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TEMPORAL ORGANIZATION: Reflections of a Darwinian Clock-Watcher

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DARWINIAN SPECTACLES

A Broken Window Leads to Darwin

This essay was prompted by the Editor's invitation to illustrate the excitement and adventure inherent in scientific work while reflecting on my own preoccupation, as an evolutionary biologist, with biological clocks. In considering the challenge, the first adventure that came to mind occurred one evening 30 years ago when a drunken graduate student, frustrated by an unwanted experimental result, attempted to throw me out of a second story window in Princeton. He didn't succeed. That seemed a good Indiana Jones start, but nothing as exciting occurred in later years, and all the adventures I can recount are less spectacular--the excitement of experiment and the hazardous fate of observation and ideas in the pursuit of understanding.

While circadian periodicities have been the immediate object of my research for 40 years, that "view of life," which Darwin so eloquently summarized in the last paragraph of "The Origin of Species," has so dominated and guided my approach that it gets substantial attention in these reflections and hopefully ties together much of what I have to say about biological clocks. This Darwinian approach to behavioral and physiological interests traces to the accidental way I became a biologist.

At 15 I kicked a soccer ball through a very large window in the Town Hall where I lived in the north of England. The only foreseeable source of the 13 shillings needed to replace it was a prize offered to local Boy Scouts for the best wild flower collection. In winning that prize, I got more money than was needed for the window and was seduced into a lasting love affair with plant

systematics (see 1, 2, 3). The reading that followed in my high school library began with Sir John Arthur Thomson's "The Great Biologists," where I discovered the previously unheard of Charles Darwin. "The Origin of Species" (a formidable task never finished in high school) gave new and exciting meaning to the affinities encountered in classifying plants. Then on to F. 0. Bower's "The Origin of a Land Flora" that had an even greater impact in making the historical process, as such, a mandatory element in my understanding of anything alive.

This was reinforced when, shortly after College, I found a primitive terrestrial bromcliad (*B. humilis)* on a dry rocky island between Trinidad and Venezuela; its remarkable root system and associated epidermal trichomes (4) immediately suggested a desert origin for those epiphytic relatives, the bulk of the *Bromeliaceae,* that exploit and adorn the canopy of rain forests elsewhere in the New World. That discovery was an instructive experience: attention to possible origins, to early selection pressures, can enhance our understanding of contemporary organization.

My high-school interest in Darwinian evolution survived an undergraduate exposure to J. W. Heslop-Harrison's Lamarckian convictions (King's College, University of Durham), flourished during graduate school (Columbia) with Dobzhansky, and matured during several later years of friendship and collaboration on a book with G. G. Simpson.

The Origin Of Organization

A whimsical analogy I enjoy and find useful sees the living world as a vast literature comprising millions of volumes, many still available but even more out of print. All are vignettes written in the universal language of nucleotide sequences. All have the same happy ending (reproduction) which, when reached, assures the volume stays in print. That ending is reached, however, in an incredible diversity of ways: in daisies, Pitcher plants, and*Sequoias;* in African butterflies whose pupae are miniature death-masks of nearby monkeys; in fish that enjoy home-delivered food while living in the cloaca of sea cucumbers; in orchids that seduce innocent wasps in illicit copulation--a little soft porn in the literature here, and outright sadism in the behavior of female mantids devouring their lovers as copulation ends--and then humans, some raising corn and cows, some writing string quartets, and others contemplating the origin of black holes as well as themselves. I once described this diversity of vignettes as a baroque variation on a common theme only to be interrupted by Margaret Mead insisting, "No! more than baroque, it's rococo.'*

Unlike its (divine) Publisher, the author of this literature is both knowable and known, and some knowledge of his style is useful to the physiologist as

well as to the naturalist. The historical process of natural selection has written all the vignettes under several pen-names: according to Dawkins (5) he is "The Blind Watchmaker," but for some time (6) I have known him as "Darwin's Demon," a physically respectable cousin of Maxwell's. Maxwell's Demon, it is recalled, stood at a closable aperture separating two gas chambers at equal pressure. By opening the door only for molecules moving in one direction he extracted work from the initial equilibrium creating negentropy in violation of the Second Law of Thermodynamics. The fallacy in this fantasy was pointed out by Szilard: the energy gained from the Demon's discriminations is wholly offset by the energy spent in acquiring information on which way the molecules are moving. Szilard's version of the Demon can indeed create negentropy locally, but only by increasing entropy in a larger context.

And so it is with Darwin's Demon, who stands on the threshold of each new generation granting favored entree to the offspring of those members of the previous generation that were the better reproducers. This single, mindless discrimination is an inevitable consequence of the reproductive process that alone assures perpetuation of the vignette, keeps it in print. The inexorable consequence of its relentless application is a local and expensive increase in negentropy within the world of life that has troubled and misled not only Alexis Carrel but Wolfgang Pauli*: there is no violation of the Second Law while the Demon continuously enhances the script that ensures reproductive success; in brief, as he generates and keeps improving the organization of living things.

'Sometime in the early 1960s Wolfgang Pauli, a visitor to The Institute for Advanced Studies in Princeton, asked our mutual humanist friend, Erich Kahler, to find a biologist with whom he could discuss evolution. Kahler steered him to me and insisted that when Pauli came to my home I was to provide him with "a hard-backed chair (to accommodate his corpus) and a bottle of good claret.** The beginning of the evening was awkward. Pauli opened with a pronouncement that, "Evolution, of course, never occurred.** After some moments of silence I asked if he could tell me how he reached that conclusion, uncommon in the scientific world. His answer was brief. "Were the passage of time to witness an increase in negentropy the Second Law would be violated; and that's not possible." The rest of the evening, with the help of the (good) claret, went more smoothly. I gave the great physicist, as humbly as I could, a brief introduction to population genetics and a sketch of how selection worked as an historical process. I also pointed out that the increase of information it engendered over time was limited to that small enclave of nature we say is living, and that Darwin's Demon is as physically respectable as Szilard's version of Maxwell's. The evening ended with a one-liner as arresting as the opener, "Well," he said, "I'm going to see Max Delbruck in New York tomorrow and I'll find out!" I was, to be sure, a little dismayed, having heard that Pauli was strong on put-downs such as, " Prof X ? He isn't even wrong!" As it turned out he was equally strong on disarming apologies. Two weeks later, after his return to Zurich, I got a letter thanking me for the discussion, which he had evidently enjoyed. He added that he had seen Delbruck in New York as planned, and had yet (two weeks later) to fully recover: Delbruck had told him that he (Pauli) and Bohr were unemployed physicists who should stick to what they knew something about. And he had the grace to tell me that.

I once asked von Neumann, given the opportunity of cocktail conversation, what he saw as the difference between order and organization, both non-random. His answer was short and to Darwinian ears sweet, "Organization has purpose; order does not." The one is information-dependent, the other is not. The primary purpose of biological organization is self-perpetuation by self-copying, and as such is the handiwork of Darwin's Demon. My choice of the von Neumann comment is deliberate: the computer revolution for which hc was so largely responsible had a major impact on the climate of biological thought at mid-century. The mere title of a book by Wiener & Bigelow, "Purposeful Machines," makes the essential point (see also, 7). The apparent inseparability of purpose and consciousness was previously responsible for a major embarrassment and impediment to the biologist that Haldane put in a characteristically pithy way, "Teleology is like a mistress to the biologist; he dare not be seen with her in public but cannot live without her." It was my intention in 1957 (8) to help get Haldane's mistress out of the closet by describing her merits as teleonomic rather than teleological. Whether or not that did help (Monod & Davis found it useful!), the commonplace nature of programmable machines at midcentury gave teleology (as teleonomy) complete respectability in the society of biological ideas. The genome was the program of a Turing machine and Darwin's Demon was the programmer.

Many of my reflections are those of a naturalist, critic of the Demon's literature: how he has coped with the challenges of recurrent adversity and opportunity in the cyclic nature of the physical environment; and how, on the other hand, he has exploited the sheer predictability of those cycles as an opportunity to serve a great diversity of other very different ends, all subservient to his ultimate purpose, which is keeping his vignettes in print.

But I also maintain that as physiologist one can turn with profit to Darwin as well as to Loeb. The Loebian insistence on reduction to physical law, fundamental in its own right, is an insufficient arsenal for explanation in biology The literature written by the Demon is no more deducible from a complete command of the nucleotide language, let alone physical law, than the works of Shakespeare or Alfred North Whitehead are deducible from a complete command of the English language. In all cases, the author's wholly unpredictable artifacts have to be addressed and understood in their own right, and with what Erich von Holst somewhere referred to as ***eine niveau adequate terminologie.*** And in that address a persisting concern with function and historical origin will yield valuable guide-posts in the challenging task of understanding what the Demon has written.

For my own part I would have been happier had "The Origin of Species'* been called "The Origin of Organization*' the non-theological explanation of biological organization (of Paley's "design") was the real Darwinian revolution, much more profound than the origin of diversity, which it incidentally entails.

TEMPORAL PROGRAMMING: Some Personal Previews

Circadian Rhythmicity

Figure 1 gives two modern examples of an observation that was first made by the French scientist, Jacques de Mairan, in 1729. Biological activities that characteristically occur once per day in nature continue in laboratory conditions of constant darkness and temperature as a persistent rhythm with a period **(τ)** that is close to but not exactly 24 hr: the period is said to be circadian (L. ***circa, dies).*** Such circadian rhythmicity has been observed at all levels of

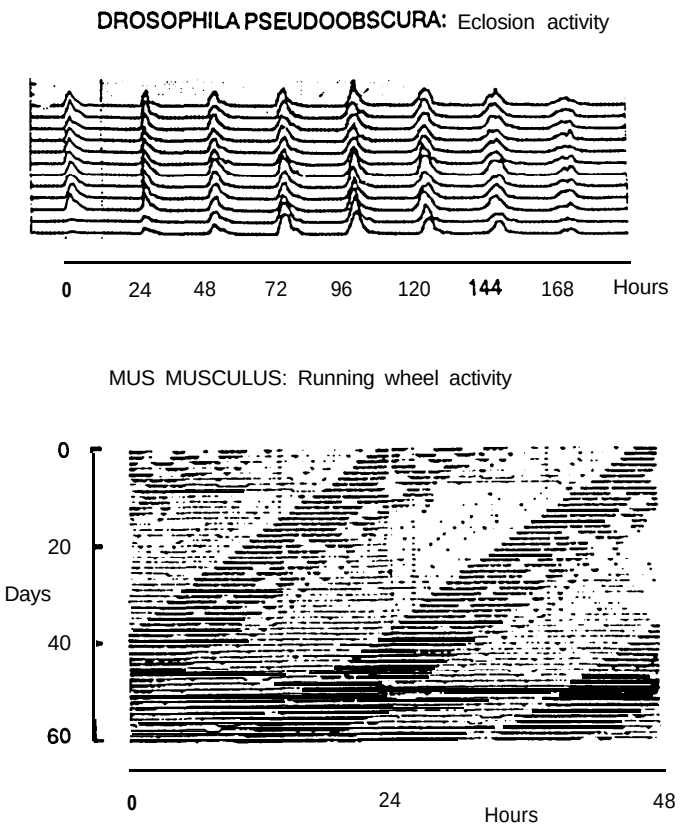


Figure 1 Circadian rhythms. (Top panel) Twelve replicate populations of ***Drosophila pseudoobscura*** pupae transferred from a 24 hr light/dark cycle into constant darkness and constant temperature 20°C. The emergence of adult flies (eclosion of pupae) occurs as a single peak of activity once per day. The period between peak medians is a few minutes longer than 24 hr (circadian). ***(Lower panel)*** Running-wheel utilization by a house-mouse (*Mus musculus*) kept in continuous darkness for 60 days. The heavy bars mark wheel utilization. The period (circadian) between onsets of activity is less than 24 hr.

organization, from the behavior of mammals, flies, and single cells, to the specific activity of enzymes, the activity of ribosomes, and the transcription of identified genes.

The endogenous nature of the rhythm is attested not only by its circadian period, but by the fact that (in suitable systems) its motion stops as soon as oxygen is withheld and promptly resumes as soon as oxygen is returned (9): and its innateness is attested in a variety of ways including many recent isolations of single gene mutations that alter its period. Circadian rhythms reflect extensive programming of biological activity that meets and exploits the challenges and opportunities offered by the periodic nature of the environment.

One of the truly remarkable features of these rhythms is their essentially indefinite persistence (>2 years in some rodents): they are driven by some self-sustaining cellular oscillation as pacemaker of the system. The stability and precision of the pacemaking oscillation are equally remarkable: the standard deviation on the average period **(τ)** of a mouse rhythm is about 1 minute, or 1/1000. A combination of this precision and the time constants involved constitute a formidable challenge to the cell physiologist.

Bananas, Mosquitoes, and Drosophila

My own introduction to biological timing came in wartime (1940s) Trinidad where, as a botanist with an undergraduate training in genetics, I had been sent to breed vegetables for the North African campaign that was, of course, already in progress! The Imperial College of Tropical Agriculture in Trinidad had a firmer grasp than the Colonial Office in London on the time-constants of such an undertaking and set me a task that lacked any redeeming relevance to the war, but was at least tractable: why were all the offspring of an interspecific banana hybrid pentaploid? Both parents were diploid. This entailed collecting banana ovaries while fending off a cloud of wasps at the top of a tall unstable ladder (adventure here!) and doing so at dawn when, I had been told, meiosis occurred. In fact it did, with the great majority of mother cells producing haploid (but sterile) embryo-sacs as one expected of an interspecific hybrid. However, in a small minority of mother cells that began meiosis much earlier than the norm, both meiotic spindles failed yielding, as a consequence, tetraploid embryo-sacs that were fully viable. The origin of pentaploids was explained (10), but I was perplexed at the restriction of meiosis to a limited time of day and even more perplexed by the spindle failures in atypically timed cells.

As soon as the banana work was finished (less than a year), I became involved, initially as a botanist, with the control of malaria on both the Naval and Airforce bases on the island. Both anopheline vectors bred in the “tanks” of water impounded by the overlapping leaves of epiphytic bromeliads, the

plants that Cladimiro Picado (11) happily described as the "overhead ponds" of tropical rain-forests, and whose historical origin I came to think was in desert conditions. Of the two anophelines breeding in bromeliads, one ***(A. homunculus)*** was largely limited to the extremely wet north-eastern end of the island. It was gradually replaced by its close sibling, ***A. bellator,*** as one moved south-west into drier rainfall zones. That clear interspecific difference in moisture requirements was again encountered in the center of the island where the species overlapped; here ***A. homunculus*** always occupied lower (wetter) strata of the forest than ***A. bellator*** (Figure 2), and equally clearly delayed its evening peak of activity to a later (moister) time. This pattern of species-specific timing persisted day after day in spite of variation in overall humidity conditions (12). If no fixed moisture level determined the timing of these evening peaks, were they dictated from within?

The same vaguely formulated proposition arose soon after the war (1946) when, as a Dobzhansky student, I worked on the daily activity cycle of ***Drosophila pseudoobscura*** and ***D. persimilis*** in the ponderosa forest near Yosemite: day after day, rain or shine, ***D. persimilis*** dominated the (moister)

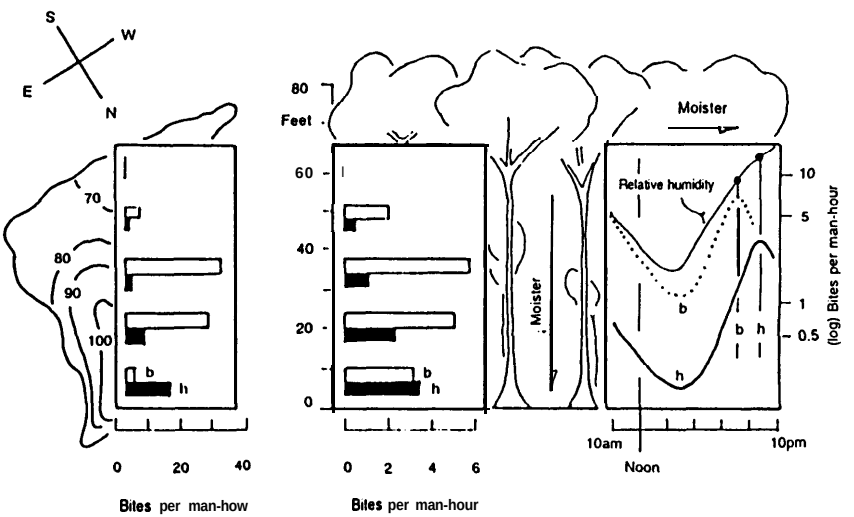


Figure 2 **Spatial and temporal differences in the distribution of two anopheline mosquitoes on moisture gradients in Trinidad, West Indies (based on data in 12). (Left panel) The abundance of** *A. homunculus* **(h) is high in the wet NE of the island, and declines towards the SW as rainfall levels fall. Its sister species,** *A. bellator,* **(b) gradually replaces it in the drier areas. (***Middle panel)* **In mid-island, where the species overlap, the interspecific difference in moisture requirement is reflected in the vertical distribution of the insects:** *A. homunculus* **is restricted to the lower, moister levels of the forest.** *(Right panel) The* **interspecific difference is again seen in the daily cycle of biting activity: the peak of** *A. homunculus* **activity occurs, day after day, at a later, cooler, moister time than that of** *A. bellator.*

morning peak of activity and ***D. pseudoobscura*** that in the (drier) evening hours.

Bunning and Kalmus

All of this led me, with pointers from my friend Marston Bates, to the papers on endogenous daily rhythms that Hans Kalmus (13) and Erwin Bunning (14) had published in the 1930s. One of the main points of their papers was to dismiss the suggestion from Rosa Stoppel(15) that those daily rhythms, which persisted in conditions of constant light and temperature, were under exogenous not endogenous control, that they were driven by some unidentified geophysical cycle (factor-x) caused by the earth's rotation. In dismissing that suggestion, both Kalmus & Bunning reported that the period of the persisting rhythm was temperature-dependent: factor-x, whose period must clearly be independent of local temperature fluctuation, could not be the driver.

lnteresting as those papers were, they failed to distract me from behavioral studies (8), which I hoped would explain the difference between ***D. pseudoobscura*** and ***D. persimilis*** in their daily activity cycles. What did change my work was listening to a lecture by Gustav Kramer in what I think was 1951.

GUSTAV KRAMER: A Biological Clock

Listening to Kramer lecture on the sun-compass behavior (16) of starlings was one of the most exciting and esthetically rewarding experiences I have enjoyed. In his most telling experiment, Kramer used a bird he had trained outdoors to go in a particular compass direction for its food reward, evidently using the sun's azimuth as a compass. It was challenged to do so inside a laboratory where an electric light replaced the real sun as direction-giver. In hour after hour, as the bird sought its target direction (where the food should be) it added 15° of arc (counter-clockwise) to the angle it made relative to the artificial sun. Three strong conclusions emerged: (a) the bird knew that the angular velocity of the sun's azimuth is, on average, 15°/hr; (b) it had access to some reliable clock to compensate for its constantly shifting (azimuth) compass; and (c) it knew it was in the northern hemisphere. Klaus Hoffmann had not then completed his crucial and beautiful experiments (17) showing the clock was indeed internal to the bird and not some external "Stoppel-dinger"; but Kramer and everyone else listening to him in 1952 was assuming that to be true, so the question was, "What is the clock?'*

I cannot recall now how many days it was after hearing him before the thought occurred to me, in fully explicit form, that Kramer's clock was based on or related to the endogenous daily rhythmicity of the Bunning & Kalmus papers of the 1930s. Didn't the expression of essentially the same period in their persistent daily rhythms itself imply that garden beans and fruit flies

could measure time-that they had a "clock'* measuring the duration of the cycle? That inference, only framed in such language after hearing Kramer, was as memorable and exhilarating as his lecture.

However, there was trouble. Bunning's initial basis for dismissing Stoppel's factor-x was his claim that temperature changed the period of the ***Drosophila*** rhythm. If the clock in Kramer's starlings were indeed the same as (even related to) the biological oscillation responsible for the flies' rhythm, its temperature-dependence would inevitably cause the birds to misjudge the position of the sun on warmer or colder days: a clock function for the oscillation demanded its angular velocity not vary with inevitable daily variation in temperature.

TEMPERATURE COMPENSATION: Functional Prerequisite

An Outhouse Experiment in the Rockies

Bunning's report was based on a very simple experiment I resolved to repeat. Abruptly dropping the temperature from 26 to 16°C when the flies were transferred to constant darkness, he found the first peak of the free-running rhythm was delayed almost 12 hr. My hope was to repeat that clearly crucial observation at Wood's Hole where several of the Princeton Department's senior members spent the summer and the facilities were excellent. However, Wood's Hole rentals in 1952 drove the family to the Rocky Mountain Biological Laboratory near Crested Butte in Colorado, where the rent was reasonable, but the facilities non-existent until I found a well-preserved outhouse (one-holer) near an abandoned mine shaft at approximately 10,000 feet. It was still erect and by now totally odorless. The walls and door were sufficiently intact that some tar paper and nails procured from Crested Butte made it a useful darkroom. Plyboard transformed the seat into an acceptable workbench. The presence of a small crystal-clear creek a few feet away provided a very stable source of low temperature-and a fine opportunity to fly-fish for trout. None of this would have been useful, however, without the pressure cooker that my wife had brought to ease the task of cooking at high altitudes. When emergence activity within them had begun, some vials of ***D. pseudoobscura*** were placed in the outhouse-darkroom and others in the pressure cooker-darkroom, which was then anchored in the creek to assure a constant low temperature. Its ventilation was effected by attaching, as a snorkel, a black rubber tube to the lid's steam outlet. To minimize distraction from trout, I limited observation to the emergence peak in both darkrooms after two days in darkness. To my surprise, because I was pessimistically expecting to confirm Bunning, the peak in the very much colder pressure cooker was only a couple of hours later than that in the outhouse: no more

than an hour or so's delay in each of the two cycles at low temperature! Crude as the experiment was, its outcome was clearly in conflict with Bunning's report of significant temperature dependence. I well remember the excitement of that afternoon in 1952; one had a bear by the tail; the angular velocity of at least one endogenous daily rhythm was, indeed, sufficiently unaffected by temperature to render it a useful clock. But what about the Bunning data?

The Follow-up in Princeton

That question was answered a few months later in Princeton where I built better facilities than the Emerald Lake outhouse offered and had the help of two able undergraduates, Lincoln Brower and Lynn Parry. This time when the culture vials were transferred from a light cycle (at 26°C) to constant darkness (at 16°C) emergence activity was assayed hourly. The initial finding showed Bunning was indeed correct: the first emergence peak at 16°C, due approximately 27 hr after entry into darkness, was 12 hr overdue. Late at night I went home disconsolate: how to explain the Colorado data? My undergraduate friends did not give up, however, and greeted me the following day with apparently perplexing data. The second peak in darkness at 16°C was only 2 hr later than that at 26°C, the same as in Colorado! Moreover, as the following days showed, the interval between all subsequent peaks at 16°C was only trivially longer than that at 26°C. Bunning had been misled by a step-induced transient that does not reflect the steady-state behavior of the pacemaking oscillation that drives the rhythm; and the Princeton data left no doubt that the oscillation was sufficiently temperature-compensated to serve as a useful clock.

Hazards in the Fate of Observations

As early as 1948, Frank A. Brown found the period of a daily rhythm of melanophore expansion in the crab ***Uca*** to be invariant over a wide range of temperatures. His paper (18), unknown to me until we met in 1955, was clear on the importance of the observation, but in overlooking the plausability of tempcraturc-independence as a functional prerequisite, Brown sought its explanation entirely in terms of external physical causation. In fact he invoked what amounted to factor-x as the cause and retained that position for many years thereafter.

In an ironically similar way, failure to anticipate temperature compensation as a functional prerequisite is what led Bunning to accept a transient as sufficient evidence of the period's temperature-dependence, which is clearly what he was looking for in his disbelief of Stoppel.

The von Frisch school's important contributions to this field illustrate a different hazard that observations face, i.e. the language used in reporting them. One of von Frisch's students, Ingeborg Beling, reported (19) a beautiful

series of experiments in which she showed that honeybees, having found an experimental food source at, say, 3 **PM,** returned to that site at essentially 3 **PM** on several successive days without reinforcement. Beling spoke of the hour at which the food was found as the "dressaurzeit" (training time) and the bee's subsequent return at the same hour as evidence of its "zeitgedachtnis" (time memory). While in Munich in 1959, I asked Martin Lindauer why they (the von Frisch laboratory) had still not reset the bee's clock with a Hoffman-like shift of the light/dark cycle. "Because," he replied, "bees don't have clocks, they have zeitgedachtnis." The impact of Beling's original (1929) language in discussing her beautiful experiments has never really been shed by the Munich workers. The use of "memory" and "training" (zeitgedachtnis and dressaurzeit) seriously distracts attention from the innate components in the overall behavior. And unlike "clock," the word "memory" fails to raise the issue of phase or local time. Why was it necessary, when the von Frisch school did eventually address the issue of local time, to fly bees from Munich to New York via Pan Am instead of the easier, and much cheaper, procedure of shifting the light cycle in the Luisenstrasse basement? It is as though the tradition of the school finds it easier to attribute memory to the "bienenvolk" than envisage a clock (something too concrete?) inside them. Their resistance to an internal clock has persisted into the 1980s, when they (20) attribute the bees' timing behaviors to control by daily variations in the earth's magnetic field. John Brady (21) has given an excellent critique of this recent resort to Stoppel's factor-x.

AN UBIQUITOUS CELLULAR CLOCK

Shortly after the ***Drosophila*** results appeared, temperature compensation was reported in several plant rhythms and, surprisingly, in mammals. The first of Michael Menaker's many contributions to the circadian physiology of mammals and birds was made in the Princeton laboratory (1959), where he found that the period of a rhythm in hibernating bats remained circadian even when their body temperature was as low as 3°C (22). In 1960, Rawson reported the equally surprising finding that the temperature compensation of a very different mammalian rhythm remained essentially unimpaired when its homeothermy was suppressed by drugs (23).

This functional prerequisite for time-measurement has since been shown to be ubiquitous in circadian systems; it is, indeed, one of their defining properties.

In one of the more rewarding adventures of that period, Victor Bruce and I (24) looked for and found temperature compensation in a unicellular system (Euglena). Shortly thereafter, Sweeney & Hastings (25) found it in another unicell, ***(Gonyaulax),*** and Ehret (26) discovered it in ***(Paramecium).*** Bruce

and I (27) deliberately sought and found it in ***Neurospora,*** as a simple eukaryote in which the genetics of the clock could be pursued. One of Bruce's students, Jerry Feldman, has done just that with great profit. And in the hands of Jay Dunlap, one of Feldman's students, ***Neurospora*** is proving a valuable tool for molecular work on the clock. While with us in Princeton, Feldman (28) also made the pioneering observation that slowing protein synthesis with cycloheximide slowed the clock, which opened up a new and major line of inquiry that has blossomed (the Feldman effect is widespread) and is returned to in the closing section of this essay.

TEMPORAL ORGANIZATION: Handiwork of Darwin's Demon

Programs Coping with the Day Outside

Much of the discussion of circadian rhythmicity at the 1960 Cold Spring Harbor Symposium was structured by a general evolutionary perspective. From life's outset, the major environmental cycles of day, tide, month, and year have confronted natural selection with windows of opportunity and hazard that recur with precisely predictable frequency; and the Demon has exploited that predictability by elaborating innate temporal programs that phase many undertakings in the life of cell or organism, metabolic or behavioral, to an appropriate time in the outside day (29). Such programs offer the clear advantage of anticipatory preparation for predictably recurrent conditions.

Filarial parasites spend most of the day in deeper tissues of their host and come into the peripheral blood stream only during the few and predictable hours when their mosquito vectors are active. That time is different in the Philippines and India (30) and the innate daily program of the two filarial populations is appropriately different.

The activity of day-active insects is very commonly restricted to the hours near dawn and sunset when the saturation deficit is lower and the hazard of water loss thereby lessened. That activity pattern persists as a circadian program in laboratory conditions of constant temperature and darkness, and there is little doubt that the timing differences I had observed between ***A. bellator*** and ***A. homunculus*** in Trinidad were genetically programmed.

The photosynthetic activity of green plants placed in constant temperature and low intensity light similarly persists as a circadian rhythm, with activity occurring only during that half-cycle when natural light is anticipated. Most spectacular is the way components of the photosynthetic process in the Caryophyllacae, Bromeliaceae (31), and other xerophytic plants are temporally programmed. The uptake of CO_2 through open stomata, which entails the hazard of water loss, is limited to nighttime when saturation deficits are

low, but there is no light to drive the photosystems; the CO_2 is initially stored in malate and released later at dawn when the now active photosystems can reduce it. Root-pressure pumping is similarly programmed in relation to the pattern of photosynthetic activity and transpiration.

The biological rhythmicity engendered by the environmental cycles becomes, itself, both challenge and opportunity for the Demon. Beling's demonstration of time-memory in honeybees is a classical case in point: a reliably phased rhythm of nectar secretion has set a premium on the insect's returning at the "right time," the day after initial discovery (32). The circadian timing of anthesis, in general, surely accounts for the circadian timing of banana meiosis that perplexed me in Trinidad.

In 1960 most of the programs coping with environmental periodicity were circadian. The years since then have produced abundant documentation of tidal, lunar, and annual programs. Stages in the development of Dietrich Neumann's intertidal midge *Clunio* are rigorously gated by a temperature-compensated 14-day clock that assures emergence of the tiny adult at low tide (33). Figure 3 from Gwinner (34) illustrates the extent to which the changing activity of a small passerine bird is programmed through the year, including the onset and duration of its migratory activity, as well as the sequence of its molts and gonadal growth. In species headed from Central Europe to South Africa, the programmed duration of nighttime flying ("zugunruhe") lasts many more days (in the Munich Laboratory) than it does in those species that (were they free) would only go to the south coast of the Mediterranean. Perhaps the most remarkable feature of these programs is their control of the bird's changing orientation to the earth's magnetic field, which it uses as reference in circumnavigating the Mediterranean. In some birds this circannual program has been read and reread for as many as 10 years in the basement of the Munich laboratory.

Programs Exploiting the Day Outside

Anticipation of a favorable time in the day outside has clearly been the Demon's goal in elaborating many circadian programs, but he has created at least as many, or more, with no such obvious function. The first such riddle in our own laboratory arose when Leland Edmunds (see 35) found DNA replication and mitosis in ***Euglena*** restricted to the darkness of night. Why? Why is meiosis in bananas restricted to the hours following dawn-and why do the meiotic spindles fail in those cells that initiate meiosis much earlier than the norm? Why is pupariation subject to strict circadian timing in ***Drosophila victoria*** but not in other ***Drosophila*** species? Why is the initiation of new developmental steps subject to similar circadian control in the straw strain of ***D. melanogaster,*** but not in other strains or species?

This challenge is nowhere stronger that in the circadian phase maps for

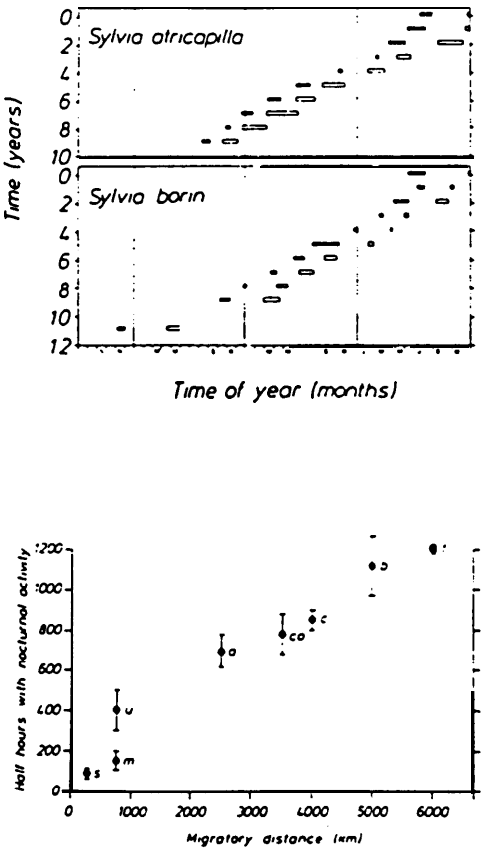


Figure 3 Circannual rhythms in small passerine birds (Warblers). (Top panel) The summer (black bats) and winter (open bars) molt in a Blackcap ***(Sylvia atricapilla)*** and a Garden Warbler (S. ***borin)*** kept for 8 and 10 years, respectively, under a constant short-day (10 light/14 dark) regime (from 34) ***(Lower panel)*** The total time spent in nocturnal migratory activit (zugenruhe) by 8 ***Sylvia*** species while in constant laboratory conditions is a function of the distance each would travel were they free to do so.

mice that Franz Halberg and his colleagues (36) have developed over the years. More than fifty physiological parameters have been assayed, each showing marked circadian periodicity with maxima at different times of day (Figure 4). For none is it clear why the particular activity is timed to its characteristic window in the outside day; what is the Demon up to?

The answer surely lies in his concern (inadvertant as usual) with temporal organization in its own right: organization that exploits the reliability of the external day as a time-reference and whose goal is an appropriate sequencing of diverse internal events rather than the concurrence of internal and external

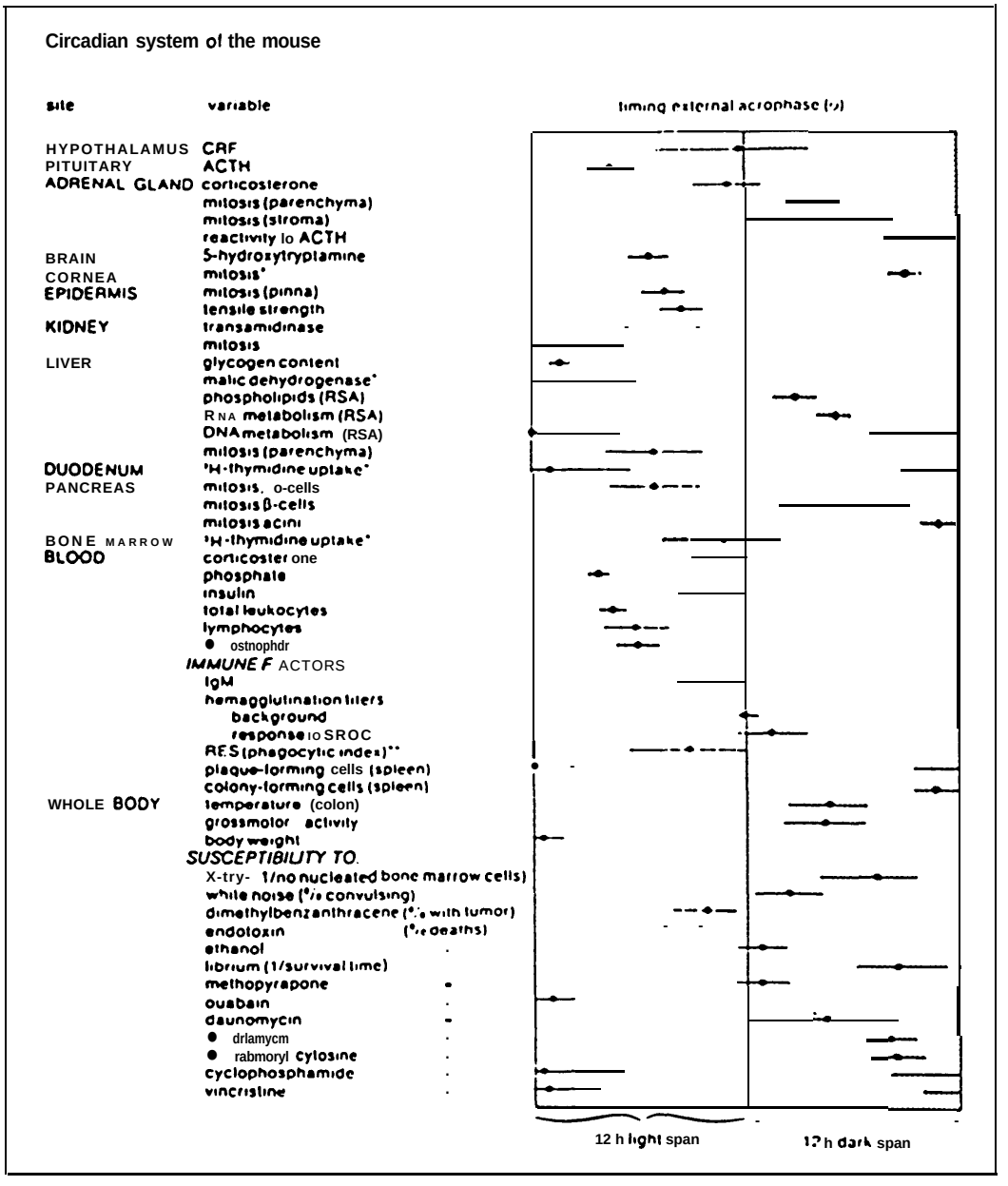


Figure 4 The circadian program of a house-mouse ***(mus musculus).*** The timing of physiological parameters in the house mouse (from 36).

events. We come back to this major role for circadian clocks in the closing section of this chapter.

Presence of such a circadian component in the organism's intrinsic temporal organization is also implied in the way abnormal entraining cycles often impair performance. The familiar stress imposed by rapid travel across time zones or shift work are examples. Both Aschoff's laboratory (37) and my own (38) have reported a negative impact on fly longevity when the insects are driven

to day lengths other than 24 hr. But the classic and most impressive evidence is still that of Fritz Went (39) and his students at the Caltech Phytotron in the 1950s, which showed the impact of disrupted days on the growth of plants. Went himself provided the clearest evidence on the crucial issue; he exposed African violets ***(Saintpaulia)*** to light cycles whose period ranged from 20 to 28 hr. In spite of each offering equal time for photosynthesis (light on 50% of the time), the cycles differed markedly in the growth they fostered. Low on the longer and shorter periods; the growth was maximal on the 24 hr cycle, which suggested that the crucial issue was resonance of a circadian component with its entraining cycle.

It is recalled that the breakdown of meiosis in banana hybrids occurred in cells that initiated the process each day some hours ahead of the majority; it is tempting to see this as a breakdown in circadian timing-the ovarian environment not yet congenial.

It was this aspect of circadian physiology that Wilhelm Hufeland (40) had in mind long ago (1798) in referring to the 24 hr period as "... die Einheit unserer naturlichen Chronologie," and what Kalmus (41) was sketching in 1935 as the "autochronie" of organisms.

SYNCHRONIZING THE PROGRAM TO LOCAL TIME

Pacemaker Entrainment: An Empirical Model

My first paper (42) reporting the ***Drosophila*** results included brief notice of their bearing on sun-compass clocks. That brought an enthusiastic response from Kramer himself who told me his Institute was pursuing the same proposition: they not only saw a functional role for endogenous daily rhythmicity as the clock in sun-compass behavior, but were already testing the idea.

In a classic experiment, Klaus Hoffmann (17) showed that the starling clock could be reset 6 hr by a 6 hr shift in the light/dark cycle to which the bird was exposed. Following the shift, the bird made a 90° error (6 x 15°) in its pursuit of the compass direction where its food reward lay. Soon after that, Kenneth Rawson, working at Kramer's Institute, began an experiment that Schmidt-Koenig (43) completed: homing pigeons subjected to a 6 hr phase advance in their daily light cycle made a similar 90° (6X 15°) counter-clockwise error in their initial headings for home: they set out from Giessen in Westphalia as if for Amsterdam instead of Wilhelmshaven. Clearly the daily cycle of light and darkness, which Aschoff calls the "zeitgeber," somehow synchronizes the clock (or program) to local time. But how?

Jurgen Aschoff (44) and I (45-47) independently introduced the oscillator

language into the discussion of circadian rhythmicity in the middle 1950s. Aschoff's brother, a distinguished engineer, introduced my friend to the idea and to relevant literature; in my case, the start came from reading a paper by John Pringle (48), in which he uses the behavior of coupled oscillators to develop a model of learning. Reading that paper, which introduced me to the entrainment phenomena, was as seminal* as listening to Kramer: when **one** oscillator is coupled to and driven by another, it assumes the period of its driver and develops a unique phase-relation to it. Here was precisely the relationship of a circadian rhythm to the environmental cycle that synchronizes it to local time: first, their coupling ensures transformation of the organism's circadian (about a day) period to precisely 24 hr and, second, in so doing establishes a unique and functionally appropriate phase-relation between them.

It was clear from the outset (ca. 1956) that to lock on to the daily cycle of light and dark, the oscillator driving the rhythm must be differentially responsive to light at successive phases of its cycle. That led to experiments with ***Drosophila,*** which used a standard brief pulse of light (15 min 50 lux) to perturb the system, otherwise free-running in constant darkness, at successively later phases of the cycle.

The results are described by a phase-response-curve (PRC) that plots the phase-shifts caused by the light at successively later phases of the free-run. The major features of the ***Drosophila*** PRC (Figure 6), reporting the effects of 15 min pulses (50 lux), are nearly universal: the half-cycle of pacemaker motion that normally coincides with the darkness of night is designated the subjective night; it is very responsive to light, which causes phase-delays in the first half of the subjective night, and then advances later. The half-cycle

*On the first day we met (1956). Victor Bruce and I found we both thought temperaturee compensation of the clock's period was based on the mutual coupling of two temperature-dependent oscillators with complementary temperature coefficients. In my case that was entirely the result of reading Pringle. Bruce and I never had suitable experimental material to test that idea in Princeton, but when Ron Konopka came to my Stanford laboratory in the early 1970s with his now famous and invaluable clock mutants, the chance was at hand to do so. My specific proposition concerned the two alleles, per^s and per^l, that determined clocks with 19 and 29 hr periods, respectively; that they corresponded with the two oscillators Bruce and I had envisaged as the mutually coupled components of wild-type (which has an intermediate period of 24 hr). The testable feature of this proposition was that per^s and per^l would not only be more temperature-dependent than wild-type, but that they would have complementary temperature coefficients: the period of one would shorten while that of the other would lengthen as temperature changed. These predictions were promptly confirmed (Figure 5) in some very satisfactory experiments (49), but it is no longer clear that this confirmation makes the basis of prediction valid: molecular analysis traces both mutations to the same exon.

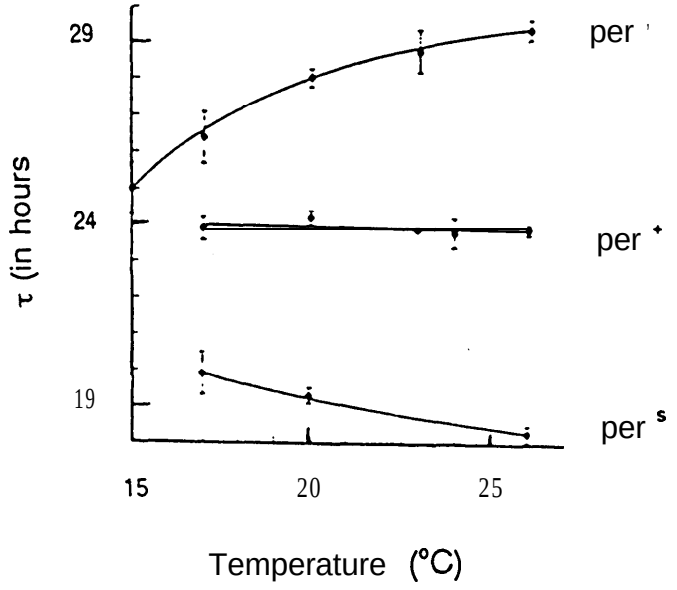


Figure 5 The effect of temperature on the period (τ) of the circadian rhythm of locomotory activity in *D. melanogaster:* in wild-type (per^{+}) and in two mutants, per^{s} and per^{l}. The period of wild-type is essentially unaffected by an increase in temperature, but that of per^{s} shortens and that of per^{l} lengthens as the temperature is increased (from 49).

designated subjective day is very unresponsive to light. All these features are rather obvious, indeed, they are analytic necessities (50).

What was not obvious is the way the phase-response-curve for a defined pulse (e.g. 15 min 50 lux) can be used to predict the phase relationship of the oscillator to an entraining cycle using that pulse. I discovered this initially by using a simple analogue device in which a circular version of the PRC was plotted on one sheet of transparent polar co-ordinate paper, and one or more light pulses were plotted on a second underlying sheet. Simulation of the oscillator's motion was effected by rotating its PRC (upper sheet) until interrupted by encounter with a light pulse on the lower sheet. The sign and amplitude of the PRC at encounter dictated a phase shift of the oscillator that I assumed occurred instantaneously. Implausible as that assumption was-a necessary oversimplification to get started-the simulations using it yielded essentially perfect predictions of the observed phase-relation between the oscillator and the light cycle that entrains it as Figure 7 attests. In steady state the light pulse in each cycle causes a discrete instantaneous phase-shift equal

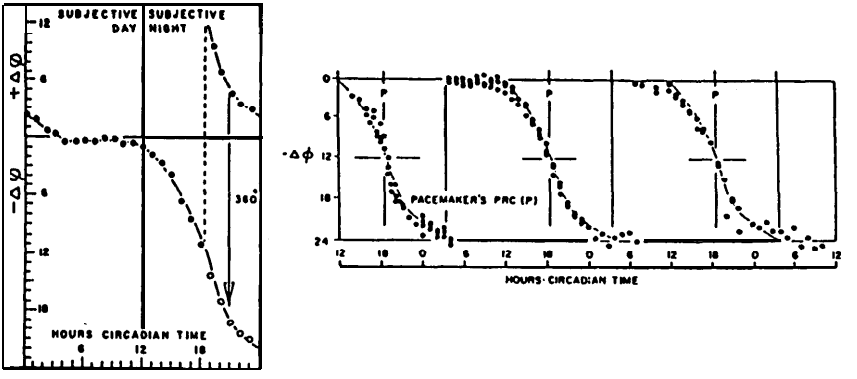


Figure 6 ***Drosophila pseudoobscura:*** phase-response-curves (PRCs) (for brief light pulses) of the pacemaker that drives the circadian rhythm of eclosion activity. (*Left*) The PRC for a pulse of white light (15 min 50 lux). See text for detail. By displacing the phase advances 360° one obtains a monotonic version of the PRC that is used in the right- hand panel. ***(Right) 72*** replicate pupal populations are released into continuous darkness after entrainment by a 12 hr light/ 12 hr dark cycle. Each population receives the standard brief light pulse, but at successively later one hour intervals. The phase-shift elicited by the pulse identifies the phase of the pacemaker's cycle at which it was given. The pacemaker's time-course is tracked through three full cycles.

to the difference between τ and T, the periods of pacemaker and light-cycle, respectively.

The PRC is a footprint, as it were, of the pacemaker's time course: the shift elicited by a strong brief pulse reliably identifies the phase of the cycle at which it was given. The right-hand panel in Figure 6 gives such a footprint of the pacemaker's time-course through three full cycles of a free-run in darkness. The upper panels in Figure 7 also give the phase-shift response (solid points) at successive hourly intervals to document the pacemaker's time-course in the entrained steady states realized by 21 and 27 hr light cycles. The curves in Figure 7 (upper panels) are predictions based on the assumption that the light pulses responsible for entrainment do indeed cause an instantaneous $\Delta\phi$ response equal to the difference between τ and T. Observation (plotted points) amply confirms that prediction.*

*As Arthur Winfree's work as a graduate student at Princeton made clear, the pulse must be above some saturation strength for the explanation of discrete entrainment developed here to be valid. His landmark analysis (51) of the effects of varying signal strength, especially in the middle of the subjective night, explained the then perplexing difference between what he calls Type-l and Type-O PRCs.

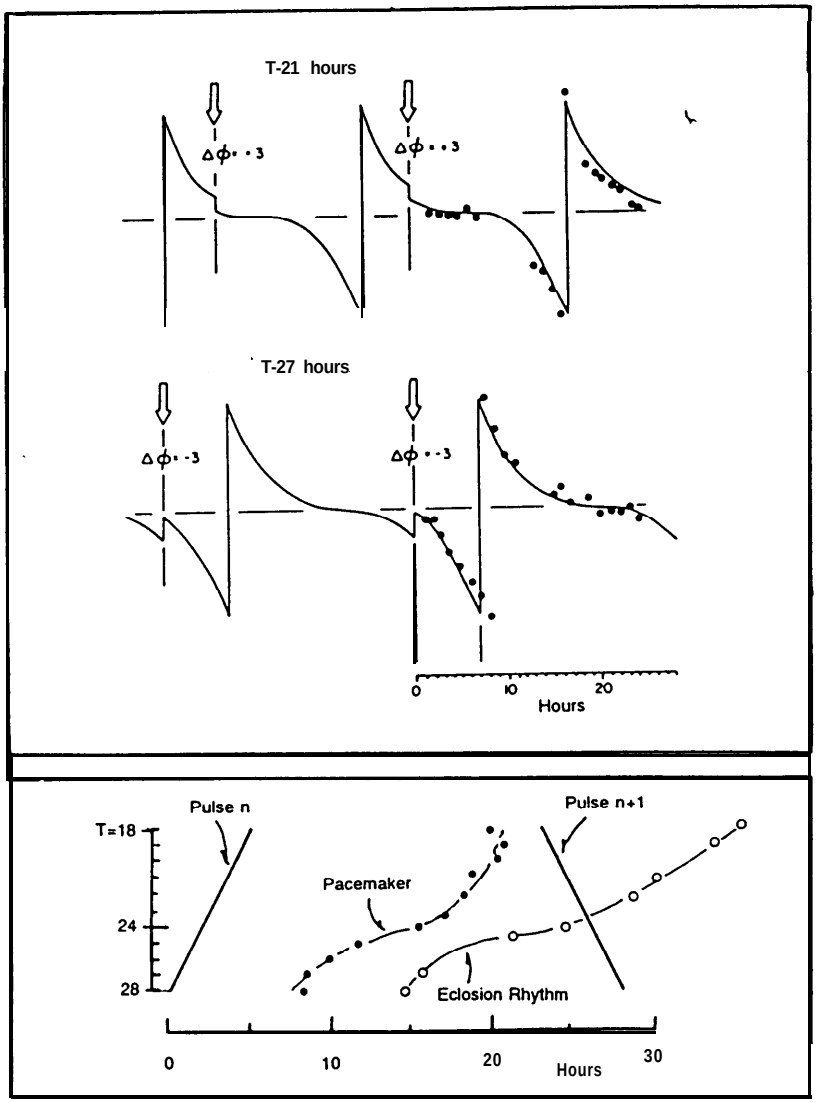


Figure 7 Entrainment of a circadian pacemaker (*Drosophila pseudoobscura*) by brief light pulses. ***Upper panels:*** The pacemaker of the pupal eclosion rhythm is entrained by cycles of brief (15 min. 50 lux) pulses of white light. The cycle length (T) in one is 21 hr and in the other is 27 hr. The solid curve represents (via its PRC) the time-course of the entrained pacemaker which undergoes a phase-shift equal to τ minus T at each light pulse. The solid points are measurements of the phase-shifts elicited by pulses at the times indicated, and match prediction closely.

Lower panel: The entrained steady states effected by cycles of brief (15 min. 50 lux) light pulses, whose periods (T) range from 18 to 28 hr. The phase of the entrained pacemaker is given as solid points that mark the time at which (middle of the subjective night) the response to the light pulse is a 12 hr phase shift. The pacemaker's phase-lag on the entraining light pulse steadily increases as the period (T) of the light cycle is shortened; the curve is predicted from the entrainment model, and the points are observed. The open points are medians of the directly observed eclosion peaks; the rhythm's phase-lag on the pacemaker also increases as T is shortened (see text).

The assumption of an instantaneous phase-shift is clearly valid and loses its implausability if one thinks of the light causing a photochemical destruction of one of the oscillator's state variables.

These successes led me to predict what the so-called limits of entrainment were for the ***Drosophila pseudoobscura*** pacemaker Since both phase-advances and phase-delays of about 12 hr were possible, I inferred one could entrain the oscillator down to a ~12 hr or up to a -36 hr cycle. Alas, while that confident assertion was in press (52) and irretrievable, we knew it was wrong. And thereby hangs a retellable tale.

Eric Ottesen, then a sophomore research assistant in my laboratory, had a computer science room-mate (Doug Sand) who laughed at the idea of our building a more complicated analogue device, and assured Eric and me that he could do better on the IBM 7090. He eventually did so, but not before his initial program (with 17 nested do-loops!) caused prolonged misery, Simplified and debugged, however, Sand's program opened up a entirely new world for us, although the first major dividend was an unwelcome bomb.

The limits of entrainment, according to the 7090, were 18 and 28 hr, not 12 and 36 hr as my paper (52), then in press, predicted. What was more, experiments promptly confirmed the IBM's prediction! Clearly something was missing from our model and that became the major concern of both of Ottesen and myself, who worked at it independently for some weeks without success until Eric alone, using high school algebra, found the answer. The slope of the PRC at the phase yielding the $\Delta\phi$ response necessary for entrainment (= τ-T) must lie between 0 and -2 for the steady state to be stable.

Delay in publishing his argument was prompted by the desire – principally mine --to have it stated in a more elegant mathematical form. This was a mistake that led to Ottesen being scooped in the open literature. Nevertheless, the excitement of discovery and the beauty of experimentally confirmed prediction (all of it an adventure) led Eric Ottensen to abandon Classics for a distinguished career in Biology and Medicine.

Challenge in Seasonal Change: Why is τ Circadian?

The difference between τ and 24 hr was, of course, a major piece of evidence in the rejection of factor-x during the 1950s. My own inclination at that time was to see τ as a tolerated approximation to the period of the outside day, sufficiently close to be entrainable by the light cycle. Remaining within the range of entrainment was all the Demon had demanded. The general idea of a tolerated approximation was made much more attractive by a delightful suggestion from Roger Revelle, whom I saw regularly at that time as a member of the NAS Oceanography Committee. Revelle's proposal was that τ was a living fossil. He had two premises. One was the chance, it so happens, that all the values of τ then known were less than 24 hr. The other was Wells' (53) now famous documentation from the growth rings of Paleozoic corals

that the period of the earth's rotation was much shorter than 24 hr: there were more than 365 rings in the annual cycle. Since then, as the earth's rotation has slowed, and the length of the day/night cycle has increased, all the Demon has demanded is, again, that the perpetually short τ remain within the range of entrainment.

Attractive as it was to an evolutionist, Revelle's idea was short-lived. The death blow came from Jurgen Aschoff, who was the first to notice that all the early measurements of τ were made on night-active species and, as newer measurements came in, the day-active species all had τ longer than 24 hr. These diurnal creatures (plant, animal, and now ***Homo sapiens)*** are clearly not accommodated by Revelle's fossil hypothesis. On the other hand, Aschoff was quick to see the important ecological regularity that was emerging and elaborated on it, at Cold Spring Harbor in 1960 (54), as the Circadian Rule. At the same meeting 1 successfully renamed it Aschoff's Rule (55).

I spent much of a 1959 Guggenheim with Aschoff in Heidelberg trying to find functional significance for my host's Rule and had the beginning of an answer in my Cold Spring Harbor paper, but it was many years before Serge Daan and 1 (50, 56) published a more satisfactory interpretation of it based on computer simulations, which used the empirical model developed for ***Drosophila.*** We found that the known differences between day- and night-active species in τ and PRC shape were such that as daylength changed seasonally, the day-active program appropriately tracked dawn, while that of night-active species tracked sunset.

I recently (57) obtained the same general result much more easily using a family of curves that Peter Kaus, a physicist friend then at RCA in Princeton, introduced to Victor Bruce and myself as early as 1956. These curves, called Kaus-Curves (sometimes Kaus-Kurves) in our group, show how the phase-lag of an oscillator on its driving cycle (e.g. light/dark) increases as the oscillator's period (τ) is lengthened; and when the strength of the coupling (C) between oscillator and light cycle is increased, how the dependence of phase-lag on oscillator period (τ) decreases.

No matter what the coupling strength, however, the phase-lag is always 90^{o} at resonance, i.e. when the oscillator's period (τ) is the same as that T of the light cycle. The consequence is a remarkable node in the family of Kaus-Kurves when $\tau = T$: to the left of that node, where $\tau < T$, an increase in coupling strength increases the phase-lag; but to the right of the node, where $\tau > T$ an increase in C decreases the phase-lag (Figure 8).

The coupling of circadian pacemakers to the light cycle is increased as daylength (photoperiod) increases in the spring, and consequently the phase-lag of those with $\tau < T$ (night-active species) increases, tracking sundown, while those with $\tau > T$ (day-active species) track sun-up. τ variation around 24 hr is clearly a non-trivial issue, and much of it has strong functional significance.

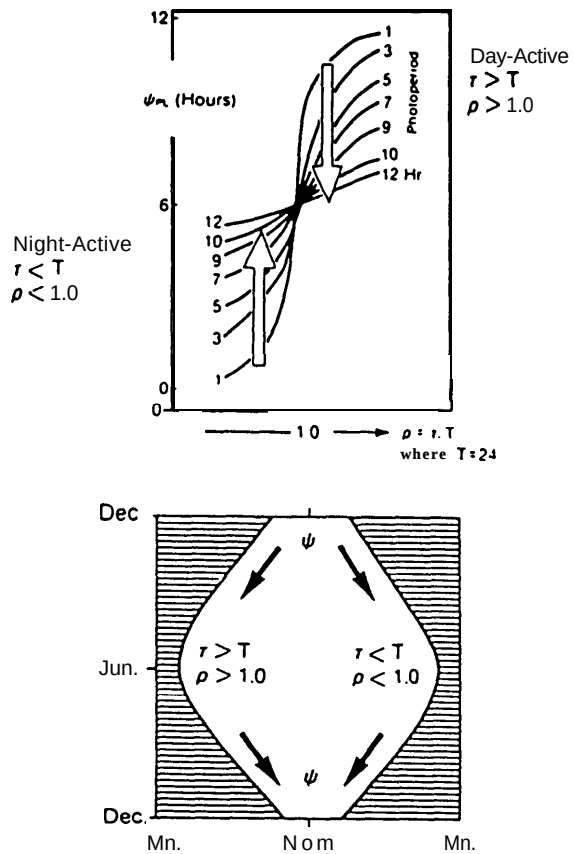


Figure 8 Aschoff s Rule (from 57). (Upper panel) A set of Kaus-Curves (see text) describes the phase-lag **(ψPL)** of a circadian pacemaker on the light cycle that entrains it, as a function of **ρ,** which is **τ/*T*.** As **τ** is increased, with *T* held constant at 24 hr, the phase-lag of the pacemaker is increased. The lag is also changed by increasing photoperiod, as shown for photoperiods of 1 to 12 hr. Increase of photoperiod reduces the phase-lag when p is greater than I .O but increases it when **ρ** is less than I .0. (Lower panel) As photopcriod changes throughout the year, the long **τ** characteristic of day-active organisms (p > I) assures their circadian program tracks sun-up; and the short **τ** characteristic of night-active animals **(ρ** < I) assures their program tracks sun-down.

Higher Latitudes: "Subjective Light Intensity"

Seasonal change in the onset of the working day is not the only challenge incurred by the Demon's use of the daily light/dark cycle as the principal entraining agent for circadian programs. The increasing duration of light in high summer poses an entirely different challenge that is only heightened the farther north (or south) one goes towards the pole.

The Pavlidis pacemaker in Figure 9 illustrates the challenge: the action of light is to bleach one of the two state variables causing the amplitude of both to be steadily depressed as the photoperiod exceeds 12 hr. Were it not for some compensatory adjustment, the very long days of the far north would almost damp out the oscillation and certainly weaken whatever signal underlies the pacemaker's timing function. There is little doubt that this potential impairment of pacemaker function by the longer days at higher latitudes is responsible (via the Demon) for the south-north cline in PRC amplitude that Kuma Takamura and I (58) encountered among Japanese races of ***Drosophila auraria.*** The light pulse used to measure the PRC has a steadily weaker effect as one goes north; it is as though what we called "subjective light intensity" declines in the north, compensating for the increase in the light's duration (photoperiod).

An increase in the amplitude of the pacemaker's free-running motion is what we believe affects this reduction in the subjective light intensity and, hence, PRC amplitude. That increase has other advantages at higher latitudes that Kyner, Takamura, and I (59) have recently sketched in some detail.

THE CIRCADIAN COMPONENT IN PHOTOPERIODIC INDUCTION

Bunning's Hypothesis: A Chilly Reception

The most important element in Erwin Bunning's papers of the 1930s was his proposition that endogenous daily rhythms played a central role in the then

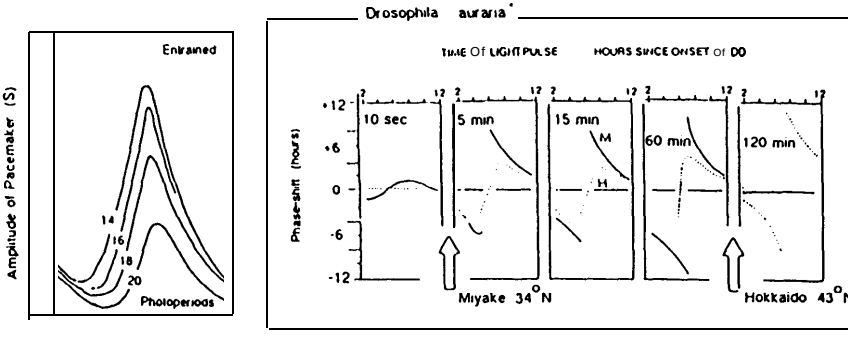


Figure 9 The effects of photoperiod and latitude on a circadian pacemaker ***(Drosophila auraria)*** (from 59). (***Left panel)*** The amplitude of one of the state variables of the Pavlidis oscillator used to simulate the behavior of the ***Drosophila*** pacemaker (see 59) is steadily decreased as the daily photopcriod is increased beyond 12 hr. ***(Right panel)*** PRCs for pulses of increasing duration applied to a southern (M; Miyake at 34°N) and a northern (H; Hokkaido at 42°N) strain of ***Drosophila attraria*** in Japan. The northern strain's response to each pulse duration is weaker than that of the southern strain. The "subjective light intensity" is lower in the north.

recently discovered phenomenon of photoperiodic induction. This idea was especially appealing to me as an evolutionist: was measurement of daylength, as a signal of season, yet another function that Darwin's Demon had found for circadian oscillations, a function additional to general programming and sun-compass orientation? My attraction to the idea increased as the empirical model of entrainment promised to yield, as it eventually did, some especially valuable observations that not only added crucial support for Bunning's hypothesis, but helped define more sharply the issues involved.

Bunning divided what we now call the circadian cycle into "photophil" and "scotophil" halves that corresponded with what we now call the subjective day and subjective night. Induction of, e.g. flowering, by a long day was attributed by Bunning to the light invading the scotophil. Initially this idea was received with some sympathy in Europe, but almost none in the United States. That was especially true in the years (1950s) following the brilliant work of Hendricks and his colleagues at Beltsville, where they identifed the pigment, phytochrome, that mediates the photoperiodic responses of green plants. Given the clock paradigm, the central issue in the 1950s was seen as a time-measurement: how did the plant (or animal) measure the duration of the daily light, or was it the daily dark period? Hendricks invoked phytochrome not only as the receptor pigment, but the clock measuring the duration of the darkness at night: he saw the thermochemical reversion of phytochrome during the dark as an hourglass process that measured night-length. The attractiveness of this was clear: phytochrome was something very concrete, indeed a molecule, whereas rhythm, so Hendricks tells us (60), was seen by his colleague Borthwick as only a word; and a word, perhaps, with a vaguely dance-hall ambience?

Circadian Surfaces

A change in the attitude of American researchers to Bunning began in 1959 when Nanda & Hamner (61) reported their now classic experiments with soybeans. Holding a normally non-inductive photoperiod constant, they could induce flowering by greatly extending the duration of the associated dark period in their exotic light/dark cycles. The several inductive cycles created this way all had periods that were modulo 24 hr. One conclusion was that the duration of neither the light nor the dark was crucial; another was that there was indeed some circadian component in the reacting system, and in this sense Bunning was correct.

Much later (62), I extended this kind of Bunning support in a re-interpretation of Beck's data, which he had taken (63) as evidence that the photoperiodic clock was an hourglass, not an oscillator. I found his data yielded a surface the co-ordinates of which, in addition to response level, were the durations of light and dark in the exotic cycles to which Beck had exposed his moths. I plotted the responses as iso-induction contours (that connect all

the cycles yielding a given response) which, to my surprise, defined a clear mountain peaking where the cycle length (combined durations of light and dark) was close to 24 hr, as though some circadian component in the insect were in resonance with the light cycle driving it (Figure 10, left).

I noticed that one transect ***(A)*** across this circadian surface yielded, as indeed it should, the standard photoperiodic response curve for diapause induction in ***Ostrinia.*** Another transect (B) corresponded with the experiments of Fritz Went, using African violets ***(Saintpaulia),*** in which he found that their growth rate was maximal when the light cycle's period was 24 hr and fell as it was either lengthened or shortened. Yet another transect (D) was the beginning of the protocol Nanda & Hamner had introduced in their ground-breaking experiments of 1959. The implication of this transect was obvious: if for every photoperiod that one used, the duration of the dark interval was extended out to, say, 72 hr, the single peak found in Beck's ***Ostrinia*** data would turn out to be only the first in a veritable range of circadian mountains.

David Saunders came to my laboratory at Stanford in 1972 just as I was about to test that proposition, using the parasitic wasp ***Nasonia,*** and he did the job using the blow-fly ***Sarcophaga,*** which he had brought with him from Edinburgh. The outcome (64) of the experiments in Palo Alto (Figure 10, right) was sufficiently rewarding to redeem the stench of ***Sarcophaga*** in our otherwise sweet-smelling ***Drosophila*** lab. I have given (62, 65) several plausible interpretations of such circadian surfaces and so has Watrus, a student of Peter Kaus, but I remain unconvinced we really understand them fully even in a formal sense.

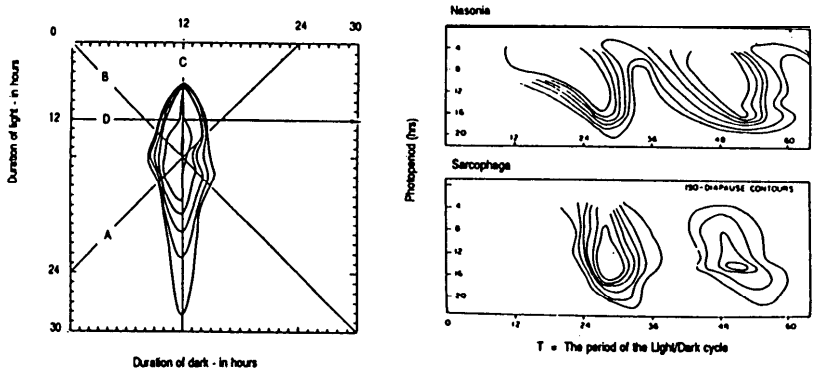


Figure 10 Circadian surfaces. (Left panel) Diapause induction in the European corn-borer (*Ostrinia nubilalis)* exposed to a wide range of exotic light/dark cycles. Iso-induction contours are plotted for response levels of 60, 70, 80, 90, 95, and 100%. The surface defined by these contours peaks near T = 24 hr. where T is the period of the light cycle (sum of the light and the dark periods) (from 62). (*Right panel)* Similar iso-induction contours for the blow fly *Sarcophaga* and the wasp *Nasonia* based on light cycles that included much longer dark periods, thus extending T, the period of the light/dark cycle, to almost 72 hr (from 64).

A Photo-Inducible Phase in the Circadian Cycle

The principal contribution of the Princeton and Stanford laboratories in developing Bunning's proposition emerged from the empirical model of entrainment. The model defined the experiments that show photoperiodic induction is not dependent on the duration of either the light or the dark in the daily cycle, but specifically on the coincidence, or non-coincidence, of light, with a very limited fraction of the subjective night. Long days are inductive not because the light lasts so long, but because, given the mechanism of entrainment, some light will coincide with the beginning or end of the subjective night. And the necessary coincidence may be very brief.

'One of the experiments that established this point involved the skeleton photoperiods discussed earlier. The two brief pulses in each 24 hour cycle define two dark intervals, each of which may be taken as the night. When one of the two intervals is shorter than 10 hr, the oscillator's subjective night always falls in the longer, no matter what the initial conditions at the onset of entrainment. If, however, the two intervals in the skeleton regime both lie between 10 and 14 hr, the subjective night may fall in either the shorter or the longer interval depending on the initial conditions. In referring to all this as the bistability phenomena, one is emphasizing that for an experimental light cycle of this unusual kind, two very different stable steady states can be realized.

My late friend Bill Hillman heard me lecture on skeleton photoperiods at Brookhaven National Laboratory in the early 1960s, before the bistability complication had been clarified. He decided, as a Bunning skeptic, to see if his own experimental material (the duckweed ***Lemna)*** would accept two-pulse skeletons as a substitute for complete photoperiods. Specifically would his short-day plant distinguish between the skeletons of 11 and 13 hr photoperiods: a complete photoperiod of 11 hr induces flowering but one of 13 hr does not.

The experimental results, replotted and related to the bistability phenomena in (66), were spectacular and perplexing to Hillman, who nevertheless recognized their clear implication of a circadian component in the induction of flowering. His assumption had been that the two-pulse per cycle regime would be taken as the skeleton of an 11 hr photoperiod, merely by having the first dark interval be 11 hr. The initial results were unclear so he then varied the time of the first pulse. The result was (one wants to say, of course) precisely what the ***Drosophila*** bistability phenomenon would have predicted. The amount of flowering was high when the initial conditions (time of first pulse and duration of first interval) assured a steady state characteristic of an 11 hr photoperiod and low when the subjective day fell in the 13 hr interval (60).

Takamura and I (67) have since obtained the same results in assaying the effect of 11 and 13 hr skeletons on the induction of diapause in ***Drosophila auraria.*** (Figure Il). In this case, two groups of pupae from the same larval

culture can be placed into the same experimental cabinet, where the two-pulse light cycle (with $T = 24$) offers the skeletons of 12 and 14 hr days, or 12 and 10 hr nights. Simply by varying the time when the two groups of pupae enter the cabinet, each of the two possible steady states can be realized: in one (12) the subjective night falls in the longer dark interval, and in the other (14) it falls in the shorter. When the incidence of diapause is assayed 10 days later, it is 27% higher in the insects on 12 (short day) than those on 14. The crucial issue is not the duration of either the light or the dark, but the phase of the circadian cycle that coincides with the brief light pulse; how far into the subjective night does the light penetrate?

This is also implied by the outcome of another exotic entrainment schedule that involves only one brief (15 min *50* lux) pulse per cycle. By making T increasingly different from τ, one can force the brief pulse further into the subjective night, and in doing so, one steadily reduces the incidence of diapause.

In 1964 Dorothy Minis and I (68) sketched a concrete version of this coincidence model: induction occurs when the maximum of a circadian rhythm in substrate concentration coincides with light that is necessary for activation of the relevant enzyme (40). This general approach, essentially pure Bunning, is now directly supported by the pioneering molecular observations of Kay & Miller (69), who find that the transcription of the *cab-2* gene in wheat is induced by photoactivated phytochrome, but the transcription is only success-

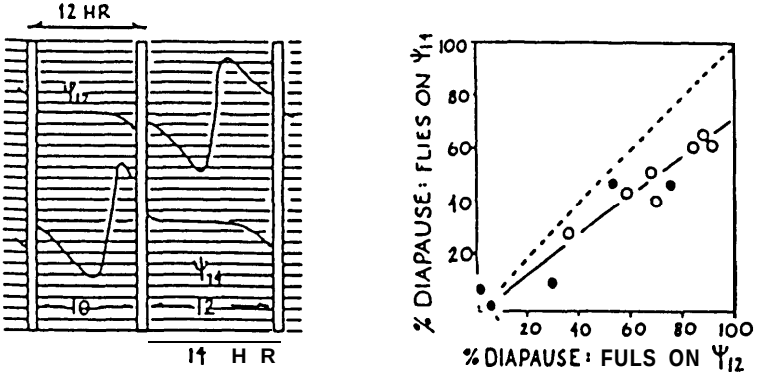


Figure 11 Photoperiodic induction and the bistability phenomena (from 67). (left) The curves are PRCs tracing the time-course of the *D. auraria* pacemaker entrained by a skeleton photoperiod regime (two I-hr light pulses per 24 hr cycle) in which the two dark intervals are IO and 12 hr, and are separated by two I-hr light pulses. Which of the two different steady states indicated in the figure is realized is determined by the initial conditions (see text). (Right) The overall level of diapause induction was varied from test to test by temperature, but in each test (a plotted point) the incidence of diapause was lower in those insects whose subjective night was compressed into the shorter dark period, as it would be on a long day.

ful when the activated phytochrome coincides with a limited phase of an ongoing circadian rhythm, the physical nature of which remains as obscure as it was when the Beltesville workers found it only a "word."

PACEMAKER AND PROGRAM : A Multi-Oscillator System

Association of Pacemaker and Photoreceptor

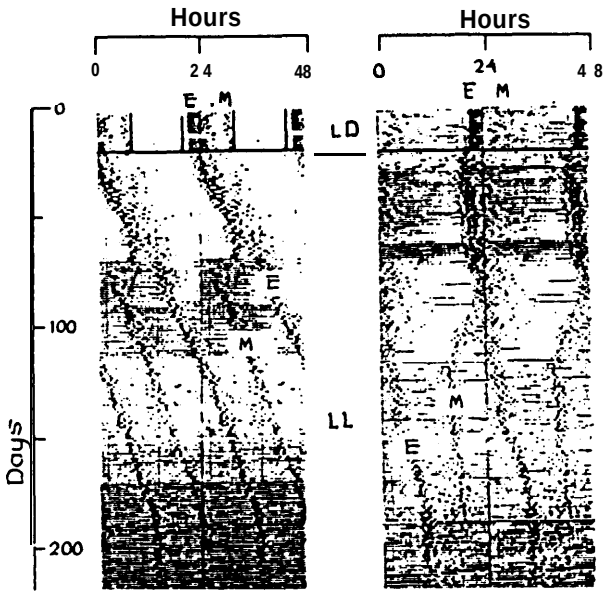
In the late 1950s, Janet Harker maintained that the oscillator driving the circadian rhythm of locomotory activity in the roach ***Periplaneta*** was housed in the insect's sub-esophageal ganglion. Enthusiastic attempts by Shephard Roberts in Princeton failed to confirm Harker (70). When Junko-Uwo came to us from Kyoto in the early 1960s, she was not only as skeptical as Roberts and myself about the Harker claim, but confident that the circadian clock of roaches would be found in their brain. In fact the experiments we did (71) indicated the optic lobes rather than the midbrain were the locus of the rhythm's bilaterally redundant pacemakers. Almost simultaneously, John Brady, a Harker student, came to the same conclusion. However, it was much later before Terry Page gave us rigorous proof based on transplantation experiments, whose serendipitous history began while he was still with us at Stanford (72).

Roaches, whose optic lobes have been surgically isolated from the midbrain, are always arrhythmic, but 30 such insects, whose utilization was inadvertantly delayed a month while Page was in Europe, were found to have normal rhythms. Histological analysis showed that regeneration had indeed occurred, which permitted the pacemakers in the optic lobes to regain control. Page then found either or both native lobes could be completely removed and replaced by implants from other roaches. Using two strains with different circadian periods (one shorter, the other longer than 24 hr) he was able to make what Menaker has since happily called a "temporal chimaera." When one lobe of a long period host was replaced with an implanted lobe from a short period donor, both periods were expressed (Figure 12, lower panel), thus demonstrating pacemaker autonomy in the implanted lobe (72).

Pacemakers of many other individual circadian rhythms have now been localized, and in all of them- insect, mollusc, reptile, bird, and mammal-- there is the same close association with a photoreceptor that we initially found in roaches, which emphasizes the dominant role of the light cycle in entraining the system.

Multi-Oscillator Structure of the Program

The first indication that control of a circadian program involves more than one oscillator came from observations Richard Swade and I (55) made on Arctic ground squirrels. Their daily band of activity was prone to break up


Hours
0
24
48
Hours
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24
48
E M
LD
LL
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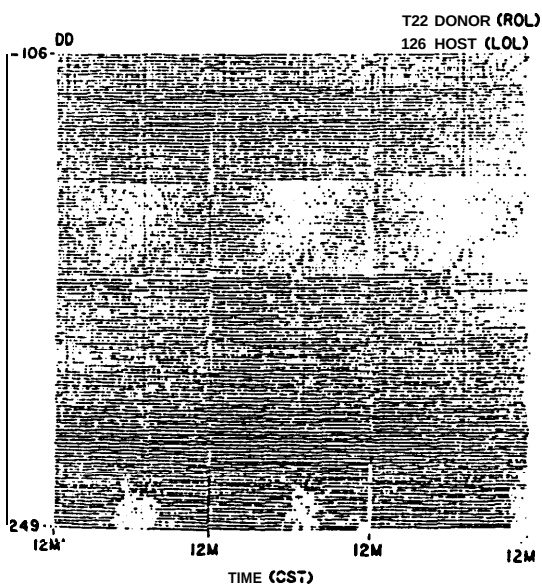
M
0
100
200
Days


T22 DONOR (ROL)
126 HOST (LOL)
DD
106
249
12M
12M
12M
12M
TIME (CST)

into two components that ran initially at two different circadian frequencies, but weeks later locked on to each other when their normal phase relation was re-established.

Such splitting of the day's activity into two oscillatory components was later encountered in more convincing detail when--once again serendipity!--the student who had tried to throw me out the window left hamsters in constant light for three months while he experimented with LSD instead of his animals. The prolonged exposure to light induced beautifully clear dissociation of morning and evening components in the daily program (9); those two components ran at very different frequencies until their original mutal phase relationship was regained or they reached 180° antiphase, which is an alternative stable steady state (Figure 12, upper panel).

Still more impressive evidence of many oscillators driving a program comes from experiments by Takahashi & Menaker (73) on house sparrows, in which the Menaker laboratory had already shown the principal pacemaker to be in the pineal gland, again the close association with photoreception. In an imaginative and elegant use of the bistability phenomenon we had found in ***Drosophila,*** Takahashi & Menaker showed that there is a host of other circadian oscillators in pinealectomised birds, each independently entrainable by the light cycle. In comparably important observations, Ishizaki and colleagues have shown that in addition to the pacemaker present in the forebrain of a ***Saturnid*** moth, there is another pacemaker, independently coupled to the light cycle, in its prothoracic glands.

In all these cases, each of the multiple oscillators is itself directly coupled to and entrained by light cycles. Control of the ***Drosophila*** eclosion rhythm, on the other hand, exemplifies multi-oscillator complexity of a different kind that has equally clear relevance to the structure of circadian programs in general. In this case, the signal that times eclosion does not come directly from the light-sensitive temperature-compensated pacemaker itself, but from a second slave oscillator that is coupled to and driven by the pacemaker. The slave oscillator differs from its pacemaker in two important respects: it is not light-sensitive, and its period is poorly temperature-compensated.

Figure 12 **Multiple pacemakers involved in a single circadian program.** (Upper panels) **Evening** (E) **and morning** (M) **components in the normal band of hamster activity (use of a running wheel) in each circadian cycle. They dissociate when the animal is exposed to prolonged constant light (LL). They continue running at different frequencies for many cycles but eventually lock on to each other again when they achieve 180° antiphase (left), or regain their original mutual phase-relationship (right) (from 9). (Lower panel) Two different circadian rhythms in one individual cockroach (Leucophea madeirae). An optic lobe from one individual whose pacemaker has a period less than 24 hr replaces the right optic lobe of a host insect whose own pacemaker has a period longer than 24 hr. When the implant has regenerated connections with the mid-brain, both periods are expressed. The animal is a "temporal chimaera" (from 72).**

This is why the daily eclosion peak is advanced or delayed slightly by temperature change, although the phase of the pacemaker is not. It also explains the temperature-induced transient (a response of the slave) that misled Kalmus & Bunning into thinking the entire endogenous daily rhythm was temperature-dependent.

The lower panel in Figure 7 summarizes one of many otherwise surprising and interesting results that are explained, even predicted, by this two-oscillator structure of the system (65) and a Kaus-Curve, which in this case concerns ψ_{sp}, the phase-lag of the slave oscillator on its pacemaker. That phase-lag is primarily a function of p, which is the ratio of the slave period over the pacemaker period, τ_s/τ_p. When entrainment by light shortens τ_p, the phase-lag of slave on pacemaker is predicted to lengthen (p has increased), but to shorten when entrainment lengthens τ_p. As the lower panel in Figure 7 shows, that is what one observes: the eclosion peak in each light/dark cycle coincides with an increasingly later phase of the pacemaker's cycle as its (pacemaker's) period is shortened by entrainment.

Collectively these observations on rodents, sparrows, moths, and flies suggest the many rhythmic components in a complex circadian program may well be timed by separate oscillators, some of them directly and others indirectly coupled to the light cycle, as the program's primary entraining agent. Even when current knowledge emphasizes the importance of a single program-pacemaker, like the supra-chiasmatic nucleus in mammals, it seems likely its control of the overall program reduces to entrainment of slave oscillations inherent in those systems it times.

While this multi-oscillator interpretation of an overall program has long seemed plausible and adequate for multicellular systems, the unicellular dinoflagellate *Gonyaulax* has eluded it. That single cell manifests five very different circadian rhythms whose maxima occur at different times of day. Phase-response-curves for the oscillation driving four of them are indistinguishable, which suggests that a common pacemaker drives all (75). While one cell/one pacemaker may have seemed plausible, it has always posed a major, if neglected, problem: how does a common pacemaker control such radically different processes as photosynthesis, bioluminescence, and mitosis? However, what is surely one of the most important developments in circadian physiology in many years appears to dispose of that problem: T. Roenneberg & D. Morse (personal communication) find that at least two of the *Gonyaulax* rhythms free-run with radically different periods, which implies that the program in this single cell clearly involves more than one pacemaker. While that disposes of the problem I thought *Gonyaulax* raised, it poses another equally challenging one: how is a light-sensitive, temperature-compensated oscillation incorporated into each of the separate components of a single cell's circadian program?

THE EVOLUTION OF CIRCADIAN ORGANIZATION

Order Precedes and Selects for Organization

The closing section of this essay, like its beginning, is Darwinian: it asks about possible origins of circadian organization as a guide to the concrete nature of its pacemaker. How did the Demon get started on programming a day within? What were the earliest selection pressures? Were they different from those prevailing today? Is their impact, given the Demon's lack of imagination, still detectable in the life of contemporary cells? Knowing his style, what can we expect?

Answers to most of these questions are necessarily conjectural, but one thing is sure: the daily cycles of temperature and light in the outside world must have imposed significant and predictable periodicity on the chemical milieu of early cells. Such order, not yet organization, would derive from inevitable variation in the temperature coefficients of the cell's constituent reactions, and the bottle-neck created by the cold at night. In a similar way the daily flood of UV and visible radiation must have created other day-night differences in the cell's photochemically reactive constituents. The resultant ***temporal order*** within the cell was probably as effective as the external cycles themselves in selecting for ***temporal programming:*** the predictable timing of one cellular event determined the optimum time for another. Such selection by temporal order internal to the cell ("The Day Within") is surely as old as that exerted more directly by the external cycles themselves ("The Day Outside").

Eukaryotic Generation Times

It seems equally clear, however, that such selection could only be effective after the cell's generation time had become as long or longer than a day because the Demon has elaborated no temporal programs whose duration exceeds the generation time of the individual organism: there are no circannual programs in organisms living less than a year; no lunar programs unless the individual lives more than a month. The nearly complete exclusion of circadian programming from prokaryotes is therefore more likely a function of their short generation time than evidence of an inadequate structural complexity. Strong support for this comes from the recent finding of a fully temperature-compensated circadian pacemaker in one prokaryote whose generation time matches that of eukaryotes (T. Kondo et al, personal communication).

In any case, the ubiquity of circadian programming in eukaryotes is a sharp reminder not just that their generation times are so much longer than those of prokaryotes, but that they cluster around the period of the earth's rotation. Lengthening its generation time must have evolved before the cell could respond to pressure for circadian organization as such. What was the Demon

up to in lengthening the cell cycle? Was some crucial step in that cycle, such as copying or reading the genome, more successfully undertaken at one time of day than another? The gating of DNA replication and subsequent mitosis to the darkness of night in many flagellates certainly encourages that possibility. Whatever the pressure may have been, it is clear that the Demon has drawn out the cell cycle of eukaryotes and in so doing met a fundamental prerequisite for the subsequent (or concurrent?) evolution of circadian programs.

Escape from Light

The idea that the daily light/dark cycle has been a source of selection as well as an entraining agent in the history of our clocks was prompted by recalling a memorable exam question in graduate school: “Write an essay on the pre-requisites for organization in a chemical system." The answer that was looked for then (it was covered in lecture) was the necessity to avoid reactions that would proceed spontaneously at physiological temperatures; “relays” in the form of enzymes are essential for the necessary control. So much for the thermochemistry of the cell; it was much later before I saw the cell’s daily bombardment by visible and UV radiation as a quite different, photochemical, threat to organization that specific protein relays cannot cope with. The Demon may have found, as usual, several ways around the problem including screening pigments, like flavins, at the cell level, as well as hair and colored skins at higher levels. A less obvious, but more intriguing, possibility was to select, whenever possible, for colorless alternatives among essential molecular devices; other, of course, than those exploited in energy-capture and vision. When colored components could not be replaced, was the last resort to restrict their involvement in the life of the cell to the darkness of night?

1 think it likely (74) that such an escape from light has played a significant, indeed major, role in the evolution of circadian organization: that the entraining action of light will prove more complex than we have previously assumed (cf 78, 79) and reflect its earlier and overlapping role as an agent of selection.

Origin of the Photoperiodic Response

A especially appealing aspect of this possibility is its bearing on the circadian component in photoperiodic induction. In selecting primarily for temporal restriction to darkness did the Demon inevitably find some photo-sensitive reactions, still on the edge of the subjective night, were better left there? Photo-activated, but only on longer days, their temporal location in the cell’s daily program provided a mechanism for sensing and responding to season. It would have been typical of the Demon to stumble on this role for daylength while responding to the different and more pervasive pressure posed by the daily bombardment of light.

Reading the Genome: Reading-Loop Oscillations

The photoperiodic phenomena themselves are most easily envisaged as the induction or suppression of a seasonally appropriate gene or genes. Is some aspect of protein synthesis impacted by light, thereby setting a premium on its relegation to darkness? Several recent developments leave no doubt that reading the genome is a central issue in circadian programming: the transcription of many genes, several in ***Neurospora (80)*** and ***Drosophila*** (R. van Gelder, personal communication) and one in wheat (69), is clock controlled. The ***Drosophila*** data from Russell van Gelder go further and encourage the idea that reading the genome has been part of the flight from light: the transcription of 19 out of 20 clock-controlled genes he has studied is restricted to the fly's subjective night. Why has the Demon left only 5% of these transcriptions exposed to light?

Other observations offer encouragement to the more radical proposition that the reading process is not just clock-controlled, but the fabric of the clock itself, as Ehret & Trucco (82) suggested years ago. The earliest of these was Jerry Feldman's pioneering finding, repeated and extended now with other drugs in a host of other organisms, that slowing protein synthesis slowed a circadian clock in ***Euglena (28).*** More recent and much more persuasive is the finding by T. Roenneberg & D. Morse (personal communication) that there are of at least two different pacemakers in the single ***Gonyaulax*** cell. But most impressive are the beautiful recent data from Hardin et al (83) on the circadian periodicity of transcript and gene product at Konopka's ***per*** locus in ***Drosophila.*** They strongly support the hypothesis that feedback of gene product on gene promoter is the core of the circadian oscillation. There is even evidence that light interferes with that loop in a way that would permit entrainment (48).

I am reminded here of two favorite lines from Eliot's "Little Gidding," "For last year's word belongs to last year's language/ And next year's word awaits another voice." Clearly our molecular colleagues have introduced "next year's words," giving us wholly new ways of framing questions and propositions one could state only vaguely in "last year's language." Russell van Gelder's data infuse new life into the escape from light adventure and my old suggestion that much circadian programming has a pacemaker-slave architecture. The new language would see it as cascading inhibition or stimulation of promoters at other oscillating (slave) loci by the gene product of a pacemaker locus itself directly entrained by light.

But strong as the promise of progress is here, it is also clear that the idea of a reading-loop oscillation has limited scope, even if valid. Tidal, lunar, and circannual clocks certainly elude such explanation and demand an entirely different fabric. On the other hand, such limitations, and there are others in

the circadian domain itself, are no reason to reject the idea as valid within a limited scope. The Demon is notoriously prone to accept any solution that meets the prevailing challenge. So while there is good reason to think reading loop oscillations are involved in much circadian programming, either as pacemaker or slave, there is equally good reason to suspect there is much more to the story (84).

Stonehenge

One of my goals in these closing sections has been to illustrate-and enjoy!-what I take to be the real adventure in the scientific enterprise, which is, among other things, a search for pattern and meaning in what one observes. It is the necessarily conjectural nature of that search, at least at the outset, which entails hazard and makes the enterprise adventurous, not only for the observer himself, but for his observations and ideas. "Escape from light" is just such an adventure, which one enjoys for its own sake, and possibly will prove useful, perhaps even correct.

I have heard that at at the end of his Caltech lectures on "Mind from Matter," Max Delbruck admonished his very bright audience not to forget those people who, 4000 years ago, built Stonehenge. They, too, were very bright and probably knew it, but did they realize how much they didn't know? Understanding circadian and circannual programs has a long way to go.

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