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CIRCADIAN PERIOD LENGTHS OF LIPID SYNTHESIS MUTANTS (*CEL*, *CHOL-1*) OF *NEUROSPORA* SHOW DEFECTIVE TEMPERATURE, BUT INTACT pH-COMPENSATION

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ABSTRACT

The influence of extracellular pH on the circadian sporulation rhythm of *Neurospora crassa* has been investigated for the mutants *chol-1* and *cel*. Both mutants have a defect in the lipid synthesis pathway and require either choline or palmitate, respectively, as supplements for normal growth. The *chol-1* and *cel* mutants also show an impaired temperature-compensation when growing on minimal medium. We investigated the possible correlation between loss of temperature- and pH-compensation in *cel* and *chol-1* similar to the correlation found earlier for the frq^7 mutant. Our results show that the *cel* and the *chol-1* mutants, although defective in temperature-compensation have an intact pH-compensation of their circadian rhythms. At present, the products of the *frq*-locus are the only components of the clock that affect the sporulation rhythm of *Neurospora* both through pH- and temperature-compensation. (*Chronobiology International, 19(3), 517–529, 2002*)

Key Words: Choline; Circadian rhythm; Clock mutants; Homeostasis of period; *Neurospora crassa*; Palmitic acid; pH; Temperature-compensation

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INTRODUCTION

Circadian rhythms are known to have regulatory clock functions in order to adapt organisms to daily and seasonal environmental changes (1–3). To act as physiological clocks, circadian rhythms contain homeostatic mechanisms which keep their period constant and compensate for external influences such as temperature, pH, or nutrient supply. Temperature-compensation (4) was first recognized as a fundamental property of circadian rhythms (5). In 1973, Pittendrigh and Caldarola proposed that temperature-compensation is only part of a more general homeostatic mechanism for the circadian clock (6).

A few model organisms have been extensively analyzed with respect to the molecular mechanisms of circadian clocks. Interestingly, in these organisms, functionally similar mechanisms for the different clocks have been found, which in all cases, involve a negative feedback regulation of certain "clock" genes (7). However, the roles of these clock genes are still not completely understood (8,9).

Neurospora crassa is a well-established model system for the investigation of circadian rhythmicity (9–11). The asexual sporulation rhythm is easily assayable in form of repeated bands of conidiospores when the mycelium is grown on agar in darkness. A variety of mutants are available which show different circadian properties such as altered period length or temperature-compensation. The circadian clock in *Neurospora* is considered to consist essentially of a transcription/translation feedback loop involving the rhythmic expression of the *frequency* (*frq*) gene, whose product, the FRQ-protein inhibits its own transcription. Additional positive and negative regulating elements have been identified (12–14), which play an important role for example in the resetting of the clock by light (13–15) and in the determination of the amplitude (16) of the rhythm. Both short and long period alleles at the *frq* locus are known, the long period mutant frq^7 showing an impaired temperature-compensation (17). The loss of temperature-compensation in frq^7 may be explained by a more stable FRQ^7 protein with an increased activation energy in FRQ^7-degradation (18). In accordance with these predictions, the half-life of FRQ^7 has been found to be approximately twice as long as the wild-type FRQ^+ half-life (19). In addition, Liu et al. (20) have recently shown, by blocking certain phosphorylation sites in FRQ, that an increased stability of FRQ leads to an increase of the period length, precisely as predicted by the Goodwin model (18).

In previous work (21), it was shown that the circadian sporulation rhythm in wild-type *Neurospora* is pH-compensated, while in frq^7 pH-compensation, like temperature-compensation, is impaired. Thus, the *frq*-mutants show an interesting correspondence between temperature- and pH-compensation, which may be linked to the stability of the FRQ-protein and its possible role for the homeostasis of the circadian period. We wondered whether a similar correlation between pH- and temperature-compensation may exist in other than *frq* clock mutants. Studies on the *chrono* (*chr*) mutant already showed (21) the existence of pH-compensation of the period while temperature-compensation was impaired. Two other interesting

Neurospora clock mutants are *cel* and *chol-1*. The *cel* strain (22) is defective in fatty acid synthesis and requires saturated fatty acids for normal growth and rhythmicity. In addition, temperature-compensation is lacking in *cel* mutants (23). The other, *chol-1*, is also a lipid-deficient mutant in which the synthesis of the phospholipid phosphatidylcholine in membranes is blocked resulting in an altered membrane lipid compositions (24,25). Unlike *frq*, the altered lipid metabolism and membrane properties in *cel* and *chol-1* are expected to influence a wide range of physiological processes. We therefore considered it promising to investigate the influence of extracellular pH on the circadian period of *cel* and *chol-1*. Here, we show that, surprisingly, both the *cel* and the *chol-1* mutants exhibit pH-compensation, in spite of the fact that both mutants have defective temperature-compensation. This indicates that even "global" changes in the lipid composition of cell membranes may not affect pH-compensation of the circadian period, while a point mutations (i.e., a single amino acid exchange) in the FRQ^7-protein affects both temperature- and pH-compensation. In this respect, *frq* plays a more central role in the control of homeostasis of the circadian period than *cel* or *chol-1*.

MATERIALS AND METHODS

Strains

All investigated strains carry the *band* (*bd*) mutation, which reduces inhibition of condition by accumulating CO_2 (26). The strains (*csp-1; bd*), (*csp-1; cel bd*), and (*csp-1; chol-1 bd*) were a gift from Dr. Patrica Lakin-Thomas (University of Cambridge). Preliminary experiments with *csp-1; cel bd* were earlier (21) performed with a corresponding strain from the Fungal Genetics Stock Center (FGSC #3484, Kansas City, KS) and showed essentially the same results as reported here.

Culture Conditions and Assays

Cultures were grown in constant light in liquid Vogel's medium (11,27) with 2% sucrose (LL medium). After inoculation of approximately 1×10^8 conidia L^{-1} in Petri dishes (90 mm diameter) containing 20 mL LL medium, the culture was exposed to white fluorescent light (25 μmol sec^{-1} m^{-2}) until a mycelium of approximately 0.5–1 mm thickness was formed. Depending on the strain, this took between 40 and 60 h. At this stage, the *cel* and *chol-1* strains were grown with supplements, i.e., the medium contained either 0.004% palmitic acid or 200 μM choline (as choline chloride), respectively. The other strains were grown without supplements. Mycelial disks were then cut out (1 cm diameter) by a cork borer and were placed in Vogel's medium with low sucrose (0.4%) at constant darkness and at 25°C. After 12 h of darkness, mycelial disks were transferred to growth tubes that contained agar, nutrients, and sometimes supplements at different pH. After

another 12 h of growth at 25°C in darkness, the growing fronts were marked at the bottom of the growth tube under a red safety light. In general, marking was repeated every 24 h for 5–6 d depending on the growth rate. Only in case of the *csp-1; chol-1 bd* mutant (when no supplement was added) the growth rate was considerably slower than for the other mutants, and the average growth rate was determined after approximately 30 d of growth in darkness at 25°C. Also for the *csp-1; chol-1 bd* mutant without supplement the daily marking indicated a practically constant growth rate (Fig. 3a).

Preparation of Growth Medium and Growth Tubes

Growth tubes with medium of different pH were prepared as described earlier (21). Supplements for the *cel* and *chol-1* strains were added to the hot agar in the form of either a 10% solution of palmitic acid in 96% ethanol or in the form of an aqueous 50 m*M* choline chloride solution, respectively. The hot agar was then transferred into the tubes (15 mL each) and autoclaved for approximately 7 min. The pH inside the agar was determined by means of a special pH electrode as described earlier (21).

RESULTS

Influence of pH on Growth Rate and Period Length in *csp-1; cel bd*

Since all mutants used in this study contain the *csp-1* mutation, we also tested the *csp-1; bd* strain as a control. The *csp-1* mutation prevents that conidia separate and become airborne. The *csp* mutation is known to shorten the circadian period by about 1 h (28,29) but should behave otherwise as a wild-type. Figure 1a and b show *csp-1; bd* growth rates and periods as a function of extracellular pH, respectively. The *csp-1; bd* growth rates clearly depend on pH with a maximum between pH 4.5–5, while the period of the circadian sporulation rhythm is pH-compensated. In the presence of palmitic acid, the *cel* mutant behaves like *csp-1; bd* or wild type, i.e., shows pH-compensation of the circadian period (Fig. 1c and d). In the absence of palmitic acid, the growth of *cel* is significantly slower, but the period is still pH-compensated (Fig. 1e and f).

Influence of Choline Concentration on Growth Rate and Period Length in *csp-1; chol-1 bd*

When *csp-1; chol-1 bd* is grown in the presence of 200 μM choline at different extracellular pH both growth rates and periods behave as wild-type and pH-compensation of the circadian period is observed (Fig. 2a and b). In the absence of choline the average growth rates are slower approximately by one order

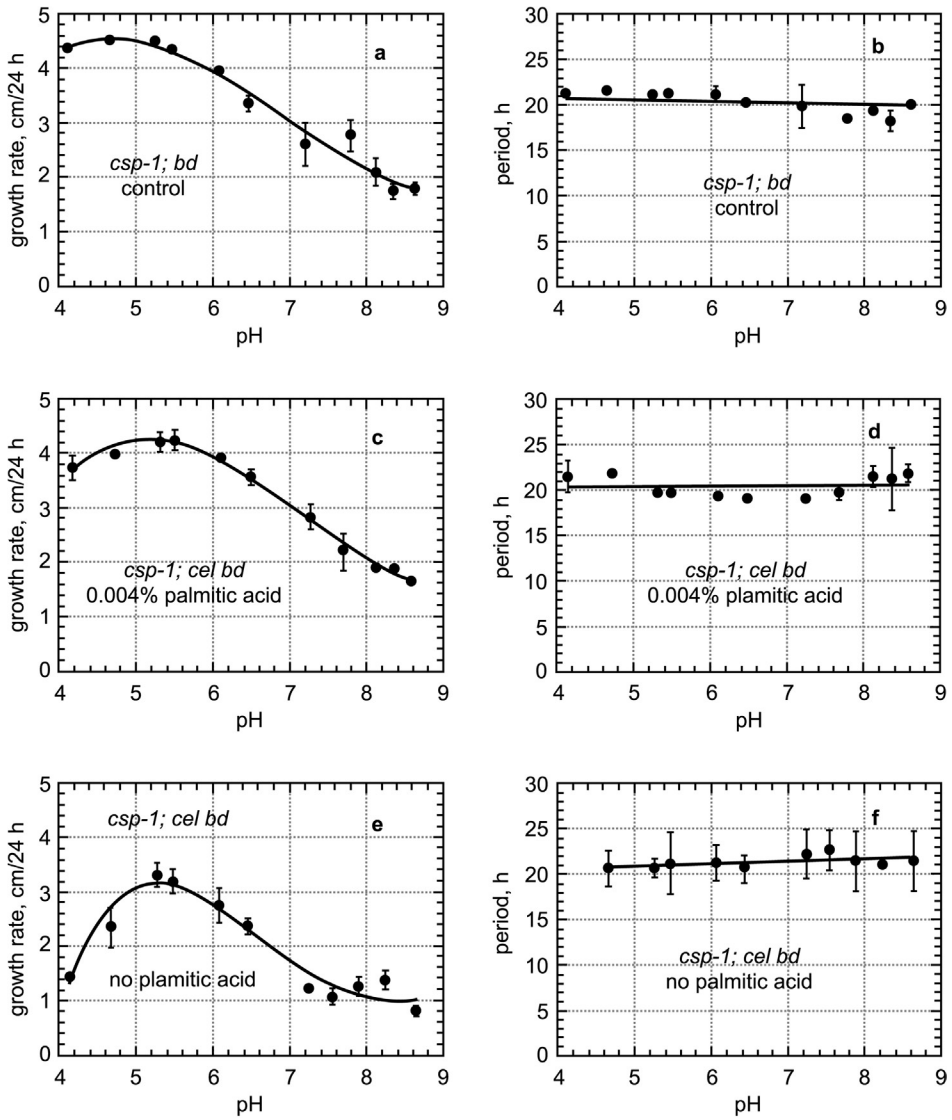

Figure 1. (a) Growth rate of *csp-1; bd* as a function of extracellular pH. (b) Period length of the *csp-1; bd* sporulation rhythm as a function of extracellular pH. (c) Growth rate of *csp-1; cel bd* as a function of extracellular pH and in the presence of 0.004% palmitic acid. (d) Period length of *csp-1; cel bd* as a function of extracellular pH and in the presence of 0.004% palmitic acid in agar. (e) Same system as in (c), but without palmitic acid. (f) Same system as in (d), but without palmitic acid.

of magnitude (Fig. 2c) when compared to the control (*csp-1; bd*) or when 200 μM choline was present in the medium. Because the mycelium was initially grown in the presence of choline, an "inital burst" of the growth rate is observed, which rapidly drops to slow linear growth probably due to choline depletion when

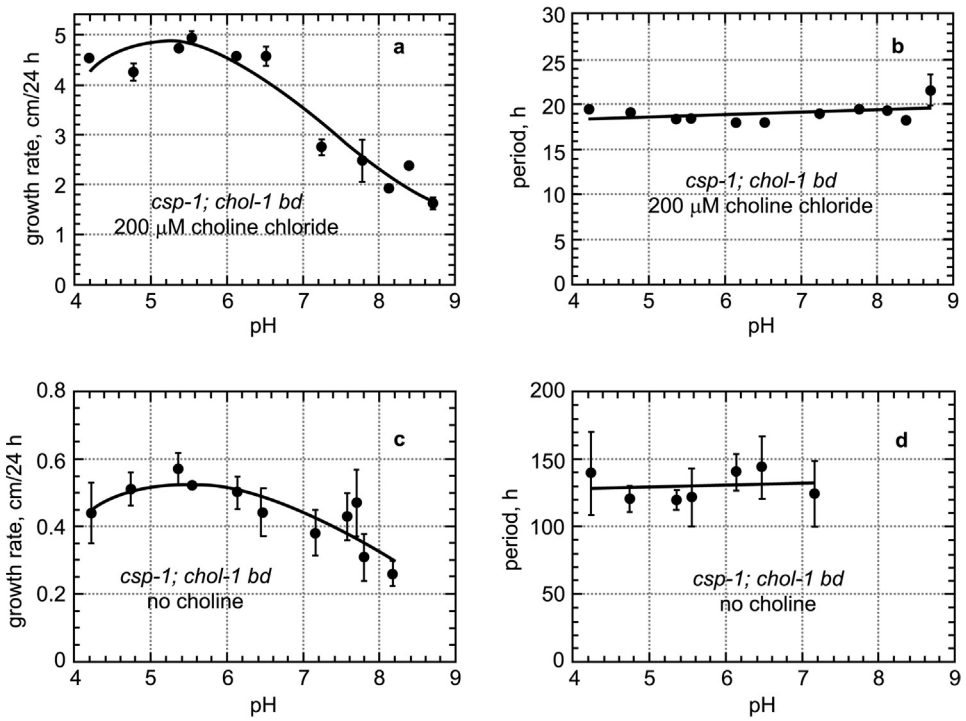

Figure 2. (a) Growth rate of *csp-1; chol-1 bd* as a function of extracellular pH and in the presence of 200 μM choline. (b) Period length of *csp-1; chol-1 bd* as a function of extracellular pH and in the presence of 200 μM choline chloride. (c) Same system as in (a), but without choline. (d) Same system as in (b), but without choline.

hyphae enter the growth tube medium without choline. Figure 3a shows the burst and the subsequent linear growth for several selected extracellular pH values. Figure 3b shows the average growth velocity during the first 18 h in the tube. The pH-profile of the burst is similar to the profile observed for *csp-1; bd* or wild-type although the growth rates in this case are not quite as high.

In the absence of choline the period length for *csp-1; chol-1 bd* is surprisingly high, around 120–130 h, but remains still pH-compensated (Fig. 2d). The influence of choline on growth and period length is indeed dramatic as can be seen in Fig. 4a and b. Figure 4a shows the growth and sporulation rhythm of *csp-1; chol-1 bd* in the presence of 200 μM choline during a six day long growth in DD, while Fig. 4b shows the same strain in DD and minimal medium (no choline) during a 30d period of growth. As seen in Fig. 4b, the rhythm becomes more "fuzzy" at higher pH and is difficult to observe for pH values higher than 7. At lower pH values, however, conidial bands in general are sharply separated. Little changes in the period have been found when the choline concentration in the medium was varied from 2.5 to 200 μM (Fig. 5). However, a jump to very high period lengths occurred when no choline was present (Fig. 6a).

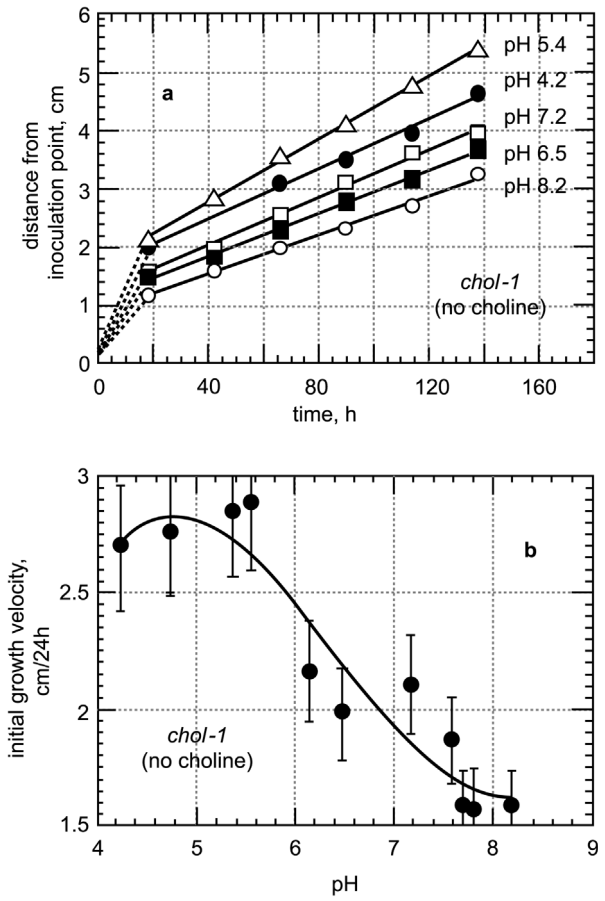

Figure 3. (a) Distance between growing mycel front and inoculation point as a function of time at various extracellular pH for *csp-1; chol-1 bd*. (b) Average initial growth rate (during first 18 h of growth) as a function of extracellular pH.

In the absence of choline, we occasionally observed a "band splitting" phenomenon. By this we mean, that even under apparently identical conditions a conidial band may sometimes split symmetrically into two parts as shown in Fig. 4c. This type of splitting has been found both for the lower and higher end of pH values where rhythmic banding is observed. The band splitting seems to occur preferentially after an induction period when rhythmic sporulation starts to develop (Fig. 4c).

DISCUSSION

Figure 6 gives an overview of pH- and temperature-compensation in the *cel*, *chol-1*, and *frq*-mutants. Comparing *cel* and *chol-1* in Figs. 1, 2, and 6a–d, we see that the absence of supplements has a considerably larger effect on the growth of

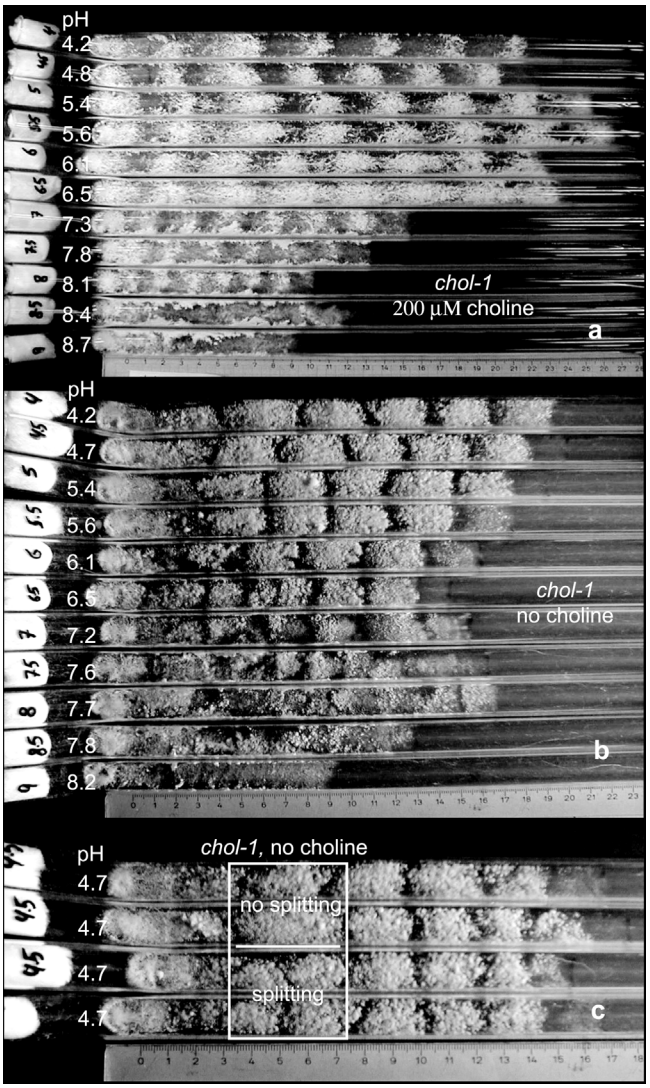

Figure 4. (a) Growth and sporulation of *csp-1; chol-1 bd* as a function of extracellular pH and in the presence of 200 μM choline. Picture was taken 6 d after inoculation. (b) Same system as in (a), but without choline. Due to the absence of choline growth rate is severely reduced. Picture was taken 30 d after inoculation. (c) In the absence of choline a splitting of conidial bands in the *chol-1* rhythm into two symmetrical parts can sometimes be observed.

chol-1 than on *cel*. In *cel*, the absence of palmitic acid decreases the growth rate by approximately 25%, while in *chol-1* absence of choline leads to an approximately 90% reduction in growth rate. While in *cel* the absence of palmitic acid has virtually no effect on period length or pH-compensation of the period (Fig. 6c), the absence of choline in *chol-1* has a dramatic effect on the period length (Fig. 6a). Similar is the effect on temperature-compensation in the absence of supplements:

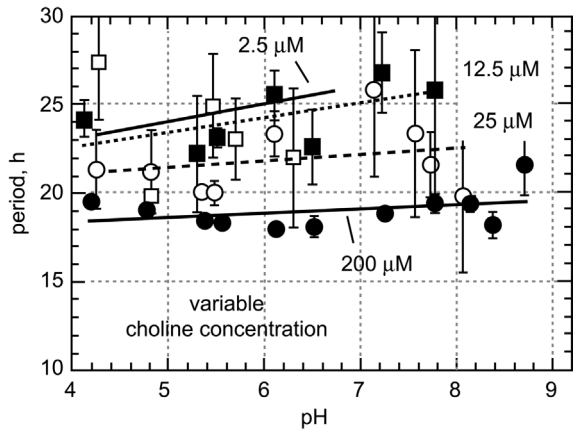

Figure 5. Period length in *csp-1; chol-1 bd* as a function of extracellular pH and varying choline concentrations. At lower choline concentrations large variations in period length are observed. Choline concentrations: 200 μM (●); 25 μM (○); 12.5 μM (■); 2.5 μM (□).

in *chol-1* a broad effect on period length over the whole temperature-range has been reported (Fig. 6b). In *cel*, however, temperature-compensation is found for most of the temperature-range (18–30°C), where oscillations can be observed. Temperature-compensation in *cel* is lost only in a small window at the lower end of the temperature scale (14–18°C, Fig. 6d), practically independent of the presence or absence of palmitic acid. It thus appears that the *chol-1* mutation affects the physiology of *N. crassa*—and hence the properties of the circadian clock—more severely than the *cel*-mutation.

Our results confirm the long period oscillations (with periods up to 100 h) that have been reported for the *chol-1* mutant (24). In fact, we observe even longer period lengths (120–140 h). Interestingly, our data [Fig. 2c and d at period $\approx$130 h, growth rate $\approx$0.5 cm/24 and extracellular pH between 5.5 and 5.8 (this pH is normally obtained when no pH adjustment of the agar is made)] fall on the same line as the period–growth rate relationship which has been described for variable choline concentrations [Fig. 3A in Ref. (24)]. Our cultures seem somehow to be more choline-starved than those in Ref. (24). This may be related to the different carbon sources used or perhaps due to variations in the agar (P. Lakin-Thomas, pers. communication). Since the growth rate is quite low in *chol-1* mutants without choline, small changes in growth rates might also influence the determination of the period lengths when the average growth is measured over a longer period (like 30 d as in this study).

As concluded from this considerable influence of choline on the period length and temperature-compensation in *chol-1* (Fig. 6a and b), we also expected a similar influence on pH-compensation of the period, as found in *frq*[7]. It was therefore quite surprising to see practically no change of period lengths when choline concentrations were as low as 2.5 μM (Figs. 5 and 6a). In the absence of choline, period lengths are although considerably larger, practically independent

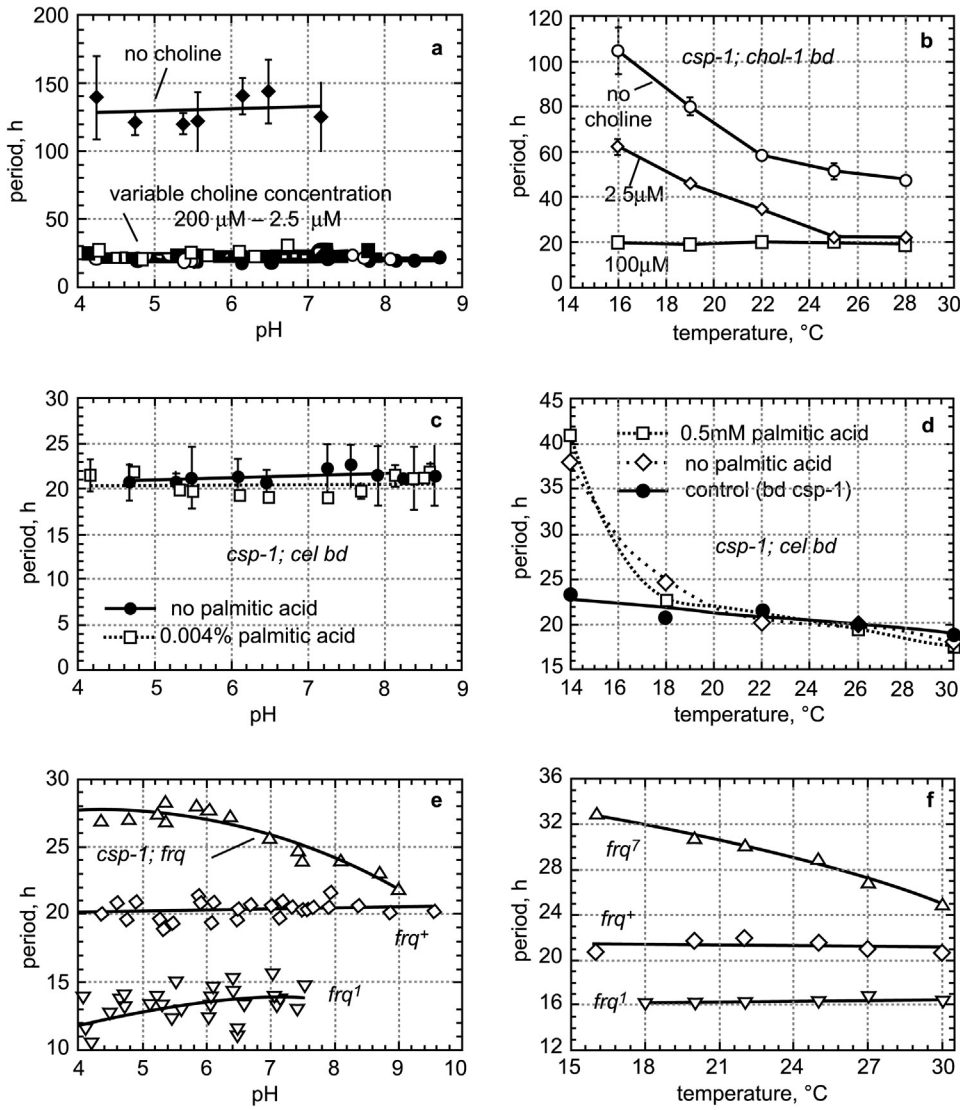

Figure 6. (a) Increase of period length in the absence of choline in *csp-1; chol-1 bd* in comparison with variable choline concentrations (see also Fig. 5). (b) Loss of temperature-compensation in *chol-1* mutant at low or no choline. Data from Ref. (25). (c) pH-compensation of the circadian period in *csp-1; cel bd* in the presence and absence of palmitic acid (see also Fig. 1). (d) Loss of temperature-compensation in the *cel* mutant. Replotted from Ref. (23). (e) Loss and presence of pH-compensation of the circadian period in *frq* mutants. From Ref. (21). (f) Loss and presence of temperature-compensation in *frq* mutants. Replotted from Ref. (17). Note the correspondence between the different mutants in (e) and (f).

of the extracellular pH (Fig. 6a), indicating that even in the absence of choline the mechanisms of pH-compensation is functioning in *chol-1*, at least at 25°C.

It may be that for temperatures other than 25°C (perhaps at the lower end of the temperature scale) one could observe a coupling between temperature- and pH-compensation in *cel* or *chol-1*. We plan to address this aspect in subsequent work. However, it is interesting that *frq*^{7} is the only mutant at present that shows a defect in both temperature- and pH-compensation (Fig. 6e and f).

Our results with *chol-1* and *cel* show that pH- and temperature-compensation mechanisms within the concept of a "general homeostasis of the circadian period" (4–6) are not always strictly coupled to each other. Temperature- and pH-compensation of the circadian period may be understood as the result of a balance between period-increasing and period-decreasing processes as temperature or pH change within cell compartments. For a network of n reactions with n individual rate constants k_i, the condition for general homeostasis of the circadian period can be formulated as a set of "balancing equations" (30), where each environmental variable ξ has to satisfy an equation of the type

$$\frac{\partial P}{\partial \xi} = \sum_{n=1}^{n} \frac{\partial f}{\partial k_i} \cdot \frac{\partial k_i}{\partial \xi} = 0 \tag{1}$$

with f describing how the period-length depends on the individual rate constants k_i. Although a set of balancing equations [Eq. (1)] must be obeyed simultaneously (for example when $\xi = \text{pH}$ and T in order to achieve temperature- and pH-compensation of a circadian rhythm), the equality conditions may be abolished independently, leading to loss of either temperature- or pH-compensation. A point-mutation that abolishes specifically either temperature- or pH-compensation may be considered as less central to the general homeostatic mechanism when compared with another mutation that abolishes both of them simultaneously. In this respect, one can argue that the *frq*-gene products—particularly FRQ protein-appear to be more central to circadian rhythmicity than *cel* or *chol-1*, because temperature-compensation and pH-homeostasis of the circadian period is influenced simultaneously in *frq*^{7} (Fig. 6), probably by the stability of FRQ, while the other mutations may change the conditions for the clock function.

On the other hand, the interpretation that the *frq*-gene has a more central position in *Neurospora*'*s* circadian clock than *chol-1* or *cel*, may be discussed with respect to recent observations that sporulation rhythms were observed even in *frq-null* strains (31–33). However, these *frq-less* oscillators (FLOs) lack essential circadian properties: the period appears to be only half of the circadian period length, i.e., 12–13 h, the rhythm shows no temperature or nutritional compensation, is not entrained by light, and the period is subject to considerable variation (9). It thus appears that only in the presence of an intact *frq*-transcription-translation feedback loop these circadian characteristics are maintained, which fits the above viewpoint of a central role of *frq*. Nevertheless, an important issue of further studies will be to understand in more detail the interactions between components of the circadian clock and their cellular environment (8,34,35).

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REFERENCES

1. Pittendrigh, C.S. Circadian Rhythms and the Circadian Organisation of Living Systems. Cold Spring Harbor Symp. Quant. Biol. **1960**, *25*, 159–184.
2. Bünning, E. *The Physiological Clock. Endogeneous Diurnal Rhythms and Biological Chronometry*; Springer-Verlag: Berlin, 1964.
3. Edmunds, L.N. *Cellular and Molecular Bases of Biological Clocks*; Springer-Verlag: New York, 1988;.
4. Pittendrigh, C.S. Temporal Organization: Reflections of a Darwinian Clock-Watcher. Annu. Rev. Physiol. **1993**, *55*, 17–54.
5. Ruoff, P. Special Issue: Temperature-Compensation of Circadian and Ultradian Clocks. Chronobiol. Int. **1997**, *14* (5).
6. Pittendrigh, C.S.; Caldarola, P.C. General Homeostasis of the Frequency of Circadian Oscillations. Proc. Natl Acad. Sci. USA **1973**, *70*, 2697–2701.
7. Dunlap, J.C. Molecular Bases for Circadian Clocks. Cell **1999**, *96*, 271–290.
8. Lakin-Thomas, P.L. Circadian Rhythms. New Functions for Old Clock Genes? Trends Genet. **2000**, *16*, 99–142.
9. Loros, J.J.; Dunlap, J.C. Genetic and Molecular Analysis of Circadian Rhythms in *Neurospora*. Annu. Rev. Physiol. **2001**, *63*, 757–794.
10. Lakin-Thomas, P.L.; Coté, G.G.; Brody, S. Circadian Rhythms in *Neurospora crassa*: Biochemistry and Genetics. Crit. Rev. Microbiol. **1990**, *17* (5), 365–416.
11. Davis, R.H. Neurospora. Contributions of a Model Organism; Oxford University Press: Oxford, 2000.
12. Lee, K.; Loros, J.J.; Dunlap, J.C. Interconnected Feedback Loops in the *Neurospora* Circadian System. Science **2000**, *289*, 107–110.
13. Heintzen, C.; Loros, J.J.; Dunlap, J.C. The PAS Protein VIVID Defines a Clock-Associated Feedback Loop That Represses Light Input, Modulates Gating, and Regulates Clock Resetting. Cell **2001**, *104*, 453–464.
14. Shrode, L.B.; Lewis, Z.A.; White, L.D.; Bell-Pedersen, D.; Ebbole, D.J. *vvd* Is Required for Light Adaptation of Conidiation-Specific Genes of *Neurospora crassa*, But Not Circadian Conidiation. Fungal Genet. Biol. **2001**, *32*, 169–181.
15. Ruoff, P.; Vinsjevik, M.; Monnerjahn, C.; Rensing, L. The Goodwin Model: Simulating the Effect of Light Pulses on the Circadian Sporulation Rhythm of *Neurospora crassa*. J. Theor. Biol. **2001**, *209*, 29–42.
16. Cheng, P.; Yang, Y.; Liu, Y. Interlocked Feedback Loops Contribute to the Robustness of the *Neurospora* Circadian Clock. PNAS **2001**, *98*, 7408–7413.
17. Gardner, G.F.; Feldman, J.F. Temperature Compensation of Circadian Period Length in Clock Mutants of *Neurospora crassa*. Plant Physiol. **1981**, *68*, 1244–1248.

18. Ruoff, P.; Mohsenzadeh, S.; Rensing, L. Circadian Rhythm and Protein Turnover: The Influence of Temperature on the Period Length of Clock Mutants Simulated by the Goodwin Oscillator. Naturwissenschaften **1996**, *83*, 514–517.
19. Ruoff, P.; Vinsjevik, M.; Monnerjahn, C.; Rensing, L. The Goodwin Oscillator: On the Importance of Degradation Reactions in the Circadian Clock. J. Biol. Rhythms **1999**, *14*, 469–479.
20. Liu, Y.; Loros, J.; Dunlap, J.C. Phosphorylation of the *Neurospora* Clock Protein FREQUENCY Determines Its Degradation Rate and Strongly Influences the Period Length of the Circadian Clock. PNAS **2000**, *97*, 234–239.
21. Ruoff, P.; Behzadi, A.; Hauglid, M.; Vinsjevik, M.; Havås, H. pH Homeostasis of the Circadian Sporulation Rhythm in Clock Mutants of *Neurospora crassa*. Chronobiol. Int. **2000**, *17*, 733–750.
22. Mattern, D.L.; Brody, S. Circadian Rhythms in *Neurospora crassa*: Effects of Saturated Fatty Acids. J. Bacteriol. **1979**, *139*, 977–983.
23. Mattern, D.L.; Forman, L.R.; Brody, S. Circadian Rhythms in *Neurospora crassa*: A Mutation Affecting Temperature Compensation. PNAS **1982**, *79*, 825–829.
24. Lakin-Thomas, P.L. Effects of Choline Depletion on the Circadian Rhythm in *Neurospora crassa*. Biol. Rhythms Res. **1996**, *27*, 12–30.
25. Lakin-Thomas, P.L. Choline Depletion, *frq* Mutations, and Temperature Compensation of the Circadian Rhythm in *Neurospora crassa*. J. Biol. Rhythms **1998**, *13*, 268–277.
26. Sargent, M.L.; Kaltenborn, S.H. Effects of Medium Composition and Carbon Dioxide on Circadian Conidiation in *Neurospora*. Plant Physiol. **1972**, *50*, 171–175.
27. Vogel, H.A. A Convenient Growth Medium for *Neurospora* (Medium N). Microb. Genet. Bull. **1956**, *15*, 42–43.
28. Dharmanda, S.; Feldman, J.F. Spatial Distribution of Circadian Clock Phase in Aging Cultures of *Neurospora crassa*. Plant Physiol. **1979**, *63*, 1049–1054.
29. Lakin-Thomas, P.L.; Brody, S. Circadian Rhythms in *Neurospora crassa*: Interactions Between Clock Mutations. Genetics **1985**, *109*, 49–66.
30. Ruoff, P. General Homeostasis in Period- and Temperature-Compensated Chemical Clock Mutants Formed by Random Selection Conditions. Naturwissenschaften **1994**, *81*, 456–459.
31. Loros, J.J.; Feldman, J.F. Loss of Temperature Compensation of Circadian Period Length in the *frq-9* Mutant of *Neurospora crassa*. J. Biol. Rhythms **1986**, *1*, 187–198.
32. Merrow, M.; Brunner, M.; Roenneberg, T. Assignment of Circadian Function for the *Neurospora* Clock Gene *frequency*. Nature **1999**, *399*, 584–586.
33. Lakin-Thomas, P.L.; Brody, S. Circadian Rhythms in *Neurospora crassa*: Lipid Deficiencies Restore Robust Rhythmicity to Null *frequency* and *white-collar* Mutants. PNAS **2000**, *97*, 256–261.
34. McWatters, H.; Dunlap, J.C.; Millar, A.J. Circadian Biology: Clocks for the Real World. Curr. Biol. **1999**, *9*, R633–R635.
35. Lillo, C.; Meyer, C.; Ruoff, P. The Nitrate Reductase Circadian System. The Central Clock Dogma Contra Multiple Oscillatory Feedback Loops. Plant Physiol. **2001**, *125*, 1554–1557.

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