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

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Abstract

The clock gene *period* (*per*) is a regulator of circadian rhythms but may also play a role in the regulation of ultradian rhythms, such as insect courtship. Males of the parasitoid wasp *Nasonia vitripennis* court females by performing series of head movements ('head-nods') and wing vibrations within repeated cycles. The pattern of cycle duration and head-nod number is species-specific and has a genetic basis. In this study, the possible involvement of *per* in regulating *Nasonia* courtship rhythms was investigated in a southern and northern European strain that differ in number and timing of courtship components. Knockdown of *per* via RNA interference (RNAi) resulted in a shortening of the circadian free running period (tau) in constant darkness (DD), and increased both the cycle duration and the number of head-nods per cycle in both strains. These results point at a role of *per* in the regulation of ultradian rhythms and male courtship behaviour of *N. vitripennis* and may contribute to resolving the controversy about the role of *per* in insect courtship behaviour.

Keywords

parasitoid wasp, circadian clock, *period* gene, courtship, *Nasonia vitripennis*, RNA interference, gene knock down, rhythmicity, ultradian rhythm.

1. 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

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be acoustic, visual, tactile and/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 a barrier for interspecies mating (Thornhill, 1979). They can also play an important role in reproductive isolation (Thornhill & Alcock, 1983; Alt et al., 1998; Greenspan & Ferveur, 2000; Talyn & Dowse, 2004).

Various behaviours, including locomotor activity and courtship, are influenced by an endogenous clock in insects and exhibit rhythmicity. Since Konopka & Benzer (1971) described the first circadian mutant of the clock gene *period* (*per*) in *Drosophila melanogaster*, many circadian clock mutants have been reported from *Drosophila* species, nematodes, mice, and other organisms (Hall, 1995; Dunlap et al., 1999; 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). A regulatory role of *per* was already demonstrated in the species-specific courtship 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 both circadian cycles and rhythmicity of ultradian courtship song components. In addition, another clock gene, *nonA*, has been implicated in shaping species-specific courtship song pulses in *Drosophila virilis* (Campesan et al., 2001). However, the existence of courtship songs rhythms in *Drosophila* is not always readily detected, in particular for the Inter-pulse-interval of courtship songs. Therefore, the existence of *Drosophila* courtship song rhythms, and hence the involvement of *per*, has been challenged (Ewing & Bennet-Clark, 1968; Kyriacou & Hall, 1980, 1982; Crossley, 1988; Stern, 2014). This challenge has been countered on the basis of methodological issues (Kyriacou & Hall, 1988; Kyriacou et al., 1990b; Alt et al., 1998), but the debate has not been resolved (Kyriacou et al., 2017; Stern et al., 2017). The possible involvement of clock genes like *per*, in rhythmic courtship components of other insect species, may help in resolving this issue. Besides a role of clock genes in insect ultradian rhythmicity, Loudon et al. (1994) reported an ultradian rhythm in the Syrian hamster affected by clock gene mutations, indicating that the phenomenon extends beyond insects. In addition, mutation of *per* have been reported to affect infradian cycles of developmental time in *Drosophila* (Kyriacou et al.,

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1990a; Srivastava et al., 2018), which is another indication that clock genes are not involved in circadian periodicity only.

Ethological studies have been conducted for more than half a century in *Nasonia* (Barrass, 1960a, b; Beukeboom & van den Assem, 2001) and its male courtship behaviour is well characterised. It consists of a repetitive pattern of specific movements with the head, termed 'head-nods', with a duration of seconds (van den Assem et al., 1980; van den Assem & Werren, 1994), which constitutes an ultradian rhythm. After mounting, *Nasonia* males start courtship by performing a series of '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, Simbolotti & Jachmann, 1980; van den Assem & Werren, 1994).

In the *Nasonia* circadian clock, *per* and *cryptochrome-2* (*cry-2*) 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; Dalla Benetta et al., 2019). Interestingly, the circadian clock of *Nasonia* exhibits different properties along a latitudinal cline in natural populations in Europe, associated with different *per* alleles (Paolucci et al., 2016). Southern wasps have a faster clock (shorter free running period, where free running period refers to the circadian activity periodicity in the absence of external cues; in constant darkness in our case) and are more active in the earlier part of the day compared to northern wasps (Floessner et al., 2019; Paolucci et al., 2019). Interestingly, *per* RNA interference (RNAi) is able to change circadian rhythms by speeding up the circadian clock (Dalla Benetta et al., 2019). In this study we assess the involvement of *per* in regulation of ultradian timing of *Nasonia*. We test whether *per* knockdown affects male courtship behaviour of two geographically distinct strains of the wasp *Nasonia vitripennis* that differ in the phase (time of the day where wasps are active) and period of the circadian clock (Paolucci et al., 2019).

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2. Material and methods

2.1. 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.

2.2. Courtship observations

Nasonia courtship behaviour can easily be observed and quantified following the procedures described by Beukeboom & van den Assem (2001). *Nasonia* have a relatively short life cycle depending on environmental temperatures and exposure to a light source. A female wasp lays eggs in a *Sarcophaga* pupae. The eggs develop into larvae within one or two days and start feeding on the *Sarcophaga* pupae. The larvae will continue to develop over the next eight to nine days, and then pupate. There are three developmental stages of wasp pupae: 'white', 'black and white', and 'black' stages, and each stage is more developed than the previous. 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 10 mm, closed off with a plug of cotton wool and previously mated females were then introduced. We used mated females in order to allow the observation of longer courtship bouts, since mated females typically do not mate again and thus do not become receptive. All males were inexperienced and one-day old adults, since male age and previous experience may have an effect on courtship performance (Beukeboom & van den Assem, 2001). Courtship by males was recorded under a stereo binocular microscope at 10× 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. Courtship was recorded for a total of 40–60 males of RNAi-treated and 40–60 untreated control males from the northern and southern strains. Courtship behaviour was observed and scored at the time of the day corresponding to high activity based on (Paolucci et al., 2019). Importantly, the scoring of the courtship was performed blind; hence, the observers did not know to which treatment group a male wasp belonged.

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2.3. Circadian activity registration

To determine daily activity patterns, 32 individual wasps, per strain and treatment, were placed individually 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 600 lum/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 males were simultaneously entrained to 4 days of LD16:08 and subsequently placed in constant darkness (DD). The entrainment light cycle LD 16:08 was chosen arbitrary and represents the diapause-preventing, rearing condition of *N. vitripennis* in the lab.

2.4. RNA extraction and 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 was extracted only from the wasp's head (where the master clock is located) between ZT 21–24 (Zeitgeber Time, ZT 0 corresponds to the time when the light turned on), which corresponds to the peak of *period* expression (Dalla Benetta et al., 2019). 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 contamination, and about 1 μg of RNA was used to synthesize cDNA with RevertAid H Minus First Strand cDNA Synthesis kit (Thermo Scientific).

2.5. Synthesis and injection of double stranded RNAs (dsRNA)

Knockdown of *per* was induced in yellow male pupae (8 days post-oviposition) following the methods described in Dalla Benetta et al. (2019,

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2020). Briefly, two PCR fragments with a T7 polymerase-binding site were transcribed in both directions using the Megascript RNAi kit (Ambion) following the manufacturer's protocol for double stranded RNA (dsRNA) synthesis using the primers listed in Table A1 in the Appendix at 10.6084/m9.figshare.14339843. Two dsRNAs (dsRNA_A and dsRNA_B, Figure A1 in the Appendix at 10.6084/m9.figshare.14339843) targeting two different regions of the *per* gene were produced. Sense and antisense RNA fragments were synthetized in separate transcription reactions. After 6 h 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.

We followed the protocol outlined by Lynch & Desplan (2006) and Dalla Benetta et al. (2020) such that males pupae were injected in the abdomen with 4 μg/μl of either *per* dsRNA_A (RNAi_A) or *per* dsRNA_B (RNAi_B) (Figure A1 in the Appendix at 10.6084/m9.figshare.14339843) and mixed with red dye (4:1 ratio). Injections were performed with Femtotips II (Eppendorf) 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°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.

2.6. Entrainment and sample collection for testing RNAi efficiency

dsRNA-injected and water-injected control males were kept after emergence under LD 16:08 at 20°C in 60 mm glass tubes, diameter 10 mm, closed off with a plug of cotton wool. Three biological replicates, each containing five wasps (total $n = 15$), were prepared for each treatment and three days post eclosion the wasps were collected at ZT0 (*Zeitgeber time*, where ZT0 represents the light on signal). To preserve the RNA, tubes with wasps were frozen in liquid nitrogen and immediately stored at −80°C.

RNA was extracted from pooled head samples and cDNA conversion was performed following the manufacturer's instructions. The cDNA was diluted 50× before use in Real-Time quantitative PCR (RT-qPCR). The RT-qPCR was performed with SYBR green (Quanta Biosciences) and Rox as

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the internal passive reference. Four 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. 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°C, 35 cycles of 15 s at 95°C, 30 s at 56°C and 30 s at 72°C. Table A2 in the Appendix at 10.6084/m9.figshare.14339843 lists the primers for *period* (*per*), *elongation factor 1*α (*ef1*α) and *adenylate kinase* (*ak*) genes.

Expression levels relative to that of the reference genes *ef1*α and *ak3* were calculated by normalizing the expression data with LinRegPCR (Ramakers et al., 2003; Ruijter et al., 2009) as described by Dalla Benetta et al. (2019). Briefly, raw fluorescence data generated by 7300 System SDS Software (Applied Biosystems) were base-line corrected and the N0 value for PCR efficiencies calculated per amplicon with LinRegPCR 11.0 (Ruijter et al., 2009). Relative expression levels were determined by dividing N_0 values of the gene of interest (*per*) by the average N_0 of the two reference genes (*ef1*α and *ak3*). These two genes were confirmed to have constant expression levels throughout the day (Dalla Benetta et al., 2019) and between treatments (Figure A2 in the Appendix at 10.6084/m9.figshare.14339843). Difference in expression of *per* between control and RNAi treatments, was statistically tested with one way ANOVA and a Tukey's multiple-comparison test in R statistical software version 3.4.1 (R Core Team, 2013).

2.7. Behavioural data analysis

The raw 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 & Helfrich-Förster (2015). We determined when wasps start to be active (onset), have the peak of activity (peak) and terminate their activity (offset) during each 24-h period, and compared this activity profile between RNAi-treated and non-treated 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

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timing of the peaks, the data are smoothed by a moving average of 30 min. Through this process, randomly occurring spikes are reduced and the real maximum of the activity can be determined. The free running period (tau, 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 in *Zeitgeber time* (ZT) and the τ values, were compared between strains and treatments. Statistical analysis, on timing of activity and free running rhythms, was performed with one-way ANOVA and 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 (R Core Team, 2013).

3. Results

3.1. Geographical differences in courtship behaviour

Male courtship behaviour, in southern and northern lines of *Nasonia vitripennis*, followed a general structure consistent with previous reports (Figure 1) (van den Assem & Beukeboom, 2004). The duration of each cycle increased steadily throughout the consecutive cycles, the duration of the first and second cycle was about 9 s and it subsequently increased up to about 11 s in the fourth cycle (Figure 1; Table A3 in the Appendix at 10.6084/m9.figshare.14339843). The highest average head-nods numbers occurred in the first cycle (4.46 $\pm$ SE 0.20 in the southern lines, 5.31 $\pm$ SE 0.36 in the northern lines), followed by a lower number in the second cycle (2.95 $\pm$ SE 0.18 for the southern and 4.23 $\pm$ SE 0.27 for the northern lines), and a gradual increase in the third and fourth cycles (Figure 1; Table A3 in the Appendix at 10.6084/m9.figshare.14339843). The southern and northern lines did not differ in the duration of the cycles ($H_2 = 2.74$; $p = 0.25$ Dunn's multiple-comparison test). In contrast, southern wasps had lower average head-nod numbers than northern ones in all cycles ($H_2 = 34.36$; $p < 0.001$; Dunn's multiple-comparison test). This means that either their nodding pace and/or their pause length was higher within their consecutive cycles (Figure 1; Table A3 in the Appendix at 10.6084/m9.figshare.14339843).

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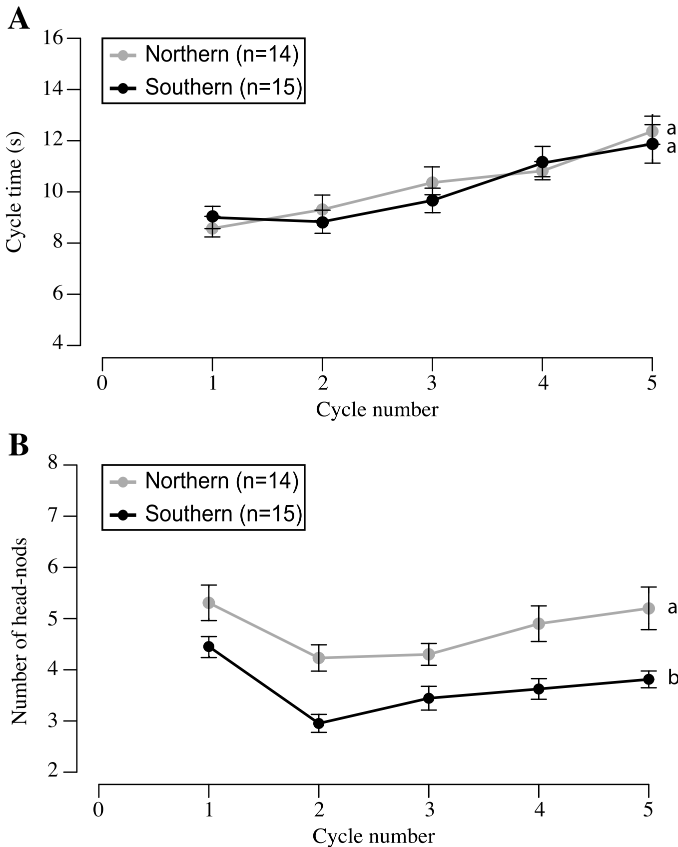

Figure 1. Male courtship behaviour of southern and northern *Nasonia* wasps. (A) Cycle duration and (B) head-nod number per cycle (average ± standard error) of the first five cycles for southern and northern males. Different letters indicate significant differences by Kruskal–Wallis test with Dunn's multiple-comparison test between southern and northern ($p < 0.001$).

3.2. 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 within the 24-h cycle (Dalla Benetta et al., 2019). The relative expression level of *per* in the dsRNA-injected wasps was significantly lower in both southern and northern ($F_{2,6} = 130.4$; $p = 1.14\text{e}{-05}$ and $F_{2,6} = 34.76$; $p = 0.0005$ respectively; one- way ANOVA and Tukey's multiple-comparison

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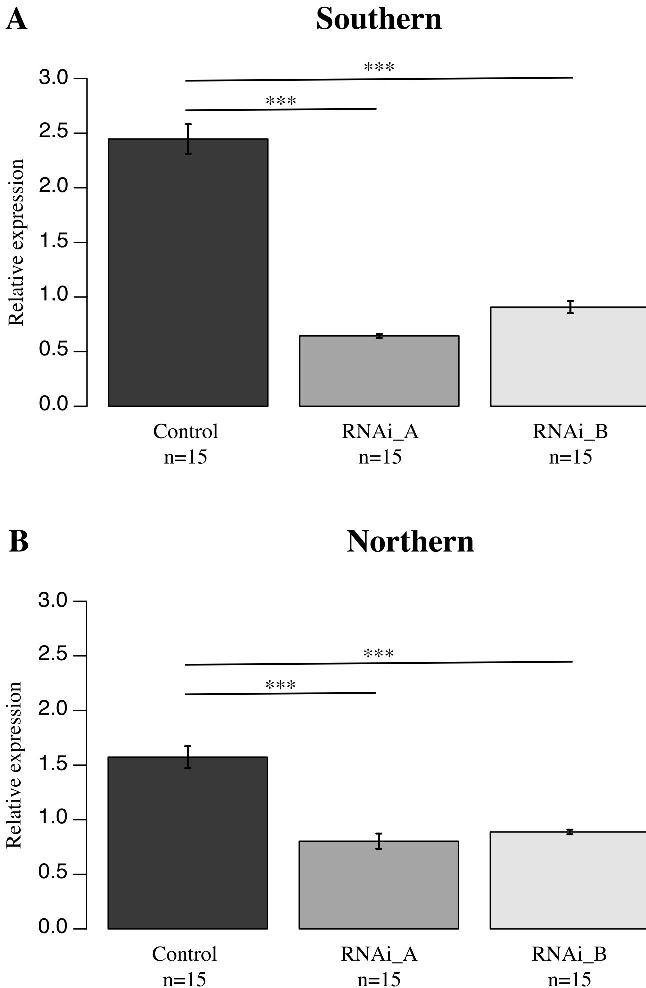

Figure 2. *Period* expression in control and *per* RNAi-treated wasps. *Per* mRNA expression in control and RNAi-injected wasps with dsRNA_A or dsRNA_B in (A) southern and (B) northern wasps. Asterisks represent significant differences between treatments ($^{***}p < 0.001$ by two-way ANOVA).

test) lines compared to controls (Figure 2). The results indicate a 50–60% efficiency of *per* knockdown via RNAi.

To test whether the endogenous properties of the circadian clock were altered by *per* RNAi, 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

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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 ($F_{2,141} = 36.36$; $p = 2.11\text{e}{-13}$ and $F_{2,132} = 80.73$; $p < 2\text{e}{-16}$ respectively; one-way ANOVA and Tukey's multiple-comparison test; Figure 3, Table A4 in the Appendix at 10.6084/m9.figshare.14339843). However, it did not affect the proportion of rhythmic wasps (Figure A3, and Tables A4 and Table A5 in the Appendix at 10.6084/m9.figshare.14339843). These results show that *per* RNAi efficiently manipulated the free running properties of the circadian clock of the wasps.

3.3. 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 (Figure 4A, B) ($H_2 = 29.00$; $p = 5.035\text{e}{-07}$; $H_2 = 8.15$; $p = 0.001$, respectively; Dunn's multiple-comparison test), as well as in head-nod numbers per cycle (Figure 4C, D) ($H_2 = 84.92$; $p < 2.2\text{e}{-16}$; $H_2 = 26.30$; $p = 1.941\text{e}{-06}$, respectively; Dunn's multiple-comparison test). The duration of the first cycle increased by more than 1 s in both lines (from 9.22 ± SE 0.20 s to 10.30 ± SE 0.38 s and 10.04 ± SE 0.20 s, respectively, in the control and the two combined RNAi treatments of northern wasps, and from 9.44 ± SE 0.21 s to 10.46 ± SE 0.37 s and 10.93 ± SE 0.28 s in the control and the two groups of RNAi-treated southern wasps). The duration of the following cycles also increased in all RNAi-treated groups (Figure 4A, B; Table A6 in the Appendix at 10.6084/m9.figshare.14339843). However, the general pattern of an increase in duration throughout consecutive cycles is maintained after *per* RNAi.

RNAi also led to an increase in the number of head-nods per cycle in both lines in all four measured head-nod cycles (Figure 4C, D; Table A6 in the Appendix at 10.6084/m9.figshare.14339843). The pattern of a reduction in the number after the first cycle followed by an increase after cycle 2 is, however, maintained. The number of head-nods in the first cycle are 5.51 ± 0.18 in the northern control wasps, 6.80 ± SE 0.36 and 6.91 ± SE 0.23 in the northern RNAi wasps, 4.88 ± SE 0.15 in the southern control males, 5.90 ± SE 0.32 and 6.08 ± SE 0.30 in the southern RNAi wasps. This is followed by a lower number in the second cycle, 4.19 ± SE 0.12 in the northern control wasps, 4.95 ± SE 0.23 and 4.65 ± SE 0.20 in the northern RNAi wasps, 3.38 ± SE 0.13 in the southern control males, 4.63 ± SE 0.32 and 4.52 ±

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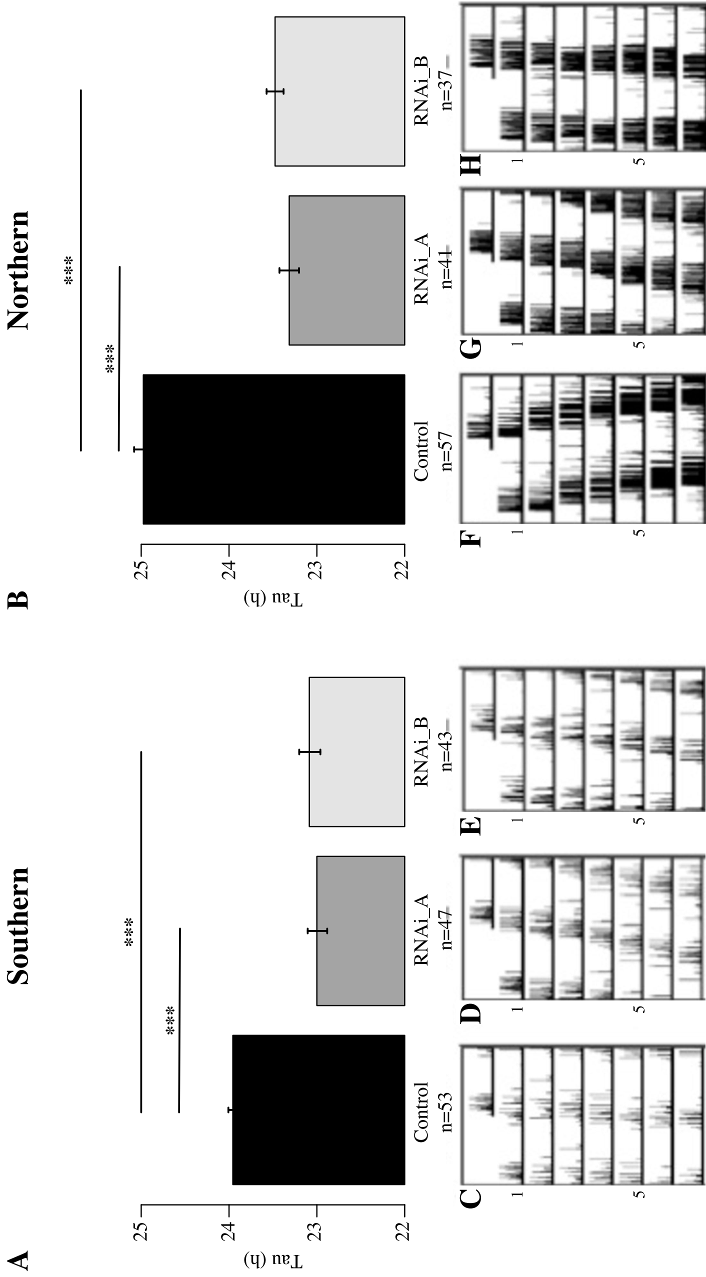

Figure 3. Circadian rhythms under constant darkness (DD) of control and RNAi-treated wasps. Free running rhythms in control and RNAi-injected wasps with dsRNA_A or dsRNA_B in (A) southern and (B) northern wasps. Double plot actograms for circadian rhythms for (C–E) southern and (F–H) northern wasps. Black bars indicate activity. Asterisks indicate significant differences (*** $p < 0.001$ by two-way ANOVA).

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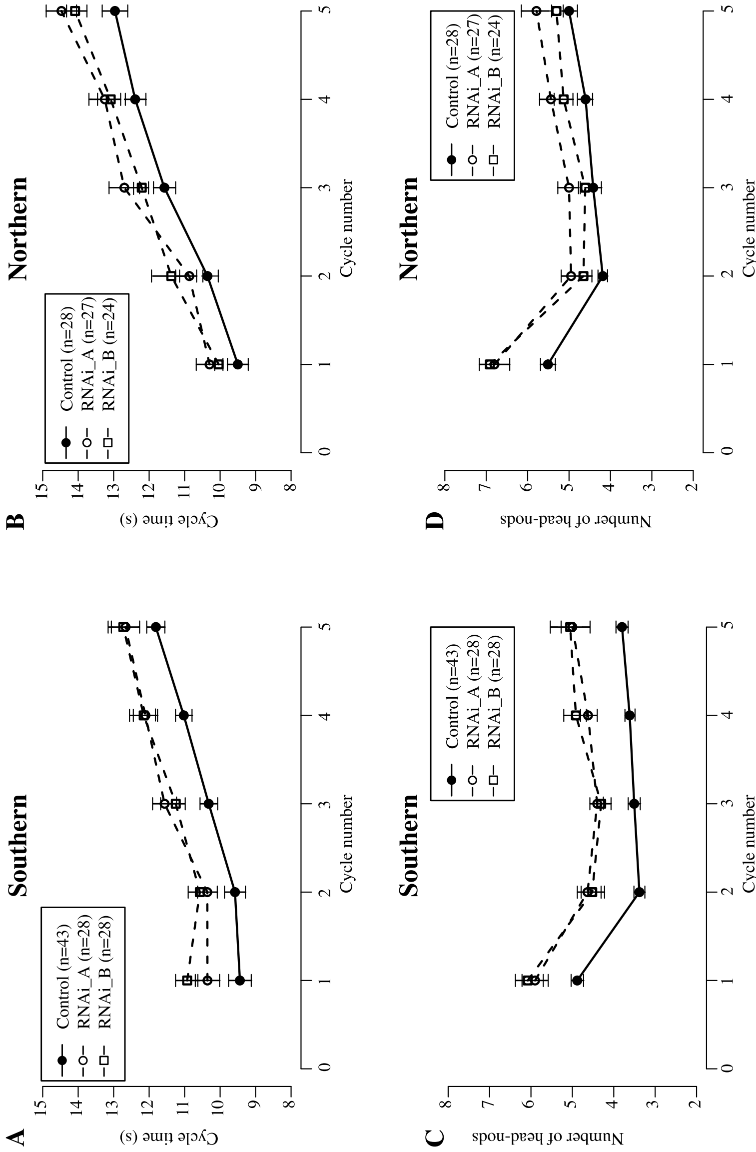

Figure 4. Courtship behaviour of control and RNAi-treated southern and northern wasps. Duration (average ± standard error) and head-nod numbers of the first five cycles of control and RNAi-treated wasps of (A, C) southern and (B, D) northern wasps. Different letters indicate significant differences by Kruskal–Wallis test with Dunn's multiple-comparison test ($p < 0.05$).

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SE 0.29 in the southern RNAi wasps. Head-nod numbers in the third and fourth cycles increase in all control and RNAi-treated lines (Figure 4C, D; Table A6 in the Appendix at 10.6084/m9.figshare.14339843).

The effect of RNAi seems slightly higher in the southern wasps, although there are no significant differences in cycle durations between southern and northern RNAi-treated wasps (Figure 4A, B; Table A6 in the Appendix at 10.6084/m9.figshare.14339843). However, the number of head-nods is increased more by RNAi in all four cycles in the southern wasps (Figure 4C, D; Table A6 in the Appendix at 10.6084/m9.figshare.14339843).

4. 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 *period* (*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. As a confirmation for effective RNAi knockdown, we recorded the circadian rhythm of treated and control wasps and found it to be significantly altered after *per* knockdown as evidenced by a shortening of the free running rhythm under constant darkness. We further found a clear effect of *per* knockdown on the ultradian rhythm of male courtship behaviour of southern and northern wasps in terms of an increase in head-nod number and cycle time. This is the first evidence that the clock gene *per* is involved in courtship behaviour in Hymenoptera. Together with previous studies in *Drosophila melanogaster* and the melon fly *Bactrocera cucurbitae*, (Kyriacou & Hall, 1980, 1982; Miyatake & Kanmiya, 2004) these data reveal a partially conserved regulating mechanism of circadian activity and courtship behaviour between Diptera and Hymenoptera.

In addition to a role of *per* in circadian and infradian rhythms of *Nasonia* (Paolucci et al., 2013, 2016, 2019; Dalla Benetta et al., 2019) this study reveals that *per* is also involved in the regulation of courtship cycles in *Nasonia*. Although a functional role of clock genes has been invoked regulating courtship song rhythms of *Drosophila* already a long time ago (Kyriacou & Hall, 1980, 1982), the rhythmicity is still being debated, mostly on methodological grounds (Kyriacou et al., 2017; Stern et al., 2017). In their original paper, Kyriacou & Hall (1980, 1982) showed that *per* regulates in a parallel fashion both circadian (tau) and ultradian (IPI) rhythms in *Drosophila*,

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whereas we found that in *Nasonia per* RNAi seems to have an antiparallel effect: it decreases tau but increases the number of head-nods and cycle time. Although the northern wasps have lower levels of *per* mRNA than southern wasps, they have longer tau. Yet, contrary to expectation, decreasing *per* mRNA levels leads to longer tau for both northern and southern wasps. This means that decreasing the level of *per* mRNA in the northern lines even more, gives them a 'southern like' tau. In contrast, the number of head-nods increases with lower levels of *per* mRNA, We have no explanation for this curious fact, but it suggests the existence of additional regulatory controls. Maybe not only the expression level, but also the specific 24 h oscillation of clock genes, can be affected in RNAi individuals (Dalla Benetta, Beukeboom & van de Zande, 2019) and affect the observed phenotypes. Interestingly, Loudon et al. (2004) also reported a reciprocal relationship between ultradian and circadian cycles in the Syrian hamster.

In *Nasonia* the rhythmic head-nods displayed 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 (Kyriacou & Hall, 1982, 1986; Ritchie et al., 1998, 1999). 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 the ultradian rhythm, it indirectly sets the pace of pheromone release (van de Assem et al., 1981) 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 possibility is that it is merely an effect of drift and has no selective history. Male courtship is essential for inducing female receptivity (van den Assem et al., 1980), but the precise number and duration of specific

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components may not be essential and merely serve to transmit pheromones to females. Direct selection on cycle time and head-nods 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 observed cline in *per* allele frequencies in *N. vitripennis* has been attributed to latitudinal selection for diapause response (Paolucci et al., 2013, 2016, 2019). Since our study directly demonstrates a role for *per* in male courtship behaviour, a genetic correlation seems the most likely explanation for the observed differences in cycle time and head-nods numbers between northern and southern wasps.

We previously showed involvement of *per* in the core mechanism of daily and seasonal timing (Mukai & Goto, 2016; Dalla Benetta et al., 2019). 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 pace 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, or through altering the action of the circadian clock. In both cases, a transcription factor in the genetic pathway of courtship behaviour may be under control of *per*, which is either up- or down regulated when *per* is knocked down (Claridge-Chang et al., 2001; McDonald & Rosbash, 2001). The route from PER via transcriptional regulator(s) to downstream courtship genes may be complex. Its elucidation requires more sophisticated genome editing experiments to fully understand the genetic regulation of ultradian rhythmicity.

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