 ± 0.20
2nd cycle	9.58 ± 0.19	10.80 ± 0.51	10.57±0.26	9.96 ± 0.27	10.87 ± 0.38	11.38± 0.35
2nd - 1st	0.15 ± 0.17	-0.04 ± 0.29	-0.36 ± 0.30	0.56 ± 0.33	0.62 ± 0.39	1.33 ± 0.36
3rd cycle	10.43 ± 0.28	11.52 ± 0.34	11.25 ± 0.38	10.94 ± 0.23	12.7 ± 0.43	12.20 ± 0.24
4th cycle	11.17 ± 0.22	12.00 ± 0.34	12.16 ± 0.45	11.59 ± 0.22	13.25 ± 0.45	13.08 ± 0.35

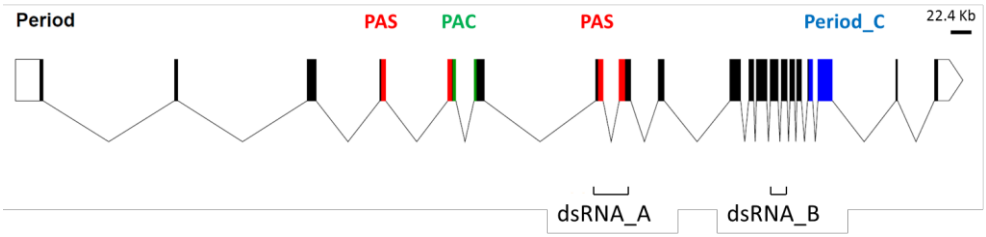

Fig S1. *Period* gene structure and location of dsRNAs
Schematic representation of *period* in *Nasonia vitripennis*. Exons are indicated with boxes and introns with lines. The total length is 22.4Kb. Red boxes indicate the PAS domains, in green the PAC domain and in blue the Period_C domain. DsRNA_A and dsRNA_B indicate the region targeted by RNAi.

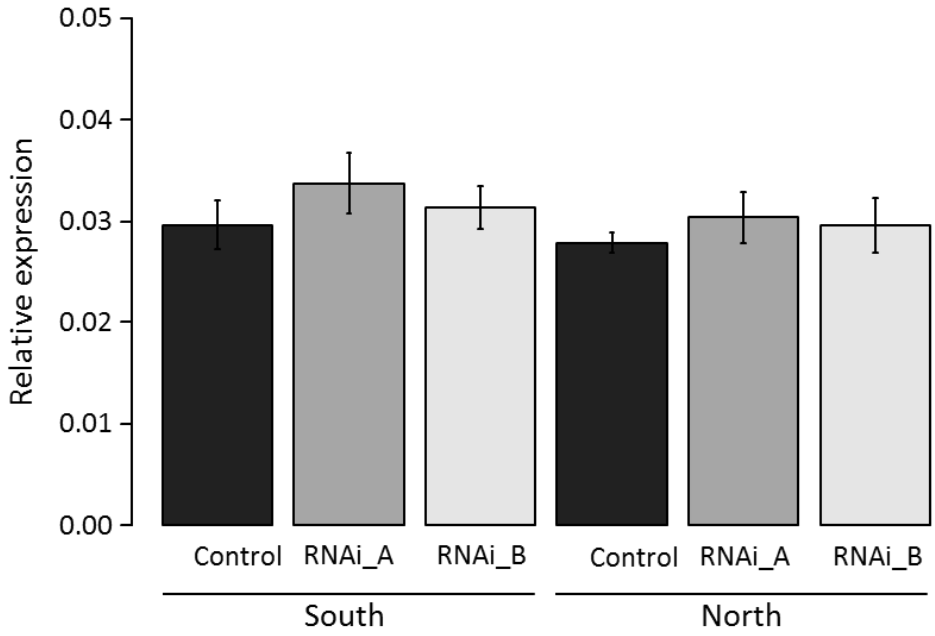

Fig. S2. Expression levels of the reference gene, *ak3*, in southern and northern lines: controls and RNAi-treated wasps
The average relative expression of *ak3* normalized against *ef1α* is compared between the southern and northern lines in controls and RNAi-treated wasps by two way ANOVA.

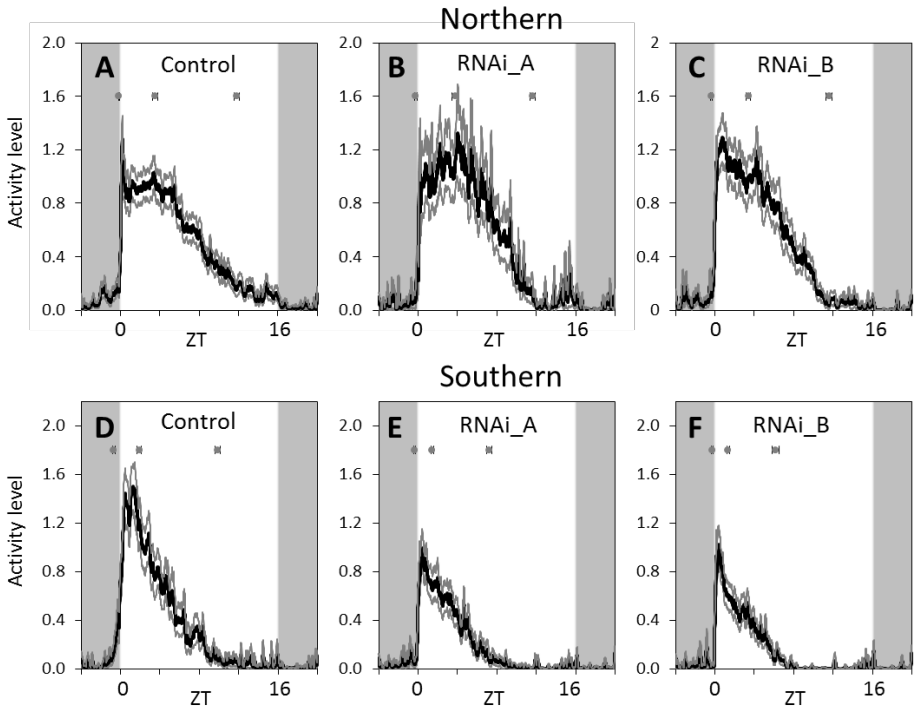

Fig. S3. Locomotor activity of control and RNAi –treated wasps

Locomotor activity profile of **(A-C)** northern wasps (control and RNAi respectively) and of **(D-F)** southern wasps (control and RNAi respectively) are shown as average of bin crosses/minute of 25-32 individuals each over 24 h periods at LD16:08. Grey shading indicates the night phase, and white indicates the day phase. *Zeitgeber time* (ZT) is given in hours on the X-axis where ZT=0 represents light on. Dots indicate respectively the average onset, the average peak ± SE and the average offset ± SE of activity.

BOX 2

Implementation of genome editing by CRISPR/Cas9 in *Nasonia vitripennis*

Elena Dalla Benetta

Anna Rensink

Background

Establishment of functional genetic approaches in new model organisms has always been a challenge. One important advance in this direction was the discovery of RNAi interference (RNAi), a mechanism that uses small RNAs processed from larger dsRNA precursors to recognize and degrade specific RNA targets (reviewed in Tijsterman et al., 2002). This method has been used in chapter 4 and 5 to knock down *per* expression, but it harbours some limitations. The interference with gene expression is transient, unlocalized, and primarily targets mRNA, and does not result in a stable genetic modification or a complete loss of function.

The ability to modify a specific genomic region offers great advantages. During the last 5 years gene editing techniques have been revolutionized by a technique that employs RNA-guided endonucleases to specifically target and modify genomic DNA (reviewed in Wiedenheft et al., 2012). The system originated as immune defence against viruses by bacteria and Archaea. The system from *Streptococcus pyogenes* involves a single CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)-associated protein (Cas9) and two RNAs (crRNA and trans-acting antisense RNA tracRNA) to build an active CRISPR/Cas9 endonuclease complex (Jinek et al., 2012). It is possible to combine these two RNAs in a single chimeric guide RNA (Known as gRNA or sgRNA) that can direct Cas9 activity to specific DNA targets (Fig. 1). The gRNA has a region of 20 nucleotides at its 5' site, that binds the target DNA and determines the target specificity. The 3' region of the gRNA corresponds to the tracRNA and is an invariable sequence required to form a complex with Cas9. The presence of the motif sequence, called PAM (Protospacer Adjacent Motif), immediately downstream the targeted sequence is required for a successful binding of the protein Cas9 to the DNA and subsequent cleavage of the target region. In the case of CRISPR/Cas9 the required PAM sequence is NGG, it can thus target only sequences that matches the motif N_{20}NGG. Cas9 cleaves both strands of the DNA within the gRNA target region, three nucleotides upstream the PAM (Jinek et al., 2012) (Fig. 1). These double-strand breaks (DSB) can elicit two types of molecular repair mechanism at the site of damage: non-homologous end joining (NHEJ) in which the broken ends are re-ligated, or homology-directed repair (HDR) in which the break is repaired using homologous DNA sequence as template (Fig. 1). In the first case, the NHEJ is error prone, and often it results in the introduction of insertion or deletions (indels) at the site of the break. It can thus be efficiently used to disrupt gene function. HDR is based on precise copying of the template and can serve to insert specific sequences that have be engineered in a donor template (Port et al., 2014) (Fig. 1).

Here, We report our attempts to implement CRISPR/Cas9 gene editing in *N. vitripennis*. We aim to disrupt the function of the gene *cinnabar* (*kynurenine 3-monooxygenase*) and *yellow* because the knock-out of these genes gives a visible phenotype

(Lynch & Desplan, 2006) which simplifies the screening of mutants. Specifically, we present (i) a method to design and generate gRNAs, (ii) an efficient method for embryo collection and (iii) a microinjection procedure and (iv) we describe our first attempts to target clock genes with CRISPR/Cas9.

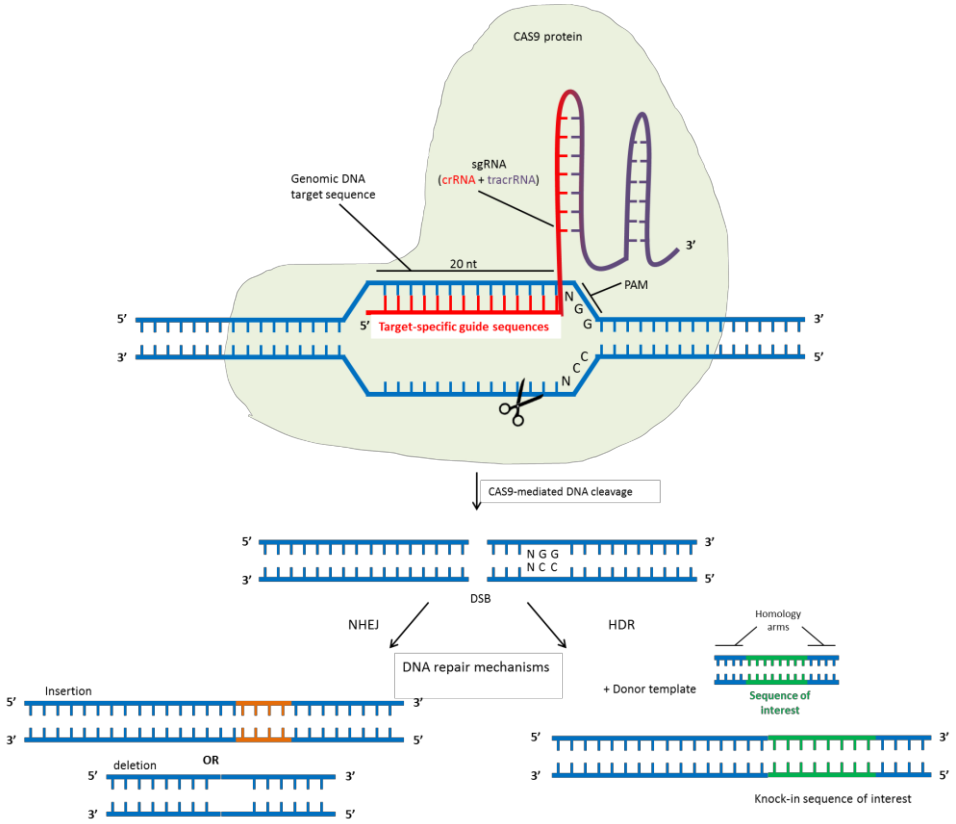

Fig. 1. The mechanism of genome editing by CRISPR/Cas9.
The genomic DNA target preceding a protospacer adjacent motif (PAM) NGG, is specified by a 20nt customized guide sequence in the sgRNA. In the cell nucleus, Cas9 protein associates with the sgRNA and binds to the target sequence, cleaving both DNA strands 3nt upstream of the PAM. Cleavage results in a DSB (double strand break) which is repaired by DNA repair mechanisms. In the absence of a donor template, error-prone NHEJ occurs which may lead to the formation of random short indels and thus frameshift mutations and disruption of gene function. If an artificial donor template is provided, for example on a plasmid containing a sequence of interest flanked by homology arms, then HDR may occur, leading to the introduction of an exogenous DNA sequence at a specified genomic location. This is the basis for performing gene knock-in, tagging, and precise pre-specified insertions or deletions using CRISPR

Methods, results and discussion

Design of gRNAs

To facilitate the implementation of the CRISPR/Cas9 technology in *Nasonia*, we selected *cinnabar* (NV14284) and *yellow* (NV16239) as target genes for mutagenesis. The gene *cinnabar* encodes an enzyme involved in ommochrome biosynthesis (Sethuraman & O'Brochta, 2005). Mutations in this locus were reported from different organisms to induce scorable eye-colour phenotypes (Paton & Sullivan, 1978). RNA interfering experiments in *Nasonia* led to a less pigmented eye-colour phenotype when the gene was silenced at the larval stage (Werren et al., 2009). The gene *yellow* encodes for an enzyme responsible for catalysing the conversion of dopachrome into 5,6-dihydroxyindole in the melanisation pathway. It is involved in pattern-specific melanin pigmentation of the cuticle during late pupal and adult stages (Han et al., 2002). In *Drosophila melanogaster yellow* mutants, the appearance of the stripes near the posterior edge of each abdominal tergite change from black to brown (Wittkopp et al., 2002). The *Nasonia yellow* gene contains the conserved functional domain Major Royal Jelly Protein (MRJP), that could be responsible for the body pigmentation of the wasps. Such a distinctive eye colour and possible body pigmentation phenotypes allow the easy screening for mutant individuals and their offspring.

To determine putative sgRNA genomic in the target genes (Fig. 2) we used CHOP CHOP server (Montague et al., 2014; Labun et al., 2016). Importantly the target sequence should be specific within the entire genome in order to avoid off-target editing. Although the targeting specificity of Cas9 is believed to be tightly controlled by the 20-nt guide sequence of the sgRNA and the presence of a PAM adjacent to the target sequence in the genome, potential off-target cleavage activity could still occur on DNA sequence with even three to five base pair mismatches in the PAM-distal part of the sgRNA-guiding sequence (Cong et al., 2013; Hsu et al., 2013; Mali et al., 2013). CHOP CHOP incorporates the *Nasonia* genomic sequences in order to check the specificity of the sgRNAs. However, specificity and activity of sgRNA is unpredictable and it is better to design and test multiple target regions.

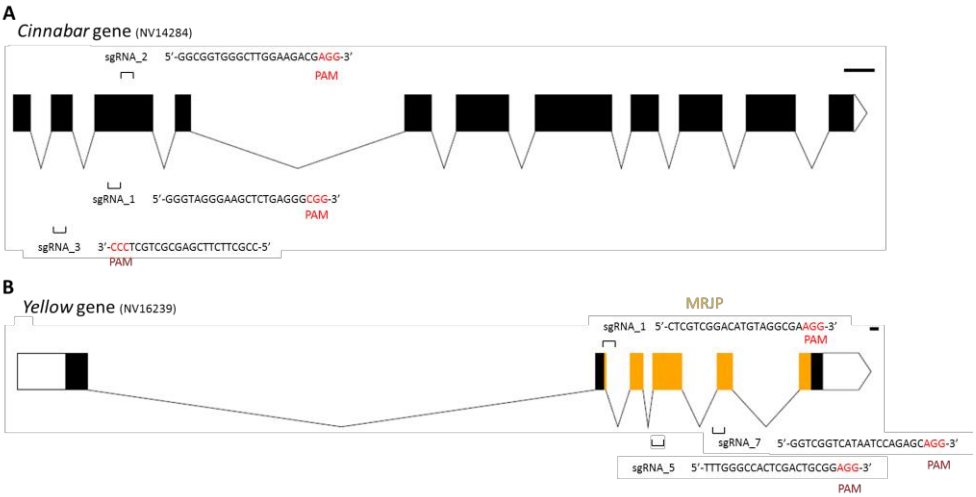

Fig 2. *Cinnabar* gene structure and location of sgRNAs
Schematic representation of **(A)** *cinnabar* in *Nasonia vitripennis* with a total length of 3066bp and **(B)** *yellow* with a total length of 10020bp. MRJP represents the functional domain major royal jelly protein. Exons are represented by boxes and introns by lines. sgRNAs indicate the target region. The required PAM region (NGG) is shown in red.

sgRNA generation

The first step to generate sgRNAs is to synthesize a DNA oligo that include the target sequence and a tracrRNA (Fig. 1). We first performed a template-free PCR to anneal two overlapping primers to produce a full-length dsDNA template according to Kistler et al., (2015) (Fig. 3). The reverse primer is universal and can be used to generate all the sgRNAs and corresponds to the tracRNA. 5'-AAAAGCACCGACTC GGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCT AGCTCTAAAAC-3'). The forward primer contains the T7 promoter upstream of the target sequence (GGN_{20}), followed by a complementary region to the reverse primer (underlined) (5'-TAATACGACTCACTATA-(GG)$\mathbf{N_{20}}$-GTTTTAGAGCTAGAAATAGCAAG-3'). The PCR reaction and conditions are reported in table1, PCR is performed with the two primers but without any other DNA template.

PCR product is then treated with proteinase K (100-200 µg/ml) and 0.5% SDS for 30 min at 50°C to eliminate enzymes such us RNase, Polymerase or other inhibitors of transcription. After this step, PCR product is purified with phenol (pH8)/chloroform extraction and ethanol precipitation.

Table. 1 Free template PCR reaction and conditions

PCR reaction		PCR program		
PCR buffer (6X)	10 µl	95°C	2 min	
dNTPs (2mM)	10 µl	95°C	20s	
$MgSO_4$ (25mM)	6 µl	58°C	10s	35 cycles
Forward primer	5 µl	70°C	10s	
Reverse Primer	5 µl			
KOD Hot start polymerase	2 µl			
Nuclease free water	Up to 100 µl			

In vitro transcription using the purified PCR template was performed with the Mega Script T7 kit (AM1333-T7, Ambion) following manufacturer's protocol (Fig. 3). The suggested template concentration for the MEGAscript reaction is 0.1-0.5 µg of purified PCR product in water or TE (10mM Tris-HCl pH7, 1mM EDTA). Optimal incubation time depends on the amplicon size and transcriptional efficiency of the template. We performed an overnight incubation. DNase treatment to remove the template DNA was performed by incubating the reaction with 1µL TURBO DNase (in the kit) for 15 min at 37°C. sgRNAs were purified with phenol (pH4)/chloroform extraction and isopropanol precipitation. Concentration of the sgRNA was measured with Nanodrop and quality was assessed in 2% TBE electrophoresis gel with guanidine thiocyanate to prevent secondary structure formation of the sgRNA. A single band of about 100nt indicates good sgRNA quality without degradation products (Fig. 4 A, B). sgRNA was then stored at -80°C.

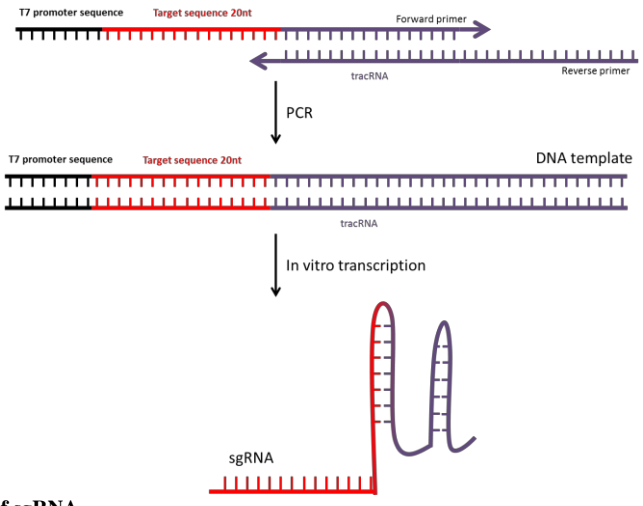

Fig 3. generation of sgRNA
Forward and reverse primers anneal in the overlapping region starting a PCR reaction without any other DNA template. The DNA template is then *in vitro* transcribed by T7 polymerase in order to generate the sgRNA.

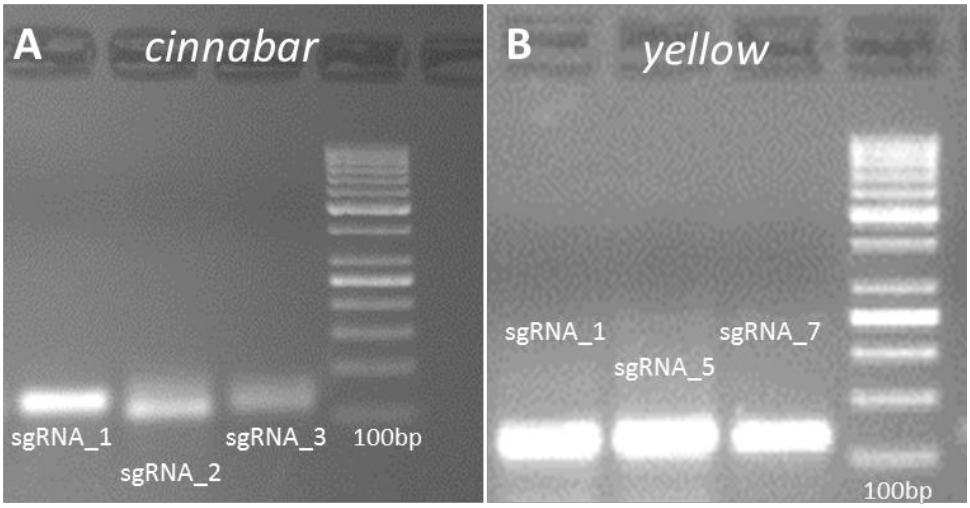

Fig 4. Quality of sgRNAs
Gel picture of sgRNAs indicating good quality without degradation products.

In vitro test

Cleavage activity of the sgRNA/Cas9 complex was assessed *in vitro*. Although the CRISPR/Cas9 complex might function differently *in vivo*, validation of sgRNAs can help to exclude sgRNAs with poor activity prior to micro-injections. Briefly Cas9/sgRNA complexes were constituted before cleavage by incubating 1µg of Cas9 nuclease (purchased from PNA-BIO), and 0.4µg of the *in vitro* transcribed sgRNA for 10 min at 37°C in reaction buffer containing BSA and NEB 3.1 buffer (50mM Tris-HCl, 10mM $MgCl_2$, 100mM NaCl, 100µl/ml BSA). Cleavage assays were conducted in a reaction volume of 10µl with Cas9/sgRNA complex and 0.4µg dsDNA substrate (PCR fragment of about 500bp) for 90 min at 37°C. The cleaved dsDNA was analysed on a 2% TBE gel.

All the three sgRNAs for *cinnabar* (Fig. 5) and three out of four sgRNAs for *yellow* (Fig. 6) were efficiently able to guide the Cas9 to the target region. The presence of multiple bands in the gel of figures 5 and 6 indicates that a DSB was generated in the target region.

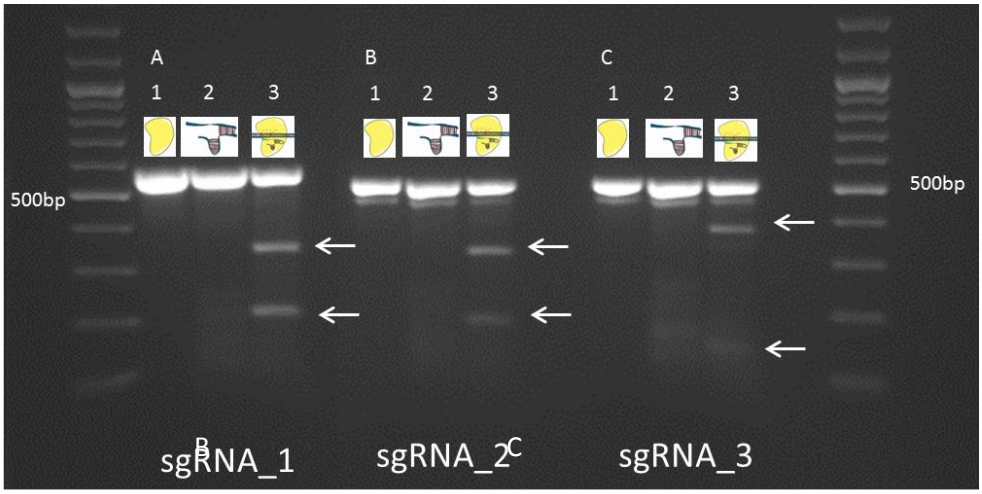

Fig. 5: ***In vitro*** **cleavage assay for** ***cinnabar*** **sgRNAs**
Successful cleavage for sgRNA_1(**A**), 2(**B**) and (**C**)3/Cas9 complex is shown in the third line of each block. First two lines of each block represent the negative controls, namely PCR template either with Cas9 (line 1) or with sgRNA(line 2).

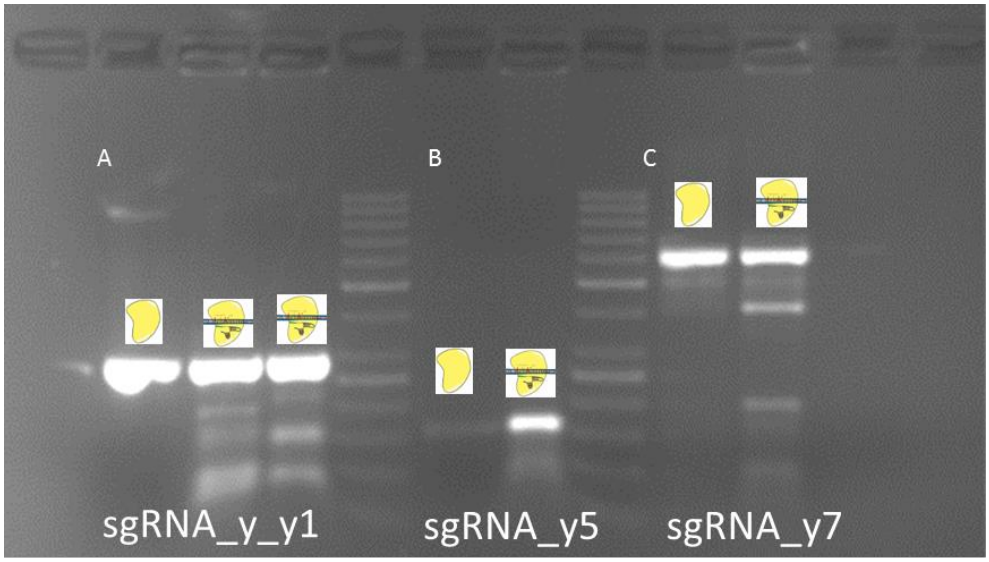

Fig. 6: ***In vitro*** **cleavage assay for** ***yellow*** **sgRNAs**
Successful cleavage for sgRNA_y and y1/Cas9 (**A**), (**C**) and sgRNA_y7/Cas9. (**B**) No cleavage for sgRNA_y5 /Cas9 complex First line of each block represents the negative controls, namely PCR template with Cas9 protein.

Embryo collection and alignment

In order to increase embryo survival and improve injection efficiency, different methods of embryo collections and alignment were tested. First, females were exposed to the posterior side of *Calliphora* spp. pupae and allowed to oviposit for 45min (Lynch & Desplan, 2006). Embryos can be brushed off the host carefully with a fine-tip paintbrush or with a needle and aligned on a slide. For ideal injections, the embryos are aligned on a barrier created by a thin piece of double-sided tape (3M/scotch brand) with a thin (approximately 5mm wide) strip of Nucleopore membrane (Whatman, Nucleopore Track-Etch) on top of the tape. A small strip (1mm) of tape was left free of the membrane. The embryos were aligned with the posterior end on the tape and the anterior side on the membrane. However, this method has several limitations. First, the toxic components of the tape could lead to unpredictable embryo mortality. Second, humidity can interfere during injection, leading to textural changes of the tape, increasing its toxicity. Desiccation of the embryo slide for 30 min before injection, as suggested in Meer et al., (1999) and Lynch and Desplan (2006), might be disadvantageous as embryonal development continues, whereas the gene editing is preferred as early in the development as possible (< 1.5h old) to facilitate germline mutation. Apart from the time window, the surface of the embryos seems to change during its development and is also influenced by its environment, changing the accessibility for the piercing of the needle. Moreover, embryos need to be transferred to pre-parasitized hosts as their original habitat, with this method embryos cannot be transferred right upon injection for being extremely fragile. They can be transferred only during early larval stage whereas the exposure to the toxic component of the tape should be limited, increasing thus their mortality.

Higher survival and more successful injections were achieved once we substituted the tape with a wet Whatman paper. The anterior end of the embryo was aligned towards the paper edge so that the posterior side of the embryo can be injected. This method allowed us to transfer the embryos to a pre-parasitized host right upon injection, limiting the exposure to toxic tapes. However, to keep the paper attached to the slide, it needs to be constantly wetted or, alternatively, can be substituted by a coverslip glued to a slide as described by Li et al, (2017). After alignment embryos are ready to be injected.

Microinjection; survival and phenotype screening

Embryos were injected in their posterior side, where germline will be located, with a vertical angle of 25-35° (Li, et al., 2017). Injections were performed with Femtotips II (Eppendorf, Hamburg, Germany) needles under continuous injection flow. To optimize sgRNA/CAS9 concentration for efficient disruption of or the candidate genes function, we started injecting sgRNA_1 in complex with Cas9 protein in various concentrations and

ratios. After some unsuccessful trials, we adjusted to the findings of Li et al., (2017) and reduced our concentration to 160 ng/µl for both the components. With this concentration embryonal survival rates of about 10% (Table 2) were achieved and are referred to as G_0. For *cinnabar* scorable eye-colour phenotype has been observed in 100% of the adult G_0 (Fig. 7A), however, mostly in mosaic patterns (i.e. single individual presents a mixture of edited and unedited cells). Mosaicism can be explain by the differential timing of action of Cas9 and differential efficiency and specificity of sgRNA in each germ cell and whether a provided repair template is used to repair the double strand break. Thus the resulting modifications present in G_0 individuals can be diverse. Moreover most of the mutations appeared to be somatic without occurring in the germline. Only 1% of mutant females efficiently transmitted the mutant phenotype to the offspring (table 2). One explanation for the higher number of somatic mosaicism can be the injection timing. For efficient germline mutation, embryos need to be in the pre-blastoderm stage.

In the case of *yellow* we recorded a high mortality of the G_0 injected wasps. Four individuals were able to pupate and showed a de-melanise phenotype (Fig. 7B). However these wasps were unable to shed their old cuticle and died entrapped in their pupal cuticle. One hypothesis could be that this lethality was caused in part by dehydration as previously reported *Tribolium castaneum* (Noh et al., 2015). Alternatively a functional *yellow* is necessary for other developmental processes.

Table 2. Embryo survival after CRISPR/Cas9 injection

Sg_RNA	No Injected embryos (G_0)	No embryo transferred (G_0)	No of adult emerging (G_0)	No of mutant G_0 (Mosaic)	No of mutant G_0 with germ line affected	Method of alignment
cn_sgRNA_1	260	50	9 (3.5%)	9	1	Tape + membrane
cn_sgRNA_1	100	100	10 (10%)	10	1	Whatman paper
y_sgRNA_7	150	150	0	4	0	Whatman paper

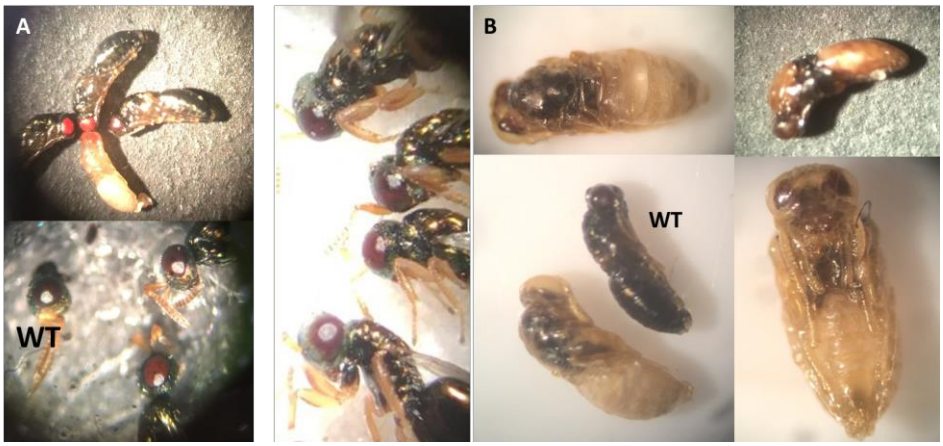

Fig.7 Phenotypic screening of G_0 injected with sgRNA/CAS9
(A) Mutants G_0 individuals show the scorable eye-color phenotype indicating the efficient mutation of the *cinnabar* locus. Different shades of red in each individual indicate the mosaic pattern of the phenotype. **(B)** Mutants G_0 pupae show a de-melanised phenotype indicating the efficient mutation of the *yellow* gene. WT represents wild type wasp.

Sequencing and T7endonuclease I assay

Sequencing of the target region can confirm the mutation induced by CRISPR/Cas9 (data not shown). However, mosaic mutations can lead to false negative results in genotypic screening of the G_0 generation, as there might be a higher rate of wild type allele over the mutated alleles, leading to a biased and possibly false negative result in amplification techniques. G_1 generation, with the mutant phenotype, gives more reliable information about the genotypes.

Alternatively to sequencing, a T7 endonuclease I assay can be used to screen the mosaic G_0 individuals for the presence of the mutation in the right target region. As described above G_0 individuals present a mixture of edited and unedited DNA (Fig. 8). The PCR products, from the genomic DNA of G_0 individuals, whose genomes were targeted using CRISPR/Cas9, is composed by at least two types of dsDNA (wild type and mutated) (Fig. 8). When PCR products ware denatured and re-annealed some of the wild type strands annealed with the mutated ones creating heteroduplex. If there is a sequence difference between the strands, the heteroduplex may show single strand loops or bubbles (unpaired regions) (Fig. 8). Afterwards, the re-annealed PCR products were incubated with the T7 Endonuclease I. This enzyme recognizes and cleaves non-perfectly matched DNA like heteroduplex. After 4h of incubation, fragments were analysed by gel electrophoresis (Fig. 9). Multiple bands in the gel indicates the presence of heteroduplex, that have been cut by the enzyme. This method can be used to screen mosaic G0 individual and give a first estimate of whether our targeting was successful or not (Fig. 9).

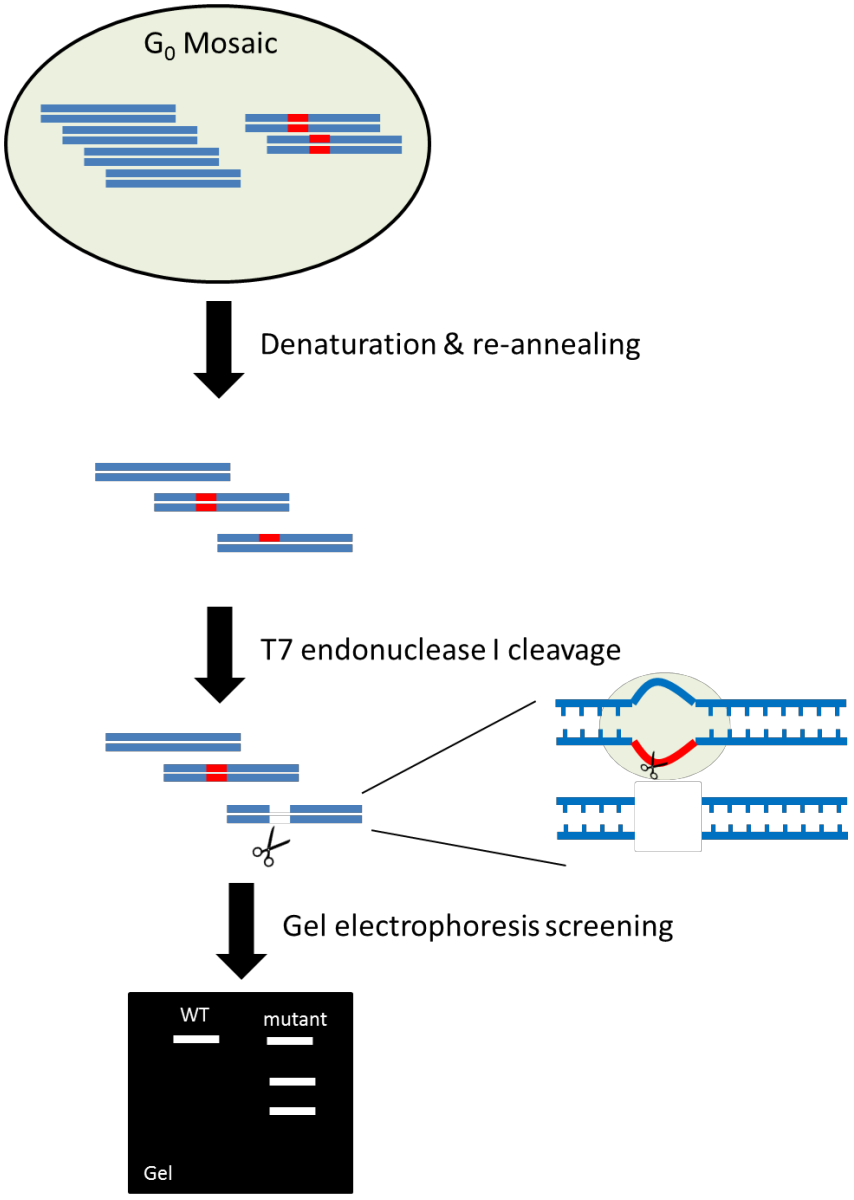

Fig. 8 T7 endonuclease I assay
PCR products are heat-denatured and then reannealed to allow correct (blue lines) and mutant (red lines) strands to re-hybridize and form heteroduplex. The heteroduplex are then cleaved by the T7 endonuclease enzyme, followed by fragment analysis by gel electrophoresis.

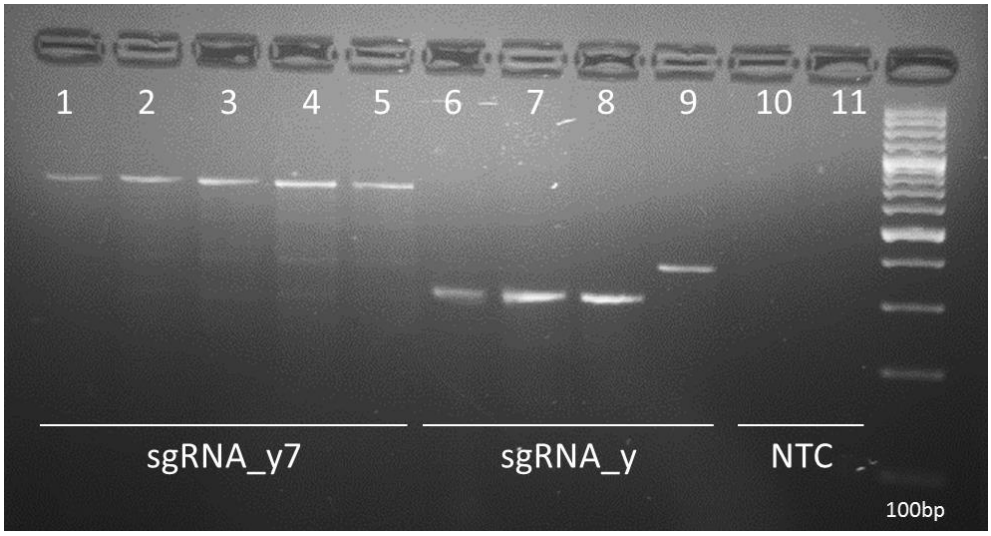

Fig. 9 T7 endonuclease I assay for *yellow* locus
Genomic DNA of the *yellow* locus from single G_0 wasps was used for the assay. Lines from 1 to 5 target region of sgRNA_y7 and from 6 to 9 target region of sgRNA_y. Individuals 2,3,4 and 5 show mismatches at the target site sgRNA_y7. NTC represents the non-template control.

Application of CRISPR/Cas9 to knock-out* period *clock gene function

Cinnabar and *yellow* are useful target genes because they facilitates the mutant screening due to their visible phenotype. However, applying the technique to other genes, that do not give a visible phenotype, might be challenging. For example, to get a knock out of the *period* (*per*) gene, G_0 mosaics will be undistinguishable from wild type, and we thus need an efficient method to select for mutants. One alternative is to induce a precise deletion of the targeted genomic region using two sgRNAs that can create a larger deletion. We thus co-injected two sgRNAs targeting the *per* locus (Fig. 10A). They potentially can generate two DSB's spanning a 600bp fragment that will be excised during the repair (Fig. 10B). Deletions created by multiple sgRNAs should be easily visualized by PCR and gel electrophoresis. *In vitro*, the two sgRNAs combination cleaved efficiently the DNA template spanning both sides (Fig. 11), but unfortunately successes were not achieved when injected in *Nasonia* embryos (G_0) (table 3). We did not identify any large deletion, however the T7 endonuclease I assay detected efficient target mutation of the G_0 (Fig. 12), namely one of the sgRNA was not efficiently working *in vivo*.

In conclusion using CRISPR/Cas9 to induce gene knock-out of genes that does not have a visible phenotype is complicated. Therefore, a knock out gene would be much easier to follow in presence of a reporter gene, inserted in the gene of interest via HRD. Introducing GFP (green fluorescence protein), for example, could help to select mutant

individuals already during the development and would make the stabilization of the mutant line much easier.

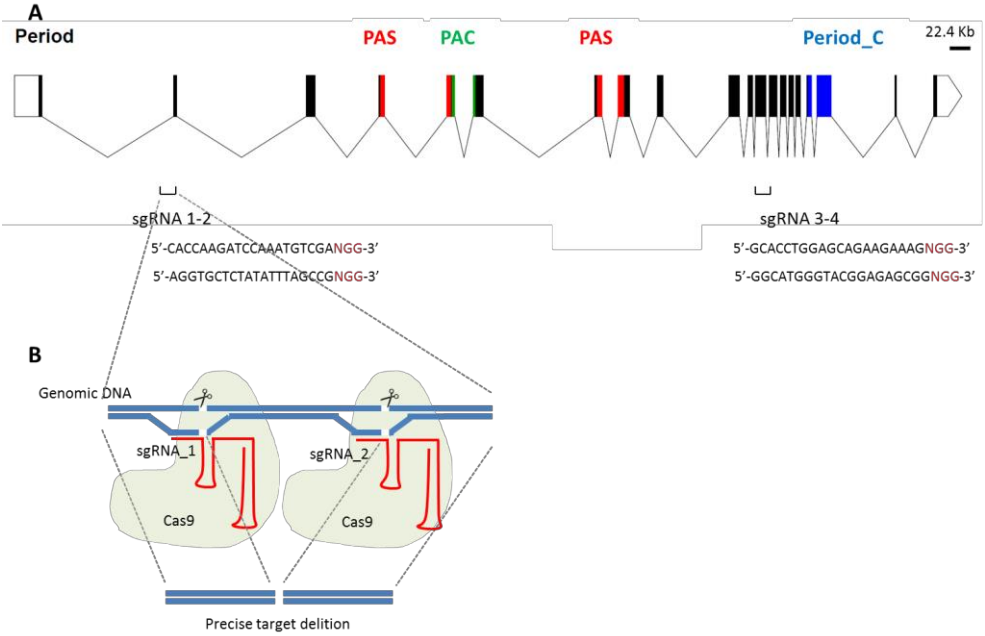

Fig. 10 *Per* gene structure and localization of sgRNA

(A) Schematic representation of *per* in *Nasonia vitripennis* with a total length of 22.4kp. Exons are represented in boxes and introns by lines. Red boxes indicated the PAS domains, in green the PAC domain and in blue the Period_C domain. sgRNA1, sgRNA_2 and sgRNA_3and sgRNA_4 indicate the target region. the required PAM region (NGG) is depicted in red. **(B)** Schematic representations of the precise deletion of *per* genomic region using sgRNA_1-2 and Cas9.

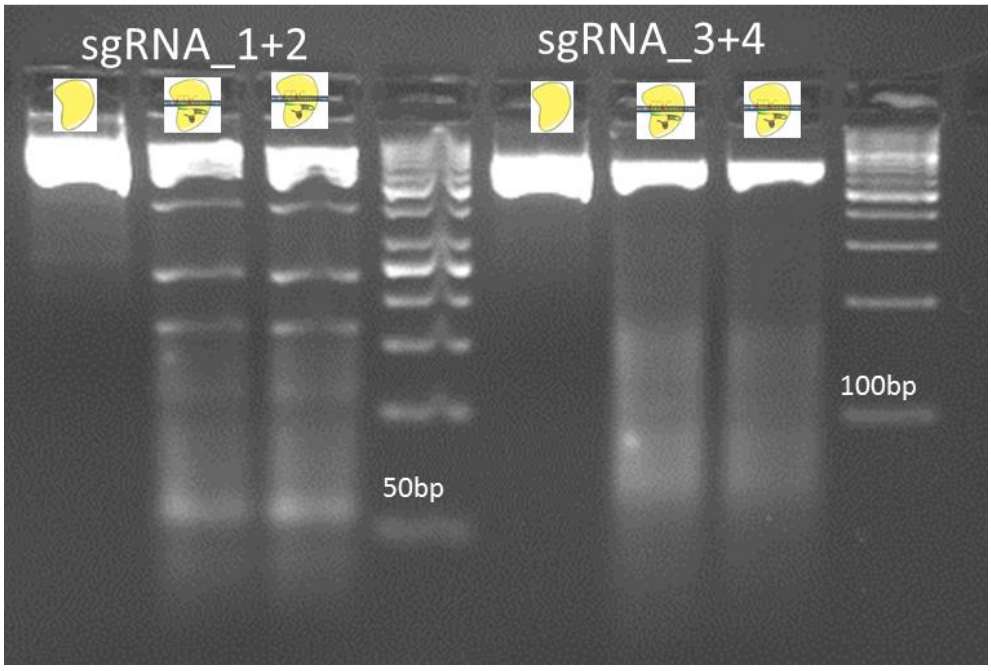

Fig.11 In-vitro test of *per*
Successful cleavage for sgRNA_1+2 /Cas9 complex is shown in the second and third lines. Successful cleavage for sgRNA_3+4 /Cas9 complex is shown in the sixth and seventh lines. Line 1 and 5 represent the negative controls without the sgRNAs.

Table. 3. Injection of *per*iod sgRNA 1+2

n° Injected embryos (G0)	n° embryo transferred (G0)	n° of adult emerging (G0)	n° of mutant G0 (showing big deletion)
450	200	100	0

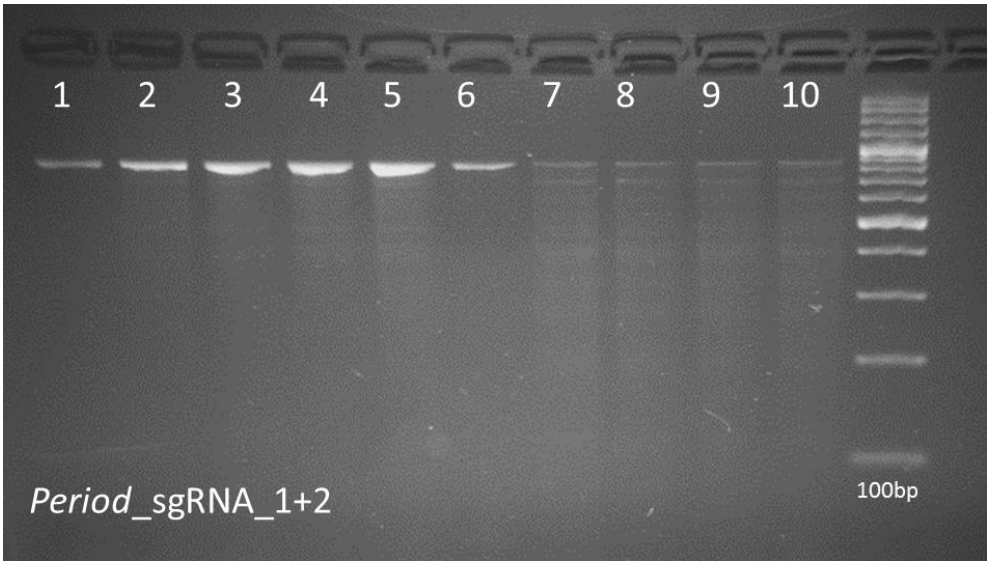

Fig. 12 T7 endonuclease I assay for *period* locus
Genomic DNA of the *period* locus from single G_0 wasps was used for the assay. Lines from 3 to 5, and 7 to 10 show mismatches at the target site sgRNA_1+2.

Conclusion

Establishment of genetic tools in new model organism has always been a challenge for functional genetics approaches. CRISPR represents a promising tool for mutagenesis in new model organisms. However, implementation of this technique in new organisms can be challenging, as a lot of steps need to be optimized to assure efficiency, survival and germ line transmission of the mutations. Here, we described step by step how to use CRISPR/Cas9 in *Nasonia* to disrupt gene function. However, much more work needs to be done in order to increase embryos survival and efficiency. Furthermore, optimization of this tool to target genes that do not generate a visible phenotype is necessary. Our last successful attempt toward this direction, in collaboration with Pegoraro Mirko and Mallon Eamonn from Leicester University, generated transgenic *Nasonia* line, using CRISPR/Cas9 mediated precise homology-directed repair (HDR) after double-stranded DNA (dsDNA) cleavage (Pegoraro et al. *in prep*). Transgenesis, represents a big step toward the establishment of *Nasonia* as an hymenoptera molecular model for many types of studies such as, epigenetics, sex determination, chronobiology, photoperiodism and much more.

Acknowledgements

We would like to thank Tim Grelling for the precious help in optimizing injection procedures, Hassan Ahmed for introducing us to CRISPR methodology, Ernst Wimmer for hosting us in his lab at Göttingen University, Giuseppe Saccone and Angela Maccariello for useful tips and to provide a customized Cas9 protein, Sander Visser for useful discussion and troubleshouting the T7 endonucleaseI assay, Leo Beukeboom and Louis van de Zande for fruitful discussion and comments on the text.

Chapter 6

General discussion

Elena Dalla Benetta

The time of day and season of the year are among the most profound environmental factors dictating daily and seasonal patterns of insect activity. Most seasonal responses in insects, such as the entry and termination of overwintering dormancy (diapause), occur at distinct moments of the year. Likewise, daily activities (such as emergence, feeding, mating, egg laying, etc.) occur at precise times each day. Additionally, ultradian rhythms (i.e. rhythms with period shorter than 20 hour) have been observed in physiological and behavioral functions, such as during specific stages of reproduction. This work contributes to the understanding of the genetics underlying biological timing in the parasitoid wasp *Nasonia vitripennis*. It adds new knowledge of how the circadian clock can be involved in different time-related traits, such as seasonal, daily and ultradian rhythms.

Geographical variation of the circadian clock in *Nasonia vitripennis*

The majority of insects living in temperate zones overwinter in a state of diapause. Diapause is a physiological state of dormancy induced by different environmental factors like photoperiod and temperature. When adult *Nasonia* females experience short day conditions, or more precisely the critical photoperiod (CPP), they initially produce normal developing larvae, but after exposure to a specific number of such light-dark cycles they switch to production of diapausing larvae. A clock mechanism is thus responsible for the timing (timer) and counting (counter) of the light-dark cycles necessary for starting the photoperiodic response (Saunders, 2013). Paolucci et al., (2013) described a positive correlation between latitude and diapause response in *Nasonia* that correlates with allelic frequencies of the circadian clock gene *period* (*per*), indicating a possible role for *per* and possibly other clock genes in photoperiodic diapause induction in *N. vitripennis* (Paolucci et al., 2016).

The circadian clock of *N. vitripennis* includes the mammalian type *cry-2*, that is part of the core feedback loop (Yuan et al., 2007; Bertossa et al., 2014). *Per* and *cry-2* represent the negative elements of the *Nasonia* circadian clock, that inhibit their own transcription by inhibiting *cycle* (*cyc*) and *clock* (*clk*). *Clk* and *cyc* represent the positive elements of the circadian clock, activating the expression of E-Box genes like *per* and *cry-2* (Hardin, 2004; Stanewsky, 2003). Geographical variation of circadian rhythms in activity has also been described for *N. vitripennis* (Paolucci, 2014), however the adaptive significance of latitude-dependent circadian traits is not well understood. Here, I described several properties of the circadian activity pattern that differ between southern and northern wasps (chapter 2). Northern wasps were mainly active at the end of the day, with a prolonged evening peak at the shorter photoperiod, whereas the southern ones showed a unimodal morning activity, with an increase of activity before the light turned on during short photoperiod. This shifted activity pattern between northern and southern wasps can

reflect local adaptation. In the south, temperatures are known to become high in the middle, late afternoon, hence shifting the activity to the coolest part of the day (namely the morning) might be a strategy of insects that live in a hot environment (Prabhakaran & Sheeba, 2012, 2013). In contrast, species that live at higher latitudes would have to cope with lower temperatures and longer photoperiods. In concordance, the tested northern lines have a reduced morning activity and have their activity peak in the second part of the day when temperatures are higher.

The different timing of activity between the two lines reflects the pace of the clock in constant darkness (DD): southern lines show shorter τ close to 24 h (faster clock), compared to northern ones that have a τ longer than 24h (slower clock). This could possibly be explained by the fact that at higher latitudes, organisms must continuously and accurately entrain to the 24-hour day, despite the sharp increase in day length during the spring. In accordance with Aschoff's rule, pacemakers with periods longer than 24 h are more efficient in tracking and interpreting the dawn, and thus photoperiodic changes (Pittendrigh & Takamura, 1989). Therefore, clocks with τ exceeding 24 hours should enhance seasonal acuity, particularly at high latitudes. This suggests that the latitudinal differences in free running period are the result of selection on the circadian rhythm mediated through selection on traits that are genetically correlated with circadian rhythms (such as seasonal response). Additionally, I found that the activity level of northern wasps was higher than southern ones, whereas southern strains tend to have higher activity when photoperiod is shorter. Such difference could result from variation in the sensitivity to light. Therefore the natural variation in circadian clock parameters described here is likely the results of different selection pressure and local adaptation.

In order to evaluate whether differential clock gene expression can explain the geographical variation in seasonal and circadian responses in *N. vitripennis,* I investigated expression patterns of candidate clock genes of wasps from different geographical origin, under different photoperiodic conditions (chapter 3). For the clock genes *per*, *cry-2*, *clk* and *cyc*, circadian expression depending on photoperiod and latitude of origin was analyzed in order to assess if clock genes may play a role in photoperiodic diapause induction in *N. vitripennis*. I found higher expression levels of the tested genes 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. In particular, *per* expression peaks at the end of the night in the southern wasps and much later (during the light phase) in the northern ones, in line with the different activity peaks and free running rhythms described above. This suggests that the different *per* alleles that are present in the southern and northern wasps, are involved in setting the pace and the phase of the clock by differential gene expression. 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, indicating that differences in transcriptional

regulation between lines and under different photoperiods may play a role in regulating seasonal adaptation.

Towards high latitude, daily and annual variation in the solar radiation leads to extreme fluctuation of many environmental factors, especially light intensity and photoperiod. Therefore, it has been argued that the light sensitivity of the circadian clock needs to be adapted in temperate zones in order to maximize an organism's fitness (Pittendrigh & Takamura, 1989; Pittendrigh et al., 1991). One mechanism for this process could be different amplitude of clock gene expression (Pittendrigh & Takamura, 1989; Pittendrigh et al., 1991) between southern and northern regions. Alternatively, it could involve different filters of the light input into the clock. Overall, the weaker expression profile of the clock genes in the northern wasps indicates the presence of a more plastic (flexible) clock in the north. Weaker clocks can more easily synchronize to different LD cycles, and more readily phase-shift to light-pulses, compared to strong circadian clocks (Vitaterna et al., 2006; van der Leest et al., 2009; Abraham et al., 2010). This could facilitate northern wasps to adapt to a more variable environment because a weak circadian clock is assumed to more easily synchronize to changing photoperiods. At the same time, weak circadian clocks are efficiently ticking under LD cycles and can serve as time reference for photoperiodism. This 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. The results of my study indicate that selection acted on modulating the expression of several clock genes. Future research should focus more on post-transcriptional regulation such as alternative splicing and protein phosphorylation in order to detect how these geographical differences in gene expression are accomplished.

Role of *period* in biological rhythms of *Nasonia vitripennis*

In order to understand the adaptive evolution of biological rhythms, it is essential to establish if and how the circadian clock is involved in the regulation of these cyclical processes. One approach towards this is to investigate the functional involvement of the clock gene *per*, both in the circadian rhythm and in the photoperiodic diapause response of *N. vitripennis* as well as in the regulation of male ultradian rhythm of courtship. I found that knock down of *per* (i) alters the daily rhythm under constant conditions (DD and LL), (ii) changes the timing of locomotor activity, (iii) influences the expression of the whole circadian clock, (iv) delays photoperiod diapause response of the wasps and (v) modifies the pace of male courtship performance.

Role of* per *in the circadian system

The role of *per* in the circadian clock mechanism of *N. vitripennis* was assessed via RNA interference (RNAi) in chapter 4. Interestingly, the knock down of *per* expression increased the speed of the clock (shorter τ) in both southern and northern lines in constant darkness (DD), and advanced the activity phase in the northern wasps in LD16:08 and LD08:16. For the first time I confirmed a functional role of *per* in the core mechanism of the *Nasonia* circadian clock. These data indicate that *per* is involved in setting the pace and the phase of the circadian clock consistent with findings in chapter 2 and 3. Furthermore, the effects of speeding up the clock in the northern lines were more pronounced, shown by an advance activity profile after *per* RNAi, with a peak of activity in the morning rather than in the evening. This larger effect on northern wasps could reflect some functional differences of *per* alleles between southern and northern lines that make northern ones more responsive to changes.

Under LL conditions, RNAi-treated northern wasps increased the duration of their free running rhythm, whereas southern ones decreased it, indicating a different effect of *per* (and of the light) between the south and north in the regulation of DD and LL rhythms. This difference could reflect the presence of a different circadian oscillator in southern and northern wasps, which phase is set by dawn in the south and dusk in the north. As proposed by Pittendrigh and Daan (1976), in the dual oscillator model, the two oscillators show different responses to light: one is accelerated and the other decelerated by constant light. One oscillator will thus shorten and the other oscillator will lengthen its period when exposed to LL (Daan et al., 2001; Pittendrigh & Daan, 1976). This difference is also visible in wildtype *Nasonia* wasps with an intact circadian clock, but as soon as I start manipulation by knocking down *per*, this differential regulation becomes more evident. These results suggest again that southern and northern wasps not only differ in the pace of their clock, but also in the phase of their circadian oscillator. However, it must be noted that this is only one of a number of possible interpretations. If these differences indeed reflect the presence of two different neuronal oscillators with different phases, further analysis should identify neurons in the brain with different circadian expression between southern and northern wasps.

I also proposed an additional role for *per* in the light input pathway, because the fraction of wasps exhibiting circadian rhythmicity under LL was higher among the RNAi-treated wasps than the control wasps. One of the mechanisms to allow northern species to adjust their light sensitivity could involve different filters of the light input into the clock (Pittendrigh & Takamura, 1989; Pittendrigh et al., 1991). Above, I mentioned that photoperiod differently affects clock gene expression patterns in the southern and northern lines that carry different *per* alleles. This points at a higher photo-responsiveness at higher latitude that, together with a weaker clock oscillation, could make northern wasps more

flexible in adjusting to a variable environment. Furthermore, our data point towards a role of *per* in the light sensitivity pathway. It would be very interesting to test whether the different *per* alleles in *Nasonia* also differ in light sensitivity as was reported for *tim* alleles in *Drosophila* (Sandrelli et al., 2007; Tauber et al., 2007), and whether the light signal is differently filtered into the clock system of southern and northern wasps.

Role of* per *in the seasonal system

Manipulation of *per* expression via RNAi also affected photoperiodic diapause response of *Nasonia* (chapter 4). Although all wasps were able to induce diapause in their offspring after *per* knock down, the timing of the photoperiodic response was delayed in southern and northern lines. This indicates that *per* knock-down is not affecting the physiology of diapause itself, but the onset of it, hence the timer mechanism, responsible for detecting the LD cycle. Additionally, our study pointed towards a geographical variation component. I found that the *per* knock down effect on northern wasps is twice as strong as on the southern ones. *Per* is important in sensing the photoperiodic changes, independently or through the circadian clock mechanism, and if it is light sensitive (directly or indirectly) as suggested from the LL data, the allelic differences between north and south might reflect the presence of a more sophisticated system in northern populations. This would allow them to detect small environmental changes, in which a small perturbation of the system generates a greater effect, and enable them to adjust their physiology and behaviour faster. In agreement to what I reported before for the "weaker" circadian clock gene oscillation in the north, these data suggest the evolution of a more sensitive clock at higher latitude that would allow northern populations to respond faster and stronger to a changeable environment.

Role of* per *in the ultradian system

The circadian clock controls a substantial proportion of transcriptional activity and protein functions (Claridge-Chang et al., 2001; McDonald & Rosbash, 2001) and its function has been associated with other time-related traits, such as courtship rhythms (Kyriacou & Hall, 1980). In chapter 5, I reported an effect of *per* RNAi on the ultradian rhythm of male courtship behaviour. *Nasonia* male courtship involves series of strong movements with the head, so-called "head-nods" that are accompanied by wing vibrations and that are interrupted by pauses. I described differences in male courtship display after *per* knock-down. In particular, knock down increased the duration of head-nod cycles and the number of head-nods per cycle, thereby changing the pace of pheromone release which occurs at each first head-nod in a cycle (van den Assem et al., 1980). Knock down of *per* also altered

the circadian rhythm of these treated males. Thus, our data show that *per* RNAi alters, in a parallel fashion, both circadian and courtship cycles in *Nasonia*. Similar findings were reported for *D. melanogaster* and the melon fly *Bactrocera cucurbitae*, in which mutation of *per* speeds up the circadian clock and also changes the periodical fluctuation of male courtship song (Kyriacou & Hall, 1980; Miyatake & Kanmiya, 2004). Our study is the first evidence that the clock gene *per* is involved in courtship behaviour in Hymenoptera, revealing a conserved regulating mechanism in mating behaviour between Diptera and Hymenoptera.

I found geographical differences in male courtship performance in the number of head-nods per cycle Although the possible adaptive significance of this variation is not known, the observed differences in ultradian rhythms may be a correlated response to selection for different *per* alleles. The cline in *per* allele frequencies in *Nasonia* has been attributed to latitudinal selection for diapause response (Paolucci et al., 2013, 2016). As this study indicates a role of *per* in male courtship behaviour, genetic correlation appears the most likely explanation for the observed differences in cycle time and headnods numbers between northern and southern wasps. On the other hand, variations in cycle time and head-nods number as part of male courtship behaviour, may serve as a cue for females to identify and choose the fittest mate. However, whether females are able to measure the timing of the courtship display and pheromone release (van den Assem & Putters, 1980) is still unknown and requires more experiments on mate choice.

In this study I showed that the ultradian rhythm of male courtship behaviour is (partially) determined by *per*. However, *per* might regulate this fast rhythm directly, due to pleiotropic function, or through the circadian clock. In both cases, whatever transcription factor is involved in the genetic pathway of courtship behaviour, it likely is either up- or down regulated when *per* is knocked down. Importantly, this study represents an additional example of the involvement of the clock gene *per*, and maybe of the circadian clock, in the timing mechanism of *N. vitripennis*.

Circadian models for seasonal adaptation in *Nasonia vitripennis*

Various behaviours are controlled by an endogenous clock in insects and exhibit circadian rhythmicity. Since Konopka and Benzer (1971) found the first circadian mutant of *period* (*per*) in *Drosophila melanogaster*, many circadian clock mutants have been reported from *Drosophila*, nematodes, mice, and other species (Hall, 1995; Dunlap *et al*., 1999; Panda et al., 2002). These mutants frequently show additional behavioural differences in time-related traits because the circadian clock controls a substantial proportion of transcriptional activity and protein functions of the whole organism (Claridge-Chang et al., 2001; McDonald & Rosbash, 2001). However, the involvement of the circadian clock in seasonal rhythms is

still debatable (Bradshaw & Holzapfel, 2010b) and the role of *period* in regulating seasonal responses is still unclear. Here, I discuss two alternative hypotheses of how *per* might regulate other time-related traits, such as seasonal diapause.

Bunning (1936) first proposed that the photoperiodic response is based on circadian functions because both phenomena react to time-giving cues from environmental light-dark cycles. In this study, I showed that *per* is important to set the pace and the phase of the circadian clock as well as to set the timing of the diapause response in *Nasonia*. These data can be interpreted in two ways: (i) there is a pleiotropic role of a single clock gene in photoperiodism or (ii) the circadian clock, as a functional module, underpins photoperiodism (Fig. 6.1). In the first hypothesis, the *per* gene can have a direct pleiotropic role in both circadian and seasonal responses, or an indirect effect by altering the expression of other genes (Fig. 6.1A). In both cases, *per* regulates the pace and the phase of the circadian clock that, in turn, generates daily rhythms (such us locomotor activity rhythms). The light information is transduced by an unknown receptor and regulates *per* gene expression differently in the southern and northern wasps. Due to this differential gene expression, the different *per* alleles in the southern and northern wasps regulate the speed and the phase of the clock differently, generating, for example, a morning activity phase in the south and an evening activity phase in the north.

Independently from its function in the circadian clock, *per* would then also regulate diapause response. The photoperiodic information is processed in the photoperiodic calendar, more precisely in the timer mechanism, which includes *per*, which expression is differently regulated in southern and northern wasps. After the processing of photoperiodic information the insect would activate an internal mechanism that leads to the production of a hypothetical substance or "diapause factor", in the counter system, which accumulates daily. This diapause factor shows a diapause-inducing effect and an internal threshold serves as a reference to determine the diapause/non-diapause developmental program (Gibbs, 1975). The accumulation rate of this factor differs between southern and northern wasps and depends on some unknown downstream effectors of PER (Fig. 1.6A). Diapause induction in *Nasonia* occurs over two generations, i.e. adult females perceive and interpret the photoperiod and transmit the information to the egg. This as yet unknown diapause factor can be any regulative elements that accumulate in the ovary or in the eggs in order to influence directly the output. The pathway between light input, PER regulation and accumulation of the diapause factor is however very complex and involves multiple gene products. Additional experiments are needed, aimed at identifying the genes involved in all the levels of this process, i. e. input pathway, core mechanism and output pathway.

In the second hypothesis, the circadian clock, as a functional module, plays a role in both processes (daily and seasonal). There are two main circadian models theorised to determine the day length: the external (Fig. 6.1B) and the internal coincidence models. The external coincidence model (Bunning, 1936) assumes the presence of one internal

(circadian) oscillator which phase is set by the light cycle (Fig. 1.4B). When the photosensitive phase of this cycle falls into the dark, owing to the shortening of the day length, a photoperiodic response is triggered. The internal coincidence model on the other hand assumes a change in phase between two circadian oscillators triggered by the change of photoperiod to be responsible for sensing seasonal changes (Pittendrigh et al., 1970; Saunders&Bertossa, 2011). *N. vitripennis* has been predicted to have this last type of clock (Saunders, 1974a) by Nanda–Hamner experiments (Nanda & Hamner, 1958). However, despite the positive Nanda–Hamner responses, several observations indicate that the external coincidence model might be the system used by *Nasonia* to detect photoperiodic changes. (i) Recent work of Vaze and Helfrich-Förster (2016) suggests that *N. vitripennis* may use a strongly damped circadian oscillator, which works in the external coincidence model. Additionally (ii) southern and northern wasps show different phase and pace of their circadian oscillators due to the presence of different *per* alleles (Paolucci et al., 2013, 2016; chapter 2, 3, 4). Moreover, (iii) changing the phase and the speed of the circadian clock, by manipulating *per* expression profile, results in an altered diapause response (chapter 4). Taken together, these results indicate the presence of one circadian oscillator that differs in phase and pace between southern and northern wasps, due to differential *per* expression. New studies, using the newly designed T-cycle experiments described by Vaze and Helfrich-Förster (2016), in conjunction with experiments aimed at identifying these oscillators at the neuronal level, may help to solve the issue and rule out one of the models.

How can the difference in diapause tendency between northern and southern wasps be explained? After the processing of photoperiodic information in the time measurement system (via internal or external coincidence models), insects accumulate a "diapause factor" in the counter system. In this model, the different phase (and maybe pace) of the circadian clock in the southern and northern wasps is differently affecting the light dependent accumulation of this factor. Under short days, both lines accumulate the substance effectively, but with different speed and different internal threshold on day x that corresponds to the switch point. Under longer days, the accumulation of the factor is much slower, probably due to different phase of the circadian oscillator and, as a consequence, diapause response is delayed (Fig. 6.1B).

In conclusion, different models can explain the involvement of the circadian clock in photoperiodism. In any case, *per* is a core component of the circadian clock generating daily rhythms in locomotor activity and it is also involved in photoperiodic time measurement in *N. vitripennis*. Several levels of regulation and interactions might be present between the perception of the light input and the output signal, which can affect the final response in different ways, making the deciphering of the molecular mechanism underlying daily and seasonal adaptation complex.

General conclusions and future direction

This work has contributed to a better understanding of the role of the clock gene *period* in seasonal, daily and ultradian rhythms. It described geographical differences in locomotor activity that reflects a latitude-dependent selection pressure. It showed how different *per* alleles can regulate several properties of the circadian clock, via differential gene expression and perhaps differential protein modulation. Most importantly it showed a functional involvement of the clock gene *per* in the circadian and in the photoperiodic time measurement of *N. vitripennis*. The function of *per* is not affecting the diapause physiology itself, indicating that natural selection acted on the timer mechanism of the photoperiodic calendar of the wasp rather than on the physiology of diapause. Additionally, it showed a role for *per* in the ultradian rhythms of male courtship behaviour.

The results give rise to many lines of future research. The identification of clock neurons and neuronal pathways generating clock gene expression will help the characterization of the molecular mechanism underlying daily rhythms and also can reveal the presence of one or multiple oscillators. Such information could be gained through whole brain *in situ* localization both at the transcript and protein levels. Additionally, investigation of post-transcriptional and post-translational regulation of *per* and other clock genes would help to better understand how phenotypic differences between latitudinal populations come about. More experiments investigating the genetic architecture of ultradian rhythmicity, e.g. in male courtship performance, would also help to better understand the evolution of such fast rhythms and their role in intra- and interspecies interactions.

The advent of CRISPR technologies will allow to introduce stable mutations in clock genes that can be inherited (M. Li, Au, et al., 2017). CRISPR has been applied to a wide range of organisms with great success, for example the mosquitoes *Aedes aegypti* (Kistler et al., 2015) and *Anopheles gambiae* (Hammond et al., 2016). Work is underway to employ this technique in *Nasonia* research. It will allow additional functional studies, like a complete knock out of *per* and the induction of targeted mutation aimed to slow down the circadian clock. Hence, applying this method could give more information about the role of *per* in regulating photoperiodic diapause response in *N. vitripennis*. Furthermore, functional studies on other candidate clock genes and/or neuropeptides as Pigment Dispersing Factor (PDF) would also give a more complete picture of the molecular mechanism underlying daily, seasonal and ultradian systems.

Finally, this study shows that *N. vitripennis* is an excellent model for studying biological rhythms. The clear photoperiodic response with distinct sensitive and responsive stages allows one to investigate different levels of regulation of this behaviour. Further genetic manipulation studies will help to decipher whether the same system regulating circadian rhythms is also involved in other time-related traits. The easily quantifiable male

courtship display can be used to study ultradian rhythm. Additionally, the widespread distribution of *N. vitripennis* provides the opportunity to analyse the adaptive value of these biological rhythms and their genetic basis. All these factors, in combination with the increasing availability of genetic tools such as CRISPR, make *Nasonia* a very powerful model for future chronobiology studies.

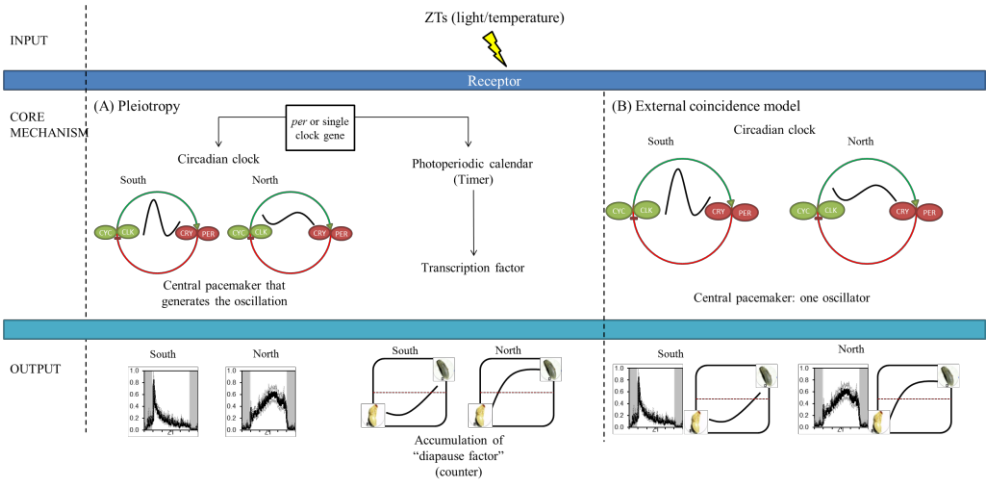

Fig.6.1. Circadian model for seasonal regulation
The environmental signals (ZTs) are transmitted by unknown receptors to a core mechanism. **(A)** in the **pleiotropy model** *period* (*per*) plays a key function in the negative feedback loop of the circadian clock and an independent role in the timer of the photoperiodic calendar. The output pathway of the circadian clock regulates daily rhythms of locomotor, morning activity in the south and evening activity in the north. Additionally per is involved in the timer mechanism of the photoperiodic calendar allowing the accumulation of an unknown diapause factor (output). Faster accumulation in the north will allow diapause induction much earlier than in the south. **(B)** Alternatively in the **external coincidence model**, the circadian clock regulates both processes. The phase of the circadian clock will determine the phase of the daily activity (morning in the south and evening in the north) and the accumulation of the diapause factor (slower in the south compared to the north).

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Summary

Since life is subject to daily cycles of light and dark, and to seasonal cycles of environmental change, caused by the rotation of the earth around its axis and around the sun. All living organisms have adapted their physiology, behaviour and metabolism in order to cope with this periodicity, especially those living towards high latitude where, owing to the Earth's axial tilt, daily and seasonal changes include more extreme fluctuations in light and temperature. The evolution of endogenous clocks has enabled organisms from bacteria to plants and animals to exhibit biological rhythms to 'time' daily (circadian) and annual events. Studies of the genetic composition and functioning of the clock reveal complexity and variation. The Nobel prize in medicine was awarded this year for the initial discovery of the circadian mechanism, It is now known that more than 50% of all genes of an organism are under the control of the circadian clock.

Insects are particularly suited for studies on biological clocks. They are widely distributed and therefore some species have adapted to a wide range of climatic conditions. The time of day and season of the year are important environmental factors dictating daily and seasonal patterns of insect activity. Most seasonal responses in insects, such as the onset and termination of overwintering dormancy (diapause), occur at specific and distinct times of the year. Likewise, daily activities (such as emergence, feeding, mating, egg laying, etc.) occur at specific and distinct times each day. Additionally, ultradian rhythms (i. e. rhythms with period shorter than 20 hour) have been observed to be essential parts of physiological and behavioural functions. This work concerns the genetics, with particular focus on clock genes, underlying biological timing in the parasitoid wasp *Nasonia vitripennis* .

Nasonia vitripennis is a small parasitic wasp that parasitizes the pupae of various fly species. It is an excellent model for chronobiology studies because it is well studied for several seasonal, circadian and ultradian biological rhythms. Its widespread distribution provides the opportunity to analyse the adaptive value of these biological rhythms and their genetic basis. *Nasonia* has a maternally and photoperiodically induced larval diapause: when adult females experience critically short daylight conditions, they produce diapausing larvae that stay inside the host pupa and only resume development when conditions are favourable. Northern European wasps induce diapause under conditions where daylight periods are still relatively long while southern European wasps require much shorter periods of daylight The period of daylight that triggers diapause induction is called critical photoperiod (CPP). Thus, at high latitudes where winter arrives earlier during the year compared to lower latitudes, the CPP is longer than at low latitudes.

Geographical variation in seasonal, correlates with the presence of different *period* (*per*) alleles. Since *per* is a clock gene regulating circadian activity, it has been hypothesised that the circadian clock is also involved in the regulation of these other time-related responses. The circadian clock is characterised by transcriptional and translational feedback loops involving different transcription factors. Positive elements, like *clock* (*clk*)

and *cycle (cyc)* enhance the transcription of other genes, like *period* (*per*) and *cryptochrome-2* (*cry-2*), that in turns act as negative elements inhibiting their own transcription. These transcriptional-translational feedback loops are regulated by the light and follow a 24h period. In order to further test the hypothesis that the circadian clock is involved in seasonal timing, I first described the natural variation in circadian rhythmicity and clock gene expression in *N. vitripennis* originating from southern (Corsica, France) and northern (Oulu, Finland) populations. *Nasonia vitripennis* is a diurnal insect with a unimodal activity pattern. It shows rhythmic activity under constant darkness (DD) and constant light (LL) conditions, that can be used to reveal important properties of the circadian clock.

In my studies I discovered several properties of the circadian activity pattern that differ between southern and northern wasps (chapter 2). Northern wasps were mainly active at the end of the day, with a prolonged evening peak at the shorter photoperiod, whereas the southern ones showed a morning activity, with an increase of activity before the light turned on during short photoperiod. This shifted activity pattern between northern and southern wasps can reflect local adaptation. In the south, temperatures are known to become high in the middle, late afternoon, hence shifting the activity to the coolest part of the day (namely the morning) might be a strategy of insects that live in a hot environment. In contrast, species that live at higher latitudes would have to cope with lower temperatures and longer photoperiods. In concordance, the tested northern lines have a reduced morning activity and have their activity peak in the second part of the day when temperatures are higher. This different timing of activity between southern and northern wasps likely reflects the pace of their circadian clock. Under constant conditions the pace of the southern clock is faster than the northern one. Interestingly, slow clocks should enhance seasonal acuity, particularly at high latitudes because they are more efficient in tracking and interpreting the dawn, and thus photoperiodic changes. Therefore, the natural variation in circadian clock parameters might be the result of different selection pressure and local adaptation.

In order to evaluate whether the observed geographical variation in seasonal and circadian responses in *N. vitripennis*, is explained by gene expression differences, I investigated expression patterns of candidate clock genes of wasps from different geographical origin, under different photoperiodic conditions (chapter 3). For the clock genes *per*, *cry-2*, *clk* and *cyc*, circadian expression depending on photoperiod and latitude of origin was analyzed. I found higher expression levels of these genes in the southern than northern wasp. I also found differences in amplitude and phase in expression profile. Expression levels and phase were differently affected by photoperiod in wasps of the two localities, in line with the different activity rhythms described above. These results suggested that *per* is involved in setting the pace and the phase of the clock by differential gene expression. Interestingly, 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, indicating that differences in transcriptional regulation between lines and under different photoperiods may play a role in regulating seasonal adaptation. Overall, I described a weaker expression profile of the clock genes in the northern wasps indicating the presence of a more plastic (flexible) clock in the north. This could facilitate northern wasps to adapt to a more variable environment because weak circadian clocks are assumed to more easily synchronize to changing photoperiods.

The role of *per* in the timing mechanism of *N. vitripennis* was assessed via RNA interference (RNAi) in chapter 4. The knock down of *per* expression increased the pace of the clock in both southern and northern lines in constant darkness (DD), and advanced the activity phase in the northern wasps. These results confirmed a functional role of *per* in the core mechanism of the *N. vitripennis* circadian clock. They also reveal that *per* is involved in setting the pace and the phase of the circadian clock. Moreover, under constant light LL conditions, northern RNAi-treated wasps slowed down their clock, whereas southern ones speeded it up, indicating a different effect of *per* (and of light) between the south and north in the regulation of DD and LL rhythms. This difference could reflect the presence of a different circadian oscillator in southern and northern wasps, which phase is set by dawn in the south and dusk in the north. The two oscillators would show different responses to light: one is accelerated and the other decelerated by constant light. These results indicate that southern and northern wasps not only differ in the pace of their clock, but also in the phase of their circadian oscillator.

Manipulation of *per* expression via RNAi also affected photoperiodic diapause response of *Nasonia*. Although all wasps were able to induce diapause in their offspring after *per* knock-down, the timing of the photoperiodic response was delayed in both the southern and the northern lines. This indicates that *per* knock-down is not affecting the physiology of diapause itself, but the onset of it, hence the timer mechanism, responsible for detecting the photoperiodic changes.

An additional type of rhythms can also be described in *N. vitripennis*. Ultradian rhythms represent all the rhythms with a period shorter that 20h. In *Nasonia* and in other insects they are part of male courtship behaviour. Courtship in many animal species consists of a repertoire of specific signals delivered by the male during the mating event. Male signals are an important courtship display in many insect species and play a role in species recognition as well as in sexual selection within species. *Nasonia* males court females by performing series of strong movements with their head, so-called “head-nods” that are accompanied by wing vibrations, and that are interrupted by pauses, together making up a series of cycles that last seconds. The rhythmic head-nods display during male courtship behaviour is important for inducing female receptivity by enabling the rhythmic release of pheromones. However, very little is known about the genetic mechanism that underlies this ultradian rhythmicity. In chapter 5, I described an effect of *per* RNAi on this ultradian rhythm of male courtship behaviour. Important courtship characteristics were

affected by *per* knock-down probably affecting the pace of pheromone release. Knock-down of *per* also altered the circadian rhythm of these treated males, indicating that *per* RNAi alters, in a parallel fashion, both circadian and courtship cycles in *Nasonia*, revealing an additional role for *per* in the ultradian rhythms of *N. vitripennis*.

As part of my studies I implemented a new genetic tool in *Nasonia*, namely CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)/CAS9 genome editing. This technique will allow to induce gene knock-out and knock-in of any gene of interest in the future. In particular, it will allow making stable lines carrying one or multiple mutations of clock genes.

In the final chapter of my thesis (chapter 6), I present, based upon my results, two alternative models of how *period* might regulate other time-related traits, such as seasonal diapause. *Per* can have a pleiotropic role and regulate diapause independently from its function in the circadian clock, or the circadian clock, as a functional module, may underpin photoperiodism. In any case, *per* is required to set the pace and the phase of the circadian clock as well as transduce the photoperiodic information for the diapause response. Under both scenarios, after the processing of photoperiodic information in the time measurement system (either via pleiotropy or through the circadian clock), the wasps accumulate a "diapause factor" and differential *per* expression might affect the light dependent accumulation of this factor. Under short days, both the southern and northern lines accumulate the substance, but with different speed. Under longer days, the accumulation of the factor is much slower and diapause response is delayed. Several levels of regulation and interaction might be present between the perception of the light input and the output signal, which can affect the final response in different ways, making the deciphering of the molecular mechanism underlying daily and seasonal adaptation complex.

In conclusion, this work contributes to the understanding of the genetics underlying biological timing of the parasitoid wasp *Nasonia vitripennis* in particular and of insects in general It adds new knowledge of how the circadian clock can be involved in different time-related traits, such as seasonal, daily and ultradian rhythms. It supports the hypothesis of an evolutionary link between the circadian clock and the seasonal photoperiodic response. Future research should identify the presence of one or multiple oscillators and focus on the neuronal architecture of the circadian clock in *N. vitripennis*. Additionally, investigation of post-transcriptional and post-translational regulation of *per* and other clock genes would help to better understand how geographical variation in rhythms comes about. More experiments investigating the genetic architecture of ultradian rhythmicity, e.g. of male courtship performance, would also help to better understand the evolution of such fast rhythms and their role in intra- and interspecies interactions. The results of this thesis facilitate the design of such experiments

Samenvatting

Dutch translation by Jenke Gorter

Het leven is onderhevig aan invloeden uit de omgeving zoals de dagelijkse licht-donker cyclus en een afwisselingen van seizoenen gedurende het jaar. Deze invloeden worden veroorzaakt door de draaiing van de aarde om zijn as en om de zon. Alle levende organismen zijn aangepast in hun fysiologie, gedrag en metabolisme om met deze periodiciteit om te gaan, maar vooral de organismen die op hoge latitude leven vanwege de extreme fluctuaties in licht-donker cyclus tussen de seizoenen, dit vanwege de kanteling in de as. De evolutie van interne klokken stelt organismen, van bacteriën tot planten en dieren, in staat om biologische ritmes te synchroniseren met dagelijkse en jaarlijkse gebeurtenissen. Onderzoek naar de genetische compositie en het functioneren van de klok hebben complexiteit en variatie onthuld. De nobelprijs voor geneeskunde is dit jaar toegekend aan de ontdekking van circadiane mechanismen, het is bekend dat meer dan 50% van alle genen van een organisme onder de controle staan van de circadiane klok.

Insecten in het specifiek zijn erg geschikt voor het onderzoek naar biologische klokken. Ze zijn wijd verspreid en sommige soorten zijn daardoor aangepast aan vele klimaten. De tijd van de dag en het seizoen van het jaar zijn belangrijke omgevingsfactoren die dagelijkse en seizoensgebonden patronen in de activiteit van insecten bepalen. De meeste seizoensgebonden reacties in insecten, zoals de start van en ontwaken uit winterslaap (diapauze), vinden plaats op specifieke momenten in het jaar. Overeenkomstig vinden dagelijkse activiteiten (zoals uitkomen/ontpoppen, voeden, paren, eitjes leggen, etc.) plaats op specifieke tijdstippen van de dag. Daarnaast zijn er ultradiane ritmes (ritmes met een periode korter dan 20 uur) die van essentieel belang zijn voor fysiologische functies en gedrag. Dit onderzoek wijdt zich aan de genetica, met focus op klokgenen, die ten grondslag ligt aan biologische timing in de parasiterende wesp *Nasionia vitripennis*.

Nasionia vitripennis is een kleine parasiterende wesp die de larven van verschillende vliegen soorten parasiteert. Het is een uitstekend model voor chronobiologische onderzoeken omdat het bekend is voor verschillende seizoengebonden, circadiane en ultradiane biologische ritmes. Het wijdverspreide voorkomen biedt de mogelijkheid om de toegepaste waarde van deze biologische ritmes en hun genetische basis te onderzoeken. *Nasonia* ondergaan diapauze als larven na maternale en fotoperiodieke inductie: als volwassen vrouwtjes kritische korte daglicht condities ervaren, produceren ze larven in diapauze die pas verder ontwikkelen en uit de gastheer tevoorschijn komen als de condities dat toelaten. Noord-Europese wespen induceren diapauze in condities waar de daglicht periodes nog relatief lang zijn, terwijl Zuid-Europese wespen veel kortere daglicht periodes nodig hebben. De daglicht periode die diapauze inductie in gang zet, noemen we de *critical photoperiod* (CPP). Kortom, bij hogere latitude, waar het eerder in het jaar winter wordt, is de CPP langer dan bij lagere latitude.

Geografische variatie in seizoenen correleert met de aanwezigheid van verschillende *period* (*per*) allelen. Aangezien *per* een klokgen is dat circadiane activiteit reguleert, is het aannemelijk dat de circadiane klok ook betrokken is bij deze andere timing-

gerelateerde reacties. De circadiane klok wordt gekarakteriseerd door een transcriptie en translatie feedback cyclus, bestaande uit verschillende transcriptie factoren. Positieve elementen, zoals *clock* (*clk*) and *cycle* (*cyc*) verhogen de transcriptie van andere genen, zoals *period* (*per*) en *cryptochrome-2* (*cry-2*), die op hun beurt fungeren als negatieve elementen die hun eigen transcriptie inhiberen. Deze transcriptie-translatie feedback cycli worden gereguleerd door licht en hebben een periode van 24 uur. Om de hypothese te testen dat de circadiane klok betrokken is bij de timing van seizoensgebonden reacties, beschrijf ik eerst de natuurlijke variatie in circadiane ritmiek en klokgen expressie in *N. vitripennis* populaties uit het zuiden (Corsica, Frankrijk) en het noorden (Oulu, Finland). *Nasonia vitripennis* is een dag-actief insect met een unimodaal activiteitspatroon. Het laat ritmische activiteit zien in constant donker (DD) en constant licht (LL), wat gebruikt kan worden om belangrijke eigenschappen van de circadiane klok te onthullen.

In mijn onderzoek heb ik verschillende eigenschappen van het circadiane activiteitspatroon ontdekt die verschillen tussen de zuidelijke en noordelijke wesp (hoofdstuk 2). Noordelijke wespen zijn voornamelijk actief aan het einde van de dag met een verlengde avondpiek bij een korte fotoperiode, terwijl de zuidelijke wespen ochtendactiviteit laten zien met een verhoogde activiteit voor de lichtperiode begint bij een korte fotoperiode. Deze verschuiving van activiteitspatronen tussen noordelijke en zuidelijke wespen kan wijzen op lokale adaptatie. In het zuiden worden de temperaturen hoog tegen het einde van de middag; daarom kan een verschuiving van de activiteit naar de koelste periode van de dag (de morgen) een strategie zijn van insecten die in deze warme omgevingen leven. Daartegenover moeten soorten die op hoge latitude leven om kunnen gaan met lage temperaturen en langere fotoperiodes. Daarmee in overeenstemming laten de noordelijke lijnen verlaagde ochtendactiviteit zien met een activiteit piek in het tweede deel van de dag wanneer de temperaturen hoger zijn. Deze verschillen in timing van activiteit tussen zuidelijke en noordelijke wespen is waarschijnlijk een reflectie van het tempo van hun circadiane klok. Onder constante condities is het tempo van de zuidelijke klok hoger dan de noordelijke. Langzame klokken zouden seizoensnauwkeurigheid verhogen, vooral bij hogere latitude, omdat ze efficiënter zonsopgang interpreteren en dus veranderingen in fotoperiode. Daarom kan de natuurlijke variatie in circadiane klok eigenschappen een resultaat zijn van verschillen in selectiedruk en lokale adaptatie.

Om vast te stellen of de geobserveerde geografische variatie in seizoensgebonden en circadiane reacties in *N. vitripennis* verklaard wordt door verschillen in genexpressie, heb ik expressiepatronen van kandidaat klokgenen van wespen met verschillende geografische oorsprong onder verschillende fotoperioden onderzocht (hoofdstuk 3). De circadiane expressiepatronen afhankelijk van fotoperiode en latitude zijn geanalyseerd voor de klokgenen *per*, *cry-2*, *clk* en *cyc*. De expressieniveaus waren hoger voor noordelijke dan zuidelijk wespen. Daarnaast heb ik verschillen gevonden in de amplitude en de fase van het expressieprofiel. Fotoperiode beïnvloedde het expressieniveau en de fase verschillend in de

wespen van twee verschillende locaties, overeenkomstig met de eerder beschreven activiteitspatronen. Deze resultaten suggereren dat *per* betrokken is bij het vaststellen van het tempo en de fase van de klok doormiddel van genexpressie. De expressie van de vier klokgenen was sterk beïnvloedt door de fotoperiode in noordelijke wespen, terwijl in de zuidelijke wespen alleen geringe effecten zichtbaar waren. Dit suggereert dat verschillen in regulatie via transcriptie tussen de lijnen bij verschillende fotoperiodes een rol speelt in seizoensgebonden adaptatie. Alles bij elkaar, heb ik een zwakker expressie profiel van de klokgenen beschreven voor de noordelijke wespen wat de aanwezigheid van een plastischere (flexibelere) klok suggereert. Dit zou de noordelijke wespen in staat kunnen stellen zich aan te passen aan een variabelere omgeving omdat zwakkere circadiane klokken makkelijker zouden synchroniseren met veranderende fotoperioden.

In hoofdstuk 4 is de rol van *per* in het timing mechanisme van *N. vitripennis* vastgesteld door middel van RNA interference (RNAi). Het inhiberen van de *per* expressie verhoogt het tempo van de klok in zowel de zuidelijke als de noordelijke lijnen in constant donker (DD) en vervroegt het activiteitspatroon in de noordelijke wespen. Deze resultaten tonen een functionele rol aan voor *per* in het kernmechanisme van de klok en bij het bepalen van het tempo en de fase in *N. vitripennis*. Daarnaast, vertraagt de klok in noordelijke RNAi-behandelde wespen onder constant licht (LL), terwijl hij versnelt in zuidelijke wespen wat op een verschil duidt van *per* (en licht) in noord en zuid met betrekking tot de regulering van DD en LL condities. Dit verschil kan een reflectie zijn van de aanwezigheid van verschillende oscillatoren in noordelijke en zuidelijke wespen waarvan de fase bepaald wordt door zonsopgang in zuid en zonsondergang in noord. De twee oscillatoren kunnen verschillend op licht reageren: de ene wordt versneld en de andere wordt vertraagd door constant licht. Deze resultaten suggereren dat zuidelijke en noordelijke wespen niet alleen verschillen in het tempo van hun klok, maar ook in de fase van hun circadiane oscillator.

Het manipuleren van *per* expressie via RNAi had ook een effect op de diapauze reactie van *Nasonia*. Ondanks dat alle wespen na inhibitie van *per* nog in staat waren om diapauze in hun nakomelingen te induceren, was de timing van de fotoperiodieke reactie vertraagd in zowel de zuidelijke als de noordelijke lijnen. Dit duidt erop dat *per* inhibitie geen effect heeft op de fysiologie van diapauze zelf, maar de start ervan, oftewel het timing mechanisme wat verantwoordelijk is voor het detecteren van fotoperiodieke veranderingen.

Een ander type ritme dat in *N. vitripennis* gevonden kan worden is het ultradiane ritme, ritme met een periode korter dan 20 uur. In *Nasonia* en andere insecten zijn zulke ritmes onderdeel van de mannelijke parings-initiatie. In veel diersoorten bestaat de parings-initiatie uit een repertoire van specifieke signalen van het mannetje. Mannelijke signalen zijn een belangrijk onderdeel van paring in veel insecten en spelen een rol in soortherkenning en seksuele selectie binnen soorten. *Nasiona* mannetjes initiëren paren door een serie van sterke hoofdknikken, zo genoemde “head-nods”, uit te voeren richting

het vrouwtje. Deze head-nods gaan gepaard met vleugel vibraties die onderbroken worden door pauzes, samen vormen ze een serie van cycli die enkele seconden duurt. De ritmische head-nods gedurende de parings-initiatie zijn belangrijk voor het induceren van vrouwelijke receptiviteit doordat het voor ritmische vrijlating van feromonen zorgt. Van de genetische mechanismen die ten grondslag liggen aan deze ultradiane ritmiek is nog weinig bekend. In hoofdstuk 5 beschrijf ik het effect van *per* RNAi op het ultradiane ritme van de mannelijke parings-initiatie. Belangrijke parings-eigenschappen zijn beïnvloedt door *per* inhibitie die mogelijk invloed hebben op het tempo van feromoon afgifte. Inhibitie van *per* verandert ook het circadiane ritme van behandelde mannetjes. Dit suggereert dat *per* RNAi in parallel circadiane en parings ritmes beïnvloedt in *Nasonia* wat wijst op een extra rol voor *per* in het ultradiane ritme van *N. virtripennis*.

Als onderdeel van mijn onderzoek heb ik een nieuwe tool geïmplementeerd in *Nasionia*, namelijk CRISPR (Regularly Interspaced Short Palindromic Repeats)/CAS9 gen editing. Deze techniek maakt het in de toekomst mogelijk om gen knock-out en knock-in te induceren voor elk gen. Concreet kan dit helpen bij het genereren van stabiele lijnen met een of meerdere mutaties in klok genen.

In het laatste hoofdstuk van mijn proefschrift (hoofdstuk 6) bespreek ik, gebaseerd op mijn resultaten, twee alternatieve modellen om te verklaren hoe *period* andere tijdgerelateerde eigenschappen, zoals diapauze zou kunnen reguleren. *Per* zou een pleiotropische rol kunnen hebben en op die manier diapauze, los van zijn functie in de circadiane klok, beïnvloeden. Als alternatief kan de circadiane klok zelf een functionele module zijn die aan de fotoperiodieke reacties onderligt. In elk geval is *per* noodzakelijk voor het vaststellen van het tempo en de fase van de circadiane klok en het doorgeven van fotoperiodieke informatie ten behoeve van diapauze. In beide scenarios, na het verwerken van fotoperiodieke informatie in het tijdmeetsysteem (via pleiotropie of de circadiane klok), accumuleren de wespen een “diapauze factor”. De verschillende *per* expressie kan de lichtafhankelijke accumulatie van deze factor beïnvloeden. Bij korte dagen accumuleren zowel de zuidelijke als de noordelijke lijnen de substantie, maar met verschillende snelheid. Bij lange dagen is de accumulatie van de factor langzamer waardoor de diapauze reactie vertraagd. Er kunnen verschillende niveaus van regulatie en interactie aanwezigheid zijn tussen het waarnemen van de licht input en het output signaal welke de uiteindelijke reactie op verschillende manieren kunnen beïnvloeden. Dit maakt het ontcijferen van het moleculaire mechanisme dat aan dagelijkse en seizoensgebonden adaptatie onderligt complex.

Concluderend, draagt dit werk bij aan het begrip van de genetica die ten grondslag ligt aan biologische timing van de parasiterende wesp *Nasionia vitripennis* in het specifiek en insecten in het algemeen. Het voegt nieuwe kennis toe aan hoe de circadiane klok betrokken kan zijn bij verschillende tijd gerelateerde eigenschappen zoals seizoensgebonden, dagelijkse en ultradiane ritmes. Het ondersteunt de hypothese dat er een

evolutionaire link is tussen de circadiane klok en fotoperiodieke seizoensgebonden reactie. Vervolgonderzoek kan nu de aanwezigheid van een of meerdere oscillatoren identificeren en focussen op de neuronale architectuur van de circadiane klok in *N. vitripennis*. Daarnaast kan onderzoek naar de post-transcriptionale en post-translationele regulatie van *per* en andere klokgenen helpen om te begrijpen hoe de geografische variatie in ritmes tot stand komt. Vervolgexperimenten naar de genetische architectuur van ultradiane ritmiek, zoals de mannelijke paringsinitiatie, zouden kunnen bijdragen aan ons begrip van hoe zulke snelle ritmes evolueren en fungeren in soortherkenning en seksuele selectie. De resultaten van dit proefschrift dragen bij aan het ontwerpen van zulke vervolgexperimenten.

Riassunto

Da quando la vita è comparsa sulla Terra, è stata sottoposta a cicli giornalieri di luce e buio e a cicli stagionali di cambiamento climatico, causati dalla rotazione della terra attorno al proprio asse e attorno al sole. Di conseguenza, tutti gli organismi viventi hanno dovuto adattare la propria fisiologia, il proprio comportamento e metabolismo per far fronte a questa periodicità. Questo è particolarmente vero per gli animali che vivono in paesi nordici (verso alte latitudini) dove, a causa dell'inclinazione assiale della Terra, i cambiamenti quotidiani e stagionali includono fluttuazioni estreme di luce e temperatura. L'evoluzione di orologi endogeni (come l'orologio circadiano) ha permesso quindi agli organismi, dai batteri alle piante e agli animali, di esibire ritmi biologici in grado di determinare la tempistica di eventi quotidiani e annuali. Gli studi sul funzionamento e sui meccanismi molecolari alla base di questi orologi biologici rivelano un'elevata complessità e variabilità, sia a livello genetico che comportamentale. L'importanza degli studi sui ritmi biologici ha portato quest'anno all'assegnazione del premio Nobel per la medicina, proprio grazie alla scoperta del meccanismo circadiano. È ormai noto infatti che oltre il 50% di tutti i geni di un organismo sono sotto il controllo dell'orologio circadiano.

Gli insetti sono particolarmente adatti per gli studi sugli orologi biologici in quanto sono geograficamente distribuiti e adattati ad un'ampia gamma di condizioni climatiche. L'ora del giorno e la stagione dell'anno sono importanti fattori ambientali che dettano quotidianità e stagionalità dell'attività degli insetti. La maggior parte delle risposte stagionali negli insetti, come l'entrata e la fine della dormienza svernante (diapausa), si verificano in periodi diversi dell'anno e allo stesso modo, le attività quotidiane (come l'emergenza dall'involucro della pupa, l'alimentazione, l'accoppiamento, la deposizione delle uova, ecc.) si verificano in momenti specifici del giorno. Oltre al ciclo circadiano, un altro tipo di ritmi, definiti ritmi ultradiani (cioè i ritmi con periodo inferiore a 20 ore), sono stati osservati in diverse funzioni fisiologiche e comportamentali durante stadi specifici della riproduzione negli insetti. Questa ricerca di Dottorato contribuisce alla comprensione dei meccanismi genetici alla base della percezione del tempo biologico in una specie di vespa parassitoide *Nasonia vitripennis*. Particolare attenzione viene data al ruolo dei geni orologio nella risposta stagionale.

Nasonia vitripennis è una piccola vespa solitaria che depone le proprie uova nelle pupe di diverse specie di mosche, dette "ospiti", dove le uova si sviluppano e da cui emergono come vespe adulte alla fine dello sviluppo. *Nasonia* rappresenta un eccellente modello per gli studi di cronobiologia in quanto esibisce diversi ritmi biologici. La sua diffusa distribuzione in varie aree del pianeta offre l'opportunità di studiare il valore adattativo di questi ritmi biologici e delle loro basi genetiche. Le femmine adulte di *Nasonia* inducono diapausa nelle uova in corrispondenza della riduzione del fotoperiodo dovuto al cambiamento di stagione. Questo fa sì che le uova depositate nelle larve ospiti si blocchino allo stadio di larva in diapausa, per riprendere lo sviluppo solo dopo l'inverno, quando le condizioni sono più favorevoli. È interessante notare che le vespe del nord

Europa inducono la diapausa molto prima, quando il giorno è ancora lungo rispetto alle vespe dell'Europa meridionale. Questo perché verso alte latitudini l'inverno arriva prima durante l'anno rispetto alle latitudini più basse.

Nasonia vitripennis è un insetto diurno con attività unimodale e mostra attività ritmica in condizioni di buio costante (DD) e luce costante (LL), rivelando importanti proprietà endogene dell'orologio circadiano. L'orologio circadiano è il meccanismo che genera la ritmicità quotidiana ed è caratterizzato da circuiti di feedback trascrizionali e traslazionali che coinvolgono diversi fattori di trascrizione. Elementi positivi, come *clock* (*clk*) e *cycle* (*cyc*), attivano la trascrizione di altri geni, come *period* (*per*) e *cryptochrome-2* (*cry-2*), che a loro volta agiscono come elementi negativi che inibiscono la loro stessa trascrizione. I ritmi ultradiani di *N. vitripennis* invece fanno parte del comportamento di corteggiamento maschile. Il corteggiamento in molte specie animali consiste in un repertorio di segnali specifici forniti dal maschio durante l'evento di accoppiamento. I segnali maschili hanno un ruolo importante nel corteggiamento in molte specie di insetti poiché permettono il riconoscimento della specie e contribuiscono alla selezione sessuale all'interno delle specie stessa. I maschi di *Nasonia* corteggiano le femmine eseguendo serie di movimenti con la testa, i cosiddetti "*head-nods*" che sono accompagnati da vibrazioni alari, e che sono interrotti da pause e che insieme formano una serie di cicli che durano pochi secondi. Gli *head-nods*durante il corteggiamento maschile sono ritmati e importanti per indurre la ricettività femminile consentendo il rilascio ritmico dei feromoni. Tuttavia, si sa molto poco sul meccanismo genetico che sta alla base di questa ritmicità ultradiana.

La variazione geografica delle risposte stagionali, giornaliere e ultradiane di *N. vitripennis* è correlata alla presenza di diversi alleli del gene *per*. Poiché *per* è un gene orologio che regola l'attività circadiana, è stato ipotizzato che l'orologio circadiano sia anche coinvolto nella regolazione di altre risposte legate al tempo. Per testare ulteriormente questa ipotesi, nel mio progetto ho inizialmente descritto la variazione naturale nel comportamento circadiano e nell'espressione genica dei geni orologio in due popolazioni di vespe originarie rispettivamente del sud Europa (Corsica, Francia) e del nord Europa (Oulu, Finlandia). Ho scoperto diverse proprietà caratteristiche dell'attività circadiana che differiscono tra le vespe del sud e del nord (capitolo 2). Le vespe settentrionali sono principalmente attive alla fine della giornata, con un picco di attività serale prolungato, mentre quelle meridionali mostravano un'attività mattutina, con un aumento dell'attività prima dell'alba. Questo diversità nel picco di attività tra vespe del nord e del sud potrebbe riflettere un adattamento locale in quanto al sud, le temperature sono generalmente alte nel tardo pomeriggio, quindi spostare l'attività nella parte più fresca della giornata (in particolare la mattina) potrebbe essere una strategia degli insetti che vivono in un ambiente caldo. Al contrario, le specie che vivono al nord, devono far fronte a temperature più basse al mattino e fotoperiodi più lunghi. In accordo con ciò, le vespe del nord hanno una ridotta attività mattutina e il loro picco di attività è spostato nella seconda parte della giornata

quando le temperature sono più alte. Inoltre questa diversa attività tra vespe meridionali e settentrionali probabilmente riflette la velocità del loro orologio circadiano. In condizioni costanti di buio, l'orologio circadiano nelle vespe meridionale è più veloce di quello delle vespe settentrionali. È interessante notare che gli orologi circadiani "lenti" migliorano l'acuità stagionale, in particolare ad alte latitudini perché sono più efficienti nel tracciare e interpretare l'alba e, quindi, i cambiamenti fotoperiodici. Pertanto, la variazione naturale nei parametri dell'orologio circadiano è probabilmente il risultato della diversa pressione selettiva e adattamento locale.

Al fine di valutare se la variabilità di espressione dei geni orologio possa spiegare la variazione geografica nelle risposte stagionali e circadiane in *N. vitripennis*, nel Capitolo 3 ho analizzato la variazione di espressione dei geni orologio nelle vespe del nord e del sud e in diversi fotoperiodi . I geni orologio *per*, *cry-2*, *clk* e *cyc*, sono stati analizzati al fine di valutare il loro possibile ruolo nell'induzione fotoperiodica della diapausa in *N. vitripennis*. Le mie ricerche hanno evidenziato livelli di espressione più alti nelle vespe del sud rispetto a quelle del nord e una diversa ampiezza e fase nel profilo trascrizionale dei geni testati. Inoltre, il fotoperiodo influenza diversamente i livelli e la fase di espressione delle vespe delle due località, in linea con i diversi ritmi di attività sopra descritti. Questi risultati hanno suggerito che il diverso assetto allelico di *per* tra le vespe meridionali e settentrionali determina delle differenze nell'espressione di tale gene, andando ad influenzare il ritmo e la frequenza dell'orologio circadiano nelle due popolazioni. Inoltre è interessante notare che l'espressione dei quattro geni orologio è fortemente influenzata dal fotoperiodo nelle vespe settentrionali, mentre nelle vespe meridionali si osservavano solo lievi effetti, indicando che le differenze nella regolazione trascrizionale tra le linee e sotto diversi fotoperiodi possono avere un ruolo nella regolazione dell'adattamento stagionale. Nel complesso, ho descritto un profilo di espressione più debole dei geni dell'orologio nelle vespe settentrionali, indice della presenza di un orologio più flessibile (nel nord). Ciò potrebbe facilitare l'adattamento delle vespe settentrionali a un ambiente più variabile, poiché si ritiene che i orologi circadiani più "deboli" siano in grado di sincronizzarsi più facilmente con il cambiamento fotoperiodico.

Per comprendere l'evoluzione adattativa dei ritmi biologici, è essenziale stabilire se e come l'orologio circadiano regoli questi processi ciclici. Il ruolo del gene orologio *per* è stato analizzato tramite la tecnica RNA interference (RNAi) nel Capitolo 4 e 5. In seguito alla riduzione dell'espressione genica di *per*, entrambe le linee analizzate hanno evidenziato un'accelerazione dell'orologio circadiano in condizioni di costante oscurità (DD), mentre solo nelle veste settentrionali si è potuta osservare un'anticipazione della fase di attività. Questi risultati per la prima volta hanno confermato un ruolo funzionale del gene *per* nel meccanismo centrale dell'orologio circadiano di *N. vitripennis*, attestandone il coinvolgimento nella determinazione del ritmo e della fase dell'orologio circadiano. Inoltre, in condizioni di luce costante (LL), le vespe del nord trattate hanno rallentato il loro

orologio, mentre quelle del sud lo hanno accelerato, indicando un diverso ruolo di *per* (e della luce) tra il sud e il nord nella regolazione dei ritmi DD e LL . Questa differenza potrebbe riflettere la presenza di un oscillatore circadiano diverso nelle vespe meridionali e settentrionali, la cui fase è stabilita all'alba nel sud e al tramonto nel nord. I due oscillatori mostrerebbero diverse risposte alla luce: uno viene accelerato e l'altra decelerato dalla luce costante. Questi risultati indicano che le vespe del sud e del nord non solo differiscono nel ritmo del loro orologio, ma anche nella fase del loro oscillatore circadiano.

Gli effetti della diminuzione di espressione di *per* mediata dalla RNAi ha inoltre influenzato la risposta fotoperiodica e l'induzione della diapausa delle vespe. Sebbene tutte le vespe fossero in grado di indurre la diapausa nella loro prole dopo il *knock-down* di *per*, i tempi della risposta fotoperiodica sono aumentati in entrambe le linee. Questo indica che il *knock-down* di *per* non ha influenzato la fisiologia della risposta stessa, ma il suo esordio e ,quindi, il meccanismo di rilevazione dei cambiamenti fotoperiodici. Tuttavia, il coinvolgimento dell'orologio circadiano nei ritmi stagionali è ancora controverso e il ruolo del gene orologio *per* nella regolazione delle risposte stagionali richiede ulteriori indagini.

Nel Capitolo 5, ho descritto un ulteriore effetto del *knock-down* di *per* sul ritmo ultradiano durante il corteggiamento maschile delle vespe. In particolare, il *knock-down* di *per* ha aumentato la durata dei cicli degli *head-nods* e il numero di cenni della testa per ciclo, modificando così il ritmo del rilascio di feromoni che si verifica ad ogni primo cenno della testa di ogni ciclo. Il *knock-down* di *per* ha anche alterato il ritmo circadiano di questi maschi trattati, alterando in parallelo, i ritmi circadiani e ultradiani in *Nasonia*. Variazioni della durata dei cicli di *head-nods* e del numero di cenni di testa, come parte del comportamento di corteggiamento maschile, può servire da segnale per le femmine nella scelta del compagno. Tuttavia, i meccanismi di scelta del partner da parte delle femmine è ancora oggetto di studio e, in particolare, ulteriori esperimenti sono richiesti per determinare se le femmine siano in grado di misurare i tempi di ogni cicli di *head-nods* durante il corteggiamento e rispondere, di conseguenza, con il rilascio di feromoni.

Una parte dei miei studi è stata dedicata alla messa a punto dell'editing del genoma tramite CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)in *Nasonia*. Questa tecnica consentirà di indurre mutazioni specifiche a livello genico, ovvero l'inserimento di qualsiasi gene di interesse nel genoma di Nasonia, consentendo, in futuro, la creazione di linee stabili che trasportano una o più mutazioni nei geni orologio.

Nel Capitolo finale della mia tesi (capitolo 6) ho presentato due ipotesi alternative su come il gene *per* potrebbe regolare altri ritmi oltre che a quello circadiano, come la stagionalità e l'induzione di diapausa. *Per* può avere un ruolo pleiotropico e regolare la diapausa indipendentemente dalla sua funzione nell' orologio circadiano, in alternativa è l'orologio circadiano, in quanto modulo funzionale, a sostenere il fotoperiodismo. In ogni caso, *per* è necessario per impostare il ritmo e la fase dell'orologio circadiano e trasdurre le informazioni fotoperiodiche per l'induzione della diapausa. In entrambi gli scenari, infatti,

dopo l'elaborazione delle informazioni fotoperiodiche nel "*timer*" (tramite la pleiotropia o attraverso l'orologio circadiano), le vespe accumulano un "fattore di diapausa" nel "contatore" e l'espressione differenziale di *per* potrebbe influire sull'accumulo dipendente dalla luce di questo fattore. Con la diminuzione del fotoperiodo all'inizio della stagione invernale, sia le linee meridionali che quelle settentrionali accumulano efficientemente la sostanza, ma con diversa velocità. Nei giorni più lunghi, alla fine della stagione invernale, l'accumulo del fattore è molto più lento e la risposta alla diapausa viene ritardata. Certamente diversi livelli di regolazione e interazione tra la percezione di luce e la risposta fotoperiodica possono influenzare la risposta finale in modi diversi, rendendo la decifrazione del meccanismo molecolare alla base dell'adattamento giornaliero e stagionale molto complessa.

In conclusione, questo lavoro contribuisce alla comprensione della genetica alla base dei ritmi biologici nella vespa parassitoide *Nasonia vitripennis*. Questo lavoro, aggiunge nuove conoscenze su come l'orologio circadiano può essere coinvolto in diverse risposte legate al tempo, come i ritmi stagionali, giornalieri e ultradiani e supporta l'ipotesi di un legame evolutivo tra l'orologio circadiano e la risposta fotoperiodica stagionale. Future ricerche dovrebbero focalizzarsi ad identificare la presenza di uno o più oscillatori endogeni e concentrarsi sull'architettura neuronale dell'orologio circadiano in *N. vitripennis*. Inoltre, l'indagine sulla regolazione post-trascrizionale e post-traduzionale di *per* e degli altri geni orologio potrebbe aiutare a capire meglio come avviene la variazione geografica dei ritmi descritti. Altri esperimenti per studiare l'architettura genetica della ritmicità ultradiana, nelle prestazioni del corteggiamento maschile, aiuterebbero anche a comprendere meglio l'evoluzione di tali ritmi "veloci" e il loro ruolo nelle interazioni intra- e inter specie.

Personal information

CV

Elena Dalla Benetta was born in Arzignano, Italy, on the 3th of July 1987. In 2011 she received her bachelor degree in Molecular Biology from the University of Padova. During this bachelor program her interest for research became strong and she immediately continued with a master in the same field. She spent the last 6 month of her Msc degree at the University of Wurzburg, Germany. There, she was introduced to the field of circadian biology in the fruit fly (*Drosophila melanogaster*). The project described the role of PDF and CRY within the l-LNvs of *Drosophila melanogaster* in adaptation to long day conditions. In 2013 she received her master degree in Molecular Biology from the University of Padova, Italy. After her graduation, she immediately started her PhD as an Early Stage Researcher at the University of Groningen, The Netherlands, within the Marie Curie initial Training Network "INsecTIME". The results of her project are presented in this thesis. During this time she has presented her work at several national and international conferences. Additionally, part of her time was devoted to assisting bachelor and master courses as well as supervising students during research projects. She will continue her career with a postdoc at the University of Claremont, Los Angeles, USA, in collaboration with the University of California San Diego (UCSD), USA, studying the mechanism of genome elimination by selfish chromosomes in *Nasonia vitripennis*.

List of Publications

Peer-reviewed

Menegazzi, P[†]., **Dalla Benetta, E[†]**., Beauchamp, M., Schlichting, M., Steffan-Dewenter, I., & Helfrich-Förster, C. (2017). Adaptation of circadian neuronal network to photoperiod in high-latitude European *Drosophilids*. *Current Biology*, *27*(6), 833–839.

Schlichting, M., Menegazzi, P., Lelito, K. R., Yao, Z., Buhl, E., **Dalla Benetta, E.,** … Shafer, O. T. (2016). A neural network underlying circadian entrainment and photoperiodic adjustment of sleep and activity in *Drosophila*. *The Journal of Neuroscience*, *36*(35), 9084–96.

In Preparation

Dalla Benetta, E., Beukeboom, L.W., & van de Zande, L. Clock gene expression profiles depend on latitude and affect photoperiodic diapause in *Nasonia vitripennis*.

Paolucci, S[†]., **Dalla Benetta**, **E[†]**., Salis, L., Doležel, D., van de Zande, L., & Beukeboom, L.W. Geographical variation in circadian clock properties of *Nasonia vitripennis*.

Dalla Benetta, E., van de Zande, L., & Beukeboom, L.W. Courtship rhythm in *Nasonia vitripennis* is affected by the clock gene *period*.

† Co-first authors

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