Commentary

Circadian rhythm in the pink–orange bread mould *Neurospora crassa*: for what?

1. Introduction

To paraphrase Theodosius Dobzhansky: Nothing in biology makes sense unless it is in the light of reproduction (Dobzhansky 1973). Does the once in ~24 hours (circadian) production of macroconidia in *Neurospora crassa* growing on an agar medium in a race tube (figure 1) or in a Petri dish as studied in the mutants *invertase* (Sargeant *et al* 1966) or *band* (Ramsdale 2001; Tan *et al* 2004; Price-Lloyd *et al* 2005) contribute to its reproductive success in nature? The characteristic pigmented macroconidia were first recognized in 1843 on mouldy bread in bakeries of Paris (Perkins 1992). Hence this fungus is popularly known as the pink or red bread-mould (Perkins 2005). It has been adopted as a model for investigating molecular mechanisms in circadian rhythms (Merrow *et al* 1999; Dunlap and Loros 2005). However, based on its growth and development on burnt sugarcane stubble (figure 2; Pandit and Maheshwari 1996) – a common substrate where this crop is grown – and reconstruction experiments using sugarcane segments and an albino mutant of the fungus, it has been deduced that the pigmented, asexually formed, airborne, multinucleate macroconidia (5–9 μm) do not ordinarily propagate *Neurospora* in nature, as has been assumed. Nor do macroconidia, though they are used as donors of male nuclei in genetic crosses in laboratories, serve as fertilizing elements in nature because the female sexual bodies (protoperithecia) are submerged inside the plant tissue. Why does *Neurospora* employ a circadian mechanism for the formation of macro-conidia that do not directly function in dissemination and survival?

The ecosystem of *Neurospora* reveals that although macroconidia have the potential for propagating *Neurospora*, these cells do not ordinarily do so. Instead, their production and liberation from infected fire-scorched vegetation create nutritional conditions and a microenvironment conducive to sexual reproduction inside tissue pockets. This requires male cells (microconidia) to be transmitted to female cells inside protoperithecia for fertilization and formation of sexual spores. For this, *Neurospora* relies on microfaunal vectors. The observed circadian rhythm in the production of macroconidia may be incidental to a microconidiation rhythm that co-evolved with an associated microfaunal vector to produce dormant spores for survival.

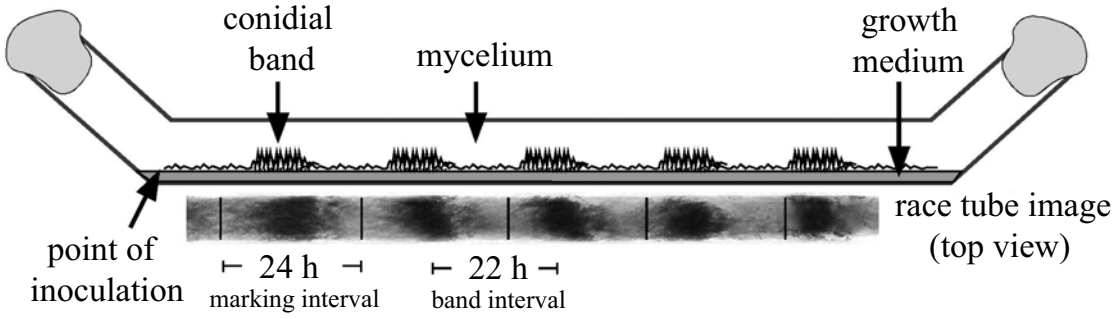

Figure 1. Circadian rhythm in macroconidia production in *Neurospora crassa*. Above, diagram of fungus growing in a race tube. Below, photograph of a race tube in top view (Based on Price-Lloyd *et al* 2005)

Keywords. *Neurospora*; circadian rhythm; microfauna; co-evolution; sexual reproduction; conidia

Figure 2. Conidiating (macroconidia) pustules of *Neurospora* growing on sugarcane stubble.

2. Natural populations: clonal or sexual?

Whether reproduction takes place by means of asexual or sexual spores can be decided only by population genetic information (Tibayrenc *et al* 1991). Genetic (heterokaryon compatibility) and molecular analyses (allozyme variation, restriction fragment length variation, DNA sequence variation) have revealed that natural populations of *Neurospora* are prevailingly sexual, not clonal (Perkins and Turner 1988; Powell *et al* 2003). Based on the results of reconstruction experiments, it has been argued that the prodigious production of macroconidia has an entirely different biological role than hitherto believed: their aerial dissemination over several weeks, and foraging by meso- and microfauna, depletes the substrate of soluble nutrients, chiefly of sugar (Pandit and Maheshwari 1996). This chemical conditioning of the substrate, for example, of burnt sugarcane stubble in agricultural fields, favours the differentiation of microconidiophores and protoperithecia by the mycelium inside the heat-killed plant tissue. The requirement of a nutrient-depleted substrate for sexual reproduction is corroborated by the need for a culture medium low in carbon and nitrogen for making genetic crosses (Davis 2000), which is consistent with the general observation that in fungi conditions for asexual and sexual reproduction are different.

3. Reproduction occurs in tissue pockets

Asexual and sexual phases of the same fungal species can be separated both in space and in time (Burnett 2003). In nature, perithecia and ascospores of *Neurospora* were reported *only* under the bark of fire-scorched trees (Kitazima 1925) or under the epidermal tissue in burnt sugarcane stubble (Pandit and Maheshwari 1996). In the latter, sexual reproduction occurred *after* macroconidia production had ceased. This may be the reason why in recent studies of *Neurospora* blooms on burnt trees following natural fires

in Europe and the USA, perithecia were not observed (Jacobson *et al* 2004, 2006). In agricultural fields of sugarcane, the meiotically produced ascospores are passively liberated into the soil by the disintegration of stubbles and presumably distributed by rain or irrigation water. Dormant ascospores are activated by furfural produced from xylan/xylose present in plant tissue following burning, which diffuses into the soil (Pandit and Maheshwari 1996). Simulation experiments with burned cane segments planted in pots in which the soil had been mixed with ascospores of a mutant strain that produces a distinct albino phenotype confirmed that the infection is by ascospores in soil, not by the airborne macroconidia (Pandit and Maheshwari 1996).

4. Implications of reproduction inside tissue pockets

The heterothallic species of *Neurospora* require *mat A* and *mat a* mating type strains for sexual reproduction. Since in nature sexual reproduction in *Neurospora* occurs within the plant tissue (Pandit and Maheshwari 2004), we may exclude a *primary* role for the externally liberated, wind-disseminated, brightly pigmented, multinucleate macroconidia (5–9 μm) in the fertilization of protoperithecia, although they have an accessory role in creating the microenvironment for sexual reproduction and in attracting animal vectors for fertilization. Rather, it is the inconspicuous, uninucleate microconidia (2.5–3.5 μm) (Maheshwari 1999) produced simultaneously with protoperithecia *beneath* the loosened plant epidermal tissue that fertilize the female sexual bodies. A microconidium needs to be transmitted to the trichogyne (a specialized hyphal cell from the protoperithecium) for donating the male nucleus to the ascogonium. The chance of a microconidium of opposite mating type contacting the trichogyne for fertilization inside a tissue pocket would be maximized if microconidiation coincided with the time of foraging of microfauna. Mites are known to cross-contaminate cultures through cotton plugs and closures (Perkins 1986), attesting to conidia being a highly palatable food for mites. While we observed nematodes and mites in *Neurospora*-infected burnt sugarcane stubbles (Pandit and Maheshwari 1996), Jacobson and co-workers observed larvae, small insects, mites and isopods beneath charred bark in the forests of North America following natural fires (Jacobson *et al* 2004). However, the role of microfauna as vectors in the life cycle of *Neurospora* and their possible role in the co-evolution of light perception and circadian rhythm in the fungus and associated microfauna was not considered previously.

The 'infection' of scorched sugarcane stubble occurs from ascospores in the soil, which are activated by furfural produced from burnt hemicellulosic substrate. The resulting hyphal growth invades the moribund plant tissue (Pandit and Maheshwari 1996). The 'infective' hypha grows inside the stubble forming subepidermal cushions of hyphae (sporodochium). The growth pressure of the aggregates of conidiophores causes the epidermal tissue to detach from the ground tissue and the conidiophores to erupt through fire-induced cracks in the tissue and liberate mature macroconidia into the air. The tissue pockets not only serve to provide protection to the fungal gametic cells from the deleterious effects of UV radiation, high temperature and desiccation, but also act as a niche for microfaunal vectors. The observed changes in infected tissue, i.e. nutrient depletion and formation of tissue pockets, suggest that, remarkably, the fungus itself creates the physiological, morphological and ecological conditions for sexual reproduction (Pandit and Maheshwari 1996). The implication of sexual reproduction occurring inside the tissue pocket and the analysis of the interaction between the fungus and the microfauna in the fire-scorched plant suggest that the interacting partners need to synchronize their activities. Shaw (1990, 1998) in Australia observed foraging activity of honey bees in the early morning hours on filter mud – a byproduct of sugar manufacture – and reported that honey bees prefer *Neurospora* conidia to pollen. Protoperithecia or microconidiophores were not sighted; as explained above, it is unlikely that sexual reproductive structures would have been formed as long as *Neurospora* blooms were visible (*http://www.fgsc.net/Neurospora/sectionB4.htm*).

5. Microconidia

Molecular analysis of the levels of *frq* messenger RNA and protein suggests that macroconidia begin to form late at night and continue to form during the early morning hours (Bell-Pedersen *et al* 1996). Since the same submerged mycelium also forms microconidia, these may also be formed during the early morning. In

our reconstruction experiments (Pandit and Maheshwari 1996), we observed microconidiophores in tissue pockets in old stubble after macroconidial blooms had ceased during the early morning hours subsequent to rainfall. Microconidiation in *Neurospora* may be a manifestation of a basic endogenous rhythm in the common mycelium, as suggested by the rhythm in the enzyme activities of glyceraldehyde-3-phosphate dehydrogenase and geranylgeranyl pyrophosphate synthase, of the cell wall protein hydrophobin (Bell-Pedersen *et al* 1996), and of pheromone peptides (Bobrowicz *et al* 2002). Sexual reproduction requires the contact of the protoperithecium (trichogyne) with a male cell. Since these structures are formed inside tissue pockets, there is an obvious need for a vector to transmit the male cells which rapidly lose viability. We believe that the coordination of microconidia production with microfaunal activity is the most likely reason for the co-evolution of circadian rhythm in *Neurospora*.

6. 'Pollination' of *Neurospora* by an animal vector

Adjacent *Neurospora* colonies on the 'infected' scorched stem may differ in mating type (Jacobson *et al* 2004, 2006), suggesting that for sexual reproduction *mat A* or *mat a* microconidia from spatially separated colonies must be brought in contact with the trichogyne of the opposite mating type. Though *Neurospora* conidia produce a diffusible pheromone capable of chemotropically attracting trichogyne (Bistis 1983), the trichogyne is too short to contact microconidia from a distant colony of opposite mating type, suggesting the need of a vector for transmitting these cells to the trichogyne, thereby affecting fertilization. *Neurospora* conidia produce a pheromone that attracts trichogyne. However, since trichogynes display chemotropism towards macroconidia of the opposite mating type, this suggests that *Neurospora* has a mechanism to also engage in fertilization by macroconidia that perchance are carried by flowing rain water or soil animals. The pheromone may be an odoriferous cue to attract microfauna as dispersers of microconidia. This assumption gains credence from the production of aroma by truffles that diffuse up through soil and are detected by sows, apparently because of its chemical similarity to the pheromone produced by a male pig. Sows were therefore used to sniff out underground truffles (*http://www.avignon-et-provence.com/provence/truffe_noire/img/truie_truffe.jpg*). Insect 'pollination' of *Epichloe typhina,* a heterothallic ascomycete that is an endophytic pathogen of grasses, has been reported (Bultman and White 1988).

7. Photobiology

Since protoperithecia formation is controlled by blue light (Sommer *et al* 1989; Linden and Macino 1997), and since in the *Neurospora* strains examined the protoperithecia are formed simultaneously with microcondiophores, the development of microconidiophores may be a blue-light response. Merrow *et al* (1999) reported that *Neurospora* could sense light as low as 8 $nEm^{-2}\ s^{-1}$ (equivalent to the light of a night with a full moon) for macroconidia formation. Springer and Yanofsky (1992) found that some developmentally controlled conidiation (*con*) genes in *N. crassa* are expressed both in macro- and microconidiation. The previously identified key genes in circadian macroconidiation, *frequency* (Merrow *et al* 1999) and *white collar* (Liu and Bell-Pedersen 2006) may have a shared function in microconidiation, but the involvement of different clock genes in microconidiation cannot be ruled out since the microconidiation pathway is distinct from the macroconidiation pathway (Maheshwari 1999).

8. Future directions

The role of the macroconidial rhythm in reproduction needs to be confirmed by comparing the numbers and concentration of conidia in the environment of the wild-type and *frq* knockout mutant conidia at different times of the day. Sugar factory filter mud dumps provide an experimental site for such a study (Shaw 1990, 1998; Rashmi *et al* 2003). On the other hand, the rhythmic production of microconidia needs to be confirmed. Culture techniques have been developed which suppress macroconidiation in *N. crassa*, allowing microconidiophores to be visualized even in wild strains (Maheshwari 1999) to determine if these display clock properties. Alternatively, pure microconidiating strains are available. The wealth

of data on the physiology and genetics of *Neurospora* (Davis 2000*)* offers a system to analyse the co-evolution of the timekeeping trait with associated microfauna. A prediction is that the circadian rhythm of various periods may be a feature of those fungi wherein a crucial event in their life cycle depends on the foraging time of associated microfauna.

Dedication

This paper is dedicated to the memory of David D Perkins, Stanford University.

References

Bell-Pedersen D, Garceau N and Loros J J 1996 Circadian rhythms in fungi; *J. Genet.* **75** 387–401

Bistis G N 1983 Chemotropic interactions between trichogynes and conidia of opposite mating type in *Neurospora crassa*; *Mycologia* **73** 959–975

Bobrowicz P, Pawlak R, Correa A, Bell-Pedersen D and Ebbole D J 2002 The *Neurospora crassa* pheromone precursor genes are regulated by the mating type locus and the circadian clock; *Mol. Microbiol*. **45** 795–804

Bultman T L and White J F 1988 "Pollination" of a fungus by a fly; *Oecologia* **75** 317–319

Burnett J 2003 *Fungal populations and species* (New York: Oxford University Press) p. 21

Davis R H 2000 *Neurospora: contributions of a model organism* (New York: Oxford University Press) p.284

Dobzhansky T 1973 Nothing in biology makes sense except in the light of evolution. *Am. Biol. Teacher* **35** 125–129

Dunlap J C and Loros J J 2004 The *Neurospora* circadian system; *J. Biol. Rhythms* **19** 414–424

Jacobson D J, Powell A J, Dettman J R, Saenz G S, Barton M M, Hiltz M D, Dvorachek W H, Glass N L, Taylor J W and Natvig D O 2004 *Neurospora* in temperate forests of western North America; *Mycologia* **96** 66–74

Jacobson D J, Dettman J R, Adams R I, Boesl C, Sultana S, Roenneberg T, Merrow M, Marques I, Ushakova A, Carneiro P, Videira A, Navarro-Sampedro L, Olmedo M, Corrochano L M and Taylor J M 2006 New findings of *Neurospora* in Europe and comparisons of diversity in temperate climates on continental scales; *Mycologia* **98** 550–559

Kitazima K 1925 On the fungus luxuriantly grown on the bark of the trees injured by the great fire of Tokyo on Sept 1, 1923; *Ann. Phytopathol. Soc. Japan* 1 15–19

Linden H and Macino G 1997 White collar 2, a partner in bluelight signal transduction, controlling expression of light-regulated genes in *Neurospora crassa*; *Fungal Genet. Biol.* **22** 98–109

Liu Y and Bell-Pedersen D, 2006 Circadian rhythms in *Neurospora crassa* and other filamentous fungi; *Euk. Cell* **5** 184–193

Maheshwari R 1999 Microconidia of *Neurospora crassa*; *Fungal Genet. Biol*. 26 1–18

Merrow M, Brunner M and Roenneberg T 1999 Assignment of circadian function for the *Neurospora* clock gene *frequency; Nature* **399** 584–586

Pandit A and Maheshwari R 1993 A simple method of obtaining pure microconidia in *Neurospora crassa; Fungal Genet. Newsl*. **40** 64

Pandit A and Maheshwari R 1994 Sexual reproduction of *Neurospora* in nature; *Fungal Genet. Newsl*. **41** 67

Pandit A and Maheshwari R 1996 Life-history of *Neurospora intermedia* in a sugar cane field; *J. Biosci.* **21** 57–79

Perkins D D 1986 Hints and precautions for the care, feeding and breeding of *Neurospora*; *Fungal Genet. Newsl.* **33** 35–41

Perkins D D and Turner B C 1988 *Neurospora* from natural populations; *Exp. Mycol.* **12** 91–131

Perkins D D 1992 The organism behind the molecular revolution; *Genetics* **130** 687–701

Perkins D D 2005 Why "red bread mould" is an inappropriate name for *Neurospora*; *Fungal Genet. Newsl.* **52** 7–8

Powell A J, Jacobson D J, Salter L and Natvig D O 2003 Variation among natural isolates of *Neurospora* on small spatial scales; *Mycologia* **95** 809–819

Price-Lloyd N, Elvin M and Heintzen C 2005 Synchronizing the *Neurospora crassa* circadian clock with the rhythmic environment; *Biochem. Soc. Trans*. **33** 949–952

Ramsdale M 2001 Fungi with a sense of time: molecular genetics of temporal organization in *Neurospora crassa*; *Mycologist* **15** 10–15

Rashmi K, Lavanya Latha J N, Sowjanya T N, Kiranmayi P, Venugopal Rao M, Menon C S and Maruthi Mohan P 2003 *Neurospora* in full bloom; *Curr. Sci.* **85** 1670–1672

Sargent M L, Briggs W R and Woodward D W 1966 The circadian nature of a rhythm expressed by an invertaseless strain of *Neurospora crassa*; *Plant Physiol.* **41** 1343–1349

Shaw D E 1990 The incidental collection of fungal spores by bees and the collection of spores in lieu of pollen; *Bee World* **71** 158–176

Shaw D E 1998 Blooms of *Neurospora* in Australia; *Mycologist* **4** 6–13

Sommer T, Chambers J A A, Eberle J, Lauter F-R and Russo V E A 1989 Fast light regulated genes of *Neurospora crassa*; *Nucleic Acids Res.* **17** 5713–5723

Springer M L and Yanofsky C 1992 Expression of *con* genes along the three sporulation pathways of *Neurospora crassa*; *Genes Dev.* **3** 1052–1057

Tan Y, Merrow M and Roenneberg T 2004 Photoperiodism in *Neurospora crassa*; *J. Biol. Rhythms* **19** 135–143

Tibayrenc M, Kjellberg F, Arnaud J, Oury B, Brenière F, Dardè M-L and Ayala F 1991 Are eukaryotic microorganisms clonal or sexual? A population genetic vantage; *Proc. Natl. Acad. Sci. USA* **88** 5129–5133

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