Ultradian Rhythms from Molecules to Mind

David Lloyd • Ernest L. Rossi
Editors

Ultradian Rhythms from Molecules to Mind

A New Vision of Life

Springer

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David Lloyd would like to dedicate this book to the memory of Gregorio Weber, Geoffrey Callely and David E. Hughes, and to two other exceptional experimental scientists J. Woody Hastings and Britton Chance on their 80th and 95th birthdays respectively.

Finally to Dr R.R. Klevecz, who made enormous contributions to our fundamental insights into the dynamic complexity of biological systems. Sadly, Bob passed away on 13.05.2008 whilst this volume was in the course of preparation.

Beauty is the proper conformity of the parts to one another and to the whole

HEISENBERG: The Meaning of Beauty in the Exact Sciences

The almost frightening simplicity and wholeness of the relationships which nature suddenly spreads out before us and for which none of us was in the least prepared

HEISENBERG in discussion with EINSTEIN

Acknowledgements

DL would like to record his indebtedness to all those who helped this volume through its production, especially Mrs. Drusilla Lewis for interpretation of my script, Dr. Douglas Murray for many of the figures and members of my family (Margaret, Alun, Siôn and Anvita) for their assistance.

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December 27, 2007

David Lloyd
Ernest L. Rossi

Contents

Introduction: The Temporal Organization of Living Systems from Molecule to Mind

D. Lloyd[1], E.L. Rossi[2] and M.R. Roussel[3]

> *It is the pattern maintained by this homeostasis, which is the touchstone of our personal identity. Our tissues change as we live: the food we eat and the air we breathe become flesh of our flesh, and bone of our bone, and the momentary elements of our flesh and bone pass out of our body every day with our excreta. We are but whirlpools in a river of ever-flowing water. We are not the stuff that abides, but patterns that perpetuate themselves.*
>
> Wiener, 1954

> *What are called structures are slow processes of long duration, functions are quick processes of short duration.*
>
> Von Bertalanffy, 1952

Images of Earth taken from an orbiting satellite emphasize the sharp transition in light intensity, temperature and relative humidity that almost every point on our planet experiences twice daily. The crescent of dawn sweeping the earth, as it has done about a million million times since life began, has imprinted itself on our evolutionary trajectory and the physiological characteristics of our ancestral lineage. We are born into regularly periodic circumstances; the capabilities of living organisms to use the separation of environmental light from dark have yielded an extraordinary repertoire of means for its exploitation. The properties of the organism are enmeshed with the planet's geophysical parameters by the circadian (τ about 24 h) clock, an internal temperature-compensated biological mechanism which is both re-settable and entrainable. Evolution of the processes whereby matching of the endogenous biochemical network to the daily cycles of night and day occurs is but one aspect of the self-optimization of the living system. The Circadian clock, ubiquitous at least from cyanobacteria to humans is underpinned by an enormously complex time structure, so that if we consider typical

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D. Lloyd, E. Rossi (eds.), *Ultradian Rhythms from Molecules to Mind*,

relaxation times of component reactions, these range over many logarithmic decades of time (Fig. 1a). We must thus be prepared to study the processes which are relevant to life on timescales ranging from attoseconds to gigaseconds and beyond, from those events completed on sub-molecular scales to rates of evolutionary change. Seen in this way, circadian control represents a very small window in an immense panoply, one that is still only dimly comprehended, because it is not studied as part of an enormously more complicated whole.

Key to the understanding of that whole is a more central hub, a more primeval time-base, one that encompasses both biophysics and biochemistry, both cellular biology and cell reproduction (Lloyd et al., 1982; Lloyd and Gilbert, 1998; Lloyd and Murray, 2005, 2006, 2007). This is the ultradian clock; more precisely designated the circahoralian clock (Brodsky, 1975), as its period is about 1 h. This time-base has been recognized only since its discovery in the soil amoeba *Acanthamoeba castellanii* in 1979, again as a temperature-compensated mechanism, which is therefore more than an oscillation and even more than a rhythm. Whereas in *A. castellanii* its period is about 69 min (Edwards and Lloyd, 1978, 1980), in other organisms this value is different. In one species of yeast, *Candida utilis*, it is 30 min (Kader and Lloyd, 1979), in another, *Schizosaccharomyces pombe*, 40 min (Poole et al., 1973). The ciliate protozoon, *Tetrahymena pyriformis* has a 50 min clock (Lloyd et al., 1978); in the flagellate *Crithidia fasciculata* it is 66 min (Edwards et al., 1975, Fig. 1b). These values have no special significance *per se*, other than as an expression of a self-optimized selection, especially appropriate to the somewhat differing networks characteristic of these different species. In humans, the 90 min basic rest activity rhythm (BRAC) first observed in 1953, by Aserinsky and Kleitman probably represents an analogous phenomenon.

Except in organisms in their cryptobiotic states, where biochemical network activities are minimized to very low levels of activity, it seems likely that this ultradian clock operates in every unicellular organism, in every metazoan and plant cell, and thus in every organism at almost all times. Its pervasive nature underlines its central importance at the core of coherence, the insistent drumbeat to which all metabolic and biosynthetic pathways, events and processes are tied. The intracellular network, involving as it does not merely sequences of enzyme-catalyzed steps, but also binding of controlling ligands both stimulatory and inhibitory, as well as protein-DNA interactions and the less well-understood protein-protein interactions is orchestrated by reference to this timekeeper.

Just as clocks can be built which indicate the year, date, hour, minute and second, living organisms also need to coordinate activities over a range of time scales. Away from the equator, the year is a biologically important time scale, and it is well known that plants and animals are very adept at preparing for the changes of the seasons. Arguably the best-studied biological clock is the circadian, which allows a variety of organisms, from bacteria to humans, to cope with the daily cycles of illumination and temperature. The circahoralian clock provides organisms with a biological hour, with consequences that are explored in detail in this book. What of biological minutes and seconds? Do our internal clocks have fast rhythms that allow for the measurement of intervals of time shorter than the biological hour of the circahoralian clock?

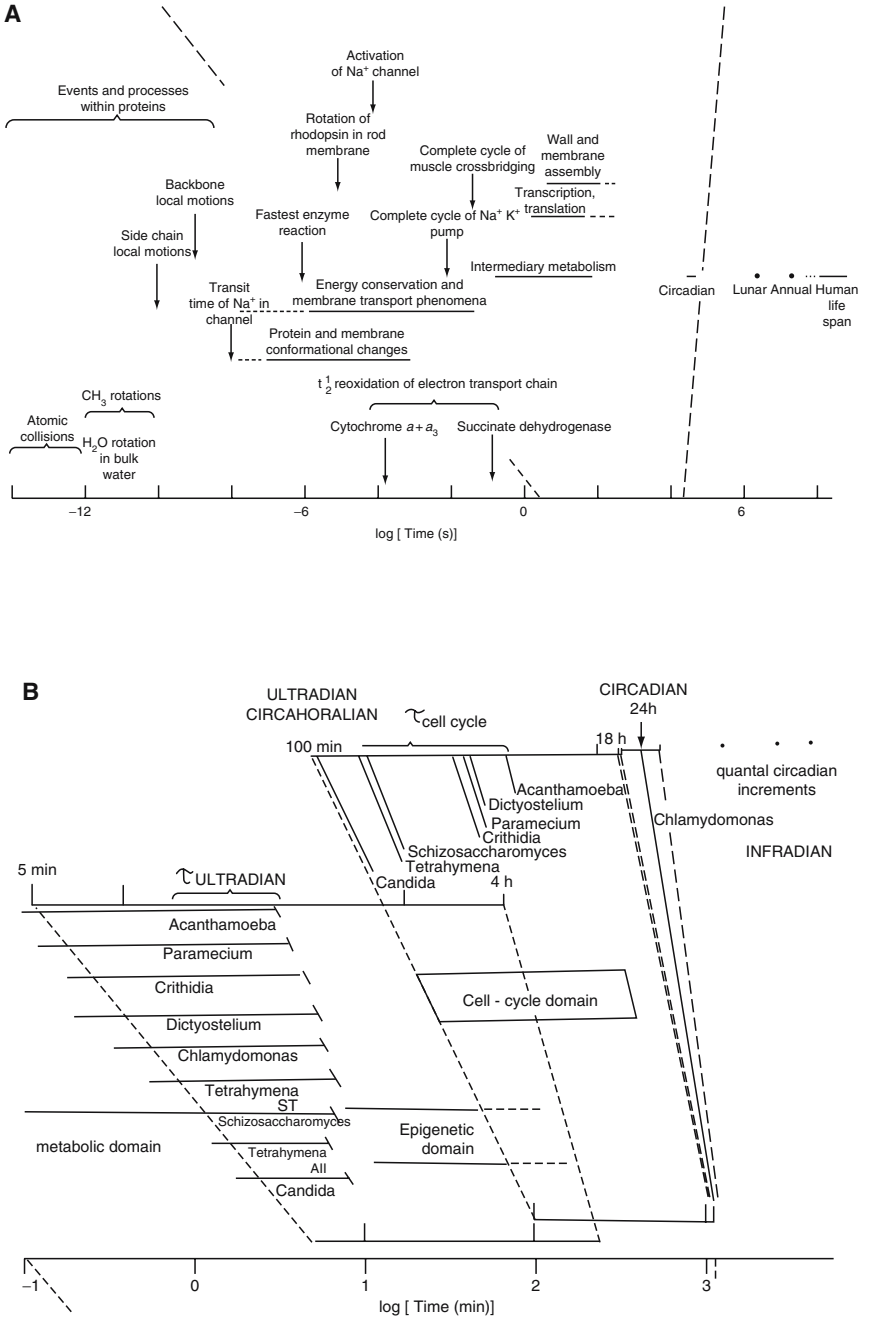

Fig. 1 The time domains of living systems: (**a**) Approximate relaxation times (to a return of 1/e of a small perturbative effect (Goodwin, 1963) are indicated. (**b**) Ultradian clock and cell division cycle times in lower eukaryotes

The biological clock may have several minute hands. Calcium oscillations have periods ranging from seconds to minutes, can be initiated by a variety of biological stimuli (hormones, etc.) and may serve as a time base for reliable signal transmission and targeting *in vivo* (Berridge and Galione, 1988; Gu and Spitzer, 1995; Hajnóczky et al., 1995; Dolmetsch et al., 1998). Oscillations in the transport of ions across mitochondrial membranes, again with periods of a few minutes, have been observed in mitochondrial suspensions (Gooch and Packer, 1974). Sustained oscillations of mitochondrial inner membrane potential, NADH and reactive oxygen species production with a similar frequency have also been observed in intact cells (Aon et al., 2003). Mitochondrial oscillations occuring in undisturbed cells have also been seen (Bashford et al., 1980; Aon et al., 2007). However, it is not inconceivable that such oscillations provide a local (i.e. within individual mitochondria) time reference on the minute time scale.

Recently, we have observed simultaneous metabolic oscillations on three, and perhaps four different time scales in a budding yeast population grown in continuous culture (Roussel and Lloyd, 2007). Figure 2 shows the three oscillatory modes we have studied in detail. The longest period observed resulted from partial cell-cycle synchrony. The well-known circahoralian rhythm also appeared clearly in our data, although it was periodically obliterated by the cell-cycle-associated

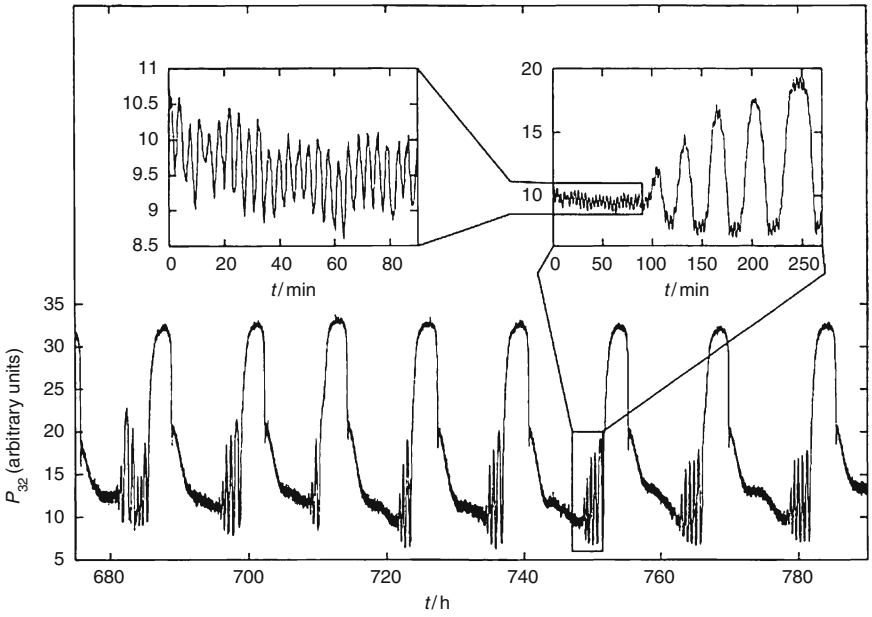

Fig. 2 The *m/z* = 32 signal from the experiment reported by Roussel and Lloyd (2007). This signal corresponds to dissolved oxygen in the fermentor medium. We see a hierarchy of oscillations associated, from slowest to fastest, with the cell cycle, the cirahoralian clock, and an as-yet unidentified biochemical oscillator with a period of approximately 4 min

oscillations. We also observed oscillations of small amplitude with a period of 4 min. These oscillations appear to have a cellular origin (i.e. they are not strictly a collective phenomenon of the culture, nor are they a physico-chemical artifact) and may be the manifestation at the population level of the mitochondrial oscillations described above.

In addition to the three periodic modes described above, we observed highly irregular fluctuations in the CO_2 and H_2S signals with a characteristic time on the order of 1 min. Unfortunately, the time scale of these fluctuations was similar to our sampling time (12 s) so that it is difficult to make any definitive statements about the nature of this oscillatory (chaotic?) mode. Interestingly, fast, irregular fluctuations (with a characteristic time of a few seconds) have also been observed in plant leaves after transfer to a low-CO_2 environment (Roussel et al., 2007). In both cases, these rapid fluctuations arise in healthy organisms. In addition to clocks with regular outputs, it thus appears that cells can display irregular oscillatory modes. Whether these modes are chaotic or stochastic is unknown at this time, as are their possible mechanisms and physiological roles, if any.

What of faster clocks, with periods on the second time scale? Due to the inherent difficulties in studying such fast rhythms, very little is known of cellular timekeeping on this time scale, and even less on subsecond time scales. Calcium oscillations operate in different tissues and situations over a range of time scales, right down to the second range (Berridge and Galione, 1988). This versatile timekeeper may thus function as the cell's second hand in some situations.

In man-made timepieces, typically there is a single fast oscillator which is geared down several times to generate second, minute, hour and day chronometers. What of biological clocks? It is known theoretically that fast oscillators can be coupled to generate slower rhythms whose period is determined in part by coupling topology (Barrio et al., 1997). There are also a few lines of evidence which suggest that the circahoralian and circadian oscillators are related in some way (Dowse and Ringo, 1992; Fuentes-Pardo and Hernández-Falcón, 1993; Lloyd and Murray, 2005). It seems unlikely however that all of the cell's timekeeping functions derive from a single primitive clock. Even circadian timekeeping may involve more than one clock in some organisms (Morse et al., 1994; Johnson, 2001; Lillo et al., 2001). The metaphor we should perhaps have in mind for a living organism's timekeeping functions is thus that of a scientific lab in which multiple timepieces exist to satisfy various needs: our data acquisition systems usually have built-in clocks. We typically have some simple bench-top chronometers for timing routine tasks. And of course, the experimenter usually possesses a wristwatch to remind him or herself of teatime.

The "time domains of living systems" presented in Fig. 1a and b are obviously a "bottom-up" perspective from molecules to cells and organisms illustrating how we typically construct a reductionist view of life. While this bottom-up perspective is impeccably scientific it is not always meaningful to the philosophical mind searching for the meaning and significance of it all for understanding human life and experience (Rossi, 2007). For meaning and understanding, our consciousness often seems to prefer a "top-down" perspective wherein we can utilize the reductionist

"facts" to construct an ever more useful comprehension of ourselves in the world. This is what we hope to achieve in section four of this volume: Ultradian and Circadian Rhythms in Human Experience.

Charles Darwin, in a little noticed statement on natural selection in chapter four of *The Origin of Species*, comments on the significance of what we now designate as ultradian and circadian rhythms in human experience.

> It may be said that natural selection is daily and hourly scrutinising, throughout the world, every variation, even the slightest; rejecting that which is bad, preserving and adding up all that is good; silently and insensibly working, whenever and wherever opportunity offers, at the improvement of each organic being in relation to its organic and inorganic conditions of life. We see nothing of these slow changes in progress, until the hand of time has marked the long lapses of ages, and then so imperfect is our view into long past geological ages, that we only see that the forms of life are now different from what they formerly were.

We take this statement by Darwin very seriously, indeed, to help us unify the bottom-up and top-down perspectives of this volume. In our final chapter we generalize the implications of Darwin's natural selection to explore a new view of the significance of art, beauty, and truth on the molecular-genomic level. We utilize current research on the evolutionary origins of consciousness that distinguishes between the experience and behavior of humans and other primates at the levels of brain anatomy, neuronal activity, and gene expression. We take a few tentative steps toward creating a new science of psychosocial genomics to resolve the now hoary nature-nurture controversy with recent epigenetics research on the molecular pathways of communication between mind, brain, body, and gene.

In all of this we must beg the reader's indulgence for we truly identify with Isaac Newton when he describes the scientific ethos.

> I don't know what I may seem to the world, but as to myself, I seem to have been only like a boy playing on the sea-shore and diverting myself in now and then finding a smoother pebble or a prettier shell than ordinary, whilst the great ocean of truth lay undiscovered all before me.

We can only hope that the creative uncertainty we all naturally experience with our bumbling efforts in the laboratory, the clinic, and public outreach will be assuaged at least in part by the earnest efforts and imagination of the excellent contributors to this volume.

References

Aon MA, Cortassa S, Marbán E, O'Rourke B (2003) Synchronized whole cell oscillations in mitochondrial metabolism triggered by a local release of reactive oxygen species in cardiac myocytes. J Biol Chem 278:44735–44744

Aon MA, Cortassa S, Lemar KM, Hayes AJ, Lloyd D (2007) Single and cell population respiratory oscillations in yeast: A 2-photon scanning laser microscopy study. FEBS Lett 581:8–14

Aserinsky E, Kleitman N (1953) Regularly occurring periods of eye mobility, and concomitant phenomena, during sleep. Science 118: 273–274

Barrio RA, Zhang L, Maini PK (1997) Hierarchically coupled ultradian oscillators generating robust circadian rhythms. Bull Math Biol 59:517–532

Bashford CL, Chance B, Lloyd D, Poole RK (1980) Oscillations of redox states in synchronously dividing cultures of *Acanthasmoeba castellanii* and *Schizosaccharomyces pombe*. Biophys J 29: 1–12

Berridge MJ, Galione A (1988) Cytosolic calcium oscillators. FASEB J 2:3074–3082

Brodsky VY (1975) Protein synthesis rhythm. J Theor Biol 55: 167–200

Dolmetsch RE, Xu K, Lewis RS (1998) Calcium oscillations increase the efficiency and specificity of gene expression. Nature 392:933–936

Dowse HB, Ringo JM (1992) Do ultradian oscillators underlie the circadian clock in Drosophila? In: Lloyd D, Rossi EL (eds) Ultradian Rhythms in Life Processes. Springer, London, pp 105–117

Edwards C, Statham M, Lloyd D (1975) The preparation of large-scale synchronous cultures of the trypanosomatid *Crithidia fasciculata* by cell-size selection: changes in respiration and adenylate charge through the cell cycle. J Gen Microbiol 88:141–152

Edwards SW, Lloyd D (1978) Oscillations of respiration and adenine nucleotides in synchronous cultures of *Acanthamoeba castellanii*: mitochondrial respiratory control in vivo. J Gen Microbiol 108:197–204

Edwards SW, Lloyd D (1980) Oscillations in protein and RNA during synchronous growth of *Acanthamoeba castellanii*: evidence for periodic turnover of macromolecules during the cell cycle. FEBS Lett 109:21–26

Fuentes-Pardo B, Hernández-Falcón J (1993) Neurobiology of the circadian clock of crayfish. Trends Comp Biochem Physiol 1:635–673

Gooch VD, Packer L (1974) Oscillatory systems in mitochondria. Biochim Biophys Acta 346:245–260

Gu X, Spitzer NC (1995) Distinct aspects of neuronal differentiation encoded by frequency of spontaneous Ca^{2+} transients. Nature 375:784–787

Hajnóczky G, Robb-Gaspers LD, Seitz MB, Thomas AP (1995) Decoding of cytosolic calcium oscillations in the mitochondria. Cell 82:415–424

Johnson CH (2001) Endogenous timekeepers in photosynthetic organisms. Annu Rev Physiol 63:695–728

Kader J, Lloyd D (1979) Respiratory oscillations and heat evolution in synchronous cultures of *Candida utilis*. J Gen Microbiol 114:455–461

Lillo C, Meyer C, Ruoff P (2001) The nitrate reductase circadian system. The central clock dogma contra multiple oscillatory feedback loops. Plant Physiol 125:1554–1557

Lloyd D, Gilbert D (1998) Temporal organization of the cell division cycle in eukaryotic microbes. Symp Soc Gen Micro 56: 251–278

Lloyd D, Murray DB (2005) Ultradian metronome: timekeeper for orchestration of cellular coherence. Trends Biochem Sci 30:373–377

Lloyd D, Murray DB (2006) The temporal architecture of eukaryotic growth. FEBS Lett 580:2830–5

Lloyd D, Murray DB (2007) Redox rhythmicity: clocks at the core of temporal coherence. Bioessays 29:1–7

Lloyd D, Phillips CA, Statham M (1978) Oscillations of respiration adenine nucleotides and heat evolution in synchrous cultures of *Tetrahymena pyriformis* ST prepared by continuous flow size-selection. J Gen Microbiol 106:19–26

Lloyd D, Edwards SW, Fry JC (1982) Temperature-compensated oscillations in respiration and cellular protein content in synchronous cultures of *Acanthamoeba castellanii*. Proc Natl Acad Sci USA 79:3785–3788

Morse D, Hastings JW, Roenneberg T (1994) Different phase responses of the two circadian oscillators in Gonyaulax. J Biol Rhythms 9:263–274

Poole RK, Lloyd D, Kemp RB (1973) Respiratory oscillations and heat in synchronously dividing cultures of the fission yeast, *Schizosaccharomyces pombe* 972h$^-$. J Gen Microbiol 77:209–220

Rossi EL (2007) The Breakout Heuristic: The New Neuroscience of Mirror Neurons, Conciousness and Creativity in Human Relationships. Phoenix, AZ: Milton H Erickson Foundation Press
Roussel MR, Lloyd D (2007) Observation of a chaotic multioscillatory metabolic attractor by real-time monitoring of a yeast continuous culture. FEBS J 274:1011–1018
Roussel MR, Ivlev AA, Igamberdiev AU (2007) Oscillations of the internal CO_2 concentration in tobacco leaves transferred to low CO_2. J Plant Physiol 164:1188–1196
Von Bertalanffy L (1952) Problems of Life. New York: Harper & Brothers, p 216
Wiener N (1954) The Human Use of Human Beings. New York: Doubleday, p 96

Part I
The Molecular-Genetic-Cellular Level

Chapter 1
The Ultradian Clock (~40 min) in Yeast

(*Saccharomyces cerevisiae*)

D. Lloyd[1], D.B. Murray[2], R.R. Klevecz[3]*, J. Wolf[4], and H. Kuriyama[5]

Abstract A precisely controlled, continuously grown, aerobic yeast culture system shows an autonomous sustained respiratory oscillation (i.e. a high amplitude change in dissolved O_2 levels: the residual O_2 that remains after the organisms use what they require). This spontaneously organized synchronous state can be maintained for extended periods (months) and continuously monitored for intracellular redox state (by direct fluorimetric output for nicotinamide nucleotides: excitation 366 nm, emission 450 nm), and dissolved gases (O_2 electrode and/or direct membrane inlet mass spectrometry for O_2, CO_2 and H_2S, sampled on a 15 s cycle). The whole culture population (~5×10^8 organisms/ml) is behaving metabolically as if it were a single cell, so that analysis for metabolic intermediates (keto and amino-acids, carboxylic acids), redox components (glutathione, cysteine, NAD(P)H and reactive O_2 species) and products (acetaldehyde, acetic acid and ethanol), with sampling at frequent time intervals gives reliable information on phase relationships on the approximately 40 min ultradian cycle. Lipid peroxidation levels indicate the changing levels of oxidative stress. Microarray analysis shows a genome-wide oscillation in transcription, with expression maxima at three nearly equally spaced intervals on the 40 min time-base. The first temporal cluster (4,679 of 5,329 genes expressed) occurred maximally during the reductive phase, whereas the remaining 650 transcripts were detected maximally in the oxidative phase. Furthermore, when fixed samples of yeasts were analysed for DNA using flow cytometry, synchronous bursts in the initiation of DNA replication (occurring in about 8% of the total population) were shown to coincide with decreasing respiration rates. A precisely defined appearance of mitochondrial energy generation-dependent conformational

*Dr Bob Klevecz passed away on 13.05.2008

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D. Lloyd, E. Rossi (eds.), *Ultradian Rhythms from Molecules to Mind*,

changes, the machinery for protein synthesis and degradation and for mitochondrial ribosome assembly are also all locked on the ultradian clock cycle phase. This indicates the pervasive and all-embracing nature of temporal co-ordination of cellular growth and division. The major players in this yeast model system are identified as some of the most highly conserved components common to all eukaryotes. Thus a primeval, fundamental and insistent drum-beat reverberates in all eukaryotic systems, in microbes, animals and plants. The ultradian clock that co-evolved with increasing atmospheric O_2 predates and underpins the circadian clock at the central core of endogenous intracellular timekeeping. It represents the prototype of all biological rhythmicity: circadian clocks evolved much more recently, almost as the icing on the cake.

Keywords Yeast, self-synchrony, respiration, redox state, metabolic switch, transcriptional regulation, cell division cycle ultradian clock

1.1 Introduction: Continuous Culture of Yeast – An Ideal System for Study

Detailed understanding of the time structure of the intricately ordered processes of energy generation, metabolic transformations, synthesis and assembly of membranes and organelles, as well as the organization of chromosomal dynamics and the cell division cycle, requires studies either in a single cell or in synchronous populations of cells or organisms. Whilst the former is even now restricted (despite enormous strides in optical microscopy, image processing, microfluidics and atomic force microscopy), and the latter is usually problematic because of the difficulties associated with the preparation of material without perturbatively disturbed "normality". However, in a seminal contribution published in 1992, the group of Kuriyama discovered a method which gave spontaneous self-synchrony of dense (~5×10^8 organisms/ml) cultures of *Saccharomyces cerevisiae* (Satroutdinov et al., 1992). The strain employed (IFO-0233, IFO, Institute of Fermentation, Osaka, Japan), is a distiller's yeast. During batch-growth on glucose-containing complex growth medium at pH 3.4 with a constant supply of air (calculated for each reactor according to its specific oxygen transfer coefficient), glucose is consumed to produce biomass, CO_2, H_2O and ethanol as well as many other fermentation products. Following ethanol utilization, depletion of trehalose and glycogen in the second stage of this diauxic growth results in the initiation of oscillatory respiration, as indicated by 40 min cycles of dissolved O_2 and CO_2 Commencement of continuous culture (Fig. 1.1) at this stage, gives a continuous supply of material for long-term monitoring of the organisms through many thousands of generations over a period of many months. The rapidly-responding probes immersed in the culture give highly sampled intervals, e.g. for NAD(P)H fluorescence 50 k samples/s, for dis-

Fig. 1.1 (**a**) Dr. Hiroshi Kuriyama working with a continuous culture of *Saccharomyces cerevisiae*: (**b**) The continuous culture system. The impellors were driven by a 24 V motor (K; green line) and controlled by a power supply(**A**). Temperature was measured using a Pt 100 probe (yellow line) and controlled (**B**) to a user set-point by a 333 W heater (pink line). A water jacket (at 10°C) consisting of rubber tubing wrapped around the glass vessel (**G**) provides cooling (not shown). pH was measured using a steam sterilizable gel electrode connected (cyan line) to a

(continued)

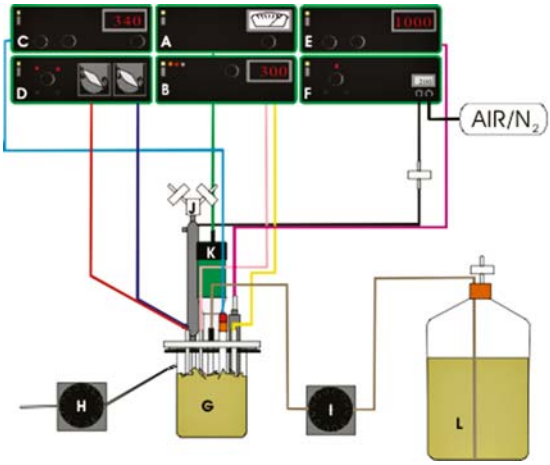

Fig. 1.1 (continued) pH meter (**C**). pH was automatically controlled (**D**) by the addition of 2.5 M NaOH (dark blue line) or 2 N phosphoric acid (red line). An oxygen meter (**E**) was connected to a polarographic oxygen electrode (magenta line). Sterile dry air was fed (black line) to the fermentor via a sparge arm, its flow rate regulated by an Aalborg mas flow controller (**F**). Waste air was passed through a condenser (10°C) and exited via two sterile filters (**J**).Sterile medium was stored in a resevoir (10 l; **L**) and pumped into the fermentor by low flow peristaltic pump (I; Microperpex, LKB) To avoid back contamination the medium was pumped through an anti-growback device prior to entry to the vessel(**G**). Waste was collected from the fermentor by a glass arm weir. A peristaltic pump (**H**; 101U Watson- Marlow) then pumped the waste into a collection vessel (white line). In addition there was a double-valved sample assembly and three auxillary ports with rubber septa that were used for extra apparatus or pulse addition of purturbating agents. The data was acquired by an in-house software that controlled the data acquistion board (DAS 16, Computer Boards) and was programmed by the user to collect and store data (up to 1 s intervals). In some experiments (not shown), a membrane-inlet mass spectrometric probe was used (for measuring gases and low molecular mass volatiles directly), either by immersion in the culture, or exposed to the headspace gas, and a rotating disc dual-channel fluorimeter (for measuring redox levels of NAD(P)H and/or flavins; Johnson Foundation, University of Pennsylvania) was linked to the vessel using light guides Standard conditions employed were: working vol. 800 ml; dilution rate, 0.085 h^{-1}; stirrer rate, 800 rpm; airflow rate, 180 ml min^{-1}; temperature 30°C (±0.2) and pH controlled at 3.4. Pulse injections were carried out through a sterile filter (0.2 µm porosity)

solved O_2 10 samples/s (Murray et al., 2007). The most convenient readout, dissolved O_2, can also be monitored by membrane inlet mass spectrometry, as can CO_2, H_2S and ethanol. Near-infra-red spectroscopy can be used on-line for ethanol, glucose, NH_3, glutamine and biomass (unpublished data). The oscillatory state of this autonomously self-sustained system is largely independent of glycolysis, and gates the cell cycle (Klevecz et al., 2004); its approximately 40 min period is temperature compensated (Fig. 1.2; Murray et al., 2001). Thus its timekeeping characteristics place it in the ultradian time domain (as defined by its characteristic of cycling many times during a day). That similar clock control can be demonstrated when ethanol is used as the dominant carbon source in the growth medium shows that these oscillations are not the well-known glycolytic or cell-cycle associated oscillations (Keulers et al., 1996).

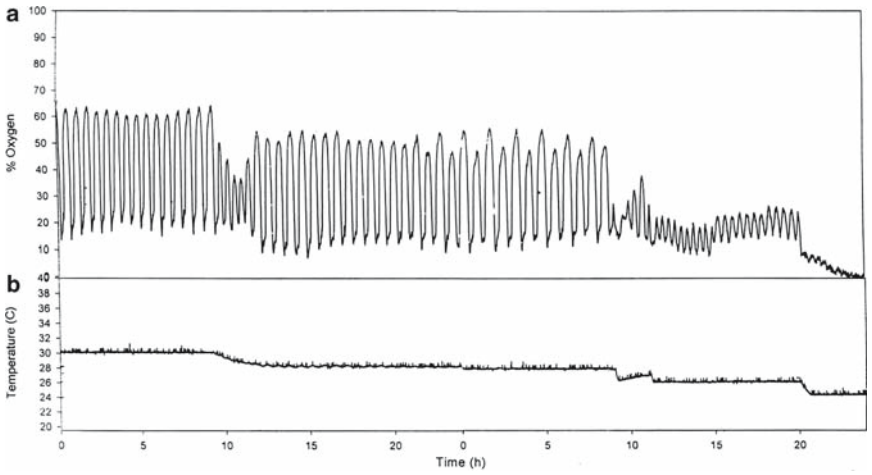

Fig. 1.2 Effects of step temperature changes on the respiratory oscillations in a continuous culture of *S. cerevisiae* grown in ethanol-containing medium. (a) Dissolved O_2, and (b) temperature were measured continuously on-line (Murray et al., 2001)

1.2 Discovery of the Ultradian Clock

It is their sustained nature that confers upon the 40 min periodicity the status of a rhythm, and temperature independence further elevates it to that of a biological clock. It thus shares important properties with the more intensively-studied Circadian clock as well as physical clocks (Lloyd et al., 2002a): The latter was first recognized as an endogenous timekeeper in 1729 (de Mairan). The Ultradian Clock, initially called The "Epigenetic Clock" was finally characterized in 1982 as a temperature-compensated ultradian timekeeper in the soil amoeba, *Acanthamoeba castellanii* (Edwards and Lloyd, 1978, 1980; Lloyd et al., 1982). Respiratory oscillations during the cell division cycle of synchronous cultures of this organism that had been produced by a minimally-perturbing size selection procedure were shown to be phase-coupled to high amplitude changes in adenine nucleotide pool sizes (ATP, ADP and AMP) and to cycles of accumulation of total cellular RNA and protein. The Q_{10} values for the periods of the O_2 consumption and protein rhythms were respectively 1.03 and 1.05 over the temperature range from 20°C to 30°C whereas the Q_{10} for the cell division cycle (16 and 7.8 h respectively) was just more than 2 over the same temperature range (Fig. 1.3a; Marques et al., 1987). These observations clearly established a timekeeping function for this Ultradian Clock, so that a quantal relationship between this clock and the cell cycle time, as earlier proposed for mammalian cells (Klevecz, 1976) and subsequently shown in lower eukaryotes (Chisholm and Costello, 1980; Lloyd et al., 1982; Homma and Hastings, 1988) is evident. Similar results previously obtained for synchronous cultures of other lower eukaryotes (the yeasts, *Candida utilis* (Kader and Lloyd,

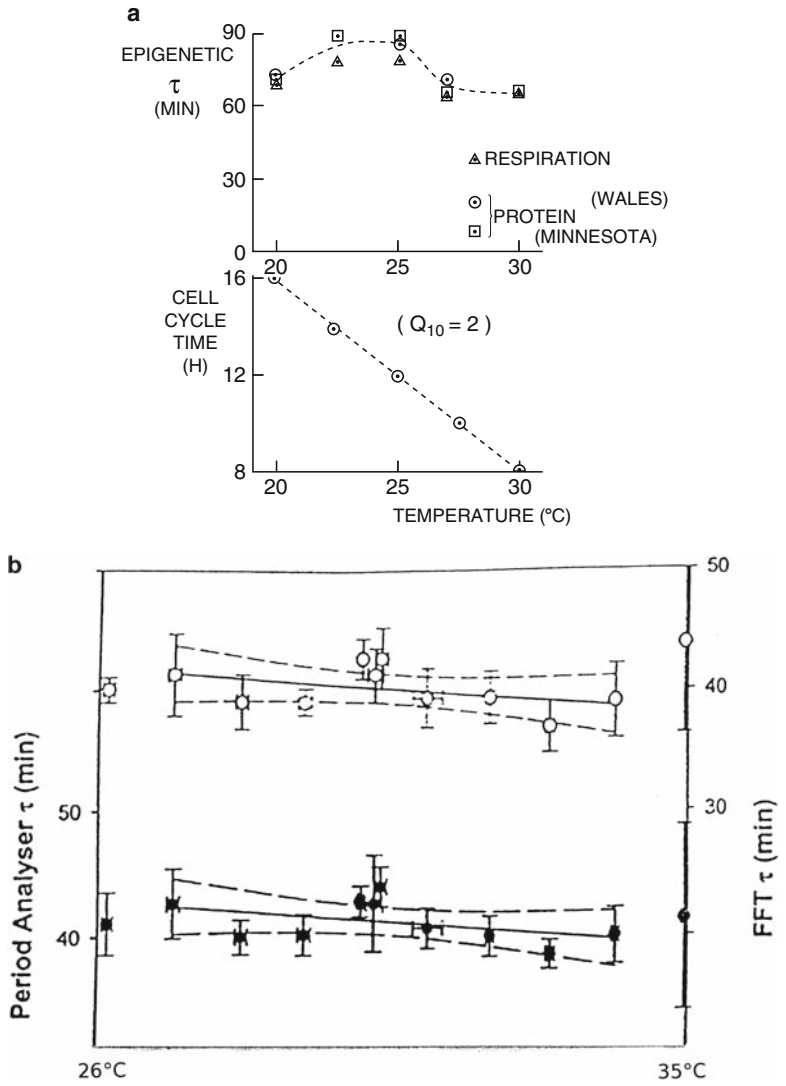

Fig. 1.3 Temperature dependence of the respiratory oscillation in (**a**) Periods of the ultradian clock-driven outputs in *Acanthamoeba castellanii*, contrasted with cell cycle times (Marques et al., 1987); analysis of protein data calculated by independent methods in Wales (**a**) and Minnesota (**b**). Periods of the respiratory oscillation in yeast calculated by Period Analyser and fast Fourier transform analysis (FFT) (Murray et al., 2001)

1979) and *Schizosaccharomyces pombe* (Poole et al., 1973), for a flagellate protist (*Crithidia fasciculata*, Edwards et al., 1975) and a ciliate (*Tetrahymena pyriformis*, Lloyd et al., 1978) indicated that the Ultradian Clock is a timer in many systems. Analogies with the 90-min BRAC (basic rest-activity cycle) observed during sleep

studies on human subjects in the Chicago laboratory of Nathanial Kleitman (Aserinsky and Kleitman, 1953) are clear, and suggest that the Ultradian Clock is a universal timekeeper from amoeba to humans. The period of the Ultradian Clock is different in each of the organisms studied, presumably representing a self-optimized value for the intracellular network that is distinctive for each species.

Of all the species studied, it seemed that the asymmetrical cellular proliferation of baker's or brewer's yeasts by budding would make studies of ultradian rhythms more complicated. However, the serendipitous observations of Satroutdinov et al. (1992) altered this view, and it soon became clear that the spontaneously self-organizing continuous culture system offered huge possibilities for continuous monitoring of the Ultradian Clock over extended time spans and by non-invasive methods. The temperature compensated nature of the yeast 40 min oscillator was intensively investigated (Figs. 1.2, 1.3b; Murray et al., 2001). Thus glucose-fed cultures showed a Q_{10} of 1.06 between 27°C and 34°C whereas ethanol-grown cultures gave a value of 0.85 (i.e. were somewhat overcompensated) Homeodynamic control as a function of dilution rate of the continuous cultures was also noted.

1.3 Metabolic Studies of the Yeast Ultradian Clock

1.3.1 Carbon Metabolism

The aerobic continuous culture of yeast can be supplied with glucose (Satroutdinov et al., 1992; Keulers et al., 1996), ethanol (Keulers et al., 1996), or acetaldehyde (Keulers and Kuriyama, 1998) as major carbon sources (in addition to those present in the yeast extract common to all the growth media employed. Whereas for growth on glucose or ethanol the period of the "metabolic" oscillation was about 40 min, with 290 mM acetaldehyde as primary carbon source it was approximately 80 min. Acetaldehyde, acetate, ethanol, dissolved O_2 and CO_2 production were all oscillatory variables (Fig. 1.4), with the acetaldehyde and acetate approximately in phase with O_2 uptake rate (i.e. these products were at a maximum during low dissolved oxygen concentration). This enhanced respiration indicated that acetaldehyde and acetate never exceeded their potentially inhibitory levels, and that this was the same irrespective of which of the three principle carbon sources were employed. Flux balance calculations indicated that ethanol production from acetaldehyde occurred periodically during the oscillation, and was accompanied by decreased acetaldehyde conversion to acetate and acetyl-CoA. It was suggested that the diminished rates of acetaldehyde flux to the TCA cycle were related to the decreased respiratory activity due to some inhibitory effect on the mitochondrial respiratory chain. The importance of acetaldehyde (but not O_2 or ethanol) as a specific synchronizing agent had been previously suspected (Keulers et al., 1996), when it was observed that high aeration rates leading to loss of CO_2 and the volatile aldehyde led to loss of the oscillatory state. The confirmation that the synchronizer

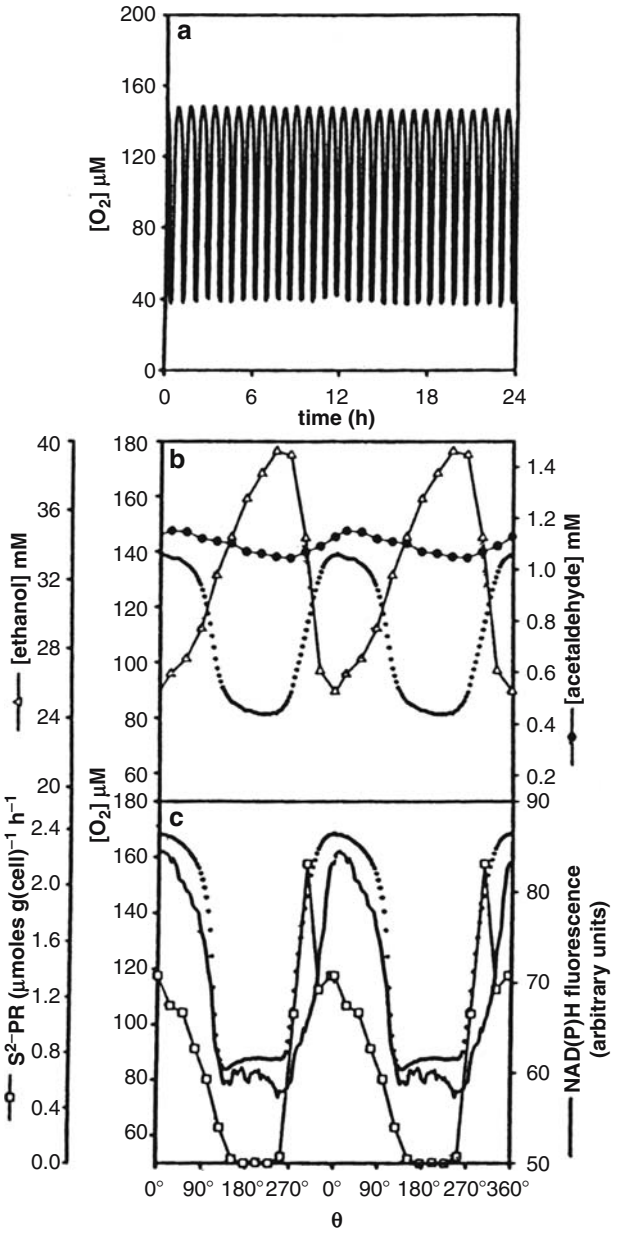

Fig. 1.4 Continuously monitored oscillatory variables (dissolved O_2 and NAD(P)H and metabolites sampled from the aerobic continuous cultures of yeast. (**a**) The respiratory oscillation. (**b**, **c**) Phase angle plots with respect to dissolved O_2 (⋯), of acetaldehyde (▲); (**b**), ethanol (●; **b**) S^{2-}PR (sulphide production rate, ■; **c**), and NAD(P)H fluorescence (-; **c**). The data are double-plotted (side-by-side) in order to clarify phase-related events (Murray et al., 2003)

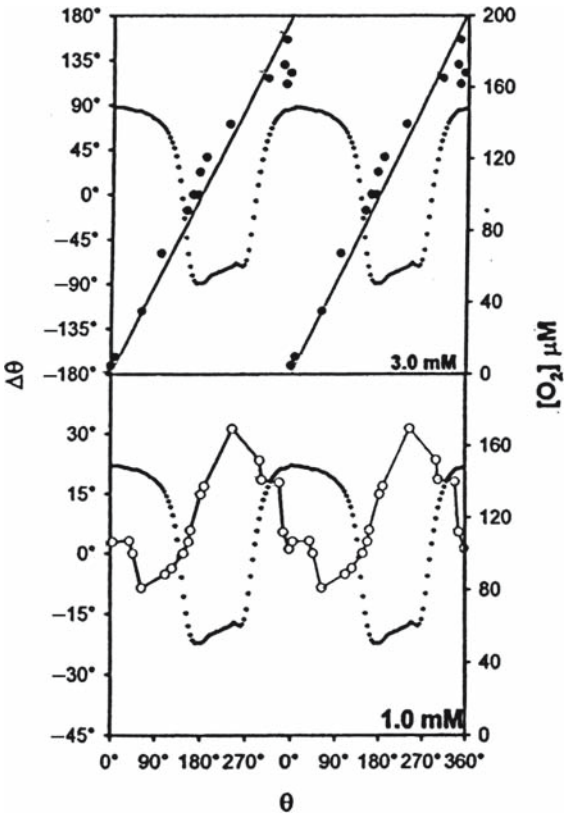

Fig. 1.5 Phase response curves for acetaldehyde phase-shifting of the respiratory oscillation in yeast. The dissolved O_2 (···) plot provides a guide to the phase of the injection. The straight line on the upper panel (3 mM) is the linear regression of the points obtained after and before the "break-point" (0°). The data are double-plotted (side by side) in order to clarify phase-related events (Murray et al., 2003)

was acetaldehyde rather than CO_2 awaited the definitive experiments of Murray et al. (2003) who demonstrated the phase-shifting capacity of acetaldehyde (Fig. 1.5, see also Chapter 3).

1.3.2 Sulphur Metabolism

A breakthrough in understanding of the mechanism of the oscillation came with the discovery that H_2S is produced at the onset of lowered O_2 uptake rates. This gas, a potent respiratory inhibitor, reached a maximum (1.5 μM) just prior to minimum respiration rates (Fig. 1.4; Sohn et al., 2000). After this, the concentration of H_2S decreased to 0.2 μM before the restoration of high respiration again. Perturbants

such as 50 μM glutathione, 50 μM $NaNO_2$ or 4.5 mM acetaldehyde transiently increased H_2S levels to more than 6 μM. Phase-shifting of the oscillation by additions of 0.77 μM $(NH_4)_2S$ enabled a phase response curve to be obtained (Murray et al., 2003) and the important conclusion that the easily oxidized, rapidly diffusing gas H_2S acts as an intercellular messenger that amplifies the respiratory oscillation. It accomplishes this by binding to the binuclear copper-haem reaction centre of cytochrome *c* oxidase, the terminal electron transport component of the mitochondrial respiratory chain. Phase-response curves indicate that it does not act by itself as a synchronizing agent rather it is implicated in amplitude modulation of the cycles.

The source of the H_2S was shown to be part of the sulphur-containing amino acid synthesis network where sulphite reductase acts on sulphite (formed by reduction of sulphate present at high concentration in the growth medium (Sohn and Kuriyama, 2001a). Pulse injection of 100 μM cysteine or methionine perturbed the respiratory oscillation and altered the timing of H_2S production (Sohn and Kuriyama, 2001b): it is considered that the feedback inhibition of sulphate uptake into the yeast by these amino acids may have a major contribution to the respiratory oscillations as suggested by mathematical modelling (Wolf et al., 2001; Henson, 2004). The high-affinity sulphate permease mRNA (both *SUL1* and *SUL2*) shows cyclic expression during the respiratory oscillation, and the mRNA expression patterns of all the enzymes involved in the sulphate assimilation pathway precede endogenous H_2S generation (Klevecz et al., 2004; Murray et al., 2007).

Glutathione addition gives a phase-related perturbation of the respiratory oscillation (Murray et al., 1999; Sohn et al., 2000) and this affects the essential role of glutathione reductase that catalyses the NADPH-dependent cycling of the GSH-GSSG system in the maintenance of the redox state of the organism (Sohn et al., 2005 a,b) Growth of a glutathione oxidoreductase disruptant confirmed this. Expression levels of GSH1 mRNA and GLR1 mRNAs (encoding γ-glutamylcysteine synthetase and glutathione oxidoreductase), as well as the oscillating activities of glutathione oxidoreductase, cysteine and glutathione were also in agreement with this proposal. Confirmation of this important conclusion came from extensive studies on the expression levels of *GSH1* and *GLR1* mRNA, as well as the activities of glutathione reductase and oscillations in cysteine and glutathione. The effects of pulse injection of thiol redox modifying agents (diethylmaleate, *N*-ethylmaleimide), of inhibitors of glutathione reductase (DL-butathionine [*S, R*]-sulphoxamine) or of glutathione synthesis (5-nitro-2-furaldehyde) further strengthened these data. The network of sulphate uptake and sulphur amino acid interactions is shown (Fig. 1.6). That this network is intricately interwoven with the regulation of redox state was further demonstrated (Kwak et al., 2003). Cellular per-oxidative adducts, as measured by the levels of lipid peroxidation products, oscillates out-of-phase with levels of dissolved O_2. Pulse addition at minima of dissolved O_2 of 100 μM *N*-acetylcysteine (which scavenges H_2O_2 and hydroxyl radicals) perturbed the respiratory oscillation and attenuated H_2S production to 63% of its normal amplitude in the next 40 min cycle. Then the respiratory oscillation damped out, only to be regained 20 h later. The non-toxic free radical scavenger, ascorbic acid as well as the inhibitor of catalase (3-aminotriazole) or superoxide dismutase (*N*-*N*[1] diethyldithio carbamate) gave further evidence for this. H_2O_2 (0.5 mM) added at a minimum of dissolved O_2

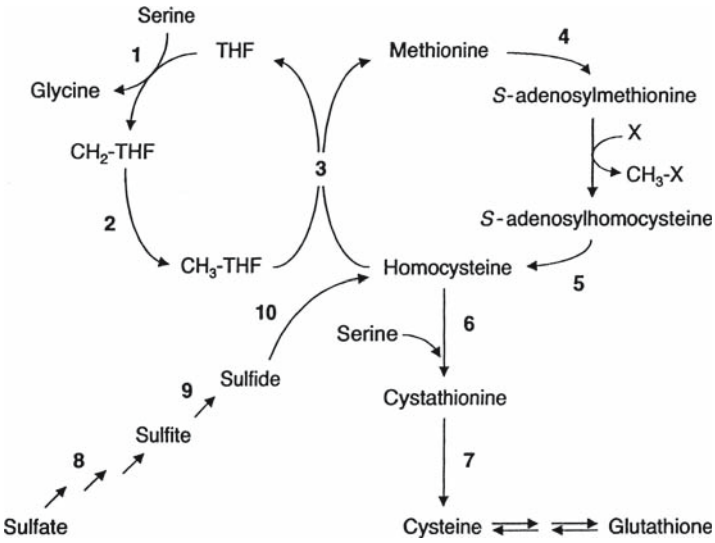

Fig. 1.6 Sulphate uptake and metabolism in yeast genes and enzymes catalyzing individual reactions are: 1. SHM1 and SHM2: serine hydroxymethyltransferase (SHMT; EC 2.1.2.1); 2. MET12 and MET13: methylenetetrahydrofolate reductase (MTHFR; EC 1.5.1.20); 3. MET6: methionine synthase (EC 2.1.1.14); 4. SAM1 and SAM2: S-adenosylmethionine synthetase (EC 2.5.1.6); 5. SAH1: S-adenosylhomocysteine hydrolase (EC 3.3.1.1); 6. CYS4: cystathionine β-synthase (CBS; EC 4.2.1.22); 7. CYS3: cystathionine γ-lyase (EC 4.4.1.1); 8. MET14: adenylsulfate kinase (EC 2.7.1.25); 9. MET10: sulfite reductase (EC 1.8.1.2); and 10. MET17: O-acetylhomoserine (thiol)-lyase (EC 2.5.1.49). "X" represents any methyl group acceptor (Chan and Appling 2003)

both perturbed the respiratory oscillation and elevated H_2S production in the subsequent cycle; 50 μM menadione, a superoxide generating agent, mimicked these effects but at two orders of magnitude less concentration, indicating that cellular reactive oxygen species defences were active throughout the cycle. However an increase ROS localised in the mitochondria caused by menadione could perturb the system at low concentrations.

Further work on the effects of glutathione perturbation on branched-chain and sulphur-containing amino acids tends to suggest that the observed effects are more closely related to amino acid metabolism and H_2S generation than with cellular redox state *per se* (Sohn et al., 2005 a,b). Confirmation of this suggestion awaited more in-depth analyses of the transcriptome and metabolome (Murray et al., 2007).

1.4 A Precise Transcriptional Oscillator Defines the Dynamic Architecture of Phenotype

It is becoming clear that regulation of levels of individual genes (and ultimately their proteins) is primarily a transcriptional process. With increasing genomic complexity of metazoans there is a dramatic increase of the fraction of the genome that

is transcribed but not translated (Taft et al., 2007). In *S. cerevisiae* the role of non-coding RNAs appears to be less dominant than in some other model eukaryotes studied, and this allows us to uncover the architecture and dynamics of phenotype unencumbered by what is estimated, in humans, to be more than a million small regulatory RNAs. Analysis of genome-wide oscillations in transcription (Fig. 1.7) reveals that the entire unicellular organism or animal cell is an oscillator, and because its trajectory can progress as series of period doublings during the course of its changing pattern of expression, it is most parsimoniously seen as an attractor (Klevecz and Li, 2007). The simulations of the dynamics that would permit period doublings yet still allow both a stable phenotype and relatively precise timekeeping have been published (Bolen et al., 1993). A chaotic attractor from which much of the unpredictability could be tuned involves appropriate arrangements of phase among the thousands of its transcriptional oscillators. In essence the maintenance of a stable phenotype requires that expression maxima in expression in clusters of transcripts must be poised at antipodal phases around the steady-state – this is the dynamic architecture of phenotype.

Recently, Li and Klevecz (2006) further characterized the first example of period doubling behaviour in the genome-wide transcriptional oscillation (Salgado et al., 2002) seen in yeast cultures growing at high cell densities and exhibiting continuous gated synchrony, and suggested that this behaviour was closely modelled by a simple modification of the Rossler attractor (Rossler, 1976). From the dynamic systems perspective we can take this rigorous model for describing the global behaviour of the timekeeping oscillator and begin to build a gene-by-gene or regulon-by-regulon dynamic systems network. The model also immediately offers an accessible graphical representation of the genome scale changes that can lead from a high frequency timekeeper to the cell-cycle and circadian rhythms. Cell-to-cell signalling in continuous cultures of *S. cerevisiae* leads to mutual entrainment or synchronization that is manifested as an oscillation in redox state (Lloyd et al., 2003) and a genome-wide oscillation in transcription and metabolism (Klevecz et al., 2004; Murray et al., 2007). In turn, this transcriptional redox attractor cycle times or gates, DNA replication and other cell cycle events. In this regard the cell cycle is a developmental process timed by the attractor. DNA replication is restricted to the reductive phase of the cycle and is initiated as levels of hydrogen sulphide rise. This is seen as an evolutionarily important mechanism for preventing oxidative damage to DNA during replication (Klevecz et al., 2004). Both exposure to drugs (e.g. Li^+ or Type A monoamine oxidase inhibitors) known to alter circadian rhythms (Fig. 1.8; Salgado et al., 2002) and deletions of known clock genes in *S. cerevisiae* yield an increase in the period of the attractor that follows a bifurcation path from 40 min to 3–4 h. This finding resonates with an earlier observation of a 3–4 h oscillation that gated cell cycle events in higher organisms (Klevecz, 1976). We suggest that the genome-wide oscillation discovered in yeast is a primordial oscillator, and that the differentiation pathways to multiple phenotypes (e.g. formation of spores or filaments) maybe through processes characterized by period doubling and period three bifurcations.

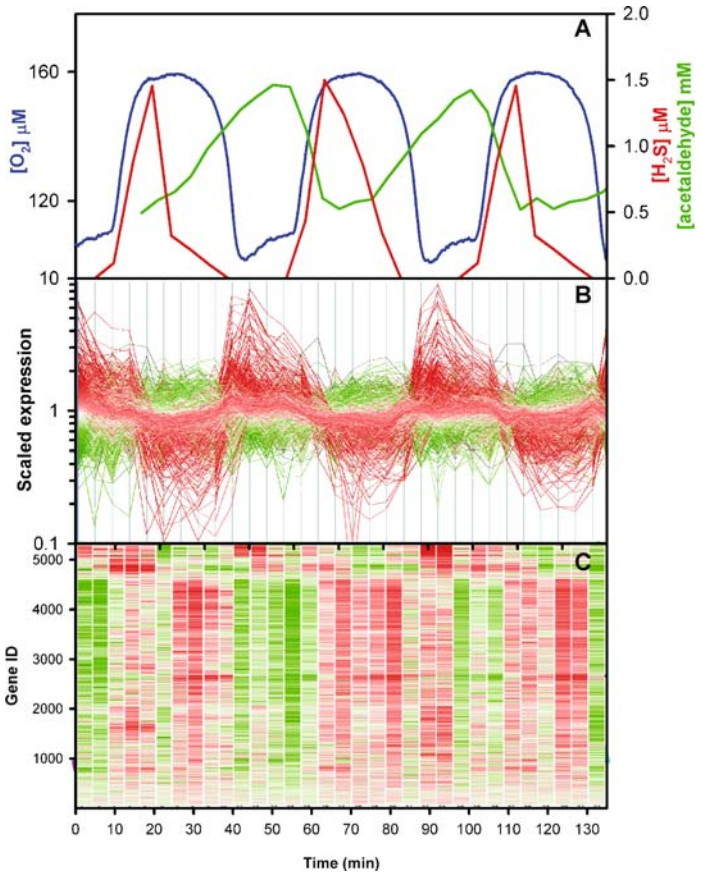

Fig. 1.7 The transcriptome of *Saccharomyces cerevisiae* in continuous culture: activity with respect to three cycles of the respiratory oscillations. (**A**) Dissolved O_2 in the culture, acetaldehyde and H_2S. (**B**) Transcripts in phase with the oxidative phase of the culture (red), and those in phase with the reductive phase (green). (**C**) The scale for the transcripts was obtained by dividing the intensity of expression of that gene at that point by the median intensity for each experiment (Data derived from Klevecz et al., 2004)

Prior to the development of methods for examining genome-wide expression, clues to the dynamic structure of the cell could only be assessed by measurement of small subsets of cellular constituents from synchronous cultures (Lloyd et al., 1982; Klevecz and Ruddle, 1968) or by intentional perturbation and measurement of the phase response curves of events such as DNA replication and cell division to the perturbation (Mitchison, 1971; Klevecz et al., 1984). These early studies provided a sketch of the dynamic architecture of phenotype and offered a rigorous alternative to simple branched sequential models based on mutational analysis of the cell cycle (Hartwell et al., 1974). Quantized generation times, together with perturbation analyses, formed the experimental foundation of efforts to synthesize a model of the cell cycle in which such disparate concepts as check points, and limit