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Adaptive significance of a circadian clock: temporal segregation of activities reduces intrinsic competitive inferiority in *Drosophila* parasitoids

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Most organisms show self-sustained circadian oscillations or biological clocks which control their daily fluctuations in behavioural and physiological activities. While extensive progress has been made in understanding the molecular mechanisms of biological clocks, there have been few clear demonstrations of the fitness value of endogenous rhythms. This study investigated the adaptive significance of circadian rhythms in a *Drosophila* parasitoid community. The activity rhythms of three sympatric *Drosophila* parasitoids are out of phase, the competitively inferior parasitoid species being active earlier than the superior competitor. This temporal segregation appears at least partially determined by endogenous periods of the clock which also vary between species and which correlate the time of activity. This earlier activity of the inferior competitor significantly reduces its intrinsic competitive disadvantage when multiparasitism occurs, thus suggesting that natural selection acting on the phase of the rhythm could substantially deviate the endogenous period from the optimal *ca.* 24 h period. This study demonstrates that temporal segregation of competing species could be endogenously controlled, which undoubtedly favours their coexistence in nature and also shows how natural selection can act on biological clocks to shape daily activity patterns.

Keywords: circadian rhythms; locomotor and egg-laying activity; *Drosophila* parasitoids; interspecific competition; *Leptopilina*; *Asobara*

1. INTRODUCTION

Many organisms, from bacteria to humans, show biochemical, physiological and behavioural daily rhythms controlled by an internal clock (Saunders 1976; Aschoff 1981; Edmunds 1988; Pittendrigh 1993). The endogenous nature of these rhythms is revealed under constant environmental conditions where the rhythms persist (free run) as self-sustaining oscillations with an endogenous period (τ) close to but different from 24 h. Under field conditions, these rhythms result from complex interactions between endogenous and exogenous factors while the natural light:dark (L:D) cycle imposes its own periodicity. Actual biological rhythms are thus entrained and show a precise 24 h period. During the last 20 years, extensive progress has been made in understanding the physiological and molecular mechanisms of circadian oscillations, which appear similar in many organisms ranging from fungi to mammals (Hall 1995; Hastings 1995; Dunlap 1996, 1998; Darlington *et al.* 1998; Schibler 1998), but the adaptive significance of circadian programmes remains unclear. The endogenous control of daily rhythms is generally thought to be a fundamental adaptation of organisms anticipating the temporal organization of the environment (Enright 1970; Daan 1981; Pittendrigh 1993), but few studies have clearly demonstrated the fitness value of this circadian programme compared to direct responses to environmental stimuli.

Another unresolved evolutionary aspect of circadian rhythms is why endogenous periods (τ) differ significantly from 24 h and why they vary within and between closely related species (Pittendrigh & Daan 1976; Petersen *et al.* 1988; Allemand *et al.* 1989; Sawyer *et al.* 1997; Czeisler *et al.* 1999). These period variations appear inconsistent with experimental evidence which suggests that fitness is at a maximum when the circadian period (τ) resonates with a 24 h environmental cycle (Ouyang *et al.* 1998). Indeed, since earth rotations have a precise 24 h period, we thus logically expect that endogenous periods differing from 24 h will have detrimental effects on fitness and should be eliminated by natural selection. A possible explanation for this variability is that endogenous circadian period variations are a by-product of natural selection on other fitness components, mainly the time of activity (phase of the rhythm). This was suggested by *Drosophila* period mutants whose rhythm phase partly depends on the period of the free-running rhythm (Hall 1995; Hamblen *et al.* 1998) and, thus, selection acting on the phase could result in indirect genetic effects on the endogenous period. Up to now, we have lacked a careful study of the relationships between the phase of the entrained rhythm, the endogenous period of the circadian oscillation and its adaptive significance in field situations.

To address this question, we studied activity rhythms in solitary *Drosophila* parasitoid species which compete for the same host and we tested the hypothesis that circadian rhythms are structured such that individuals of otherwise competitively inferior species reduce their competitive deficit by being active earlier each day than their intrinsically superior competitors. Solitary parasitoids are wasps which lay their eggs on or in the bodies of

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other arthropods, each juvenile parasitoid feeding on a single host until it matures into a free-living adult (Godfray 1994; Quicke 1997). Parasitoid activity rhythms are known to influence their reproductive success in isolation, since locating hosts requires some degree of synchronization with their host's activity (Vinson 1981; Godfray 1994). However, when the same host individual is parasitized by two solitary wasp species (multiparasitism), the outcome of the intrinsic competition between parasitoid larvae depends on the interval between ovipositions (Van Strien-Van Liempt 1983) and, thus, the earlier species, which anticipates the activity of its competitor, should be at an advantage. We employed the three wasp species which commonly parasitize *Drosophila* larvae in southern Europe. Multiparasitism on *Drosophila* is quite common (Allemand *et al.* 1999) and, among the three parasitoids, *Leptopilina boulardi* is the intrinsically dominant competitor, whereas *Leptopilina heterotoma* and *Asobara tabida* are less abundant and prove poorer direct competitors (Carton *et al.* 1991; Allemand *et al.* 1999). We first studied their locomotor activity rhythms to determine the endogenous nature of the rhythms and the value of the circadian period for these three species. In a second series of experiments, we measured the oviposition rhythm, a more fitness-related trait on which unfortunately the circadian period cannot be measured for technical reasons and we tested how the time of oviposition activity could modify the outcome of intrinsic competition.

2. MATERIAL AND METHODS

(a) *Strains and culture conditions*

In the south of Europe, *L. heterotoma*, *L. boulardi* and *A. tabida* are the most common species which parasitize *Drosophila* larvae living in fermenting fruits, mainly *Drosophila melanogaster* and *Drosophila simulans*. The three wasp species used in the experiments originate from Antibes (south of France). Wild females caught in the same traps (sympatric species) were used to initiate laboratory strains maintained under a 12 L:12 D photoperiod at 22 °C. A 12 L:12 D cycle corresponds to the autumn natural L:D photoperiod, the season when the species are most abundant. The parasitoids were cultured on a laboratory strain of *D. melanogaster* fed with a standard diet (David 1962).

(b) *Locomotor activity*

Activity rhythms were continuously measured at 22 °C for females first exposed to a 12 L:12 D photoperiod for three days (entrained rhythm), then to constant darkness for seven days (free-running rhythm). The locomotor activity of each of 28–33 females was checked every 5 min using an automatic video infrared recording device for each species (see Allemand *et al.* (1994) for further details). A computer determined whether or not each female had moved during the video recording 12 times per hour and their activity was then quantified by binary data (1 = active and 0 = not active). The hourly activity was then calculated as the percentage of active recordings among the 12 hourly ones. The endogenous period (τ) was calculated from the data under constant darkness using the χ^2-periodogram method (Sokolove & Bushell 1978). The daily pattern of activity under a 12 L:12 D photoperiod was established as follows. We calculated the hourly percentage of the total activity recorded over the corresponding 24 h period for each female on each day and the individual pattern was estimated as the mean curve over the three successive days. Each species was characterized by averaging the 28–33 individual curves. This method reduces any bias resulting from daily or individual variations in the overall activity of the females. The profile of the rhythms was then characterized by the mean hour of activity peaks (phase of the peaks). Under a 12 L:12 D photoperiod, the data are given as a function of the environmental cycle with light on at 08.00 and off at 20.00 (universal time). Under constant darkness, activity is plotted as a function of the circadian time (t_C), where the timescale of each individual covers its own endogenous period (see Saunders 1976). The beginning of the free-running experiment, which started at 20.00 (light off), is termed t_{C0}. Each circadian unit is equivalent to $\tau/24$ h where τ is the period of the free-running rhythm. The curves were thus normalized to take into account the individual and species variation in the endogenous period.

(c) *Oviposition activity*

Females were individually housed in 5 cm diameter Petri dishes with a droplet of honey and a 15 mm diameter cup of diet containing 60 second-instar larvae of *D. melanogaster*. Batches of hosts were renewed every 2 h and then placed in individual vials for development (22 °C and 12 L:12 D). Emerging flies were counted and a comparison with the fly numbers emerging from the control (unparasitized) batches of hosts gave an estimation of the number of hosts actually parasitized. The experiments were performed under the same conditions as for locomotor activity (22 °C, 12 L:12 D and light on at 08.00 and off at 20.00). The insects were illuminated with infrared light which they did not perceive allowing host renewal in the darkness. Twenty-eight females were followed for two days for each species and only individuals which oviposited in hosts on each of the two days were used for the analysis. Oviposition activity was calculated every 2 h as the percentage of the total number of hosts parasitized over the 24 h of the experiment. The rhythm phase was calculated as the mean hour of the two daily maxima. The correlation between the locomotor and oviposition activities was found from the summed locomotor activities over 2 h intervals.

(d) *Multiparasitism experiments*

To determine whether differences in the adult activity phases play a role in the outcome of intrinsic competition between parasitoid larvae, we let isolated females of two different wasp species successively infest the same host and, after development, we examined how the outcome of competition depended on the delay between the two ovipositions. The experiments were performed at 22 °C under a 12 L:12 D photoperiod. *Asobara tabida* was excluded from these experiments since it suffers high mortality due to the immune response by the host (Kraaijeveld & Van Alphen 1995). Second-instar *D. melanogaster* larvae were placed in a Petri dish with agar and yeast and parasitized by one *L. heterotoma* female. Oviposition by *L. heterotoma* was performed between 10.00 and 12.00 and confirmed under a binocular microscope. Each parasitized host was removed and placed in a second Petri dish for parasitism by *L. boulardi* 0–2, 2–5 or 5–8 h after the first species of parasitoid had oviposited. Multiparasitized hosts were then put into separate vials for development. The outcome of larval competition was determined by checking which wasp species emerged from each multiparasitized host. The percentage of *L. boulardi* emerging was compared to the expected value of 50% under symmetrical competition for each treatment.

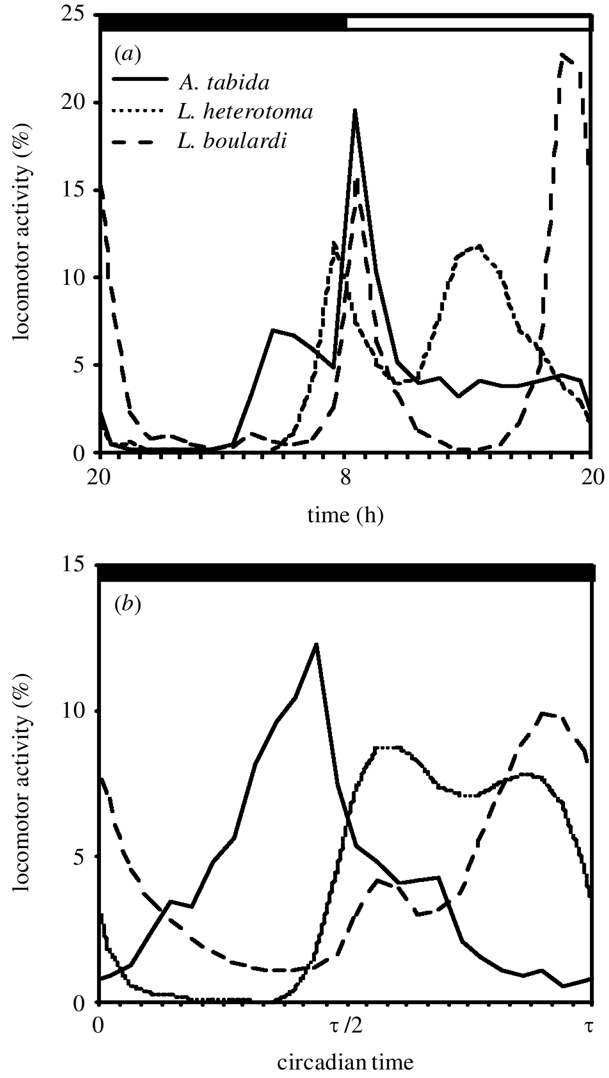

Figure 1. Daily pattern of locomotor activity in three *Drosophila* parasitoids: *A. tabida*, *L. heterotoma* and *L. boulardi*. (*a*) Mean curves of the rhythms measured for three days under a 12 L:12 D photoperiod (light on at 08.00 and off at 20.00). (*b*) Mean curves of the rhythm measured for seven days under constant darkness (activity is plotted against circadian time, see §2).

3. RESULTS AND DISCUSSION

We first studied the locomotor activity of adult female wasps under both L:D and constant darkness conditions. The three parasitoid species show striking differences in their profiles of entrained and free-running rhythms of locomotor activity, with a clear tendency for different parasitoids to be active at different times of the day (figure 1). Under a 12 L:12 D photoperiod (figure 1*a*) all species have bimodal rhythms, each activity peak being out of phase between species. The first peaks of activity occur several hours before and shortly before light on in *A. tabida* and *L. heterotoma*, respectively and at light on in *L. boulardi*. The temporal segregation was maximal for the second peak of activity, with *A. tabida* mostly active at light on, *L. heterotoma* half-way into the light phase and *L. boulardi* just before dark. The circadian nature of these rhythms is revealed by their persistence under constant darkness with periods significantly departing from 24 h (figure 1*b* and table 1). Under constant darkness conditions, each species displayed its own endogenous oscillation which differed characteristically from its profile in the L:D experiment. *Leptopilina heterotoma* remained bimodal, whereas the first peak of *L. boulardi* was greatly reduced and *A. tabida* exhibited only one main bout of activity. This reduction or virtual absence of peaks which were normally exhibited at light on indicated that this activity under L:D is exogenously determined (by the direct effect of the light switched on) and that the endogenous profiles are unimodal. Therefore, the three species temporally segregated both exogenously and endogenously.

Moreover, in accordance with theoretical predictions of the relationship between phase and the endogenous period (Aschoff 1981; Pittendrigh 1981), the free-running experiment indicated that the endogenous period varies between species in parallel with the rhythm phase (table 1). *Asobara tabida* has a short free-running period ($\tau = 21.3$ h), shows clear anticipation in constant darkness conditions and is active very early in the L:D cycle. Conversely, *L. boulardi* has a longer free-running period ($\tau = 24.3$ h), weaker anticipation in permanent darkness and later activity under L:D conditions. These results are consistent with studies on the *per* gene in *Drosophila*. The mRNA-encoded proteins of the mutant per^S cycle, with a shorter period and earlier peak than the wild-type, result in a phase advance in locomotor activity (Hall 1995; Hamblen *et al.* 1998). If indeed the circadian period determines the timing of activities (Aschoff 1981; Pittendrigh 1981), then selection acting on the phase of the rhythm (phenotypic expression) could result in variation in the endogenous period (genetic basis), which may vary between species in order to time their activities optimally. Figure 1*b*, where the activity patterns are corrected by the circadian periods, indicates that the phase angle between circadian oscillation and the L:D cycle also varies between species, thus confirming our results that temporal partitioning is endogenously controlled.

It could be argued that locomotor activity is not an accurate predictor of parasitoid fitness since it may only be loosely associated with actual oviposition. We therefore observed the daily oviposition rhythms under a L:D cycle, a trait more tightly linked to reproductive success. Although there are differences in the detailed patterns of the locomotor and ovipositional activities, the temporal order of the two is the same between species (figure 2 and table 1). The two earliest species are *A. tabida* and *L. heterotoma*, which mainly parasitize hosts in the morning and show a significant correlation between locomotor activity and oviposition ($r = 0.63$ and $p = 0.03$ and $r = 0.79$ and $p = 0.002$, respectively). *Leptopilina boulardi* oviposits later at the time of its main peak of locomotor activity, but the correlation in activities is not significant ($r = 0.18$ and $p = 0.58$), apparently due to the morning exogenous peak in locomotion under L:D conditions. Thus, temporal segregation of the species is confirmed for parasitoid oviposition. The circadian period of the oviposition rhythm cannot be measured, but we can assume that the period is the same between rhythms as it was demonstrated in *Drosophila* mutants for other different rhythms such as emergence and locomotor activity (Konopka & Benzer 1971).

Table 1. *Parameters of the activity rhythms of competing* Drosophila *parasitoids*

(The data were analysed using ANOVA and the *p*-values are given (last line). Differing superscript lower-case letters represent significant difference between means within columns ($p < 0.05$).)

	locomotor activity rhythm			oviposition rhythm
	phase peak 1 (L:D) hours ± s.e	phase peak 2 (L:D) hours ± s.e	period (τ) hours ± s.e	phase hours ± s.e
A. tabida	$4.54^{a} \pm 0.19$	$8.11^{a} \pm 0.06$	$21.33^{a} \pm 0.16$	$11.62^{a} \pm 0.86$
	($n = 28$)	($n = 28$)	($n = 6$)	($n = 13$)
L. heterotoma	$7.29^{b} \pm 0.15$	$13.8^{b} \pm 0.2$	$22.66^{b} \pm 0.06$	$12.14^{a} \pm 0.58$
	($n = 30$)	($n = 30$)	($n = 30$)	($n = 28$)
L. boulardi	$7.88^{c} \pm 0.14$	$18.7^{c} \pm 0.1$	$24.30^{c} \pm 0.11$	$16.81^{b} \pm 0.52$
	($n = 33$)	($n = 33$)	($n = 28$)	($n = 21$)
	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$

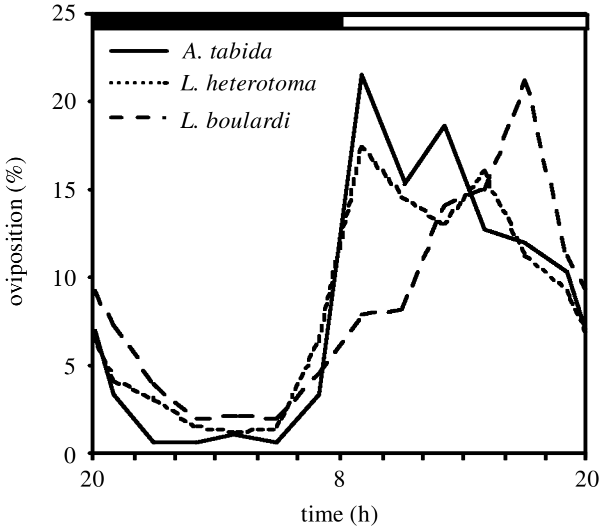

Figure 2. Temporal organization of parasitoid oviposition. The mean curves of the oviposition rhythms of the three *Drosophila* parasitoids (oviposition was measured at 2 h intervals).

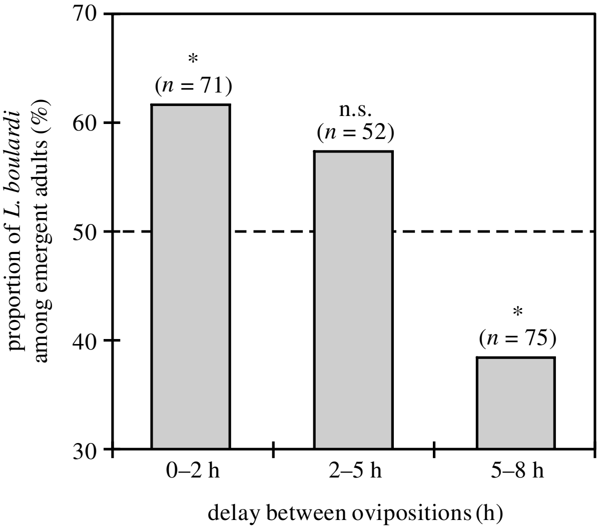

Figure 3. Reversal of the outcome of intrinsic larval competition in multiparasitized hosts with increasing delay between ovipositions by *L. heterotoma* and *L. boulardi*: with a delay of 0–2 h *L. boulardi* outcompetes *L. heterotoma* ($\chi^2 = 4.07$ and $p = 0.04$), with a delay of 2–5 h the probability of a competitive victory is not significantly different from 50% ($\chi^2 = 1.23$ and $p = 0.27$) and with a delay of 5–8 h *L. heterotoma* out-competes *L. boulardi* ($\chi^2 = 3.85$ and $p = 0.05$). The dotted line is the expected value under symmetrical competition (50%).

Finally, to determine whether the differences in the rhythm phases were sufficient to counteract the intrinsic competitive differences between the parasitoids, we investigated how the temporal organization of parasitoid oviposition affected the outcome of larval competition within multiply parasitized hosts. For short intervals (0–2 h), *L. boulardi* usually out-competes *L. heterotoma* (figure 3), confirming the higher intrinsic competitiveness of the former (Carton *et al.* 1991). For 2–5 h intervals, the competition is more balanced and for longer intervals (5–8 h), *L. heterotoma* most often eliminates *L. boulardi*. This shift in parasitoid success on a scale of several hours corresponds to the delay we observed in the activity patterns of the two species (table 1). Therefore, by characteristically attacking hosts several hours earlier in the day, *L. heterotoma* reduces its intrinsic competitive disadvantage to *L. boulardi*. The fact that the overall outcome of competition in multiparasitized hosts is probabilistic and that selection acts to maintain the differences in the activity periods is supported by field data showing that 44% of second-instar *Drosophila* larvae bore more than one parasitoid larva ($n = 201$, the population at Valence, south of France).

4. CONCLUSION

Oviposition is connected with locomotor activity in the three species of parasitoids. The circadian period of the endogenous oscillation, which correlates positively with the rhythm phase, is probably involved in the determination of the time of activity, consistent with previous results on the dependence of phase on period (Aschoff 1981; Pittendrigh 1981). Thus, although the free-running rhythm does not express itself in the wild, its endogenous period could be indirectly selected because of its involvement in the phase of the entrained rhythm. In the *Drosophila* parasitoid community, larval competition could act as a selective pressure on the phase of the adult parasitoid rhythm and, thus, on the endogenous period which varies accordingly between species. However, this trait is exposed to other selective pressures, particularly those responsible for fitness gain when the circadian period is close to 24 h (Ouyang *et al.* 1998). The endogenous period of each species would thus correspond to a balance between these opposite pressures, leading to values departing from 24 h. Different evolutionary outcomes are expected depending on species-specific features, namely competitiveness, the temporal availability of resources and predation. In *Drosophila* parasitoids, the higher the competitiveness, the closer the expected period to 24 h through direct fitness maximization; the lower the competitiveness, the shorter the expected period through selection for an early activity phase. However, why the activity of the superior competitor *L. boulardi* occurs late in the afternoon remains unexplained. Possible explanatory hypotheses are the consequences of a longer endogenous period, better availability of a host despite the absence of a circadian rhythm in *Drosophila* larvae (Sawin *et al.* 1994) or the result of asymmetrical reciprocal selective pressures between competitors.

This study indicates that, in *Drosophila* parasitoid communities, competition is the dominant selective force acting on activity rhythms. This confirms previous results which have demonstrated a different pattern of activity in *L. heterotoma* populations originating from more northern parts of Europe where its competitor *L. boulardi* is absent (Fleury *et al.* 1995). The overall result of natural selection leads to temporal segregation of competing species which could promote their coexistence in the wild. The fitness gain obtained by species which anticipate the activity of their competitors demonstrates the adaptive significance of the endogenous control of daily rhythms. Moreover, the relationship between the phase of the rhythm and the endogenous period of the clock, suggesting that period could be selected through the phase, which is directly exposed to natural selection, gives a possible adaptive explanation for endogenous circadian period variation among closely related species.

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