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Neurospora crassa –
A Model System for Photoperiodism and Circadian Rhythm Research

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1. Introduction

1.1 Photoperiodism

The rotation of the Earth around the Sun gives rise to the procession of seasonal changes. The hallmark of seasonal progression is changing daylength. The word "photoperiodism" is derived from the Greek for "light" and "duration of time", which refers to the phenomenon whereby day- or night-length changes. The proportion of light and darkness is used to initiate a cascade of time-of-year specific responses.

In many organisms, a photoperiodic response is seen as a physiological change, which promotes reproduction in the most promising time of the year. Photoperiodic responses are common amongst organisms from the equator to high latitudes and have been observed in a wide range of types of organisms from arthropods to plants, e.g. diapause and flowering. These biological processes are triggered as soon as the actual daylength or nightlength reaches certain duration, known as the critical photoperiod. This value varies with species, but is typically between 10 hours and 14 hours of light. Even in tropical latitudes where the seasonal daylength changes are small, they are used to synchronize reproductive activities with annual events such as dry or rainy period (Thomas, 1998) (Figure 1.1).

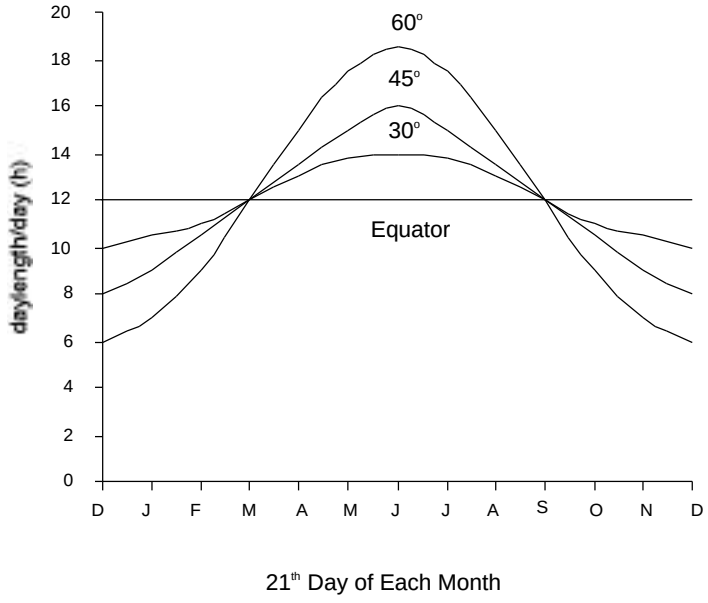

Figure 1.1 Day length over the course of a year at different latitudes (Redrawn after Palmer, 1976).

1.1.1 Specific examples of photoperiodism

Plants are known in which at least the following processes are controlled by photoperiod: flowering, tuber formation, seed germination, vegetative development, tissue differentiation and induction or termination of dormancy of buds, bulbs, etc. (Thomas, 1998). In plants, photoperiodic perception occurs in the young expanded leaves (Lang, 1965; Vince-Prue, 1975). The first clear observation of photoperiodism was reported in tobacco *Nicotiana tabacum*, which only flowered and set seeds when given 10 hours light and 14 hours of darkness per day in the lab, while those living under the long days in the field remained vegetative (Garner, 1920). Following this study, it was found that chlorosis and decreased growth of tomato plants caused by continuous light could be prevented by subjecting the plants to light-dark cycles in the context of 24 hours (Hillman, 1956), which demonstrated a protective effect of plants' responses to photoperiod. After the molecular genetic identification of photoreceptors and light-signaling components in plants, further progress in photoperiodism has been made. Most recently, the expression of the floral regulator gene CONSTANS shifts either at dawn or at dusk, depending on the daylength, which suggested that the photoperiodic perception in *Arabidopsis* was mediated by adjustments in the phase of entrainment to the daily light:dark cycle (Roden, 2002; see 1.3).

Following the first publication describing photoperiodism in plants, it was recognized that the annual cycle of the appearance and egg deposition of the plant lice, *Aphis forbesi*, was controlled by photoperiod (Marcovith, 1923). Many insect and mite species, go through a stage called diapause during their development, which is under photoperiodic control (Figure 1.2, Lee, 1955; Adkisson, 1964).

Twice per year, with great regularity, many birds migrate. By exposing captive juncos to cycles of lengths consistent with springtime, the male birds' testes increased in diameter to reproductive size (Rowan, 1925). In some species, such as the Japanese quail, an increase in photoperiod beyond 11.5 hours induced gonadal maturation (Follett and Maung, 1978), and the gonadal maturation was maintained indefinitely in long photoperiods, or even in constant light. Gonadal regression occurred with a decrease in photoperiod. However, in other species, such as song sparrows (Wingfield, 1993) and house sparrows (Dawson, 1998), the gonadal maturation induced by increasing photoperiod became photorefractory during exposure to long photoperiods. Many songbirds exhibit apparent seasonal variation in song behavior, with highest

rates of singing in spring when the males sing to defend their territories and/or attract females (Ball, 1999). Annual changes controlled by photoperiod, such as gonadal maturation and molt, were also found in tropical birds (Gwinner and Scheuerlein, 1999; Bentley, 2000).

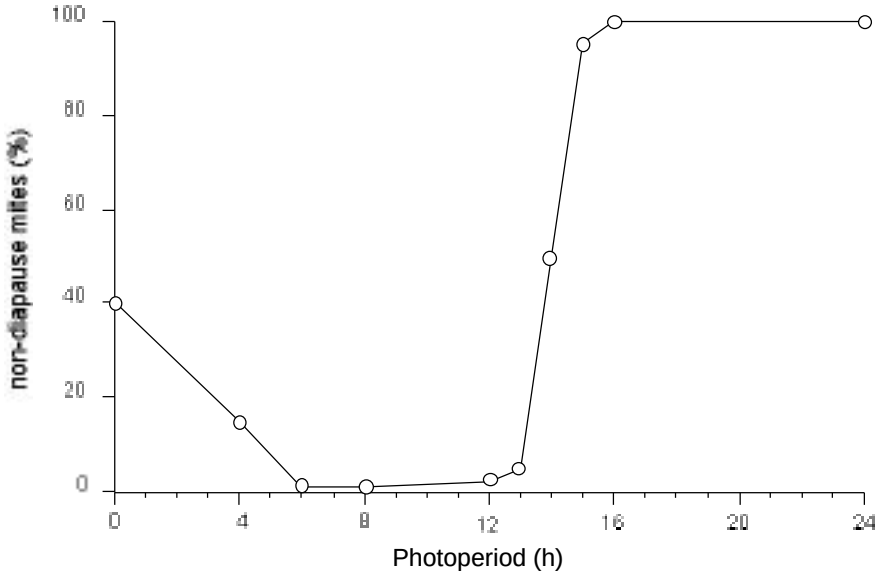

Figure 1.2 Photoperiodic response of diapause in the red spider mite *Metatetranychus ulmi* (after Lees, 1960)

Most nontropical rodent species respond to the photoperiod with changes in physiology and behavior (Bronson and Heideman, 1994). Despite seasonal fluctuations in various external factors, such as ambient temperature, rainfall, and humidity, photoperiod appears to be the primary environmental cue used by most mammalian species to coordinate behavioral and physiological responses (Bronson, 1989). Hamsters are one of the most extensively studied animal models for photoperiodism. It has been demonstrated that the weight of male hamsters' testes responded to photoperiods — by increasing the photoperiod from 12 hours to 12.5 hours per day, the increase is over 3-fold in size (Figure 1.3; Elliot, 1976).

When the photoperiod decreased, the testes showed dramatic decreases in weight, and also in sperm production (Binkley, 1990). Short daylength enhanced aggression in male Syrian hamsters (Garrett, 1980), and this increased aggression was inversely related to circulating concentrations of testosterone. The same results were also found in male Siberian hamsters (Jasnow, 2000).

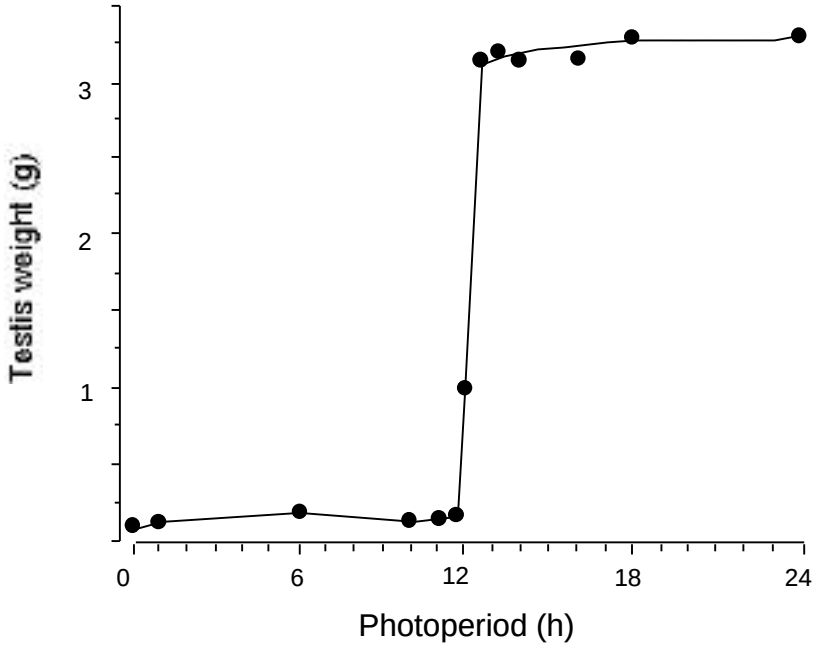

Figure 1.3 Hamster testicular response to photoperiods (T=24h). Each time point represents the mean paired testis weight of a group of hamsters subjected to the indicated photoperiod for about 3 months. Photoperiods of at least 12.5 hours per day were required for maintenance of the testes. The critical daylength was between 12 and 12.5 hours. (Redrawn after Elliott, 1976)

Based on a worldwide selection of statistics, it was demonstrated that the annual rhythms of human conception rates were correlated with photoperiod. Since 1930 this influence has almost disappeared due to the introduction of electric lamps and industrialization (Roenneberg and Aschoff, 1990). Studies on suicide revealed a peak incidence in the Northern Hemisphere between late spring and early summer (Kevan, 1980; Aschoff, 1981), which gave rise to the hypothesis that in some mood disorders, the perception and response to photoperiod was an important factor. Seasonal affective disorder (SAD or winter depression), characterized by recurrent episodes of depression in the autumn and winter and euthymia or hypomania in the spring and summer (Rosenthal, 1984), is dramatically affected by the photoperiod. Seasonal changes in the social activity of humans have also been related to the change of daylength. For instance, the incidence of violent crimes in the Northern Hemisphere showed an annual rhythm with a peak in July and August and a nadir from December to February. The converse rhythm was observed in the Southern Hemisphere. In both cases, the rhythms were found to correlate significantly with the change of photoperiod (Schreiber, 1997).

Studies on photoperiodism afford the opportunity to probe the nature of the annual clock itself, and to understand the effects of the photoperiod on many aspects of organisms for economic interests and health benefits.

1.2 Circadian Rhythm

Though there have been abundant evidence of photoperiodism, its mechanism is still unknown. Circadian clock is assumed the fundamental to two major hypotheses of photoperiodism: external coincidence and internal coincidence (see 1.3). To approach the questions of photoperiodism of *Neurospora crassa*, it is essential to study circadian rhythm.

1.2.1 Circadian rhythm is common in nature

The term circadian rhythm, proposed by Halberg, refers to biological rhythms, which have a period of about a day ("*circa diem*"). These rhythms generally persist in constant conditions with a period that is approximately 24 hours. The period in such conditions is called the free-running-period (FRP).

The first observation of circadian rhythm was reported more than two hundred years ago by the French astronomer de Mairan (de Marian, 1729). By placing a household plant, the *mimosa*, into a light-tight desk, he found that leaf opening in the morning and closing in the evening continued as if it were still in a light-dark cycle. This was the first experimental evidence of the endogenous rhythmicity in the absence of the external cues. Circadian rhythms have now been observed in all phyla, including microorganisms such as algae, fungi, and protozoa, and have been reported among prokaryotes.

Circadian rhythms are controlled by an endogenous clock, which regulates such diverse processes as cell division, photosynthesis, body temperature, and sleep/wake cycles. In the laboratory, circadian rhythms are typically determined/analyzed in continuous dark or light and constant temperature. They can be synchronized to various periods using artificial light-dark or temperature cycles. These external signals, called zeitgebers (time-givers), also can include food-intake and social cues (Moore-Ede, *et al*., 1982).

1.2.2 Circadian rhythms of human

The circadian aspects of various diseases have been observed and used for treatment in traditional Chinese medicine (Miraculous Pivot, ~1000 BC). The first observation of circadian rhythms in humans by western scientists was made in 1866, when William Ogle noted that fluctuations in body temperature varied in synchrony with day and night (Ogle, 1866). The circadian rhythm of hundreds of biological variables in humans has been revealed (Moore-Ede, *et al*., 1982). These functions are found in both physiological (e.g., body temperature, hormone production, sleep-wake cycles) and psychological (e.g., cognitive performance, memory) outputs. Modern medicine has also recognized the biological rhythms of the body: the frequency of heart attacks peaks between 6 am and noon (Rocco, 1987), asthma attacks are most prevalent at night (McFadden, 1988), the blood glucose concentration peaks late at night or early in the morning and insulin secretion peaks in the afternoon (Nejean, 1988).

1.2.3 Properties of circadian rhythms

Circadian rhythms in all described systems share a common set of properties, including:

- a rhythmicity, which is independent of frequency;
- a period in the circadian range, which is determined in constant conditions as free running period (FRP), and is usually close to 24 h;
- amplitude sufficiently robust to drive output rhythms;
- self-sustainment, referring the fact that the rhythmicity is endogenous and sufficiently sustained to continue unabated;
- temperature compensation, which describes the rough consistent of the period over a wide range of temperatures;
- entrainability, which means the clock can be synchronized by external signals such as light or temperature.

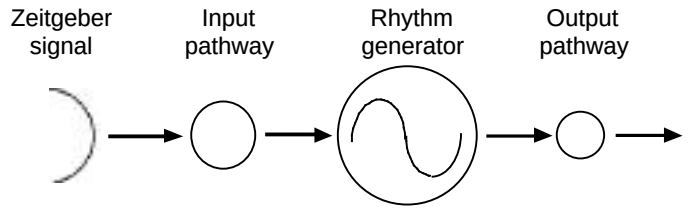

Figure 1.4 Cartoon of a general circadian system. Circadian systems are often described as consisting of three parts: input pathways that perceive and relay external signals that entrain the oscillator; a central oscillator or rhythm generator that generates rhythms; and output pathways that create overt rhythms.

1.2.4 General description of circadian system

Conceptually, circadian systems must be "built" out of similar components. On a very simple level, the circadian system can be visualized as a three-component system: an input pathway to transmit a signal from the environment to the rhythm generator, a central oscillator to generate rhythms, and an output pathway through which the rhythm is expressed (Figure 1.4).

This characterization is oversimplified but has proven conceptually useful. At molecular level, a circadian clock includes multiple photoreceptors, transcription-translation feedback loops, and output pathways, e.g. overexpression via Eboxes. Negative feedback loops are at the heart of oscillatory processes and have been found in all circadian clocks investigated to date. In all clock feedback loops described so far, all negative elements and some of the positive elements cycle at the transcription/translation level (Figure 1.5).

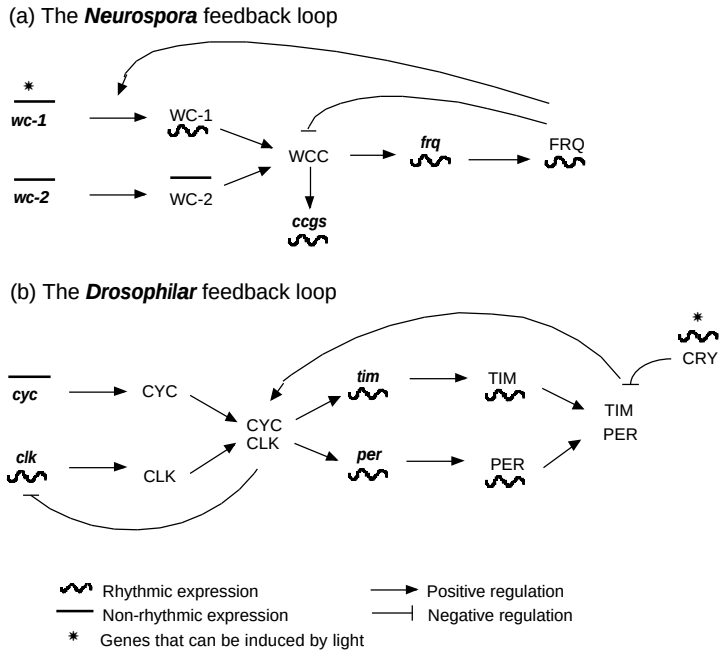

Figure 1.5 Negative feedback loops in circadian systems. (a) The *Neurospora crassa* transcription/translation feedback loop. Levels of the critical clock gene *frequency* (*frq*) RNA and FRQ protein depend on WHITE COLLAR-1 (WC-1) and WC-2, which form the WHITE COLLAR complex (WCC). WC-1 levels depend on FRQ. The net effect is two interlinked regulatory loops. Light reaches the system through WC-1, the blue light photoreceptor. *ccgs* are clock-controlled genes, some of which are light induced. (b) The *Drosophila melanogaster* transcription/translation feedback loop. CLOCK (CLK) and CYCLE (CYC) are factors that activate transcription of *period* (*per*) and *timeless* (*tim*) transcription. PER and TIM proteins feed back negatively on their own transcription by interfering with this process. CLK and CYC, in turn, negatively regulate *clk* RNA expression, resulting in two interlinked regulatory loops. Light reaches the system through CRYPTOCHROME (CRY). (Redrawn after Merrow and Roenneberg, 2001)

1.3 Mechanism of Photoperiodism

Although photoperiodism has been extensively studied at the physiological level, the underlying mechanism is still unknown. Two major hypotheses have been proposed to describe the mechanism of photoperiodic response: external coincidence and internal coincidence (Figure 1.6). The circadian system is fundamental to both of them.

In the external coincidence model, responses are triggered when the photo-inducible phase coincides with the circadian rhythm. Light will evoke a photoperiodic response only if it falls during a specific phase of the circadian cycle (Bünning, 1960; Pittendrigh, 1966).

In the internal coincidence model, the phase relation between two endogenous circadian oscillators varies to determine the response to photoperiod. Light is the zeit-geber for both oscillators, but the phase of each oscillator is also effected via a mutual coupling between the two oscillators (Boulos and Rusak, 1982; Daan, 2001).

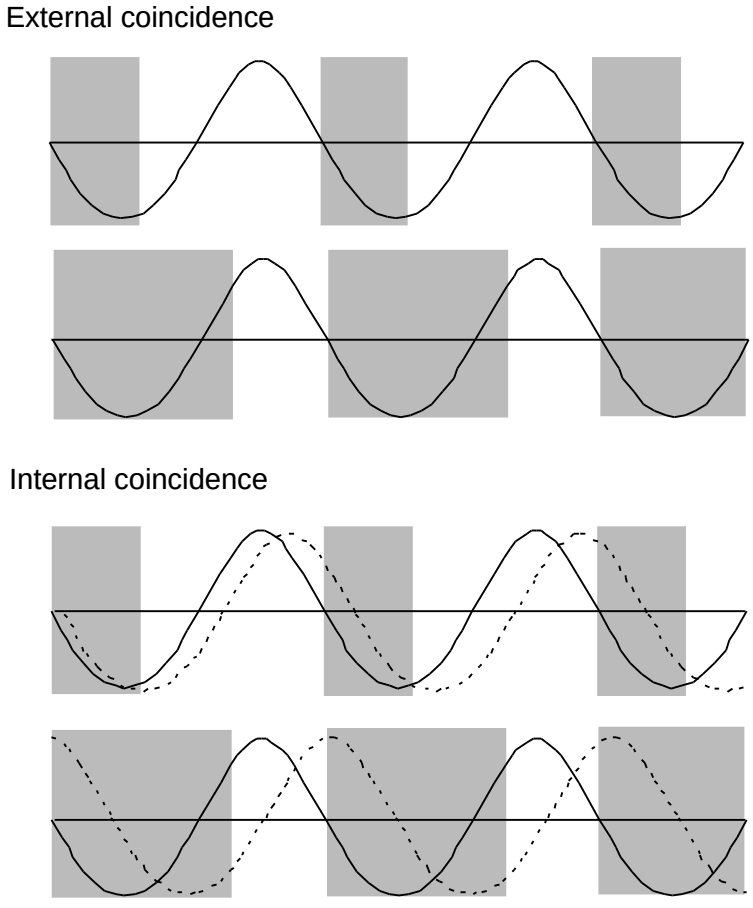

Figure 1.6 Two models of photoperiodic time measurement. In the external coincidence model (top), photoperiodic responses are triggered when light perception coincides with the sensitive phase of a circadian rhythm. In the internal coincidence model (bottom), photoperiodic responses depend on the synchronization of two distinct circadian oscillators. When the daylength is long, the two rhythms are entrained to a similar phase; when the daylength is short, the phase of the two rhythms are further apart, thus inhibiting their co-action. Grey areas indicate darkness; white areas indicate light. (Redrawn after Davis, 2002)

The external coincidence model was supported by results of night-break experiments, in which an extended dark period was interrupted by a light pulse at different phases of the dark. A light break in the middle of a long dark period gives rise to the shortest dark period causing the maximal response, and the results were interpreted in terms of the external coincidence hypothesis (Bünning, 1960) (Figure 1.7).

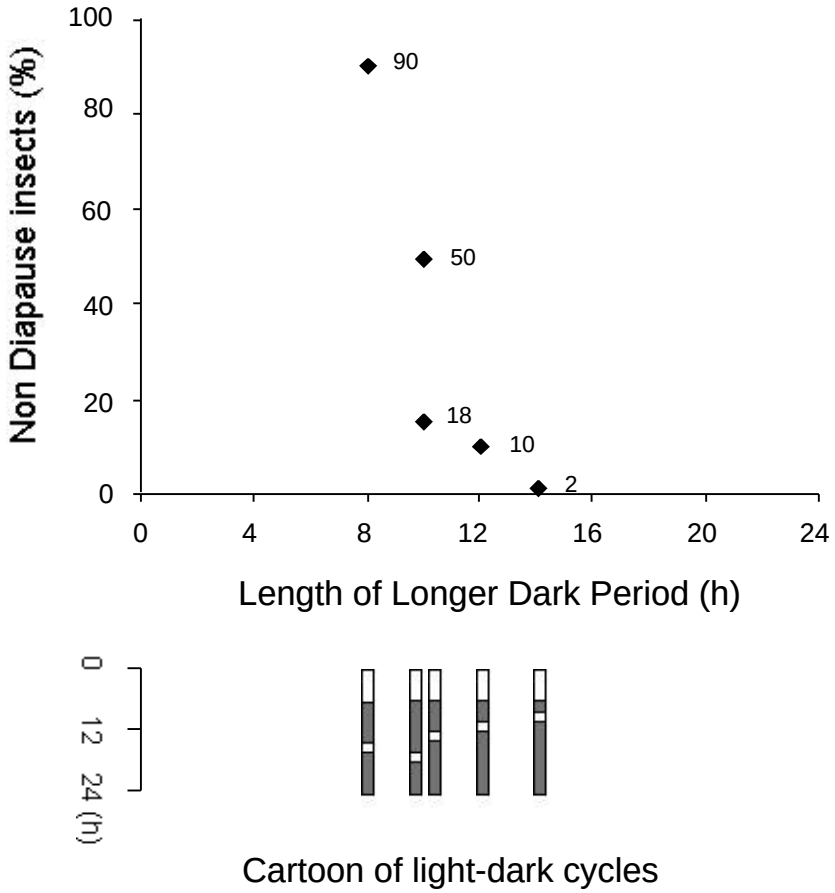

Figure 1.7 Diapause-inhibiting effects of night-breaks during the dark period. Diapause of the insect, *Pieris brassicae*, was inhibited maximally when the short light pause fell in the middle of the night period, giving the shortest nightlength, thus longest daylength. The results indicated that the length of night was important, not merely the length of the light incubation (Redrawn after Bünning, 1960). Cartoons of the different light (white) and dark (grey) incubations are shown in the bottom panel. Experimental time (h) is represented on the Y axis.

Similar results in night-break experiments were found in many other organisms' responses, such as flowering in plants, testes of hamsters (Binkley, 1990), sperm production of male juncos (*Junco hyemalis*), egg-laying activity of chickens, etc. (Bünning, 1958).

1.1.3 *Neurospora crassa* – a potential model to attack questions of photoperiodism at molecular level

We suggest that the circadian model organism of the simple fungus *Neurospora crassa* has the potential for attacking the question of photoperiodism at the molecular and genetic levels. The heterothallic filamentous fungus, *Neurospora crassa*, is potentially an excellent model system for investigating the mechanism of photoperiodism because it has a well characterized circadian system, its genome is well-characterized by mutations and the genome is now fully sequenced (Galagan, *et al*., 2003).

This thesis describes photoperiodic responses in *Neurospora crassa* for the first time.

1.1.3.1 The life cycles of *Neurospora crassa*

The filamentous fungus, *Neurospora crassa* has two types of development – asexual and sexual. The bright orange asexual spores (conidia) germinate if supplied with humidity and appropriate nutrition. The initial germ tube extends to form a hypha, and continues to grow by tip extension and by branching to form a vegetative mycelium. After nutrients exhausted, aerial hyphae form from the mycelia, and, conidia develop above the substrate by budding and segmentation (Springer, 1993).

Ascospores are the products of the sexual cycle of *Neurospora*. The sexual cycle of *Neurospora*, a heterothallic species, requires the presence of both mating types: *A* and *a*. Either of the two mating type strains may present itself as a female parent by forming protoperithecia, the spherical multicellular female structure, which has a specialized hypha, called a trichogyne, which fuses with a conidium or hypha of the opposite mating type. The fertilized protoperithecium, called perithecium, enlarges and darkens around 48 hours after the fertilization at 25^{o}C. Around 7 days after the perithecia appear, black mature ascospores are discharged. Germination of ascospores represents the initiation of the next vegetative or asexual cycle. *Neurospora* favors one or the other life cycle by using external clues, such as nutrition – reduced nitrogen and carbon, for instance (Westergaard, 1947), and temperature (Nelson, 1996). While conidia can't survive in nature for long, ascospores, with their thick walls, are capable of remaining viable for up to years until activated by heat or chemicals. Visible black perithecia of *Neurospora crassa* in nature were found on burned-over stumps of vegetation following the presence of the conidia colonies (Kitazima, 1925)(Figure 1.8).

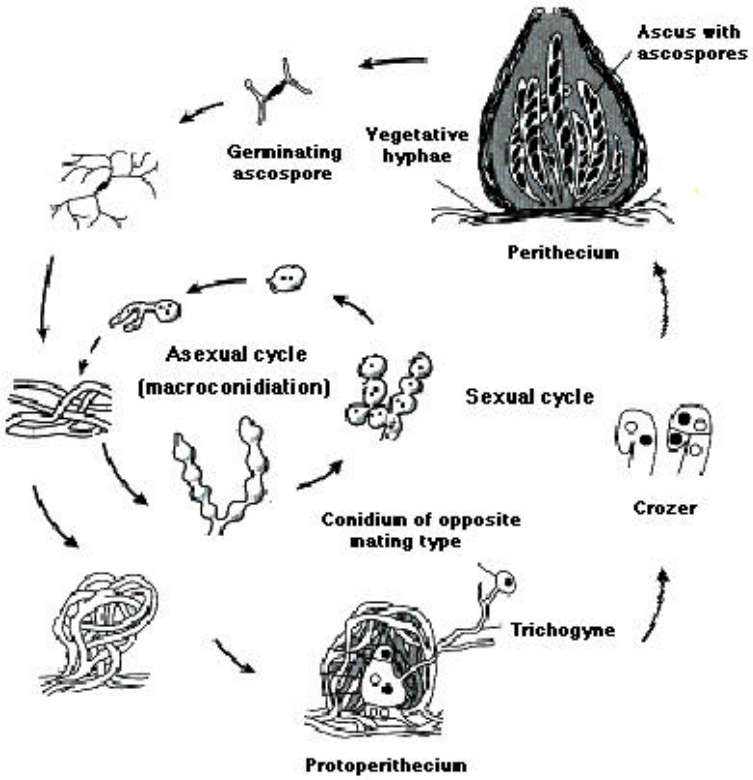

Figure 1.8. Life cycles of *Neurospora*. The asexual cycle, the inner circle, depicts the formation of macroconidia from aerial hyphae and their germination to form a new mycelium. The outer sequence depicts the sexual cycle, originating with a protoperithecium, its fertilization via its trichogyne by a conidium of the opposite mating type. The sexual cycle culminates in the formation of asci, containing ascospores. (Redrawn after Davis, 2000).

1.1.3.2 The conditions of *Neurospora crassa* in nature allow a photoperiodic response

Neurospora strains are found over a large range of latitudes from the equator to Alaska (Jecobson, D., personal communication). Colonies of *Neurospora* are typically among the first species to appear in burned sugar cane fields, which indicate favorable nutritional conditions: carbon, nitrogen, phosphate, etc. Most of the *Neurospora* strains that are used in laboratory experiments are derived from strains collected in Louisiana, where throughout the course of the year, daylength and temperature change by as much as 4 h and 30°C, and rainfall differs by 100 mm per month, while humidity varies largely throughout the course of the day but not over the course of the year. A photoperiodic response would enable accommodation to seasonal changes in the environment.

The molecular mechanism of circadian rhythm of *Neurospora crassa* has begun to be described last decade. The critical clock gene *frequency* (*frq*) has been cloned, and WHITE COLLAR-1 (WC-1) has been identified as the blue light photoreceptor of *Neurospora crassa*. There could be many advantages to approach the questions of photoperiodism at molecular level using *Neurospora crassa* as a model system.

1.4 Circadian Rhythms of *Neurospora crassa*

1.4.1 Circadian rhythm of conidiation of *Neurospora crassa*

The circadian rhythm in *Neurospora crassa* is routinely assayed by a conidiation rhythm on agar media. The circadian system regulates distinct conidiation regions (band) when growing on race tubes. A less dense mycelial growth and few conidia characterize the regions between the bands. These alternating band and interband regions leave a "fossil " record of the rhythm. (Figure 1.9)

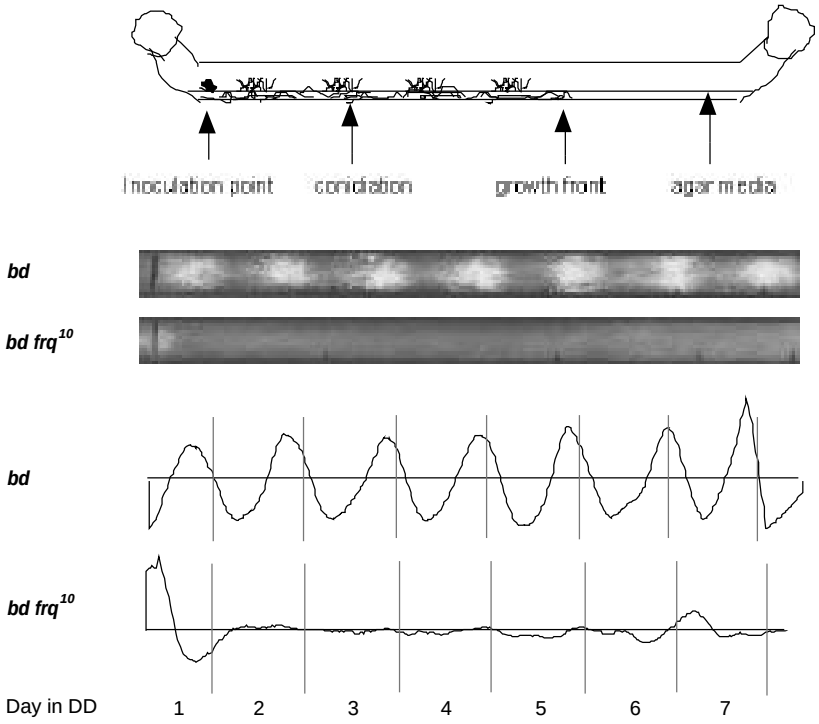

Figure 1.9 Conidiation in race tubes. The race tube was injected with 6 ml of standard racetube media. Conidia were inoculated and allowed to germinate. Hyphae of *Neurospora* started growing from one side of the race tube. During incubation in experimental conditions (DD, light-dark cycles, or temperature cycles), the growth fronts were marked. Race tubes were assayed with the “Chrono” program (Roenneberg, University of Munich; see 2.2.5). In constant darkness, the FRQ-sufficient strain, *bd* was rhythmic; the FRQ-deficient strain, *bd frq*^{10} was arrhythmic.

1.4.2 The molecular mechanism of circadian rhythms in *Neurospora crassa*

1.4.2.1 The "central" components of the oscillation

WC-1 and WC-2 are key components in the molecular feedback loop of the *Neurospora* clock (Crosthwaite, 1997). WC-1 and WC-2 mutants are arrhythmic in DD. A WC-1 and WC-2 complex (WCC) also mediates blue light signaling. Both proteins have dimerization domains (called Per-Arnt-Sim, PAS), which are involved in the formation of the WCC (Cheng, 2002). WC-1, was recently reported to function as a blue-light photoreceptor in *Neurospora* (He, 2002; Froehlich, 2002) using FAD as a photopigment. It is part of the molecular clock network because it binds to the *frq* promoter and activates its transcription.

The critical clock gene *frequency* (*frq*) was identified in a mutant screen (Feldman, 1973). The free-running-period (FRP) of *frq* mutants varies from 16h (*bd frq*^{1}) to 29h (*bd frq*^{7}). *bd frq*^{9} (a point mutant) and *bd frq*^{10} (a knockout mutant) are null mutants and are arrhythmic, except in special conditions (Loros, 1986, Aronson, 1994b). Overexpression of *frq* RNA and protein also results in arrhythmicity. These tools have also allowed demonstration of the negative feedback effect of FRQ on its own transcription. Thus, FRQ fulfills a negative feedback role, while WC-1 and WC-2 are positive effects, resulting in the oscillations that are observed in the clock components (Aronson, 1994a; Aronson, 1994c; Dunlap, 1996). *frq* transcripts encode two forms of FRQ protein, a long form of 989 amino acids (lFRQ) and a short form of 890 amino acids (sFRQ). Both FRQs are required for robust physiological rhythms. Both *frq* mRNA and FRQ protein cycle in constant conditions (Aronson, 1994a; Garceau, 1997). The *frq* transcript is expressed rhythmically with a period length reflecting that of the overt rhythm (Aronson, 1994c). Negative feedback of FRQ occurs relatively rapidly within 3 hours (Merrow, 1997).

In the *Neurospora* circadian feedback loop (Figure 1.10), WCC activates expression of the *frq* gene. *frq* mRNA translates into FRQ protein which then feeds back to suppress the activation (Aronson, 1994c). WC-1 and WC-2 are required as positive elements for maintaining *frq* mRNA and protein levels, and in the absence of functional WC-1, *frq* and FRQ expression are low (Crosthwaite, 1997; Lee, 2003; Merrow, 2001). WC-1 protein is rhythmic in constant darkness, though its mRNA is constitutively expressed. However, levels of WC-1 protein are FRQ-dependent, creating a second feedback loop (Lee, 2000).

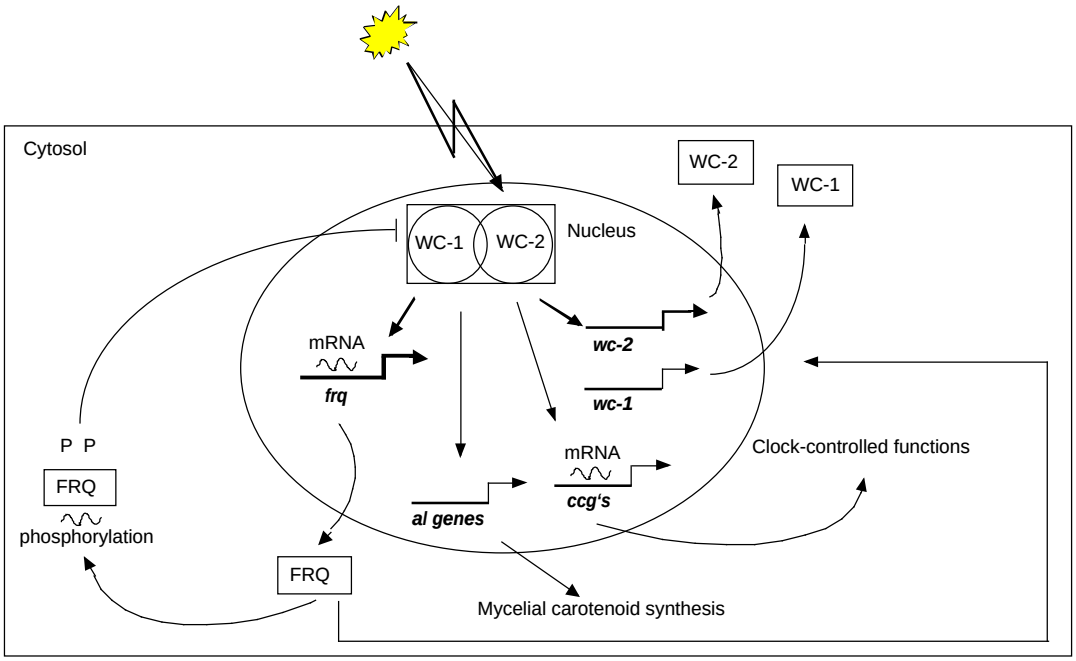

Figure 1.10 Negative feedback loop of *Neurospora crassa*. In the nucleus, the expression of *frq* mRNA is activated by WC-1 and WC-2 complex (WCC). *frq* mRNA then encodes FRQ protein. In the cytosol, FRQ is progressively phosphorylated, and negatively feeds back to its own transcription by interfering with WCC activity. The second role of FRQ is to promote the synthesis of WC-1 from existing *wc-1* message. *wc-1* and *wc-2* message levels and WC-2 protein show no significant rhythmicity. WCC also initiates *al-3* transcription, which gives rise to carotenoid syntheses. The rhythmically expressed clock-controlled genes (*ccgs*) are output pathways and contribute to rhythmic control of various cellular progress, e.g. metabolism and behavior. (modified after Bell-Pedersen, 1998; Dunlap, 1999; Loros, 2001)

1.4.2.2 The molecular hallmarks of the *Neurospora* clock cycle

The expectation is that the molecular components of a circadian system will be rhythmically expressed in constant conditions, as is the overt rhythms. This is indeed the case in *Neurospora crassa*. It has been demonstrated that *frq* mRNA starts rising at around the end of the subjective night, and peaks at around CT4 (ET10) (Aronson, 1994c; Crosthwaite, 1997), FRQ protein appears after around dawn and enters the nucleus after synthesis (Garceau, 1997; Luo, 1998). Its peak is at around CT8 (ET14), about 4 hours after *frq* mRNA reaches its maximum (Garceau, 1997). The subsequent decrease (within 3 hrs) (Merrow, 1997) either directly or indirectly represses the levels of *frq* mRNA. Phosphorylation also plays a critical role in regulation of FRQ. FRQ

phosphorylation triggers its degradation, and the stability of FRQ is an important factor in determining the period length of the *Neurospora* circadian clock (Liu, 2000;, Görl, 2001). FRQ is gradually phosphorylated, and when FRQ becomes heavily phosphorylated, its levels begin to decrease. Once the level of FRQ falls below a critical point, *frq* isn't repressed efficiently and starts to be activated by positive factors *wc-1* and *wc-2* (Crosthwaite, 1997) and the next cycle starts.

1.4.3 Circadian gated light signaling in *Neurospora crassa*

The filamentous fungus *Neurospora crassa* synthesizes carotenoids in response to a few cues, including development and light. In the asexual cycle, conidia and mycelium of wild type strains produce orange, carotenoid pigments. Carotenoids are formed in the isoprenoid pathway originating from mevalonic acid (Harding, 1980). Biosynthesis of carotenoids in mycelia is induced by light, while in conidia the carotenoid synthesis is not wholly light dependent. In the darkness, the mycelia of *Neurospora* contain only a small amount of carotenoids. The albino genes *al-1* and *al-2* encode the carotenogenic enzymes phytoene desaturase and phytoene syntheses, respectively (Harding, 1981). Nitrogen limitation can lead *al-1*, *al-2* expression in mycelia (Sokolovsky, 1992). These nutritional conditions also regulate *ccg-2*. Induction of carotenoids in mycelia by light requires the clock proteins WC-1 and WC-2 but not FRQ (Merrow, 2001), and light-dependent carotenoid synthesis is entirely absent in *wc-1* and *wc-2* mutants (Dehli-Innocenti, 1984;Linden, 1999; Merrow, 2001).

Some of the light responses of *Neurospora crassa*, such as *wc-1*, *frq*, *al-1* RNA and light-induced mycelial carotenogenesis, are circadianly regulated (Merrow, 2001) (Figure 1.11). The amplitude of *wc-1* response to light in the subjective day is less than half when compared with the subjective night (Figure 1.11A). In contrast to *wc-1*, *frq* RNA is induced to approximately the same maximal levels when exposed to light at the opposite circadian times (Figure 1.11 B) (Crosthwaite, 1995). However, *frq* levels are circadian (Aronson, 1994c; Garceau, 1997; Merrow, 2001), so *frq* is already at different levels at the beginning of light incubation. The light induction of *al-1* RNA resembles the pattern of *wc-1* induction, but the maximal level of subjective night is 10 fold of that of subjective day (Figure1.11C). Light-induced mycelial carotenoid production is also circadian, reaching its maximum before subjective dawn, and decreasing to its basal level at approximately mid of subjective day (Figure 1.11D).

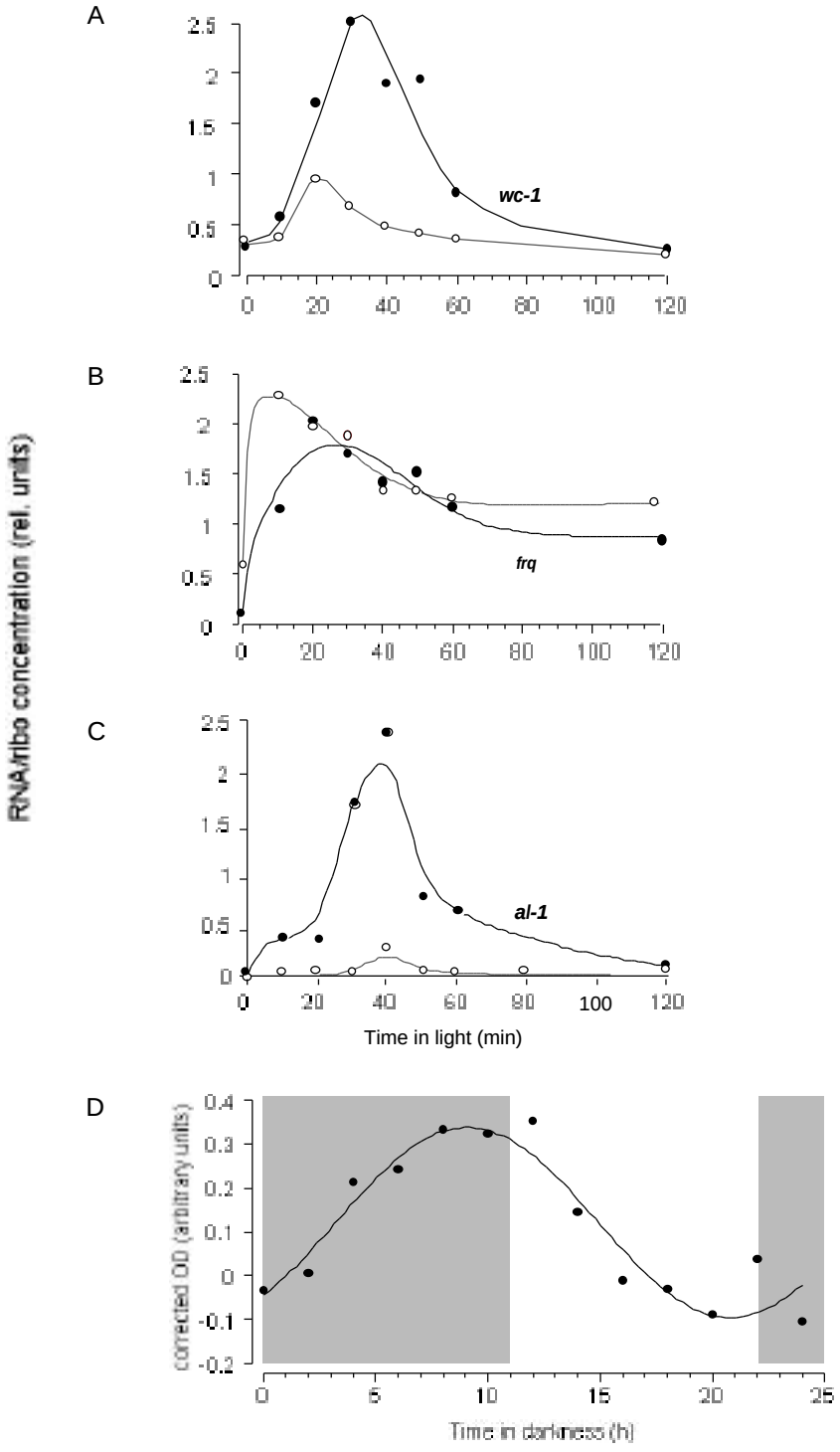

Figure 1.11 Circadian regulation of light responses. Time of day-specific light induction of *wc-1* (A), *frq* RNA (B) and *al-1* RNA (C). Mycelial pads were incubated for 16 h (~ET11, open circles) and 27 h (~ET23, filled circles) in the dark and then exposed to light (0.4µE/m^2/s). (D) Light-induced mycelial carotenogenesis over the course of a circadian cycle. (Redrawn after Merrow, 2001)

2. Materials and Methods

2.1 *Neurospora crassa* Strains

The band (*bd*) mutation allows visualization of rhythmic conidiation in race tubes. For true wild-type strains, conidiation is usually inhibited by the accumulation of CO_2 in the race tubes (Sargent, 1972).

- *bd a* 30-7 is the standard laboratory *bd* strain, with a free-running-period (FRP) of approximately 22h (see Table 2.1 for a list of all strains used in this study).
- *bd frq*^{1} is a short period mutant (FRP=16h), and *bd frq*^{7} is a long period mutant (FRP=29h). Both mutants are the results of single point mutations (G to A transition) in the *frequency* gene (Merrow, 1994).
- *bd frq*^{10} is an arrhythmic mutant (Loros, 1986; Merrow, 1994; Aronson, 1994a). Deleting almost the entire *frq* open reading frame (ORF) by homologous recombination yielded the arrhythmic mutant *bd frq*^{10}.
- The *white-collar-1* mutant (*bd wc-1*) was obtained by crossing the RIP mutant (Talora, 1999) onto a *bd* background. Its hyphae appear white due to the deficiency of light inducible carotenoid synthesis, though its conidia produce carotenoids as wild type or *bd* strains.
- *fluffy A 7430* and *fluffy a 7431* were obtained from the FGSC (Fungal Genetics Stock Center, Department of Microbiology, University of Kansas Medical Center, Kansas City, Kansas, USA). *fluffy* strains do not produce macroconidia (thus they are regarded as aconidial), and are highly fertile (Lindegren, 1933). They were used for visual assays of protoperithecia – the female structure of *Neurospora crassa* -- and long term incubation in Vogel's media, where generation of conidia was undesired.

fluffy mutants were obtained from crosses between *fluffy* strains and *bd* mutants:

- *bd fluffy* (CR28 -23) is from the cross between *fluffy A 7430* and *bd a 30-7*.
- *bd fluffy frq*^{1} (CR29 -13) is from the cross between *fluffy A 7430* and *bd frq*^{1}*a*.
- *fluffy frq*^{1} (CR38 - 89) is from the cross between *fluffy a 7431* and *bd frq*^{1} *A*.
- *fluffy frq*^{10} (CR30 -10), *bd fluffy frq*^{10} (CR30 - 2), and wild type *frq*^{10} (CR30-112) are from the cross between *fluffy A 7430* and *bd frq*^{10}.
- *fluffy wc-1* (CR35-1) is from the cross between *fluffy a 7431* and *bd wc-1*.

Table 2.1 Strains used in this study

Strain	Genotype	Phenotype	FRP (h)	Reference
frq^{+} *30-7*	*bd*	conidial	22	Aronson, 1994a
frq^{1}	*frq*^{1}; *bd* FRQ G487D	conidial	16	Feldman & Hoyle, 1976; Merrow, 1994
frq^{7}	*frq*^{7}; *bd* FRQ G464D	conidial	29	Loros, 1986; Aronson, 1994b; Merrow, 1994
frq^{9}	*frq*^{9}; *bd* FRQ S667Stop	conidial	arrhyth-mic	Loros, 1986; Merrow, 1994
frq^{10}	*frq*^{10}; *bd* *frq* Deletion	conidial	arrhyth-mic	Aronson, 1994a
wc-1#7	*wc-1; bd*; *wc-1* Deletion with RIPing	mycelia light insensitive	arrhyth-mic	Talora, 1999; Dragovic, 2002
K93-2A (de-rived from *74OR23-1A*)	wild type	conidial	n/a	Prokisch, personal communication
fluffy A *7430*	*fl*; A	aconidial	n/a	FGSC
fluffy a *7431*	*fl*; a	aconidial	n/a	FGSC
bd fluffy (CR28-23)	*fl*; *bd*	aconidial	n/a	this study
bd fluffy frq^{1} (CR29-13)	*fl*; *frq*^{1}; *bd*	aconidial	n/a	this study
fluffy frq^{1} (CR38-89)	*fl*; *frq*^{1}	aconidial	n/a	this study
fluffy frq^{10} (CR30-10)	*fl*; *frq*^{10}	aconidial	n/a	this study
bd fluffy frq^{10} (CR30-2)	*fl*; *frq*^{10}; *bd*	aconidial	n/a	this study
fluffy wc-1 (CR35-1)	*fl*; *wc-1*	aconidial	n/a	this study

n/a: not available

All *fluffy*, *bd*, *frq* and *wc-1* mutants were determined according to expected phenotypes and genotypes (see Tables 2.2 - 2.4).

Table 2.2 Screening *fluffy frq* mutants

mutant	phenotype	growth rate*	PCR**
bd fluffy	aconidial	slow	amplified with primers frqwt 5' and flbd 3' (same as *bd*)
bd fluffy frq^{1}	aconidial	slow	amplified with primers frq1 5' and flbd 3' (same as *bd frq^{1}*)
fluffy frq^{1}	aconidial	fast	amplified only with primers frq1 5' and flbd 3' (same as *bd frq^{1}*)

* The slow growth rate reflects the band mutation.

** See 2.3.8 for primers' sequences.

Table 2.3 PCR for screening *frq* mutants

5 min. 94^{o}C		
30 cycles:	15 seconds 94^{o}C	denature DNA
	15 seconds 66^{o}C	annealing primers
	15 seconds 72^{o}C	extension primers
4 min. 72^{o}C		final extension
cool to 4^{o}C		

Table 2.4 Screening for *fluffy frq^{10}* mutants

mutant	phenotype	growth rate	Hygromycin resistant*
bd fluffy frq^{10}	aconidial	fast	+
fluffy frq^{10}	aconidial	slow	+
frq^{10} (wt)	conidial	fast	+

* The *frq* knockout was generated by replacement of FRQ ORF with a Hygromycin resistance cassette (Aronson, 1994b).

fluffy wc1 was determined according to its phenotype: it was aconidial and the mycelia had no carotenoids induced in response to light after a 24 hour exposure. Positive and negative controls were included in this screen.

The wild type strain, *K93-2A* is isogenic to strain *74OR23-1A*.

2.2 Physiological Methods

2.2.1 Strain maintenance

All strains were inoculated into slants with Vogel's minimal agar media (appendix) from the original stocks at the same time, then allowed to grow on the bench for 7 days before using or being wrapped individually with Parafilm for long term storage. For storage, all conidial strains were preserved at –20°C, while all aconidial strains were held at –70°C. To avoid the accumulation of background mutations, no further subcultures were made. When cultures with an unknown history were used, experimental results were unpredictable, especially for experiments investigating sexual reproduction.

All working slants (the second generation) were kept at 4°C in the dark, and were discarded after 2 months.

All conidia or hyphae for experiments were from 7- to 8-day old flask cultures (the third generation) with Vogel's minimal agar media, inoculated from working slants (the second generation).

2.2.2 Growth conditions

Neurospora crassa can grow in many ways in the laboratory: in slants (or petri dishes, or Erlenmeyer flasks) with agar media, it grows by extension on the surface and into the media. In the glass tubes with agar media, and turned up at both ends, called race tubes, the linear growth of the hyphal front in one dimension allows the study of circadian rhythm (see section 2.2.5). In stationary liquid culture in petri dishes, *Neurospora* forms a mycelial mat on the surface of the medium, followed by subsurface growth, and formation of aerial hyphae on the upper surface and on the walls of dishes. Conidia develop on the branches of hyphae. In this study, wild type (*K93-2A*) and *fluffy* mutants were used to study mycelial carotenogenesis in stationary liquid culture (in modified Westergaard's media) in order to avoid conidial development during long term incubation, which occurred very often with *bd* mutants. Mycelial mats of both wild type and *fluffy* strains were investigated under the microscope. No conidia or pro-conidia were found in any mats following the incubation, in all cases. In agitated liquid cultures in Erlenmeyer flasks, conidia germinate into hyphae in about 4 hours. This growth condition generally yielded a large amount of mycelia.

All experiments in these studies were carried out at 25-26^{o}C, except temperature cycles (see 2.2.11).

2.2.3 *Neurospora* cultures

Fresh conidia for inoculation of experiments were prepared by growth in 500 ml flasks with 100 ml Vogel's minimal agar media (appendix) for 7-8 days in the lab at room temperature. To harvest the conidia, about 50 ml sterile H_2O was added to a flask culture. The flask was put on a shaker for more than 10 minutes, after which, the conidial suspension was filtered through a funnel with a 4-layer sterile compress. The concentration of conidia was determined by quantitating the optical density of the suspension with spectrophotometer at 420 nm (when OD_{420} equals 1, the concentration of conidia is 2.86 X 10^8/ml).

To prepare homogeneous hyphal suspensions of the aconidial *fluffy* strains, the *fluffy* strains were grown in 1,000 ml flasks with 150 ml Vogel's minimal agar media (appendix) for 7 to 8 days. 70 – 80 ml sterile H_2O was poured into a flask culture, then it was shaken by hand for about 5 minutes. The hyphal suspension was distributed equally into two 50 ml single-use tubes, then was suspended with a 10 ml glass syringe (Poulten & Graf GmbH, Germany) and a needle (2.00 x 50 mm, unimedâ , Switzerland) by plunging 10 full times. The solution was then sheared with a 10-ml single-use syringe (B½BRAUN, Germany) and a smaller needle (1.20 x 40 mm, B½BRAUN, Germany) for another 10 full times. The needle was replaced by a still smaller one (0.90 x 40 mm, Henke Sass Wolf GmbH, Germany) with which the hyphae were plunged another 10 full times. The hyphal suspension was filtered through a sterile funnel with 4 layers of compress. In order to inoculate equal amounts of hyphae to each petri dish with either solid or liquid media, there should be no visible masses of hyphae. If there was observed, the process was repeated until a homogeneous hyphal suspension was obtained.

2.2.4 Conidia counting

2.2.4.1 Preparation of slants

Each test tube (100 X 14 mm) was filled with 2 ml Vogel's minimal agar media (appendix), and plugged with cotton. After autoclaving, test tubes were slanted to about 30 degree when the media was still hot. Slants were kept at 4^{o}C before use. For con-

idia counting experiments, each slant was inoculated with 8.02 X 10^4 conidia (10 – 20 µl). All slants were then transferred to different photoperiodic cycles at constant temperature (25°C) and laid horizontally with the angled surface facing upwards toward the light source. This is the first experimental day.

2.2.4.2 Conidia harvesting and counting

On the 7th day, conidia were washed out of each slant with 1 ml sterile H_2O by pipetting and vortexing. This was repeated a total of three times. The suspension of each slant was then filtered through a lab-made filter (a 2-ml Eppendorf tube filled with 0.1 g cotton) into a clean test tube. The filter was washed with 1 ml sterile H_2O two times. The volume of the suspension was recorded for each sample.

5 µl of each suspension was placed on a slide and pictures were recorded with the video-camera (Panasonic, BP100) attached to the microscope (12.5 X18 X6.3 magnification, Leitz, Dialux 20). Brightness and contrast were adjusted so that only conidia were visible on the screen. After specifying a size threshold, the conidia were counted automatically by "VideoAnalysis" (Roenneberg, University of Munich).

The counts were normalized according to the total volume of each sample.

2.2.5 Race tubes

The glass tubes turned up at both ends, called race tubes (30 cm long, 1.3 cm in diameter), were filled with 6 ml of molten race tube media (appendix). After autoclaving, plugs were inserted at both ends of the tube in order to prevent contamination. After cooling, conidia were inoculated at one end of a race tube. They were allowed to germinate in constant light overnight (> 20 hours). The growth front of the mycelia on the race tubes was marked with a pen before they were transferred to experimental conditions. Thereafter, the race tubes were marked every other day.

When the mycelia reached the other end of the race tubes, the tubes were scanned (scanner: AGFA SNAPSCAN 1236, Germany; program settings: Greyscale; Imagine Resolution: 150 dpi) from underneath, and saved as a PICT file, then analyzed with the "Chrono" program (Roenneberg, University of Munich).

2.2.6 Sexual crosses

Neurospora utilizes both asexual and sexual reproduction for its propagation. When grown on nitrogen and carbon starvation media**,** the sexual cycle is favored (Wester-

gaard and Hirsch, 1954). Nitrogen is commonly a limiting nutrient for *Neurospora* in nature (Davis, 2000). Thus, despite few observation of sexual crossing in nature, it likely occurs. The mating type loci in *Neurospora* are called *A* and *a*. Either strain may present itself as a female parent by forming a multicellular protoperithecium. The fertilizing agent is a nucleus of the opposite mating type supplied as a conidium or hypha. About one week after the protoperithecia are fertilized, perithecia enlarge and darken, and ascospores are shot to the lids of petri dishes.

For the purpose of genetic crossing, it is critical to isolate single (uninuclear) ascospores. Ascospores were collected by pipetting 100 µl sterile H_2O onto the lid with spores on it, then suspending and plating them on a 4% agar petri dish. Individual spores were picked with a sterile needle and inoculated into minimal slants individually. After heat shock at 60°C for 30 minutes, slants were incubated first at 30°C overnight, then on the bench top. Mycelia were observed within 3 days.

2.2.7 Protoperithecia counting

2.2.7.1 Preparation of conidia for inoculation

For counting protoperithecia of conidial strains (as compared to aconidial strains), conidia concentration was quantitated with a “Coulter” counter (Coulter Z2, Beckman). The conidial suspension was diluted into Isoton II (1:1000) and quantitated. A size threshold of 5 to 9 µm in diameter was used (Davis, 2000). 2,000 conidia (diluted into 40 µl sterile H_2O) were inoculated onto each petri dishes (ø35 mm) with 2 ml modified Westergaard’s agar media (appendix).

2.2.7.2 Processing and counting of protoperithecia from conidial strains

In each experiment, 3 petri dishes were transferred to each experimental condition. After 3 to 5 days (varying according to strains), protoperithecia can be recognized under the microscope as water droplet-like structures (Raju Namiboori, personal communication) (Figure 2.1). After 96 hours (4 days) incubation, the cultures were analyzed. The protoperithecia were immediately fixed with 4% formaldehyde (in 1 X PBS) for 20 minutes, then rinsed with 1 X PBS 3 times before being stained with staining solution (appendix) by gentle shaking for 20 minutes. The petri dishes were destained with destaining solution (appendix) for 40 minutes with shaking. The destaining solution was drained and protoperithecia were counted with the microscope

(10 X 20 magnification, WILD HEERBURGG). 10 fields (0.09cm^2/field) of vision were selected at random. The size threshold of 120 μm in diameter of protoperithecia was used by measuring the protoperithecia with a known scale inside the microscope. All cultures of all experiments were masked for double-blind counting.

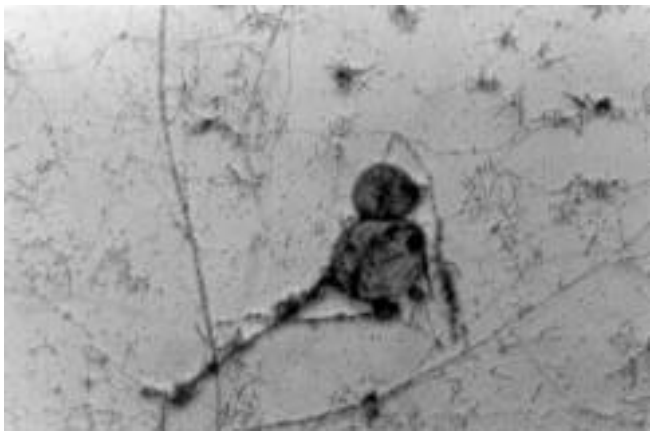

Figure 2.1 Protoperithecia of *Neurospora crassa*. Conidia were inoculated on the plate with modified Wsetergaard's agar media, and incubated in DD for 6.5 days. The imagine (10 X 32 magnification) was taken after the plate was stained and destained.

2.2.7.3 Preparation and counting of protoperithecia from aconidial strains

For counting protoperithecia of the aconidial *fluffy* strains, 40 μl or 92 μl homogeneous hyphal suspension (see 2.2.3) was inoculated on each petri dish (ø53 mm) with 6 ml standard Westergaard's agar media (appendix). The incubation period and fixing processes were the same as those for conidial strains (2.2.7.2). Since the *fluffy* strains are highly fertile, the "VideoAnalysis" program (Roenneberg, University of Munich) was utilized to count the large number of protoperithecia efficiently. 5 different views (1.4cm X1.3cm of each) of each petri dish were chosen at random and collected using a camera attached to the microscope, and saved as PICT file. After setting the proper size threshold, the protoperithecia were counted. The data was analyzed and plotted with Microsoft Excel.

2.2.8 Spore counting

Conidia were inoculated at the edge of each petri dish (ø53 mm) containing 4 ml standard Westergaard's agar media (appendix) with 0.1% sucrose to reduce conidia, and mycelia were allowed to grow over the surface of the agar media. On the 5th day, all cultures were fertilized with a conidial suspension (OD_{420}=0.5, 100 μl/petri dish) of the opposite mating type. Lids were changed and inspected with a microscope for spores after the perithecia turned black in 3 to 5 days. Spores were washed off from

each lid (500 µl H_2O, 2 times) and put into an Eppendorf tube with 100 µl 0.1% agarose. Spores were centrifuged to the bottom of the tubes, then suspended in 10 µl 0.1% agarose. The entire 10 µl was transferred to a slide and spores were counted with the microscope (12.5 X 6.3 magnification; Leitz, Dialux 20). Before pipetting, tips were dipped into 1% SDS to prevent the spores from sticking to the tips.

Perithecia were counted with the "VideoAnalysis" program (Roenneberg, University of Munich).

2.2.9 Carotenoid assay

2.2.9.1 Media for carotenoid assay

For investigating photoperiodism of light-induced carotenogeneses in *Neurospora*, tissue was grown in modified Westergaard's media (Innocenti, 1983) (appendix). 25 ml of media was used per ø90 mm petri dish. The main difference between this modified Westergaard's media (Innocenti, 1983) and the standard Westergaard's media (Westergaard, 1947) is the trace elements (see Table 2.5).

Table 2.5 Trace elements used in modified and standard Westergaard's media

modified Westergaard's media		standard Westergaard's media	
$Na_2B_40_7.10H_2O$	5.25 mg/L	H_3BO_3-anhydrous	0.05 g/L
$CuCl_2.2H_2O$	100 mg/L	$CuSO_4.5H_2O$	0.25 g/L
$FeCl_3.6H_2O$	200 mg/L	$Fe(NH_4)_2(SO_4).6H_2O$	1.0 g/L
$Mo_70_{24}.4H_2O$	20 mg/L	$MnSO_4.5H_2O$	0.05 g/L
$MnCl_2.4H_2O$	20 mg/L	$ZnSO_4.7H_2O$	5.0 g/L
$CoCl_2$	2 mg/L	$Na_2MoO_4.2H_2O$	0.05 g/L
		citric acid.H_2O	5.0 g/L

2.2.9.2 Inoculation and harvesting

3.5 X 10^6 fresh conidia or aliquot (200 - 300 µl) of homogeneous *fluffy* hyphal suspension (see 2.2.3) were inoculated to each petri dish. Samples were incubated in experimental conditions for 120 hours (5 days) before being harvested. Tissue was frozen in liquid nitrogen immediately after drying on paper towels, and was stored at –20°C until the carotenoid extraction.

2.2.9.3 Carotenoid extraction

When extracting carotenoids, the tissue was thawed at room temperature for 10 minutes, then blotted on paper towels until the change of the weight was less than 1 mg. The dry tissue per petri dish normally weighed around 50 mg. The tissue was put into a 2 ml tube (RENNER GmbH) with 500 µl Methanol (MERCK), 750 µl isohexane (MERCK), ca. 0.15 g beads and ca. 0.3 g sand. The tissue was homogenized with "FastPrep FP120" (SAVANT, Instruments, INC. Holbrook, NY) at setting of 6.5 for 40 seconds. All samples were centrifuged at 4ºC for 5 minutes. The clear supernatant (carotenoid isohexane extraction) was quantitated with a spectrophotometer (Beckman, UV-DU-64) at 445 nm. Values above 1.0 were diluted to ensure accuracy. All readouts were normalized to 150 mg dry tissue according to their dry weight.

2.2.10 Light-dark cycles

All light-dark cycles were performed in rooms at a constant temperature of 25-26ºC. All photoperiod cycle experiments with high light levels (3µE/m^2/s) were carried out in light-tight boxes with an air-circulating fan, a white fluorescent strip lamp (OSRAML, 10W) and a layer of diffuser (Cinegel #3026, Rosco) to improve light distribution. All photoperiod cycle experiments with low light (30 nE/m^2/s) were carried out in light-tight boxes with three "night-light" fluorescent lamps (KEMA, 0.18W). Temperature inside the boxes was monitored with a thermometer (Wilh Lambrecht, Göttingen). Light intensity was determined with an IL1400A Radiometer/Photometer (International Light).

2.2.11 Temperature cycles

In all temperature cycles, race tubes were placed in boxes filled with water. Two waterbaths alternately (according to the protocols) circulated warm or cool water through metal tubes at the bottom of the boxes to achieve proper temperature for experiments. All temperature cycles were held in rooms at 25-26ºC, with the cool portion of the incubation at 22ºC and the warm one at 27ºC.

2.3 Molecular Methods

2.3.1 DNA preparation

2.3.1.1 Genomic DNA preparations

Tissue was grown in a 100 ml Erlenmeyer flask with 40 ml Vogel's minimal media (appendix) for 2 days with shaking. For harvesting, the tissue was dried and frozen in liquid nitrogen. The frozen tissue was ground with a mortar and pestle in liquid nitrogen with about 100 µl sand, then transferred to a 2 ml Eppendorf tube with 0.8 ml 2 X CTAB buffer (Zolan and Pukkila, 1986; Taylor and Natvig, 1987) (appendix). The tube was vortexed and incubated at 60°C for 30 minutes. 0.8 ml Chloroform/Isoamyl/alcohol (24:1) was added to the tube at room temperature and mixed on a shaker for 15 minutes. The sample was centrifuged at 8,000 rpm for 10 minutes. The aqueous phase was transferred to another tube and the chloroform extraction was repeated. 1 µl RNase A (10mg/ml) was added to 1 ml of aqueous solution and incubated at 37°C for 10 minutes. An equal volume of isopropanol was added at room temperature and mixed until the DNA precipitated. The sample was centrifuged at 14,000 rpm for 5 minutes to pellet the DNA. The aqueous phase was removed and the DNA pellet was washed with ice cold 70% ethanol. Excess ethanol was removed, and the pellet was air dried briefly with a vacuum centrifuge. The DNA pellet was suspended with 50 to 100 µl sterile H_2O.

2.3.1.2 Amplification of DNA with Polymerase Chain Reaction (PCR)

Polymerase Chain Reaction (PCR) can be used to directly amplify specific DNA sequences in a complex mixture when the ends of the sequences are known. This technique can therefore be use to detect known mutations (e.g., after crossing *frq* mutations onto other mutant backgrounds).

In this study, genomic DNA of *Neurospora crassa* was supplied as a template. DNA was first heated at 94°C to denature into single strands. Primers, two synthetic oligonucleotides complementary to the ends of the target DNA fragment of interest, were added in large excess of the denatured DNA. To anneal primers and DNA, the temperature was lowered according to the melting temperature of the primers (typically, annealing is performed at 5°C below the melting temperature). The bound primers were extended with a supply of deoxynucleotides and a temperature-resistant

DNA polymerase, Taq polymerase (FINNZYMES), at 72°C. The entire mixture was heated to melt the newly formed DNA duplexes. 30 to 35 cycles were repeated for one PCR.

Typically, the reaction was carried out in 100 µl Eppendorf tubes. One reaction tube contained 0.1 ng genomic DNA as template, 50 pmol each of forward primer and reverse primer, 5 µl of 10 X buffer (FINNZYMES), 1µl dNTP (10 mM, FINNZYMES), 0.5 µl Taq polymerase (2U/µl, FINNZYMES), and sterile H_2O. The final volume is 50 µl.

2.3.1.3 Prepare plasmid DNA from *E.coli*

Mini-preparation of plasmid DNA

To prepare a small amount of plasmid DNA (mini-preps), individual colonies were picked with a sterile tooth-stick, and inoculated into a test tube with 2 ml LB_{Amp}-media (Luria Bertani media, appendix). Cultures were incubated overnight at 37°C with shaking (~160 rpm). The next day, 1 ml of each sample was centrifuged at full speed (14,000 rpm) for 1 minute. The aqueous supernatant was poured off, and the pellet was suspended in 100 µl of GET (appendix) and 150 µl alkaline lysis buffer, and incubated on ice for 5 minutes. To precipitate DNA, 120 µl of 3M NaAcO was added to each sample and incubated on ice for 5 minutes. 370 µl phenol/chloroform/ IAA (25:24:1) was added and samples were mixed by vortex to extract the protein into the phenol. Samples were centrifuged at 14,000 rpm for 3 minutes. The supernatant was transferred to a new tube, and 1 µl RNase A (ribonuclease) was added and incubated at 37°C for 10 minutes. 1 ml ice cold 100% ethanol was added into each sample and mixed, then samples were incubated on ice for 5 minutes. Samples were centrifuged at 4°C, 14,000 rpm for 5 minutes. The supernatant was removed, and each pellet was washed with 500 µl ice cold 70% ethanol. The pellets were briefly dried in a vacuum to deplete the alcohol. Each pellet was suspended in 50 µl sterile H_2O. Plasmid DNA was stored at -20°C.

Midi-preparation of plasmid DNA

To prepare large amount of plasmid DNA, “Jetstar Plasmid Purification Kit” (KRD) was employed.

When correct clones were recovered, large amounts of plasmid DNA were prepared. As starter culture, 1.5 ml of the LB_{Amp}-media culture was inoculated into a 1,000 ml flask with 250 ml LB_{Amp} media. The large culture was incubated at 37°C with vigorous shaking (~300 rpm) for 12 to 16 hours.

2.3.2 Quantitation of DNA concentration

2.3.2.1 Quantitation of DNA with spectrophotometer

DNA concentration can be quantitated by its absorption spectrum at 260 nm (Beckman, UV-DU-64). DNA was diluted so that the reading fell between 0.1 and 1.0. The DNA concentration is 50 μg/ml when OD_{260} equals 1.0.

2.3.2.2 Quantitation of DNA with gel electrophoresis

1 μl and 2 μl of a DNA ladder (New England BioLabs) were loaded on either side of a sample. The DNA concentration was calculated according to the brightness of the bands in comparison with the ladder.

2.3.3 Plasmid DNA cloning

2.3.3.1 Cloning pKSbar2cpc1frq

frq ORF was inserted into vector pKSbar2cpc1. The cpc1 promoter in this construct is mutated, such that it drives high, constitutive levels of expression. EcoRI and Cla I sites in the vector were unique. pKSbar2cpc1 was digested first with EcoRI. After gel purification, the linear pKSbar2cpc1 was digested with Cla I. One “arm” of the insert was digested with EcoR I and Sph I, the other “arm” was digested with Cla I and Sph I. The vector and the two arms were ligated in a single reaction.

Plasmid pCRM119 was digested with EcoR I and Sph I for one of the inserts (the 5′ end of the gene and the UTR). In order to obtain the rest of the ORF and the 3′ end UTR, pCRM119 was digested with Cla I and Sph I. After incubation at 37°C for 2 hours, all digested plasmids were loaded on a 1% agarose gel in TAE buffer (Tris Acetate-EDTA) and run at constant voltage (100V) for 2 hours. Inserts and vector fragments were purified from the gel and precipitated to concentrate. The concentration of each insert and vector was determined by electrophoresing 1 μl of each on a 1% agarose gel (see 2.3.4.2). Each insert was combined with the vector at an 8:8:1 ratio for ligation. A control ligation sample was also prepared with only digested vector.

The two samples were incubated at 16°C overnight. The next day, the two samples were precipitated before being transformed into *E.coli* by electroporation (see 2.3.3). The cells were plated on a LB_{Amp}-agar media plate, and incubated at 37°C overnight. More than 100 colonies appeared on the plate with DNA while 6 colonies appeared on the vector control plate. 16 colonies were picked and inoculated into 2 ml LB_{Amp}-media and incubated at 37°C overnight with shaking (~300 rpm).

DNA from each colony was obtained by Mini-preparation (see 2.3.1.3). The DNA was then digested with EcoR I for verification. DNA with bands at 6.3 kb, 1.5 kb, 1.1kb and 1.0 kb was pKSbar2cpc1frq. The plasmid DNA was sent for sequencing (Top Lab, GmbH) for confirmation.

DNA of pKSbar2cpc1frq was transformed into *bd* frq^{10} spheroplasts (see 2.4.2). Colonies were picked and inoculated into 2 ml Vogel's media (appendix) with 0.3% Basta, then the surface of conidia were inoculated into LB_{Amp}-media to germinate conidia. Race tubes and Western blots were used to confirm the overexpression of FRQ (see 3.7.1).

2.3.3.2 Cloning the 5' end of the *wc-1* ORF

In order to characterize the *wc-1* RIP strain (Talora,et al.,1999), the 5' end of the *wc-1* ORF of the *wc-1* RIP strain was cloned into pSK-II for subsequent sequencing. The 5' end of the *wc-1* ORF of the *bd* strain was also cloned as a control.

Genomic DNA of the *bd wc-1* RIP strain was used as a template for amplification by PCR (see Table 2.6), while DNA of the *bd* strain was used as control. Primers were Ywc-1(5') and Ywc-1(3') (see 2.3.8).

Table 2.6 PCR for amplification of *wc-1* ORF

5 min. 94°C		
35 cycles:	1 min. 94°C	denature DNA
	1 min. 52°C	annealing primers
	1.5 min. 72°C	extension primers
4 min. 72°C		final extension
cool to 4°C		

Cla I and Xho I sites are unique in the vector pSK II. Xho I site is also unique in the *wc-1* ORF. One of the 3 Cla I sites in the *wc-1* ORF is 688 bp upstream of the Xho I site, which is the closest one to Xho I site. So the PCR products as well as pSK-II were digested with Cla I and Xho I at 37°C for 2 hours. The insert and vector were gel purified by loading on a 1.5% agarose gel in TAE buffer (Tris Acetate-EDTA) and run at constant voltage (100V) for 1.5 hours. The 688 bp fragment was cut out of the agarose gel and put into a lab-made filter filled with sterile glasswool in an Eppendorf tube, then centrifuged briefly at 8,000 rpm. DNA was precipitated with 500 µl 100% ethanol and incubated at –70°C for 20 minutes. DNA was then pelleted by centrifugation. The aqueous solution was poured off, and the pellet was washed with 1 ml 70% ice cold ethanol and air-dried. 50 µl sterile H_2O was added to suspend the DNA pellet. For ligation, the purified DNA of the insert (688 bp) and vector (2961 bp) were added at 5:1, according to their concentration, together with T4 DNA ligase (New England BioLabs)and T4 DNA ligase buffer (New England BioLabs). A vector control ligation sample was also prepared, where only digested vector was added to T4 ligase and buffer. The two samples were incubated at 16°C overnight. The next day, the two samples were precipitated, washed and re-suspended in water to remove salts. DNA of each sample was then transformed into *E.coli* by electroporation. The cells were then plated on a LB_{Amp} agar plate with IPTG (1 mM, AppliChem) and XGal (20mg/ml, AppliChem) for "Blue/White Screen". The plates were incubated at 37°C overnight. The next day, 10 individual white colonies were picked and inoculated into 2 ml LB_{Amp}-media (appendix), and incubated at 37°C overnight with shaking (~300 rpm). In white colonies the ß-galactosidase gene is interrupted by the insert, while in blue colonies the gene is intact.

DNA from each colony was obtained by Mini-preparation. The DNA was digested with BssH II for verification. Two bands appeared as expected at 861 bp, and 2788 bp. All colonies apparently contained the insert from the *wc-1* RIP strain ligated with the vector from pSK-II. The plasmid DNA was sent for sequencing (Top Lab, GmbH).

2.3.4 Transformation of *E.coli* by electroporation

For each electroporation, a 0.2 cm electroporation cuvette (Bio-Rad) was cooled on ice. *E.coli* cells were thawed on ice. The electroporation instrument (BioRad Gene

Pulser, Bio-Rad) was set at 2.5KV, 200 ohms, 25 microfaradays. 1 µl DNA from ligation sample or negative control sample was added to 40 µl *E.coli* electrocompetent cells, and cells were immediately placed into the ice-cold cuvette. The electric pulse was delivered (note that the pulse time should be 4 to 5 msec, if it is not, then something is wrong). The cells were immediately added into 1 ml SOC (appendix) in a sterile test tube and recovered for 1 hour at 37°C (shaking at 100 rpm). Cells were transferred to a 1.5 ml Eppendorf tube and centrifuged briefly. Most of the supernatant was removed, and cells were suspended with the remainder of the media (< 100 µl). Cells were plated on a petri dish with LB_{Amp}-agar media.

2.3.5 Protein analysis

2.3.5.1 *Neurospora* protein extraction

Frozen tissue was homogenized in a mortar with a pestle and ~100 µl sand, then immediately filled into Eppendorf tubes with 300 – 350 µl protein extraction buffer (appendix). The ground tissue was mixed with buffer completely by vortexing, then incubated on ice for 20 minutes. To prevent proteolysis, 10 µg/ml leupeptin (USB), 10 µg/ml pepstatin (USB) and 1mM PMSF (Phenylmethansulfonyl fluoride, Fluka) were added into protein extraction buffer just before the extraction. If the process lasted longer than 30 minutes, the proteolysis inhibitors were added again. Samples were centrifuged at 4°C, 14,000 rpm for 30 minutes, and the clear supernatant of each sample was harvested and stored at –20°C.

2.3.5.2 Establishing a standard curve for quantitation of protein extracts

The protein quantitative assay is based on the Bradford dye-binding procedure (Bradford, 1976), which measures the color change of Coomassie Brilliant Blue G-250 dye when it binds to protein. Different volumes of a stock solution of γ-globulin (Protein Standard I, bovine γ-globulin, Bio-Rad) were added to 1 ml of a 1:5 dilution of DC™ Protein Assay (Bio-Rad), then all samples were quantitated with a spectrophotometer (Beckman, UV-DU-64) at 595 nm to establish a standard curve.

2.3.5.3 Quantitation of protein concentration

The total protein was first diluted with protein extraction buffer (1:15), then 15 µl of the protein was added into 1 ml 1:5 dilution of DC™ Protein Assay (Bio-Rad), and

the absorption was measured with a spectrophotometer (Beckman, UV-DU-64) at 595 nm. All protein samples were quantitated using the standard protein samples at the same time. The protein concentration was calculated by comparison with the standard curve.

2.3.5.4 SDS-PAGE

The protein molecules were separated with SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE). SDS (sodium dodecyl sulfate) is a detergent (soap). Polyacrylamide gels form covalent cross-links between polymer strands. N,N′-methylenebisacrylamide was used as the cross-linker. The purpose of this method was to separate proteins according to their size.

When preparing gels (see Table 2.7), the vertical slab gels were cast between a pair of glass plates for support. By putting two spacers at each side, a chamber was constructed. The resolving gel (9.5 x 14 x 0.2 cm) was poured into the chamber first, and polymerized with a layer of Isopropanol on the top of the gel to prevent formation a meniscus. A stacking gel (2.5 x 14 x 0.2) was poured 45 minutes after the resolving gel was poured. A Teflon comb was inserted into the stacking gel to create wells for loading protein. Before loading protein into each well, any excess stacking gel was removed.

Table 2.7 Components of SDS-PAGE

Resolving gel:	-- 7.5% (w/v) Polyacrylamide (29.2 g Acrylamide, 0.8 g N,N′-Methylenebisacrylamide dissolved into 100 ml H_2O, and filtered)
	-- 375 mM Tris/HCl pH 8.8
	-- 0.1% (w/v) SDS
	-- 0.1% (w/v) APS (ammonium-peroxodisulfate)
	--0.1% (v/v) TEMED
Stacking gel:	-- 5% (w/v) Polyacrylamide
	-- 60 mM Tris/HCl, pH 6.8
	-- 0.1% (w/v) SDS
	-- 0.05% (w/v) APS
	-- 0.1% (v/v) TEMED

200 µg total protein of each sample was loaded. Protein was mixed with 1 to 1.5 X Laemmle buffer (appendix) and incubated at 95ºC for 5 minutes to denature protein. Samples were centrifuged briefly before loading. The prepared gel was placed in the electrophoresis apparatus, in which the gel was mounted between two chambers filled with electrophoresis buffer (appendix). 10 µl of a molecular weight standard (Precision Protein™ Standards, Bio-Rad) was loaded, while a negative control sample appropriate for the target protein was also loaded. Electrophoresis was run at 80V for approximately 1.5 hours, then changed to 125V after the protein samples entered the resolving gel. The gel was run approximately 3 hours or more until the dye front exited the gel.

2.3.5.5 Protein transfer from gel to nitrocellulose membrane

This method was based on the electrophoresis transfer to nitrocellulose membrane described by Towbin (1979). For Western blotting, protein molecules must be transferred to a membrane. The acrylamide gel was separated from the glass plates and overlaid with a piece of nitrocellulose transfer membrane (PROTRAN®, Schleicher & Schuell) pre-wetted in blotting buffer (appendix). The resolving gel together with the membrane was placed between double layers of Whatman paper (GB 002 Gel-Blotting Paper, Schleicher & Schuell) soaked with blotting buffer. This sandwich was put between two pads, into a cassette and then put into the blotting chamber (Trans-Blot® Electrophoretic Transfer Cell, Bio-Rad) filled with blotting buffer. The blotting lasted 2 hours at 800 mA. The membrane was rinsed briefly with H_2O to remove excess SDS, which would interfere with Ponceau staining. The membrane was stained with Ponceau S solution (appendix) for 15 to 20 seconds. The membrane was left to dry after the excess Ponceau was rinsed away with H_2O.

2.3.5.6 Probing the membrane

The membrane was soaked in TBS (appendix) for 10 minutes to remove the Ponceau. The membrane was incubated with 5% milk (Magermilk in TBS) for 1 hour to block the nitrocellulose. The milk was filtered before use. The milk blocking solution was poured off and the membrane was incubated with specific primary antibody (1:20 dilution in 5% milk) with gentle shaking at room temperature for 2 hours. The primary antibody was removed and the membrane was rinsed with TBS. The secondary antibody (Bio-Rad, Goat Anti-mouse) which targets was diluted in 5% milk (1:5,000)

and allowed to incubate with the membrane at 4°C overnight with gentle shaking. The next day, the secondary antibody was drained, and the membrane was washed with TBS 3 times to remove excess antibody.

2.3.5.7 Developing the membrane

The membrane was placed in a plastic bag and the excess buffer was removed. 750 µl of each luminol solution (Lumi-Light^{Plus}, Roche) was mixed well and spread over the membrane. The excess luminol was removed after 2 minutes. The membrane was placed in a cassette and exposed to film (Fuji Medical X-Ray film 100) for various times, to generate an ordered series of exposures for reliable quantitation.

Table 2.8 Antibodies used in this study

antibody	antigen	origin	targeted protein
αFRQ 3G11	MBP-FRQ (989aa)	mouse, monoclonal	long form FRQ (989aa)
αFRQ 4D11	MBP-FRQ (65-989aa)	mouse, monoclonal	long & short forms FRQ
αWC-1 4H4	GST-WC-1-C200	mouse, monoclonal	WC-1

2.3.5.8 Analysis of the blots

All films were scanned (scanner: AGFA SNAPSCAN 1236, Germany) and saved as PICT files. The density of each band was analyzed with the "VideoAnalysis 1.8" program (Roenneberg, University of Munich).

The pixel densities of each film were transferred to "Kleidagraph (3.0.1)" where gel-saturation curves were plotted using the following equation: $(m_1*(((m_0+m_3)/m_2)/(1+((m_0+m_3)/m_2)\text{^}2)\text{^}0.5+m_4;m_1=2;m_2=1;m_3=1;m_4=-1)$. For gel-saturation curves, zero was regarded as the minimal level, while the quantification of the film exposed for longest period was the maximal level. The proper exposure time for all curves at the logarithmic phase was chosen when the relative amount of protein was calculated in "Microsoft Excel 98". The relative amount of specific protein was then calculated according to the gel-saturation curves, and normalized to Ponceau staining for accuracy. (Merrow, 2001)

2.3.6 RNA analysis

All pipet-tips and buffers for RNA preparation were sterile and RNase free. The gel tray, blotting and hybridization equipment was used for RNA only. This protocol is modified from the method of Sambrook (1989).

2.3.6.1 *Neurospora* RNA extraction

The frozen tissue was ground with a mortar, a pestle and ~ 100 µl sand under liquid nitrogen to prevent RNA degradation. The ground tissue was transferred to a 2 ml Eppendorf tube with 450 µl Aqua-Roti$^{®}$-Phenol (Roth) and 450 µl RNA extraction buffer (appendix), votexed and shaken vigorously at room temperature for up to 30 minutes. Samples were centrifuged at 4^{o}C, 14,000 rpm for 20 – 30 minutes. The supernatant was removed to another tube with 450 µl Chloroform:Isoamyl/alcohol (Sigma), mixed by vortexing, then put on a shaker for 5 minutes before centrifuging at 4^{o}C, 14,000 rpm for 10 minutes. The supernatant was transferred to another Eppendorf tube with 40 µl 3M NaAcO. 1 ml ice cold 100% ethanol was added. The sample was incubated at –20^{o}C for 1 to 2 hours or at -70^{o}C for 30 minutes. The sample was centrifuged at 4^{o}C, 14,000 rpm for 20 – 30 minutes. The supernatant was removed, and the RNA pellet was washed with 1 ml ice cold 70% ethanol. After removing all ethanol, the pellet was air-dried briefly with a centrifuge vacuum. The dry pellet was suspended in 50 to 100 µl sterile H_2O.

2.3.6.2 Quantitation of RNA concentration

2 µl RNA was diluted into 998 µl H_2O (1:500), and quantitated with spectrophotometer at 260 nm and 280 nm. When the OD_{260} is 1, the RNA concentration is 40 µg/µl. The optimal ratio of OD_{260} : OD_{280} should be between 1.8 – 2, which indicates the absence of contaminating phenol. The concentration of RNA was determined, and RNA was diluted to 2 µg/µl.

2.3.6.3 RNA gel electrophoresis

Gel electrophoresis of RNA

When preparing an RNA agarose gel (100 ml, appendix). When the molten agarose gel was cooled (~60^{o}C), 2 µl Ethidium Bromide (10 mg/ml) and 5 ml formaldehyde (37%(v/v)) were added and mixed well before pouring into a gel tray. A comb was

immediately inserted in the tray to create wells for RNA loading. The gel was solidified for 1 hour.

In this study, 30 µg RNA of each sample was loaded onto the gel. 15 µl of each sample was mixed with 34 µl of a RNA pre-mix (appendix). The sample mixture was incubated at 70°C for 10 minutes, then chilled on ice for 2 minutes. A maximum of 44 µl of each sample mixture was loaded in each gel. The electrophoresis was run at 70V for 4 – 5 hours or at 20V overnight (>16 hours). The gel was placed on a UV lamp to take a picture. Bands of rRNA should be present at equal density from lane to lane if the quantitation was done well. As well, the upper ribosomal band should be denser than the lower one, which indicates the quality of the RNA is good.

2.3.6.4 Northern blot: Transfer of RNA from the gel to a nylon membrane

After electrophoresis, formaldehyde was washed away with 10 X SSC (appendix) for 2 x 15 minutes. For blotting, a piece of glass was placed across two basins filled with 10 X SSC. A long piece of Whatman paper (GB 002 Gel-Blotting Paper, Schleicher & Schuell) soaked with 10 X SSC was laid on the glass with two ends dipping in 10 X SSC to act as a wick. The gel was put upside down on the glass, and a piece of nylon membrane (Hybond-N, Amersham Corp.) was placed on it. There should be no bubbles in between the glass and the paper, the paper and the gel, and the gel and the membrane. Two layers of Whatman paper were put on the membrane, then some paper towels were placed on them. On the top, about 1 kg weight was evenly distributed to improve the blotting efficiency. The gel was blotted for 24 hours at room temperature. The membrane was rinsed briefly with sterile H_2O, and crosslinked with UV light (UV Stratalinker, Stratagene) for 120 seconds, then allowed to dry at room temperature. The membrane should be stored in the dark.

2.3.6.5 RNA hybridization

The membrane was incubated with 15 ml prehybridization solution (appendix) in a glass tube in a hybridization oven (Bachhoffer) at 60°C for 30 minutes or longer. The *wc-1* riboprobe was generated from pGEM4wc-1 using 0.2 – 1 µg linearized plasmid, 4 µl 2.5 mM transcription buffer (Promega), 2 µl 0.1 M Dithiothreitol (DTT, Sigma), 0.6 µl RNasin (Boehringer), 4 µl [α-^{32}P]dUTP, 2.4 µl 0.1 mM UTP, 1 µl T7-Polymerase (Promega) and 19 µl H_2O. The reaction was incubated at 37°C for 1 hour.

The riboprobe was purified with a column (Nuc trap probe purification columns, Stratagene). After counting the probe, 2.5 X 10^6 cpm/ml were added to the hybridization bottle. The membrane was hybridized at 60°C overnight. The next day, the membrane was washed with 2 X SSC, 0.1% SDS twice for 15 minutes at room temperature, then twice in 0.2 X SSC, 0.1% SDS for 15 minutes at 68°C. The membrane was then placed in a plastic bag, and the excess buffer was removed. The membrane was placed in a cassette and exposed to phosphorimager screens for various periods of time (30 minutes to 24 hours). The quantitation of RNA expression was analyzed with the "GelAnalysis" program (Roenneberg, University of Munich).

2.3.7 RNA analysis with TaqMan RT-PCR

Real-Time PCR is designed to collect data as the reaction is proceeding, which is more accurate for DNA and RNA quantitation. Real-Time PCR monitors the fluorescence of the reporter dye emitted during the reaction as an indicator of the production during each PCR cycle. Reverse transcription of total RNA to cDNA was carried out with random hexamers or sequence-specific reverse primers. All RNA preparations were digested with RNase free DNase to avoid genomic DNA contamination.

2.3.7.1 RNA digestion with DNase

RNA extraction was performed as described in the previous section (2.3.6.1). Even the best RNA extraction will be contaminated with genomic DNA, which would give a false positive result by increasing the specific signal. A pre-mix of DNase was prepared which contained 30.8 µl 10X DNase I buffer (appendix), 9.24 µl DNase I Enzyme (10U/µl, amplification grade), and 114 µl H_2O (RNase free). For the DNase digestion of each RNA preparation, 10 µl RNA (0.2µg/µl) was mixed with 10 µl of DNase pre-mix, so that a total 2 µg RNA was added for a final volume of 20 µl.

2.3.7.2 Reverse transcription

All reagents were from "TaqMan Reverse Transcription Reagents" and "AmpliTaq Gold® PCR Master Mix" kits (Applied Biosystems). A pre-mix for reverse transcription was prepared containing: 1X TaqMan RT buffer, 5.5 mM $MgCl_2$, 500 µM of each dNTP mix, 2.5 µM oligonucleotide (rN6/d(T)16/Spec.), 40 U RNase inhibitor (20 U/µl), 125 U Reverse Transcription Enzyme (50 U/µl), and RNase-free H_2O. 20 µl of DNase I digested RNA was added to a 0.2 ml Eppendorf tube with 80 µl of pre-

mix. The final volume of each sample was 100 µl. Reverse transcription was performed as described in Table 2.9.

Table 2.9 Incubation program for reverse transcription

25°C	10 minutes
42°C	30 minutes
95°C	5 minutes

After reverse transcription, cDNA was diluted to 200 µl by adding 100 µl RNase-free H_2O. Primers were mixed with a SYBR Green RT-PCR Master Mix (SYBR Green Mix, Applied Biosystems): 12.5 µl 2X SYBR Green, 0.5 µl forward primer (200 nM), 0.5 µl reverse primer (200 nM), and 1.5 µl H_2O. The total volume was 15 µl. 10 µl cDNA dilution was added to the SYBR Green mixture into a well of a 96-well reaction plate. The RT-PCR conditions are listed in Table 2.10.

Table 2.10 RT-PCR program

Stage 1	
	Repetition 1
	Temperature 50°C
	Time 2 minutes
Stage 2	
	Repetition 1
	Temperature 95°C
	Time 10 minutes
Stage 3	
	Repetition 40
	Temperature 95°C
	Time 15 seconds
Finish at 60°C, 1 minute	

2.3.8 Primers used in this study

The names and sequences of primers used in this study are listed in Table 2.10.

Table 2.10 Primers in this study

name	sequence	purpose
frqwt 5′	5′- GTT GCT CCG GAT GCG GAA G	for *fluffy* mutant screening
flbd 3′	3′- TTT CGG AGA CAG CTT GGC G	for *fluffy* mutant screening
frq1 5′	5′- GTT GCT CCG CAT GCG GAA A	for *fluffy frq*^{1} mutants screening
frq-FO sy1	5′-CGC CTT GCG CGA GAT ACT AG	for RT-PCR of *frq*
frq-RE sy1	5′-TCC CAG TGC GGA AGA TGA AG	for RT-PCR of *frq*
Ywc1 (5′)	5′-TCT GCT TGA GTG ACA GC	for *wc-1* cloning
Ywc1 (3′)	5′-CGG AGC CAC TAT CCA TG	for *wc-1* cloning
wc1-one-sy2-FO	5′-CCG ACT GGC ACA AAC AAT CC	for RT-PCR of *wc-1*
wc1-one-sy2-RE	5′-CGT CTG CGT TCT CCA AAA GC	for RT-PCR of *wc-1*

2.4 Genetic Methods

Spheroplasts of *bd frq*^{10} were prepared for plasmid DNA transformation with the method of Vollmer and Yanofsky (1986).

2.4.1 Spheroplast Preparation

About 100 ml sterile H_2O was added to a 7-day old *bd frq*^{10} flask culture, and the conidial suspension was passed through sterile cheesecloth to remove mycelial fragments. The conidial suspension was allowed to rehydrate at 4°C overnight.

The concentration of conidia was quantitated with a spectrophotometer (Beckman, UV-DU-64) at 420 nm. 2 X10^9 conidia were obtained (the following amounts of chemicals and buffers were based on this). Conidia were centrifuged (Heraeus) at 4^{o}C, 2000 rpm for 5 minutes, and the supernatant was poured off. Conidia were resuspended in 50 ml of wash solution (appendix). The conidia were incubated at 30°C, 200 rpm for 20 minutes, then centrifuged at 2,000 rpm for 5 minutes. The supernatant was removed. The conidia were washed once in 50 ml 1M Mg_2SO_4, then centrifuged again at 2,000 rpm for 5 minutes. The pellet was suspended in 20 ml 1M Mg_2SO_4. 4 ml lysing enzymes (Sigma, 10 - 25 mg/ml in 1 M sorbitol) were added into the con-

idial suspension and the conidia were incubated with shaking (100 rpm) at 30° for 40 minutes. The lysing enzymes contain cellulase, protease, and are often used for cell-wall digestion. The completion of spheroplasting was checked with a microscope (Leitz, Dialux 20) after 20 minutes, and thereafter every 5 minutes. When the conidia popped after adding water, the spheroplasting process was completed. This is the crucial step. If overdone, the efficiency of transformation would decrease. The spheroplasts were centrifuged at 4°C, 1,000 rpm for 10 minutes, and the pellet was washed once in about 20 ml of 1M sorbitol, then washed again in about 20 ml of STC (appendix). The washed pellet was resuspended in 16 ml STC, 4 ml PTC (PEG-Tris-Calcium chloride, appendix) and 200 µl of DMSO (Dimethyl sulfoxide, Sigma D2650). The suspension was stored as aliquots at -80°C. 0.1 ml of spheroplast suspension was used for each transformation. Frozen spheroplasts remained competent for transformation for up to 3 months. Longer storage results in less efficient transformation.

2.4.2 Transformation of DNA into Spheroplasts

The DNA should be digested so that it is linear, and purified, in order to maximize the efficiency of the transformation. The spheroplasts were thawed on ice, then added to the linear DNA. The suspension was incubated on ice for 30 minutes. Then, 10 volumes of PTC (appendix) were added and incubated at room temperature for at least 20 minutes. The efficiency of transformation increases with a prolonged incubation period (for instance, first incubate on ice for 45 minutes, then 30 minutes at room temperature). The spheroplasts were mixed gently by inversion with 10 ml of Top Agar (appendix) at 50°C, and the mixture was plated on a petri dish with 20 ml Bottom Agar media (appendix) with 3% Basta. The petri dish was incubated at 30°C for 48 hours. Conidia remained colonial due to the presence of sorbose in the agar. Colonies were picked up with a sterile needle and inoculated into 1 ml Vogel's media with 0.3% Basta, then incubated at 30°C for 3 days until conidia were observed. Mutants were checked with race tubes and by Western Blotting for the appropriate insertion and expression.

2.5 Time Scales

Unless elsewhere mentioned, “external time”(ET) was used in this study. External time is the number of hours x 24/T elapsed since the middle of the dark period, where T is the duration of the LD cycle in hours, and external time 0 is exactly the middle of darkness (Daan, 2002).

Circadian time (CT) is used to compare the properties of circadian rhythms from organisms or strains with different endogenous periods whereby the period is divided into 24 equal parts with each part defined as 1 circadian hour (Bell-Pedersen, 1998). Thus, CT0 is subjective dawn and CT12 is subjective dusk.

3. Results

3.1 Photoperiodic Responses in a 24-hour Day

3.1.1 Phase of conidiation in different photoperiods

As a first approach to investigate photoperiodic responses in *Neurospora*, the phase of conidiation of the *bd* strain (*bd* 30-7, FRP=22h) was measured in different photoperiods, in the context of 24-hour day. Experiments were performed with two light intensities. Conidiation was stably entrained even when photoperiods were as short as 30 minutes (the shortest photoperiod tested. Except for photoperiods less than 4-hour, the onset of conidiation of *bd* occurred almost always began at around midnight. (Fig. 3.1; Fig. 3.2)

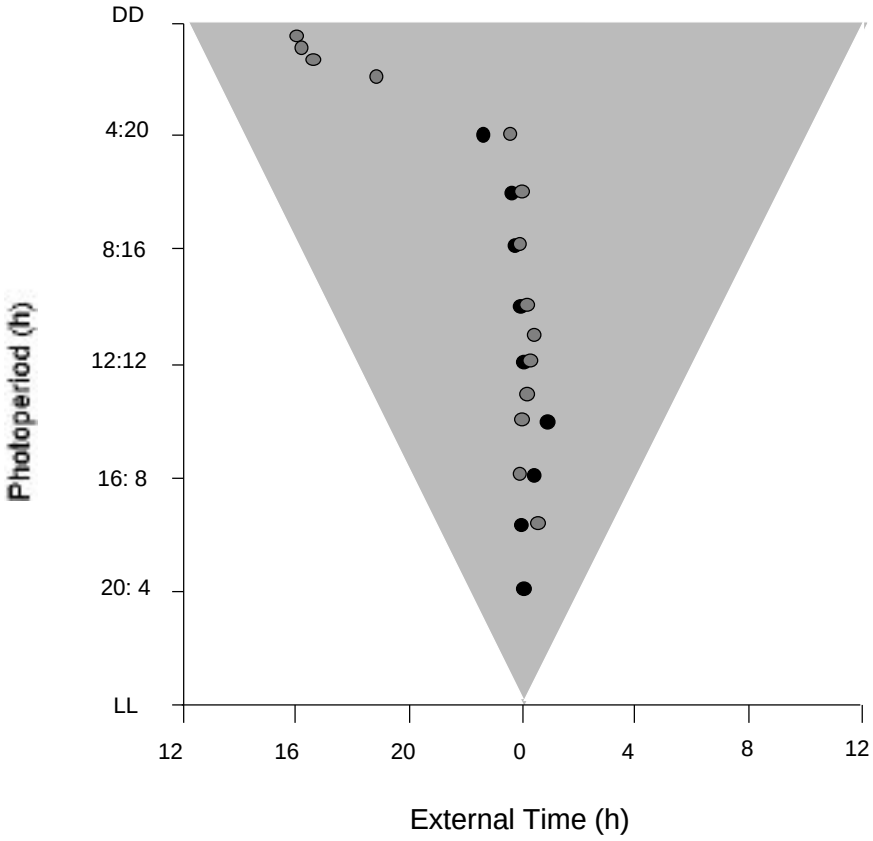

Fig.3.1 Entrained phase of conidiation: *bd* in different photo-periods (T=24h). Onset of conidiation of *bd* (*bd* 30-7) in photoperiods of either low light (fluorescent light, 30nE/m^2/s, striped circles) or high light (fluorescent light, 3μE/m^2/s, filled circles) was stably entrained, even in photoperiods as short as 30 minutes. *Neurospora* failed to establish stable entrainment in photoperiods longer than 20 hours. The onset of conidiation was pegged to midnight (ET0) in all photoperiods between 4 and 20 hours, independent of light intensities. Each time point represents the average of 5 (low light) or 7 (high light) independent experiments with 6 race tubes within each experiment. LD cycles are portrayed with grey representing darkness and white representing light.

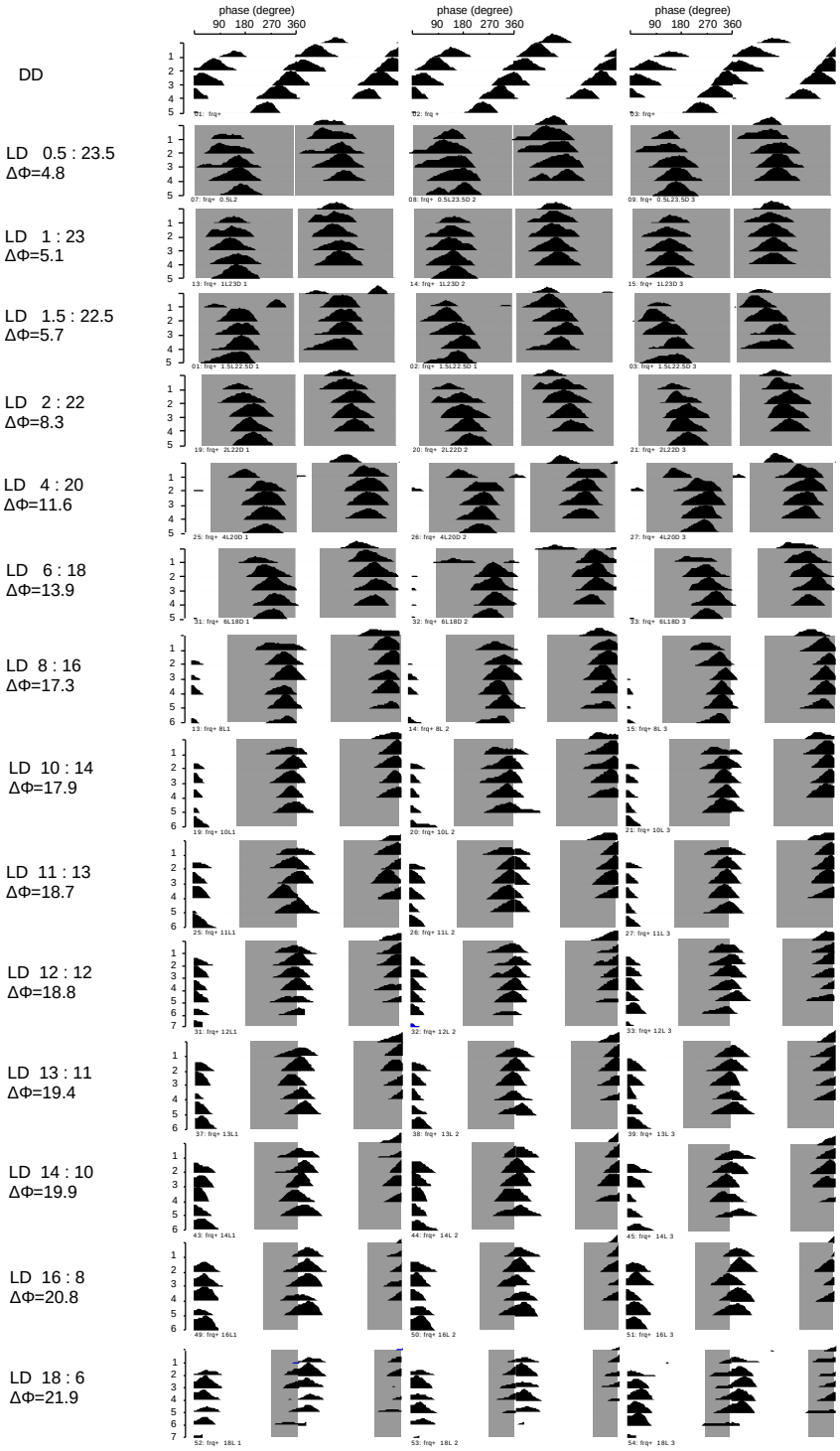

Fig. 3.2 Phase of conidiation of *bd* in different photoperiods (T=24h, fluorescent light, 30nE/m2/s). Conidiation (black areas) of *bd* (*bd* 30-7) was double plotted (the data from 2 cycles were graphed in a continuum, starting from the top left of each graph with the 1st cycle, and moving down by one cycle on each line of the vertical axis). Double plotting the data in this way allows trends to be visualized across midnight. Grey areas indicate darkness; open areas indicate light. The length (hours) of light and darkness in each photoperiod (LD) as well as the average phase angle between the onset of entrained phase of conidiation and the dark to light transition in each photoperiod is indicated at the left (ΔΦ). Note that the banding in different photoperiods is independent of L→ D or D→ L transition.

The same experiments were performed with period mutants of the *frq* gene, *bd frq*^{1} (FRP=16h) and *bd frq*^{7} (FRP=29h). For the majority of the photoperiods, the onsets were again independent of dawn and dusk. The short period mutant, *bd frq*^{1}, began to make conidial bands approximately 2 hours before midnight and the long period mutant *bd frq*^{7} approximately 5 hours after midnight. At least 4 hours photoperiod was required to stably entrain *bd frq*^{7} (Fig. 3.3).

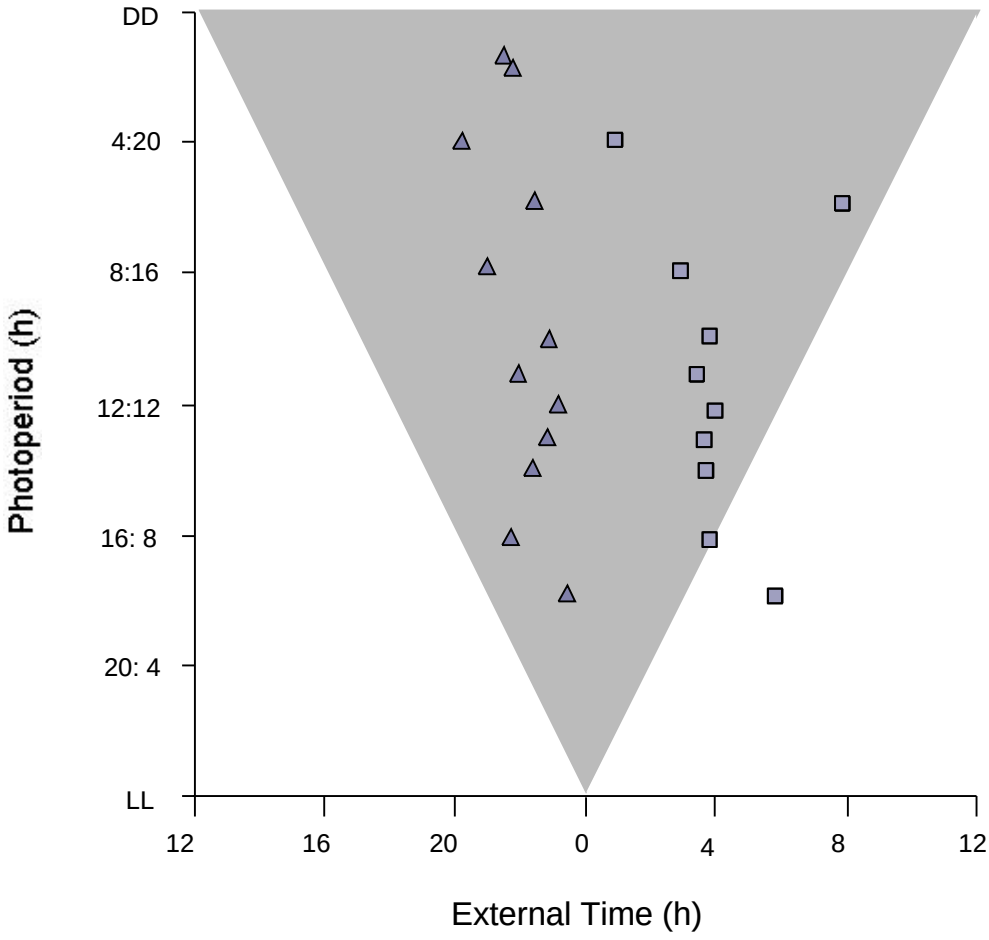

Fig. 3.3 Entrained phase of conidiation: *bd frq*^{1} and *bd frq*^{7} in different photoperiods (T=24h, fluorescent light, 30nE/m^{2}/s). The two period mutant strains, *bd frq*^{1} (FRP=16h) and *bd frq*^{7} (FRP=29h), responded differently than *bd* (FRP=22h). The onset of conidiation of *bd frq*^{1} (striped trangles) occurred around 2 hours before midnight; onset of conidiation of *bd frq*^{7} (striped squares) was consistently approximately 5 hours after midnight. Each time point represents the average of 2 (for *bd frq*^{1}) or 4 (for *bd frq*^{7}) independent experiments with 6 race tubes for each experimental condition within each experiment. Grey area indicates dark phase; open areas indicate light phase.

3.1.2 Photoperiodic response of the formation of asexual spores – conidia

For investigating the photoperiodic response of asexual development of *Neurospora*, conidia production was assessed in different photoperiods (T=24h) (Fig. 3.4).

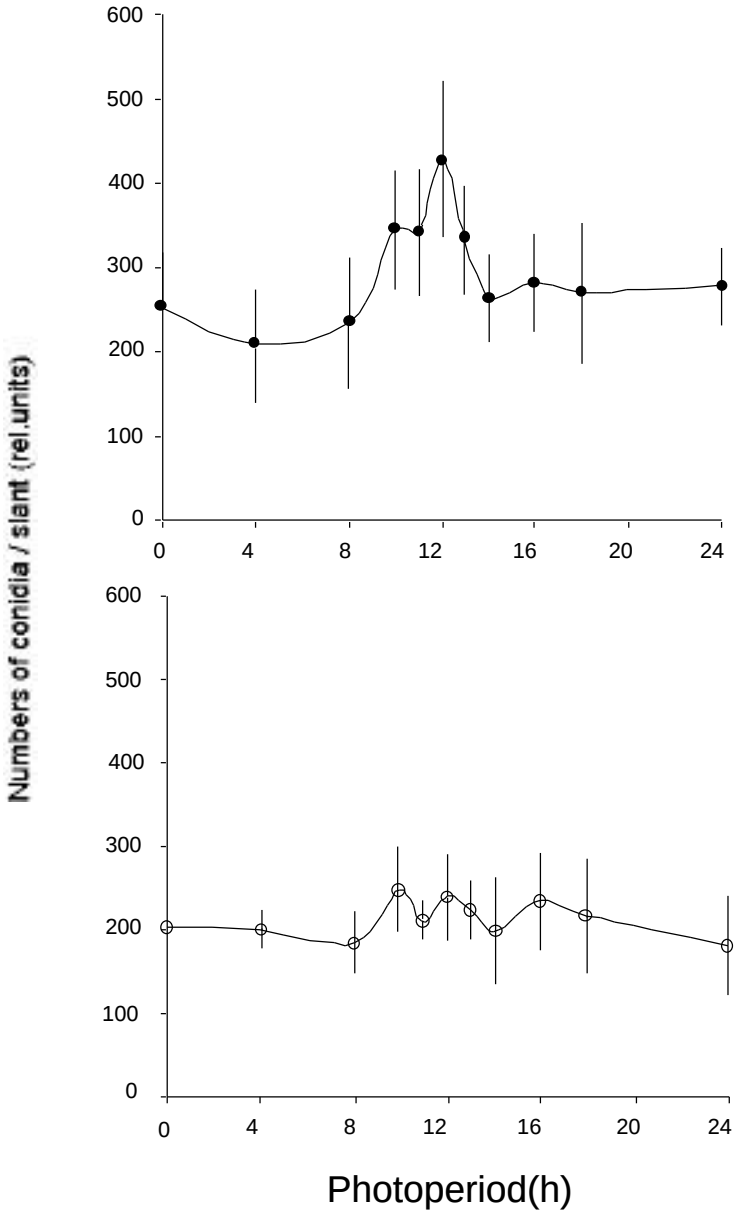

Fig. 3.4 Conidia production in different photoperiods (T=24h, fluorescent light, 30nE/m2/s). Conidia produced in different photoperiods by *bd* (filled circles) and by *bd frq*^{10} (open circles) were collected from 3 slants (Vogel's minimal agar media) and counted (see 2.2.4). Each time point represents the mean ± SD of 45 counts (3 independent experiments with 3 slants for each time point within each experiment, five double-blind counts per slant). Individual counts were averaged for each slant, and normalized for the experiment.

The results of counting conidia showed that about twice as many conidia were produced by the FRQ-sufficient strain *bd* in a photoperiod of around 12 hours compared to shorter and longer photoperiods. The number of conidia of the FRQ-deficient strain *bd frq*^{10} showed no significant relationship to photoperiod, and it was overall less than *bd*.

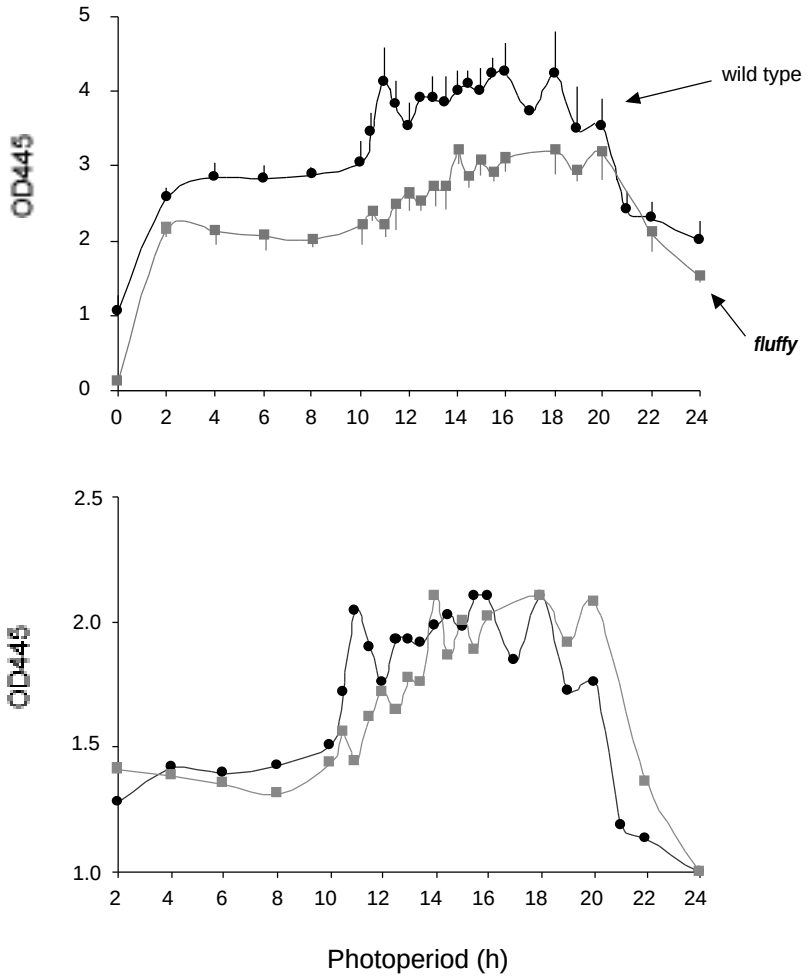

Fig. 3.5 Light induced mycelial carotenoid production in different photoperiods (T=24h, fluorescent light, 3μE/m^2/s). (A) Light-induced mycelial carotenoids of wild type *Neurospora* (*K93-2A*, filled black circles) (not carrying the *bd* mutation) as well as *fluffy* (*fluffy a 7431*, filled grey squares) was enhanced at specific photoperiods. In DD, both wild type and *fluffy* strains produced mycelial carotenoids at basal levels. Between 2- and 10-hour photoperiods, the amount of carotenoids remained unchanged. Carotenoid production started to increase with photoperiods longer than 10 hours, stayed high until 20-hour photoperiods, and decreased in longer photoperiods. Note that the value of carotenoids in constant light was lower than in all photoperiods. Each time point represents the mean ± SD of 15 measurements (for wild type) or 12 measurements (of *fluffy a 7431*) (5 independent experiments for wild type and 4 independent experiments for *fluffy a 7431*, 3 samples in each photoperiod within each experiment). (B) All values were nomalized to that of constant light, to high light the photoperiodic response of carotenoid production.

3.1.3 Photoperiodic response of light induced mycelial carotenoids

Light-induced mycelial carotenoid production was assayed in different photoperiods. Because mycelial carotenoid production requires light, a "jump" from 0 to 1.5 (OD_{445}) occurred when samples of both FRQ-sufficient and FRQ-deficient strains grown in constant darkness were compared to those that had a photoperiod of 2 hours (shortest photoperiod tested). Levels of light-induced mycelial carotenoid production of the FRQ-sufficient strains remained unchanged between 2 and 10 hours of photoperiod, then increased and stayed high until the photoperiod was longer than 20 hours. At photoperiods longer than 20 hours or in constant light, mycelial carotenoid production declined. Interestingly, the level of mycelial cartenoids in LL was lower than all other photoperiods (Fig. 3.5). The aconidial *fluffy* strains were used to investigate light-induced mycelial carotenoid production without generating conidia (Figs. 3.5 - 3.7).

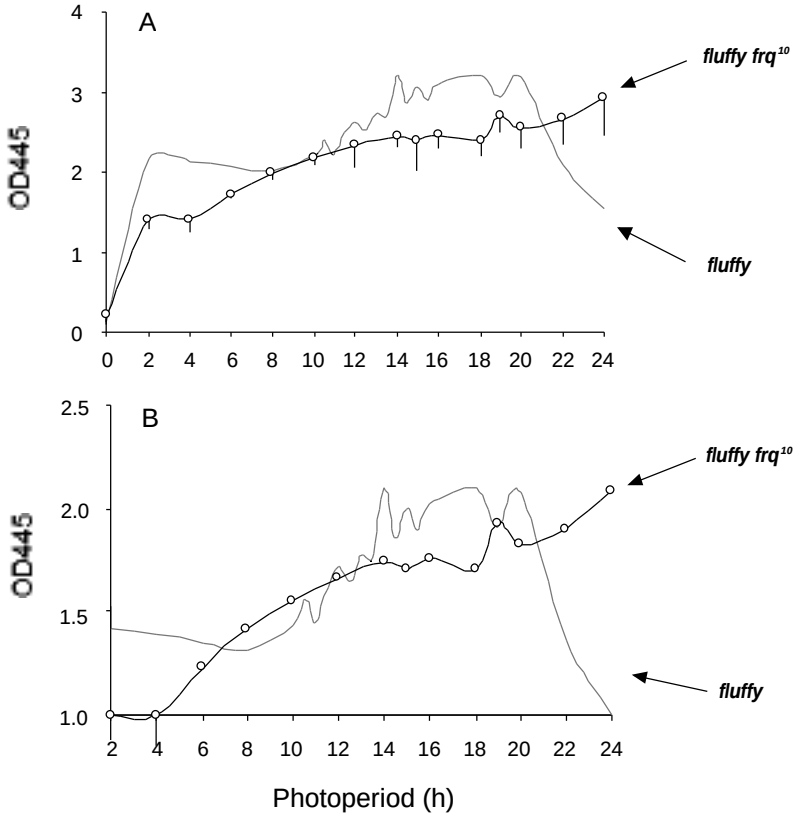

Fig. 3.6 Light induced mycelial carotenoids in different photoperiods (T=24h, fluorescent light, 3μE/m^2/s). (A) The FRQ-sufficient strain *fluffy* (*fluffy a 7431*) showed a photoperiodic response in carotenoid production. In contrast, carotenoids of the FRQ-deficient *fluffy* mutant (*fluffy frq^{10}*, open circles) accumulated according to the amount of light they received. Each time point of *fluffy frq^{10}* represents the mean ± SD of 6 measurements (2 independent experiments with 3 samples for each time point within each experiment). The photoperiodic response of *fluffy* (see Fig. 3.5) was redrawn for comparison. (B) All values of *fluffy* from (A) were normalized to those in LL, and all values of *fluffy frq^{10}* were normalized to that in 2-hour photoperiods.

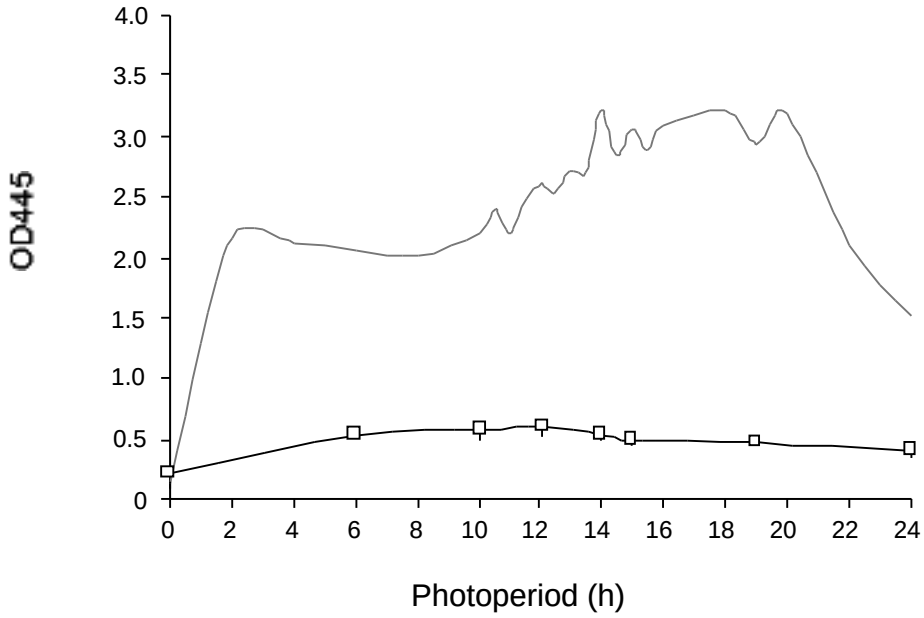

Fig. 3.7 Light induced mycelial carotenoids of *wc-1* mutant in different photoperiods (T=24h, fluorescent light, 3μE/m^2/s). Carotenoid production of the *wc-1* mutant (*fluffy wc-1*, open squares) did not show any apparent response to light. The photoperiodic response of *fluffy* (see Fig. 3.5) was redrawn for comparison. Each time point represents the mean ± SD of 3 samples for each time point within each experiment.

In contrast, light-induced mycelial carotenoid production of the FRQ-deficient strain, *fluffy frq*^{10}, accumulated according to the amount of light they received. The level of mycelial cartenoids of *fluffy frq*^{10} in LL was higher than all other photoperiods (Fig. 3.6).

Mycelial carotenoid production of the wc-1 mutant (*fluffy wc-1*) did not show any apparent response to light (Fig. 3.7).

3.1.4 Photoperiodic response of protoperithecia development

The effect of photoperiod on sexual development of *Neurospora* was investigated by counting the protoperithecia (Fig. 3.8).

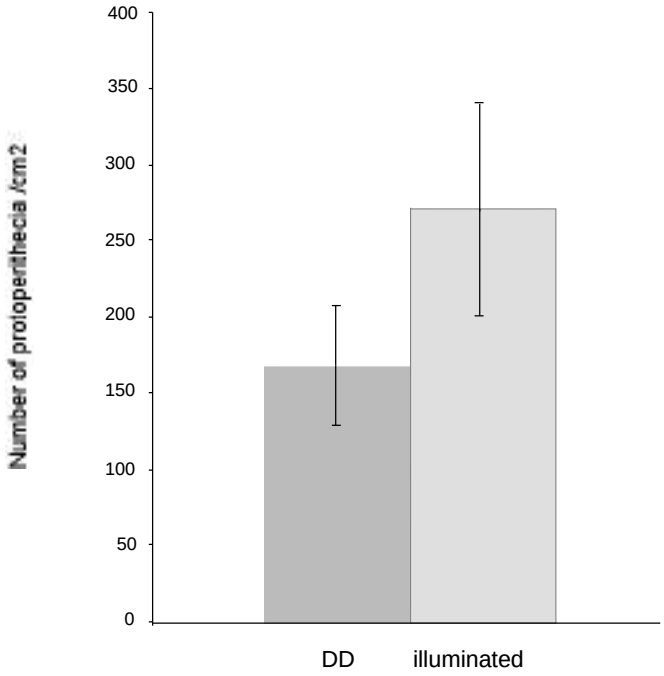

Fig. 3.8 Light induction of protoperithecia. Protoperithecia of *bd* (*bd* 30-7) were induced by brief light exposure (fluorescent light, 3μE/m^2/s, 2 minutes, striped bar) which were almost 2-fold of those in DD (grey bar). Each bar represents the average of 6 petri dishes (with 11 counts/petri dish), i.e. 66 counts contributed to the mean ± SD. 2,000 conidia were inoculated to each petri dish with modified Westergaard's agar media and incubated in DD for 72 hours. 6 petri dishes were exposed to light for 2 minutes. 48 hour after the illumination, all petri dishes including 6 controls in DD were counted under the microscpoe, 11 fields (0.09 cm^2/field) of vision selected at random.

Protoperithecia development of two FRQ-sufficient strains, *bd* and *bd frq*^{1}, showed a photoperiodic response. The amount of protoperithecia of *bd* strain increased constantly, with photoperiod reaching a maximum at 14-hour photoperiods, and decreased in longer photoperiods. Again LL counts were lower than in all photoperiods for FRQ-sufficient strains, similar to DD levels. The short period mutant *bd frq*^{1} responded to photoperiods similar to *bd*, but peaked at a shorter photoperiod, 12-hour. In contrast, the FRQ-deficient strain, *bd frq*^{10} showed no significant response to photoperiod and produced overall much less protoperithecia (Fig.3.9).

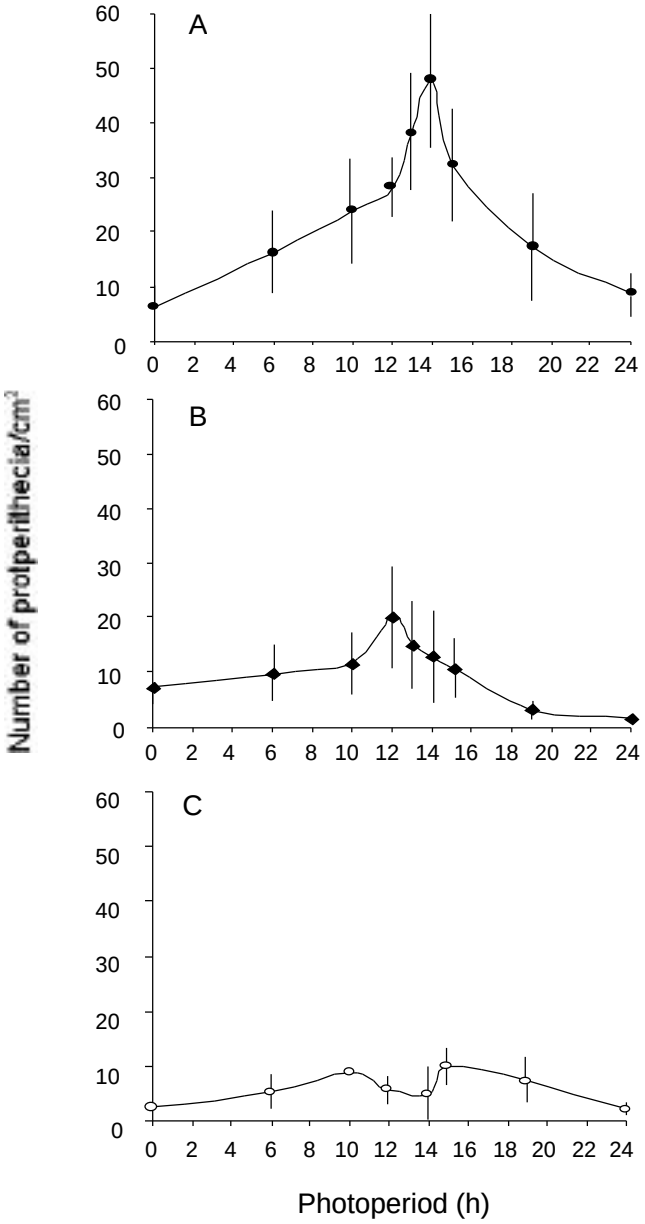

Fig. 3.9 Development of protoperithecia in different photoperiods (T=24h, fluorescent light, 3μE/m^2/s). (A) The results showed that about 10-fold more protoperithecia were produced by the *bd* strain (*bd* 30-7, FRP=22h, filled circles) in the 14-hour photoperiod compared to DD or LL. (B) The protoperithecia of the short period clock mutant *bd frq*^{1} (FRP=16h, filled diamonds) also responded to photoperiods but peaked at around 12-hour photoperiod, and the amplitude of the response was smaller. (C) The protoperithecia of the FRQ-deficient mutant *bd frq*^{10} (open circles) showed no significant response to photoperiod. 2,000 conidia of each strain were inoculated on each petri dish with modified Westergaard's agar media and incubated for 4 full photoperiod cycles (96 hours) prior to counting. For *bd*, each time point represents mean ± SD of 150 counts (5 independent experiments with 3 petri dishes per photoperiod within each experiment, 10 blind counts/petri dish). For *bd frq*^{1}, each time point represents mean ± SD of 60 counts (2 independent experiments with 3 petri dishes per photoperiod within each experiment, 10 blind counts/petri dish). For *bd frq*^{10}, each time point represents the mean ± SD of 30 counts (1 experiment with 3 petri dishes per photoperiod, 10 blind counts/petri dish).

The aconidial and highly fertile *fluffy* mutants, which provided the possibility for counting protoperithecia at a clean background, were used to investigate the protoperithecia in photoperiod, and same results were obtained (Fig. 3.10).

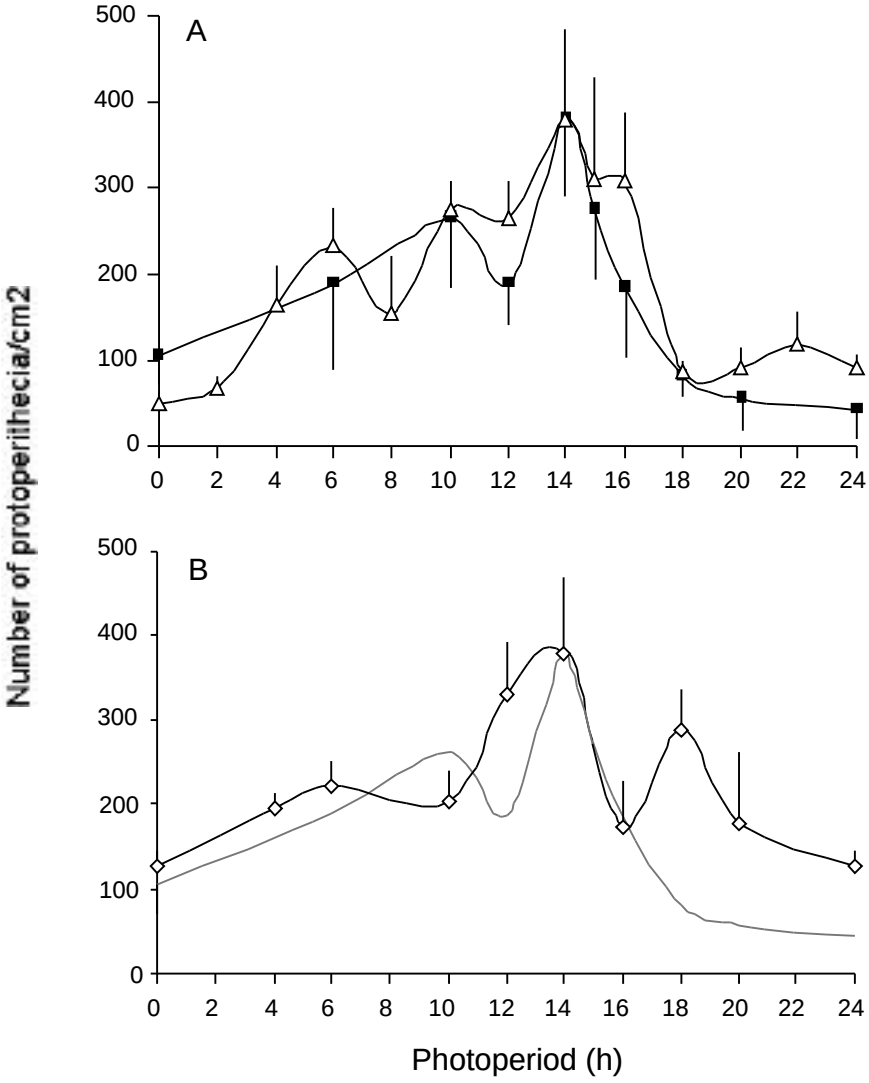

Fig. 3.10 Development of protoperithecia of *fluffy* strains in different photoperiods (T=24h, fluorescent light, 3µE/m^2/s). (A) *fluffy* (*fluffy a 7431*, filled squares), *fluffy bd* (open triangles) and (B) *fluffy bd frq*^{1} (open diamonds) showed photoperiod-specific response. All strains produced approximately 4-fold more protoperithecia in 14-hour photoperiod compared to DD or LL. 96 µl of homogeneous hyphal suspension was inoculated to each petri dish (Ø53mm) with 6 ml of standard Westergaard's agar media. Each time point represents mean ± SD of 15 counts (1 experiment, 3 petri dishes of each strain per photoperiod, 5 blind counts/petri dish). Number of protoperithecia of *fluffy bd* and *fluffy bd frq*^{1} were normalized to the maximum of *fluffy* for comparison.

Unlike the FRQ-suffcient strains, the FRQ-deficient strain, *fluffy frq*^{10}, as well as the WC-1-deficient strain, *fluffy wc1*, showed no significant photoperiodic response. (Fig. 3.11)

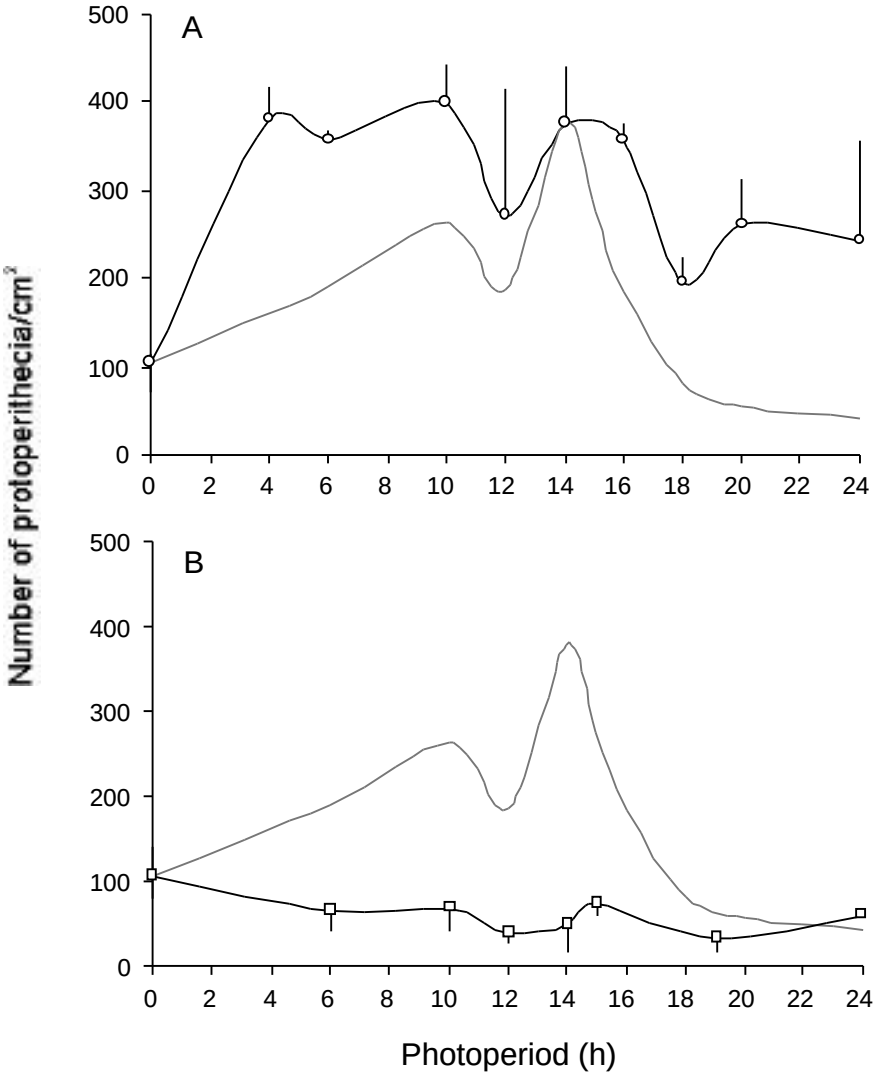

Fig. 3.11 Development of protoperithecia of *fluffy* strains in different photoperiods (T=24h, fluorescent light, 3µE/m^2/s). (A) Unlike the FRQ-sufficient strain *fluffy* (*fluffy a 7431*, filled squares), protoperithecia of the FRQ-deficient strain *fluffy frq*^{10} (open circles) were induced by light and remained as high in all photoperiods as the FRQ-sufficient strain at its maximum. (B) Protoperithecia were not light-induced in the *fluffy wc-1* strain (open squares). 92 µl of homogeneous hyphal suspension was inoculated to each petri dish (Ø53mm) with 6 ml standard Westergaard's agar media. Each time point represents mean ± SD of 15 counts (1 experiment, 3 petri dishes of each strain per photoperiod, 5 blind counts/petri dish). Numbers of protoperithecia of *fluffy frq*^{10} and *fluffy wc-1* were normalized to that of *fluffy* in DD for comparison.

3.1.3 Photoperiodic response of ascospore-shooting

When protoperithecia were fertilized with the conidia of the opposite mating type, ripe perithecia started to shoot ascospores in around 7 days. Ascospores in different photoperiods were collected, counted and normalized to the number of perithecia. The FRQ-sufficient strains (*bd a* and *bd A*) showed a photoperiodic response (maximum at 14-hour) with almost no spores produced in either DD or LL. In contrast, the FRQ-deficient strains showed no photoperiodic response (Fig.3.12).

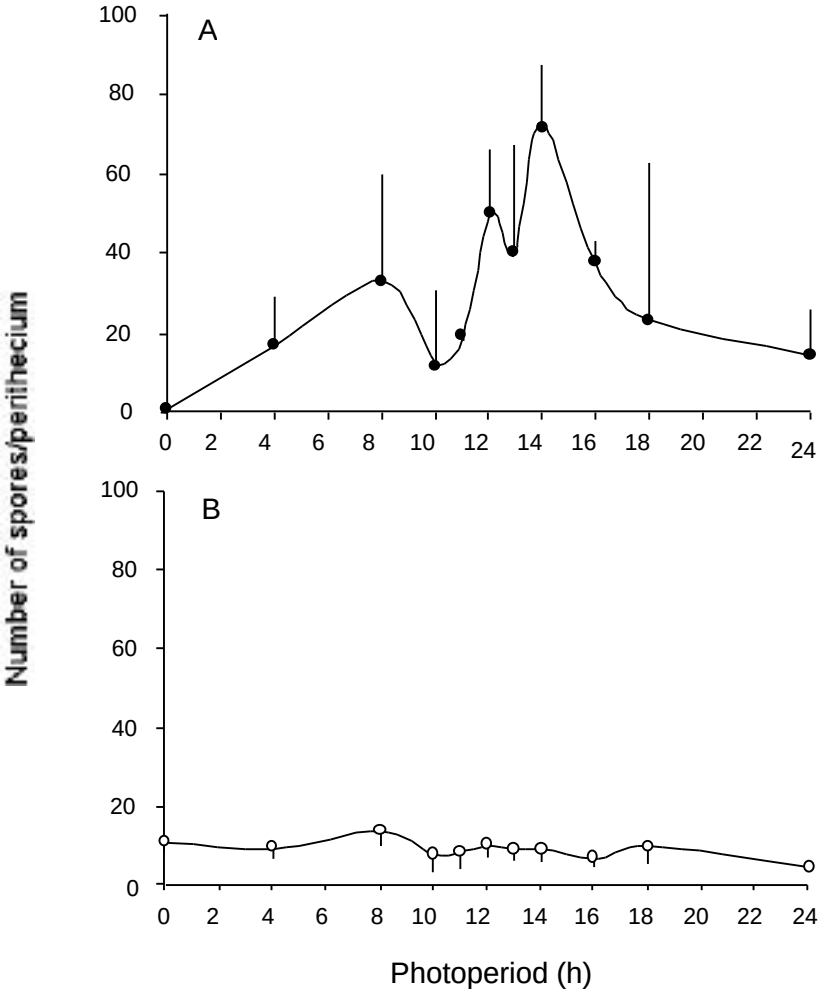

Fig. 3.12 Spore-shooting in different photoperiods (T=24h, fluorescent light, 3μE/m^2/s). (A) In photoperiod cycles, perithecia of FRQ-sufficient strains (*bd a* and *bd A*) produced sexual spores while no spores were shot in DD and LL in spite of forming perithecia. Approximately 80 spores per perithecium were produced in a 14-hour photoperiod. (B) In contrast, the FRQ-deficient strains (*bd frq^{10} a* and *bd frq^{10} A*) produced approximately 10 spores per perithecium in all conditions tested, indicating both an activation (in photoperiods) and an inhibition (in constant conditions) of the FRQ-clock on spore production. Each time point represents mean ± SD of 3 counts (1 experiment, 3 petri dishes of either FRQ-sufficient strains or FRQ-deficient strains).

3.2 Physiological responses in a non-24-hour photoperiod (T=18h)

3.2.1 Influence of photoperiod on carotenoid production

In the context of 18-h cycles, carotenoid production showed a clear photoperiodic response which was different from the response in the context of 24-h cycles.

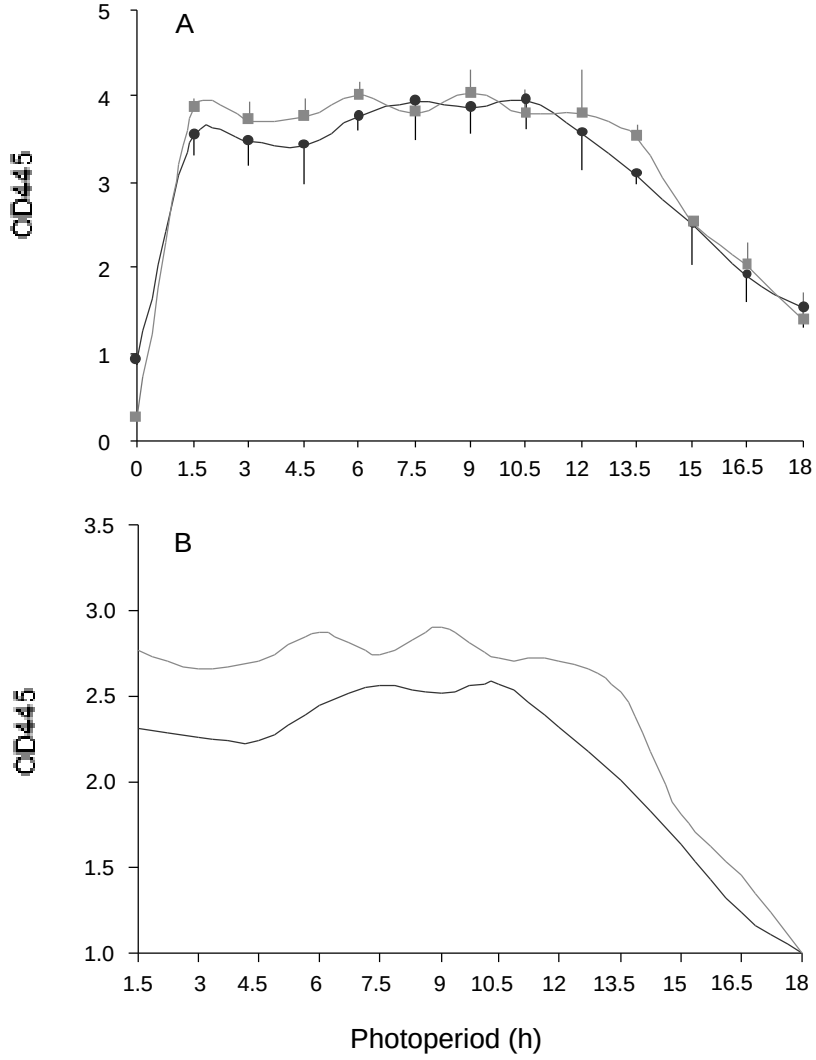

Fig. 3.13 Photoperiodic response in an 18-hour cycle: Light-induced mycelial carotenoid production (fluorescent light, 3µE/m^2/s). (A) Light-induced mycelial carotenoids of wild type *Neurospora* (*K93-2A*, filled black circles) and *fluffy* (*fluffy a 7431*, filled grey squares) reached maximal levels within 1.5-hour photoperiods – the shortest one tested. Between 1.5 and 13.5 hours photoperiods, carotenoids remained unchanged at high levels, and then started to decrease. Note that the value of carotenoids in LL was again much lower than in all photoperiods. For wild type, each time point represents mean ± SD of 9 measurements (3 independent experiments, 3 petri dishes per photoperiod with each experiment). For *fluffy*, each time point represents the mean ± SD of 3 measurements (1 experiment, 3 petri dishes per photoperiod). (B) Values from (A) were normalized to those in LL, omitting the DD results.

Light-induced mycelial carotenoids did not increase gradually to maximal levels in the context of 18-hour photoperiod. Rather, carotenoids were induced by light to maximal level already at the shortest photoperiod tested (1.5-h), and remained high until the photoperiod exceeded 13.5-h. The concentration of carotenoids in LL was again lower than those in all other photoperiods (Fig.3.13).

3.2.2 Photoperiodic response of protoperithecia development

When the response of photoperiod on protoperithecia formation of *bd* strains was measured in the context of 18-hour cycles, the response was similar to that in 24-h cycles. LD proportions were calculated from 24-h photoperiod experiments.

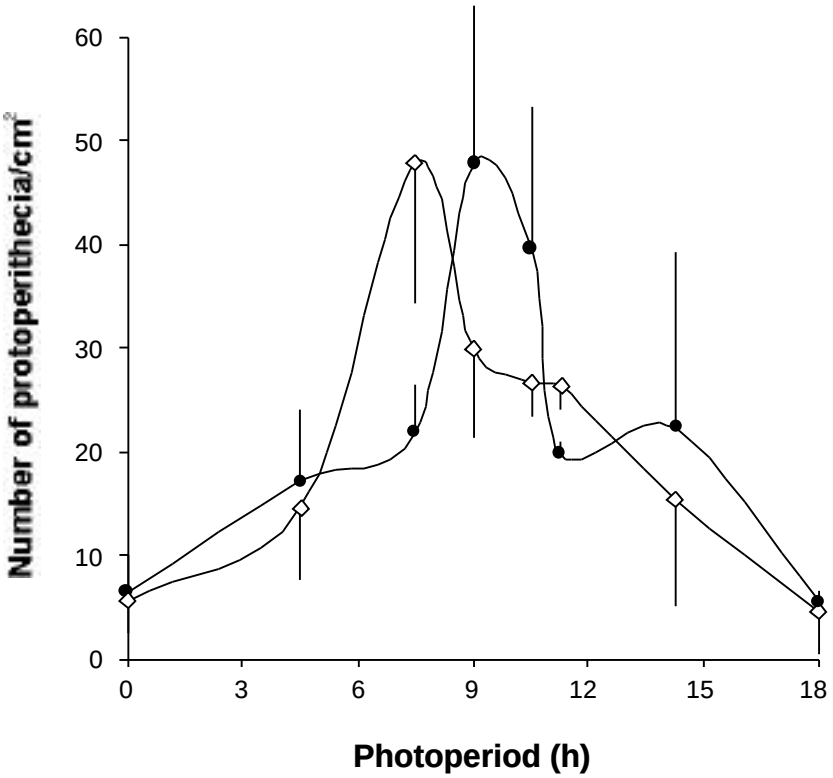

Fig. 3.14 Photoperiodic response in an 18-h cycle: Development of protoperithecia (fluorescent light, 3μE/m^2/s). Approximately 10-fold more protoperithecia were produced by the *bd* strain (*bd* 30-7, filled circles) around 9-h photoperiod compared to DD or LL. The protoperithecia production of the short period clock mutant, *bd frq^1* (open diamonds) peaked around 7.5-h photoperiod. Each time point represents the mean ± SD of 30 counts (1 experiment, 3 petri dishes of each strain per photoperiod, 10 blind counts/petri dish). 2,000 conidia of either *bd* or *bd frq^1* were inoculated on each petri dish with modified Westergaard's agar media and incubated for 96 hours (i.e., 5.3 full photoperiod cycles) prior to counting.

In the context of 18-hour cycles, the maximal protoperithecia production by *bd* strain (FRP=22h) was at around 9-h photoperiod. Again, the short period clock mutant *bd frq*^{1} (FRP=16h) reached its maximal level at a shorter photoperiod (7.5-h) (Fig.3.14).

3.3 Physiological Responses in Night-break Experiments

Classical night-break protocol was used to investigate the mechanism of photoperiodism. A typical night-break experiment starts with a main light period followed by an extended dark period which is interrupted by a shorter light pulse at different phases of the dark.

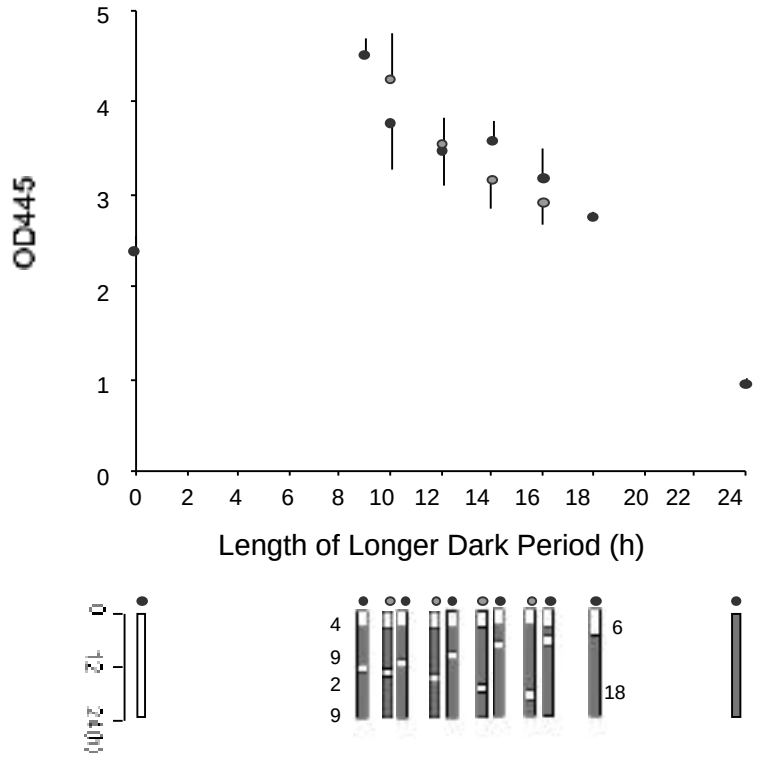

Fig. 3.15 Light-induced mycelial carotenoid production in night-break experiments (T=24h, fluorescent light, 3μE/m^2/s). The photoperiodic response of *Neurospora* measured the duration of interrupted darkness, as shown with light-induced mycelial carotenoid production of wild type *Neurospora* (*K93-2A*). All photoperiods contained the same total length of light and darkness, 6 and 18 hours respectively. Values of carotenoid production in DD, LL and the only full photoperiod LD 6:18 (at 18-h point) were shown as controls. Cartoons of the different light (white) and dark (grey) distributions are shown in the bottom panel. Each time point represents mean ± SD of 6 measurements (2 independent experiments, 3 petri dishes within each experiment). Data of other strains were presented in following figure (Fig. 3.15 a, b, c, d).

3.3.1 Light-induced mycelial carotenoids in night-break experiments

Light induced mycelial carotenoid production was investigated in night-break experiments. All light-dark cycles contained 6 hours of light and 18 hours of darkness, however, their distribution within the 24-h cycle was changed, while all cycles began with 4 hours of light and an additional 2-hour was "moved" through the 18-hour dark period (Fig 3.15; 3.15 a, b, c, d).

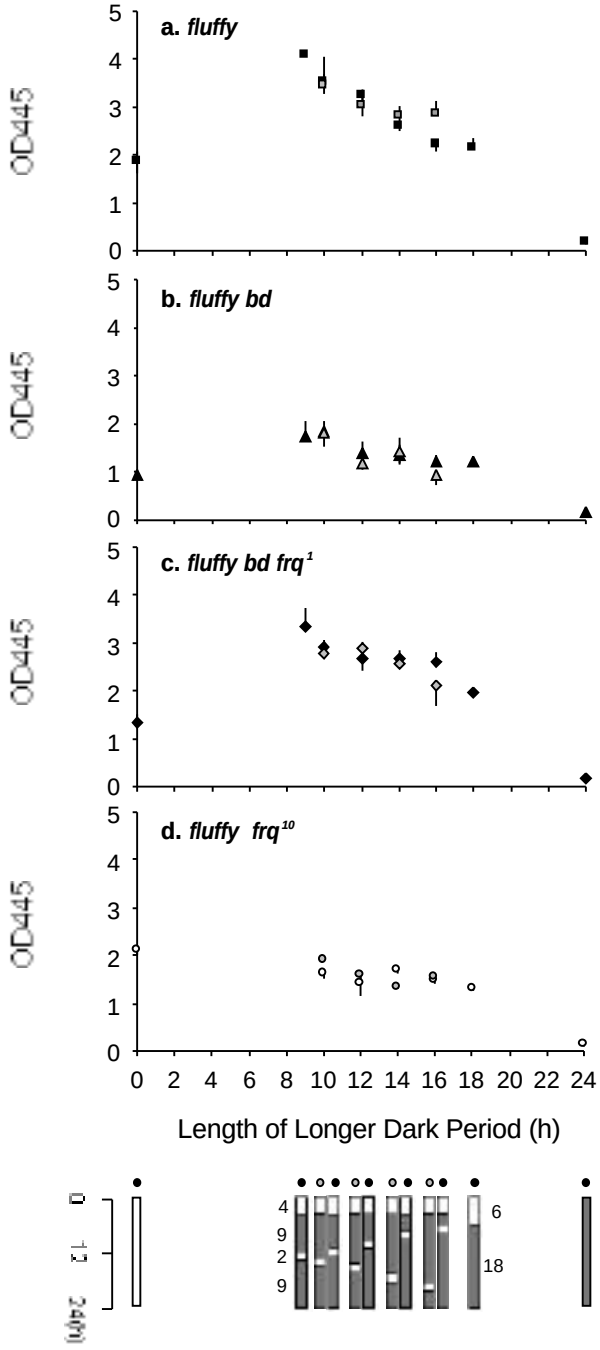

Fig. 3.15 a-d. See text for details.

All FRQ-sufficient strains tested gave similar responses to the different light interruptions. For all FRQ-sufficient strains, light-induced mycelial carotenoids reached maximal levels when the short light pause fell in the middle of the 18-hour dark period, i.e., the shortest uninterrupted dark period (9 h). Carotenoid production in the complete photoperiod (LD 6:18), which had the longest length of darkness, was always lower than in interrupted scotoperiods. The FRQ-deficient mutant, *fluffy frq*^{10} showed the weakest differences between the different conditions.

3.3.2 Protoperithecia development in night-break experiments

Protoperithecia development was investigated with the same protocol in night-break experiments, and the response was similar to that of carotenoids. (Fig. 3.16A, B)

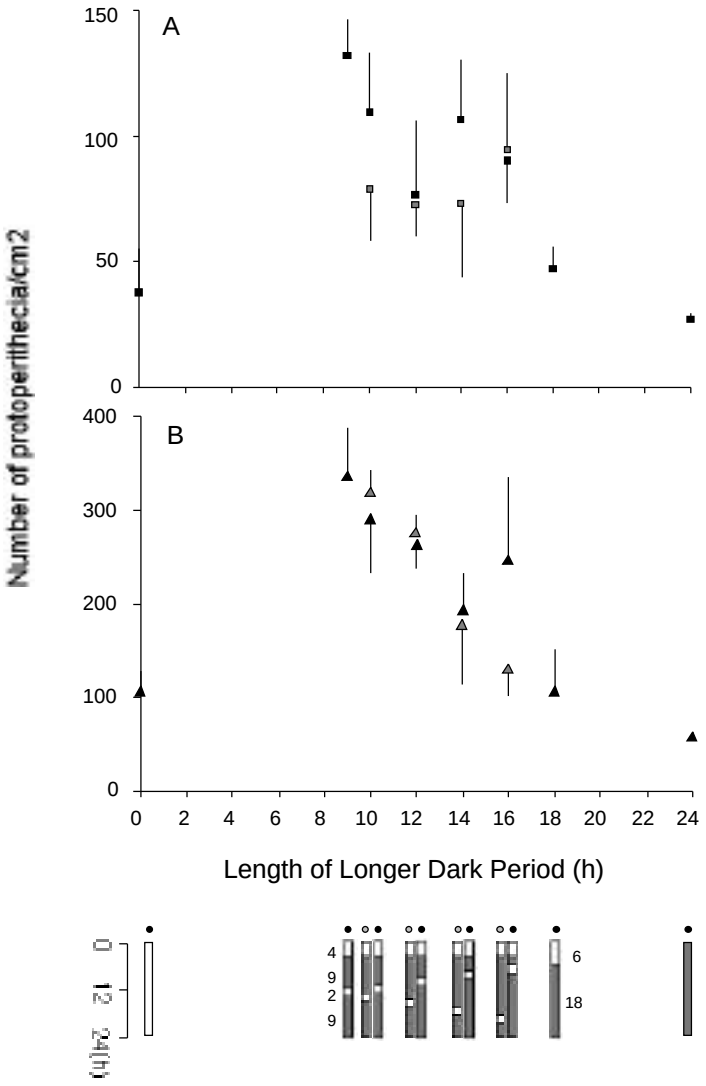

Fig. 3.16 Development of protoperithecia in night-break experiments (T=24h, fluorescent light, 3µE/m^2/s). (A). The photoperiodic response of *Neurospora* measured the duration of interrupted darkness, as shown with the protoperithecia development of *fluffy* (squares). All photoperiods contained the same total length of light and darkness, 6 and 18 hours respectively. Numbers of protoperithecia in DD, LL and the complete photoperiod LD 6:18 (at 18-h point) were shown as controls. (B) *fluffy bd* (triangles) responded to interrupted photoperiods as did *fluffy*. 92 µl of homogeneous hyphal suspension of either *fluffy* or *fluffy bd* was inoculated to each petri dish (Ø53mm) with 6 ml standard Westergaard's agar media. Cartoons of the different light (white) and dark (grey) distributions are shown in the bottom panel. Each time point represents mean ± SD of 15 counts (1 experiment, 3 petri dishes of each strain with 5 blind-count/petri dish).

The responses of the protoperithecia development to interrupted photoperiods were very similar to those for carotenoid production.

3.4 Molecular Profiles in Different Photoperiods

Data from physiological experiments indicated that FRQ, the key component of the circadian clock, was involved in photoperiodic time measurement. It was of interest to investigate the daily expression profiles of molecular clock components.

3.4.1 Expression of light-regulated mRNA expression in photoperiods

3.4.1.1 *frq* mRNA regulation in photoperiods

The transcription of the critical clock gene *frequency* in different photoperiods was assayed with “Real-Time PCR” (RT-PCR). The results showed that *frq* mRNA was induced by light rapidly to its maximal level, within 15 minutes after lights-on, and then adapted to a lower, saturation level within 2 hours. After lights-off, *frq* mRNA rapidly decreased within 2 hours. This kinetic was seen in all the photoperiods tested, from 4-h up to 20-h of light per 24-h cycle. In 4-h photoperiod cycle, in which the night is long, *frq* mRNA levels rose again at midnight, while in 10-h photoperiod cycle, *frq* mRNA levels came up again near the end of darkness. In 14-h and 20-h photoperiod cycles, in which nights are shorter, *frq* mRNA expression was still at the basal levels at the end of darkness (Fig. 3.17).

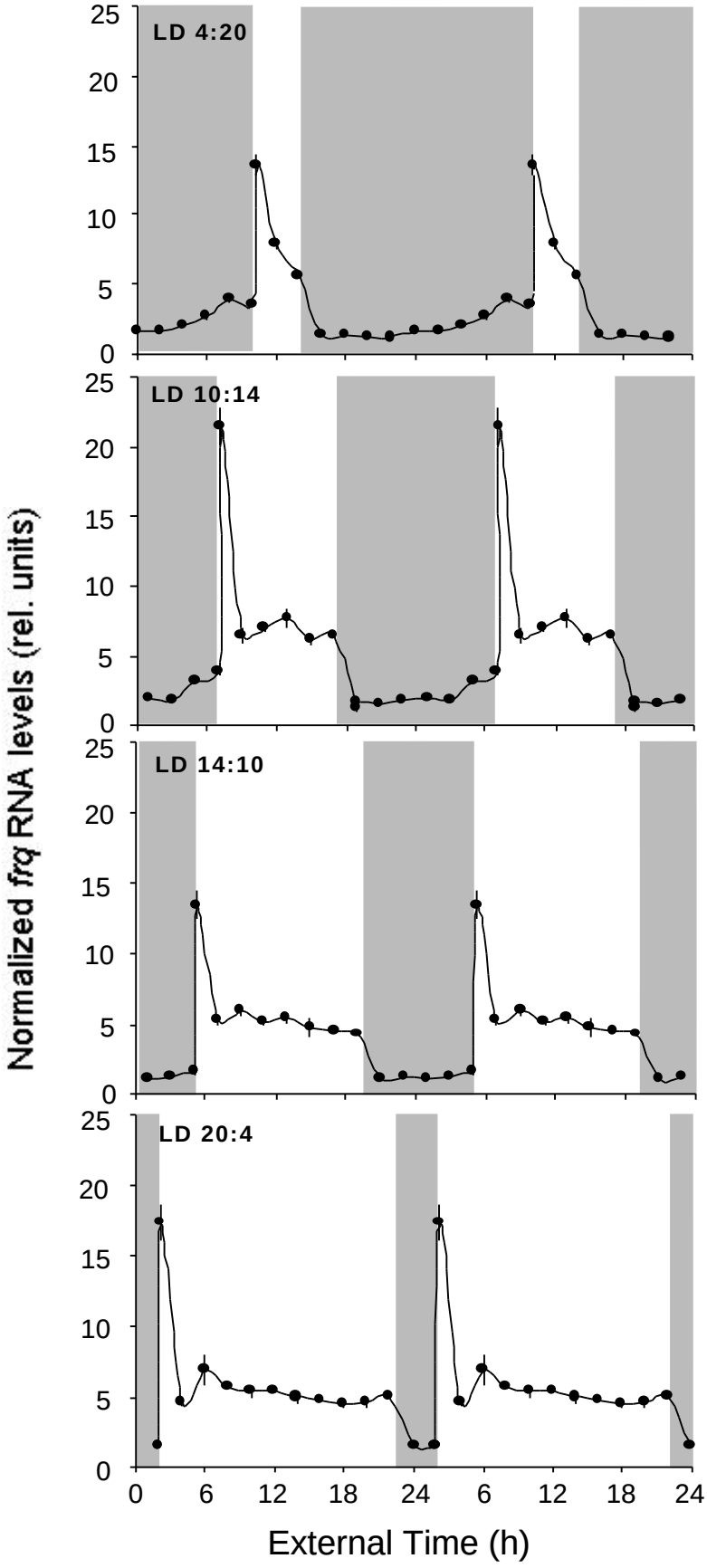

Fig. 3.17 *frq* mRNA expression in different photoperiods (T=24h, fluorescent light, 3µE/m^2/s). The clock gene *frq* in wild type *Neurospora* (*K93-2A*) was induced by light to its highest level within 15 minutes and then dropped to a lower, sustained adaptation level (note that *frq* was induced to higher levels by light in LD 10:14 photoperiod compared to other photoperiods tested). After lights-off, *frq* mRNA dropped exponentially to low levels. In LD 4:20 cycle, in which the night was long, *frq* mRNA levels rose again at midnight; in a LD 10:14 cycle, *frq* mRNA levels came up again near the end of darkness; in a LD 14:10 cycle, at almost the end of the darkness. In LD 20:4 cycles, which the night was much shorter, *frq* mRNA was still at the basal levels at the end of darkness. *frq* mRNA levels were normalized to ribosomal rRNA (mRNA/rel.). Tissue was grown in modified Westergaard's media in different photoperiods for 5 full cycles before harvesting. Each time point represents mean ± SD of triplicates of each RNA sample. Grey areas indicate darkness; white areas light.

3.4.1.2 *wc-1* mRNA regulation in photoperiods

The *wc-1* gene encodes the blue light photoreceptor of *Neurospora*, and the protein is another central player of the transcription/translation feedback loop of the *Neurospora* circadian clock. *wc-1* mRNA expression was first analyzed in LD 12:12 by Northern blotting.

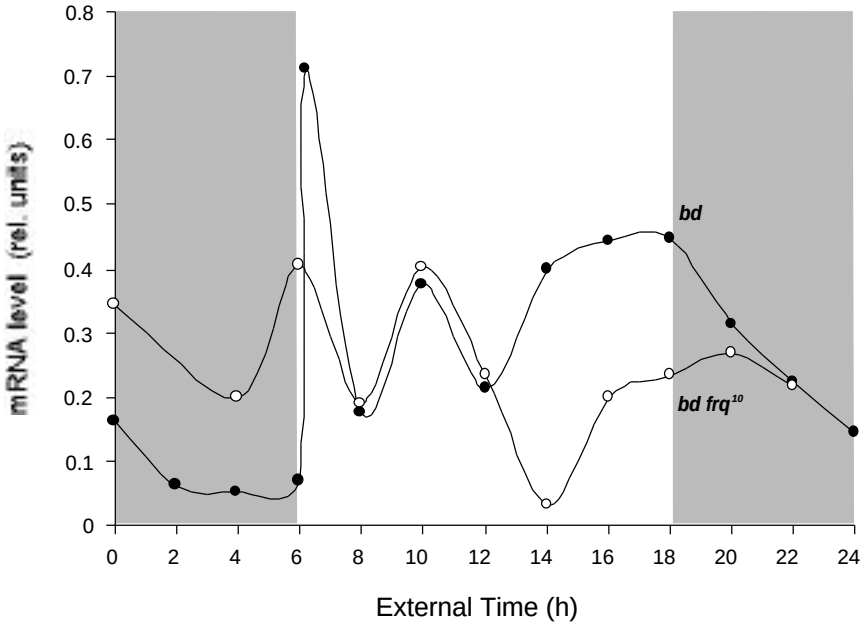

Fig. 3.18 *wc-1* mRNA expression in a 12:12 LD cycle (fluorescent light, 2µE/m^2/s). *wc-1* mRNA in FRQ-sufficient strain (*bd*, filled circles) was induced within 15 minutes in light, followed by a rapid adaptation. Compared to *frq* mRNA, *wc-1* mRNA levels decreased much slower after light-off, and were still at the basal level at the end of darkness. *wc-1* mRNA in FRQ-deficient strain (*bd frq*^{10}, open circles) was analyzed in the same experiment, and showed no apparent regulation by light. Tissue was grown in Vogel's media in LL for 12 hours before transferred to light or dark phase of LD cycles. All samples were harvested within 12 hours. *wc-1* mRNA of the indicated time points were prepared and analyzed by Northern blotting, and the signals were normalized to rRNA (RNA/ribo.rel.units). Grey areas indicate dark period; open area indicates light period.

The results of the Northern blotting of *wc-1* mRNA in the FRQ-sufficient strain (*bd*) in LD 12:12 showed that *wc-1* mRNA was induced by light to peak level within 15 minutes, and like *frq* mRNA, returned to a lower adaptation level. It decreased further in dark phase and reached the lowest level at the end of darkness. *wc-1* mRNA in the FRQ-deficient strain (*bd frq*^{10}) was not significantly regulated by light (Fig. 3.18).

The tissue for the Northern blot was generated in Vogel's media, but the experiments in photoperiods of protoperithecia development and carotenoid production were done using modified Westergaard's media. Therefore, the *wc-1* RNA was re-investigated systematically using tissue grown in modified Westergaard's media in different photoperiods. The regulation of *wc-1* mRNA expression in modified Westergaard's media was analyzed by RT-PCR in the same series of photoperiods as shown in Fig 3.17.

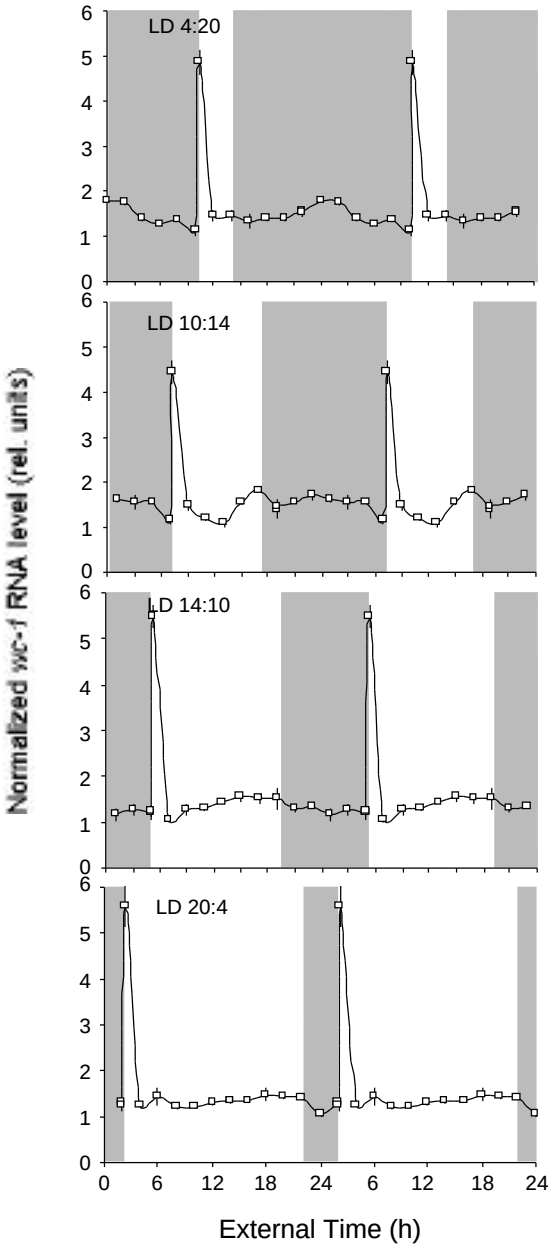

Fig. 3.19 *wc-1* mRNA expression in different photoperiods (T=24h, fluorescent light, 3μE/m^2/s). Like *frq*, *wc-1* mRNA in wild type *Neurospora* (*K93-2A*) was also induced by light to peak levels within 15 minutes after lights-on. However, within 2 hours of light induction, *wc-1* mRNA decreased to its basal level in all photoperiods tested. *wc-1* mRNA signals were normalized to ribosomal rRNA. Tissue was grown in modified Westergaard's media for 5 full cycles before harvesting. Each time point represents mean ± SD of triplicates of each RNA sample. Grey areas indicate darkness; white areas light.

wc-1 mRNA levels, which, like *frq*, were rapidly induced by light within 15 minutes. Unlike *frq* in the same experiment and unlike *wc-1* in Vogel's media, *wc-1* mRNA rapidly adapted to the lowest levels which were the same in darkness. Thus, the light responses of *wc-1* depended on medium composition, and the response to lights-off was absent (Fig. 3.19).

3.4.2 Expression of FRQ protein in photoperiods

3.4.2.1 FRQ expression in Vogel's media in light:dark cycles

The regulation of FRQ protein was first analyzed in different photoperiods (LD 12:12 and 14:10) in Vogel's media. All previous measurements of FRQ protein were recorded in cultures from constant light or constant darkness (see numerious references).

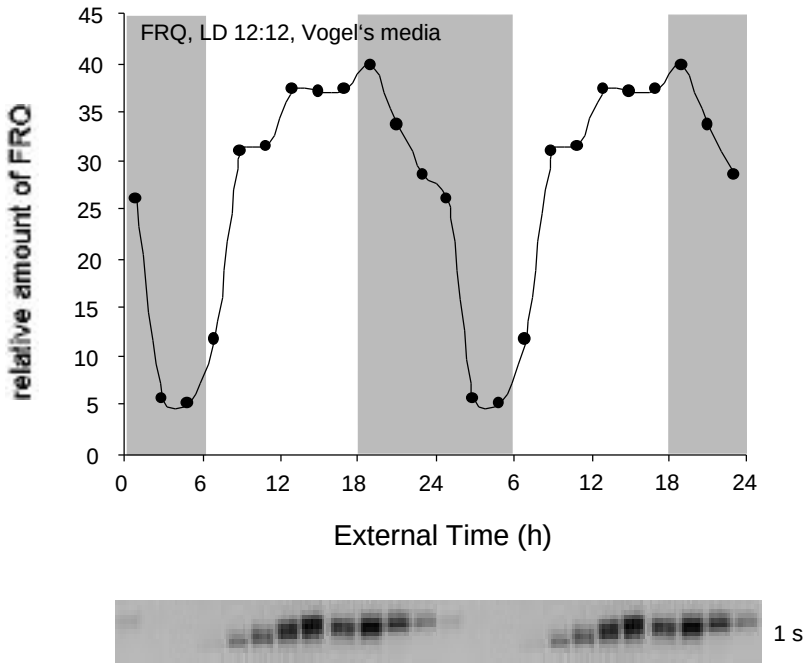

Fig. 3.20 FRQ regulation in LD 12:12 (fluorescent light, 2.2µE/m^2/s). Quantification of Western blots of FRQ (*bd* 30-7) showed FRQ levels increased steadily in light, and steadily decreased in darkness, reaching the lowest level approximately 1 hour before lights-on. Pads were generated by growth at 30ºC for 72 hours following inoculation. Two discs cut from the pads were placed into a flask containing 100 ml Vogel's media and grown with shaking (120 rpm) in LL for 2 hours before transfer to LD 12:12. Duplicate sets of flasks were transferred to reciprocal LD 12:12 cycles, so that the tissue could be harvested within 12 hours of development. Harvests began 45 hours incubation in LD cycles. Immunodetection was performed using a monoclonal antibody (αFRQ 3G11) which detects the long form of FRQ protein. Relative amounts of FRQ for each time point were analyzed on Western blots. Exposure time (1 second) of the Western blot is indicated at right.

In 12-h photoperiods, in Vogel's media, FRQ levels were induced by light within 1 hour. Unlike *frq* RNA profiles, FRQ increased continuously in light. FRQ levels decreased steadily in darkness, and reached the lowest level approximately 1 hour before lights-on. As showed in the Western blot, after lights-on, low molecular weight species of FRQ appeared first and gradually developed to higher molecular weights and became phosphorylated progressively; after lights-off, FRQ disappeared first from low molecular weight as it consolidated to high molecular weight, and then degraded almost entirely. Phosphorylation of FRQ is responable for these molecular weight changes (Garceau, 1997) and dictates stability of FRQ (Fig. 3.20) (Görl, 2001).

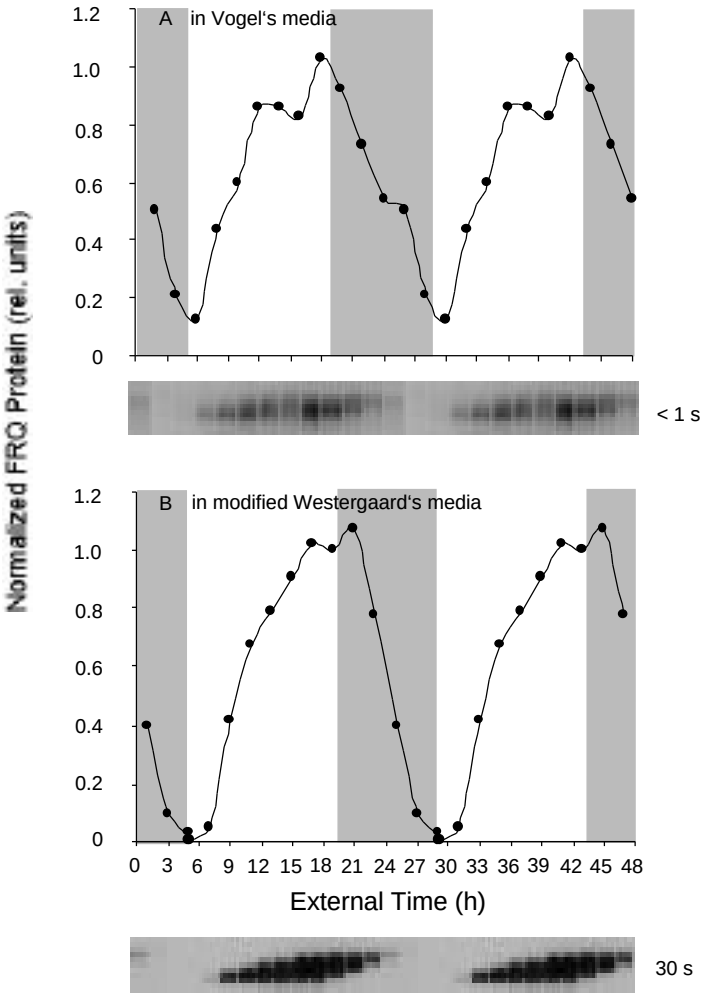

Fig. 3.21 FRQ expression in LD 14:10 in different media (fluorescent light, 3μE/m^2/s). (A) Results from the quantification of Western blots of FRQ (*bd* 30-7) in Vogel's media showed that FRQ increased steadily in light and decreased after 1 hour in darkness. (B) FRQ of wild type *Neurospora* (*K93-2A*) in modified Westergaard's media showed similar responses to LD 14:10 photoperiods. Note that in these media the maximal levels of FRQ in the two FRQ-sufficient strains were similar, and the amplitude of FRQ expression in modified Westergaard's media was higher. Tissue in Vogel's media was grown as same as described in Fig. 3.20 (i.e., in flasks containing 100 ml Vogel's media for 45 hours before harvest, etc.). In modified Westergaard's media, tissue was grown in petri dishes for 5 full LD cycles before harvesting. Immunodetection was performed using a monoclonal antibody (αFRQ 4D11) which detected both long and short forms of FRQ protein. FRQ levels on each blot were normalized to the ponceau staining. FRQ levels from different blots were normalized to the same scale when one sample of each blot was compared on a single blot and quantitated in comparison with each other. Exposure time of each blot is shown at right.

3.4.2.2 Comparison of FRQ expression in FRQ-sufficient strains in the same photoperiod (LD 14:10) in Vogel's versus modified Westergaard's media

As for the mRNA profiles, the expression pattern of FRQ in the photoperiodic response must be investigated in the same media which was used to determine the photoperiodic responses of carotenoid production, protoperithecia development and spore-shooting. For media comparison, FRQ expression was assayed in the same photoperiod (LD 14:10) in both modified Westergaard's media and Vogel's media. The main difference between these two media is that the former one has poor nitrogen and limited carbon source, the latter one is rich in these nutrients.

In both Vogel's media and modified Westergaard's media, FRQ showed similar responses in a LD 14:10 cycle. FRQ was induced by light and increased continuously in light and decreased in darkness. As in the LD 12:12 cycle, (Fig.3.20), FRQ appeared at low molecular weight first after lights-on, and migrated gradually to higher molecular weight, due to progressive phosphorylation. After lights-off, FRQ moved gradually from low molecular weight to high molecular weight forms. FRQ was almost undetectable approximately 1 hour before lights-on in Vogel's media, and approximately between 3 hours before lights-on to 15 minutes after lights-on in modified Westergaard's media (Fig. 3.21). In the experiment in Vogel's media, the *bd* strain (*bd* 30-7) was used. In modified Westergaard's media, the wild type strain (*K93-2A*) was used.

Next, the FRQ expression in different photoperiods was determined. In contrast to the regulation of *frq* RNA, FRQ protein of the wild type *Neurospora* (*K93-2A*) was induced by light within 15 minutes only in the short photoperiod (LD 4:20). In longer photoperiods, FRQ started to increase after 2 hours of light. Except for the longest photoperiod (LD 20:4), FRQ levels kept rising in the light phase and even during the first 2 hours in the darkness. Thereafter, FRQ decreased constantly in the darkness. In the longest photoperiod (LD 20:4) tested, FRQ did not increase significantly for 6 to 8 hours in light, and already started to decrease 2 hours before the light went off. The amplitude of the oscillation was the largest in the 14-h photoperiod, and was smallest in the 20-h photoperiod. In the 14-h photoperiod, FRQ decreased to undetectable levels at the end of dark period (Fig. 3.22). Western blots of FRQ in different photoperiods were presented in Fig. 3.23.

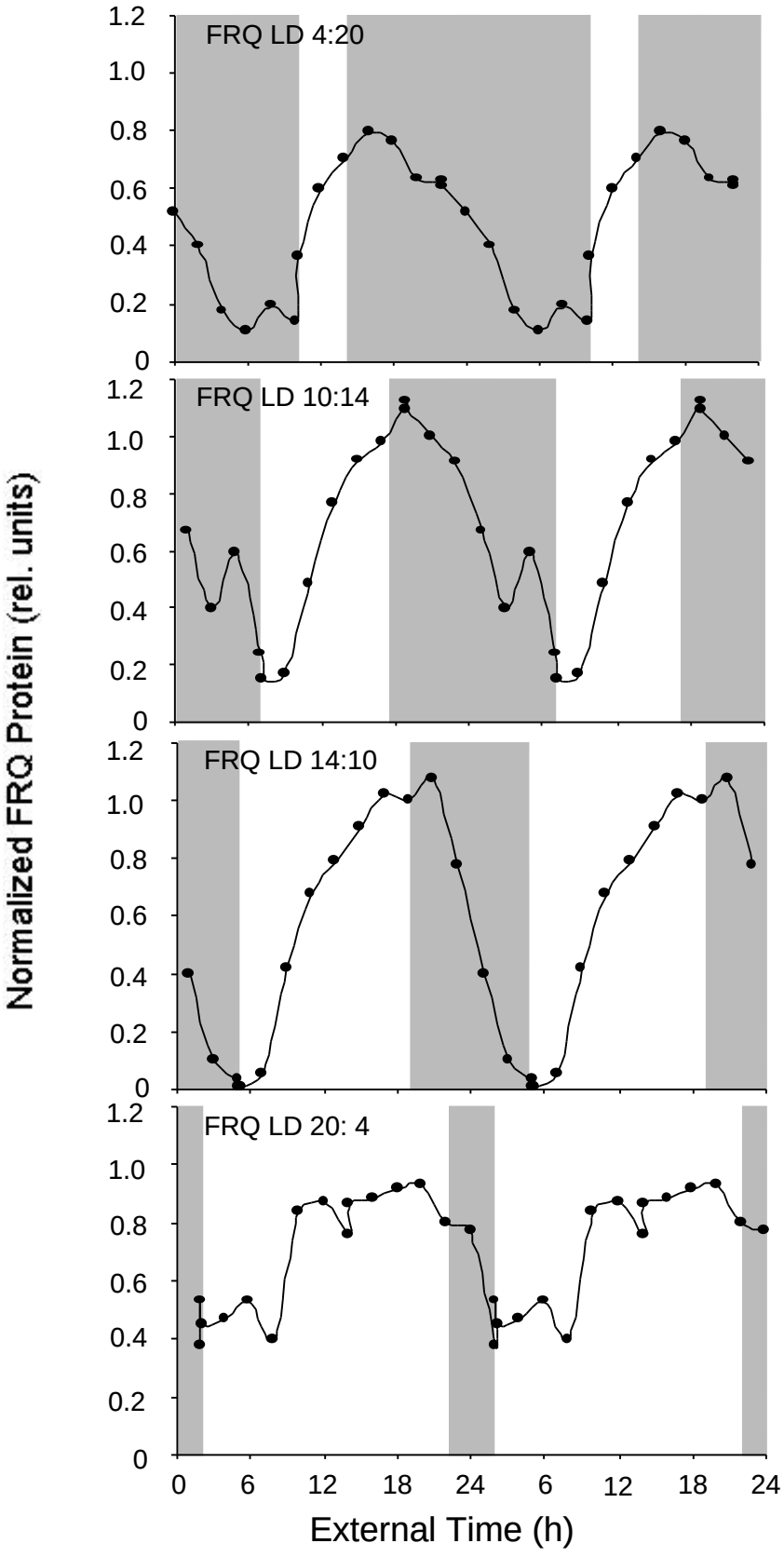

Fig. 3.22 Time courses of FRQ protein in different photoperiods (T=24h, fluorescent light, 3μE/m^2/s, wild type *Neurospora K93-2A*). In the shortest photoperiod (LD 4:20) tested, FRQ was induced by light within 15 minutes. In longer photoperiods, how-ever, FRQ levels only started to increase after 2 hours of light. After initial light induction, FRQ levels kept rising in the light phase in all photoperiods with the exception of the longest photoperiod (LD 20:4) tested. In 14-h photoperiod, the amplitude of FRQ changes was largest. After lights-off, FRQ levels declined in all photoperiods, however, with different kinetics. Note that the exponential decline is steeper in 10-h and 14-h photoperiods than in 4-h photoperiod, which has longer night. Tissue was grown in modified Westergaard's media for 5 full LD cycles before harvesting. Immunodetection was performed using a monoclonal antibody (αFRQ 4D11) which detects both long and short forms of FRQ protein. FRQ levels of each blot were normalized using ponceau staining. FRQ levels from different blots were normalized to the same scale when one sample of each gel was compared to a single blot and quantitated with each other.

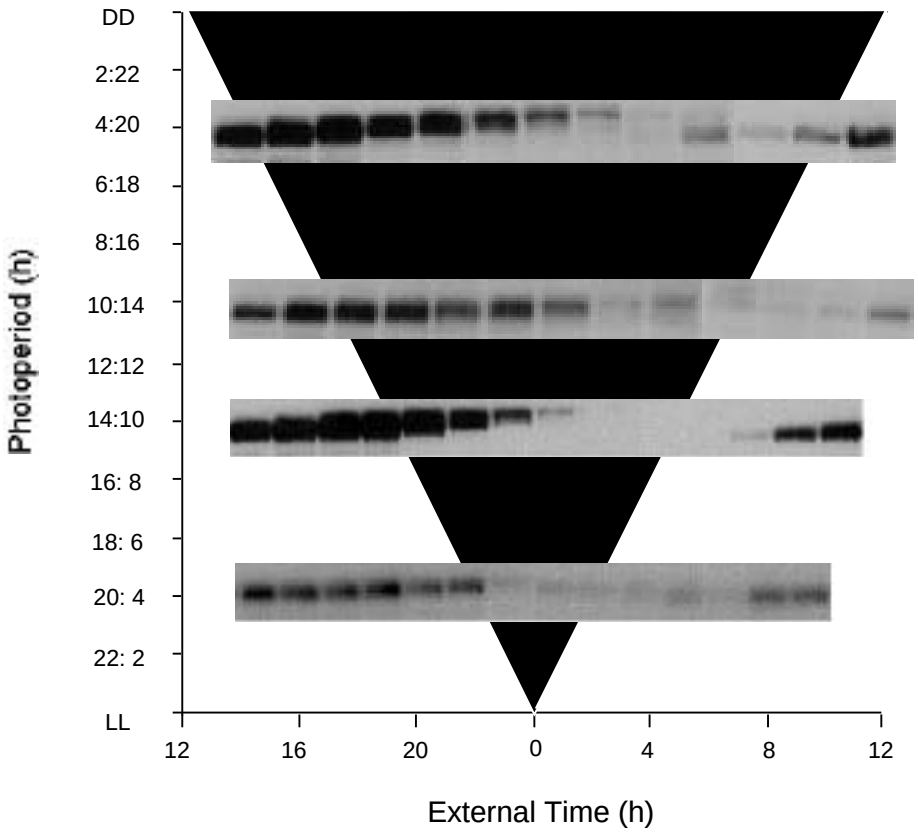

Fig. 3.23 Western blots for FRQ in different photoperiods (T=24h, fluorescent light, 3μE/m^2/s, wild type *Neurospora K93-2A*). The Western blots that gave rise to the quantifications shown in Fig. 3.22 as showed here, so that the progression of FRQ phosphorylation could be seen over time in different photoperiods. The time when the unphosphorylated FRQ appeared at low molecular weight was not keyed to the dark to light transitions. Immunodetection was performed using a monoclonal antibody (αFRQ 4D11) which detected both long and short forms of FRQ. All Western blots were exposed for 30 seconds. Black area indicates darkness; white areas indicate light.

3.5 Nutritional Effects on the Response to Photoperiod

Nutrition has many influences on the development of *Neurospora*, from metabolism to reproduction. It is essential to examine the nutritional effects on photoperiodic response, especially nitrogen and carbon.

3.5.1 Nitrogen and carbon limitation effects on phase of conidiation

In modified Westregaard's agar media with 2% sucrose, the band formed by conidia in the conidiation of *bd* strain (*bd* 30-7, FRP=22h) was longer than in standard race tube media, which contains 0.3% glucose and rich nitrogen source. In modified

Westregaard's agar media, the free-running rhythmicity of *bd* in DD was not as clear as it in standard race tube media, in which a 22-h rhythm presented distinctly. More importantly, the onset of conidiation in modified Westregaard's agar media occurred in the light phase -- 9.5 hours earlier than it in standard race tube media which was in the dakness. (Fig. 3.24)

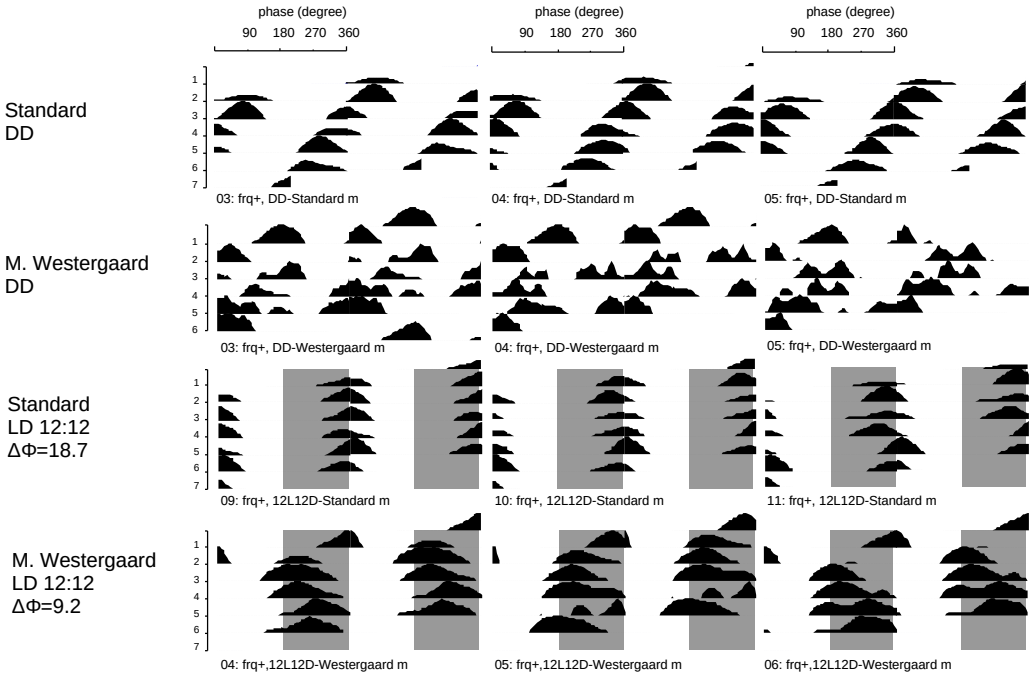

Fig. 3.24 Nutritional effects on the phase of the conidiation rhythm in *Neurospora* (T=24h, fluorescent light, 3μE/m^2/s, *bd* 30-7). In LD 12:12, the onset of conidiation with standard race tube media appeared at around midnight, while in modified Westergaard's media it occurred near the end of the light phase. Conidiation (black areas) was double plotted (see Fig. 3.2). Grey areas indicate darkness; open areas indicate light. The medium used in race tubes is indicated at left panel as either "Standard" (standard race tube media) or "M. Westergaard" (modified Westergaard's media), together with hours after lights-on until the onset of conidian (ΔΦ).

3.5.2 Effect of nitrogen and carbon limitation on light-induced mycelial carotenoids

Carotenoids extraxted from tissue grown in modified Westergaard's media with poor nitrogen and carbon sources showed a strong photoperiodic response and high levels of carotenoid production. In contrast, when carotenoids were extracted from the tissue grown in Vogel's minimal media with high carbon and nitrogen sources, there was no

apparent photoperiodic response. Carotenoids were induced by light, although overall carotenoid production was much lower in Vogel's minimal media (Fig. 3.25).

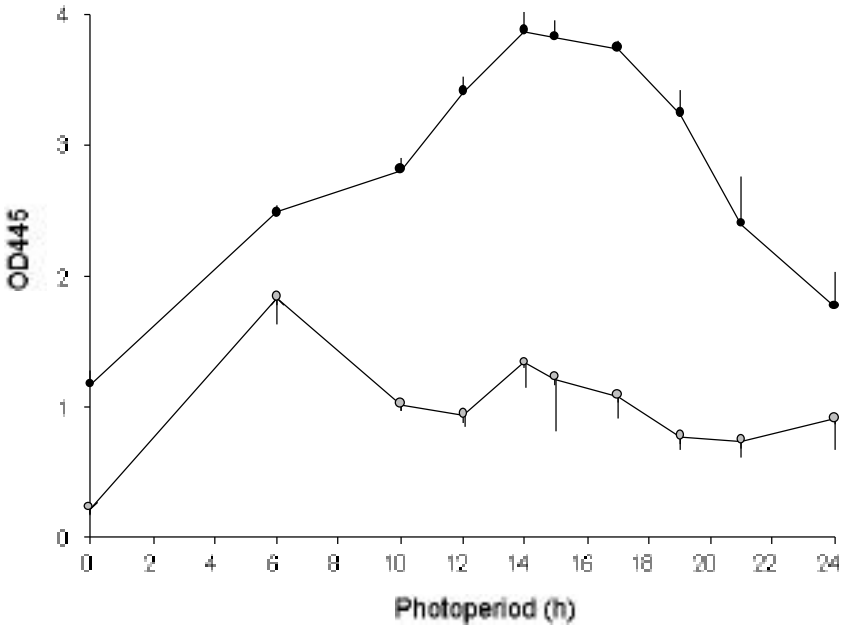

Fig. 3.25 Nutritional effects on the photoperiodic response of carotenoid production (T=24h, fluorescent light, 3μE/m^2/s). Carotenoids extracted from tissue of wild type *Neurospora* (*K93-2A*) grown in modified Westergaard's media (filled circles) showed a strong photoperiodic response and accumulated to high levels. In contrast, extractions from tissue grown in Vogel's minimal media (striped circles) showed no significant response to photoperiods and were at overall lower levels. Tissue was grown in either modified Westergaard's media or Vogel's minimal media in petri dishes for 5 full cycles before harvesting. Each time point represents the mean ± SD of 3 measurements (1 experiment, 3 petri dishes with either modified Westergaard's media or Vogel's minimal media).

3.6 *Neurospora* in Thermoperiods

To investigate the response of *Neurospora* in different duration of low and high temperature thermoperiods, racetube experiments of both FRQ-sufficient strain (*bd*) and FRQ-deficient strain (*bd frq*^{10}) were carried out with standard race tube media in the context of 24-hour cycles. All cycles performed in constant darkness. The phase of conidiation of the FRQ-sufficient strain (*bd*, FRP=22h) was stably entrained at the most extreme thermoperiods tested (WC 4:20 to WC 22:2) (Fig. 3.26 -- 3.28).

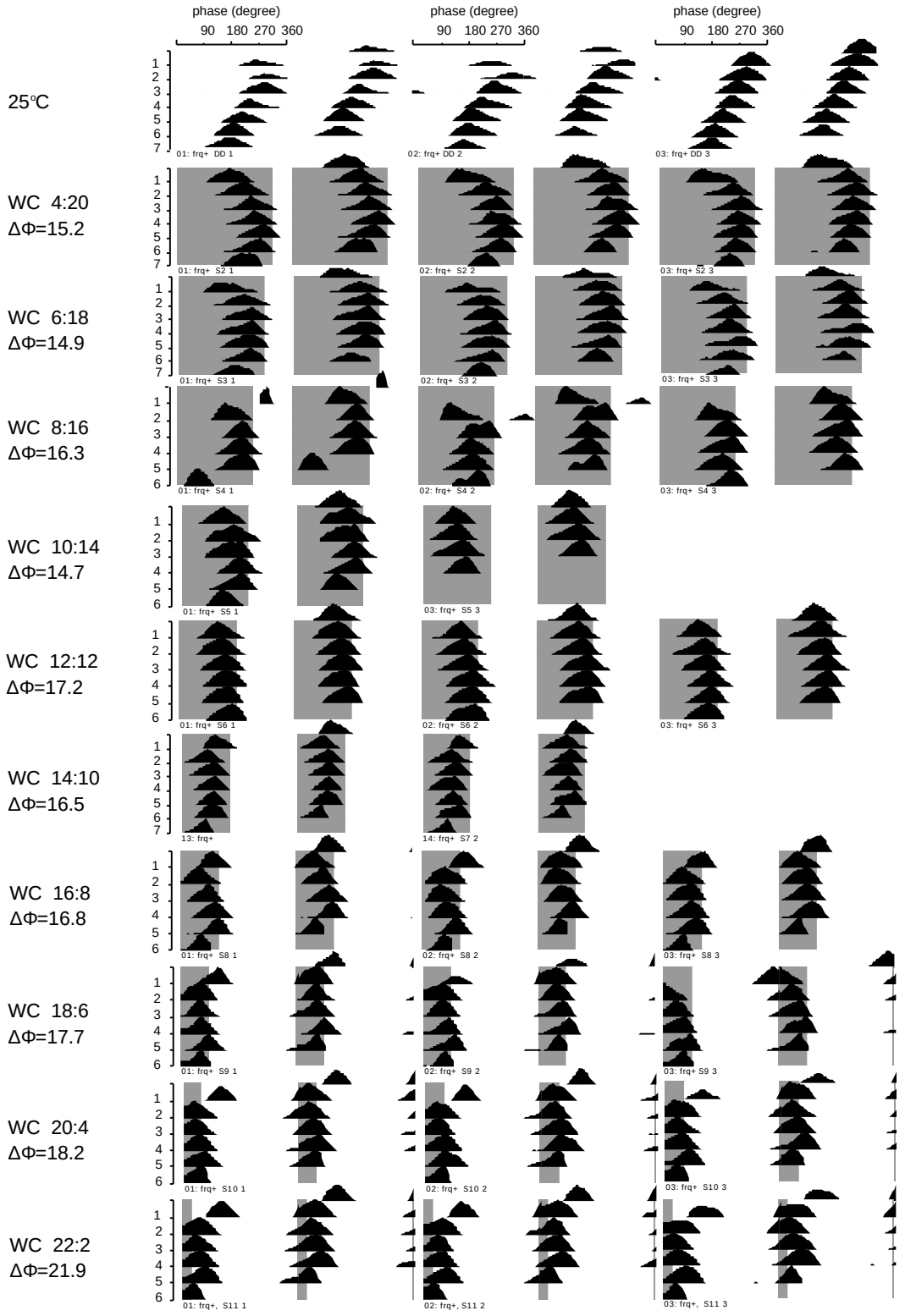

Fig. 3.26 Phase of conidiation of the FRQ-sufficient strain (*bd*) in different thermoperiods (22-27°C in constant darkness). Conidiation (black areas) of FRQ-sufficient strain (*bd*) was double plotted (see legend of Fig. 3.2). Grey areas indicate low temperature (22°C); white areas indicate high temperature (27°C). Thermoperiods are indicated at the left as WC (e.g., WC 12:12), together with the average phase angle between the onset of conidiation and the onset of the low temperature portion (ΔΦ).

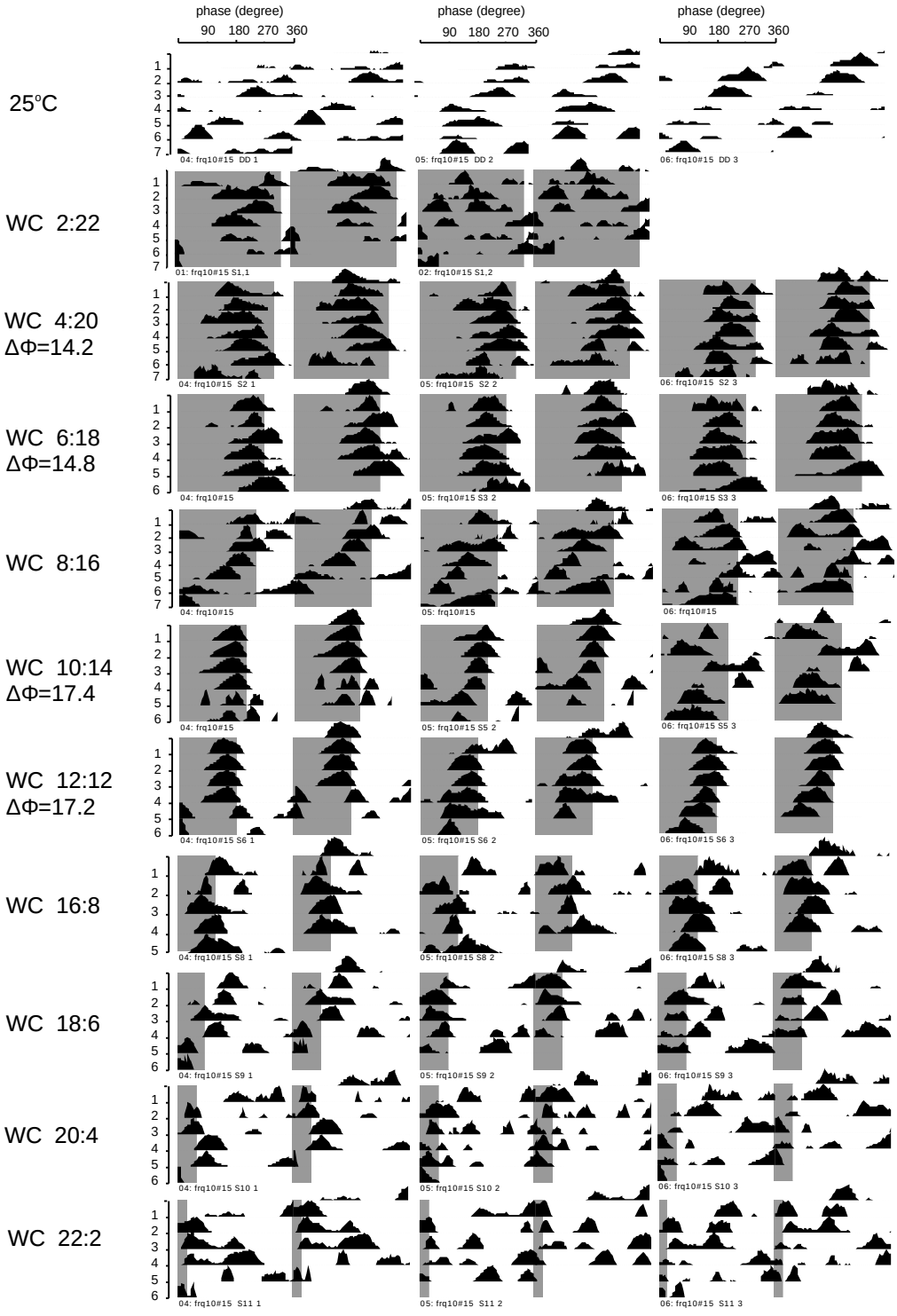

Fig. 3.27 Phase of conidiation of the FRQ-deficient strain (*bd frq*^{10}) in different thermoperiods (22-27°C in constant darkness). Conidiation (black areas) of FRQ-deficient strain (*bd frq*^{10}) was double plotted (see legend of Fig. 3.2; 3.26).

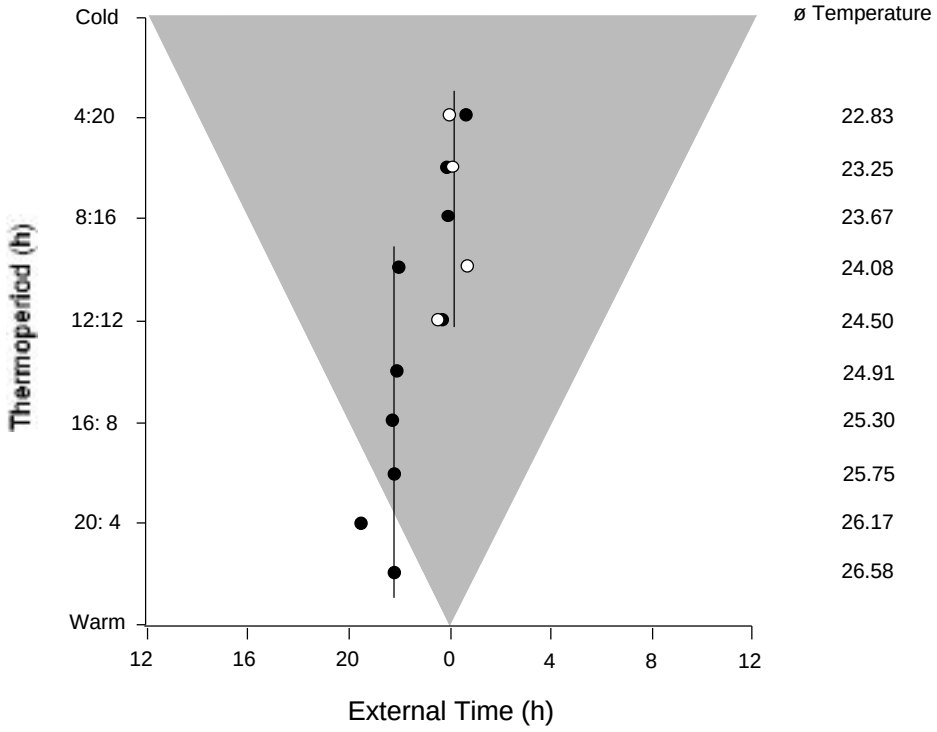

Fig. 3.28 Entrained phase of conidiation of *bd* and *bd frq^{10}* in different thermoperiods (T=24h, 22-27°C). Onset of conidiation of FRQ-sufficient strain (*bd*, filled circles) in the thermoperiods shifted from midnight to approximately 2 hours earlier, as the average temperature increased. Conidiation of the FRQ-deficient strain (*bd frq^{10}*, open circles) could also be entrained and its onset of entrained conidiation occurred almost at midnight throughout the thermoperiod cycles tested. This change in phase might correlate with the shortening FRP that occurred at higher temperatures. Each time point represents the average of 3 race tubes of each strain in 1 experiment. Grey area indicates low temperature phase; white areas indicate high temperature phase. The average temperature ((length of high temperature x 27°C+ length of low temperature x 22°C)/24) of each cycle is presented at right panel.

Phase of conidiation of the FRQ-sufficient strain (*bd*) was stably entrained by temperature cycles, but the onset of conidiation shifted from around midnight, in cycles with a greater proportion in the cold incubation, to approximately 2 hours before midnight in cycles with proportionally more time spent in higher temperature. The average temperature changed according to the different lengths of the high and low temperature. In contrast to the entrainment in photoperiods, the phase of conidiation of the FRQ-deficient strain (*bd frq^{10}*) could also be stably entrained in temperature cycles when the high temperature duration was shorter than or up to 12 hours.

3.7 Results of Cloning

3.7.1 Cloning of pKSbar2cpc1frq

In order to investigate the role of FRQ in the photoperiodic response further, the *frq* open reading frame (ORF) was inserted into the vector pKSbar2cpc1, which contains the cpc1 promoter. The hypothesis was that overexpression of FRQ would lead an alteration in the photoperiodic responses. The cpc1 (cross pathway control 1) promoter is highly regulated in *Neurospora*, but in pKSbar2cpc1 vector, it has been mutated so that it drives high, constitutive levels of expression. The *Bar2* gene confers resistance to the pesticide Basta (phosphinothricin or glufosinate ammonium).

The vector pKSbar2cpc1 was digested with EcoR I and Cla I (5.1 kb). One of the two inserts was digested from plasmid pCRM119 with EcoR I and Sph I (1.4 kb). Another insert was digested from plasmid pCRM119 with Cla I and Sph I (4 kb). After ligation, the plasmid pKSbar2cpc1frq was 10.5 kb. pKSbar2cpc1frq was transformed into *bd frq*^{10} spheroplasts (see 2.4.2). After transformation, 10 colonies were picked and inoculated into Vogel's media with 0.3% Basta. Conidia were tested for FRQ protein by Western blot (Fig.3.29). No overexpression of FRQ was found in either Western blotting or phase of conidiation in race tubes (data not shown).

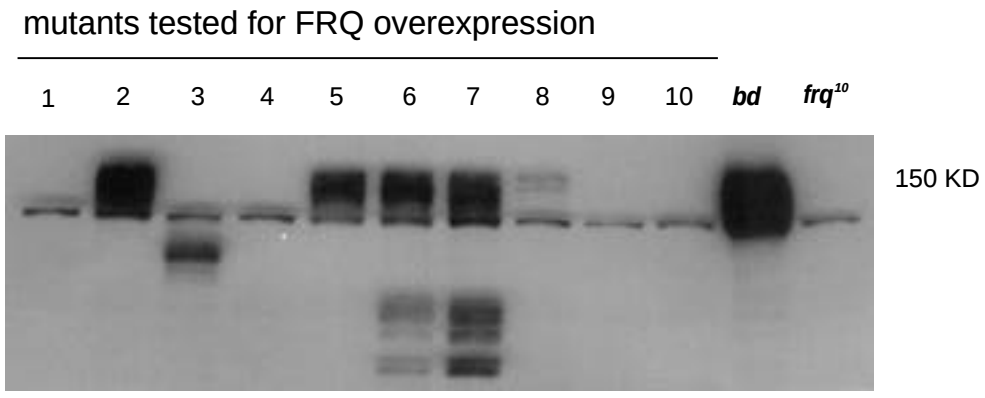

Fig. 3.29 Western blot of mutants with pKSbar2cpc1frq. No overexpression of FRQ was found in these 10 mutants transformed with pKSbar2cpc1frq. Total protein extracted from each mutant's tissue was loaded on the same gel together with FRQ from *bd* as positive control and from *frq10* as negative control. In some of these mutants, FRQ was detected at different positions compared to the positive control. Tissue was grown in Vogel's media in light for 2 days with shaking. Immunodetection was performed using a monoclonal antibody (αFRQ 4D11) which detected both forms of FRQ.

3.7.2 Cloning the 5' end of the *wc-1* open reading frame (ORF)

To verify the *bd wc-1* strain (Talora, 1999; Dragovic, 2002), the beginning of *wc-1* ORF of that strain was cloned into pSK-II for subsequent sequencing. The *wc-1* ORF of *bd* strain was also cloned as control.

Fig. 3.30 Verification of the *bd wc-1* mutant. (A) The Western blot showed no detectable WC-1protein in the *bd wc-1* mutant (right panel) compared with *bd* strain (*bd* 30-7, left panel). Immunodetection was performed using a monoclonal antibody (αWC-1 4H4). The blot was exposed for 15 seconds. (B) The area surrounding the putative translation start site (grey box) for *wc-1* was cloned and sequenced from the *bd wc-1* mutant. The sequence in the top line corresponded to the *wc-1* DNA sequence of *bd* strain, and the mutant sequence was immediately below it. Asterisks indicate the location of point mutations, and the last row of sequence corresponds to the putative translation of the sequence of protein, which reflects silent mutations until the stop codon.

Results of sequencing the clone of the *bd wc-1* mutant showed that there were several point mutations at the beginning of the open reading frame, and the first stop codon appeared just 15 amino acids after the starting codon. The Western blot for WC-1 protein indicated that no WC-1 protein was detectable in the *bd wc-1* mutant (Fig. 3.30).

4. Discussion

Photoperiodism is one of the most fascinating biological questions: the reproduction of practically all organisms, ranging from single cell algae to humans is controlled by a temporal machinery optimizing the time of annual events (Bünning, 1960; Thomas, 1998). The use of daylength (or nightlength) allows organisms to assess the season-of-year and to process relatively predictable annual variations with a reliable environmental cue – the photoperiod. Our knowledge about this phenomenon is abundant, yet no ideal molecular model exists to explain the mechanisms. There have been recent studies of photoperiodic gene regulation in the plant model *Arabidopsis*, which, compared to *Neurospora crassa*, is more complicated for studying photoperiodism at the molecular level. We assumed that the circadian model organism of the simple fungus *Neurospora crassa* is a potential for the question: 1). The circadian clock is well described in the molecular and genetic levels (e.g., the critical clock gene *frq*, the blue light photoreceptor WC-1, the basic transcription/translation feedback, etc.); 2). Its relatively small genome (43Mb) has been published (Galagan, *et al*., 2003); 3). There are wealth of genetic and biochemical tools available.

In nature, photoperiodism is particularly important for reproduction of organisms to ensure the optimum survival of their progeny. Most of the current knowledge of photoperiodism is about the reproduction or related function (e.g., puberty). Examples of photoperiodically regulated physiology include flowering in plants, diapause in insects and testes size variation in hamsters.

Neurospora crassa has two types of development – asexual and sexual cycles. The bright orange asexual spores (conidia) germinate if supplied with humidity and appropriate nutritious (e.g., carbon and nitrogen).

In this study, *Neurospora crassa* shows different physiological responses in various aspects of its development to different photoperiods, including asexual and sexual reproduction, and light-induced carotenoid production. Investigation at the molecular level also showed distinct responses of key clock components to different photoperiods.

4.1 Physiological Responses of *Neurospora* to Photoperiods

4.1.1 Physiological responses to a 24-h photoperiod

4.1.1.1 Photoperiodic regulation of phase of conidiation

As the first approach, the phase of conidiation of *Neurospora crassa* was investigated in different photoperiods (T=24h). The entrained phase of conidiation of the FRQ-sufficient strain *bd* (FRP=22h) was pegged to the midnight (ET0), which did not maintain a constant phase relationship with either light to dark or dark to light transitions (Fig. 3.1, 3.2).

In the same experiments, onsets of the phase of conidiation of the two period mutants were also independent of dawn and dusk. Onsets of the short period mutant *bd frq*^{1} (FRP=16h) were approximately 2 hours before midnight and the long period mutant *bd frq*^{7} (FRP=29h) approximately 5 hours after. *bd frq*^{7} required at least 4 hours photoperiod to be stably entrained. (Fig. 3.3)

Data of the phase of conidiation indicated that circadian mechanism was involved in the responses to photoperiods, and *Neurospora* seemed measure the length of darkness. The data reported here contrast a previous report showing that a minimum of 2 hour was required for stably entrainment of the phase of conidiation of *bd* and *bd frq*^{7} in 24-h LD cycles (Chang, 1997). In these experiments, *bd* The same report showed that 4-h darkness (20-h photoperiod) was the minimum requirement for entrainment of *bd* and *bd frq*^{7} (~210μE/m^{2}/s, Chang, 1997) which corresponds with what was found in this study (Fig. 3.1, 3.2, 3.3).

4.1.1.2 Photoperiodic regulation of asexual and sexual reproduction of *Neurospora crassa*

Asexual reproduction of *Neurospora crassa* was investigated by assessing the production of conidia – the asexual spores – in different photoperiods (T=24h).

Conidia production of the FRQ-sufficient strain *bd* showed a clear response to photoperiods, where about twice as many conidia were produced by in a photoperiod of around 12 hours compared to either shorter or longer photoperiods. This indicated that 12-h was the optimum photoperiod for asexual reproduction in *Neurospora crassa*. Most of the *Neurospora* strains that are used in laboratory experiments have been collected in Louisiana, where average photoperiods are 10 to 14 hours. In con-

trast, the FRQ-deficient strain *bd frq*^{10} showed no significant conidiation response to photoperiod, and it was overall less than *bd* (Fig. 3.4).

Data of the asexual spores (conidia) reproduction indicated that one of the key components of the circadian clock FRQ was essential for the photoperiodic response and conidia production.

Neurospora uses sexual reproduction in certain conditions. Reduced nitrogen and carbon (Westergaard, 1947) and temperature (Nelson, 1996) promote sexual reproduction (even though some conidia continue to be produced). The sexual cycle of *Neurospora* requires the presence of both mating types: *A* and *a*. Either of the two mating type strains may serve as a female parent – depending on which one is presented first – by forming the female structure, a protoperithecium. Conidia or hyphae of the opposite mating type act as the male parent and fertilize the protoperithecia. In this study, the development the protoperithecia in different photoperiods was investigated. By counting the numbers of protoperithecia in different photoperiods, it was found that the female parent of the FRQ-sufficient strains showed a strong preference for certain photoperiods. This finding holds for both the *bd* mutants and the aconidial *fluffy* mutants, which were highly fertile and provided clear view for protoperithecia counting. The number of protoperithecia of the FRQ-sufficient strains reached a maximum in 14-h photoperiods, and decreased in longer photoperiods. The maximum photoperiod (14-h) of protoperithecia development was different from the asexual development (12-h), indicating that the photoperiodic response of the sexual vs. sexual cycles might utilize different programs. LL counts were lower than in all photoperiods tested for FRQ-sufficient strains, similar to DD levels. In contrast, the FRQ-deficient strains (*bd frq*^{10} and *fluffy frq*^{10}) showed no apparent response to different photoperiods. However, protoperitherica of the FRQ-deficient strains were not completely blind to light because in LD cycles the number of protoperithecia was higher than in DD, and was smaller in LL. The *wc-1* mutant *fluffy wc-1* showed no response to photoperiods, and the number of protoperithecia was low in all LD cycles and in LL, which indicated that the development of protoperithecia was not affected by light when the blue light photoreceptor WC-1 protein was absent. (Fig. 3.9, 3.10, 3.11)

When counting ascospores – the progeny released by fertilized, mature perithecia – in different photoperiods, the response was found to be similar to that for protoperithecia development. The pair of FRQ-sufficient strains (*bd a* and *bd A*) pro-

duced approximately 80 spores per perithecium in 14-h photoperiod while almost no spores were shot in DD and LL. Again, the pair of FRQ-deficient strains (*bd frq*^{10} *a* and *bd frq*^{10} *A*) showed no apparent response to different photoperiods by producing approximately 10 spores per perithecium through all conditions tested (Fig. 3.12).

Previous studies on diapause of the insects, *Ostrinia nubilalis*, showed a similar response (Beck, 1962), which the percentage of diapause reached the maximum in approximately 13-h photoperiod and decreased in longer photoperiods and the value in LL was lower than in all photoperiods. Some photoperiodic responses do not return to low levels in constant conditions – testes of hamsters. As we learn more details about photoperiodism in *Neurospora*, we may be able to classify its photoperiodism with a subset of others.

Thus, data discussing asexual and sexual development in different photoperiods indicated that FRQ was one of the key components for photoperiodic timing in sexual reproduction. FRQ is also a key circadian clock component in *Neurospora*.

4.1.1.3 Photoperiodic regulation of carotenoid production of *Neurospora crassa*

In nature, UV light induced photooxidative reactions cause damage to molecules and affect the integrity of cells and tissues. For many organisms, carotenoids assume several roles in natural photosynthesis. Carotenoids are efficient antioxidants, scavenging single molecular oxygen and peroxyl radicals generated during photooxidation (Li, 1995; Davis, 2000). It was hypothesized that light-induced mycelial carotenoids of *Neurospora* should vary in response to different photoperiods together with developmental hallmarks (asexual and sexual reproduction) possibly to serve a protective function.

Carotenoid production by FRQ-sufficient strains, including wild type *Neurospora* (*K93-2A*) and *fluffy* (*fluffy a 7431*) showed a significant response to photoperiod. In 2-h to 10-h photoperiods, the mycelial carotenoids were induced by light but did not increase or decrease. Carotenoid production was enhanced again when the photoperiod was longer than 10 hours, which demonstrated that 10-h was the critical photoperiod for carotenoid production. The decreasing carotenoid production in photoperiods longer than 20-h indicated that the enzymatic pathway leading to carotenoid production was down-regulated or repressed. Interestingly, unlike its response of the asexual and sexual reproduction, carotenoid production of the FRQ-deficient strain (*fluffy frq*^{10}) was induced in amounts roughly proportional to amounts of light. No de-

creases were observed in long photoperiods, and the value in LL was higher than all other photoperiods. This finding suggested that in the absence of negative feedback of FRQ, expression of the a*l-3* gene, which is involved in carotenoid syntheses, was activated by WCC constantly, in an unregulated manner (Fig. 1.10). Compared to wild type *Neurospora*, the *wc-1* mutant (*fluffy wc-1*) showed no significant response to different photoperiods.

Carotenoid production in photoperiods corresponded with the previous demonstration that the induction of carotenoids in mycelia by light required the clock proteins WC-1 and WC-2 but not FRQ (Merrow, 2001), and light-dependent carotenoid synthesis is entirely absent in *wc-1* mutants (Degli-Innocenti, 1984;Linden, 1999; Merrow, 2001).

4.1.2 Physiological responses in a non-24-h photoperiod (T=18h)

To further investigate the photoperiodic timing mechanism, for which two major hypotheses have been proposed – external coincidence and internal coincidence – a non-24-h photoperiod was employed. As 18-h photoperiod was used in conjunction with *bd frq*^{1} because the ratio of FRP to T (cycle length) was the same as that for *bd* in 24-h photoperiod (22 to 24 vs. 16 to 18). LD proportions were calculated based on 24-h photoperiod experiments.

When carotenoid production of the FRQ-sufficient strains (*K93-2A* and *fluffy*) was first tested in 18-h photoperiod, it showed different response from its in 24-h photoperiod. In the 18-h photoperiod carotenoids were induced to maximum already at the shortest photoperiod tested (1.5h), and remained high until the photoperiod exceeded 13.5-h. Unlike in 24-h photoperiod, no apparent critical photoperiod could be determined in the photoperiodic response carotenoid production (Fig. 3.13).

When the development of protoperithecia was measured in 18-h photoperiod, the response curve was similar to that in 24-h photoperiod with the maximum at 9-h photoperiod for *bd* strain and at 7.5-h for *bd frq*^{1} (Fig. 3.14).

The different responses of carotenoid production and the development of protoperithecia in the 18-h photoperiods reinforce the idea that there is more than one program to generate the outputs of photoperiodic responses. The mechanisms behind these differences remain to be explored, as they are in no way fully understood.

4.2 Physiological Responses in Night-break Experiments

The classical night-break protocol has been developed to investigate photoperiodism. It has been demonstrated that diapause in insects, the testes size of hamsters, flowering in plants, sperm production of male juncos (*Junco hyemalis*), and egg-laying activity of chickens, all responded to interrupted LD cycles in a similar way (Bünning, 1958). They clearly showed that the responses did not depend on *how much* light was perceived but *when* the light fell. It is essential to investigate whether *Neurospora crassa* would respond in night-break experiments similar to these other photoperiodic model organisms.

When light-induced mycelial carotenoid production of *Neurospora crassa* was assayed using night-break protocol, it was found that all FRQ-sufficient strains tested gave similar responses to the different night interruptions. Carotenoid production reached the maximal levels when the short light pulse fell in the middle of the 18-hour dark period, i.e., the shortest interrupted darkness (9 h). Carotenoid production in the uninterrupted photoperiod cycle (LD 6:18), which had the longest dark period, was always lower than in interrupted scotoperiods. The FRQ-deficient mutant, *fluffy frq*^{10} showed the weakest responses to night-breaks (Fig 3.15, 3.15a, b, c, d).

When the development of protoperithecia was measured with the same protocol, it gave a similar response to interrupted LD cycles. (Fig. 3.16)

Results of night-break experiments demonstrated that it was not the length or dose of the light that was critical, but the length of the darkness. One possible interpretation of these findings is that these responses are initiated when the light perception coincides with a sensitive phase of a circadian rhythm. Again, the FRQ-deficient strain failed to gave significant response to interrupted LD cycles indicating that FRQ is one of the key components of photoperiodic timing mechanism. The difference of the slope of different FRQ-sufficient strains' responses might be due to the subtle difference in genetic background.

4.3 Molecular Profiles of Photoperiodic Responses

4.3.1 Transcription/translation of *frq* in different photoperiods

The molecular mechanism of circadian rhythm and the interaction of its central components is still an open question. When the protocols of the previously published data

were studied, it was found that, in almost all the cases, the cultures of *Neurospora crassa* had been incubated in LL or single LD cycle before harvested either in LL or DD. It was assumed that the rhythmic autoregulation of *frq* transcription/translation in constant conditions should be comparable to LD (Collett, 2002).

This study, for the first time, provides evidence that *frq* transcription/translation is entrained by LD. Its mechanism could be much more complicated than was previously thought (see enormous references).

In this study, it was revealed that after entrainment in different photoperiods (from LD 4:20 to LD 20:4) for 5 full cycles, *frq* RNA was induced by light to its maximal levels within 15 minutes. *frq* RNA rose again at around midnight in the very short photoperiod (LD 4:20), near the end of the darkness in LD 10:14, and almost at the end of darkness in LD 14:10. In LD 20:4, the longest photoperiod testes, *frq* RNA remained at basal levels at the end of darkness. This finding demonstrated that *frq* RNA did not always rise at around the end of the night in all conditions (Fig. 3.17).

In contrast to RNA levels in different photoperiods, FRQ protein appeared at different rates, relative to the onset of the light incubation. In short photoperiod (LD 4:20), FRQ appeared at a remarkable level, within 15 minutes (ET 10.25) in light. In longer photoperiods, FRQ appeared much later, 4 hours after lights-on in LD 10:14 (ET11), and 6 hours after lights-on in LD 14:10 (ET11). In the longest photoperiod test (LD 20:4), FRQ was always at present, although it decreased during the short dark period. FRQ protein levels reached maximum just after lights-off in all photoperiods with the exception of the longest photoperiod (LD 20:4), which was different from previously assumed that it peaked at around CT8 (ET14), about 4 hours after *frq* mRNA reached its maximum (Garceau, 1997) (Fig. 3.22; 3.23).

4.3.2 *wc-1* expression in different photoperiod

It was known that the RNA levels of *wc-1* were not rhythmic in constant darkness (Lee, 2000; Merrow, 2001).

Data from Northern blotting showed that in LD 12:12 *wc-1* RNA of the FRQ-sufficient strain (*bd*) was induced by light to the maximum within 15 minutes, and returned to the adapted level, and decreased further in the dark period. In contrast, the FRQ-deficient strain (*bd* frq^{10}) was not significantly regulated by light, although it had been reported to be induced by light in this strain (Fig. 3.18; Merrow, 2001).

When *wc-1* RNA in the same series of experiments as *frq* RNA was assayed, it showed that its levels were induced by light to their maximum within 15 minutes after lights-on, but decreased to a low adapted levels within 2 hours of light induction, and *wc-1* RNA did not show an apparent, further decrease in darkness in all photoperiods tested. In short, *wc-1* RNA seemed did not respond systematically to different photoperiods (Fig. 3.19).

4.3.3 FRQ expression in different strains in different media

Previous data on the regulation of FRQ expression were based on the experiments with *bd* in Vogel's media. It would be important to investigate the regulation of FRQ in the nitrogen and carbon starvation media – the modified Westergaard's media, which was used to assay carotenoid production and protoperithecia development in different photoperiods. Meanwhile, it's also interesting to see whether there is any difference of FRQ regulation in different FRQ-sufficient strains, such as *bd* (e.g., for phase of conidiation assay) and wild type *Neurospora crassa* (*K93-2A*; e.g., for carotenoid production assay).

In the same photoperiod (LD 14:10), in both Vogel's media and modified Westergaard's media, FRQ levels in the two FRQ-sufficient strains increased continuously in light but decreased in darkness, and reached the similar maximal levels, which indicated the overall kinetics of FRQ regulation were very similar despite the differences of strains and media (Fig. 3.21).

Data from this experiment suggested that the nutrition and the different genetic background (only *bd* mutation) between the two FRQ-sufficient strains do not change FRQ expression patterns in a photoperiod of LD 14:10.

4.4 Nutritional Effects on the Response to the Photoperiod

The albino genes *al-1*, *al-2* and *al-3* encode the carotenoid biosynthetic enzymes phytoene desaturase, phytoene synthase and geranylgeranyl pyrophosphate synthase, respectively. Nitrogen limitation can stimulate *al-1* and *al-2* expression in mycelia (Sokolovsky, 1992). After the photoperiodic response of sexual reproduction of *Neurospora* was uncovered, it was essential to investigate other readouts with the same modified Westergaard's media used for sexual cycles.

When phase of conidiation was assayed with modified Westergaard's agar media, it was found that the free-running rhythmicity of *bd* in DD became difficult to visualize compared to what is seen on Vogel's agar media. More interestingly, in LD 12:12, the onset of conidiation with Vogel's agar media appeared at around midnight (Merrow, 1999), while with modified Westergaard's agar media, it shifted to near the end of the light phase (Fig. 3.24).

When the carotenoid production in different photoperiods was measured with both Vogel's minimal media and modified Westergaard's media, it was found that with the former there was no significant response, while apparent photoperiodic response was obtained with the latter one (Fig. 3.25).

Data of the nutritional effects on photoperiodic response suggested that nutrition might play a role at the level of the output pathway, but that the central timing mechanism, at least using FRQ as a readout, is intact (e.g. Fig. 3.21 FRQ in LD 14:10 in different media).

4.5 *Neurospora* Response to Thermoperiods

It has been demonstrated that the FRQ-deficient strain *bd frq*^{9} (a frameshift mutation) could establish a stable phase relationship of conidia production in a temperature cycle, which indicated that FRQ was unnecessary for entraining an oscillator with generic circadian properties in temperature cycles (Merrow, 1999). It was interesting to see whether FRQ was necessary in response to thermoperiods.

In this experiment, the FRQ-sufficient strain *bd* as well as the FRQ-deficient strain *bd frq*^{10} (a knockout) were used to assay the phase of conidiation in different thermoperiods (T=24h, 22-27^{o}C). It was found that the phase of conidiation of *bd* could be entrained stably in all thermoperiods tested. Interestingly, the phase of conidiation of the FRQ-deficient strain *bd frq*^{10} could also be stably entrained in some, though not all, temperature cycles (T=24h) tested. The onset of conidiation of *bd* in the thermoperiods shifted from midnight to approximately 2 hours before midnight, as the average temperature increased. The onset of the entrained conidiation of *bd frq*^{10} appeared around midnight (Fig. 3.26, 3.27, 3.28), where it could be determined.

This change in phase could correlate with the subtleshortening free-running-period which occurs in higher temperatures, because the circadian clock is not fully temperature compensated (Aronson, 1994a).

5. Summary

The data from this study demonstrates for the first time that *Neurospora crassa*, a circadian model organism, has a photoperiodic clock. The experiments provide the opportunity for further research on photoperiodism. Evidence of this study supports the external coincidence hypothesis that photoperiodism shares the same mechanism with circadian rhythms. The central components of the *Neurospora* circadian clock, such as FRQ and WC-1, are also essential for photoperiodic responses. The dissociation between *frq* RNA and FRQ protein in different photoperiods suggests that the transcriptional/translational regulation is far complicated. The shift of the maximum of conidial production compared to protoperithecia development suggests the different favourite season for the asexual and sexual reproduction of *Neurospora crassa*.

However, there is a difference between circadian rhythm and photoperiodism -- circadian rhythm is endogenous, which remains at constant conditions (DD or LL); in contrast, photoperiodism requires external signals – light and darkness.

Further research on photoperiodism of *Neurospora* must be carried out to study the gene(s) which is/are critical for photoperiodic responses, the molecular mechanism of transcriptional/translational regulation of critical clock components in photoperiods, the generation of the oscillator, and the mechanism which differentiates many outputs of photoperiodic responses.

5. Zusammenfassung

Die Daten dieser Studie zeigen zum ersten Mal, dass Neurospora crassa, ein Modellorganismus der circadianen Forschung, eine photoperiodische Uhr besitzt. Die Experimente eröffnen die Möglichkeit für weitere Forschung auf dem Gebiet des Photoperiodismus. Die Belege dieser Studie unterstützen die externe Coinzidenz Theorie, die besagt, dass Photoperiodismus die gleichen Mechanismen nutzt wie die circadianen Rhythmen. Die zentralen Bestandteile der Uhr von Neurospora, wie zum Beispiel FRQ und WC-1, sind auch für photoperiodische Antworten unabdingbar. Die Dissoziation zwischen *frq* RNA und FRQ Protein in verschiedenen Photoperioden lässt vermuten, dass die transkriptionelle bzw. translationelle Regulierung noch viel komplexer ist. Die Verschiebung des Maximums der Produktion von Konidien im Vergleich zur Entwicklung von Protoperithecien ist ein Hinweis auf die unterschiedlich bevorzugte Jahreszeiten für asexuelle und für sexuelle Reproduktion von Neurospora crassa.

Es gibt jedoch einen Unterschied zwischen circadianen Rhythmen und Photoperiodismus - Circadiane Rhythmen sind endogen, sie bleiben in konstanten Bedingungen (zum Beispiel DD oder LL) erhalten; im Gegensatz dazu sind für Photoperiodismus externe Signale erforderlich: Licht und Dunkelheit.

Die Forschung über Photoperiodismus bei Neurospora muss weitergeführt werden, um die Gene zu untersuchen, die für photoperiodische Antworten, den molekularen Mechanismus der transkriptionellen/translationalen Regulation essentieller Uhr-Bestandteile in Photoperioden, die Erzeugung des Oszillators, und den Mechanismus, der die vielen Ausgänge der photoperiodischen Antworten unterscheidet, entscheidend sind.

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7. Appendix

Blotting buffer

50 mM	Tris
384 mM	glycin
20%	methanol

Bottom Agar

10 ml	Vogels'
440 ml	H_2O
7.5 g	Bacto-Agar
91 g	sorbitol

add 50 ml 10 X FIGS after autoclaving (~75°C).

2X CTAB buffer

10 ml 1M Tris-HCl pH 7.5 or 8.0	100 mM
2 g CTAB	2 %
28 ml 5M NaCl	1.4 M
4 ml 0.5 M EDTA pH 7.5 or 8.0	20 mM
1 g sodium bisulfite	1 %
H_20 to 100 ml	

first dissolve the CTAB in 58 ml H_2O, then add the salt and other ingredients.

50 X Denhardts

5 g	Ficoll
5 g	Polyvinylpyrrolidone
5 g	BSA (Bovine Serum Albumin)

fill RNase-free H_2O up to 500 ml, and filter, store at –20°C

DNA/RNA loading dye

0.5 ml	glycerol
0.0372 g	EDTA
0.04 g	Bromphenol Blue (LMW)
0.04 g	Xylene Cyanol (HMW)
0.5 ml	H_2O

Destaining solution

10%(v/v)	$C_2H_4O_2$
40%(v/v)	methanol

10X DNase I buffer

200 mM	Tris/HCl pH 8.3
500 mM	KCl
20 mM	$MgCl_2$

Electrophoresis buffer

50 mM	Tris/HCl pH8.3
384 mM	glycin
0.1% (w/v)	SDS

10 X FIGS

100 g	L(-)sorbose
2.5 g	D(-)fructose
2.5 g	glucose
500 ml	H_2O

autoclave

GET

25 ml	40% glucose
2.5 ml	1M Tris PH 7.5
2.0 ml	0.5M EDTA PH 8.0
70.5 ml	H_2O

add 3 mg/ml lysozyme before use

Laemmle buffer

60 mM	Tris/HCl pH6.8
2% (w/v)	SDS
5% (v/v)	b-Mercaptoethanol
10% (v/v)	glycerin
0.2% (w/v)	Bromophenol Blue

LB_{Amp}- media (Luria Bertani media)

5 g	Tryptone
3 g	yeast extract
5 g	NaCl
500 ml	H_2O

(add 100 µg/ml Ampicillin after autoclaving)

LB_{Amp}- agar media (Luria Bertani agar media)

5 g	Tryptone
3 g	yeast extract
5 g	NaCl
7.5 g	Bacto-Agar
500 ml	H_2O

(add 100 µg/ml Ampicillin after autoclaving)

Mg Solution

12 g	$MgSO_4$
9.5 g	$MgCl_2$
100 ml	H_2O

filter sterilize (0.45 micron filters, Schleicher & Schuell)

Modified trace elements

5.25 mg	$Na_2B_4O_7.10H_2O$
100 mg	$CuCl_2.\ 2H_2O$
200 mg	$FeCl_3.\ 6H_2O$
20 mg	$MnCl_2.\ 4H_2O$
20 mg	$(NH_4)_6.Mo_7O_{24}.\ 4H_2O$
2 mg	$CoCl_2$

fill H_2O up to 1L

modified Westergaard's salts (5 X)

in 250 ml	
KNO_3	1.25 g
KH_2PO_4	1.25 g
$MgSO_4$	0.625 g
$CaCl_2$	0.125 g
NaCl	0.125 g
biotin	6.25 µg

modified Westergaard's media

2%	sucros
1 X	modified Westergaard salts
1µl/ml	trace elements

adjust pH to 6.5

modified Westergaard's agar media

2%	sucros
1 X	modified Westergaard salts
1µl/ml	trace elements
1.5%	Bacto-Agar

adjust pH to 6.5

20 X MOPS

92.4 g	MOPS
33.3 ml	3 M NaAcO
40 ml	0.5 M EDTA

fill H_2O to 1 L, and adjust pH to 7.0 with 10 M NaOH

Minimal media

2%	Bacto-Agar
2%	glucose
1 X	Vogels' salts

10 X PBS

80 g	NaCl
2 g	KCl
11.5 g	$Na_2HPO_4.7H_2O$
2 g	KH_2PO_4

fill H_2O to 1L

PMC

40%(w/v)	PEG 4000
50 mM	$CaCl_2$
10 mM	MOPS pH6.3

Polyacrylamide solution

29.2 g	acrylamide
0.8 g	N, N′-Methylene-bis- acrylamide

fill H_2O up to 100 ml, and filtered with a faltenfilter paper after dissolved, store at 4ºC for up to 2 months

Ponceau solution

0.2%	Ponceau S
3%	Trichloroacetic (TCA)

Prehybridization solution

100 ml	50 X denhardts
250 ml	formamide
25 ml	1 M Tris pH 7.5
29.2 g	NaCl
0.5 g	sodium pyrophosphate
5 ml	10mg/ml denatured salmon sperm DNA
90 ml	H_2O

Protein extraction buffer

1.2 g	Hepes
10 ml	10% Glycerol anhydrous
2.7 ml	5 M NaCl
1 ml	0.5 M EDTA pH 8.0
90 ml	H_2O

PTC

40%(w/v)	PEG 4000
50 mM	$CaCl_2$
50 mM	Tris/HCl pH 8.0

Race tube media

2%(w/v)	Bacto-Agar
0.5%(w/v)	arginine
0.3%(w/v)	glucose
1X	Vogels’ salts
1 ng/ml	biotin

RNA agarose gel

1.2%	agarose
1 X	MOPS
RNase free	H_2O

RNA extraction buffer

2 ml 0.5M EDTA pH8.0
10 ml 1 M Tris pH 8.0
40 ml 10% SDS
12 ml 5 M NaCl
36 ml H_2O (sterile)

RNA loading dye

50% (v/v) glycerin
1 mM EDTA
0.4% (v/v) Bromphenol Blue
0.4% (v/v) Xylene Cyanol.

RNA pre-mix

for each RNA loading sample
2.75 µl 20XMOPS
2.75 µl 37% (v/v) formaldehyde
27.5 µl formamide
1 µl RNA loading dye

10 X SDS PAGE running buffer

144.13 g glycin
30.28 g Tris
10 g SDS
fill H_2O up to 1L

SOC

2.5 g yeast extract
10 g tryptone
0.25 g NaCl
fill H_2O up to 500 ml and autoclave, then add 2.5 ml Mg solution and 5 ml 2M glucose

20 X SSC

525.6 g NaCl
264.6 g NaCitrate
fill H_2O up to 3L. adjust pH to 7.0 with HCl

Staining solution

0.1%(w/v) Cooomassie-Blue G250
10%(v/v) $C_2H_4O_2$
40%(v/v) methanol

Standard trace elements

0.05 g/L H_3BO_3-anhydrous
0.25 g/L $CuSO_4.5H_2O$
1.0 g/L $Fe(NH_4)_2(SO_4).6H_2O$
0.05 g/L $MnSO_4.5H_2O$
5.0 g/L $ZnSO_4.7H_2O$

0.05 g/L	$Na_2MoO_4.2H_2O$
5.0 g/L	citric acid.H_2O

Standard Westergaard's salts (5X)

in 500 ml	
KNO_3	2.5 g
KH_2PO_4	2.5 g
$MgSO_4$	1.25g
$CaCl_2$	0.25 g
NaCl	0.25 g
standard trace elements	0.25 ml

Standard Westergaard's agar media

2%	sucrose
5 µg/ml	biotin
1X	standard Westergaard's salts
1.5%	Bacto-Agar

adjust pH to 6.5

STC

1 M	sorbitol
50 mM	$CaCl_2$
50 mM	Tris/HCl pH8.0

STE

2 ml	5 M NaCl
2 ml	1 M Tris pH7.5
2 ml	0.5 M EDTA
94 ml	H_2O

Tank blotting buffer (Western blot)

288 g	glycin
60 g	Tris
2 L	Methanol

fill H_2O up to 10L

10 X TBS

90 g	NaCl
200 ml	1 M Tris pH7.4
2.5 ml	20% Tween 20

fill H_2O up to 1L

Top Agar

10 ml	50 X Vogels'
91 g	sorbitol
14 g	Bacto-Agar
450 ml	H_2O

add 50 ml 10 X FIGS after autoclaving (~75^{o}C)

50 X Vogels' salts

Na_3 citrate . 5.5 H_2O ($2H_2O$)	150 g (125 g in 775 ml dH_2O)
KH_2PO_4 (anhydrous)	250 g
NH_4NO_3 (anhydrous)	100 g
$MgSO_4$. $7H_2O$	10 g (4.88 g $MgSO_4$)
Cacl2. $2H_2O$ ($0H_2O$)	5 g (3.8 g)
Trace elements solution	5.0 ml
Biotin(0.1 mg/ml)	2.5 ml
Total volume	1000 ml

In 750 ml H_2O dissolve successively. Make sure sompound dissolved completely before adding the next ingredient. Adjust pH to 5.8. Add 2 ml chloroform for preservation

Vogel's media

0.5%	glucose
0.5%	arginine
1X	Vogel's slats
0.1µg/ml	biotin

Vogel's minimal media

2%	glucose
1X	Vogel's slats

Vogel's minimal agar media

2%	glucose
2%	Bacto-Agar
1X	Vogel's slats

Wash solution

in 50 ml H_2O

87 µl	B-mercaptoethanol
500 µl	0.5M EDTA pH 8.0

8. Abbreviations

al-1	albino-1
al-2	albino-2
al-3	albino-3
Amp	Ampicillin
bd	*Band*
bp	base pair
ccg	clock controlled gene
CLK	CLOCK
CRY, cry	CRYPTOCHROM
CTAB	cetyltrimethylammoniumbromid
CT	circadian time
CYC.cyc	CYCLE
DD	constant darkness
DNA	dexyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid disodium salt dihydrate
ET	external time
E.coli	Escherichia coli
FRP	free-running-period
FRQ, frq	FREQUENCY
g	gram
kb	kilobase
KD	kilodalton
l	liter
LB	Luria-Bertani
LD	light:dark
LL	constant light
M	molar
mM	micromolar
mg	microgram
ml	milliliter
mRNA	message RNA
nE	nano Einstein
nm	nanometer
OD	optical density
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresid
PCR	polymerase chain reaction
PER, per	PERIOD
rel.	relative
RIP	Repeated point mutation
RNA	ribonucleic acid
rpm	rotations per minute
rRNA	ribosomal RNA
RT-PCR	realtime - polymerase chain reaction
s	second
SDS	sodium dodecyl sulfate
T	cycle length
Taq	Thermus aquaticus

TEMED	N,N,N′,N′ - Tetramethyl-ethylendiamin
TIM	TIMELESS
µE	micro Einstein
UV	ultraviolet
v/v	volumn per volumn
w/v	weight per volumn
w/w	weight per weight
WC	warm:cool
WC-1, wc-1	WHITE-COLLAR-1
WC-2, wc-2	WHITE-COLLAR-2

PUBLICATIONS:

“A photoperiodic clock in *Neurospora crassa*”, Ying Tan, Zdravko Dragovic, Martha Merrow, and Till Roenneberg, in preparation

“Light reception and circadian behavior in 'blind' and 'clock-less' mutants of *Neurospora crassa*”, Zdravko Dragovic, Ying Tan, Margit Görl, Till Roenneberg, and Martha Merrow (2002), *EMBO Journal*, 21, 3643-3651

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