Latitudinal variation in circadian rhythmicity in *Nasonia vitripennis*

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Running title

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ABSTRACT

Many physiological processes of living organism show circadian rhythms governed by an endogenous clock. This clock has a genetic basis and is entrained by external cues such as light and temperature. Other physiological processes exhibit seasonal rhythms, that are also responsive to light and temperature. We previously reported a natural latitudinal cline of photoperiodic diapause induction in the parasitic wasp *Nasonia vitripennis* in Europe and a correlated haplotype frequency for the circadian clock gene *period* (*per*). To evaluate if this correlation is reflected in circadian behaviour, we investigated circadian locomotor activity of seven populations from the cline. We found that the proportion of rhythmic males is higher than females in constant darkness, and that mating decreased rhythmicity of both sexes. Only for virgin females *tau* increased weakly with latitude. Wasps from the most southern locality had an overall lower level of circadian locomotor activity and an onset, peak and offset of activity earlier during the 24 h period, than wasps from the most northern locality. We evaluate this variation in rhythmicity as a function of *period* haplotype frequencies in the populations and discuss its functional significance in the context of local adaptation.

INTRODUCTION

The daily rotation of the Earth around its axis causes oscillating photoperiods that have led to the evolution of a large variety of activity patterns of organisms. Many behavioural and physiological activities, like mating, feeding and resting, show distinct oscillating rhythms with a peak of activity at a specific moment during the light-dark (LD) cycle. These are driven by an endogenous clock, that is reset daily (entrained) by the prevailing LD cycles, runs with an intrinsic period of approximately 24 hours in constant darkness (DD) (Pittendrigh et al., 1991). The length of this endogenous rhythm is called the *free running period* (*tau*, τ).

Day length (photoperiod) oscillates also seasonally and is an important cue for season-dependent behaviour, like migration in birds, hibernation in mammals and diapause in insects (reviewed in (Bradshaw and Holzapfel, 2007). Additionally, daily photoperiods depend on latitude, being almost constant near the equator and are increasing in variation towards higher latitudes. Hence, depending on latitude, organisms will experience different photoperiods over the year. Given the sensitivity of the circadian clock to light dark fluctuations, it is conceivable that it also plays a role in seasonal rhythmicity (Bunning, 1960). There is accumulating evidence for a role of the circadian clock genes in photoperiodism in many species (Saunders, 2010; Koštál, 2011; Mukai and Goto, 2016; Urbanová et al., 2016; Dalla Benetta et al., *in press*) and several studies have shown that seasonal responses differ geographically as result of variation in photoperiodic conditions (Hut et al., 2013). Nevertheless, it is still unclear whether the observed natural variation in photoperiodic response is controlled by specific circadian clock properties as a whole, such as the pace and the phase of the endogenous clock, or by pleiotropy of individual clock genes (Hut and Beersma, 2011; Dalla Benetta et al., *in press*).

Larval diapause of the parasitoid *Nasonia vitripennis* is maternally induced following a certain number of days (switch point) of a given critical photoperiod (CPP) and shows a robust clinal photoperiodic response (Paolucci et al., 2013; Saunders, 2013). Apparently, a clock mechanism is responsible for the timing and counting of the light-dark cycles necessary for proper starting of the photoperiodic response (Saunders 2013). Under long photoperiods, the switch point to start inducing diapause occurs late in life or not at all (Saunders, 1968). Interestingly, the observed cline in photoperiodic diapause induction (Paolucci et al., 2013, 2016) is also present in haplotype frequency distribution of the circadian clock gene *period*. Therefore, correlated latitudinal variation in circadian rhythms can be expected in natural populations.

To investigate if the observed cline of *per* haplotypes is reflected in circadian rhythms in *N. vitripennis*, natural variation in circadian activities, that are known to be regulated by the gene *period* (Dalla Benetta et al., *in press*), were analysed in populations from different latitudes. We determined the proportion of rhythmic individuals of seven populations collected along the European cline from Corsica to northern Finland. We first tested for clinal variation in free running period *tau.* We then analysed the timing and level of locomotor activity for the most southern and most northern population. Our data indicate that activity level, activity timing and average free-running rhythm differ between southern and northern lines of *N. vitripennis*, suggesting latitude-dependent effect on circadian clock consistent with clinal *per* haplotypes.

MATERIALS AND METHODS

Experimental lines

To study variation in locomotor activity in *Nasonia vitripennis*, we used isofemale lines established from natural field collected populations (Paolucci et al., 2013). These lines originated from seven European sampling locations (OUL (Finland, Oulu): 65°3'40.16"N, 25°31'40.80"E; TUR (Finland, Turku): 61°15'40.53"N, 22°13'23.96"E; LAT (Latvia): 56°51'22.56"N, 25°12'1.38"E; HAM (Germany, Hamburg): 53°36'23.62"N, 10°10'17.74"E; SCH (Germany, Schlüchtern): 50°19'56.10"N, 9°30'47.00"E; SWI (Switzerland): 46°44'9.14"N, 7°6'57.34"E; COR (France, Corsica): 42°22'40.80"N, 8°44' 52.80"E). Wasps were maintained on *Calliphora* spp. pupae as hosts in mass culture vials under LD 18:06, 20 ± 1°C, to minimise diapause induction. For establishing free running periods under constant darkness (DD) 17-25 isofemale lines from each location and 4-8 individuals from each isofemale line were used (797 females and 715 males). As some individuals died before all data were collected, only 1072 individuals could be used for locomotor activity analysis: 548 females (163 virgin and 385 mated) and 544 males (122 virgin and 402 mated).

For assessing timing and level of activity under different conditions (see below) we used virgin females from five isogenic lines from Oulu, Finland ("northern" lines) and Corsica, France ("southern" lines) each. These isogenic lines were established from isofemale lines by mating one virgin female with one of her sons, followed by 7-8 generations of brother-sister mating.

Locomotor activity recording

To quantify animal movement over time, individuals were collected one day after emergence (mated group) or collected as pupae and allowed to develop into adults at room temperature (virgin group) and kept either at LD16:08 or LD08:16 based on experimental group. Adults were then individually transferred without anesthetization to glass tubes (diameter 5mm x height 70mm) that were half filled with an agar gel containing 30% sugar. Trikinetics *Drosophila* activity monitors 2 (DAM2) (www.trikinetics.com) were used for activity registration with 32 wasps per monitor. All assays were performed in light tight boxes in temperature-controlled environmental chambers with 20°C and 50% humidity. The light source in the box consisted of white light with a maximum light intensity of about 200 lum/sqf. The Trikinetics system monitors how many times per minute each individual wasp interrupts an infrared light beam that passes through the centre of the glass tube. To determine free-running period under constant darkness (DD), wasps were first entrained under LD16:8 for 4 days and then subjected to 10 days of DD. To compare daily activity profile under long and short photoperiods, adult wasps were recorded for 10 days in either LD16:08 or LD08:16 regime.

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 were eye inspected and dead animals were omitted from further analysis. Under constant darkness (DD) it was possible to measure the period of activity (τ) with periodogram analysis available in ActogramJ, which incorporates chi-square test (Sokolove and Bushell, 1978). Linear mixed effect models were used to test the effect of location, latitude, sex and mating status on the percentage of rhythmic individuals and on the length of the free running period *tau*, with isofemale line nested into location as random effect (package *nlme*). Only rhythmic individuals were included in these tests. All statistical analyses were performed with R statistical software (version 3.4.1, R Development Core Team 2012).

Under LD conditions the average activity was calculated as described by Schlichting and Helfrich-Förster (2015). The first 4 days of entrainment were excluded for the analysis. To determine the onset and offset of activity on each 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 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 min. Through this process, randomly occurring spikes are reduced and the real maximum of the activity can be determined. The average phase of the onset, peak and offset, represented in *Zeitgeber time* (ZT, where ZT 0 represents the time when the light turned on), was compared between different lines and treatments.

The activity level of each individual was defined as an average of the locomotor activity based on 60 minutes/bins. Activity level was determined separately for light phase and dark phase at long day (LD16:08), short day (LD 08:16) and constant dark (DD) regimes. Average activity levels were compared between lines and photoperiods by ANOVA with a Tukey's multiple-comparisons test.

RESULTS

Rhythmicity and free running periods

The percentage of rhythmic individuals ranged from 75-84% and did not show a latitudinal cline. Overall, there was a significant difference in the proportion of rhythmic males (0.90) and rhythmic females (0.68) (GLM, effect of location x sex: $\chi^2 = 65.71$, $P<0.01$), with small but significant differences between locations (GLM, effect of location x sex: $\chi^2 = 33.00$, $P < 0.01$). Within each sex, there was a significant effect of location (GLM, effect of location: for females $\chi^2 = 13.28$, $P < 0.05$; for males $\chi^2 = 13.46$, $P < 0.05$), but no significant correlation with latitude, both for the overall data (GLM, effect of latitude: $\chi^2 = 1.77$, $P = 0.18$) and for the sexes separately (GLM, effect of latitude: for females $\chi^2 = 1.65$, $P = 0.19$; for males $\chi^2 = 0.16$, $P = 0.68$).

There was a significant difference in rhythmicity between mated and virgin individuals. 58% of mated females were rhythmic compared to 92% of virgin females (GLM model, effect of mating status within females: $\chi^2 = 50.25$, $P < 0.01$) (Fig. 1). For males, 97% of virgin individuals were rhythmic and 88% of mated ones (GLM model, effect of mating status within males: $\chi2 = 12.18$, $P < 0.01$). There was, however, no interaction effect between mating status and sex (GLM, effect of mating status x sex: $\chi^2 = 1.57$, $P = 0.20$). Again, no latitudinal cline of rhythmicity was detected, when mating status was taken into account (GLM, effect of latitude: for virgin individuals $\chi^2 = 2.22$, $P = 0.13$; for mated individuals $\chi^2 = 1.60$, $P = 0.20$) (Fig. 2.). These results indicate that there is variation between populations, sexes and mating status in proportions of rhythmic individuals but this variation is not following a geographical cline.

Among all the tested individuals there was a large variation in *tau*, which ranged from 22 hours of the southern Corsica lines to 27.5 hours of the northern Oulu lines. Overall the free running period was shorter for individuals of southern latitudes and increased towards the north (Fig. 3). Females had a longer *tau* compared to males (24.39 ± 0.04 and 23.97 ± 0.04 h, respectively) and virgin individuals had longer *tau* compared to mated individuals (24.49 ± 0.05 and 24.00 ± 0.03 h, respectively). Sex, mating status and locality had significant effects on *tau* (LME model, effect of sex: $LRT = 2.22$, $P = 0.13$; effect of mating status: $LRT = 44.32$, $P < 0.01$; effect of location: $LRT = 38.38$, $P < 0.01$). The effect of location was significant for virgin females (LME, effect of location: $LRT = 14.56$, $P < 0.05$) and mated males (LME, effect of location: $LRT = 13.02$, $P < 0.05$) but not for mated females (LME effect of location: $LRT = 12.02$, $P = 0.897$) and virgin males (LME, effect of location: $LRT = 4.19$, $P = 0.895$). A very shallow but significant latitudinal cline in *tau* for virgin females (LME, effect of latitude: $LRT = 4.28$, $r^2 = 0.20$, $P < 0.001$) was detected, but not for males and mated females (Fig. 3). The average *tau* for virgin COR females was 24.6 ± 0.12 h and for OUL 25.42 ± 0.11 h corresponding to a difference of 49.2 minutes.

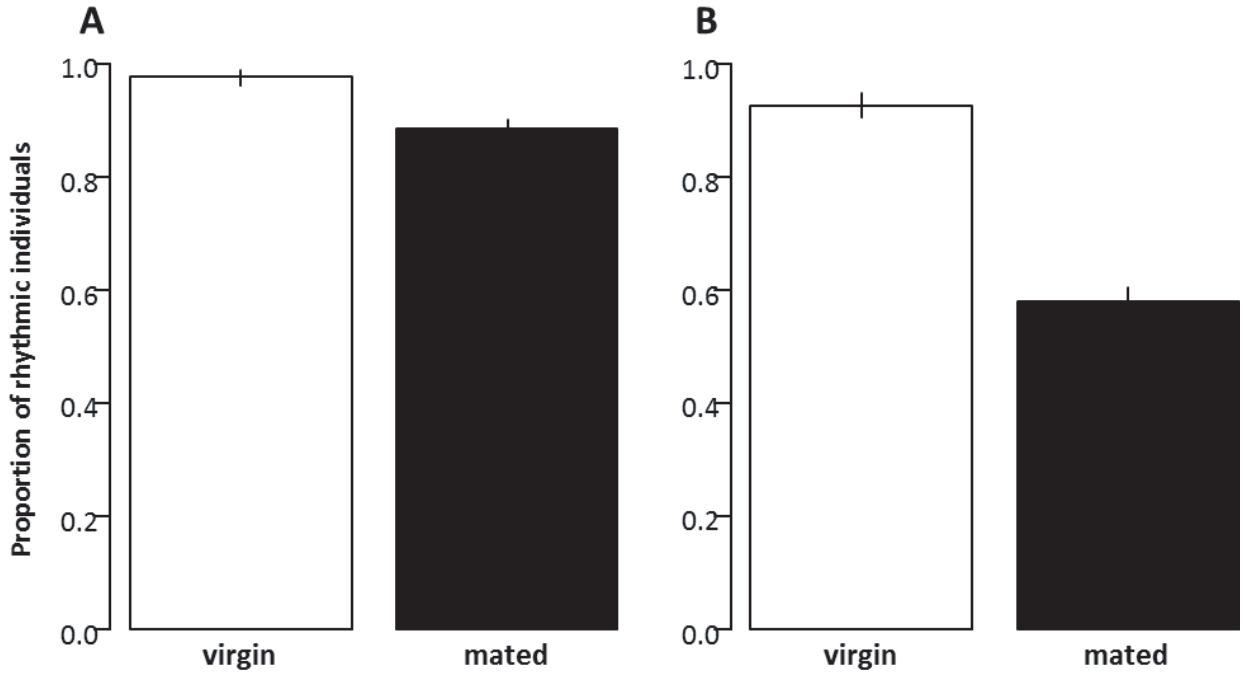

Fig. 1 Effect of mating status on proportion of individuals with internal rhythmicity
Rhythmicity in *Nasonia vitripennis* individuals measured as proportion of individuals with internal rhythmicity under constant darkness in **(A)** males and **(B)** females. Rhythmic individuals are those individuals for whom a significant periodicity could be detected across several consecutive days in constant darkness and tau could be measured. Bars indicate standard errors. There is a significant difference between sexes and mating status (see text).

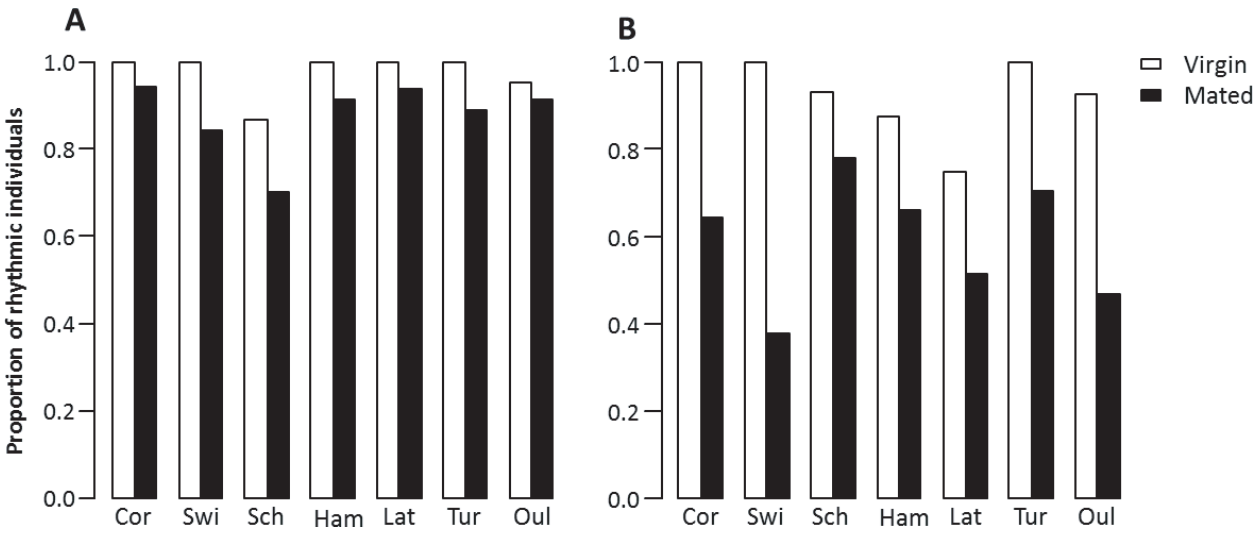

Fig. 2 Rhythmicity under constant darkness of wasps originating from the European latitudinal transect
Proportion of rhythmic *Nasonia vitripennis* individuals in populations originating from seven locations in Europe in **(A)** males and **(B)** females. Locations along the x-axis are arranged from lower to higher latitude, see text for locality details.

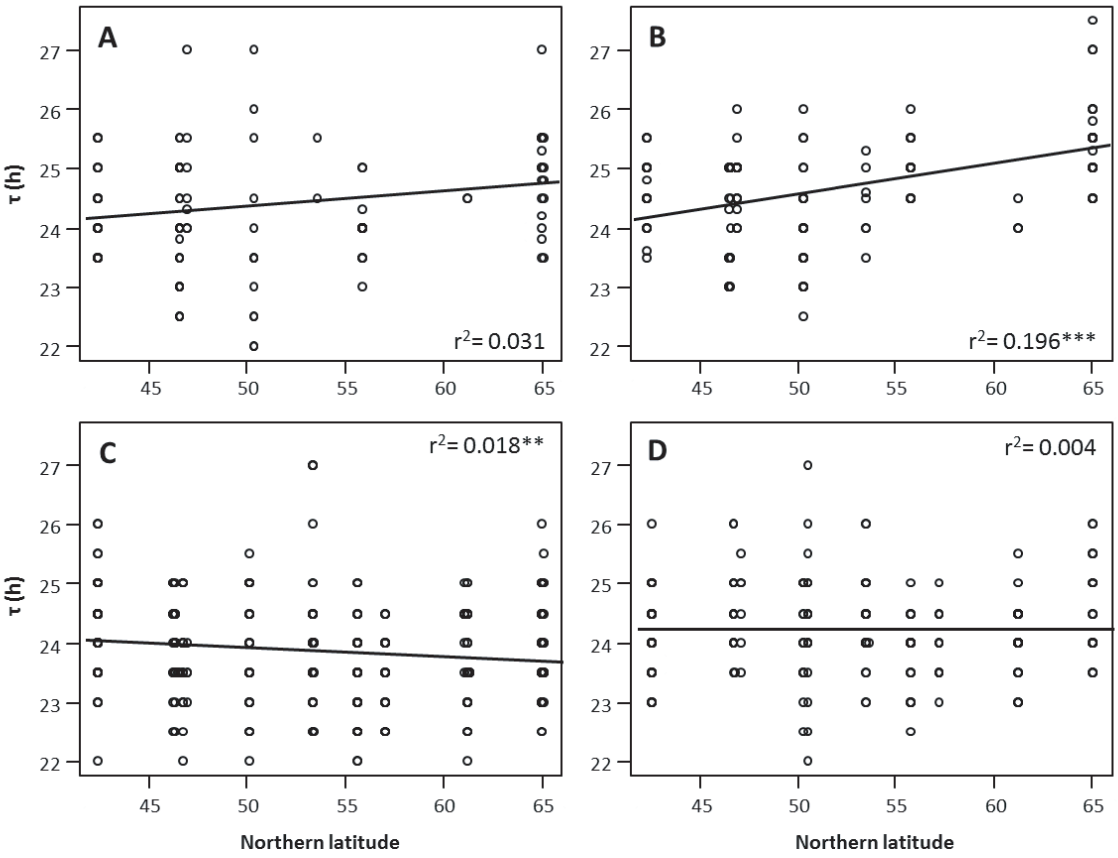

Fig. 3 Free running period (τ) under constant darkness (DD) as a function of latitude
Free running period (τ) of **(A)** virgin males, **(B)** virgin females, **(C)** mated males and **(D)** mated females in *Nasonia vitripennis* populations collected along a latitudinal gradient in Europe. Asterisks indicate a significant effect of location along the cline (*** *P*<0.001 and ** *P*<0.05, linear mixed effect model)

Activity timing

Virgin females from 5 isogenic lines established from populations of the two extremes of the sampling range were exposed to a light dark regime of either LD16:08 or LD08:16h for 4 days. Under LD16:08, both southern and northern wasps displayed a unimodal activity pattern (Fig. 4), but with significant differences in the timing of onset, peak and offset of activity (Table 1, Table S1). Southern wasps started activity on average around ZT 0, which is about two hours earlier than northern wasps (Fig. 4, Table 1, Table S1). Southern wasps displayed maximum activity around ZT 5, while northern wasps peaked at ZT 8 (Fig. 4, Table 1, Table S1). Offset of activity was around ZT 13 and ZT 16 for southern and northern wasps, respectively (Fig. 4, Table 1, Table S1). Thus, southern wasps were more active in the first half of the light period and northern wasps towards the end of the day.

Under shorter photoperiod LD08:16 southern wasps started their activity also earlier than northern wasps (Fig. 4, Table 1, Table S2). Onset of activity was already when the light was still off, around ZT 21.5. Northern wasps became active at ZT 0 (Fig. 4, Table 1, Table S2). The peaks of activity differed about one-and-half hour, at ZT 2.5 and ZT 4 for southern and northern wasps, respectively (Fig. 4, Table 1, Table S2). Offset of activity was at ZT 8 for southern wasps. Northern wasps prolonged activity for more than two hours into darkness, until ZT 10.5 on average (Fig. 4, Table 1, Table S2). Thus, under both light regimes there is a difference in phase of activity between southern and northern wasps. The consequence is that in short photoperiod southern wasps start activity in the dark and finish in the light phase, whereas northern wasps start activity at the beginning of the light phase and continue in the dark.

In agreement with the results obtained for the isofemale lines, the southern and northern isogenic lines differed in free-running rhythms under constant conditions (Fig. 4, Table1). 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 ($P < 0.001$).

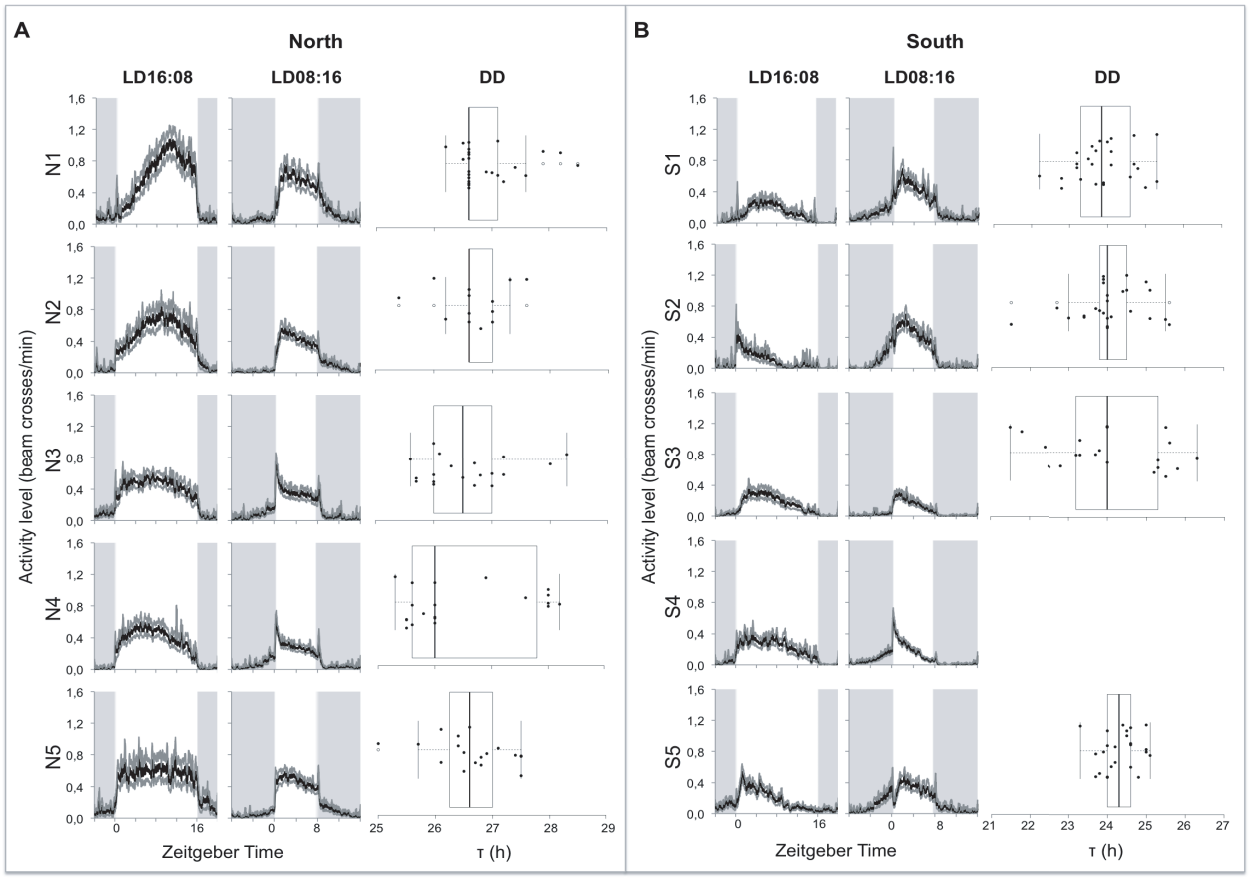

Fig. 4 Locomotor activity of northern and southern wasps under different LD cycles

(A) Locomotor activity profiles for isogenic lines derived from Oulu (65°) (N1, N2, N3, N4, N5) and (**B)** from Corsica (45°) (S1, S2, S3, S4, S5) at long (LD16:08) and short (LD 08:16) 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 of light turn-on. Activity is calculated as average of bin crosses/minute of 25-32 individuals each over 24 h periods. Box plots represent the free running rhythms in constant darkness (DD). Box plots depict the median (thick horizontal line within the box), the 25 and 75 percentiles (box margins) and the 1.5 interquartile range (thin horizontal line). Note different scale on x-axis for A and B panels.

Activity levels

Activity level was measured for southern and northern wasps under two LD cycles and constant darkness (DD). Generally, activity of southern wasps was lower than that of northern ones (Fig. 5, Table 2). Under LD16:08 the night activity was very low in all lines but still lower in southern than northern wasps. Southern wasps had an average activity level of 2.16 + 0.23 per hour bin during the night and 11.22 + 0.52 during the day, compared to northern wasps with 4.44 + 0.38 and 31.19 + 1.06 for dark and light phase respectively (ANOVA with a Tukey's multiple-comparisons test, $P < 0.001$) (Fig. 5, Table 2).

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. 5B). Although this is a significant increase (ANOVA with a Tukey's multiple-comparisons test, $P < 0.001$), not all southern lines differed in activity between long and short photoperiod (Table 2). In contrast, none of the northern lines differed in activity level between the two photoperiods. Despite the larger increase in activity level under short photoperiod of the southern lines, the northern wasps remained, on average, more active than the southern ones during the light phase (ANOVA with a Tukey's multiple-comparisons test, $P < 0.001$). Under constant conditions of DD, southern wasps always displayed a lower activity level (11.49 + 0.51) than northern wasps (19.97 + 0.90) (ANOVA with a Tukey's multiple-comparisons test, $p<0.001$) (Fig. 5C, table 2). Taken together, these data indicate that activity level is inherently different between southern and northern lines.

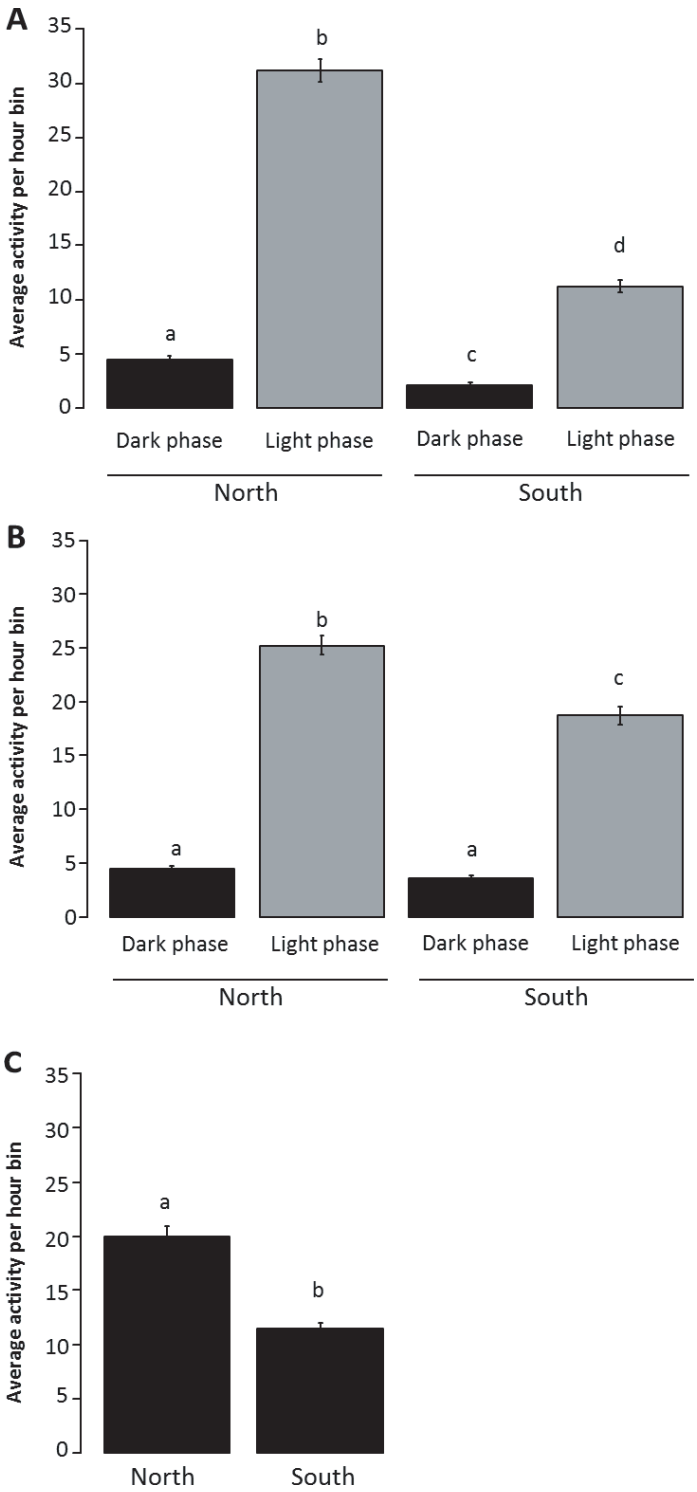

Fig. 5 Activity level of northern and southern lines in the dark and light phase

Activity level of northern and southern wasps **(A)** under LD16:08 during the dark and light phase. **(B)** under LD08:16 during the dark and light phase and **(C)** under constant darkness (DD). Different letters indicate significant differences ($P<0.001$, ANOVA with a Tukey's multiple-comparisons test).

Table 1: Timing of onset, peak and offset of activity for Corsica (45°) (S1, S2, S3, S4, S5) and Oulu (65°) (N1, N2, N3, N4, N5) wasps under long (LD16:08) and short (LD08:16) day conditions and free running rhythm (τ) under constant condition (DD). ZT (h) is zeitgeber time in hours. Different letters indicate significant differences (*P*<0.05, ANOVA with a Tukey's post hoc multiple-comparisons test).

	LD16:08			**LD08:16**			**DD**	
Lines	Onset ± SE	Peak ± SE	Offset ± SE	Onset ± SE	Peak ± SE	Offset ± SE	τ ± SE	Arrhythmic
	ZT	ZT	ZT	ZT	ZT	ZT	h	%
N1	3.1 ± 0.3	10.8 ± 0.3	18.7 ± 0.6	0.1 ± 0.1	4.5 ± 0.3	11.6 ± 0.4	26.9 ± 0.1	19.4
	(a)	(a)	(a)	(a)	(a)	(ab)	(a)	
N2	1.7 ± 0.4	8.2 ± 0.4	16.2 ± 0.3	23.9 ± 0.2	4.51± 0.2	11.92 ± 0.38	26.7 ± 0.1	55.2
	(ab)	(b)	(b)	(a)	(a)	(b)	(a)	
N3	2.29 ± 0.42	7.2 ± 0.2	17.9 ± 0.2	22.7 ± 0.3	3.15 ± 0.2	10.6 ± 0.3	26.57 ± 0.17	36.7
	(b)	(bc)	(bc)	(ab)	(b)	(a)	(a)	
N4	1.3 ± 0.2	6.7 ± 0.3	14.3 ± 0.3	22.6 ± 0.3	3.12 ± 0.2	10.7 ± 0.4	26.47 ± 0.25	40.7
	(bc)	(bcd)	(c)	(ab)	(b)	(a)	(a)	
N5	1.3 ± 0.5	6.4 ± 0.7	14.6 ± 0.6	22.9 ± 0.2	2.8 ± 0.2	8.7 ± 0.4	26.7 ± 0.1	41.7
	(bc)	(cd)	(bc)	(ab)	(b)	(c)	(a)	
Overall N	**2.0 ± 0.2**	**7.9 ± 0.2**	**16.1 ± 0.2**	**23.4 ± 0.2**	**3.5 ± 0.1**	**10.5 ± 0.2**	**26.8 ± 0.1**	**43.5**
S1	1.1 ± 0.4	6.4 ± 0.4	12.7 ± 0.4	21.7 ± 0.2	2.9 ± 0.2	8.7 ± 0.2	23.8 ± 0.1	27.0
	(ab)	(cd)	(c)	(bc)	(b)	(c)	(b)	
S2	0.1 ± 0.1	5.3 ± 0.2	13.1 ± 0.2	22.3 ± 0.2	3.0 ± 0.3	8.6 ± 0.1	24.7 ± 0.2	17.0
	(c)	(d)	(c)	(bc)	(b)	(c)	(c)	
S3	23.8 ± 0.2	5.8 ± 0.3	14.2 ± 0.3	21.1 ± 0.2	1.5 ± 0.2	7.5 ± 0.3	24.4 ± 0.4	51.0
	(c)	(cd)	(c)	(c)	(c)	(c)		
S4	23.9 ± 0.3	5.4 ± 0.4	13.1 ± 0.4	21.9 ± 0.3	3.2 ± 0.3	9.0 ± 0.2	-	100
	(c)	(d)	(c)	(bc)	(b)	(c)	(b)	
S5	23.7 ± 0.2	3.6 ± 0.2	13.2 ± 0.3	21.1 ± 0.2	0.9 ± 0.1	7.8 ± 0.1	24.3 ± 0.1	22.6
	(c)	(e)	(c)	(c)	(c)	(c)	(b)	
Overall S	**0.1 ± 0.1**	**5.3 ± 0.2**	**13.2 ± 0.2**	**21.6 ± 0.1**	**2.3 ± 0.1**	**8.3 ± 0.1**	**24.3 ± 0.1**	**38.7**

Table 2: Activity level for Corsica (45°)(S1, S2, S3, S4, S5) and Oulu (65°) (N1, N2, N3, N4, N5) wasps under LD16:08, LD08:16 and DD conditions. Activity is calculated as number of beam crosses in hour bin. Different letters indicate significant differences between lines (*P*<0.05) and asterisks indicate significant differences between LD16:08 and LD08:16, and between DD and LL conditions (*P*<0.001).

	LD16:08				LD08:16		DD
	Light phase	**Dark phase**	**Light**	**Dark**	**Light phase**	**Dark phase**	**DD 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)
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)
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)
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)
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)
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)
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)
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)
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)
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)
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)

DISCUSSION

We found natural variation for several properties of circadian locomotor activity of *Nasonia vitripennis*. Despite the proportion of rhythmic individuals being generally high, we observed significant differences between females and males: males are on average more rhythmic than females and have shorter free-running periods (*tau*). Similar differences between both sexes were observed in the laboratory strain *N. vitripennis* AsymC (Bertossa et al., 2013). Virgin individuals of both sexes are highly rhythmic in constant darkness. The difference in rhythmicity between males and females is more apparent in mated individuals. Moreover, mated males maintain a high level of rhythmicity (although lower compared to virgin males), whereas most females lose their internal rhythmicity after mating. Similar effects of mating status on rhythmicity were found in the ant species *Camponotus compressus,* in which ovipositing queens exhibited arrhythmic locomotor activity during the egg laying phase and restored rhythmicity afterwards (Sharma et al., 2004). In addition, we found a significant interaction between locality and mating status on proportion of rhythmic individuals; for example, the effect of mating status in females of the SWI population is larger than in the SCH population. This might reflect standing genetic variation for rhythmic behaviour within and among populations. It is also possible that environmental factors affect the rhythmic locomotor activity as was recently shown for the northern fly species *Drosophila montana*, in which the proportion of rhythmic individuals was higher at lower temperature (Kauranen et al., 2012). It would be worthwhile to investigate how environmental conditions (temperature and light intensity among others) influence circadian locomotor activity behaviour of males and females in more insect species and test whether the level of variability will differ between populations from different geographical regions. Such studies could reveal differential selection pressures for stability of the circadian clock under different conditions.

We also found differences in free running rhythms (*tau*, τ) between sexes and locations. Towards the south, wasps have a faster clock with τ close to 24h, whereas wasps towards northern latitudes have a slower clock with τ longer than 24h. For virgin females, we observed a weak, but significant, latitudinal cline for τ, increasing towards higher latitudes. The presence of a positive latitudinal cline from south to north in DD rhythm was previously reported for *Drosophila* species, but only few studies have addressed variation of free running rhythms within a species. For example, in the plant *Arabidopsis thaliana* the free running period under DD increases towards northern latitude, and correlates with clinal variation in seasonal flowering time regulated by photoperiodic cycles (Michael,

2003). In insects, similar results (*i.e*. longer τ towards northern latitude) were reported from the mosquito *Culex pipiens* (Shinkawa et al., 1994) and the linden bug *Pyrrhocoris apterus* (Pivarciova et al., 2016). This suggests that the latitudinal differences in free running period are the result of selection on traits that enable local adaptation. One possibility is selection for phase of activity in which a faster clock corresponds to earlier activity phase whereas and a slower clock to later activity phase.

The pace of the circadian clock of *Nasonia* females can reflect the timing of locomotor activity under LD conditions. Indeed, we observed a positive correlation between the activity phase and free running rhythm of the wasps (i.e. wasps with shorter τ have earlier activity peak) (Fig. S1). Southern and northern wasps displayed profound differences in their daily locomotor activity. Southern wasps were mainly active in the morning with an increase of activity before the light turned on during short photoperiod, whereas northern ones present a unimodal evening activity, with a prolonged evening peak at the shorter photoperiod. This shifted activity pattern between southern and northern 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 response of insects that live in a hot environment (Prabhakaran and Sheeba, 2012; 2013). In contrast, species that live at higher latitudes have to cope with lower temperatures and longer photoperiods (Helfrich-Förster et al., 2018). Indeed, the northern *Nasonia* lines have a reduced morning activity with their activity peak in the second part of the day when temperatures are higher. Similar differences in activity patterns between southern and northern populations have been reported for *Drosophila*, albeit between *Drosophila* species rather than populations within species (Helfrich-Förster et al., 2018; Prabhakaran and Sheeba, 2012; 2013). However, the overall activity profile of *Nasonia* is rather broad compared to the more precisely timed behaviour of *Drosophila melanogaster*, possibly reflecting a stronger selection on the *D. melanogaster* than *Nasonia* activity phase. On the other hand *Nasonia* presents a strong photoperiodic response (Saunders 1968). Interestingly, a latitudinal cline similar to the observed cline in τ for virgin females, has been previously reported by Paolucci et al. (2013, 2016) for the frequencies of *period* (*per*) haplotypes correlating with the cline for photoperiodic diapause induction. This supports the hypothesis that *per* participates in photoperiod measurement by fine-tuning its critical day length to latitude-dependent needs. Consequently, the cline in free running period would reflect a mere "side effect" of the selection pressure. In congruence with this hypothesis, previous experiments demonstrated that the critical day length of *Nasonia* diapause was affected when

per expression and τ were altered (Dalla Benetta et al., *in press*) suggesting an involvement of the clock gene in the photoperiodic timer of *Nasonia.*

We also investigated differences in level of activity between selected northern and southern lines. Overall, higher level of activity was observed in the northern lines compared to the southern ones, whereas southern lines tended to have higher activity when photoperiod is shorter. Such differences could result from variation in the sensitivity to light. For example, northern individuals might be more sensitive to light and thus show a stronger response, whereas southern lines compensate for bright illumination experienced in the Mediterranean by reduced light sensitivity.

In summary we described natural variation on the pace, phase and level of daily rhythms between southern and northern *N. vitripennis* lines. Interestingly, many traits related to circadian activity show a high level of plasticity, which allows flexibility in daily activities depending on internal conditions (e.g. mating status) or external environmental conditions (e.g. light dark cycle, presence of food). However, variation between geographic locations is maintained even in the plastic response to different stimuli suggesting that natural selection acts on the response of the circadian system to the environment and not on the circadian clock *per se*. Apparently, more detailed functional experiments are required to reveal the exact molecular mechanism underpinning circadian clock, photoperiodic timer and their mutual connections.

References

Bertossa RC, van Dijk J, Diao W, et al. (2013) Circadian rhythms differ between sexes and closely related species of *Nasonia* wasps. *PloS one* 8(3): e60167.

Bradshaw WE and Holzapfel CM (2007) Evolution of Animal Photoperiodism. *Annual Review of Ecology, Evolution, and Systematics*. DOI: 10.1146/annurev.ecolsys.37.091305.110115.

Bunning E (1960) Circadian rhythms and the time measurement in photoperiodism. *Cold Spring Harbor Symposia on Quantitative Biology*. DOI: 10.1101/sqb.1960.025.01.026.

Helfrich-Förster C, Bertolini E and Menegazzi P (2018) Flies as models for circadian clock adaptation to environmental challenges. *European Journal of Neuroscience*. DOI: 10.1111/ejn.14180.

Hut RA and Beersma DGM (2011) Evolution of time-keeping mechanisms: early emergence and adaptation to photoperiod. *Philosophical Transactions of the Royal Society B: Biological Sciences*. DOI: 10.1098/rstb.2010.0409.

Hut RA, Paolucci S, Dor R, et al. (2013) Latitudinal clines: an evolutionary view on biological rhythms. *Proceedings. Biological sciences / The Royal Society* 280(1765): 20130433.

Kauranen H, Menegazzi P, Costa R, et al. (2012) Flies in the north: locomotor behavior and clock neuron organization of *Drosophila montana*. *Journal of biological rhythms* 27(5): 377–387.

Koštál V (2011) Insect photoperiodic calendar and circadian clock: independence, cooperation, or unity? *Journal of insect physiology* 57(5): 538–556.

Michael TP (2003) Enhanced fitness conferred by naturally occurring variation in the circadian clock. *Science*. DOI: 10.1126/science.1082971.

Mukai A and Goto SG (2016) The clock gene *period* is essential for the photoperiodic response in the jewel wasp *Nasonia vitripennis* (Hymenoptera: Pteromalidae). *Applied Entomology and Zoology*. DOI: 10.1007/s13355-015-0384-1.

Paolucci S, van de Zande L and Beukeboom LW (2013) Adaptive latitudinal cline of photoperiodic diapause induction in the parasitoid *Nasonia vitripennis* in Europe. *Journal of evolutionary biology* 26(4): 705–718.

Paolucci S, Salis L, Vermeulen CJ, et al. (2016) QTL analysis of the photoperiodic response and clinal distribution of *period* alleles in *Nasonia vitripennis*. *Molecular ecology* 25(19): 4805–4817.

Pittendrigh CS, Kyner WT and Takamura T (1991) The amplitude of circadian oscillations: Temperature dependence, latitudinal clines, and the photoperiodic time measurement. *Journal of biological rhythms*. DOI: 10.1177/074873049100600402.

Pivarciova L, Vaneckova H, Provaznik J, et al. (2016) Unexpected geographic variability of the free running period in the linden bug *Pyrrhocoris apterus*. *Journal of biological rhythms* 31(6): 568–576.

Prabhakaran PM and Sheeba V (2012) Sympatric Drosophilid species *melanogaster* and *ananassae* differ in temporal patterns of activity. *Journal of biological rhythms*. DOI: 10.1177/0748730412458661.

Prabhakaran PM and Sheeba V (2013) Insights into differential activity patterns of drosophilids under semi-natural conditions. *Journal of Experimental Biology*. DOI: 10.1242/jeb.092270.

Saunders DS (1968) Photoperiodism and time measurement in the parasitic wasp, *Nasonia vitripennis*. *Journal of Insect Physiology*. DOI: 10.1016/0022-1910(68)90060-7.

Saunders DS (2010) Controversial aspects of photoperiodism in insects and mites. *Journal of insect physiology* 56(11): 1491–1502.

Saunders DS (2013) Insect photoperiodism: measuring the night. *Journal of insect physiology* 59(1): 1–10.

Schlichting M and Helfrich-Förster C (2015) Photic entrainment in *Drosophila* assessed by locomotor activity recordings. *Methods in Enzymology*. DOI: 10.1016/bs.mie.2014.10.017.

Schmid B, Helfrich-Förster C and Yoshii T (2011) A new ImageJ plug-in 'ActogramJ' for chronobiological analyses. *Journal of biological rhythms* 26(5): 464–467.

Sharma V, Lone S, Goel A, et al. (2004) Circadian consequences of social organization in the ant species *Camponotus compressus*. *Naturwissenschaften*. DOI: 10.1007/s00114-004-0544-6.

Shinkawa Y, Takeda S, Tomioka K, et al. (1994) Variability in circadian activity patterns within the *Culex pipiens* complex (Diptera: Culicidae). *Journal of medical entomology* 31(1): 49–56.

Sokolove PG and Bushell WN (1978) The chi square periodogram: its utility for analysis of circadian rhythms. *Journal of theoretical biology* 72(1): 131–160.

Urbanová V, Bazalová O, Vaněčková H, et al. (2016) Photoperiod regulates growth of male accessory glands through juvenile hormone signaling in the linden bug, *Pyrrhocoris apterus*. *Insect Biochemistry and Molecular Biology*. DOI: 10.1016/j.ibmb.2016.01.003.

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