CHAPTER 1

ENDOCRINE CONTROL MECHANISMS AND MODELING RHYTHMIC BEHAVIORS

THE modeling of biological clocks using physical machines, chemical reactions, and electronic circuits that one observes in the papers presented at the twenty-fifth Cold Spring Harbor Symposium on Quantitative Biology in 1960, which was devoted to biological clocks, presupposes that such clocks exist in organisms and that their behaviors can be experimentally studied and modeled. Therefore, before physiologists began to even think about modeling clocks, they had to make the conceptual leap from focusing their research on the physiology of stimulus-response systems that showed cyclical behaviors to thinking about those behaviors as characteristic rhythms, and then to looking for organic structures that have rhythmic functions. Put another way, attention to physiological systems that regulate observed rhythmic behaviors would have to shift to investigation of rhythmic controlling mechanisms, which then could be studied abstractly as oscillators and clocks.

Much of the early work on endocrines and their roles in controlling retinal pigment migration and color changes in some arthropods and lower-order vertebrates began with questions of how such changes were controlled, and experimental physiologists directed their research programs toward understanding how neural or endocrine control systems translated stimuli into responses. Daily patterns in the variability of the color changes, known since the turn-of-the-century studies by Frederick Keeble and Frederick Gamble, were at first viewed as undesirable experimental complications, aggravating assessment of stimulus-response studies. Only gradually did these researchers

come to recognize the rhythmic nature of these changes as a characteristic biological feature, the explanation of which challenged materialist-mechanist philosophy and bore on discussion about how instinctual behaviors were learned and transmitted from generation to generation. How these physiologists got from probing systems for controlling chromatophore dilation and contraction to thinking about the controlling endocrine glands as themselves rhythmic mechanisms, as oscillating biological clocks, is a prerequisite for modeling and finding such mechanisms.

If organic mechanisms that produce rhythms could be isolated in specific parts of organisms, they could in principle be identified by histologists, and physiologists could then study them experimentally by stimulating them in various ways and observing how the rhythms change. But even if one could locate clock structures, looking into them to discern their component structures and how these worked was beyond the technical capability of biologists before the development of molecular biology and molecular genetics in the 1970s and the innovation of tools such as genetic knockouts, cloning, and sequencing in the 1980s. In the meantime, the clock mechanisms were treated as "black boxes." Their functions could be studied from the outside, without opening them up to see the actual parts, so to speak. In this way relationships between inputs to the mechanism and the resulting outputs from it could be determined, permitting inferences about its structure.

The speed of the clock regulating an organism's rhythm could be altered experimentally by altering its inputs—namely, environmental conditions or triggering factors (mainly changes in illumination, humidity, or temperature), and then the effects of these changes on the clock could be gauged by corresponding changes in its outputs (its rhythmic behaviors). Adjusting the inputs was likened to setting the clock so that its hands pointed in the right directions; observing the outputs was compared to looking at the motion of its hands, the outward effects of the hidden timing mechanism. The analogy was imperfect, but it served as an important reminder that there might not be a perfect connection between what was going on inside the box (the timing mechanism) and what was observed from its exterior. Also, its use is an indication of how vested researchers were in the clock as a metaphor for biological rhythmicity.

Without inspection of the inner workings of the organism's clock, the only way to discern how it might be functioning internally was through carefully designed experiments that controlled the inputs and then interpretation of the resulting changes in outputs. From this it was possible to construct both virtual and physically real models, the behaviors of which could be compared to the natural and experimental phenomena. In the case of a clock, one can adjust it to a new phasing by resetting its hands each day—for example, to

point to "dawn" or "dusk" as the beginning of the organism's resting period, or one can do this by adjusting its speed to coordinate the clock's rhythm with that of a key environmental cycle. Colin Pittendrigh, one of the leading voices in biological rhythms research in the 1950s and the chief organizer of the 1960 Cold Spring Harbor Symposium on biological clocks, favored models in which transitions in illumination or other factors set or rephased clocks, and his German colleague and collaborator in this effort, Jürgen Aschoff, favored models in which cumulative exposure to these factors (e.g., daylight) altered the clock's speed. Different sorts of models were brought to bear on how these different modes of clock synchronization might be accomplished—by regularly resetting the clock or adjusting its speed to keep it in tune with key environmental factors.

Once biologists recognized rhythms as important biological features, they sought to fit them into the overall picture of organic evolution. For this reason researchers experimentally adjusted those environmental inputs that they presumed affected organisms' temporal adaptation and conveyed fitness, chiefly natural daily cycles of illumination, humidity, or temperature. In the case of illumination, the environmental factor most commonly altered in chronobiological experiments, researchers generally altered the lengths of the light and dark periods (the L:D cycle), which together constituted the experimental day length. For example, a twelve-hour light period followed by a twelve-hour dark period (denoted as L:D = 12:12) evenly divided the twenty-four-hour "day" into experimental daytime and nighttime spans, sometimes called phases. The term *phase* is also used in a more technical sense, to mean a time relation between a rhythm and some fixed time (e.g., dawn, midnight, or the time of day an experiment commenced), which is not to be confused with day and night phases. Similarly, an L:D = 8:16 experiment would indicate eight-hour light phases alternating with sixteen-hour dark phases, simply altering the illumination balance of the (8 + 16) twenty-four-hour day. Another variable that could be altered is when the transitions between the light and dark phases began with respect to the natural day, by shifting the schedule. An extreme example of this is an inverted schedule (D:L), which is useful for studying the effects of night work on human physiology, for example, but is more generally a technique for experimenting with the black box clocks to make inferences about their operations. With such an abrupt shift, observing how the biological clock adjusts to the new schedule—by leaping forward or falling back, as it were—and how rapidly it succeeds in making the adjustment provided modelers with important parameters.

Pittendrigh and many other chronobiologists assumed that the biological clocks of many species are adjusted by natural L:D transitions and that their clocks could be experimentally studied as black boxes by experimentally

varying these conditions or by altering the corresponding temperature and humidity cycles, depending on which variables were being studied. But one could also see how clocks respond to unnatural L:D regimens—L:D = 18:18 (a thirty-six-hour "day"), for example, or L:D = 6:6 (a twelve-hour "day"), or even continual light (denoted simply L:L) or continual darkness (D:D). The point of these experiments was not to observe how organisms' rhythms adjust to natural rhythms but, rather, to study the properties and operations of the biological clocks as mechanisms.

One important experiment used in biological rhythms research is to synchronize or "entrain" the subject's biological rhythm to a regular L:D cycle and then subject it to continual light or dark. This was what first alerted scientists to the existence of internal biological rhythm—the rhythm that persists when the presumed stimulus is stopped (see many historical examples in volumes I and II). For modeling the biological clock mechanism, the key point is that it has a natural speed determined by its physicochemical parameters and that these can be controlled or experimentally modified (by the administration of chemical toxins, for example, or by changing the ambient temperature at which the clock operates) but also to see how the mechanism responds to different triggering or resetting stimuli. The stimuli that acted to reset or synchronize biological rhythms were variously referred to as *timing cues*, *synchronizers*, or *pacemakers* among English speakers, but eventually the international scientific community adopted the German term *Zeitgeber* (meaning "time giver").

Experimental changes of state of the suspected Zeitgeber, usually an L:D change, were typically rather abrupt, likely because this was practical in the early days of artificial electrical illumination, and this became standard practice. Perhaps for this reason, early twentieth-century botanists and zoologists studying rhythms, in addition to tinkering with the L:D phases, also subjected their specimens to light "pulses"—namely, very short-duration exposures to fully bright light in what was otherwise total darkness. Conversely, lights could be turned out for short times in otherwise continual light to produce "dark pulses." The point was to see how the black boxes responded to single, abrupt resetting signals, the length of which could not by itself convey to the subject any appropriate rhythmic information. Erwin Bünning used light pulses to reset the timing of daily plant-sleep (nyctitropic) movements of bean leaves in the 1930s. But in the 1950s researchers realized that the point at which the Zeitgeber was applied to specimens during their natural or experimentally entrained L:D cycle determined whether the timing of the clock's rhythm, its "phase" with respect to its original state, was advanced or delayed, with the result that "dawn" or "dusk" activities were pushed earlier or later on subsequent days. In the late 1950s it became apparent to researchers that a

graph of the clock's time shift, plotting the advance or delay against the time in the original cycle at which it was subjected to a resetting pulse, was characteristic of the organism under study. This was standardized as the phase-response curve (PRC), and the similar shapes of these curves for various species permitted researchers to gain insights into the nature of the biological clocks. The history of the PRC is therefore an important part of the story.

THE SEARCH FOR CONTROL MECHANISMS LEADS TO THE SEARCH FOR CLOCKS

When the English experimental physiologist Lancelot Hogben recognized the rhythmic nature of the alternating dispersion and concentration of pigment in the chromatophores of certain kinds of crustaceans, lizards, and amphibians, which produces changes of coloration, he was looking for the mechanism by which this behavior was controlled. He continued to orient his research in this way in a series of papers over the next several years. He termed this "The Pigmentary Effector System" in his 1924 monograph with that title, and he maintained this phrase in multiple research papers. He designed his experiments in the context of the early development of endocrinology and framed them within debates about whether such behaviors were regulated by neural systems or humoral (endocrine) systems. This was fundamentally a discussion among physiologists, and Hogben, like Elmer Perkins, John Walsh, G. H. Parker, and other biologists working on organisms' color changes in the 1920s and 1930s, was looking for *control systems* in the context of the stimulus-response paradigm that was current in early twentieth-century biology. Consequently he did not conceive of these controls as intrinsically rhythmic, as *clock mechanisms*. Similarly, when Elmer Perkins and Gottfried Koller were snipping the eyestalks off crustaceans in 1928, they were looking for a gland, a *control mechanism* that regulated chromatophore expansion and contraction.

After moving to Cape Town and resuming the research he had begun in England, Hogben and his South African collaborator David Slome were still looking for control systems, postulating separate systems for expansion and contraction. They were not looking for clocks. Their work aimed to apply the pigmentary effector system concept to the South African claw-toed frog *Xenopus lævis* and to confirm that the endocrines produced in the pituitary gland, which they had used to effect chromatophore expansion and contraction, played a necessary role in color change. In their 1929 paper "The Time Factor in Chromatic Response of *Xenopus Lævis*," they proposed two separate hormone control mechanisms, one for expansion and another for contraction. They called these "mechanism B" and "mechanism W" and associated them with "B substance" and "W substance," respectively, locating the mechanisms that produced these substances in separate parts of the pituitary gland. They were clearly thinking about control mechanisms and not clocks; rhythms

were not under scrutiny. However, they had observed "a decided tendency for the melanophores to be more contracted at midnight," which they interpreted as a feature of the frog's pigmentary response system and thought that it was in all likelihood conditioned by the daily alternations of light and dark in its environment.[1] In 1931 they localized the relevant endocrine production to the hypophysis (pituitary gland), but with no argument for its rhythm or the rhythmic function of the gland.[2] Similarly, John Z. Young investigated color changes in lamprey eels in 1935 and posited the existence of an "inner mechanism" that governed the rhythmic changes, but locating it did not seem to interest him or other marine biologists, who were intent on the physiology of color change and not its rhythmicity per se.[3]

Perhaps it was the idea of isolating the source of the substance controlling rhythmic changes of coloration to a specific organ that shaped a rethinking of such structures not as control mechanisms but as autonomous timing mechanisms. The idea of a biological clock as a metaphor to characterize biological rhythms was not new in the twentieth century. The general notion that biological rhythms were a function of a living clockwork of some sort was implicit in Julien-Joseph Virey's description of the *horloge vivante* (a living clock) already in 1814, and various references to insects and plants having an *Uhr* (clock) by Oskar Wahl, Hans Kalmus, and others in the early 1930s signify that biologists for many years had been thinking about rhythmic behaviors as timed by a clock of some sort.[4] It was not until later in the decade, however, that Kalmus and other rhythms researchers began to consider locating the clock mechanisms in specific organs.

In 1938 Kalmus noted that using a ligature to constrict the flow of fluids or impulses through parts of the body suggested that the organ controlling color change in the walking-stick insect *Dixippus* was located in its head. The next year he published the results of controlled illumination experiments on the timing of *Drosophila* eclosion, when the adult fly emerges from the pupa, which demonstrated that whatever mechanism controlled eclosion must be located in the head portion of the pupa, indicating that he was in fact intentionally looking for the "central clock" (*Zentraluhr*).[5] This promising line of investigation was abruptly interrupted when he was removed from his laboratory in Prague as a non-Aryan and he fled to London. Upon resuming work there, with the larvae of the salamander *Axolotl*, he found that surgical removal of its hypophysis eliminated the persistence of daily rhythmic behavior in constant darkness, which was evident in intact controls. This implies that he was attempting to continue his pursuit of the clock in the Galton laboratory.[6] When the laboratory was bombed during the Blitz and was forced to relocate, Kalmus apparently did not continue to search for specific timing organs.[7]

At the same time that Kalmus was beginning to locate the rhythmic control in *Drosophila* in Prague, Harvard invertebrate zoologist John Welsh began to think about crustacean rhythms as generated by some kind of endogenous biochemical oscillator, just about the time that World War II began to sidetrack fundamental biological research worldwide. Swedish invertebrate endocrinologist Bertil Hanström, who visited the Marine Biology Laboratory at Woods Hole, recommended looking for it in the suboesophageal ganglion, but by 1941 Welsh had not localized a biochemical oscillator to any specific center and was instead thinking about rhythm as a systemic relationship rather than as the function of a specific organ.

In Cambridge, England, Kenneth Mellanby was also contemplating a timing mechanism, in the cockroach, but his publications do not indicate he attempted to find it. He had presented at the second meeting of the International Society for the Study of Biological Rhythm (ISSBR), which met in Utrecht in 1939 just weeks before the German invasion of Poland triggered the war in Europe, and published a study of the rhythms of the cockroach *Blatta orientalis* in the *Journal of Experimental Biology* in 1940. Donald Gunn, E. W. Bentley, and Dennis W. Ewer were also working on the rhythms of insects in England at that time. Gunn, at the University of Birmingham, published the results of his experiments with the activity rhythms of *Blatta orientalis* in the journal that year, and he collaborated with Bentley and Ewer on a paper on the beetle *Ptinus tectus*, which appeared in the journal in 1941. So there was a small, but active group of rhythms researchers in Cambridge and Birmingham studying the rhythmic behavior of insects, in particular cockroaches, when the exigencies of wartime generally suppressed research programs unrelated to the military emergency. The cockroach was a good choice for such research, owing to its striking daily rhythm of activity onset under natural conditions and the ease with which it can be raised and studied in the laboratory. This research provided a good foundation for resumption of chronobiological study at Cambridge after the war, notably by John L. Cloudsley-Thompson and Janet Harker, both of whom became leaders in the field during the 1950s and wrote textbook introductions to biological rhythms in the 1960s.

Cloudsley-Thompson was a student at Cambridge when World War II commenced, and he broke off study to serve with the Seventh Armored Division in the North African desert campaign and then took part in the invasion of Normandy.[8] His time in the desert engendered a keen interest in the adaptations of insects and other animals in order to survive in the harsh climate, which called for avoiding the midday heat and the evolution of strategies and structures for conserving body moisture. When he returned to Cambridge

after the war, he undertook to publish several studies of myriapods (centipedes and millepedes) while writing his doctoral thesis. The first two of these concerned the peculiar migratory behavior of millepedes and where they fit into ecology, but a third turned to their rhythmic behavior, in which he employed the standard methods of experimental control of changes in illumination and temperature and the use of the aktograph to make a time record of the insects' movements. This was the first of three articles he would write on the diurnal or daily rhythm of insects, and he pointedly situated his work in the context of biological rhythms research by Welsh, Orlando Park, John Calhoun, and Bentley, Gunn, and Ewer. He determined that all four species exhibited diurnal rhythms, but that the two tropical species were more sensitive to shifted temperature cycles than to shifted light cycles, a characteristic he attributed to their evolution in dark forest habitats.[9]

After submitting his thesis in 1950, Cloudsley-Thompson was appointed to the Department of Zoology at King's College, University of London, where he published a second paper on diurnal rhythms (this time pertaining to the wood louse *Oniscus asellus*) before turning to the rhythms of the cockroach *Periplaneta americana*. He published the results of this work in 1953, situating it in the context of Gunn's research on cockroaches. He thanked Gunn both for useful advice and for lending him an aktograph to use to verify the accuracy of the one he had earlier constructed for his millepede work.[10]

There is not much strikingly new in Cloudsley-Thompson's article, which mainly verifies earlier findings by Mellanby and by Gunn and his collaborators, but it is exemplary in comparing behavior under eighteen-hour L:D regimens (9:9 and 3:15) to behavior observed with twenty-four-hour (3:21 and 21:3) and forty-eight-hour (24:24) regimens, affirming Gunn and Mellanby's finding that the activity period is concentrated mainly in the hours after the onset of darkness and that eighteen-hour cycles were less effective for entraining the roaches than the longer cycle lengths. Following up on a question posed by Gunn in 1940, whether light acts through the compound eyes to effect synchronization of the behavior rhythm, Cloudsley-Thompson found that both removal of and painting over the eyes abolished rhythmicity, but so did painting over other light-sensitive organs called ocelli, implying a complex entrainment pathway in this insect.[11] In 1952, two years after Cloudsley-Thompson left Cambridge for London, Harker began her studies on cockroaches at the University of Cambridge, where she successfully adapted a technique called parabiosis, joining two living cockroaches to explore the role of endocrines in the control of activity rhythms, and the surgical "remove and replace" method to isolate and explore the physical source of daily behavior rhythm.

JANET HARKER AND SURGICAL METHODS TO LOCATE THE CLOCK

Harker completed a master's degree in science at the University of Sydney in 1949 and pursued a PhD in zoology at the University of Manchester. She completed her thesis in 1951 ("A Study of the Factors Affecting the Distribution of the Fauna of a Moorland Stream") and was appointed assistant lecturer in zoology at Manchester. She soon resigned to accept a position as lecturer at Girton College, University of Cambridge, in the fall of 1952.[12] As the title of her thesis implies, Harker's original project at Manchester was an ecological study of four species of mayfly nymph, but in the course of her research she extended it to include the biological rhythmicity that these insects exhibited, which she also explored experimentally: "All four species showed a diurnal rhythm with the greatest peak of activity in the early morning. Neither light nor temperature affected the rhythm once it was established."[13] She was thus becoming interested in biological rhythms about the same time as Pittendrigh on the other side of the Atlantic Ocean, and they would read and react to each other's scientific work over the next decade or so. As a result of experiments she conducted with artificial lighting conditions, she concluded that "the rhythm of activity was in no way altered by the reversals of illumination," but elsewhere she noted that an experiment where darkness was imposed from 10 a.m. to 4 p.m. established a rhythm in the nymphs that was different from that of the controls.[14]

Another experiment may have led Harker to conclude that the daily rhythms of insects are inculcated in early development, an idea that ran contrary to the notion of an inherited clock and that was later sharply criticized by Pittendrigh. Taking mayfly eggs immediately after oviposition and keeping them in constant light (L:L) for seven months, she "found that the rhythm had either been broken or not developed. There was no rhythm in the activity at all, and the total activity had dropped considerably."[15] However, exposure to normal L:D alternations for one day was sufficient to initiate a rhythm that persisted when the insects were returned to L:L. Her reference to studies of time memory in bees by Ingeborg von Stein-Beling (1935) suggests that Harker may have been thinking of the persistence of rhythm as a learned behavior that was durably impressed in early development. References to work by Kalmus (1938), J. S. Szymanski (1914), and Frank Lutz (1932) indicate that she was aware of some of the general literature on biological rhythms, but she also referred to more immediately local work on insect rhythms, a paper by Mellanby (1940) and the collaboration by Bentley, Gunn, and Ewer (1941).

Harker's publication of some of the results of her doctoral research in the *Journal of Experimental Biology* the following year (1953) indicates that it was the mayflies' daily rhythms that had captured her interest. The methods she

used to study the mayfly rhythms were standard in biological rhythms research at the time—namely, controlling or altering environmental factors (mainly illumination and temperature) to study their effects on the manifest rhythmic behaviors. In this paper she cited only two previous publications, one of which was her own, and did not engage in any experimentation with or speculation on endocrines or other internal physiological factors, implying that she was not informed of the work of Hogben and others on endocrine controls of rhythmic behavior at that time.[16] With her appointment to Cambridge in 1952 she switched over to working with cockroaches. Several factors may have contributed to this fortunate choice, one of which was that there were others in England who had worked on or were working on the activity rhythms of cockroaches—notably Gunn at the University of Birmingham, whose work she had studied while she was a graduate student at Manchester, and Cloudsley-Thompson.

Harker's first paper after her move to Cambridge (1954) reported the preliminary results of her surgical experiments on the American cockroach *Periplaneta americana*, which aimed to elucidate the role of hormones in producing activity rhythms in roaches. Employing the method of parabiosis, she removed the dorsal chiton of two roaches, of which one had been synchronized to a 12:12 light-dark cycle and the other had been raised in continual light and expressed no activity rhythm, and surgically joined them back to back. This permitted the couple to share body fluids without any neural connections. She then removed the legs of the rhythmic top cockroach to prevent them from interfering with the couple's movements and observed that the pair moved about with the rhythm of the legless top roach. Harker deduced from this that the temporal controls were transmitted humorally and not neurally, probably by a hormone.[17]

It seems likely that the immediate model for Harker's new research direction was Dietrich Bodenstein's 1953 study of the role of hormones in cockroach molting, since she stated in a later paper that she had used Bodenstein's surgical method and cited his 1953 paper on the use of parabiosis on cockroaches.[18] Like Harker, Bodenstein had opened a window in the chiton on the backs of the roaches and glued them together, back to back, to enable the paired specimens to share a common fluid circulation but without interconnecting their nervous systems. The method was clearly useful for physiologists who were attempting to discern neural control from humoral control, as any hormone produced in one roach would be shared with its parabiotic mate, which should react to it accordingly. The American cockroach nymph molts ten times before emerging as an adult, and Bodenstein was able to show by surgical removal of glands in some instances and parabiotic joining of juveniles to mature roaches in others that a hormone secreted from the *corpora*

allata of the juvenile—small organs near the brain—suppressed the metamorphosis of the later-stage roach, causing supernumerary moltings and delaying its metamorphosis into an adult.[19] This may have suggested to Harker that a structure controlling insects' rhythmic activity behavior might similarly be identified.

It is easy to see how Bodenstein's research would have come to the attention of Harker, whose work at Manchester had also been on the life cycle of insects. Bodenstein's parabiosis would have been an obvious choice for study of endocrine mechanisms that control development and could be adapted to search for the clock that regulates daily activity rhythm, such as she had observed in mayflies. What she added in her further experiments, reported in 1955, was the transplantation of specific endocrine organs from donor roaches into hosts in which these organs had been ablated, in order to ascertain their function. This is a variation on the "remove and replace" method that endocrinologists used to verify the specific functions of glands—remove the gland and remove the function; restore it and restore the function.

Having determined from her parabiosis experiments that the mechanism for daily activity rhythms most likely involved the secretion of a hormone, she turned to search for the organ that secreted the hormone, finding a likely suspect in the suboesophageal ganglion: "Implanting a sub-oesophageal ganglion from a cockroach which has a normal rhythm into an intact cockroach which has been kept in constant darkness or light and has lost its rhythm," she wrote, "causes the implanted cockroach to take up a normal rhythm" (see figure 1.1, upper histogram).[20] Moreover, her experiments affirmed Cloudsley-Thompson's finding that the insect's eyes and ocelli were somehow involved in regulating rhythms, since ablation or painting them black could rephase the rhythm or destroy it altogether. She found that other major secretory organs—the *corpora allata*, *corpora cardiaca*, as also the roach brains themselves—did not transfer rhythmic behaviors when she transplanted them, which reinforced her conclusion that the suboesophageal ganglion was the organ chiefly responsible for the rhythm and that this was controlled somehow by the ocelli.[21] She was closing in on the timing mechanism of the cockroach and was on the track of how changes in light, mediated by the light-sensitive ocelli, might be linked to it.

Harker's work through 1956 was narrowly focused, and her research publications contain remarkably few authoritative citations to the work of her colleagues and predecessors. This changed as she prepared an extensive review article on diurnal rhythms in animals for *Biological Reviews* in 1957, which referenced 346 articles and another 20 in an addendum made just prior to publication in 1958. Encouraged by her wide reading of the biological rhythms literature to view her own work in a larger perspective, she was careful at that

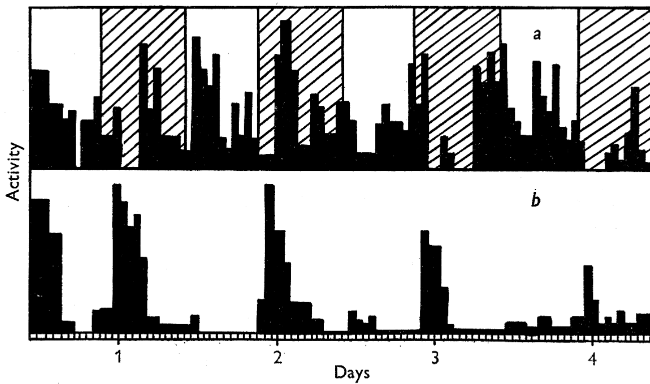

Fig. 5. (*a*) The activity of a headless *P. americana* in alternating light and darkness. (*b*) The activity in continuous light of a headless cockroach into which had been implanted the sub-oesophageal ganglia from a normally rhythmic cockroach.

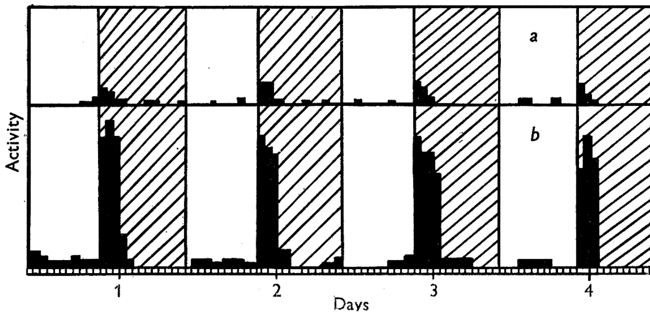

Fig. 6. (*a*) The activity of an entire *P. americana* into which had been implanted two sub-oesophageal ganglia from normally rhythmic cockroaches. (*b*) The activity of a headless cockroach into which had been implanted two sub-oesophageal ganglia.

FIG. 1.1. Cockroaches, nocturnal animals, exhibit strongly marked activity onset after dusk and therefore are useful models for chronobiological research. Figure 5a from her 1956 paper in the *Journal of Experimental Biology* shows the somewhat asynchronous activity of the headless roach, lacking the distinct periods of nighttime activity and daytime quiescence characteristic of healthy roaches under natural conditions of illumination, which exhibited marked rhythmic behavior (5b) when she implanted a suboesophageal ganglion that she had removed from the head of a roach that had been entrained to natural alternations of light and dark (L:D). The lower figure shows the narrowing of the activity period when two ganglia are transplanted into an intact roach (6a) and headless roach (6b), suggesting that the donor ganglia interfered with the host ganglia, but successfully produced a rhythm in the headless roach. These experiments strongly suggested that the suboesophageal ganglia were biological clock mechanisms or essential parts of such mechanisms. Janet E. Harker, "Factors Controlling the Diurnal Rhythm of Activity of *Periplaneta americana* L.," *Journal of Experimental Biology* 33, no. 1 (1956): 230, figs. 5 and 6.

point not to assert that the suboesophageal ganglion was *the* biological clock but, rather, stated that it was "only part of a chain of intermediaries, all of which must be present for the rhythm to be expressed."[22] In other words, she

was being very cautious about distinguishing the clock from its rhythmic expression, its "hands." She explicitly stated in the summary that "the 'physiological clock' controlling 24-hour rhythms has not been found" but that hormones appeared to be the intermediaries in the system. Her final point was that the new interpretation of the experimental evidence to date was that a twenty-four-hour clock exists in all animal cells, but that some "cell or group of cells may constitute a 'physiological clock' regulating certain activities" and that there may be several such clocks functioning simultaneously in the animal.[23]

When she later that year (1957) submitted to the *Journal of Experimental Biology* her findings of a relationship between induced dysrhythmia and carcinogenesis, which in a sense was an unexpected byproduct of her search for the clock, she abandoned her earlier caution: "In earlier papers . . . it has been shown that the sub-oesophageal ganglion of *Periplaneta americana* L. undergoes a 24 hr. rhythm of secretory activity. . . . Furthermore, . . . *the animal into which it is implanted will take up a locomotor activity rhythm in phase with the secretory rhythm of the implant*, provided that the animal is not already showing a strong rhythm. *Now that this autonomous 'clock' has been found* it may be possible to upset the cycle of 24 hr."[24] From the portion of this quotation that I have italicized for emphasis, it is clear she now asserted that both the period length of the activity rhythm and the phase of activity onset that were programmed into the donor's suboesophageal ganglion by the experimental L:D rhythm were transplanted with the ganglion from the donor roach to the host roach, which had been rendered arrhythmic by removal of its suboesophageal ganglia. Moreover, she believed she had found the clock. She did not report whether she had checked the rhythm and phase of the host roach prior to rendering it arrhythmic, which logically should not have mattered once its clock-ganglia were removed. But this omission raised a red flag for Pittendrigh as he evaluated her experimental report.

SCIENTIFIC RESPONSES TO HARKER'S WORK

The significance of Harker's experimental results was recognized almost immediately. Her surgical "remove and replace" method and the method of parabiosis, which she had learned from Bodenstein and now applied to the search for the clock, had demonstrated that the endogenous rhythm programmed into an organism by controlling the L:D regimen of its environment could be isolated to a specific organ. When that organ was transplanted into another individual, its functions went with it. It appeared as if she had found the clock mechanism—or at least a part that housed it.

In an article they contributed to an edited volume, *Rhythmic and Synthetic Process in Growth* (1957), Princeton University researchers Colin Pittendrigh

and Victor Bruce hailed her "recent and elegant experiments," which they interpreted as supporting their own hypothesis that the clock "is physically located in the nervous tissue of this species. Harker has demonstrated that headless (but living) roaches are arrhythmic but reacquire a rhythm following implantation of a sub-oesophageal ganglion from a rhythmic donor roach. . . . This is one of the most remarkable experiments in the field of persistent daily rhythms."[25] The authors identified the fact that the implanted ganglion brings with it the *phase* of the donor's rhythm as the crucial finding. This point was not clear to them from their reading of Harker's paper, and they added in a footnote that they had interpreted Harker's results as meaning that both donor rhythm *and* its phase were transplanted to the host, and that if only the former, then her results were less convincing.[26]

Impressed as they were by Harker's surgical prowess and findings, Pittendrigh and Bruce observed that her isolation of the clock in the roach's neural system, in a neurosecretory gland, did not imply that this was true of other species, inasmuch as a wide variety of organisms without nerves exhibit rhythmicity. Moreover, the fact that unicellular organisms manifest rhythms implies that rhythms are generated at the cellular level.[27] In other words, the suboesophageal clock was not a generalizable mechanism and therefore not easily put into an evolutionary context. They repeated this point in the *American Naturalist* that same year: "Harker demonstrated that motor activity in headless roaches is non rhythmic although they remain alive and active for some time. She also demonstrated that transfer of the sub-oesophageal ganglion from a rhythmic donor roach restores the rhythm in the headless roach. It is equally clear from the work on plants and microorganisms, however, that one need not look to the complexity of the nervous system to find the necessary or essential features of the clock."[28] They were looking for something more basic, a mechanism that was primitive enough in its origins that it can be found in unicellular organisms and which has been selected for organic functions in more complex animals, a clock that had homologous features across species. To this end they had wondered if Harker's "remove and replace" method might be put to use in localizing the clock within the cell: "An obvious first question is 'nucleus or cytoplasm?' . . . In short, we need techniques analogous to Harker's in which we demonstrate clock autonomy in a manipulatable part."[29]

Milton Fingerman, an invertebrate zoologist who had trained in Frank Brown's laboratory, applied Harker's method to the grasshopper *Romalea*, noting that "the current trend is to elucidate the mechanism of rhythmical behavior."[30] Accordingly, Fingerman and his collaborators followed Harker's lead and found that, after the suboesophageal ganglia had been removed, the hoppers "exhibited very little activity throughout the 24-hour day," from

which they concluded that "the subesophageal ganglion was essential for the visible expression of the locomotor activity rhythms." However, the authors do not seem to have grasped the essential importance of Harker's transplantation experiments, which had been noted by Pittendrigh and Bruce, because they subsequently implanted *three* suboesophageal ganglia into *each* of three hoppers that previously had had their own removed. They found that "the implants neither increased the total activity nor induced an apparent locomotor activity rhythm," seemingly without consideration for the donors' period lengths or their phasing and the possibility that the rhythmic outputs of multiple ganglia might confound each other if they were not synchronized.[31] The conclusion of their paper shows that they accepted the legitimacy of Harker's findings but also recognized a key limitation: "The seat of expression of 24-hour rhythmicity of spontaneous locomotor activity may be the subesophageal ganglion of *Romalea* just as shown by Harker (1956) for the cockroach *Periplaneta*. However, these results do not prove that the subesophageal ganglion of *Romalea* is the center of 24-hour rhythmicity but only that the center of rhythmicity of locomotor activity must operate through the subesophageal ganglion that exercises normal control over locomotion."[32] They were suggesting that the ganglia might be the clock's hands but not the timing mechanism itself. However, this would not explain the success of Harker's transplantations in transferring the rhythm.

The conclusions Harker had drawn about the role of the suboesophageal ganglion and her early ocelli experiments remained little changed in her presentation at the Cold Spring Harbor Symposium in 1960, in which she mainly recounted her discoveries from 1953 to 1958 (see figure 1.2). One new experiment suggested that there might be two clocks at work in the cockroach. She developed a technique for chilling the ganglia in situ, which resulted in a delayed activity rhythm. Examining the timing of the secretions of the suboesophageal ganglia with respect to the light/dark cycle, she ascertained that there is a time at which the rhythmic secretion will begin and initiate the activity rhythm, but there is also a window of time during which the sequence can be reset by an artificial light/dark shift, and that the timing of this window seemed to be regulated by an independent clock.[33] This was an idea that pleased Pittendrigh, who had earlier elaborated such a theory with his colleague Bruce.[34]

Given the striking nature of Harker's results—showing that roach rhythms could be transplanted by removing the ganglia and introducing them to a new host—it is not surprising that her presentation of her work at the Cold Spring Harbor Symposium was received with excitement. A decade after the event, Richie Ward, a scientific popularizer who interviewed Pittendrigh and other symposium attendees for his book *The Living Clocks* (1971), was told

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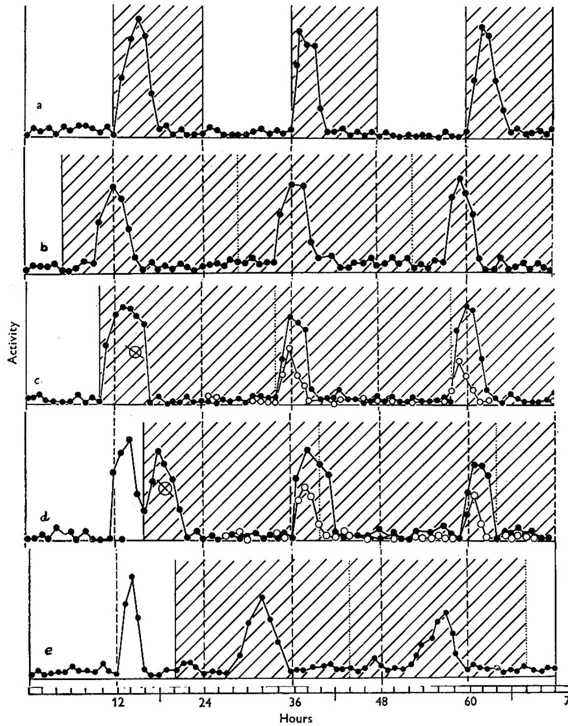

FIGURE 3 *A–E*. The activity of cockroaches when (*a*) onset of darkness occurs at the "normal" time—control experiment. Activity when darkness occurs (*b*) 7 hr., (*c*) 4 hr. earlier than normal. Activity when darkness occurs (*d*) 4 hr., (*e*) 8 hr. later than normal. ⊘, Time at which ganglion was removed and implanted into arrhythmic animal. ○——○, The activity of the implanted animal. (Redrawn from Harker, 1960.)

FIG. 1.2. Janet Harker reported at the 1960 Cold Spring Harbor Symposium on Biological Clocks on her successful experiments synchronizing cockroaches to shifted schedules and then transplanting their suboesophageal ganglia into headless, arhythmic roaches, demonstrating that the animals so implanted exhibited activity rhythms in phase with those of the donors. Graphs 3d and 3e of her presentation show that when she transplanted the suboesophageal ganglion of a rhythmically entrained roach into a headless, asynchronous roach, the recipient adopted the period and phase of the donor's rhythm. Shep Roberts attempted to replicate this experiment, unsuccessfully, in the early 1960s. Janet E. Harker, "Endocrine and Nervous Factors in Insect Circadian Rhythms," *Biological Clocks: Cold Spring Harbor Symposia on Quantitative Biology* 25 (1960): 281, fig. 3.

that when Harker concluded her presentation audience members leapt to their feet and applauded, an event that he noted was rare in "the normally staid atmosphere of scientific meetings."[35] Nevertheless, the discussion that immediately followed Harker's presentation at the Cold Spring Harbor meeting, part of which was edited for inclusion with the proceedings, points to some disagreement among audience members over the validity of her methods and

about the need for repeated confirmation or, better yet, statistical assurances of experimental findings in the field.

Good science requires independent verification of experimental findings, and Pittendrigh may have simply been raising the general point after her presentation that the work of Harker and her English colleague Cloudsley-Thompson had not been confirmed, but he had specific qualms about their findings in mind. He and Cloudsley-Thompson exchanged a few words about accepting the latter's claim for the role of the ocelli in mediating light as a Zeitgeber in the American roach *Periplaneta*, and Pittendrigh pointed out that his graduate student at Princeton, Shephard (Shep) Roberts, had not found this to be true of another roach, *Leucophaea*. Pittendrigh bristled at Cloudsley-Thompson's assertion that statistics were "irrelevant in discussing qualitative experiments that show a causal explanation of observed effects," and Harker bristled at Pittendrigh's implication that her findings, which were based on few observations, were not sufficient evidence. Pittendrigh, perhaps sarcastically, observed that "Miss Harker did, nevertheless, assure us that the reproducibility of the critical observations was 100%," perhaps hinting at his continued unease with her work, which was noted already in his cautious footnote in 1957 (mentioned above).[36]

I suspect that after learning of Harker's surgical methods and reading her conclusions, perhaps already in 1957, Pittendrigh began to reproduce her transplantation experiments in order to verify the identity of the suboesophageal ganglion as even a part of the clock, and that at the time of her presentation at the Cold Spring Harbor Symposium his laboratory had not yet reached closure on this. Apparently he put Roberts to work on the problem, for in a latter recollection he stated that "enthusiastic attempts by Shephard Roberts in Princeton failed to confirm Harker."[37] Roberts recounted his effort to replicate Harker's results in his presentation at the 1964 summer school on biological clocks, which was hosted by Aschoff in Feldafing, Bavaria. His paper begins: "Certainly, one of the most striking reports in the literature pertaining to insect rhythms is that of Harker (1956), who claims to find a 'clock' in the sub-esophageal ganglion of the cockroach, *Periplaneta*. Animals made arrhythmic by decapitation regained their locomotor rhythms after implantation of ganglia from normally rhythmic donors." The key to the demonstration, he noted, was that "in order to unequivocally demonstrate that such an implanted ganglion maintains an autonomous rhythmicity . . . explicit information about the phase of the donor and host rhythms is required," observing that, "apparently, this important qualification was not immediately recognized by Harker, although in a later paper (Harker, 1960) it was stated that host animals had taken on the phase of the donor rhythms." Roberts then referred to the inability of Fingerman and his colleagues to reestablish the

activity rhythm in the grasshopper after removal and replacement of the suboesophageal ganglia, and he recounted his own failure (1959) to establish rhythmicity in decapitated roaches after implanting ganglia from rhythmic donors.[38]

Roberts was even more outspoken in his criticism of Harker's methodology the following year. "Employing techniques essentially identical to Harker's, and using the same species of roach," he wrote, "I have been unable to find any evidence to support the original claim. . . . I have been unable to confirm either Harker's original observations indicating (1) that the subesophageal ganglion functions as a clock or, (2) that the *corpora cardiaca* are necessary for the maintenance of locomotor rhythms."[39] He did not see any consequential flaws in her procedures but could not reproduce her results. Recalling the footnote about the crucial importance of phase information in Pittendrigh and Bruce's 1957 paper, Roberts complained that "apparently, the important prediction of phase transfer was not immediately recognized by Harker and her original data preclude its unequivocal demonstration." In later papers she had asserted that the phase was transferred with the ganglion, but her experiments had failed to consider the phase of the host roach's activity rhythm prior to decapitation, which she supposed to render it arrhythmic, in which case any subsequent rhythm must reflect that of the donor: "Without such evidence one can only conclude that the ganglion is a necessary factor for the expression of a rhythm," and not for its timing.[40] This is basically a restatement of the conclusions of Fingerman and his colleagues noted above.

Such results were not wholly satisfactory, for a failure to confirm Harker's findings is not the same as confirming her failure. A more decisive approach to proving her wrong was to find the clock elsewhere. About the time Roberts reported his inability to reproduce Harker's experiments, a visiting researcher in Pittendrigh's laboratory at Princeton, Junko Nishiitsutsuji-Uwo from Kyoto University in Japan, determined that the roach's clock was actually a part of its brain, locating it in the midbrain optical lobes, and that these bilateral organs constitute redundant pacemakers. John Brady, one of Harker's former students, was likewise following up on doubts about her findings and arrived at this same conclusion almost simultaneously.[41]

The discovery that the cockroach's clock is part of the optical lobe of the brain effectively discredited the claim Harker made in 1957 that she had found the biological clock in the cockroach, and this was duly noted in the scientific literature. In 1970 Cloudsley-Thompson wrote that "considerable doubt has been cast upon the validity of the work of Harker (1964) and on the neurohormonal control of cockroach rhythms, by Roberts (1966) and other workers who have been unable to duplicate her results."[42] Brady summarily reported in his 1979 textbook *Biological Clocks*: "It was once thought (and is still claimed

in some textbooks) that this rhythm is controlled by a hormone secreted rhythmically by a small group of neurosecretory cells in the sub-oesophageal ganglion. This has since been shown many times to be most unlikely," and "it therefore appears that the optic lobes contain a vital component of the circadian system that controls behaviour. All the ganglia contain neurosecretory tissue, but none elicit rhythms when transplanted into other cockroaches."[43]

Although Harker's experimental findings were ultimately rejected, they played an important role in the search for the clock. Her basic methodology had been employed—and even refined—in this follow-up work by other rhythms researchers. As a result of her experiments chronobiologists became convinced that, in some cases at least, it was possible to find the clock, remove it, and tinker with it to study its function. Michael Menaker, a rising star in the field during the 1960s, made extensive use of this method for studying the role of the pineal gland as a clockwork in birds.

Following up on Nishiitsutsuji-Uwo's work, Terry Page explicitly tracked the function of the optical lobes by selecting roaches with different free-running biological rhythms under constant conditions for transplantation into an arrhythmic host. He transplanted one lobe removed from a long-period roach and another lobe removed from a short-period roach, so that both clocks were running in the host roach at different speeds, and then put the host roach into continual darkness. The result was one roach with two sets of activity rhythms, demonstrating that each lobe carried its own clockwork.[44]

The remove-and-replace transplantation technique was also employed by Hans-Georg Schweiger to investigate the role of the nucleus in determining circadian rhythm of the large unicellular alga *Acetabularia* in 1971, moving toward Pittendrigh's goal of finding the essential features of the clock across species.[45] One essential feature that was not to be found by this surgical method was how the clock synchronized with the rhythmic cycles of its environment. Characterization of the relationship between rhythmic behaviors and environmental cycles resulted in the creation of a standard descriptive tool for chronobiologists, the phase-response curve.

THE PHASE-RESPONSE CURVE

The phase-response curve (PRC) was developed in the 1950s to characterize how biological rhythms are reset by Zeitgeber to synchronize them with environmental rhythms that are important to the organism's (and hence the clock's) survival. The most common of these rhythms is the daily alternation of daylight and darkness, adaptation to which has produced diurnal and nocturnal species. Chronobiologists theorized two ways that an organism's clock might be synchronized with the environment, either its speed can be

regularly adjusted or its phase can be regularly reset. By analogy to a mechanical clock or watch, the former corresponds to setting it to run faster or slower by adjusting the length of the pendulum or the tension on the torsion spring, and the latter corresponds to moving the hands to reset the phase without adjusting the mechanism's speed. The former method results in adjusting the period length of the daily cycle and the latter regularly adjusts the clock's phase, bringing it "into phase" or synchrony with the environmental cycle. Experimental resetting of biological rhythms revealed that how quickly they come into phase depends on the point in their cycle at which the clocks are subjected to a reset stimulus. Researchers can plot how quickly a biological clock resets to conform to a new biological rhythm by constructing what is called a period-response curve (τ-RC) or a phase-response curve (PRC), the former applicable to speed adjustment and the latter to phase adjustment.

Assuming that biological clocks are not very useful to organisms unless they are synchronized with key regular changes in the environment, chronobiologists reasoned that understanding how biological rhythms respond to resetting stimuli is as important as how the clock keeps time. The latter requires looking into the mechanism and studying its molecular parts, which was not technically possible in the 1950s. But the interaction of the mechanism with its environment could be studied by treating it as a black box, adjusting the input stimulus and observing the output response, in this case varying the timing and duration of the Zeitgeber to see what effect this might have on the period and phasing of the clock—that is, the observed biological behavior.

Most rhythmic phenomena of interest in the organic world recur on a daily basis, and the stimulus for this was assumed to be the primary characteristic of the environment, the light/dark cycle, though regular daily changes in temperature or humidity were conceivably more important under certain circumstances—on the floor of a thick rainforest, for example, where daily changes in illumination are not apparent. The PRC was therefore first developed in connection with daily rhythms, which is where PRCs find their general application. They are often used to characterize the results of experiments designed to shift the daily rhythm of specimens by using a pulse resetting signal or Zeitgeber.

Kalmus was using light pulses to synchronize the eclosion rhythm of *Drosophila* in the 1930s, and it was in the context of continued work on *Drosophila* eclosion in the 1950s by William Brett and Colin Pittendrigh and its extension to hamsters by Pittendrigh's student John Burchard and to flying squirrels by Patricia DeCoursey that the PRC began to take shape as an explicit graphic form. By their very nature, triggering pulses are long enough

in duration to be effective, but they are very short with respect to the clock's period. A light pulse may be several minutes or even an hour, but this is a small fraction of the natural circadian period, and the length of the pulse is not especially significant, only that it be long enough to act as a trigger. This carried with it the implication that it was the abrupt transition from dark to light (or light to dark) that was the effective Zeitgeber and not the pulse length per se. For this reason, and because dawn and dusk were observed to be significant transition times in the activity of many animals, chronobiologists developed the PRC earlier than the τ-RC. The latter required imagining how the length and intensity of exposure to light might speed up or slow down the clock, which was harder to model experimentally and did not follow logically from the use of pulses as Zeitgeber. Moreover, as chronobiologists began to construct PRCs for many and diverse organisms, they quickly realized that PRCs were of similar shape, suggesting that phase-resetting was a common property of biological clocks, which warranted investigation in its own right. Before considering the historical development of the PRC, it is helpful to establish how important it was to those investigating biological clocks and to become familiar with some of its technical details, for it is in these details that its importance lies.

An organism's internal clock, even if it is running with precisely a twenty-four-hour period, needs to be rather continually reset to the current conditions if the important environmental factors are connected with dawn or dusk. It was becoming clear to most researchers that these times either are important or are a proxy for changes in other important factors connected with the onset of day and night (for example, temperature and humidity). It is supposed that *Drosophila* eclosion happens mainly near dawn because this gives the emergent day-active adult insects time to adjust to their new environments during a moist time of the day, prior to the daytime decrease in humidity and increase in temperature that would desiccate them on emergence. Other creatures clearly are tuned to dusk—for example, the rather abrupt commencement of flying squirrels' activity with sundown and the scurrying of cockroaches in the early night, typical for nocturnal animals. Some changes are less apparent without careful study. For example, the rhythmic decline in kidney activity during the night does not depend on whether a person is asleep but on the phasing of the body's clock, as studies of urine-secretion rhythms demonstrated. But few clocks have a precise solar-day timing. Their natural periodicity varies considerably from person to person and from mouse to mouse and needs routine adjustment to bring it back in phase. Moreover, dawn does not occur at the same time of day throughout the annual period. Such daily changes are not sudden in nature, and presumably

whatever mechanism adjusts the clock can do so gradually as the days and nights progress. But we can impose more abrupt changes experimentally in order to study how the clock responds.

Logically, there are two ways one can adjust the phase of a clock (as distinct from changing its speed); one can advance the hands or turn them back. It works this way on many digital clocks and coffee makers, too; one can step the clock forward or backward to set the time or rephase the clock with the time standard. The purpose of the PRC is to study the biological clock's entrainability by external stimuli, to characterize how quickly the subject organism can shift schedules and whether the shift is achieved by advancing the schedule or delaying it. Brady put this succinctly in his 1979 textbook *Biological Clocks*, using the example of the nocturnally active cockroach:

> The cockroach has a differential phase-shifting ability across the 24 h: at some points in its circadian cycle it can make bigger phase-shifts to a new Zeitgeber than it can at others. . . . This differential phase-shifting to Zeitgeber falling at different times in the rhythm's cycle is typical, indeed diagnostic, of all endogenous circadian and circa-tidal rhythms. . . . The usual procedure is to place a series of individuals in constant darkness, let their rhythms free run, and then at different points (phases) in their free-running cycle expose each individual (or group of individuals) to a single "pulse" of light of, say, 1 h duration.[46]

Although this procedure in no way resembles natural conditions, which are never constant and changes are seldom abrupt, it quickly became a standard tool for analysis in chronobiology. Light pulses can be used to study nocturnal animals that are experimentally kept in continual darkness (D:D) or continual very low illumination, when the pulse triggers the onset of the "daytime" rest phase, or applied to diurnal animals, when the pulse signals the wake-up time. This is the most common method, but when organisms are experimentally maintained in continual light of normal intensity (L:L), the lights can be switched off for short durations to create dark pulses, and one can also use abrupt changes in temperature as pulses and, in principle, any other functional Zeitgeber. Importantly, PRCs can be constructed for organisms that do not function well in continual darkness for very long, notably those requiring photosynthesis for energy, either by using dark pulses or by using bright light pulses during otherwise constant dim illumination. The unicell dinoflagellate *Gonyaulax* is one such organism, which has been very important for rhythms research but which perishes if deprived of light for more than a couple of days. The PRC has been and remains an important tool for biological rhythms

research, and the history of its development is therefore of particular relevance here.

The complexity of Brady's description of the phase shifting of the cockroach can be unpacked if we realize that the PRC is a stimulus-response graph, one indicating the shifts in the phase of the subject organism's natural rhythm in the absence of environmental Zeitgeber (i.e., in constant conditions) plotted against the varying times during its cycle when it is exposed to a single pulse of such a phase-setting signal. In its developed standard form, these two variables are plotted on a simple x–y coordinate graph, where the observed phase shift is plotted on the ordinate (y axis) as an advance or delay of a significant rhythmic change in behavior that serves as the rhythm's marker variable, with reference to the comparable metric in an unshifted control—that is, when the marker would have occurred without the stimulus. The amount and direction of this phase shift depends on the point in the subject's cycle at which the pulsed Zeitgeber is experienced, which is plotted on the abscissa (x axis). The PRC therefore graphs the length of the phase adjustment that the clock makes in response to when in its cycle it is shifted (see figure 1.3 for a good example of such a graph).

An example of how this works will make this more intelligible. Consider the flying squirrel (for which a PRC is given in figure 1.6 below). This is a nocturnal animal that naturally begins its active phase rather abruptly in the early nighttime. When kept in continual darkness, it will react to a pulse of light during the early nighttime (dark) as if it is still daytime and begin to shift its schedule accordingly by delaying its phase. The amount of this shift increases early in the night, reaching a maximum delay around the time of the animal's normal onset of nighttime activity (marked "onset") and then tapers off toward morning. However, sometime around when dawn should occur, the squirrel reaches a point where the light pulse will produce no more delay effect, after which it will react by advancing the phase during the time the squirrel should normally be entering is daytime resting phase.[47] The situation is similar for plants and humans. An endogenous clock is predicated on the assumption that the clock will govern the rest/activity rhythm even in the absence of actual dark/light cycles, which is why shift work is problematic for humans; the phasing of our clocks persists for days on a daytime schedule, leaving some of our body systems asleep while we are working at night, until we adjust to the new activity rhythm.

Prior to the development of genetic mutants and microbiological demonstrations of genes and gene expressions, which permitted researchers to study the mechanisms inside the black boxes, the PRC and the related τ-RC were the chief quantitative experimental characterizations available for studying biological clocks. On the basis of these response curves, it was possible to

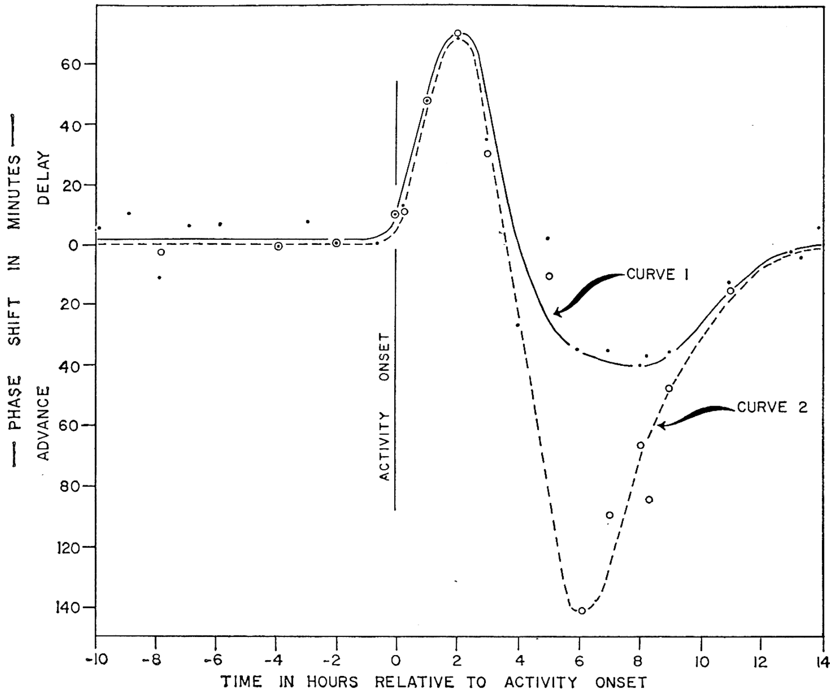

Fig. 2 Light response curve. Phase shifts resulting from the 10-minute light exposures are graphed with respect to time of the light before or after activity onset for hamster no. 708. Curve 1 indicates the immediate shift, and Curve 2 the steady state shift. See text.

FIG. 1.3. The PRC (phase response curve, called a light response curve in this 1964 example by Patricia DeCoursey) plots the shift in animal activity against the experimental Zeitgeber or timing cue. In this case, hamsters were kept in continual darkness and exposed to ten-minute light pulses at varying times with respect to the animals' activity onset time, which is then plotted as 0 on the abscissa, and the resulting shift in the successive time of activity onset was then plotted on the scale of the ordinate. The graph of this particularly rhythmic hamster shows that light pulses falling up to ten hours prior to the activity onset time did not shift the period of the hamster's internal clock, while light pulses coming up to about four hours after the onset time delayed subsequent onset times, with a maximum delay occurring when the pulse was about two hours after activity onset. A light pulse more than four hours and up to fourteen hours after activity onset causes the animal's clock to advance. Curve 1 on this graph represents the calculated immediate effect of the light pulses and curve 2 the shift settled into after several cycles, when transient effects calmed down. The difference between these curves indicates that the clock mechanism responded almost immediately to delaying shifts but took longer to accomplish advancing shifts. Patricia DeCoursey, "Function of a Light Response Rhythm in Hamsters," *Journal of Cellular and Comparative Physiology* 63 (1964): 192, fig. 2.

place limitations on possible structures and make inferences about them without actually "seeing" them. They therefore served an important heuristic function.

Looking back on the development of the PRC at the end of the twentieth century, two prominent rhythms researchers noted its historical significance for understanding biological rhythms. Serge Daan wrote in 1998 that "perhaps the greatest success of the PRC entrainment model was the PRC itself. It gave the field its first-rate, clearly defined, experimental tool for probing the physiology of circadian systems."[48] His colleague Carl Hirschie Johnson was just as blunt a year later: "Until recent advances in the identification of molecules that we believe may function as components of the circadian clockwork, PRCs have been our only gauges of the mysterious inner workings of this biological timepiece and remain an important tool."[49] We turn now to explore the history of its development.

LIGHT PULSES AND THE DEVELOPMENT OF THE PHASE-RESPONSE CURVE

There are varied accounts of the genesis of the PRC, perhaps because its mature form as a graphic representation took shape from experimental phase-shifting practices that several laboratories were using over a period of years in the mid- to late 1950s. In 1991 John Palmer attributed the concept of the PRC to H. Marguerite Webb and credited the parallel use with temperature pulses to Grover C. Stevens, both of them Frank Brown's students, thus situating the fundamental idea for the PRC in Brown's laboratory in the late 1940s and early 1950s: "Webb's doctoral work in 1948 gave chronobiology the first data, *describing what has since come to be called the light phase-response-curve* (she also described what are now called 'transients'); and Stevens did the same for the temperature phase-response-curve."[50] Moreover, Palmer noted that Webb was using light pulses in D:D to study phase shifting in both the solar and lunar (tidal) rhythms of the fiddler crab, leading to his comment that "this study shed the first light on the multiple and opposite responses of rhythms to light pulses, now summarized by the classic phase-response curve known to underlie the entrainment of virtually all circadian rhythms."[51] Palmer was a well-established scholar, and looking back on the contributions of Brown's laboratory to the field of chronobiology and being one of Brown's students himself he may have been partisan in this account. Nevertheless, his claim for the innovation of the PRC bears examination.

Note that Palmer carefully said Webb had generated phase-response *data* and *described the phase-response curve*, but he did not say she called it that. It is clear that Brown's students were using light pulses to stimulate phase shifts (the data that Palmer refers to here) and that figuring out how to display this data led to the construction of phase-response curves, but were they

constructed by Brown's students? The distinction is important, because beyond being simply a convenient means of displaying data, the standardized PRC itself became a characteristic of clocks in general and the clocks of individual species in particular, which is what Daan meant by calling it a tool for probing the physiology of circadian systems.

Examination of Webb's 1950 paper "Diurnal Variations of Response to Light in the Fiddler Crab, *Uca*" reveals that she used single light pulses of six hours or longer simply to effect shifts in the crabs' chromatophore rhythms in order to study the stability of the period and persistence of the new phase. Webb noted that the response to the pulse depended on the animal's activity phase—light pulses were effective during the subjective night but not during the subjective day phase—but she made no attempt to determine systematically the relationship between pulse timing and amounts and direction of phase shift, which is the point of the PRC.[52]

A similar awareness of the sensitivity of the rhythm's phase to the timing of changes in illumination is expressed in a 1952 paper by Brown and another of his students, Margaret Hines. One of their experiments entailed maintaining a group of crabs in continual darkness and then subjecting them to a one-hour pulse of light at different times during the daily cycle and pulsing a complementary group kept in continual light with an hour of darkness. They concluded that "*the response of a change from light to darkness* of *Uca* which have been maintained for 10–58 hours in darkness and then treated with 1 hour of illumination *varies with the phase in the diurnal cycle*," but again there was no systematic effort to generalize a phase-response study method.[53]

This passage makes it clear that Brown and his students were using light pulses to synchronize the crab's chromatophore rhythm, and the italicized portion indicates that they had apprehended the basic principle behind the PRC before Pittendrigh engaged in rhythms research, which by my reckoning must have been no earlier than 1952. More interesting, in light of Pittendrigh's later ownership of *Drosophila* eclosion studies, is a similar reference in Brown's 1954 *Scientific American* article to experiments conducted on *Drosophila* by his graduate student William Brett: "Yet if a group of fly larvae being raised in darkness are exposed to light for even as brief a period as one minute, *the mature flies will tend to emerge* (several days later) *at a time of day which is correlated with the time of day when they were given the light flash*."[54] Brett, who completed his doctoral dissertation at Northwestern University in June 1953, was clearly using light pulses to shift the eclosion rhythm in *Drosophila* at about the same time as Pittendrigh was beginning to work on this same rhythmic phenomenon.

Brett published his research in 1955 in the *Annals of the American Entomological Society* as an article with the same title as his doctoral thesis, "Persistent

Diurnal Rhythmicity in *Drosophila* Emergence," pointing out there that the paper reproduced content from his doctoral dissertation. In fact, the paper is in all essential aspects a verbatim replication of the dissertation, from beginning to end, complete with all illustrations and captions. I presume that the experimental results Brown referred to in his 1954 *Scientific American* article refer to the experiments that Brett completed in the winter of 1952–1953 and reported in his dissertation. Citing Kalmus's use of a single light pulse to trigger *Drosophila* eclosion, Brett stated that "it would be interesting to learn whether the phases of the subsequent emergence rhythm were related in any definite manner to the time of day of the single light period," a vague reference to what amounts to a phase-response study.[55]

Still, he made no attempt to apply the method systematically to generate a body of phase-response data that could constitute a PRC or to construct such a graph. Instead he explored the use of various pulse durations, from one minute to two hours. He applied the two-hour pulse at 6 p.m. and the others at both noon and midnight in order to judge qualitatively the differing phase responses. He concluded that "in every instance there was a direct correlation between phases of the rhythm and the time of day the particular culture was illuminated."[56] It seems that, although they used pulses to effect phase shifts in rhythms in the late 1940s and early 1950s and understood that the pulses produce different phase-shift responses when applied to crabs at different phases of their free-running rhythm and to asynchronous *Drosophila* populations, Brown and his students made no attempt to formalize the method to produce PRCs and to use PRCs to characterize the clock systems.

In 1998, seven years after Palmer's attribution of the phase-response concept to Brown's laboratory, Daan, who had worked as a postdoc with Pittendrigh and Aschoff and had come to know the PRC and τ-RC intimately, identified the PRC as emerging "virtually simultaneously" in work being done by John Ely Burchard in Pittendrigh's laboratory in Princeton (PhD 1958), by Woody Hastings, who was using the light-pulse technique to study *Gonyaulax* in collaboration with Beatrice Sweeney (1958), and by DeCoursey in Ken Rawson's laboratory at the University of Wisconsin (PhD 1959).[57] This agrees well with the genealogy given a year later by Johnson, but Johnson stressed that "the first journal publication of phase-shifting data that was plotted into the now-familiar form of a phase-response curve (PRC) appeared . . . [in a paper by] Hastings and Sweeney, 1958."[58] This judgment is supported by Hastings's own recollection in 2001 that this was the first PRC, a claim he repeated in 2007.[59] The development of the PRC is thus historically complex and somewhat contested and bears closer scrutiny.

First, Daan's attribution of the PRC to Burchard's doctoral dissertation fits the evidence. Burchard clearly plotted his data on the phase-shifting response

of hamsters, induced by four-hour and twelve-hour light pulses, as a PRC.[60] It is unclear whether Burchard innovated this diagram or if it was already in use in Pittendrigh's laboratory, but examination of when Pittendrigh appears to have first used it suggests that Pittendrigh may have adopted it from Burchard.

Pittendrigh's much later recollection of his early study of *Drosophila* implied that the PRC emerged from the experiments he was doing to elaborate an oscillator model to explain the timing of eclosion: "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 . . . 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)."[61] As is now very clear, Pittendrigh's use of light pulses to trigger the circadian rhythm of *Drosophila* eclosion was not new. Bünning had already (in 1935) reported his use of single light pulses to synchronize the eclosion of *Drosophila* populations that had been made asynchronous through generations of maintenance in continual dim light, and a concern for the ability of brief pulses to synchronize organisms is implicit in his and Kurt Stern's earlier revelation that Rose Stoppel had inadvertently synchronized her plants by pulsing them with red light during her experiments and with that had introduced an unsuspected Zeitgeber. Kalmus also used this method in his study of *Drosophila* eclosion in 1935. Pittendrigh may not have been familiar with these 1935 papers when he began to use light pulsing, although as a graduate student working on *Drosophila* behavior under the direction of Theodosius Dobzhansky, he should have read widely on fruit flies. Moreover, he later recalled that while he was a student doing fieldwork in Yosemite National Park, he realized that two species of *Drosophila* had different activity periods and that a friend, Marston Bates, pointed him to the papers of Bünning and Kalmus.[62]

Pittendrigh's field observations may have suggested to him the possibility that *Drosophila* navigated by "sun-compass clocks," which he had heard about in a lecture on bird navigation by Gustav Kramer, as he recalled later in life. But his first paper specifically on the timing of *Drosophila* eclosion, "On Temperature Independence in the Clock System Controlling Emergence Time in *Drosophila*" (1954)—in which he cited the work of Bünning, Kalmus, and Kramer—emphasizes the temperature independence of the rhythm as an argument for an inherited and entrainable endogenous oscillator as opposed to a "learning model" that Kalmus had defected to (in Pittendrigh's opinion). Hastings identified Kalmus's work on *Drosophila* eclosion as the main target of Pittendrigh's 1954 paper and pointed out that Pittendrigh had not given

Kalmus proper credit for identifying the temperature independence of eclosion rhythm, once transient periods settled down after an experimental temperature change, ostensibly the main thesis of Pittendrigh's paper.[63] Pittendrigh's reading of Bünning's papers had revealed to him Bünning's struggle with Stoppel on the issue of endogenous clocks in plants, and the demonstration of temperature independence of timing in a cold-blooded (*poikiothermal*) animal was crucial to Pittendrigh's decades-long controversy with Brown on this matter, which he was already gearing up for in this paper.[64] In this paper he reported the use of single light and temperature pulses to synchronize *Drosophila* eclosion, showing that the period did not depend on temperature, and he argued that the information about period length could not have been imparted to the previously asynchronous population by the brief pulse.[65]

It is also possible that Pittendrigh learned the use of light pulsing from Brown's laboratory (where it was being used as an experimental tool well before Pittendrigh began to study biological rhythms) by Webb and, more recently, by Brett for the research reported in his 1953 dissertation. Inasmuch as Brown was not convinced that there *were* endogenous clocks, he had little incentive to develop a tool to probe them, unlike Pittendrigh. Brett, however, was open to the existence of the endogenous clock and followed the line of inquiry into the rhythm of eclosion that was begun by Bünning, W. N. Scott, and Kalmus, verifying their results using pulse triggering. But then he went further. His experiments suggested to him that it was the dark-to-light transition edge of the pulse that was operative in entrainment and that the length and intensity of the light were not crucial factors in setting the clock. He stated this clearly in terms that directly liken the inner timer to an ordinary alarm clock: "The dark to light change is the same as setting the alarm which, as long as the clock is running, will continue to ring at the same time each day." He went on to say the alarm could be reset several times in one day, within the practical developmental limits of *Drosophila* pupae, concluding that "as in the case of an alarm clock, when the alarm is no longer being reset (placed in constant darkness) the alarm will continue to ring at the time of the last setting."[66] Like Bünning and Kalmus, Brett argued that an endogenous clock was responsible for this behavior, because no rhythmic information could possibly be conveyed by the brief and varied pulse lengths. But, unlike his predecessors, he clearly stated that the eclosion rhythm was *not* dependent on ambient temperature: "The length of the light cycle of *Drosophila* is a function of the temperature but *there is no apparent effect upon the frequency of the rhythm of emergence in these experiments*. In other words, at several temperatures the rhythm continued to have 24-hour cycles."[67] The temperature independence

of the clock in organisms that do not maintain a steady body temperature is an important feature of endogenous biological timing mechanisms, and Brett clearly observed this in his 1953 doctoral dissertation.

Pittendrigh also used light pulses to synchronize *Drosophila* eclosion for a paper he presented at a symposium on perspectives in marine biology that was held at Scripps Institute of Oceanography in late spring 1956, in the same way as he had reported in 1954, as an important piece of evidence that eclosion rhythm represents a group synchronization of the daily rhythms that are inherent in individual *Drosophila*. Here he called for "the use of single perturbations as an experimental tool for the study of rhythms," which he thought "has been severely neglected," and this indicates he was thinking about phase response as an important oscillator characteristic.[68] His interpretation of phase resetting by this method implied different advances and delays of rhythm, which comprise the PRC, but he emphasized that rephasing was accomplished through transient rhythms with longer or shorter periods, and he did not elaborate the method as a tool.

Pittendrigh does not appear to have begun constructing PRCs until the end of the decade, recalling later that he began to graph the PRC in polar coordinates as a means to study the relationship between the entraining light pulses and his understanding of the clock:

> 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. . . . the simulations using it yielded essentially perfect predictions of the observed phase-relation between the oscillator and the light cycle that entrains it.[69]

No vestige of this circular version of a phase-response graph appears in the papers published by Pittendrigh and Bruce during this period, so further illumination of Pittendrigh's recollection of the genesis of the PRC must await archival revelation.

As Daan reported, Hastings and Sweeney did in fact in their 1958 paper "On the Luminescence Rhythm of *Gonyaulax*" plot the phase-shifting effect of single three-hour pulses of light on the luminescence rhythm of a population of the single cell bioluminescent algae, which were free-running in D:D after having been synchronized to L:D = 12:12 (see figure 1.4). However, the graph is not quite in the standard form in which PRCs would be presented later, with phase shifts plotted as advances or delays as a function of the circa-

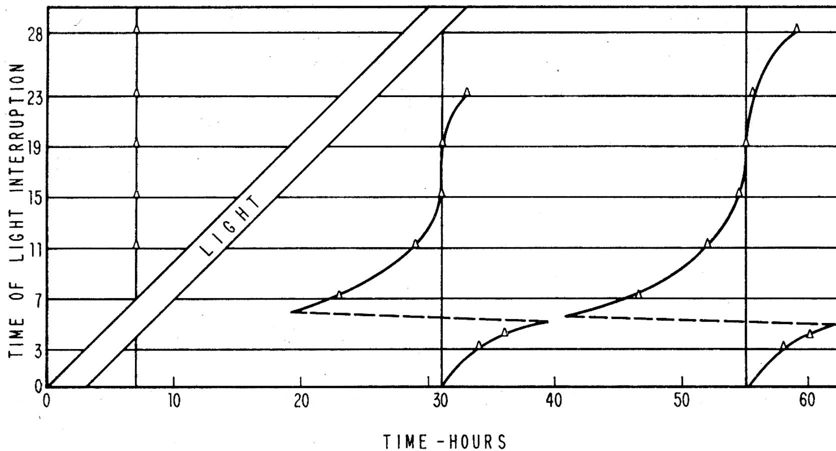

FIG. 1.4. Figure 8 of Woody Hastings and Beatrice Sweeney's 1958 paper in the Woods Hole *Biological Bulletin* displays the essential information of phase response experiments with their model organism, *Gonyaulax*, but not in the format that the mature phase response curve (PRC) would attain. Here, test groups of the bioluminescent population were put into continual darkness (D:D) after twelve hours of illumination and then subjected to three-hour light pulses at various times into the extended D:D period. The vertical lines indicate the time of maximum circadian luminescence for the control group, which was not subjected to a light pulse but remained in D:D, and thus the expected time of recurring maximal luminescence for the strobed groups. The diagonal white line represents the sliding three-hour pulse window. The phase advance and delay is shown here as a horizontal displacement between the time of the subgroup's maximum luminescence and when it would have been expected without the pulse—that is, the time of the control group's maximum. One can see the advantages of the straightforward phase advance/delay PRC graph as it was later developed, but this graph must be seen as a precursor in this process. J. Woodland Hastings and Beatrice M. Sweeney, "A Persistent Diurnal Rhythm of Luminescence in *Gonyaulax polyedra*," *Biological Bulletin* 115, no. 3 (1958): 449, fig. 8.

dian time of the shifting pulse, but this information is easily recoverable from their graph, which plots time of pulse (ordinate) against time of maximum luminescence for sample populations that were exposed to a single pulse at four-hour intervals.[70] They stated that the research for this paper was in part carried out at Northwestern University, where Hastings was on the faculty until 1957, when he took a position at the University of Illinois, Urbana. This chronology is important, inasmuch as it locates the application of pulse resetting and the development of phase-response data by Hastings and Sweeney in 1957 in the same institutional context as Brown, where we know light pulsing was a standard research method.

Hastings was not working on biological rhythms per se when he joined Brown's department, but as a colleague in the same department he likely was aware of Brown's work on rhythms. Many years later Hastings recalled meeting Brown when he arrived at Northwestern: "Brown was a highly

accomplished and well-respected invertebrate endocrinologist who had been studying the remarkable daily rhythm of pigmentation change in the fiddler crab, *Uca* with a superimposed tidal rhythm. Brown was personable and interesting, and his lectures were engaging."[71] This suggests that Hastings was becoming familiar with what Brown and his students were working on in his laboratory. However, we should note that much of the same information appears in a paper published the previous year by Pittendrigh and Bruce, where the phase shifts they induced in the *Drosophila* eclosion rhythm are similarly evident, plotted on the abscissa for sample populations of flies in D:D that were pulsed at two-hour intervals (see figure 1.5). Nevertheless, advances and delays are not clearly distinguished on these graphs, and the difference is not made explicit in the accompanying text.[72] The paper reports a presentation the authors made at a July 1956 conference, which implies they were thinking about plotting phase shifting at about the same time as Hastings and Sweeney, perhaps a year before. Taken together, these papers document the early development of the PRC graph in the 1956–1957 period, but it had not yet been given its standard form, which can be discerned in Burchard's May 1958 doctoral dissertation.

DeCoursey, who had studied the activity rhythm of the flying squirrel *Glaucomys* for her August 1959 doctoral dissertation at the University of Wisconsin, kept animals that had been synchronized to an L:D cycle in continual darkness and subjected them to light pulses at various times of their activity cycle in order to see how the timing of the pulses altered the times they began their pronounced evening activity in subsequent cycles. She plotted this data as a PRC for an article dated August 24, 1959, that appeared in the January 1, 1960, issue of *Science* (see figure 1.6). This may be the earliest appearance in print of the PRC plotted in standard form. She cited Burchard's 1958 doctoral dissertation, noting he had shown a rhythm of sensitivity to "standard light exposures of several hours' duration" in the golden hamster, implying that this form had originated with him. She presented the same PRC at the Cold Spring Harbor Symposium in the summer of 1960, clearly depicting the morning transition from a shift delay to a shift advance. As the opening paragraph of her paper makes clear, understanding the mechanism of entrainment was her chief objective, and this is what the PRC was developed to explore.[73] Her use of ten-minute light pulses and the singular focus of this article on systematic study of phase response argues that she clearly understood the construction of PRCs to be a significant, general research tool.[74] She closed her paper in *Science*: "A daily rhythm of light sensitivity [which is what the PRC shows] also serves as a basis for the interpretation of many previous studies of the effect of light upon the activity cycles of rodents."[75]

Pittendrigh's presentation at the Cold Spring Harbor Symposium, "Circa-

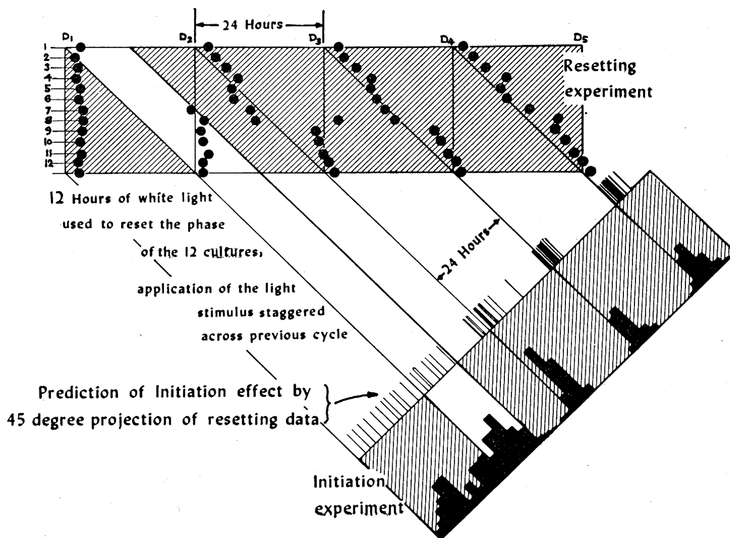

Fig. 5. The effects on the *Drosophila* eclosion rhythm of single perturbations with light (12 hours duration).

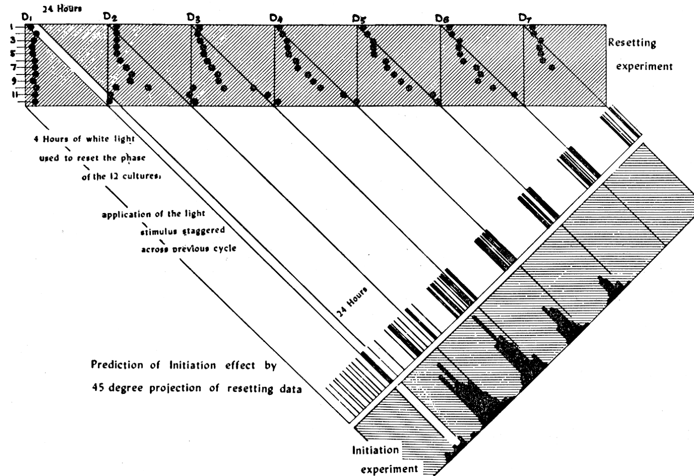

Fig. 6. The effect on the *Drosophila* eclosion rhythm of single perturbations with light (4 hours duration).

FIG. 1.5. Colin Pittendrigh and Victor Bruce were experimenting with shifting the phase of the diurnal rhythm of eclosion of *Drosophila* pupae about the same time as Woody Hastings and Beatrice Sweeney were doing their phase-shifting experiments with *Gonyaulax*. Figures 5 and 6 from their 1957 paper illustrate the shifts produced in *Drosophila* kept in continual darkness (D:D) when then subjected to twelve-hour and four-hour light pulses, respectively. The black dots represent the time of eclosion of the different test groups, which were subjected to delay or advance by the light pulses, beginning at the leading edge of the diagonal white zones on the graphs. The vertical lines at twenty-four-hour intervals represent the time at about the expected eclosion maximum of a population not subjected to a light pulse. The strength of this method of charting is that it portrays the rate at which the populations normalize to the shifted schedule (i.e., conformity of the black circles to the diagonal black lines) and how the diagramed population corresponds to the experimental results, shown as an eclosion histogram at a 45° angle to the resetting graph. Colin S. Pittendrigh and Victor G. Bruce, "An Oscillator Model for Biological Clocks," in *Rhythmic and Synthetic Process in Growth*, ed. Dorothea Rudnick (Princeton, NJ: Princeton University Press, 1957), 96–97, figs. 5 and 6.

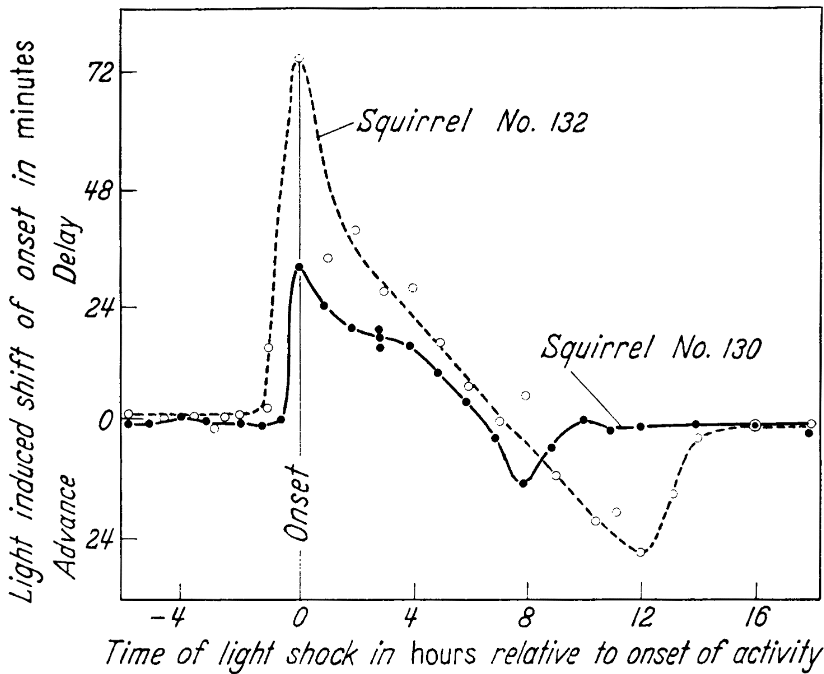

FIG. 1.6. Patricia DeCoursey published a mature version of the phase response curve (PRC) from her 1959 doctoral dissertation in a 1961 paper. In figure 11 she graphed the experimental results from subjecting two flying squirrels that were kept in continual darkness to ten-minute light pulses. An important point of this demonstration is that there is individual variability in the degree of phase response (amplitude on this graph), but that the essential timing characteristics—that is, the shape of the PRC—is very similar across the species. Patricia J. DeCoursey, "Effect of Light on the Circadian Activity Rhythm of the Flying Squirrel, *Glaucomys volans*," *Zeitschrift für vergleichende Physiologie* 44, no. 4 (1961): 347, fig. 11.

dian Rhythms and the Circadian Organization of Living Systems," included a section on "the comparative study of response curves for single light signals." Here, embedded in his figure 14 (see figure 1.7), is a composition of PRCs for *Drosophila*, the hamster, and the flying squirrel. He attributed the *Drosophila* eclosion data to his earlier published studies, but as we have seen, the PRC is not fully formulated in these. Pittendrigh's curves for the hamster and flying squirrel were based on data he took from the doctoral work of Burchard and DeCoursey respectively, so he may have adopted the standard form PRC from his student's research.[76]

VARIABLE CLOCK SPEED AND PERIOD RESPONSE

Pittendrigh put the PRC to specific work in the paper he presented at the Cold Spring Harbor Symposium—namely, to argue that phase resetting is a

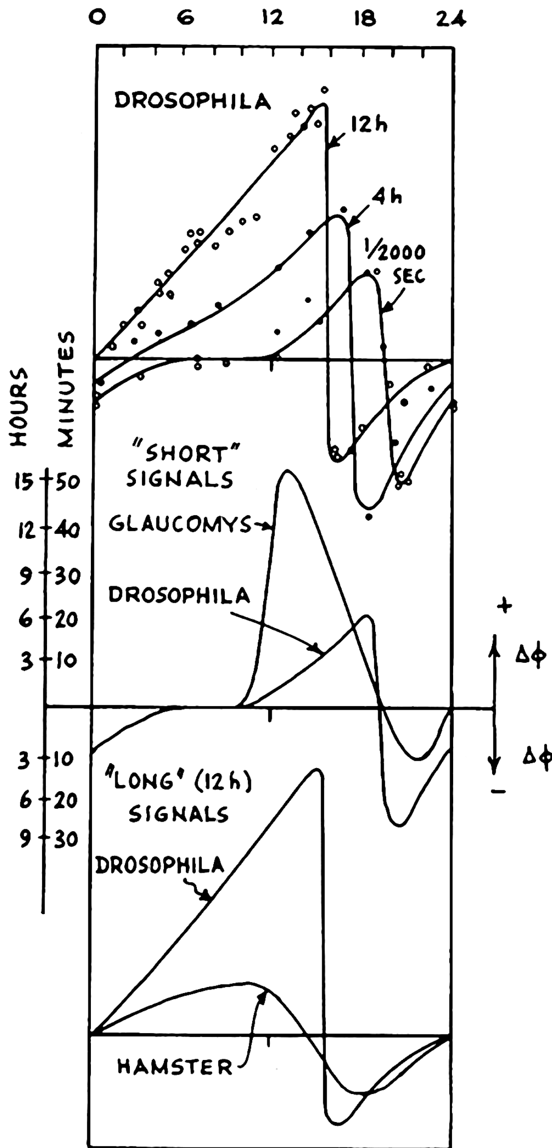

FIGURE 14. Curves for the response to light of *Drosophila*, *Glaucomys*, and *Mesocricetus* (hamster). The response

FIG. 1.7. By the time of the 1960 Cold Spring Harbor Symposium on Biological Clocks, Colin Pittendrigh had adopted what was becoming the standard phase response curve (PRC) diagram, shown in his figure 14, where he plotted the effects of different light-pulse lengths on *Drosophila* eclosion (top), and compared the PRC for *Drosophila* with those of the hamster and flying squirrel, drawing on Patricia DeCoursey's work at the University of Wisconsin. Colin S. Pittendrigh, "Circadian Rhythms and the Circadian Organization of Living Systems," *Biological Clocks: Cold Spring Harbor Symposia on Quantitative Biology* 25 (1960): 175, fig. 14.

threshold process, triggered by the light/dark "sunset" transition when the photoperiod is at least twelve hours. This rejects a competing hypothesis that light acts continuously on the organism's clock, speeding it up or slowing it down to adjust its period to the environment.[77] This latter idea was presented at the Cold Spring Harbor Symposium by Aschoff, leading to his development of a period-response curve (τ-RC) as a measure of the effect of Zeitgeber on clock speed.

As Aschoff and his German colleague Rütger Wever saw it, a Zeitgeber—light, for example—could act on the endogenous clock in one of two ways or as a combination of these. First, it could be the transitions from dark to light and light to dark that were effective—that is, the experience of dawn and dusk, respectively. This is the model favored by Pittendrigh—namely, that the hands of the clock were pushed forward or backward by relatively sudden transitions in the Zeitgeber, which reset the clock to keep it synchronized with the relevant environmental rhythm (such as the alternation between daylight and night). Aschoff called this the differential effect, and it is the assumption underlying Pittendrigh's model for oscillators. It was a natural for explaining pulse-phasing experiments, since pulses are abrupt—if brief—transitions from one state to another, whether they are light pulses, dark pulses, or temperature or humidity pulses. A second possibility was that the level and duration of the Zeitgeber were collectively effective, as one experiences more daylight in the North on a summer day than on a winter day, for example. The assumption here is that an organism's biological clock is more or less continually readjusting its speed, owing to its differential exposure to the Zeitgeber (e.g., light), in both duration and intensity. In this case some integration (i.e., continual summation) process was implicated. Aschoff called this the proportional effect. The historian of technology might suspect that the contemporary development of edge-triggered and level-triggered digital electronic circuits and voltage-controlled frequency oscillators may have influenced Aschoff's and Wever's modeling, or Bruce's for that matter.

Certainly the language and intellectual construction of some of these oscillator models do suggest familiarity with electronic computer design, as does the application of electronic circuits and wave-form generators to model biological clocks. An integrative (proportional) model would instinctively appeal to researchers who had neon-bulb, capacitive-discharge relaxation oscillators or inductive-capacitive tank oscillators (L-C circuits) in mind, models that by 1960 were commonplace in rhythms studies. The possibility that edge-triggered semiconductor gates and square-wave generating flip-flop circuits used in digital computer design provided Aschoff some guidance is suggested by the use of square-waves to illustrate the difference between level-

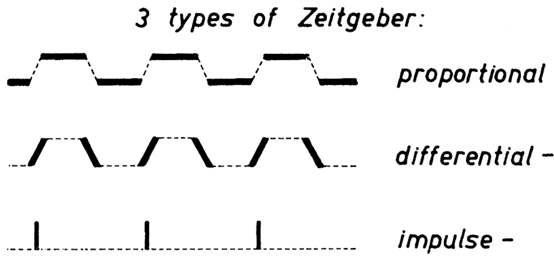

Figure 11. Simplified scheme of 3 possible types of effective Zeitgeber components.

FIG. 1.8. Jürgen Aschoff proposed in his contribution to the 1960 Cold Spring Harbor Symposium that exogenously generated Zeitgeber, or triggering signals, might act on the biological clock mechanism either as a function of a transition, say from light to dark (L:D, a differential effect), or as a function of accumulated quantity or proportion of some sort (proportional). Aschoff's inclusion of this illustration of square waves with pulse lengths (top) and level transitions (middle) suggests that he was influenced by models based on digital computer circuitry. Scientists familiar with this might have tended toward the differential effect, inasmuch as digital switches are typically triggered by leading-edge or trailing-edge transitions in, for example, voltage levels. The early audio-coupled computer modems also used such a differential system, alternating between two fixed tones. But some observed rhythmic phenomena suggested that some sort of accumulative, proportional effect of exposure to light, which could be modeled mathematically as an integration—the length of the pulse rather than its edge transitions—might be the effective synchronizer. Jürgen Aschoff, "Exogenous and Endogenous Components in Circadian Rhythms," *Biological Clocks: Cold Spring Harbor Symposia on Quantitative Biology* 25 (1960): 19, fig. 11.

triggered and pulse/edge-triggered entrainment in figure 11 of his paper (see figure 1.8).[78]

One advantage of the proportional-effect Zeitgeber model is that it fit well with the relaxation-oscillator model that many chronobiologists adopted following their introduction to it by Balthasar van der Pol at the 1939 meeting of the ISSBR. In this model, the Zeitgeber (say, illumination) is accumulated or summed until it reaches a threshold and triggers a sudden, irritable release of stored energy. The model was compatible with the idea that such reactions were "irritable" in nature, which had a long tradition in biological speculation, a kind of vitalistic "mechanism" originating in the seventeenth-century observations of Francis Glisson and enjoying longevity in Albrecht von Haller's doctrine of irritability.

In particular, Aschoff's research had revealed that it was not just the time in the rhythmic cycle when a stimulus was presented that was important but also the length and intensity of the stimulus pulse, agreeing in a general sense with Bünning's ideas about how light stimulus interacts with biological clocks

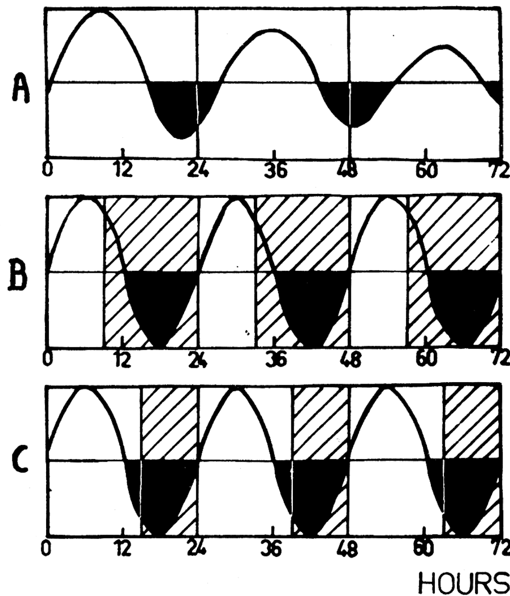

FIGURE 20*A*–*C*. The clock causes the alternation of half cycles with qualitatively different light sensitivity (white versus black). *A:* The free running clock in continuous light or continuous darkness. Cycles not exactly 24 hours. *B:* Short day. *C:* Long day. Long-day conditions allow the light to fall into the "black" half cycle.

FIG. 1.9. Erwin Bünning hypothesized that the seasonal rhythms of plants and animals are set by an interaction between incident light and their native circadian clocks. This figure from his contribution to the 1960 Cold Spring Harbor Symposium illustrates this principle. During short days, proportionally less of the circadian "day" part of the cycle would be exposed to light (b), and during long days, some of the light would fall into the "night" portion of the cycle (c). Erwin Bünning, "Circadian Rhythms and Time Measurement in Photoperiodism," *Biological Clocks: Cold Spring Harbor Symposia on Quantitative Biology* 25 (1960): 253, fig. 20.

to control photoperiodic response in plants and animals (see figure 1.9). This principle explained the different effects of illumination on day-active and night-active animals, which was summarized in what Pittendrigh denoted "Aschoff's rule": Day-active (diurnal) animals become increasingly active when kept in L:L, and their "spontaneous frequency," defined as the ratio between the active and rest portions of their cycle (α/ρ), increases with increased intensity of illumination. This was a level effect, not an edge-triggered transition effect. Nocturnal animals show the opposite response, the intensity of the light depressing their activity and decreasing the α/ρ ratio.

Continual darkness has just the opposite effect, lengthening the period of the diurnal animal and shortening it for the nocturnal.[79] This makes sense, of course. Humans naturally get sleepy when the lights go out, and a bright light sends nocturnal cockroaches scurrying for their hideaways. Indeed, Aschoff wrote, "what does 'level' mean in the circadian oscillation of an organism? The terms 'light-active' and 'dark-active' are meaningful only in the way that light increases activity (or the level) in light-active animals and decreases it in dark-active animals. Without such a causal connection between intensity of illumination and level of excitement (or activity) there would be no separation between light- and dark-active animals."[80]

It followed from this that the intensity of the Zeitgeber must act to speed up or slow down the clock; a continuous adjustment of angular velocity, in the language of oscillators, rather than the abrupt edge effects one might deduce from pulse phasing. Likewise, the duration of the Zeitgeber, the length between dawn and dusk, should be of consequence in this model, where adjustment of angular velocity depends on a summation of light exposure in some way, even if this takes the shape of the duration between two light pulses in an experiment. This would help explain photoperiodic effects that were observed experimentally—namely, the use of a pair of pulses to create what was called a skeleton day length, a functionally equivalent stand-in for continual day-length illumination. In 1960 Aschoff admitted that both phase adjustment and velocity adjustment might be at work in actual entrainment.

From the standpoint of oscillator mechanics, phase setting and velocity adjustment achieved the same results, but the choice carried with it an implicit commitment to the nature of the biological mechanism. Otto Schmitt, a biophysicist at the University of Minnesota with a long-standing preoccupation with the physics of biological systems, participated in the 1960 Cold Spring Harbor Symposium and offered this observation during the discussion period following Sweeney's paper:

> It is perhaps worthwhile to examine this relationship specifically and to emphasize that these nomenclatures are fully interchangeable and that the choice of one over the other is dictated primarily by the mathematical model of oscillation that the author has in mind. If he thinks of the rhythmic process as originating in an intrinsic or extrinsic oscillator of constant frequency which passes on its signal to an overt manifestation through a series of delaying or modifying processes, then he will probably utilize the phase shift nomenclature. If he thinks of the oscillation as being autonomous and essentially free running but controlled or modulated by environmental parameters, then he will probably utilize the variable frequency nomenclature.[81]

This was an electrical engineer's take on the problem, speaking in terms of an oscillator's frequency rather than its velocity.

Working as a postdoc with both Pittendrigh and Aschoff, Daan later relabeled these alternatives "non-parametric entrainment," which was a discrete or "phasic" adjustment of the clock, and "parametric entrainment," which was continuous or "tonic." The term *tonic* harks back to the early modern doctrine of irritability, when stimulus was thought to affect the "tone" of the body's fibers, which then affected the circulation of fluids and physiological function in general.[82] It therefore carried with it a historical plausibility as a vitalist-mechanist explanation. But for Daan, the similarity of experimental conditions to actual natural conditions was a compelling factor. He gravitated toward Aschoff's proportional or parametric model in part because a central weakness of pulse phasing was that it did not represent natural conditions. The natural situations in which organisms find themselves are characterized by widely varying light conditions, from sunny days to gloomy ones, and burrowing day-active animals, for example, do not experience a "dawn" Zeitgeber until after they have left their lairs, by which time they are already awake: "While Aschoff's model allowed qualitative predictions on the timing of animal behavior in the natural environment . . . the PRC model has never been tested under natural LD situations."[83]

There was no decisive evidence for the parametric-entrainment or proportional model until Daan investigated the rhythm of the European ground squirrel in the 1990s. Just as described hypothetically above, this animal emerges from its burrow two or three hours after dawn and returns two hours before dusk and thus never experiences the natural cosmic light/dark transitions, only those it imposes by its actions; they "virtually never in their whole life see the twilight transitions, which are so crucial in PRC entrainment."[84] For this animal, entrainment must draw phasing information from the light it is exposed to while it is aboveground, a summative or level effect, and for this to provide reliable synchronization in naturally variable daylight conditions, the information must somehow be integrated over many days.[85] Interestingly, the τ-RCs that can be experimentally constructed for these animals are similar in shape to those for other rodents, raising the possibility that some combination of edge-triggering phase shift and level-triggering angular velocity adjustment of the clock might be at work, analogous to adjusting an analog clock's escapement spring or pendulum length (speed) at the same time as moving its hands ("phasic" reset) to the "correct" time. But, for Daan, as for many evolutionary biologists, the velocity-adjustment parametric model had more verisimilitude and attested to the power of natural selection to produce good clocks:

> I believe that the notion of PRC entrainment by single, discrete phase shifts has suggested to us a mechanism far too coarse for circadian pacemakers. The adjustment of τ [period] by continuous action of light may well turn out to be a fundamental functional property of these pacemakers, allowing animals to finely tune their intrinsic period to that of the earth's rotation without needing to perturb the system each day. . . . In contrast to the prevailing opinion in our field, I am convinced that evolution has not satisfied itself with making sloppy biological clocks, with periods deviating from 24 h, which need resetting once or twice each day. No, evolution has gone the whole hog and has taken care that these clocks under natural conditions run at exactly 24 h without the need for disturbing daily corrections.[86]

An important part of the history of chronobiology is how biologists—who recognized that some organic phenomena are rhythmic and that these rhythms are not simple responses to stimuli but inherent characteristics of seemingly all organisms—moved beyond the phenomenology of rhythms to modeling them, to searching for the mechanisms that produce rhythms, and ultimately to probing these mechanisms with the tools of microbiology and molecular genetics. This transition from searching for stimulus-response control mechanisms to modeling clocks marks an essential transformation from physiology to chronobiology, although it would not be called by this name until the late 1960s. The seminal Twenty Fifth Cold Spring Harbor Symposium on Quantitative Biology, at which researchers interested in biological rhythms from various disciplinary backgrounds—botanists, zoologists, mathematicians, and engineers—presented their wide-ranging experiments and offered theoretical interpretations of their results gave biological rhythmicity a publicly visible place in biological science.

One aspect of these initial stages of development was the reorientation of physiologists' approach to phenomena that they casually observed to be rhythmic, but which were being investigated as responses to stimuli controlled by specific mechanisms. They now came to recognize that the rhythms themselves were important properties of plants and animals and that the organs or parts generating them were significant objects for biological study. Once formulated as scientific objects, biological clocks could be characterized and experimented with as black boxes, opaque mechanisms that could be analyzed

by controlled experiments, by varying experimental inputs, and by observing corresponding changes in behaviors, be they motor activities, color changes, or variations in subtle physiological functions such as the excretion of potassium ions in the urine.

Once the concept of the biological clock as an organic mechanism was articulated, biologists realized that the ability of biological clocks to respond to environmental signals that help coordinate or synchronize them with key rhythmic changes in the environment, anticipation of which might convey fitness, was of central importance. How clocks interact with the environment became a subject of study in leading laboratories. A first step was to describe the relationship between synchronizing signals provided by the environment—the Zeitgeber that "entrain" the clocks to rhythms with particular frequencies and phasing—and the function of the clocks by observing organisms' behaviors.

One protocol that was developed already in the 1920s with plants and used extensively in the study of *Drosophila* eclosion rhythm in the 1930s and 1950s was the use of light (or dark) pulses to reset the rhythms of specimens that were otherwise kept in continual darkness or continual light. This protocol led directly to the development of the PRC in the late 1950s to describe organisms' responses to abrupt transitions in Zeitgeber. This may have influenced Brett to liken phase resetting to setting the alarm on an alarm clock and Pittendrigh to emphasize edge-triggered oscillators as models for biological clocks. Pittendrigh's preference for a transition-triggered phase reset was, as Daan later observed, likely a result of his choice of *Drosophila* eclosion as a model, as this produced relatively clean "dawn" threshold effects when subject to short light pulses.[87] But it was also a logical conclusion: "the *pattern* of the curve (which is all that different species share) has to be as it is: only a morning advance and an evening delay will give a stable equilibrium no matter what the *shape* of the curve."[88]

The PRC therefore emerged into daylight as a refined tool in standard form at least by 1958 in the doctoral work of Burchard, which DeCoursey quickly adopted, using it in print for the first time in her January 1960 paper in *Science*. But its gestation began earlier and appears to have been a logical outcome of the use of light pulses as a technique for studying how clocks are reset by Zeitgeber. This technique was in use in several laboratories by the time Burchard and DeCoursey normalized the data it produced as the PRC. Hastings and Sweeney were using this technique to study the shift in peak luminescence of populations of the bioluminescent alga *Gonyaulax* in 1958. But they did not at that time graph the results of these experimental shifts in what became the standard form of the PRC.

Sweeney and Hastings included phase response in their 1960 Cold Spring Harbor paper, but in the form of a graph of the effect of a light pulse on *Drosophila* eclosion that they had appropriated from a 1958 paper by Pittendrigh, Bruce, and Peter Kaus, and they did not construct a standardized PRC. The fact that for years they had been using the experimental protocols that had led Burchard and DeCoursey to formalize the PRC but did not themselves deploy it until after 1960 suggests that their attention was not focused on phase response as a signature phenomenon of biological oscillators but, rather, on other properties of biological rhythms. The title of their paper for the Cold Spring Harbor Symposium, "Effects of Temperature upon Diurnal Rhythms," points to the crucial importance of the clock's response to temperature.