Functional Ecology 2008, **22**, 19–29 doi: 10.1111/j.1365-2435.2007.01343.x

REVIEW

Genomic approaches for studying biological clocks

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Summary

1. Time is an important dimension for any ecological niche.
2. Most higher organisms show adaptations that are related to daily or seasonal timing, and these adaptations are regulated by endogenous clocks. At the molecular level, these clocks are encoded by a network of proteins interacting with each other and with their own transcripts.
3. Recent expression studies suggested that a large fraction of the transcriptome and the proteome both in mammals and insects may show significant daily oscillations. Here, we review some of the recent genomic approaches to studying circadian clocks, including transcriptomic, proteomic and quantitative trait loci (QTL) analyses, in a wide variety of organisms, from plants to mammals.
4. We also discuss some of the methodological problems that are inherent in these types of studies. Understanding how the circadian system interacts with the environment at the molecular level is perhaps the most important challenge of chronobiology and we anticipate future developments with these methods using experimental paradigms that are more environmentally and ecologically focused.
5. The identification of novel clock genes using more ecologically relevant experimental designs will provide a reservoir of genetic variation whose function can be studied in natural populations.

Key-words: circadian, genes, seasonal, quantitative trait loci, transcriptome

Introduction

Time is an important domain for defining the ecological niche of a species. It is not difficult to see how an organism can adapt to its environment merely through shifting the time at which an activity occurs, rather than changing its habitat, or evolving its behaviour *per se* or its morphology. For example, organisms can adapt to a hot environment by becoming nocturnal and thereby escaping the desiccation that is imposed by the sun during the day. In contrast, they can escape harsh winters with a physiologically protective seasonal diapause (Tauber, Tauber & Masaki 1986). Natural selection might also act to change the temporal phasing of behaviour during the day, so for example, the timing of courtship for sympatric sibling species may be several hours out of synchrony, thereby contributing to maintaining the species barrier (Sakai & Ishida 2001; Miyatake *et al.* 2002; Tauber *et al.* 2003).

The timing of many daily processes such as foraging, feeding or rest can be determined or modulated by the endogenous circadian clock, which is ubiquitous in higher organisms and is also present is some prokaryotes. Equipped with internal time mechanisms, organisms can anticipate the daily changes in their habitat (temperature, light, predation risk) and modify their behaviour/physiology accordingly, rather than merely responding to external stimuli. This becomes particularly important in temperate zones where the daylength changes significantly during the year (Winfree 1987). Clearly, natural selection has favoured the evolution of such a clock mechanism, although experiments designed to test this seemingly obvious idea, are somewhat thin on the ground. Perhaps the best example is in Cyanobacteria, the photosynthetic prokaryote, in which mutations exist which lengthen or shorten the endogenous (or 'free-running') circadian period (Mori & Johnson 2001). If a short period mutant is placed in a corresponding short period world, it will out-compete (in a Darwinian sense) the wild-type (which has a normal 24-h rhythm). Similarly, a long period mutant placed in a long period environment, will also out-compete the wild-type. When these variants are competed in a normal 24 h world against the wild-type, the latter reigns supreme (Ouyang *et al.* 1998), revealing a selective advantage for an organism to resonate with the solar cycle. Such quasi-ecological experiments on the relationship between endogenous periods and the environment are rare, but dramatically informative (see also Sawyer *et al.* 1997).

The identification of the first circadian-clock mutants in *Drosophila* finally nailed to rest the idea that circadian rhythms were imposed by some subtle, external, cycling geophysical variable (Konopka & Benzer 1971). These *period* mutants changed the endogenous free-running circadian

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period of the flies, one variant to 19 h, another to 29 h, while the third allele was arrhythmic. While one might reasonably argue that an arrhythmic mutant was simply not responding to an external environmental factor, it was difficult to see how changes in the circadian period could be based on misreading geophysical information. The molecular basis of the clock has subsequently been extensively studied in flies, *Neurospora* (Dunlap & Loros 2006), *Arabidopsis* (McClung 2006), Cyanobacteria (Williams 2007) and model vertebrates (Cahill 2002; Reppert & Weaver 2002), with some progress made on some other non-model organisms (Chang *et al.* 2003; Meireles-Filho *et al.* 2006; Reppert 2006; Rubin *et al.* 2006). At least in higher animals, the take home message has been that the clock molecules themselves have been highly conserved through the latter stages of evolution, that is, probably since the Cambrian (Tauber *et al.* 2004). At the level of gene regulation, the discovery of positive and negative circadian autoregulatory loops appears to be a general phenomenon common to all circadian systems.

How the clock works: from flies to mice

The *period* (*per*) gene in the fly was identified at the molecular level more than 20 years ago. Since then, a number of other components of the clock have been identified, mostly through forward genetic screens. Briefly, the *per* and *timeless* (*tim*) genes act as negative autoregulators, and their expression, at both transcript and protein levels in their brain and other tissues, waxes and wanes with a 24-h rhythm, even under constant conditions (reviewed in Hall 2003). These two clock proteins negatively regulate their own genes by interacting with two positive bHLH PAS transcription factors, CLOCK (dCLK) and Cycle (CYC or dBMAL1), which bind to the *per* and *tim* promoters at hexameric E-boxes (*CACGTG*). The PAS dimerization motif is also found in PER itself, which is one of the founder members of the PAS family of proteins. The expression of CLK has also been reported to cycle (Bae *et al.* 1998; Lee, Bae & Edery 1998) via positive and negative regulation by two further transcription factors, PDP1ε and VRILLE (Cyran *et al.* 2003; Glossop *et al.* 2003). However, more recent work suggests that the relevant cycling is in the phosphorylated isoforms of CLK (Houl *et al.* 2006; Yu *et al.* 2006). CLK may provide the intersection point of two molecular loops, providing additional cohesion to the system. The stability of the PER and TIM proteins is modulated by various kinases, including DOUBLETIME (DBT or Casein kinase 1ε), CASEIN KINASE 2 and SHAGGY (Glycogen synthase kinase 3) (Hall 2003) (Fig. 1).

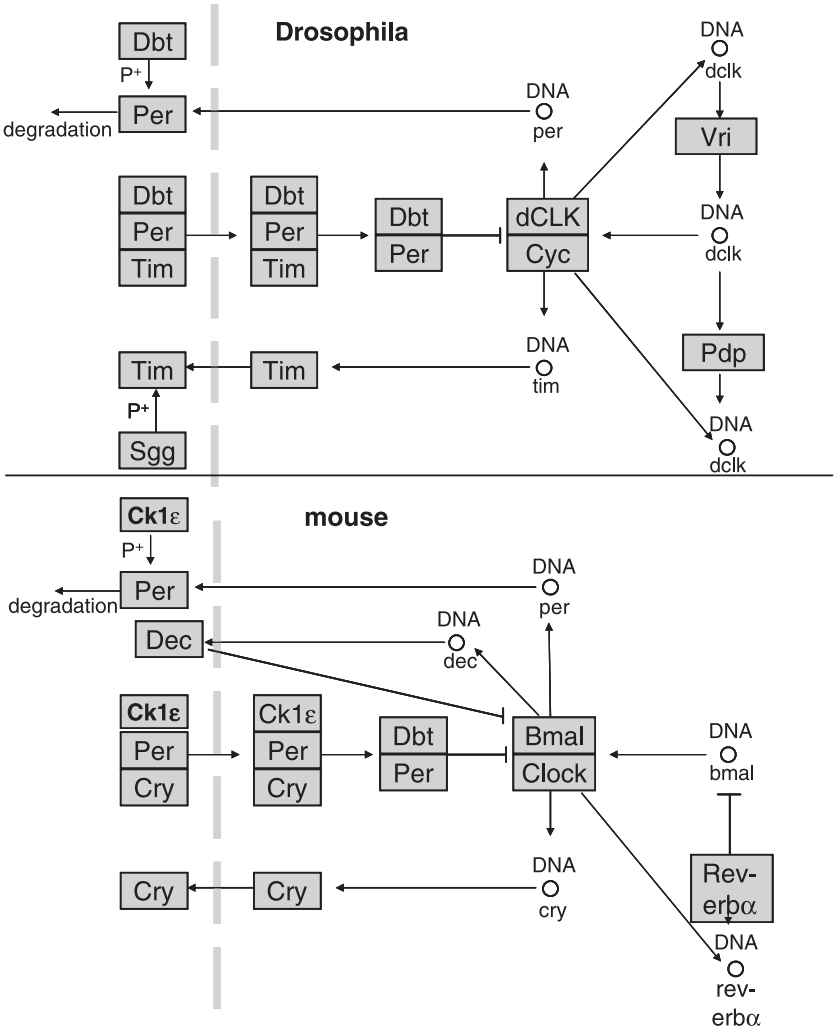

Fig. 1. The circadian molecular clock. Diagrams are taken from the KEGG PATHWAY data base (http://www.genome.jp/kegg/pathway.html), representing molecular interaction networks in the circadian clock of *Drosophila* (path: dme04710) and the mouse (path: mmu04710). Symbol key: →, activation; —|, inhibition; +p, phosphorylation. The grey dashed line represents the barrier between the cytoplasm (left) and the nucleus (right).

In addition, a blue-light photoreceptor, CRYPTOCHROME, mediates the circadian clock's light response, and when activated by light, attaches itself to TIM, and degrades it rapidly. In the absence of CRY in the fly, the circadian clock has problems resetting itself to light stimuli, even though the canonical visual input pathway mediated by the rhodopsins, can partially compensate. Needless to say, when both CRY and the opsins are removed with the use of mutations, the fly clock can barely entrain itself to light (Hall 2003).

This brief review of the circadian clock mechanism in the fly ignores the evolutionary tinkering that has apparently been at work with these components to generate the vertebrate system. The similarities and differences between the insect and vertebrate clocks, and indeed, among insects and vertebrates, has been the focus of several recent studies, so we will not labour them here (Zhu, Conte & Green 2003; Rubin *et al.* 2006). However, the main differences concern the role of CRY, which in the mouse, serves as the major negative regulator of PER and itself (Clayton, Kyriacou & Reppert 2001). Indeed, there are two types of CRY's, one serving the negative autoregulator function in the clock, and the other, functioning as the dedicated circadian blue-light photoreceptor. In mice the two paralogous *Cry* genes, both serve as negative regulators (Shearman *et al.* 2000). This is in contrast to Lepidoptera, for example, where the two *Cry*s mediate both regulator and photoreceptor functions (Zhu *et al.* 2005; Yuan *et al.* 2007). In fact it gets even more complicated, because in *Drosophila*, in peripheral organs, CRY may act within the oscillator mechanism itself, perhaps as a negative regulator (Stanewsky *et al.* 1998; Krishnan *et al.* 2001). In addition, in certain regions of the fly brain CRY may act as a repressor of CLK (Collins *et al.* 2006). These studies reveal that tissue-specific, *trans*-acting factors may determine exactly how CRY works in the fly.

Other differences between fly and mouse clocks include the numbers of paralogous clock genes, for example, mouse has four *Per* genes, two *Cry*'s, and a number of paralogues of *Clock* and *Bmal1* (Tauber *et al.* 2004). The fly, however, has two *tim* genes (Benna *et al.* 2000; Gotter *et al.* 2000), whereas mouse has only one, the ancestral *tim2* (or *timeout)* gene, from which *tim* probably duplicated, and which serves vital developmental as well as clock related functions (Benna *et al.* 2000; Gotter *et al.* 2000; Barnes *et al.* 2003; Unsal-Kacmaz *et al.* 2005). In contrast, the fly *tim* gene can be mutated without any serious adverse effects on viability; the clock simply stops working (Sehgal *et al.* 1994). The fly *tim2* gene has yet to be studied comprehensively, but like its mouse equivalent, knockdown is lethal (F. Sandrelli and R. Costa, pers comm.). Other differences worth noting include fly *Clk* mRNA cycling but not *cyc* (*Bmal1*) (Lee *et al.* 1998), whereas in the mouse it is *Bmal1* that cycles, and *Clk* does not (Honma *et al.* 1998; Shearman *et al.* 1999). Cycling of *Bmal1* in the mouse is maintained by the balance between Reverbα and Rorα transcription factors (Preitner *et al.* 2002; Sato *et al.* 2004), which contrasts with PDP1ε and VRI in the fly. Interestingly, in other insects such as honeybees and sandflies, like mice, it is *cyc* that cycles rather than *Clk* (Meireles-Filho *et al.* 2006; Rubin *et al.* 2006).

These species differences in clock gene regulation have evolved over hundreds of millions of years, and it is very difficult to say with any certainty whether any of these changes reflect differential ecological adaptations. Rather they may be the endpoints of historical changes, for example, duplications of entire sub-genomic regions in vertebrate lineages (Ohno 1970), that carried along clock genes with them, and then allowed the clock paralogues to diverge and take on slightly different functions (Tauber *et al.* 2004). Among more closely related species, for example insects, it may be that changes in the usage of CRY's can be understood in terms of current ecological requirements although exactly how and why, remains to be determined. Why, for example, did the mosquito *Anopheles gambiae* maintain two *Cry* genes that encode both types of roles, negative regulator and photoreceptor (Zhu *et al.* 2005), whereas *Drosophila* apparently only maintained the blue-light receptor function?

ECOLOGICAL ASPECTS OF CHRONOBIOLOGY

One of the most important characteristic of the circadian clock is temperature compensation, which refers to the ability of the pacemaker to maintain a constant circadian period over a broad range of temperatures, that is, to function as a clock rather than a thermometer. Although the molecular mechanism of temperature compensation is still not clear, it is however evident that natural populations bear molecular variation (polymorphism) in circadian-clock genes allowing adaptation to local microclimates. Within the *Drosophila melanogaster per* gene, a region of threonine–glycine (Thr–Gly) repeat shows length polymorphism in different natural populations and the frequency distribution of the two most common length variants, $(Thr–Gly)_{17}$ and $(Thr–Gly)_{20}$ follows a latitudinal cline in Europe (Costa *et al.* 1992). Further studies revealed that flies with the $(Thr–Gly)_{20}$ allele, the more common allele in the north of Europe, show better temperature compensation than flies carrying $(Thr–Gly)_{17}$, the dominant allele in southern Europe (Sawyer *et al.* 1997). Thus, $(Thr–Gly)_{17}$ is better adapted to warmer environments (Mediterranean) than $(Thr–Gly)_{20}$, which in turn is better adjusted to harsher northern environments with larger daily and seasonal temperature fluctuations. Furthermore, a similar latitudinal cline in Australia for the $(Thr–Gly)_{20}$ variant has been reported from flies collected and analysed in the early 1990s (Sawyer *et al.* 2006). This cline was not as robust as that in Europe, possibly reflecting the 100 or so years since this species had been introduced to this continent (David & Capy 1988). In contrast, Weeks, Mckechnie & Hoffman (2006) claimed that they could not find a Thr–Gly cline in their Australian collections when they sampled 10 years later and we refer the interested reader to the correspondence between the two groups (Kyriacou, Peixoto & Costa 2007; Weeks, Mckechnie & Hoffman 2007).

The *per* polymorphism was also shown to be implicated in thermal adaptation on a much smaller geographical scale, in 'Evolution Canyon' in Israel (Zamorzaeva *et al.* 2005). There is a considerable difference in solar exposure between the two

opposite slopes of the canyon, resulting in the south-facing slope being more 'African' and the north-facing being more 'European' in abiotic (e.g. temperature fluctuations) and biotic factors. Consistent with the hypothesis that thermal selection may be important in Thr–Gly length variation, $(Thr–Gly)_{20}$ is more common in the north-facing slope and $(Thr–Gly)_{17}$ prevails in the south-face slope, even though the major allele in Israel is, as expected, the 'Mediterranean' $(Thr–Gly)_{17}$.

The major Thr–Gly length variants found in Europe and Australia have 14, 17, 20 and 23 repeats (Costa *et al.* 1992; Sawyer *et al.* 2006). This 'periodicity' of $(Thr–Gly)_3$ which separates the different peptides is interesting, because conformational analysis reveals that this moiety generates a type 2 β-turn (Castiglione-Morelli *et al.* 1995). Thus these natural Thr–Gly peptides within PER differ from each other by one complete turn. Furthermore, the temperature compensation of the 14-17-20-23 series of Thr–Gly variants is surprisingly linear in that the 14 and 17 alleles are overcompensated (the higher the temperature the longer the period), the 20 is almost perfectly compensated, whereas the 23 is under-compensated, with a period shortening on increasing temperature (Sawyer *et al.* 1997). European flies which carry much rarer alleles with 15, 18, 21 or 24 repeats, and are consequently out-of-phase with the common variants, numerically and conformationally, have generally poorer temperature compensation which does not conform to the predictable relationship observed with the in-phase alleleic series (Sawyer *et al.* 1997). Perhaps that is why they are rare in temperate regions such as Europe? This idea is supported by the observations that in tropical subequatorial Africa and northern Australia, where seasonal temperature variation is minimal, the frequencies of these rare out-of-phase variants is higher than in Europe, suggesting an absence of significant thermal selection (Sawyer *et al.* 2006; Weeks *et al.* 2006). While these studies implicate thermal selection to be important in shaping the length of the Thr–Gly region, the difficult task of examining the Darwinian fitness of the different length variants under daily and seasonally fluctuating environments that mimic true ecological conditions, remains to be done. In addition, while the conformational analysis of Thr–Gly peptides has proved to be revealing, the biochemical basis for the effects on temperature compensation of Thr–Gly length variation is still a mystery.

The circadian clock has been the target of natural selection for adaptations that allow animals to cope not only with the time structure of the environment but also with intra- or inter-species competitors. The partitioning of time as an ecological resource was demonstrated in two sympatric spiny mouse species (Kronfeld-Schor *et al.* 2001). The common spiny mouse (*Acomys cahirinus*) is nocturnal while the golden spiny mouse (*A. russatus*) is diurnal. The two species are ecologically similar and experiments showed that *A. russatus* shifts its locomotor activity as a response to the presence of *A. cahirinus* to reduce competition. In the laboratory, however, *A. russatus* revert to a nocturnal rhythm, revealing an unusually plastic character displacement in response to community-level interactions. Studies in the honeybee reveal another form of circadian plasticity (Bloch & Robinson 2001). Foragers (older) bees show higher level of per expression than non-forager younger bees (Toma *et al.* 2000). Honeybees that become foragers can revert to nursing in response to colony conditions. Bloch & Robinson (2001) showed that foragers reverting to nursing lost their circadian rhythmicity and adopted arrhythmic behaviour to match their round-the-clock brood care obligations.

Seasonal timing is another example of chronobiological research that has important ecological implications. Most organisms use daylength measurement to monitor the annual change of day (photoperiod) and to anticipate the coming season. Whether the photoperiodic timer is linked to the circadian pacemaker (known as the 'Bünning hypothesis') is a matter of intensive debate. At least in plants, the causal connection between the two clocks has been demonstrated (reviewed in Imaizumi & Kay 2006). In *Arabidopsis*, flowering time is triggered by long days and the link between the two clocks is provided by CONSTANS (CO), which is under circadian control. CO is a transcriptional activator that induces the expression of the floral activator FLOWERING LOCUS T (FT), when photoperiod is sufficiently long to coincide with a specific phase of the circadian cycle (Yanovsky & Kay 2002). In insects, some evidence suggests that *timeless*, which encodes a light-sensitive, circadian clock protein, represents, a possible link between the circadian clock and the photoperiodic timer that drives short-day induced winter diapause (Pavelka, Shimada & Kostal 2003; Mathias *et al.* 2005), a relationship that has recently received considerable support in *D. melanogaster* (Sandrelli *et al.* 2007; Tauber *et al.* 2007).

However, the most dramatic example of clock genomics impinging on a phenotype of ecological interest, is the work of Reppert and colleagues on the migration and navigation of the North American monarch butterfly (*Danaus plexippus*). These butterflies migrate from the northeastern corner of the USA and southern Canada, up to 4000 km to their wintering grounds in Mexico in the autumn (reviewed in Reppert 2006). The butterflies are already in diapause, conserving energy for the long trip, but in the spring, they mate, travel to the southern United States to lay eggs on milkweed, and as milkweed develops up the eastern USA, the butterflies by late summer have reached the northern point from which their great or great-great grandparents had migrated the season before (Reppert 2006). This remarkable and beautiful biological phenomenon involves photoperiodicity and time compensated sun compass navigation, and the question arises whether either or both require the circadian clock? For the latter the answer would appear to be in the affirmative as disruption of the clock with the use of constant bright light, also blocks the time-compensation component of navigation (Froy *et al.* 2003). Monarchs are able to use polarized light for time compensated navigation, and the relevant receptors have been identified in the dorsal rim of the eye (Reppert, Zhu & White 2004; Sauman *et al.* 2005). Tantalisingly, axons from these receptors terminate in an optic region called the medulla where CRY1 (the *Drosophila*-like CRY) fibres also converge,

suggesting a cross-talk between circadian and polarized light pathways (Sauman *et al*. 2005).

In addition, four neurons which co-express monarch PER, TIM and CRY1, and likely represent the clock neurons because of robust PER cycling, have been identified in the pars lateralis (PL; Sauman *et al*. 2005). The same molecules are found in the neurosecretory cells of the pars intercerebralis, which is connected to the PL by a CRY1-staining fibre, but in the PI, there is no robust PER cycling. A model has been proposed whereby photoperiodic information is conveyed to the PL by the CRY1 pathway, and then again by CRY1 fibres to the PI (Reppert 2006), which in turn controls juvenile hormone, the regulator of diapause (Herman & Tatar 2001). In indirect support of this model, recent observations reveal that a TIM–CRY interaction is important for changing levels of diapause in *D. melanogaster* (Sandrelli *et al*. 2007).

A number of studies have demonstrated that population expansion to northern latitudes involves adaptation of the photoperiodic timer leading to latitudinal clines in the incidence of diapause in drosophilids (e.g. Lankinen 1986; Schmidt *et al*. 2005; Lankinen & Forsman 2006). In insects, this may reflect the constraint of temperature on reproduction, leading to earlier diapause (and shorter breeding season) in northern populations. The latitudinal cline in the photoperiodic response can correlate with circadian photoresponsiveness (the extent to which flies respond to light entrainment) with reduced light-sensitivity in northern populations. This is presumably an adaptation to the detrimental effect of the extended summer light exposures on the circadian clock (Pittendrigh & Takamura 1989; Pittendrigh, Kyner & Takamura 1991). Recent studies in *D. melanogaster* appear to confirm this general idea (Sandrelli *et al*. 2007; Tauber *et al*. 2007).

In mammals, seasonal reproduction has also been studied with reference to clock genes. In the Soay sheep, for example, the hormonal balance changes between summer and autumn leading to changes in reproductive behaviour, and this is mediated by night time melatonin secretion from the pineal which provides a photoperiodic readout (Lincoln, Andersson & Hazlerigg 2003). This melatonin signal is decoded by a local timer in the pars tuberalis (PT) in the pituitary which is rich in melatonin receptors as well as canonical *Per*, *Clock*, *Bmal1* and *Cry* expression (Lincoln *et al*. 2006). The PT secretes a prolactin releasing factor that engages the release of prolactin in the adjacent pars distalis, which acts as a slave to the PT pacemaker (Lincoln *et al*. 2006). These clock genes have been implicated as part of the PT time decoding programme, and a number of speculative hypotheses have been put forward based on photoperiodic changes in these genes expression profiles (Messager *et al*. 1999; Lincoln *et al*. 2003).

Genomic studies of circadian clocks

Over the past few years, genomic studies have contributed to a more global analysis of circadian mechanisms. These types of experiments range from QTL investigations that aim to locate more clock genes, to transcriptomic and proteomic analyses that examine clock gene and protein expression. Most of these do not have any particular ecological axe to grind, yet they are interesting, if only to reveal how circadian regulation percolates through to every aspect of biological function.

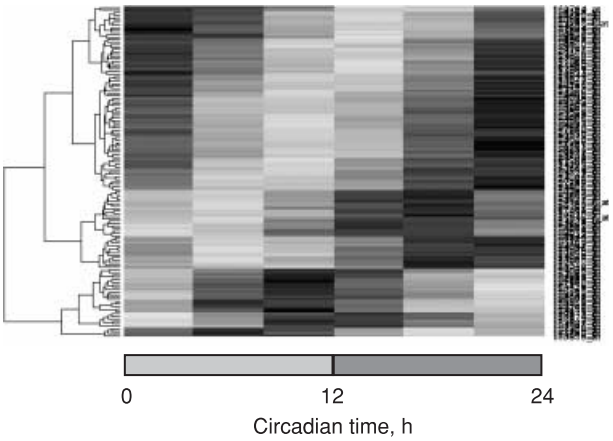

Fig. 2. Microarray analysis of circadian expression. An example of a heat map, showing the circadian change in expression level for 373 *Drosophila* genes (Data from McDonald & Rosbash 2001). These genes were classified as rhythmic (Fisher *g* test, $P < 001$) using the R package GeneTS (Wichert, Fokianos & Strimmer 2004). Expression levels have been scaled for each row (gene). The dendrogram on the left is used to sort the genes based on phase of expression. White and black represent high and low expression, respectively. Flies were maintained in continuous darkness. Circadian time 0 and 12 are the onset of the subjective day and night, respectively, as represented by the horizontal bars.

GLOBAL EXPRESSION STUDIES USING MICROARRAYS

The development of microarrays has provided the opportunity to interrogate the expression level of thousands of genes simultaneously, and circadian biologists have not been slow to apply these methods. The experimental protocol usually involves attempting to identify transcripts whose expression follows a circadian profile. Organisms are sacrificed at fixed intervals (e.g. 3 or 4 h) for one to several days 2–3 days under constant conditions, RNA is extracted, converted to cDNA and hybridized with the microarray probes.

To date, over 25 circadian microarray studies have been published in model organisms such as mouse (Akhtar *et al.* 2002; Panda *et al.* 2002; Storch *et al.* 2002; Ueda *et al.* 2002a), rat (Grundschober *et al.* 2001; Duffield *et al.* 2002; Hirota *et al.* 2002; Humphries *et al.* 2002; Kita *et al.* 2002) and *Drosophila* (Claridge-Chang *et al.* 2001; McDonald & Rosbash 2001; Ceriani *et al.* 2002; Lin *et al.* 2002; Ueda *et al.* 2002b) (Fig. 2). Plant studies have also been carried out in *Arabidopsis* (Harmer *et al*. 2000; Schaffer *et al*. 2001) and *Neurospora* has been used as a fungus model (Nowrousian *et al.* 2003). A few of these studies were based on cDNA microarrays, representing 1000–8000 genes, but most studies used commercial oligonucleotide arrays (Affymetrix) where up to 15 000 genes can be

interrogated per chip (Duffield 2003). Using these commercial platforms means that the list of cycling genes can be compared between studies.

Let us be partisan and take one example from our own laboratory (Akhtar *et al.* 2002), which represented an early circadian *in vivo* microarray analysis of the vertebrate transcriptome. With vertebrates, you have to select the tissue you wish to study (in flies one usually simply takes heads, and thereby the expression of genes in many different cell types is mixed). The mouse liver is large, is relatively homogeneous in cell type, and produces lots of RNA compared to the suprachiasmatic nucleus (SCN, the central brain oscillator in mammals). Furthermore, it is known that the levels of mRNAs of the canonical clock genes, *Bmal1*, *mPer* and *mCry*'s show circadian cycling so they can act as positive controls (Schibler 2006). Using a purpose built cDNA glass microarray, Akhtar *et al.* interrogated a few thousand genes for cycling, and found that almost 10% of the sequences that gave good liver signals, showed statistically significant rhythms. When they repeated this on the hypothalamus, that includes the SCN, they found that about 5% of the transcriptome cycled, yet only about 20 cycling genes were common to both the liver and hypothalamus, although this included several canonical clock genes. This remarkable result has been replicated several times in independent studies, and has revealed that only about 10% of the cycling transcriptome is shared among tissues. This has led some of us in this business to speculate that there may be no such thing as a 'constitutively expressed gene', in that every gene may have a particular tissue in which it cycles (Akhtar *et al.* 2002; Duffield 2003).

When one digests the cycling gene lists, it is clear that cycling transcripts converge on every possible biological function. In the Akhtar *et al.* study, cycling mRNAs were discovered from genes involved in the cell cycle (*wee1*, *cyclinD1*), apoptosis, metabolism and so on. The cell cycle genes that show circadian rhythms in expression are particularly interesting, and subsequently the relationship between the circadian clock and cell cycle has been further explored, as has the relevance of circadian apoptotic and cell cycle genes to the development of cancer (Fu *et al.* 2002; Matsuo *et al.* 2003; Unsal-Kacmaz *et al.* 2005).

However, circadian transcriptomic studies do not usually stop at simply generating a list of genes. Akhtar *et al.* also manipulated the clock by ablating the SCN (to create SCNX mice), and asking whether the circadian rhythms in gene expression in the liver were altered? In these animals, the rhythms of cycling liver genes now appeared flat. This could have been because the SCN in these animals normally sends a signal to the liver to maintain peripheral gene expression cycling, and in its absence, the rhythms decay, or it could have been that different individual mice that made up each time point, were now desynchronized from each other and in different phases, giving the appearance of 'flatness'. In fact, it's probably the latter, as peripheral rhythms can run independently of the SCN as they contain all the canonical genes required to run the clock (Yoo *et al.* 2005). The SCN provides coordinating signals to the periphery to maintain a coherent phase relationship. One of these signals involves glucocorticoid (Reddy *et al.* 2007).

These studies reveal some of the types of questions that can be asked with this technology. With flies for example, a common manipulation is to examine the cycling transcriptome from the head in wild-type flies compared to arrhythmic clock-mutant animals. What is usually found is that the vast majority (or all) of transcripts do not cycle in the mutants (e.g. Claridge-Chang *et al.* 2001; McDonald & Rosbash 2001). However, what is clear is that many hundreds of genes whose mRNAs do not cycle normally, can be either significantly up- or down–regulated in the mutants (Claridge-Chang *et al.* 2001; McDonald & Rosbash 2001; Lin *et al.* 2002). Thus canonical clock genes that are transcription factors (or are least transcriptional regulators), appear to also regulate many other downstream components that may have very little or nothing to do with circadian rhythms.

One challenge of circadian expression studies is to distinguish between oscillating genes which are important for the clock function (core clock genes) and those which are driven by the clock (the so called output genes). Several approaches have been used to tackle this problem. Some studies have tested the oscillation in the transcriptome of animals kept in continuous darkness (representing the 'free-running' clock) with the transcriptome of animals kept in light–dark cycles (whose oscillating transcripts are enhanced by the additional effect of light entrainment). Several studies attempted to profile the transcriptome of clock mutants (Claridge-Chang *et al.* 2001; McDonald & Rosbash 2001; Lin *et al.* 2002; Wijnen *et al.* 2006). In *Drosophila*, abolishing core circadian genes such as *Clk* or *tim* eliminated all detectable transcriptional circadian rhythm, suggesting that only a single transcriptional clock is present in the fly, in contrast to other model systems (e.g. cyanobacteria, fungi and plants) where multiple clocks exist (Bell-Pedersen *et al.* 2005; Pregueiro *et al.* 2005).

In theory, if the experiments have been done in a similar fashion, there should be an impressive correlation between the lists of cycling genes from any pair of studies. More often that not, this does not happen (e.g. Jackson & Schroeder 2001). It is possible that the inconsistencies between different studies result from different conditions or different strains of animals used. However, the main culprit is the different algorithms used to analyse the data. The major problem of microarray experiments is the cost, and typically only limited numbers of slides (time points), usually from 3–4 h intervals in 2–3 day experiments, are being used. This causes a computational problem as the sensitivity (power) of rhythm-detecting algorithms, such as spectral analysis, is poor (unlike rhythm analysis of behavioural activity where long time-series with hundreds of time-points are available). The same data set, normalized and analysed by different algorithms can produce non-overlapping lists of oscillating genes (Walker & Hogenesch 2005).

Recently, a different algorithm for meta-analysis of available data in *Drosophila* has been developed and suggests that the overlap between different studies is larger than previously thought (Wijnen *et al.* 2006). The data from different

experiments was concatenated to a single time series, which was then subjected to spectral analysis. The distribution of the Fourier scores at different given periods, which was compared with a null distribution of the permutated data set, revealed a significant excess of genes with Fourier components of 24 h. This study showed that the consensus between the different microarray studies is far broader than initially assumed (see above). Also, by comparing data from normal and mutant flies that were either free-running in constant conditions or entrained by light, this study identified a large number of genes that showed non-circadian, but light-driven expression (Wijnen *et al.* 2006).

While much attention has been devoted to circadian microarray studies, mRNA cycles are only part of the story, and a cycling transcript does not necessarily mean a cycling protein, or *vice versa.* An initial study of the circadian proteome has identified cycling proteins within mouse liver (Reddy *et al.* 2006). These cytosolic proteins represented about 20% of the detectable soluble fraction, and corresponded to a wide range of known metabolic functions. In addition, a number of novel cycling proteins were identified. Proteins were shown to cycle in different phases, with some enzymes within a particular pathway such as ureagenesis cycling coordinately or in antiphase. Enzymes involved in sugar metabolism peaked at night, reflecting the metabolism of nocturnal feeding in the mouse. For some proteins, differentially phosphorylated isoforms of the same protein cycled in antiphase. Intriguingly, only about half of the cycling proteins were encoded by genes whose mature mRNAs also cycled (Reddy *et al.* 2006). In other words, about half the cytosolic proteome probably shows circadian rhythms due to post-transcriptional or post-translational modifications. At present levels of proteome detection, it is impossible to ask the reverse question, that is, what proportion of cycling transcripts have a cycling protein, but this study serves to highlight that there is more to circadian 'omics' than 'transcriptomics'.

QUANTITATIVE TRAIT LOCI (QTL) ANALYSIS

QTL mapping is another approach used by chronobiologist in genomic scans for loci involved in the circadian clock. These studies have confirmed previously known clock loci as well as identifying novel clock genes in *Arabidopsis* (Swarup *et al.* 1999) and the mouse (Suzuki *et al.* 2000; Shimomura *et al.* 2001). Briefly, QTL mapping is based on an analysis of the phenotype of any series of genetically segregating lines, F2s, backcrosses, or recombinant inbred lines (RILs), for which a comprehensive set of molecular markers is available (Falconer & MacKay 1996). If we take RILs for example, each line represents different combinations of the genomes of the parental lines used to derive the RIL. Correlating the phenotype and the marker information of each strain allows the identification of chromosomal regions within the genome that contribute to circadian variation. Examination of clock-mediated leaf movement in *Arabidopsis thaliana*, revealed considerable natural variation in three circadian parameters: period, phase and amplitude (Michael *et al.* 2003). Using the Columbia (Col)-Landsberg-erecta (Ler) set of 76 RILs, five significant QTLs have been identified. One of the QTL affecting the period included a gene called *APRR7* and further experiments demonstrated how different natural alleles of *APRR7* (and of other *APRR* paralogues) conveyed different circadian periods, and may provide primary sources of natural variation for local adaptations. A further *Arabidopsis* QTL study, using the Ler and Cape Verde Island accessions as starting material, specifically focused on the phase of the cycle using a reporter assay. Four QTLs were identified, of which one on chromosome 5 contained novel polymorphisms in two genes already known to be relevant to circadian function, *SIGNALING IN RED LIGHT REDUCED 1* and *PSEUDORESPONSE REGULATOR 3.* Two other QTLs contained no known clock-relevant loci (Darrah *et al.* 2006).

In *Arabidopsis* several QTLs, have been identified that are involved in circadian temperature compensation (Edwards *et al.* 2005). None of these QTLs co-map onto the positions of known core circadian genes. Thus the variation available in natural isolates for temperature compensation either reflects the properties of genes acting in *trans* to the clock loci, or perhaps that these QTLs are identifying new clock genes that are important for buffering plants circadian responses to temperature, or that in the particular parental strains used, there was no relevant clock gene polymorphism. However, one of these QTLs includes *GIGANTEA* (*GI*), a flowering-time gene (Gould *et al.* 2006). *GI* buffers the period of rhythmic leaf movements and the amplitude of the cycling core molecular components *TOC1*, *LHY* and *CCA1* to changes in temperature, and also extends the temperature range (both high and low) at which the plant maintains rhythmicity in leaf movements (Gould *et al.* 2006). Another QTL contained the locus *FLOWERING LOCUS C*, which was involved in the temperature response at high temperatures (Edwards *et al.* 2006).

Turning to mammals, a genome-wide trait analysis of 196 F_2 hybrid mice, investigating five different circadian phenotypes (circadian period, strength of rhythmicity, phase of entrainment, activity level and dissociation of rhythmicity) identified 14 loci as having a significant contribution (Shimomura *et al.* 2001). Except in two cases, these loci did not co-localize with any of the known circadian genes, demonstrating the value of this approach for the discovery of new genes. More importantly, these authors also carried out a genome-wide interaction analysis. Large numbers of pairs of loci were identified that seemed to interact epistatically, having larger effects on circadian behaviour than either of the loci alone. The message here is that in the study of a complex phenotype such as circadian behaviour, the effect of a given allele should be evaluated in the context of the allelic combinations present in a specific genome. The role of epistatic interactions also may explain why the phenotypic variation seen in the RI lines exceeds the variation observed in the parental lines in this study. This so-called transgressive segregation was also found in other studies (e.g. in *Arabidopsis*, Swarup *et al.* 1999; Michael *et al.* 2003) and is possibly the

result of epistatic interactions that increase the phenotypic variation in the progeny.

Another study used QTL mapping to study circadian photosensitivity in mice (Yoshimura *et al.* 2002). Photosensitivity is assessed by applying brief light-pulses at different phases to animals that are in constant conditions. Most organism respond by shifting their activity, so that early at night, a light pulse may delay circadian cycles by a couple of hours, whereas a light pulse late at night will usually generate an advance in the behavioural rhythm. A retinally degenerated mutant with attenuated photosensitivity was used as one of the parental lines in an F2 crossing scheme. A few significant QTLs were identified on different chromosomes, suggesting that photosensitivity is a polygenic trait. None of the genes within the QTLs encoded either a photopigment or structural component of visual receptors, suggesting that variation in photosensitivity might be mediated by signal transduction genes.

QTL mapping was also used to identify loci involved in seasonal timing. For most organisms light is the primary cue for seasonal timing (reviewed by Tauber & Kyriacou 2001) and the question of whether the circadian clock is involved in photoperiodism is a subject of much current interest. A recent study in the rainbow trout tested the role of several circadian (and circannual) genes in the seasonal timing of spawning (Leder, Danzmann & Ferguson 2006). In this investigation the polymorphism in the candidate genes was applied to existing QTL date sets for spawning, and interval mapping was used to associate gene polymorphism and timing of spawning. The most significant QTL identified, explaining 20% and 50% trait variability in females and males, respectively, encompassed the gene *Clock* (although the effect was not shown to be unequivocally localized to that specific gene). In *Arabidopsis*, QTL analysis of daylength sensitivity for seasonal flowering time led to the identification of a natural polymorphism (involving a single amino-acid replacement) in *Cry2* (El-Din El-Assal *et al.* 2001), which plays a photoreceptor rather than a clock role in plants.

Conclusions

From our brief description of the 'omic' studies outlined above, we can see that there is considerable natural genetic variation underlying circadian phenotypes. However, if we look a little deeper into the implications of some of these studies, we can see perhaps that they provide some food for ecological thought. Let us begin with the photosensitivity QTL study in mice described above. It has been argued that circadian photosensitivity needs to be modulated so that animals are less sensitive to the exotic photoperiods that are routinely experienced in the higher, temperate latitudes (Pittendrigh *et al.* 1991). At such extreme photoperiods (even in Leicester, latitude 52·6 N, midsummer photoperiods reach almost LD 20 : 4), circadian behaviour can be disrupted (Shafer 2001). Thus any genes that might contribute to photosensitivity will be candidates for examining interspecific, or more importantly, intraspecific variation, particularly on a geographic scale. Might the genetic variation of such photosensitive genes be expected to show clines within a species? For example, we already have examples of clock gene variation in the repetitive region of *per* in *D. melanogaster* that shows clinal variation, and this variation appears to be maintained by balancing thermal selection (Costa *et al.* 1992; Rosato *et al.* 1997; Sawyer *et al.* 1997, 2006). Once identified, clock-related genes that lie within a QTL will provide a rich source of natural variation that can be systematically assayed in different environments, thereby providing the link between circadian genomics and circadian ecology.

Second, and again with reference to QTL studies, the finding that epistatic interactions in clock phenotypes in mice may be significant, is of general interest to ecologists and population biologists. What needs to be considered here is that these effects are discovered by crossing together two inbred mouse lines, each of which is circadian quasi-normal. In other words, epistasis reflects the function of a peculiar combination of natural allelic variations that must have come together in the wild forebearers of today's extant inbred lines (or perhaps accidentally came together during the inbreeding process). Such epistatic interactions among loci could be maintained by selection in natural populations. Once identified, these loci would again provide opportunities for assessing the variation at these loci in natural populations, with the possibility of correlating such variation with particular selective agents. In addition, if genes acting epistatically in a pairwise manner can be identified with QTLs, one wonders what a major mutational event in one such locus might generate in terms of a clock phenotype, irrespective of variation at the other locus? It may be useful to carry out directed mutagenesis in order to observe more clearly any major effects on the phenotype, bearing in mind that even under such a programme, the particular mutations obtained may not necessarily affect the phenotype as expected (DeBruyne *et al*. 2006). Similarly, it is conceivable that this approach of 'forward genetics', that has been so successful in identifying clock genes by mutagenesis, may not pinpoint genes that have any functional relevance at the level of natural variation.

Turning to the transcriptomic work described above, the past few years have seen some of the basic analyses performed for circadian phenotypes in a variety of model organisms. Again these studies as they stand do not have direct ecological implications, yet future work might be channelled along such lines. For example, a transcriptomic analysis of an organism under different photoperiods might produce some interesting candidate genes that might be relevant to photoperiodic modulation of circadian behaviour, and experimental designs could include photoperiodically induced phenotypes such as diapause or overwintering. This could be done in plants, relevant tissues in photoperiodic mammals or in insect brains. Similar experiments might be designed to examine candidate genes for temperature compensation. For example the amplitudes of the cycling trajectories of *per* and *tim* transcripts in *D. melanogaster* move in opposite directions when the temperature is raised. This 'balancing' effect may have some relevance to temperature compensation of the clock (Majercak

et al. 1999; also see Gould *et al.* 2006 for a similar example in *Arabidopsis*). A transcriptomic study might be designed to examine which cycling genes show such opposite effects with temperature change, as an initial entrée into a traditionally very difficult research area.

There are other biological rhythms, such a tidal (12·4 h), lunidian (24·8 h) or semi-lunar rhythms (15 days), that are present in non-model intertidal organisms such as Crustacea. These are as yet untouched by any kind of genetic or molecular analysis, yet identifying transcripts that cycle with the corresponding periods, would generate an enormous amount of interest in the circadian and ecological communities, and again provide the initial material for dissecting out these important and as yet unexplored cyclical phenomena. These experiments would not be trivial, as generating microarrays from non-model organisms is a long-term commitment, yet such studies may provide an initial way forward in this intriguing area. One way of bypassing these species barriers might be to use a model organism array platform and perform cross-species hybridizations. A number of such studies exist and this may represent a useful emerging approach, although interpretation of the results is not so straightforward (reviewed in Bar-Or, Czosnek & Koltai 2006).

Consequently, we predict that in the next few years, progress will be made along all these various lines of research. In the final analysis, however, each single candidate gene, once it has been established as a contributor to the phenotype under study, will have to be analysed in terms of its expression patterns, its interactions with other loci, and its evolution. In terms of the latter, population geneticists will have a goldmine in which to explore functional natural variation at these loci, and determine whether or not selection is shaping these patterns of polymorphisms. The tools required for these kinds of statistical analyses, so called 'neutrality tests' are themselves constantly evolving. However, there are a number of 'classical' and well-tried and trusted tests that can determine whether selection is acting on the variation at the locus in question, and what type of selection, whether balancing or directional, might be at work (e.g. Hudson, Kreitman & Aguade 1987; Tajima 1989). Once selection is established, then the next step is to focus on the putative selective agents that are shaping variation at the locus of interest, with inevitable and direct ecological implications.

Acknowledgements

ET and CPK thank NERC and BBSRC for research support. CPK acknowledges a Royal Society Wolfson Research Merit Fellowship. We thank two anonymous referees for helpful comments on the manuscript.

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Received 8 December 2006; accepted 5 September 2007
Handling Editor: Richard Preziosi