

Demasking biological oscillators: Properties and principles of entrainment exemplified by the *Neurospora* circadian clock

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Edited by Joseph S. Takahashi, Northwestern University, Evanston, IL, and approved April 14, 2005 (received for review March 8, 2005)

Oscillations are found throughout the physical and biological worlds. Their interactions can result in a systematic process of synchronization called entrainment, which is distinct from a simple stimulus-response pattern. Oscillators respond to stimuli at some times in their cycle and may not respond at others. Oscillators can also be driven if the stimulus is strong (or if the oscillator is weak); i.e., they restart their cycle every time they receive a stimulus. Stimuli can also directly affect rhythms without entraining the underlying oscillator (masking): Drivenness and masking are often difficult to distinguish. Here we use the circadian biological clock to explore properties of entrainment. We confirm previous results showing that the residual circadian system in *Neurospora* can be entrained in a mutant of the clock gene *frequency* (*frq⁹*, a strain deficient in producing a functional FRQ protein). This finding has implications for understanding the evolution of circadian programs. By comparing data sets from independent studies, we develop a template for analyzing, modeling, and dissecting the interactions of entrained and masked components. These insights can be applied to oscillators of all periodicities.

clock gene | light | masking | model | temperature

The periods of biological oscillations, based on biochemical and neuronal processes or even predator-prey interactions, range from milliseconds to years (1). A subset of these oscillators are synchronized (entrained) to cycles that themselves can be endogenous or environmental. The most important environmental cycles that synchronize endogenous oscillators are the tides, the rotation of the earth, the lunar cycle, and the seasons. Without information about the environment's temporal structure (in constant conditions), the corresponding biological oscillators can run "free" with their endogenous period, approximating but not necessarily identical to that of the environmental cycle. These four circa-rhythms (circatidal, circadian, circalunar, and circannual) serve as endogenous clocks to organize the internal temporal program in accordance with and in anticipation of exogenous changes.

Entrainment of any oscillator (from mechanical to biological) works through the same principles. Depending on its continually changing phase, an oscillator responds differently to an entraining stimulus (2): At some phases, the oscillator is advanced and at others it is delayed or it may not respond at all. These response characteristics, which can be drawn as a phase response curve, are determined by a number of properties: (i) the period, (ii) strength, and (iii) duration of the entraining stimulus as well as the (iv) period and (v) inherent responsiveness of the oscillator to the entraining stimulus. This basic principle has numerous consequences:

1. The period of an entrained oscillator (at least when averaged over several cycles) is identical to that of the entraining cycle and its phase relationship (Ψ) to the entraining stimulus

- remains constant (in case of the circadian clock, the time of the human temperature minimum could be related to dawn).
2. An oscillator may go through several cycles before it reaches stable entrainment (in the case of the circadian clock, we experience these "transients" during jetlag).
3. When oscillators are submitted to entraining cycles of different periods (T), Ψ can change systematically, ranging from relatively more delayed in short cycles to relatively more advanced in long cycles (see examples in Fig. 2) (3). The relationship between Ψ and T is not necessarily linear. The slope of $\Psi = f(T)$ depends on the properties (i-v) listed above and, therefore, does not necessarily show a 1:1 relationship.
4. When the entraining stimulus is very strong (or if the oscillator is very responsive), the slope of $\Psi = f(T)$ can approach zero, i.e., Ψ does not change with T . Under these conditions, the oscillator is said to be driven by the entraining stimulus.
5. Oscillators can only be entrained within a given range of periods (known as range of entrainment), which again depends on the listed properties (i-v). When the period of the entraining cycle is close to half of the oscillator's own period, the oscillator may entrain to every second entraining cycle (known as frequency demultiplication).
6. Entrainment is not restricted to self-sustained oscillators (such as intact circadian clocks). Any oscillator, even one that is damped with no self-sustained period, can be entrained with the characteristics described above (consequences 1-5).
7. Beyond their entrainment function, entraining stimuli can have acute effects on rhythms that are controlled by the oscillator (e.g., locomotor activity of mice is acutely suppressed by light). This process is called masking, and it is part of how an organism adapts to environmental changes (4). Masking makes investigations of the underlying oscillator more difficult.

By far the best understood biological oscillator is the circadian clock. Although its investigation in constant conditions is important to understand the mechanisms that generate the self-sustained oscillation, an understanding of how biological clocks function under entrained conditions is the key for understanding their full biological significance (2, 5). Circadian clocks entrain to environmental cycles through signals called zeitgeber (e.g., light or temperature). The principles of entrainment have been extensively studied by the pioneers of circadian research (6) and can be applied at all levels, from behavior and physiology down to the molecular clocks of individual cells (7).

Although the fundamental principles of entrainment have been published in the annals of circadian research (6, 8, 9), there still remains some confusion. A recent paper (10) repeated investigations of entrainment in *Neurospora* by temperature

This paper was submitted directly (Track II) to the PNAS office.

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cycles, concluding that it occurs only when the molecular feedback loop involving the clock gene *frequency* (*frq*) is intact. The authors' interpretations contradict results that we published 6 years ago (11).

Here, we use the comparison between the two data sets to highlight complications that arise when studying entrainment and to define general properties and principles of entrainment and masking. We demonstrate masking and drivenness and show how entrainment and masking influence the rhythm's waveform and consequently affect different phase reference points. Finally, we present methods and models that help to distinguish between masking and entrainment.

All points are illustrated by using *Neurospora crassa*, an excellent and tractable genetic model system for rhythms research. This simple fungus can serve as a powerful tool for understanding the general mechanisms of biological oscillators and their entrainment, which can be extrapolated and serve as a road map for describing biological oscillators of all frequencies.

Materials and Methods

Strains and Media. The strains and media used here are as described in ref. 11. The *frq⁹* strain carries a frame shift mutation resulting in a premature stop codon. The phenotype of the *frq⁹* strain is indistinguishable from the null mutant, *frq¹⁰* (10, 12–16). For reasons of simplicity, we will therefore refer to this strain as *frq^{NULL}* and to the *frq*-sufficient strain as *frq^{WT}*.

Temperature and Air-Flow Cycle Conditions and Protocols. The temperature cycles are as described in ref. 11. Note that the temperature is regulated in large enclosed water baths, ensuring 100% humidity and smooth, gradual and slow temperature transitions. So-called air-flow cycles were performed by periodically passing air through race tubes attached to an aquarium pump (375 ml/min). In the experiment shown here, air-flows were introduced for 2 h every 18 h. Except for the air flow, race tubes were kept in constant conditions (darkness and 25°C).

Data Analysis. To exclude all subjective decisions on phase calculations, we performed a completely automatic reanalysis of previously published experiments (11, 16). Omitting the first and the last day of each recording, daily profiles of *Neurospora*'s banding rhythm (similar to those shown in Fig. 4) were calculated from the raw data (i.e., without smoothing or trend correction) of density scans of single race tubes. The time bases of the daily profiles corresponded to the respective cycle length (*T*). A two-component cosine function was fitted to each profile. Composite cosine fits using the fundamental and the first harmonic are extremely powerful in describing most waveforms (17). The average correlation coefficient of these least-square fits was $r = 0.93 \pm 0.07$. Troughs, onsets (upward transition through the zero line of the cosine fit), peaks, and offsets (downward transition through the zero line of the cosine fit) were calculated based on the cosine fits (Table 1; see also Fig. 2).

Phase reference points were plotted in degrees (360° per cycle, as used in ref. 16) rather than real hours (as used in refs. 10 and 11). In the entrained state, circadian oscillators and their outputs are fully adjusted to the zeitgeber cycle, regardless of cycle length. Thus, using degrees is more appropriate to represent the data. It eliminates arbitrary decisions as to which part of the zeitgeber the calculated phases should be related to (e.g., onset of cold versus onset of warm phase, see figure 2B of ref. 10). In addition, using degrees allows the correct use of standard deviations: If the timing of a phase marker varies (e.g., between individual race tubes), differences measured in hours (and not degrees) misrepresent the variance in short versus long cycles.

To identify subtle responses to a stimulus, it is helpful to record as many responses as possible and compile them into a single profile. We used this method to investigate the response

Table 1. Statistical analysis of the data shown in Fig. 2

Strains	This study			Pregueiro et al. (10)	
	Slope	<i>r</i>	<i>P</i>	Slope	<i>P</i>
<i>frq^{WT}</i>					
Trough	26.0	0.93	<0.001		
Onset	23.6	0.98	<0.001		
Peak	25.9	0.98	<0.001		
Offset	26.2	0.90	<0.001		
<i>frq^{NULL}</i>					
Trough	20.4	0.86	<0.001		
Onset	20.5	0.85	<0.001		
Peak	21.6	0.83	<0.001		
Offset	20.7	0.83	<0.001		
<i>frq^{WT}</i>					
Trough	1.5	0.95	<0.001	1.70	<0.001
Onset	1.1	0.97	<0.001	1.05	<0.001
Peak	1.0	0.97	<0.001	0.80	<0.001
Offset	0.8	0.96	<0.001	0.91	<0.001
<i>frq^{NULL*}</i>					
Trough	1.6	0.91	<0.001	0.20	0.001
Onset	1.3	0.86	<0.001	0.50	<0.001
Peak	1.2	0.79	<0.001	−0.05	n.s.
Offset	0.9	0.72	<0.001	0.16	0.02

Slopes, regression coefficients, and significance levels are calculated by using the results of each single race tube ($n = 60$), not the means \pm SD as used in Fig. 2. Strains are shown with phase reference points; the values in the upper half of the table are in degrees, and the values in the lower half of the table are in real hours. Probabilities in our data ranged from $P \leq 10^{-12}$ to 10^{-247} . n.s., Not significant.

*Values for *frq^{NULL}* under Pregueiro et al. (10) include *frq⁹* and *frq¹⁰*.

to short temperature cycles ($T = 12$ h), which we then plotted as two consecutive 12-h cycles. We compiled 88 responses (each of 24-h or two 12-h warm:cold cycles) (see profiles in Fig. 4 A, B, D, and E) and 54 responses (nine of these cycles from six race tubes) (see Fig. 4 C and F).

Results and Discussion

Clock Mutant (*frq^{NULL}*) Strains Entrain to Temperature Cycles. As described above, every oscillator can be entrained given the right conditions. The question at hand is whether there is entrainment in *Neurospora* without *frq*. It has long been known that rhythms in the circadian range can be recorded when *frq^{NULL}* strains are kept in constant conditions (12): Their conditional arrhythmicity appears to be more a special case than a rule (18–21). Conditional arrhythmicity has also been shown for clock mutants in other organisms; e.g., *Per2* mutant mice are arrhythmic in constant darkness but not in constant light (22). In addition, circadian oscillations have recently been shown in *frq^{NULL}* *Neurospora* strains by following the activity of nitrate reductase (23) and RNA profiles (24) as read-outs. Thus, *frq^{NULL}* strains possess a residual circadian system that can be entrained given the right zeitgeber stimulus. But what are appropriate zeitgebers for the *frq^{NULL}* residual *Neurospora* clock? Light apparently is not useful because *frq^{NULL}* strains are partially blind (11, 18, 25, 26).

We used temperature cycles (alternating between 22°C and 27°C) of different length ($T = 16$ –27 h) as zeitgebers and recorded the rhythmic production of asexual spores (conidia) as a read-out of *Neurospora*'s circadian clock. Under these conditions, *frq^{NULL}* strains entrain with the characteristics described in the introduction (see ref. 11 and the examples in Fig. 1). The signature feature indicating systematic entrainment is that the entire biological oscillation progressively moves earlier with respect to the physical oscillation (temperature) when cycle length (T) is increased. Previously, we showed the dependence

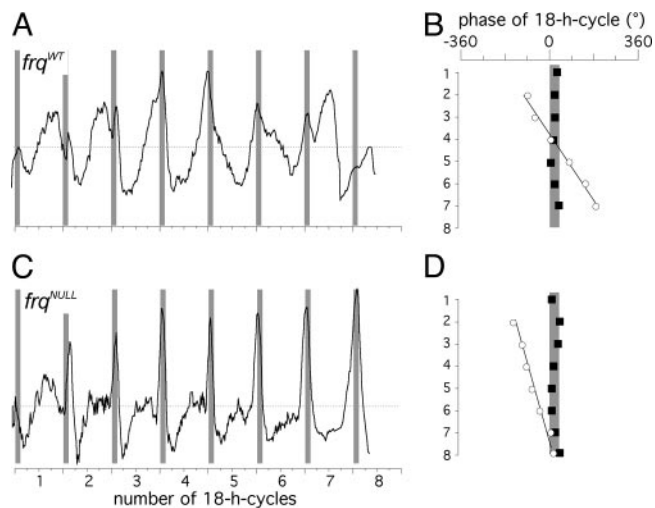


Fig. 3. Masking effects of air-flow on the conidiation rhythm in *frq*^{WT} (A and B) and *frq*^{NULL} (C and D) strains. A pump attached to a timer supplied a gentle air-flow through the race tubes for 2 h every 18 h (vertical gray bars). (A) Time series of the conidiation rhythm in *frq*^{WT} (average of four race tubes; data are plotted as deviation from the 18-h mean). (B) Phase plot of the maxima of the endogenous (○, 21-h period) and masked (■, 18-h period) component calculated from the time series shown in A. (C) Time series of the conidiation rhythm in *frq*^{NULL} strains (average of four race tubes). (D) Phase plot of the maxima of the endogenous (○, 19-h period) and the masked (■, 18-h period) component calculated from the time series shown in C. Phases for the maxima of the endogenous component were calculated by the demasking procedure described for Fig. 5. Note that all graphs use an 18-h time base.

Because the distinction of drivenness and masking concerns each of the alternative hypotheses, we must first explore acute effects on conidiation (masking) and drivenness of the clock. The following examples show masking by a stimulus that does not entrain the circadian clock, masking in the context of frequency demultiplication, and, lastly, a model that combines an endogenous oscillator and a masking component in the context of T-cycles within the range of entrainment. The latter two examples also provide methods for demasking endogenous oscillators.

Masking Without Entrainment. The discrepancies between our results and those of the recent study (10) indicate differences in experimental conditions. The experimental setups were distinct: whereas we used a closed water bath system to keep humidity constant, air-controlled chambers were used in the recent study where temperature changes are accompanied by changes of relative humidity. That air composition within race tubes affects the conidiation rhythm has been known since the first use of the *band* mutation, which was discovered because it allows the visualization of circadian bands of dense conidia formation despite CO₂ accumulation (32).

To investigate the possibility of air acutely affecting (masking) the circadian conidiation rhythm, we performed a series of experiments where air was passed through race tubes by a pump attached to a timer. The example shown in Fig. 3 represents a T-cycle experiment with a period of 18 h, with air flow for 2 h in each cycle. As can be seen in Fig. 3 A and B, two components oscillate with different periods. The period of one of the components is identical to that of the T-cycle (18 h), and the other is close to *Neurospora*'s free-running, circadian period (21 h). *Neurospora*'s conidiation rhythm is not entrained by the rhythmic air-flow, yet the 2-h pulses of air have a direct (masking) effect on conidiation density. When the two components merge, the amplitude increases either because the two components are additive or because the masking response is

itself regulated by the oscillator. That the sensitivity of acute responses to a stimulus can be under circadian control has been shown in several different model systems (e.g., refs. 25 and 33). The masking effect of the air-flow is much stronger in the *frq*^{NULL} strain than in the *frq*^{WT} strain (Fig. 3 C and D), but two rhythmic components (masked and endogenous) can still be distinguished with periods of 18 and 19 h, respectively. These results show that masking, which is known to exist in many other circadian systems (4, 34–37), also exists in *Neurospora*.

Masking in the Context of Frequency Demultiplication. As described in the Introduction, circadian clocks can entrain to a multiple of zeitgeber cycles when the cycle length is close to a half (or to a third or fourth, etc.) of the endogenous period. This observation depends on a robust circadian oscillator, which is neither driven nor masked by the zeitgeber stimulus. It is surprising, then, that in *frq*^{WT} *Neurospora* no demultiplication occurs in 12-h cycles that use light as a zeitgeber (11). Furthermore, in light:dark cycles of different lengths, conidiation occurs ≈5–6 h after dusk, i.e., Ψ does not change with T (11). These observations suggest that the *Neurospora* clock is driven by light cycles; it was only when we explored further, by investigating *Neurospora* in different photoperiods, that we were able to show that the underlying circadian oscillator is not driven (38). Thus, apparent drivenness can be probed further with different conditions, e.g., by different zeitgeber strengths.

We had observed that *frq*^{NULL} strains apparently fail to demultiply in 12 h (6 h each of 22°C and 27°C) temperature cycles (16, 39), a finding repeated by ref. 10. By using a new form of analysis dedicated to identify subtle response characteristics (see *Materials and Methods*) and by changing zeitgeber strength, we can demonstrate that masking obscures frequency demultiplication of the residual circadian system in *frq*^{NULL} strains. Fig. 4 A shows 24-h profiles (compiled from raw data) for 11 race tubes for the *frq*^{WT} strain in a 12-h temperature cycle. When the profiles are averaged (Fig. 4 B) robust demultiplication is obvious, accompanied by a smaller masking component (indicated by the two arrows). By decreasing the amplitude of the temperature cycle (as well as the mean temperature), the demultiplication becomes even clearer as the masked component almost disappears (Fig. 4 C). Fig. 4 D and E shows the 24-h bins and their average for the *frq*^{NULL} strain. Although conidiation responds to each temperature change, the conidiation patterns in the successive 12-h cycles are clearly distinguished in shape and amplitude. This so-called beat indicates the demultiplication of an oscillator that entrains to 24 h rather than to 12 h. As for the *frq*^{WT} strain, the demultiplication becomes clearer in the lower amplitude temperature cycle (Fig. 4 F). Modeling the *Neurospora* circadian system based on our data (11, 16) suggests that “the wild-type circadian period of *Neurospora* is encoded in the residual network of the null *frq* mutants” (40).

These examples show the importance of experimental explorations and detailed analysis when distinguishing between drivenness and masking. Entrainment is a complex phenomenon, and conclusions based on the absence of an entrainment quality are, therefore, premature and unsupported unless the absence persists under a variety of different conditions and/or evaluation procedures.

A Model for Demasking the Circadian Component. Although we find that the slopes of the relationship between Ψ and T are close to parallel for all phase reference points in the *frq*^{NULL} strain, they vary greatly in the study by Pogueiro *et al.* (10). Different slopes for different phase reference points arise when the wave form of the rhythm changes in different T-cycles. Changes in waveform suggest the influence of two processes, e.g., an endogenous rhythm, the phase of which varies with T , and a masking component that is phase-locked to T (i.e., always occurs at the

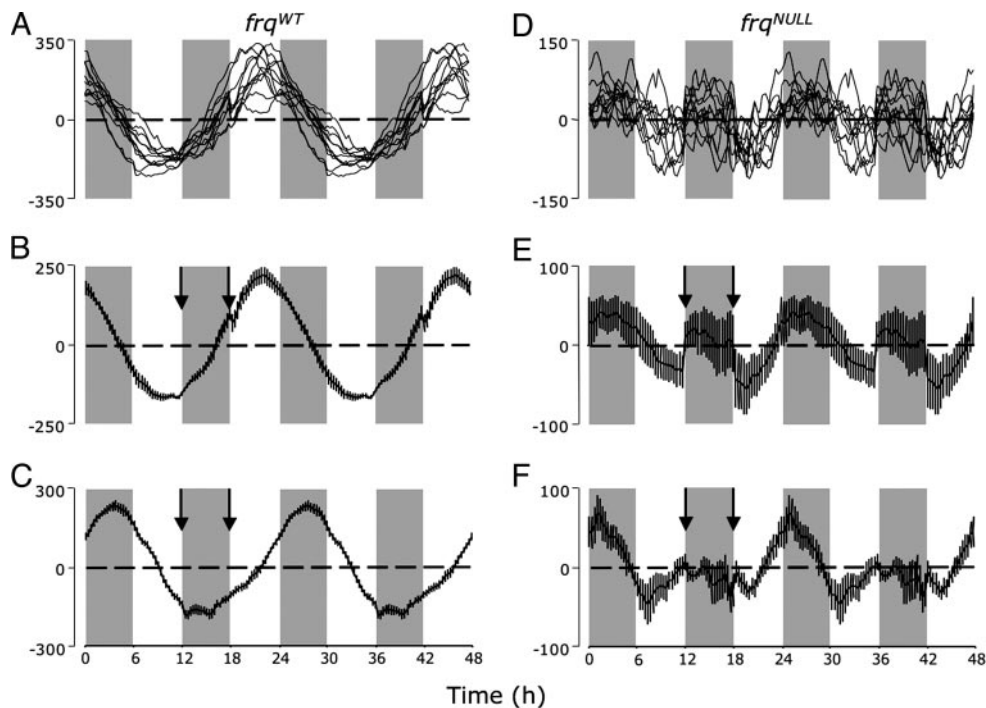


Fig. 4. Frequency demultiplication in 12-h temperature cycles using different mean temperatures and different temperature amplitudes. Profiles of condensation density of *Neurospora* exposed to temperature cycles alternating between 6 h of cold (gray areas) and 6 h of warm (white areas; temperatures are indicated for each graph). (A, B, D, and E) Results from temperature cycles of 22°C:27°C experiments. In A and D, the density tracings for 11 race tubes were broken up into 24-h segments and averaged to daily profiles for each race tube, which are further averaged to the profiles shown in panels B and E (\pm SEM; see *Materials and Methods*) (C and F) Results from temperature cycles from 20°C:23°C experiments. A similar analysis is shown for the lower amplitude and lower mean temperature cycle (in this case, six race tubes per strain contributed to the averaged profiles, see *Materials and Methods*). (A–C) The *frq*^{WT} strain. (D–F) The *frq*^{NULL} strain. Arrows indicate evidence of masking. Data are double plotted to facilitate visualization.

same phase of the cycle). We have shown here that a residual circadian oscillator exists in *frq*^{NULL} strains (Figs. 1 and 2) and that acute (masking) responses to air changes (Fig. 3) and/or temperature (Fig. 4) are far stronger in these strains than in *frq*^{WT} strains. These results indicate that the data of Pregeloro *et al.* (10) are in accordance with the third hypothesis: that systematic entrainment to temperature is present in *frq*^{NULL} strains but that it is masked. Masking would also provide an answer to the two initial questions, why slopes differ between phase markers in the results of Pregeloro *et al.* (10) and why onsets are the most similar phase markers between their data and ours.

To test this hypothesis, we used a simple mathematical model comprising a masked component (phase-locked to the temperature cycle, thin curves in Fig. 5 *Left*) and a sinusoidal component representing the circadian clock (thick curves in Fig. 5 *Left*). The endogenous component was moved for each respective cycle length according to phase angles predicted by the *frq*^{WT} strain in both studies and by our results for the *frq*^{NULL} strain. To model the actual time series, the two components were simply added (Fig. 5 *Right*). The resulting rhythms clearly change shape in different lengths (T) of the zeitgeber and match those published for the *frq*^{NULL} strains in ref. 10. Even small details of the rhythms' waveforms and phases of the four reference points are predicted by this modeling exercise.

By directly applying our model to the data published by Pregeloro *et al.* (10), it should be possible to demask the phase angles of the endogenous component. We traced the time series shown for the *frq*^{NULL} strain in figure 2a from ref. 10 and correlated them with the model. The model's endogenous component was moved so that the modeled and published data gave the best (least-square) correlation (Table 2). The slope of the relationship between Ψ and T derived from the demasked phase angles for $T \leq 24$ is 16.3°/h (0.78 h/h). This value changes to 12.1°/h (0.56 h/h) when the $T = 26$ is included. Note that the peaks of the *frq*^{WT} strain for $T > 24$ in the recent study (see figures 2B and 3B in ref. 10) behave differently compared with ours. The modeled slope for $T \leq 24$ is flatter than that for the *frq*^{NULL} strain found in our data set (indicating a stronger

zeitgeber), and the slope including $T = 26$ is close to that found for onsets by Pregeloro *et al.* (10).

The results of this demasking procedure show that the differences between the two studies can be analytically explained by hypothesis *iii*, which suggests an entrainment pattern that is

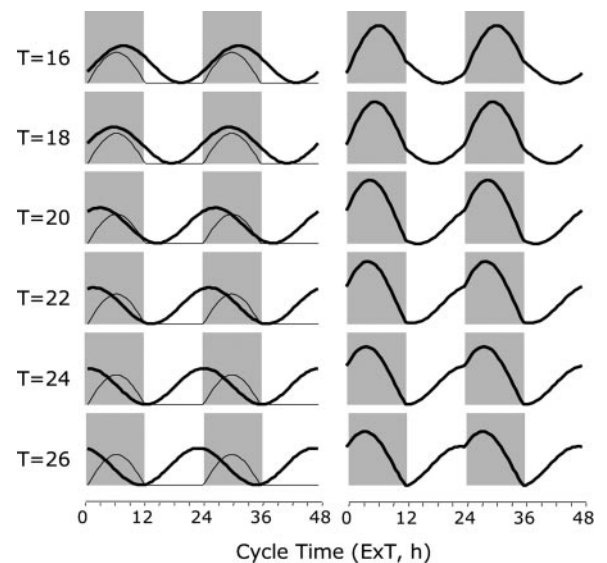


Fig. 5. Modeling an endogenous (oscillator) component and a masked component in temperature cycles of different lengths (T is indicated on the left). (*Left*) The two components are shown separately (thick lines, endogenous; thin lines, masked). (*Right*) The two components are combined (by simple addition). Data are plotted based on cycle time [expressed as external time (EXT); see ref. 43]. Although the endogenous component progressively moves to earlier phases in reference to the temperature cycle, the masking component remains phase-locked. The resulting curves in *Right* show how the waveform of the rhythms changes systematically with cycle length (T). The model shown here can be used as a demasking procedure whereby the endogenous component is moved until correlations with the actual data give the best, least-square fit. For results of applying this procedure to the data by Pregeloro *et al.* (10), see Table 2.

Table 2. Model results

T , h	$\psi_{\text{peak}, \circ}$	$\psi_{\text{peak}, \text{h}}$	r
16	112.50	5.00	0.991
18	83.33	4.17	0.990
20	42.00	2.33	0.979
22	21.82	1.33	0.986
24	-20.00	-1.33	0.989
26	9.23	0.67	0.981

Results of applying the demasking procedure (shown in Fig. 5) with the traced data for $\text{freq}^{\text{NULL}}$ from ref. 10. Peak phases of the endogenous component resulting from the best fit procedure are given in degrees and real hours for each cycle length (T); model fit (r) values indicate the qualities of the respective fits.

obfuscated by masking components. Note that the difficulties arising from masking can be problematic in any entrainment protocol of any oscillator. In the case discussed here, peaks are more heavily masked than other phase reference points, which is also indicative in the statistical analysis of the recently published data (Table 1; see also table 1 in ref. 10): Peak is the only reference point that does not change significantly with T in the $\text{freq}^{\text{NULL}}$ strain.

Based on our analytical approach, we conclude (contrary to

the claims made in ref. 10) that our finding of entrainment to temperature cycles has been replicated independently in the three laboratories represented in that paper. The rudimentary circadian system in $\text{freq}^{\text{NULL}}$ strains entrains to different T-cycle lengths with systematically changing phase angles. Conidiation shows a strong masking component under the conditions used by Pogueiro *et al.* (10), and (owing to the masking component) onsets are, in this case, the best markers to estimate the behavior of the underlying circadian oscillator.

Evolution of the four circa-oscillators (tidal, daily, lunar, and annual) helped organisms adapt to a rhythmically changing environment. The recent characterization of oscillations with very short periods in yeast [regulating processes from metabolism to gene expression (41)] suggests that other biological oscillators evolved as a fundamental mechanism that avoided chaotic coordination between cellular subsystems by (mutual) entrainment (42). Thus, understanding properties of biological oscillators and their entrainment is important far beyond the field of circadian research. By using the circadian clock, we have described a set of approaches and an exploration of formalisms that can be adapted to rhythms of all frequencies.

This paper is dedicated to Serge Daan on the occasion of his 65th birthday for contributing so much to the understanding of entrainment. This work was supported by the Deutsche Forschungsgemeinschaft and the Dr.-Meyer-Struckmann-Stiftung.

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