Behavioral Neurogenetic Studies of a Circadian Clock in *Drosophila melanogaster*

Thesis by

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To

the fond memories

of

Yuk-Kwang Wong, grandaunt

and

King-Yee Orr, father

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Abstract

The circadian clock controlling the locomotor activity of the adult fruitfly, *Drosophila melanogaster*, is studied in one wild-type and five clock mutant strains. Locomotive activity of individual flies are monitored using arrays of infra-red beams and detectors. It is found that the temperature compensation mechanism is intact in the mutants *And* and Clk^{KO6}, is slightly defective in the mutant per^{s} and is grossly defective in the mutants per^{l1} and per^{l2}. In the per^{s} and per^{l1} mutants, this defect is enhanced when both eyes and major parts of both optic lobes are eliminated by a genetic mutation (*sine oculus*). The inter-individual variation of periods in a strain is found to increase much more than linearly with the average period of the same strain. The interaction between the *And* and the *per* loci and that between the *And* and Clk^{KO6} loci are found to be either very weak or non-existent (effects of mutations additive), whereas the interactions among the various alleles in the *per* locus are found to be strong (effects of mutations non-additive).

Ten 'Phase Resetting Curves' (PRC) obtained with saturating light pulses for six strains of flies at various temperatures are presented. All the ten cases exhibit basically 'type-1' resetting behavior (average slope = 1). Comparisons of the PRC's for per^{s}, per^{l1} and wild-type at 17° C. suggest that the mutations per^{s} and per^{l1} change the period of the circadian clock by differentially shortening and lengthening, respectively, the duration of the 'subjective day' phase of the oscillation . Comparisons between the PRC's for per^{s} at 17°C, 22°C, and 25°C. and comparison between the wild-type PRC's at 17°C and 22°C. do *not* reveal major changes in the temporal structure of these two circadian clocks over the stated temperature ranges .

The responses of one wild-type and five mutant circadian clocks to sustained dim light of the range 5×10^{-4} lux to 50 lux at 22 °C. are studied. In each strain, a

critical 'window' of light intensity is found within which a variety of unstable clock features, including arrhythmia, are observed. The light intensity at which this critical window occurs in each of the mutant is 5 to 10 times lower than that in the wild-type. Responses from a ERG-defective mutant (*norpA*) are found to be qualitatively, but not quantitatively, similar to that of the wild-type. Responses from an eyeless and ocelli-less mutant (*sine oculus*) indicate that both period changes and arrhythmicity can be elicited by light in the absence of the compound eyes and ocelli. However, the sharp dependence of the occurences of these phenomena on light intensity is lost in this mutant.

Arguments are presented to suggest that none of the four mutations -- *And* , Clk^{KO6}, per^{s}, and per^{ll} -- cause changes of period by mimicking the effects of tonic light on the *Drosophila* circadian system.

The phase resetting curves (PRC) and the dim light responses described above are found to be incompatible with a particular model of the Velocity Response Curve (VRC) theory to inter-relate the phasic to tonic effects of light, in which the tonic effect of light is assumed to be the result of a summation of the effects of a contiguous series of single light pulses, taking into account adaptation.

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Chapter 1

General Introduction

1.1 Observations on circadian rhythms

The first scientific observation of the existence of a daily rhythm was described in 1729 by the French astronomer de Marian in a report (ref.1-1) which showed that plants maintained in constant darkness and relatively constant temperature continued to demonstrate diurnally periodic leaf movements. This phenomenon later attracted the attention of many botanists in the 19^{th} century, including De Candolle, Sachs, Darwin, and Pfeffer. The efforts of these investigators, especially Pfeffer (ref.1-2), were primarily concerned with the interaction of the leaf movement rhythm in plants with light and temperature. Their results generally indicated that these rhythms *did* persist in darkness and in constant temperature, but opinions were divided as to whether such rhythms were *internally* generated in the plants or *externally* driven by a 'factor X' in the environment which had not yet been detected by the experimenters. It was not until 1930 that Bunning and Stern (ref.1-3) pointed out that the periods of most 'daily' rhythms observed are not precisely 24 hours but rather *circa* –24 hours with a range from 22 to 26 hours and thus brought severe difficulty to the 'factor X' theory. To this date, this observation has remained as one of the most forceful indications to practically all [1] researchers in the field that the observed rhythms are of an endogenous nature. Probably because of the uncertainty concerning the endogenous nature of the observed daily rhythms from 1729 to 1930, the question of whether these rhythms were of any use -- and therefore had selective value -- to the organism was not raised by any of the investigators in this period, including Darwin himself.

1. For the interpretation of this fact by the views of a very minor group in the field, see ref.1-4

1.2 Analysis of the functions of circadian clocks

Just like the shadow of an object cast by the sun can be considered as a chronometer only in the case of a sundial[2], likewise an observed oscillation, even when proven to be endogenously generated in an organism, can only be considered to be a biological *clock* when it is shown to serve a *function* of measuring time for the organism. Such a function was first sought for by Bunning (ref.1-6) in 1936 when he suggested, but did not prove, that a relationship existed between the daily rhythms of leaf movements in the plants and their ability to measure day length as expressed in the control of flowering. This suggestion by itself brought forth two new concepts in the field : (1) that the observed daily rhythms in organisms might not be the self-sustained oscillators themselves, but rather the output of such oscillators; (2) that these underlying oscillators serve useful functions to the organisms in which they are contained. Subsequent to the original proposal by Bunning, circadian clock features have indeed been found in the measurement of day length not only in the flowering of plants (ref.1-7) but also in the diapause of insects (ref.1-8). However whether the same circadian clock is involved in both the daily leaf movement rhythm and the photoperiodic timing, as originally proposed by Bunning, has not been established.

The search for adaptive values in the formal properties of circadian clocks have also been extended to the eclosion rhythm in insects by Kaus (ref.1-9), and to the locomotor activity of rodents by Pittendrigh and Daan (refs.1-10,1-11,1-12,1-13,1-14). In these studies, the authors extended the concept of the function of biological clocks to include, not only measuring the passage of time, but also the recognition of local time. That is to say, the circadian clock might be responsible for the synchronization (phase-locking) of the physiological activities of an organism to the 24-hour cycle in the environment. An obvious extension to this function of the

2. Analogy borrowed from Bunning (ref.1-5)

biological clock is to also demand it to play the role of a synchronizer for the various physiological processes in the internal milieu of an organism -- i.e. for the establishment of an internal temporal order. This last possibility is being actively pursued at a phenomenological level by Pittendrigh (ref.1-15) and at a physiological level by Moore-Ede (ref.1-16). Finally, biological clocks were shown to be involved in the orientation behavior of birds (refs.1-17,1-18) and of arthropods (ref.1-19). However, whether such clocks possess the same properties as the circadian oscillators being discussed has yet to be clearly established.

By searching for functions of circadian oscillations, the above analyses, most of which are formal in nature, have served very well to pinpoint many general properties of the circadian clock. These include the general precision of circadian oscillations compared to other known biological oscillations, their strong interactions with light, and their general homeotasis of period. (Of these properties, the temperature compensation of circadian clock period has attracted the most attention of investigators in the field and will be discussed in section 2.2 of this thesis.) However, these analyses have not yet provided any unequivocal insights about the concrete physical mechanisms underlying any particular circadian clock.

1.3 Analysis of the mechanisms underlying circadian clocks

Since the beginning of the 1960's, the field of circadian biology, formerly populated mainly by botanists and zoologists, has attracted investigators from diverse backgrounds, which include mathematics, biochemistry, neurophysiology, endocrinology and genetics. The interests of these investigators, which are currently by far the majority in the field, have been focused not so much on 'what does the circadian clock do ?', but rather, 'how does it do it?'. Mechanisms underlying the clock have been sought for along several separate lines of research: (1) studies of the dynamics of the clock, (2) analysis of the physiological organization of the clock, (3) searching for concrete biophysical mechanisms, and (4) anatomical localization of the clock. These are discussed separately below.

1.3.1 Studies of the dynamics of the clock

Since the period and phase of circadian clocks are well known to be strongly influenced by light (see chapter 3 and chapter 4 of this thesis), many investigators have sought insights of the clock mechanism by observations of the dynamics involved in the entrainment and phase-shifting of circadian rhythm by light. As these attempts are mostly *ad hoc* models proposed to explain particular sets of experiments, the nature of the models are as diverse as the experimental phenomena observed. Thus, in the interpretation of the phase-shifting phenomenology of the *D. pseudobscura* eclosion rhythm, Ottesen, Pavlidis and Pittendrigh (ref.1-20) found it adequate to describe the circadian clock as a simple one-parameter oscillation where the phase could be instantaneously reset ('popped') from some state to another. From studies on the resetting (ref.1-72) and entrainment (ref.1-73) behavior of the circadian rhythm of adult emergence from the pupal case in the *Pernyi* silkmoth, Truman (ref.1-73) found evidence to support a 'relaxation oscillator' model in which the cycle could be broken down to a charging phase

and a discharging phase. He further suggested that, under entrainment of certain photoperiods, this oscillator in fact operated in an 'hour glass' mode (in which the 'hour glass' was 'turned over' at every 'dusk'). To incorporate the phase-shifting phenomenology in the *D. pseudobscura* eclosion clock with its behavior under changes of ambient light and temperature levels, Pavlidis (ref.1-21) found a limit cycle model of the clock to be most satisfactory. Most recently, by examining the resetting of *amplitude* as well as phase of the *D pseudobscura* eclosion clock and by examining the slopes of resetting curves in general, Winfree (ref.1-22) presented arguments to nullify all of the above models and suggested (on rather weak arguments) a multi-oscillator model for the circadian clock.

In the opinion of the author, the major contribution of this line of research has been the demonstration of the possibility of using the light responsiveness of the circadian system as an operational definition of the state of the underlying clock (see chapter 3). There should be no doubt to any one that this operational definition is no more than an approximation of the underlying physical process. The apparent confusion in this sub-field of circadian research seems to arise from attempts to search for the next higher level of approximation of the state of the clock -- *in its mathematical form.* All the prevailing mathematical approaches tend to represent the circadian clock as a *continuous*, multi-parameter oscillation in a one- or multi- dimensional phase space (refs. 1-21, 1-23, 1-24) . To the degree that different biological processes may be involved at different phases of the circadian cycle, there is a danger that these mathematical models totally misrepresent the reality of the situation. Thus, until more information about the concrete mechanism underlying circadian oscillations is known, rigorous arguments on the alternative mathematical models concerning the dynamics of the circadian clock do not seem fruitful.

1.3.2 Analysis of the physiological organization of circadian systems

Perhaps the most prominent outcome of researches aimed at elucidating the organization of the circadian clock at the physiological level in recent years is the convergence of observations made in diverse organisms that clearly indicate that there exist more than one circadian clock in a single metazoan organism. These observations fall into two distinct groups: (1) Evidence based on the existence of more than one oscillator controlling the expression of a *single* overt rhythm ; and , (2) Evidence based on the existence of different oscillators controlling the expressions of *different* overt rhythms.

Evidence of the existence of more than one oscillator controlling the expression of a *single* overt rhythm comes mainly from observations in the 'splitting' of locomotor activity in several vertebrates -- squirrels (ref.1-25), tree shrews (ref.1-26), hamsters (ref.1-27), starlings (ref.1-28), and lizards (ref.1-29) -- into two or more groups, which showed up as bands in actograms, with each group of activity running at a different frequency. While, in some cases (as in the rodents) , such bands of activity were observed to eventually merge or lock on to a particular phase relationship; in other cases (as in the lizards) , the differences in frequencies were observed to persist for many cycles so that the bands of activity represented on an actogram actually 'criss-crossed' one another in a very striking fashion. Even though the 'splitting' behavior appeared to be very similar in the different species, the stimuli that were found to be effective in eliciting this behavior differed widely from one species to the next. Thus 'splitting' could be induced by a step-up in ambient light level to the squirrel, by a step-down in ambient light level to the tree shrew, by testosterone injection into the starling, and by pinealectomy in the lizard. Recently, Pickand and Turek (ref.1-74) have provided evidence that suggests bilateral representation of circadian oscillators in the suprachiasmatic nuclei in the hamster can account for the splitting of activity rhythm due to change of ambient

light levels in this species. Among invertebrates, a roughly analogous example of internally desynchronized oscillators was reported in the beetle, *Blaps* (ref.1-30), where circadian oscillations of the sensitivities of the two compound eyes were observed to drift from totally in phase to 180 degrees out of phase in the course of an 18-day freerun. Similar desynchronization of the spontaneous rhythms of compound action potential (CAP) of the two eyes in Aplysia, which were shown to be circadian oscillators themselves (ref.1-31) , has also been observed (ref.1-32) .

Evidence of the existence of different oscillators controlling the expressions of *different* overt rhythms was initially observed in studies in which the activity rhythm and rectal temperature in man were monitored under free running conditions (ref.1-33). It was observed in one subject that, for the first two weeks in free run, the two rhythms ran in phase and with a common period of 25.3 hours, but, starting on the 15^{th} day and continuing on to the end of the fourth week, the period of the activity rhythm was lengthened to 33.4 hours while the period of the rectal temperature rhythm stayed at the original period. Thus, the two rhythms monitored were drastically decoupled from one another. A second observation on the internal desynchronization of two or more physiological processes was made in the squirrel monkey (ref.1-16). In this case, the rhythms of feeding and of colonic temperature of an animal were found to freerun with periods of 25.0 and 25.2 hours, respectively, while the rhythms of urinary potassium concentration and of water excretion of the same animal were found to have periods of 20.6 and 20.9 hours, respectively.

The findings described above, taken as a whole, have brought forth a new and fundamental insight of the physiological organization of circadian systems, and have opened up new avenues for many circadian 'model-builders' (see dicussion of ref.1-14 for a mini-review). Unfortunately, systematic follow-up investigations of each of the above cases are still lacking. At least part of the reason for this stagnation seems to be the difficulty involved in consistently reproducing the 'splitting'

phenomenon under a controlled environment in all the cases.

1.3.3 Searching for concrete biophysical mechanisms

Research on the physical basis of circadian rhythms have been aimed at determining the possible involvement of subcellular components (from DNA, RNA, proteins, cAMP to membrane and mitochondria) in the oscillating process. A rather standard protocol has been followed by most investigators. Briefly, a chemical agent is considered to have an effect on the clock process if application of the agent to an organism causes a change in period and/or phase in the overt rhythm . Dose response curves are normally obtained and specificity of the action of the agent also needs to be established. A brief summary of the main results from this line of research follows. (See refs. 1-35, 1-36, 1-37 for more exhaustive reviews.)

The observations that circadian rhythms have been observed in nondividing cells (ref.1-7), anucleate cells (ref.1-39), and cells that have been treated with inihibitors of DNA synthesis (ref.1-40) have been generally taken as strong evidence that DNA replication is not required for the generation of circadian rhythms. However, opinions on whether RNA synthesis is required for the generation of circadian rhythms are more diverse. The applications of inhibitors of DNA transcription to such systems as *Acetabularia* (ref.1-40), *Aplysia* (ref.1-41), *Gonyaulax* (ref.1-42), and *Neurospora* (ref.1-43) have been shown to cause arrhythmicity or phase shifts in some of the rhythms monitored but to have no detectable effects on others. Since the loss of overt rhythmicity cannot be taken as indication of an arrhythmic state in the oscillator, and since consistent and comprehensive phase responses have not yet been reported in a single system, these results are not sufficient to assess the role that RNA synthesis plays, if any, in the generation of circadian oscillation. Two reports have been often cited to rule out the involvement of RNA synthesis in the clock mechanism; the first is the observation of a circadian rhythm in

photosynthesis in enucleated *Acetabularia* (ref.1-39) and the second is the report on the persistence of circadian rhythm in enzyme activity in human red blood cell suspension (ref.1-38). However, the first report did not explore the possibility of certain DNA segments or long-lived mRNA in the cytosol, and the second report has not been reproducible (ref.1-55).

The role of protein synthesis in the generation and maintenance of circadian rhythms has been more clearly demonstrated. In general it has been shown that inhibitors of organelle protein synthesis (e.g. chloramphenicol and streptomycin) have no effects on the expression of circadian rhythms (refs. 1-34, 1-45), whereas inhibitors of cytosol protein synthesis (e.g. cycloheximide and puromycin) are able to change the periods of circadian rhythms when applied continuously and are able to induce both positive and negative phase shifts when applied in pulse form (refs. 1-34, 1-40, 1-42, 1-48). It is not yet possible to decide from these studies whether protein synthesis is involved as part of the oscillating mechanisms, or a protein directly involved in the oscillating mechanism has a very short life, or the effect of blocking protein synthesis on the clock is an indirect one.

The possible involvement of membrane in circadian rhythm has found strong support in two different reports. The first is the finding (ref.1-49) that a ten fold increase in extracellular potassium concentration for a period of four hours can phase advance or delay the spontaneous CAP rhythm in the eye of *Aplysia* depending on the phase at which the pulse is given. The second is the observation that the period of conidiation rhythm in a *Neurospora* mutant which is defective in a fatty acid synthetase can be drastically lengthened (up to period length of 40 hours) by manipulation in the growth media of the concentration of unsaturated fatty acids. Many models (refs. 1-51, 1-52, 1-53, 1-54) have been postulated to explain the generation of circadian oscillation in terms of membrane components and membrane related phenomena (with 'keywords' like pumps, channels, ions gradient, transport,

cooperative phenomenon). Some of these models, (e.g. ref.1-51), have the merit of helping to unify many features observed in circadian clocks -- their temperature compensation of period, their long time course, their sensitivities to light. However, none of these models provide predictions that are specific enough to help designing a new experiment.

1.3.4 Anatomical localization of clock sites

The anatomical localization of circadian clocks has been a popular approach among researchers using arthropod, birds and mammals as experimental subjects (see refs. 1-56, 1-57, 1-58 for reviews) . The protocol most often used is to demonstrate the loss of overt rhythmicity of an experimental animal after a certain organ, nerve tract, or nucleus in the nervous system has been lesioned. One then tries to restore rhythmicity to such a lesioned and arrhythmic animal by the transplantation of a corresponding intact part from a control animal to the former. Successful restoration of rhythmicity in the experimental animal, including preservation of the phase of the original rhythm in the control animal, is then taken to be strong evidence that the involved anatomical site contains at least one essential component of the circadian clock. Furthermore, it would be inferred that the coupling between the clock and the overt rhythm monitored is via a hormonal channel. During the past ten years, this approach has enjoyed a number of notable successes, which will be discussed in section 2.3 . In one case (ref.1-59), a tentative 'circuit diagram' can even be drawn relating the clock to the input and output pathways -- at least as a working model.

1.4 Genetic approaches to the study of circadian clocks

Genetic studies of the circadian clock have followed several approaches. The first approach involves examination of the differences between clock phenotypes (e.g. periods, phase, waveforms) of different strains among a species and observation of the inheritance patterns when the strains are crossed (refs. 1-60, 1-61, 1-62) . The results, at least in some cases, indicate that there is more than one gene involved in the phenotypes studied. A second approach is to use selection procedures in the laboratory to screen for a particular clock attribute, as is well exemplified by the selection of strains of *D. pseudobscura* with 'early' and 'late' eclosion times by Pittendrigh (ref. 1-63). A difficulty in the selection experiments is also the uncertainty in the number of genes involved in the effect being selected for and this makes further analysis rather complex. As a result, a third approach has gained popularity in the last decade or so in which the effect on the clock due to a single gene mutation is sought for.

Conceptually, single gene mutations that affect clock parameters can fall into three classes. First, the effect on the clock observed may be a nonspecific (pleiotropic) action of the mutation. A hypothetical example would be that the clock mechanism was based on some membrane-related phenomenon in a certain group of neurons in an organism: a mutation that affected some general membrane property in all neurons in the organism would then also affect the oscillating mechanism. While these are *bona fide* changes in clock properties, the non-specific nature of the cause makes this class of mutants less attractive from an experimental point of view.

The second class of mutations includes those that cause changes in clock parameters because a specific step in the mechanisms making up the 'clock pathway' is altered as a result of the mutation(s). With a sizeable number of these muta-

tions in hand, one can then hope to carry out the kind of 'genetic dissection' analyses that have proven so successful in the illumination of metabolic pathways (ref.1-64). Specifically, molecular genetic and biochemical techniques can be used to isolate products of the wild-type and mutant clock genes. It is likely that such information would give unique insight into the mechanism underlying the circadian clock. Secondly, mutants can be searched for that have altered physiological responses to various environmental changes (entrainment, phase-resetting, response to temperature changes) to reveal the structure and the dynamics of the system. Thirdly, clock mutations can be analysed as to their 'conventional genetic ' relationship with one another, such as complementation and dosage effects. We note that it is the working hypothesis of most 'clock geneticists' that clock mutations obtained, until proven otherwise, fall into this class.

The third class of clock mutations to be discussed is actually a subset of the mutations in the second class, but it is treated separately because its classification arises from concepts in the field of behavioral genetics rather than circadian research. One working hypothesis of a behavior geneticist (ref.1-65) is that a given behavior can be decomposed to its constituent components (which we shall call 'functional units of behavior' here) each of which is specified by a group of genes. By isolating mutants that are defective in one or more of the key control elements in these groups of genes, one can therefore hope to modify or even delete the corresponding functional units at the behavioral level so that a relatively complex behavior can be 'dissected' into simpler subunits in this way. Even though examples of genetic manipulation of functional behavioral units as outlined above are still lacking, yet the discovery by Rothenbuhler (ref.1-66) that a certain 'hygienic' behavior in the honey bee could be decomposed into two units (uncapping of pupal case and removal of dead pupae) each of which could be blocked by mutation of a separate recessive gene indicates that this approach is a feasible one. In the

perspective of clock genetics, one hopes that the circadian oscillator is so structured that mutations could be obtained which would drastically alter or abolish a *functional unit* of the basic 'pathway'. One possible example of such functional decomposition of the circadian clock is the division of the cycle into 'subjective day' and 'subjective night' phases (or possibly even finer segments). The possibility that each of these phases could be controlled by different genes in *Drosophila* is explored in Chapter 3 of this thesis.

Currently, single gene clock mutations have been found in three organisms -- the photosynthetic flagellate, *Chlamydomonas reinhardi* (ref.1-67), the bread mold, *Neurospora crassa* (ref.1-68), and the fruit fly, *Drosophila melanogaster* (ref.1-69).

Two short period and four long period mutants have been found in *Chlamydomonas* which affect the circadian rhythm of the populational phototactic response. Progress in the analysis of these circadian mutants has been slow, at least partly because of the extremely difficult genetic work involved in this organism.

So far, seven long period and five short period mutants have been isolated in *Neurospora*. The rather well known genetics and the large body of information on the biochemistry of this system make it a very promising model system for the elucidation of the biochemical basis of circadian rhythm.

Of the three organisms in which clock mutations have been found, *Drosophila* has the most well known genetics (ref.1-70). It is the only system that shows relatively complex behavior and thus is the organism from which one would expect a relatively more interesting answer to the ultimate question of the function of the circadian clock. Also, the *Drosophila* mutants are the only clock mutants that possess a nervous system. The neuroanatomy of this organism is well studied (ref.1-71) and the neurogenetics of this organism has recently attracted the attention of many

investigators (ref.1-44). Initial indications that the circadian oscillator of this organism was contained in the brain (ref.1-45) have been substantiated by recent transplantation experiments (ref.1-46). Cellular histochemical mosiac techniques (ref.1-47) are available for further localization of the site of oscillation. Finally, *Drosophila* clock mutants are the only ones whose clock features can be studied on the basis of individual organisms rather than of a population ; and the locomotor activity rhythm of adult *Drosophila* (see appendices of this thesis), with which one can assay the circadian oscillator, provides a resolution of measurement not approached by the other two clock genetic systems.

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Chapter 2

Characterization of the Phenotypes of Five Clock Mutations in *Drosophila melanogaster* and their Mutual Interactions

Summary

(1) The temperature dependence of clock period length of one wild-type and five clock mutant strains of *D. melanogaster* is examined. It is found that the temperature dependence of period in the two mutants *And* and Clk^{KO6} is similar to that of the wild-type, but the temperature dependence of period in the three *per* locus mutants, per^s, per^{l1}, and per^{l2}, is much larger than that of the wild-type (Fig. 2-1). Furthermore, the degree of such temperature dependence varies greatly from one animal to another in per^{l1} and per^{l2}. (Fig. 2-3)

(2) Both inter- and intra- individual stability of periods are studied. A general positive correlation is found between the inter-individual variability of periods of a strain and the average period of the same strain. (Fig. 2-2) The average precision of individual activity rhythm, normalized as percentage of average period, is found to be 1.4 % for the wild-type strain, 1.3 % for per^s, 1.2 % for per^{l1}, 1.3% for per^s/per^{l1}, 1.7 % for Clk^{KO6}, and 2.6 % for *And*.

(3) The defect in temperature compensation in per^s and per^{l1} is found to be enhanced when both eyes and major parts of both optic lobes in an animal are eliminated by a genetic mutation (Tables 2-1 and 2-2)

(4) Complementation of the per^0 and per^{l2} alleles to the other *per* alleles, including per^+, is studied. The results indicate that, generally, the average period of the per^x/per^0 animals is about 1 hour longer than that of the homozygous per^x animals, (where $x = s, l1,$ *or* +). However, most per^{l2}/per^0 animals are arrhythmic. (Table 2-3) The complementation pattern of per^{l2} is found to be drastically different from that of per^{l1}, but is almost identical to that of per^0. (Table 2-4)

(5) Periods of recombinants between the *per* and the *And* loci are analysed. The results suggest that interaction between these two loci are either very weak or non-existent. (Table 2-5)

(6) Complementation studies indicate that the mutant Clk^{KO6} is, like all the other *Drosophila* clock period mutants examined so far, 'semi-dominant' to the wild-type allele (Table 2-6). The interaction between Clk^{KO6} and *And* is also very weak or non-existent (Table 2-5).

(7) The implications of these findings are discussed.

2.1 Introduction

In the fruitfly *Drosophila melanogaster*, ten mutations have been induced by EMS [1] that affect the circadian rhythm of the animal. Three of the mutations, per^s,per^{l1}, and per^0, isolated by Konopka and Benzer (ref.2-1), were shown by these authors to affect the circadian clock(s) controlling both the pupal eclosion rhythm and the adult locomotor activity rhythm. per^s shortens the normal 24-hour period, per^{l1} lengthens it, and per^0 completely abolishes the rhythms. These three mutations behave as alleles of a single locus in the 3B1 region near the distal tip of the X-chromosome ,to which they have been extensively mapped (ref.2-1). A second long period mutant(*And*), isolated in 1976 by R. Smith and the author, was later mapped to about the mid point of the X-chromosome (ref.2-5). This mutation also affects both the eclosion and activity rhythms (ref.2-5). A third long period mutant and a second arrhythmic mutant, isolated ca. 1979 by R. Konopka through an activity rhythm screen, have both been shown to be allelic to the per^{l1} and per^{01} loci, respectively, and have thus been named per^{l2} and per^{02}, respectively. A seventh clock mutant on the X-chromosome was isolated in a locomotor activity screen in 1980 (Konopka and Orr, unpublished results). This mutant, tentatively named Clk^{KO6}, has been roughly mapped to a location near, but clearly not identical to , the *per* locus. (Konopka and Orr, unpublished observations.) The eclosion rhythms of these last three mutants have not yet been tested. Three more mutants -- *psi*1 and *gat* on the second chromosome, and *psi*2 on the third chromosome -- have recently been obtained through eclosion rhythm screens by R. Jackson (ref.2-6). The mutants *psi*1 and *psi*2 are defective in the phase relationships of the overt rhythm to the environmental light cycles and the mutant *gat* is defective in the gating of the eclosion peaks so that the overt rhythmicity is lost after a couple of cycles in constant darkness. That it is the circadian clock itself, rather than just the

1. ethyl methane sulfonate

output of the clock, that is affected in these last three mutants is suggested by the fact that in, all these mutants, a slight increase of free-running period (about 1 hour) is also observed.

In this chapter, we present results from our efforts to characterize the effects of six of the above ten mutations on the circadian system of *D. melanogaster* , as assayed by the locomotor activity rhythm in the adult animals. Throughout this study, we frequently exploit the fact that *Drosophila* is the *only* organism in which both clock mutations and analysis of the clock features of *individual* organisms, rather than those of a population, are available.

2.2 Temperature dependence and stability of periods

The phenomenon of temperature independence of the period in the *D.pseudobscura* circadian clock system was first established by Pittendrigh (ref.2-7) in 1954. The possibility that circadian systems are temperature *compensated*, rather than temperature *independent* was later proposed by Hastings and Sweeney in 1957(ref.2-8), who based their proposal on the observation that , in the dinoflagellate *Gonyaulax polyedra* , the clock period is in fact *longer* at higher temperature. That circadian clocks are not temperature *independent* are further suggested by two more lines of evidence. First, it is well known that circadian systems can be phase-shifted by temperature steps and pulses (ref.2-9,ref.2-10), suggesting that they are sensitive to temperature changes. Secondly, for many organisms, such as *Euglena* (ref.2-11) and *Neurospora* (ref.2-12), the apparent temperature insensitivity of clock period is effective only within a limited range of temperatures. The manner in which temperature compensation of clock period is carried out is unknown . The efforts described in section 3.4 of this thesis were aimed at differentiating between two definite types of models of this mechanism. The purpose of part of the investigations in this section is to ask whether this widely observed homeostatic property, which most investigators take as part of the definitions of a circadian oscillator, is susceptible to disturbance by the clock mutations under study.

One other general property of circadian clocks that emerges from the comparative studies of circadian rhythms in different species of nocturnal rodents by Daan and Pittendrigh (ref.2-25) is the relationship between the inter- and intra-individual stability of periods. These authors found that the further away from 24 hours the average period of a species is, the less precise is the individual clock in that species. These results are interpreted in terms of an entrainment strategy in that nature is selecting for oscillations that can more easily entrain, with a specific

phase relationship, to the 24 hour cycle that exists on earth. Here, we attempt to find out whether this functional relationship has a mechanistic base by seeking a relationship between single clock precision and average period lengths which are produced by single gene mutations.

2.2.1 Temperature Dependence of the Clock Period

Fig. 2-1 shows the distributions of periods , as measured by periodogram analysis, of the free-running activity rhythm in darkness of the wild-type strain Canton-S and five clock period mutants at three different temperatures -- 17°C., 22°C., and 25°C. [2] The results indicate that the period of the wild-type clock is very well, but nevertheless not completely, compensated in this temperature range -- the clock running on the average about 0.4 hour faster at 25°C. than at 17°C. This gives a change of period of 1.4% over an 8-degree span, which is approximately equal to a Q_{10} of 1.02 , showing a very well-compensated system compared to the ranges of values obtained in other circadian systems. (refs.2-8,2-23)

The period distributions of the *per* mutants, however, show that the temperature compensation mechanism is affected in all of these three period mutants. Of the three, the per^s mutant is affected least, with the clock running about 0.8 hour faster at 25°C. than at 17°C. This gives a 4.4% change in period over a 8-degree span (Q_{10}= 1.05), compared to the 1.4% in the wild-type.

The results for per^{l1} reveal a more severe defect in the temperature compensation mechanism. The group-averaged period of this mutant is increased from 27.8 hours at 17°C. to 30.5 hours at 25°C., giving a 9.7% change in period over an 8-degree

2. Practical considerations fix the temperatures studied to this limited range. Below 17°C., the animals are very inactive and therefore give very poor rhythms. Above 25°C., it is very hard to keep animals hydrated and also moulds tend to develop in the food and frequently lead to animals' death. 22°C. is found to be the optimal temperature both for the longevity of the animals and the sharpness of their rhythms For a given animal, the amount of activity per cycle tends to increase with temperature in the 17°C to 25°C. range. However, at a given temperature, the total amount of activity per day varies greatly from animal to animal (2 to 3 fold).

span and an approximate Q_{10} of about 0.88. Thus, this mutant clock actually runs significantly *more slowly* at a higher temperature. We note that even though the Q_{10} values of 0.88 for per^{l1} and 1.05 for per^{s} indicate an increase in temperature dependence on period in these mutants when compared to the wild-type, yet these Q_{10} values are still within the range of most other wild-type circadian systems (refs.2-8, 2-9, 2-23) that are less temperature compensated than the *Drosophila* system.

The results for per^{l2} are slightly more complicated. At both 17°C. and 22°C., the period distributions of this mutant are similar to those of per^{l1}, except that, of the population examined,a significant fraction (10% at 17C. and 30 % at 22°C.) are arrhythmic. [3] At 25°C., 19 out of 29 (70%) animals examined are arrhythmic, and the 8 animals that are rhythmic show greatly different periods.

On the other hand, the period distributions of the Clk^{KO6} mutant indicate a strong temperature compensation in period (Q_{10} = 1.02) just as in the case of the wild-type strain, even though the average period is about 1.6 hours shorter than the wild-type strain at all temperatures measured.

Likewise, the period distribution of the *And* mutant also indicates strong temperature compensation in period. In fact, with the relatively big spread in period distribution in this mutant, there is no significant difference observed in the average period at the three temperatures observed (P=0.71, 0.87, 0.60 for the 17°C/22°C, 17°C/25°C, 22°C/25°C comparisons, respectively; Student's t-distribution test).

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3. It is important to point out that, under normal situations and with most of the strains of Drosophila studied, there is a small but significant portion of the animals (estimated to be about 10 %) that would give arrhythmic behavior under free-running conditions. In these cases, it is usually discovered at the end of a run that the food has dehydrated or become mouldy, or that the run tube has become moist and sticky, or that the animals are moribund. The records from these animals are routinely discarded. The arrhythmia cases reported here for the per^{l2} mutant do not include such records.

2.2.2 Inter-individual Stability of Periods

From fig. 2-1, it is obvious that there is a rather larger spread in periods for some strains of flies than others. This is examined further in fig. 2-2, where the average period of a strain is plotted against the standard deviation of the period distribution of that same strain. A general relationship seems to emerge in that the distribution in period tends to be larger as the average period of a strain increases. For wild-type, per^s and per^{l1} , the spread in distribution is about the same at all temperatures examined. For Clk^{KO6} and *And* , there is a temperature dependence, with *no.*6 showing a larger spread at 25°C. and *And* showing a larger spread at 17°C. In the extreme case of the per^{l2} at 25°C, where the majority of the flies are arrhythmic, the spread in individual periods is the biggest-- there being a 6 hour difference between the fastest and slowest running clocks!

Fig.2-3 shows the periods of 16 per^{l1} and 6 per^{l2} individuals which are first run at 25°C. and then transferred to 17°C. Within the group of per^{l1} animals, the distribution of periods ranges from 26.5 hours to 28.5 hours in 17°C. and from 29.5 hours to 32.5 hours in 25°C, confirming the defect in the temperature compensation in individual animals. However, the severity of such defect is not uniform among all the individuals studied. For example, while the period of one animal is lengthened by 2.5 hours (from 27.0 to 29.5 hours), there exists another whose period is lengthened by as much as 4.5 hours (from 26.5 hours to 31.0 hours) at the higher temperature.

The behavior of the 6 per^{L2} individuals is even more drastic. While the periods of two animals remain unchanged at the two temperatures, the periods of two other animals are drastically lengthened, and yet another two animals lose overt rhythmicity altogether at the higher temperature.

Thus, in these two mutants, the Q_{10} of each individual at this temperature range can differ drastically from one another. Furthermore, there seems to be no

relationship between the Q_{10} of an animal and its free-running period relative to those of other animals at the same temperature. Put another way, the relative order of period lengths of a population of clocks in these two mutants is not preserved when going from one temperature to another.

2.2.3 Intra-individual Stability of Periods

The previous section describes data which indicate that, although the clock periods in wild-type and the short period mutants are rather stable from one animal to the other, the clocks of the long period mutants show signs of instabilities exhibited by (1) a great variations of periods, (2) different Q_{10} values for different individuals, and (3) some animals tend to become arrhythmic under some situations. In this section, we try to estimate the stability (i.e. the precision) of the clock within an individual animal.

A general problem in the determination of the precision of a circadian clock is the difficulty in distinguishing the properties of a directly observable rhythm (the locomotor activity rhythm in our case) and those of the clock that drives it. Here, we shall estimate the precision of the activity rhythm itself, which then serves as a upper bound for the precision of the circadian clock. That is, we assume that the clock is more precise than , or equally precise as, the rhythm it drives.

As we mentioned in section 2.2.1, the viability and other general conditions of the animals in the activity monitor set-up seem to be optimal at 22°C. (at least in the hand of the author); we thus assume that analysis of the rhythm at this temperature would give the most accurate estimate of the clock. As a measure of precision of the rhythm, we take the standard deviation of a linear regression fit of the phase reference points (offsets) of five consecutive cycles of an animal's activity rhythm -- defined as the 'S' factor. (See section 6.4 for definition of phase reference points, and calculation of the 'S' factor.)

Fig.2-4 shows distributions of such 'sharpness of rhythm' estimates for six strains of flies at 22 °C. It shows that the sharpness of the wild-type rhythm, as defined above, is 0.33 hours on the average. (That is, on the average, an animal would stop its locomotor activity at 20 minutes from the expected 'mean' time-- as calculated from a linear regression fit of 5 such consecutive offsets.) Normalizing this average value of sharpness as a percentage of the average period of the wild-type strain (23.9 hours) , we obtain a precision estimate of 1.4 %. Similar estimates for the other strains are as follow: 1.3% for per^s, 1.7% for Clk^{KO6}, 1.2% for per^{l1}, 1.3% for per^s/per^{l1}, and 2.6% for *And* . Thus, with the exception of *And*, the precision of the various individual mutant clocks seems not to be affected. We do not know whether the relative 'sloppiness' in the rhythm of *And* reflects a basic feature of the clock, the driven rhythm, the coupling between the two, or a combination of all of these factors.

Since the methods used for current analysis requires the activity patterns to be relatively smooth to give meaningful phase reference points , and since the activity patterns of most per^{l2} animals are rather 'bursty' in nature, the records of this mutant are not included in this analysis.

Fig.2-5 examines the question of whether, in per^{l1} , the sharpness of the rhythm is related to the period of the clock. The results shown indicate that, both at 17°C. and 25°C., a relation between the two does not seem to exist.

2.3 Effects of eyes and optic lobes on the periods of two clock mutants

Anatomical localization of the circadian oscillator(s) in multi-cellular organisms has been a popular, and extremely fruitful, approach in the recent literature of circadian biology. Well known examples of localized circadian oscillators include the eyes of the sea mollusc *Aplysia* (ref.2-14), the pineal gland of the house sparrow *Passer domesticus* (ref.2-15), and the suprachiasmatic nuclei in the hamster *Mesocricetus auratus* (ref.2-16). Both the *Aplysia* eye and the bird pineal demonstrate robust oscillations in organ culture that can be monitored quantitatively by either electro-physiological, biochemical or endocrinological methods. In insects, the localization of circadian oscillators has not been carried out to such a precise extent. However, two pioneer studies have provided important information. By micro-operation in the brain of the cockroach *Leucophaea madeirae*, Nishiitsutsuji-Uwo and Pittendrigh (ref.2-17) demonstrated that the intact connection of the brain of this animal to at least *one* of its optic lobes is required for the expression of the circadian rhythm of locomotor activity. Working with the eclosion rhythm of the giant silkmoth *Hyalophora cecropia*, Truman (ref.2-18), on the other hand, demonstrated, through transplantation of differently transsected partial brains to brainless animals, that the circadian rhythm of eclosion of this species can be expressed in the absence of the optic lobes. But a bilateral transsection separating the medial and lateral group of neurosecretory cells in the brain proper would abolish the rhythm. In short, the simplest working hypothesis generally adopted by current workers in the field is that, in the cockroach, the circadian oscillator controlling the activity rhythm resides in the optic lobes, while, in the moth, the circadian oscillator that control the eclosion rhythm resides in the lateral mid-brain.

Owing to the small size of *Drosophila*, micro-operations of the kind mentioned above are hard to perform in a reliable fashion. Yet, genetic techniques can be adopted for the localizaton of the circadian clock. Thus, genetic mosiacs are being

used to map the site of the circadian clock in the brain of the fly (ref.2-19).[4] Also, mutations that cause part of the brain to be missing can be used in eliminating possible sites of the circadian clock in the fly.

In *D. melanogaster* , a mutation on the third chromosome, called *sine oculus* (*so*) was isolated by Melani in 1939 (ref.2-22). This mutation causes a consistent absence of the ocelli and occasional absence of the eyes and also major parts of the optic lobes -viz. the lamina and the medulla. Earlier work by Konopka (ref.2-21) showed that the circadian rhythmicity of the locomotor activity of this mutant is basically normal. In this section, we report observations on the effect of this mutation on the temperature compensation mechanism of the two clock mutants per^s and per^{l1}.

2.3.1 Effects on the period of per^s at 22° C.

Fig.2-6 shows distributions of periods for five categories of per^s flies. All of the animals carry the 'normal' per^s mutation on the X-chromosome (on both X-chromosomes in cases of female animals). The flies in category (a) have the *so* mutation on only one of the third chromosomes and is *so*-plus on the other chromosome, which is a 'SM5' balancer. This strain is a by-product of the crosses used in obtaining the eyeless per^s flies in the other categories[5] , and, except for the heterogosity of the *so* genes, is genetically very similar to the latter. Since *so* is recessive, the phenotype of this category of flies is wild-type, retaining the full set of ocelli and full-size eyes and optic lobes. The fact that the average period of this group of flies is 18.8 hours, compared to 19.0 hours for 'normal' per^s flies at this temperature (P=0.15 with Student's *t*-distribution test; see Table 2-1), indicates that the genetic manipulations involved in preparing the flies used in this study do not introduce any significant variation in genetic background that causes changes in period lengths of

4. Earlier genetic mosiac work (ref.2-20) have already mapped the site to the brain.
5. See section 6.1 for details of the crosses involved.

the circadian clock.

The flies in categories (b),(c),(d),and (e) are genetically identical and differ only in the degree of expression of the homozygous *so* genes. Thus, the flies in group (b) have both eyes present(albeit in a reduced form), while flies in group (c) and group (d) have only one eye on the left and on the right, respectively. The other eye, together with most of the optic lobe (lamina and medulla) is missing for these two groups of animals. The animals in group (e) are most severely affected. They completely lack eyes (and the associated optic lobes) on both sides.[6] Since the eyes and optic lobes are major structures on the head of a fruit fly, the heads of these animals are drastically reduced. However, the degree of reduction of the head size of this animal varies considerably from animal to animal, with the animal shown in the picture in fig.2-6 being rather average. Whether the size of the heads of these animals reflects the size the residual lobes is not known. We note that in all of these animals the ocelli are missing.

It is obvious from the figure that the average periods of animals which retain the left, the right, and both eyes and the optic lobe are practically identical to the control. However, the distribution of periods of the complete eyeless animals is manifestly different from the other categories in two aspects. First, the inter-individual variations of periods are close to double those of the others, with periods spanning from 16.5 hours to 20.5 hours. Secondly, the mean period of these flies is 1.2 hours less than those of all the other categories. Fig.2-7 shows a record of such an animal free-running in darkness for 14 cycles. The record is artificially broken up into two segments of 7 cycles each. Periodogram analysis shows the period is 17.5 hours for both segments, indicating that the shortened period is not a transient

6. In the majority of these 'totally eyeless' animals, it is often found that a small group of ommatidia are left on one or both sides of the head. It is not known whether these isolated ommatidia are functionally connected to the brain. Only flies that are totally devoid of ommatidium are used under group (e) in this study.

effect but can be sustained up to 14 cycles. Also to be noted is that the phase of the rhythm, which can be seen most conveniently in the simulated actogram plot, has offsets that project back to about 6 hours after the constant light to constant darkness transition. In comparison, a similar value for a regular per^s animal would be less than 1 hour. The histograms given in fig.6-7 in the Appendix show that this difference between the two groups is very robust. We do not know at this stage whether the absence of the eyes and most of the optics lobes in per^s causes a change in the coupling between the light-dark transition and the clock or in the coupling between the clock and the output system to bring about such drastic phase differences.

2.3.2 Optic lobes and temperature compensation for per^s

In the previous section, we have provided evidence that the bilateral lack of optic lobes and eyes cause both a 'loosening-up' of inter-individual stability of period and a shortening of average period in per^s at 22°C. Since in section 2.1, we have also shown that the periods of the *per* mutants are rather sensitive to temperature, it is of interest to see how the optic-lobe effect and temperature effect interact. Fig.2-8 shows the behavior of the clocks of 6 per^s animals. Three of these animals (Fig.2-8b) lack eyes and optic lobes on one side and the other three (Fig.2-8a) lack eyes on both sides. These animals are transferred through three different temperatures, and the periods of their rhythms at each temperature is shown in the diagram. The results show that all 6 animals are quite normal at 17°C., with periods of 19 hours. However, for the 3 totally eyeless animals, the period decreases to 17.0 or 17.5 hours at 22°C., and finally at 25°C., the periods stay at between 17.5 to 18.0 hours. It is not clear from the limited sample whether the 0.5 hour increase in period from 22°C. to 25°C. is a real temperature effect or it is due to 'spontaneous' drift in period or an 'after-effect' of the 22°C. to 25°C. step-up in temperature.[7] Nevertheless, regardless

of the cause of these changes, it seems safe to state that the difference in periods between the clocks of the eyeless and 'normal' per^s animals seen at 22°C. does not get noticeably enhanced at a higher temperature. The situation is different for the 3 'half-eyeless' animals. Here, the 17°C. to 22°C. transition causes no change in period for one animal and only 0.5 hour change in the other two , while the 22°C. to 25°C. transition causes another 0.5 hour decrease in period in all three. We note that of the many 'normal' per^s animals observed at 25°C. , none shows a period as short as 18 hours, yet this is observed for two of the three half-eyeless animals here. It thus seems that the relative lack of temperature compensation has been slightly enhanced at 25°C. even for the half-eyeless per^s mutants.

To complement the single animal results presented above and to avoid the possibility of after-effects as well as the difficulty of maintaining these generally less viable mutants in a long run, a separate study was carried out in which animals were transferred from 22°C. to different temperatures in a 'parallel' fashion. The results, shown in table 2-1, agree with the single animal experiments rather well. Thus, the average period of the totally eyeless animals is 18.7 hours at 17°C., being 0.8 hours shorter than the normal-eyed per^s flies. This 0.8 hour difference increases to 1.4 hours at 22°C. , as already noted in fig.2-8; and, as in the individual animal cases, there is no significant further shortening of the period at 25°C. The average period of the half-eyeless animals, again as in the individual animal cases, is intermediate between the totally eyeless and the 'fully-eyed' animals at all temperatures.

2.3.3 Optic lobes and temperature compensation for per^{l1}

Since the results from the previous section show that there is some component of the optic lobe the absence of which seems to enhance the defect in temperature

7. After-effects are noticeable, but normally small, changes in the structure of a circadian system (most commonly manifested as a slight change in period) that is caused by a phasic environmental disturbance. (See ref.2-3 for general discussions.) Examples of rather drastic after-effects due to a light pulse are given in the last section of Chapter 3 in this study.

compensation in per^s, and since the results in section 2.1.1 show that such a defect is even more drastic in per^{l1}, we undertake a similar study on the latter mutant. Unfortunately, in the strain of per^{l1} ; *so*/*so* mutant that is available in this study, the expression of the *so* gene is such that there are practically no half-eyed animals (most probably owing to background effects). Also, since this strain of flies was made by Dr. R. Konopka many years ago and has existed in the lab as a separate strain since then, it is difficult to assess the effect of genetic background on the period. For the above two reasons, only direct comparison of periods of the fully-eyed and totally eyeless animals is possible. The results, shown in table 2-2, indicate that at 22°C., the two groups of animals show indistinguishable averages of periods (P=0.54 with Student's t-distribution test). However, raising the temperature to 25°C. seems to introduce two effects in the totally eyeless mutants. First,the spread of distribution of periods of this group of animals increases by more than two fold compared to the value at 22°C. Secondly, at this temperature, the average period of the totally eyeless mutants is about 2 hours more than that of the fully-eyed group (P=0.001 with Student's t-distribution test). Thus, the total absence of eyes and nearly total absence of the optic lobes seem to amplify the lack of temperature compensation of this strain as well, except that this amplification takes place at a higher temperature than is the case in per^s.

2.4 Genetic interactions of the clock mutations

One hope shared by circadian geneticists is to be able to analyze the structure of circadian clocks by using mutations to block specific 'clock pathways', as the method has been so successfully applied in the elucidation of biochemical pathways. One first step toward this goal is to determine whether the clock genes isolated so far act independently of or in conjunction with each other. To this end, we present, in this section, results from studying the clock phenotypes of heterozygous mutants at the *per* locus and double mutants between the various clock loci on the X-chromosome.[8] Since the mutation Clk^{KO6} is very near *per*, recombination studies between the two loci is not yet possible until a finer map location of this mutation is available.

2.4.1 Interaction among the per alleles

In their initial report of the *per* mutants, Konopka and Benzer (ref.2-1) reported on the complementation relationship between the three mutants per^s, per^{l1}, and per^0 and between these mutants and the wild-type allele. They classified these mutants as follows: per^s being semi-dominant, and both per^{l1} and per^0 being recessive.

Here, we report on further analysis of these complementation relationships, with emphasis on (1) the effects of per^0 on the other alleles and (2) a comparison between per^{l1} and per^{l2}.

2.4.1.1 Effects of per^0 on other 'per' alleles

Table 2-3 lines (a) through (f) show comparisons of the clock phenotypes of each period mutant at the *per* locus to the phenotypes of heterozygotes formed by combining the corresponding mutation with the per^0 allele. [9] The results indicate that,

8. We characterize clock phenotypes by presence and absence of rhythmicity of locomotor activity , and, if rhythmic, the period of the rhythm.

9. Strictly speaking, since all of the heterozygous mutants are females, the controls should be homozygous females of the corresponding mutants. Due to the constraint of time, however, results

at all temperatures examined, the average period length of the per^s/per^0 flies is increased by 1 hour when compared to the per^s / Y control ($P< 0.001$ in all three cases with Student's t-distribution test). Similarly, at 22°C. and at 25°C., the average period length of per^{l1}/per^0 is increased by 1 hour when compared to the per^{l1} / Y control ($P< 0.03$). But at 17°C., the average period of the heterozygous mutant becomes slightly *shorter* ($P=0.02$) than that of the control. The effect of the per^0 on the per^{l2} allele is to increase the spread of inter-individual periods of the animals that are rhythmic at 17°C. At 22°C. and at 25°C, most of the heterozygous animals are arrhythmic.

Lines (g) and (h) of Table 2-3 show the complementation relationship of per^0 to the per^+ allele. It again shows that the heterozygous mutant has average periods that are 0.6, 1.4, and 0.9 hour longer ($P=0.09$, $P< 0.01$, $P< 0.01$) than those of the per^+ / Y control at 17°C., 22°C. , and 25°C. respectively. Also shown in line (i) of Table 2-3 are results for the heterozygous animals with one wild-type X-chromosome over an aberrant X-chromosome in which a region on the chromosome that includes the *per* locus is missing. The resulting lengthening of 0.7 hour and 1.4 hour of the average period of these heterozygous animals compared to that of the per^+ / Y control, at 17°C.and 25°C. respectively, is compatible with the view that the per^0 allele behaves as a deletion in its effects on the circadian clock (ref.2-1,ref.2-4).

Finally, we note that a significant number of heterozygous animals with one per^0 allele are arrhythmic. This phenomenon is particularly acute in the case where the second chromosome is a per^{l2}.

2.4.1.2 Interaction of per^{l2} with other 'per' alleles

Since per^{l2} causes roughly the same amount of period lengthening as per^{l1} at 17°C.

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from the study shown in Fig.2-1, which involves only males, are used for control here. Where the figures for females are available, they either are not significantly different from those of the male counterparts or the directions of the differences are to enhance the effects discussed here.

and 22°C. (ref. fig.2-1), and since this mutation is allelic to per^{l1}, the possibility is raised as to whether the two mutations are the same. Two facts argue to the contrary. First, both the drastic spread in the distribution of periods at 25°C. (Fig.2-1) and the lack of temperature compensation in individual animals (Fig.2-3) are very different in the two strains. Secondly, the fact that a significant fraction of per^{l2} animals are arrhythmic within the temperatures range studied is something not observed in per^{l1} (at least in the hands of the present investigator). This second point, however, lacks strength in that arrhythmicity can be induced in an animal because of defects in the coupling between a driven rhythm and its driver or in the driven process itself. It is thus desirable to have other independent indications of the 'arrhythmic-like' nature of the per^{l2} mutation. The experiments the results of which are presented in Table 2-4 address this issue. In lines (a) through (h) of Table 2-4, the behavior of the per^{l1}, per^{l2}, and per^{0}, alleles , when complemented by per^{+} or per^{s} , respectively, is examined. It is observed that, from the expression of clock phenotypes in both cases, the behavior of per^{l2} resembles that of per^{0} much more closely than that of per^{l1}. Thus, per^{l2}, like per^{0}, when combined with the per^{s} , results in a period length which is about 1 hour longer than the homozygous per^{s} animals at all temperatures, whereas per^{l1}, when combined with per^{s}, causes period lengths up to about 5 hours longer than the homozygous per^{s} mutants at 25°C. Note that the Q_{10}'s of the periods of the per^{s}/per^{0} and the per^{s}/per^{l2} animals are greater than 1 (i.e.more 'per^{s} like'), while those of the per^{s}/per^{l1} animals are less than 1 (i.e. more 'per^{l1} like'). In this sense, then, per^{l2} is a 'weaker' allele than per^{l1}.

Lines (i) and (j) of Table 2-4 shows the behavior of the heterozygous per^{l1}/per^{l2} mutants. The results show that, once again, per^{0} and per^{l2} give very similar period lengths when combined with per^{l1}. However, there is one difference between the two heterozygous mutants: whereas the rhythms expressed in all the per^{l1}/per^{l2}

animals observed are in fact much sharper than the average, a significant number of per^{l2}/per^{0} animals are arrhythmic at 17°C. and at 25°C.

2.4.2 Interaction of the 'And' and 'per' loci

Fig.9 shows the combined results of two experiments in which double mutants of the *And* and *per* loci are obtained through recombinations of the *And* mutant and per^{s}, per^{l1} and per^{l2} mutants respectively. [10] The results indicate that the two clock phenotypes segregate independently, as expected.[11] In Table 2-5 we examine the periods of the three double mutants over three different temperatures. The question we ask here is whether the period of a double mutant is reasonably close to a value which is derived by assuming a simple addition of the individual mutant effects. The results indicate that for the per^{l1} *And* double mutant, the predicted and actual values agree very well at all temperatures studied, whereas the actual value for the per^{s} *And* double mutant agrees well with the predicted value at 17°C. but become about 1 hour shorter than the predicted value at 22°C. and 25°C. Likewise, the predicted and actual value for the per^{l2} *And* double mutant agree well at 17°C. and at 22°C. We note that 26 out of 28 such double mutants run at 25°C. are arrhythmic, which is consistent with the behavior of the per^{l2} single mutants at this temperature. In summary, the above results suggest that the degree of interaction between the *per* and the *And* loci, if there is any, appears to be extremely weak.

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10. Advantage is taken of the fact that the *per* locus is very close to the *white* locus and *And* is practically inseparable from the *dusky* locus. The mutation *white*, when homozygous, produces white-eyed flies and the mutation *dusky*, when homozygous, produced flies with short wings. Thus, starting with white-eyed, dusky *And* animals and mating them to red-eyed, normal-winged per^{x} animals would give *F*1 females heterozygous with the two X-chromosomes. A single cross-over of the X-chromosomes in these females will give *F*2 double clock mutants identifiable by red eyes and short wings. (See caption to Fig. 2-9)

11. Two side points in the histograms in Fig. 2-9 are to be noted. First, for reasons unknown, the *white* mutation causes a 0.5 hour shortening of period both by itself (as seen in the w dy+ animals) and on the *And* background (as seen in the w dy animals). Secondly, both the mutations *white* and *yellow*, in the hand of the author, always introduce greatly increased percentages of arrhythmic animals, which is likely the reason for some of the arrhythmic cases reported in the histograms. (Some of the arrhythmic cases in the per^{l2} column may be attributable to this mutation itself.)

2.4.3 Interaction of Clk^{KO6} with other alleles

Table 2-6 examines the recessive/dominant nature of the mutation Clk^{KO6} The results presented indicate that period of the heterozygous Clk^{KO6}/Clk^{KO6+} is intermediate between the homozygous mutant and wild-type.

Table 2-5j-l examines the interaction between the Clk^{KO6} and *And* loci.[12] The results show very little, or no, interaction between these two clock loci.

12. Since Clk^{KO6} is mapped very close to the *per* locus, it is possible to construct Clk^{KO6} *And* double mutants using the same method as described in the last sub-section, even though the exact location of of this mutation is not yet known.

2.5 Discussion

Of the five clock period mutants studied in section 2.2, it is found that the temperature compensation mechanism is intact in one short period mutant (Clk^{KO6}) and one long period mutant (*And*). It is interesting to note that the other three mutations that affect this homeostatic mechanism all map to the same locus, suggesting that this locus may play a more crucial role in the *D. melanogaster* circadian system. On the other hand, the fact that one can obtain mutations that change the period of the clock without affecting the temperature compensation mechanism suggests that, not only does this mechanism work in a *circa –dian* regime, but it can also work when the period of oscillation is moved away from the normal 24-hour value by up to about 2 hours each way. It would be very interesting to note, in the period mutants to be isolated in the future, (1) whether all mutations in the *per* locus will be defective in this mechanism, and, if so, whether the defect is always more severe in the long period mutations; and (2) whether a pattern exists in which this mechanism is defective whenever the mutant period deviates from the normal 24-hour value by more than a certain amount. It is worthwhile to note that, in a recent investigation of the temperature compensation in *Neurospora* clock mutants, Gardner and Feldman (ref.2-23) report that this mechanism is also defective in the long period *frq* mutants [13] while relatively intact in the short period *frq* mutants.

The results on the inter- and intra- individual stability of clock phenotypes indicate that precision of circadian rhythm can be preserved even when the period length is caused to deviate up to 5 hours away from the natural value of 24 hours. Thus, whatever component of the circadian clock on which selection has been exerting pressure to ensure proper entrainment, it is not likely to be the same as the component that is affected by the mutations under study (with the exception of

13. *frq* is a clock locus in *Neurospora* analogous to *per* in *Drosophila* in that it is at this site that most of the clock mutants are found and that the mutants that are found at this locus consist of both long and short period phenotypes. (ref.2-24)

And).

The results in section 2.3, showing the enhancement of the defective temperature compensation mechanism in per^s and per^{l1} animals when both eyes and major parts of both optic lobes are absent, suggest, for the first time , that whatever mechanism underlies the temperature compensation of period, it may involve components that are anatomically discrete. The following *ad hoc* model is one way to explain these results. The circadian clock system in *Drosophila* consists of at least two components: a major component that is contained in the brain proper and an ancillary component that is contained in the optic lobes. (Both of these components are assumed to be present bilaterally and the ancillary component in each optic lobe is assumed to be connected to the major components in both side of the brain.) In both the wild-type and the mutant animals, the period of the circadian rhythm is determined by the interaction of the major and ancillary components. The *per* mutations affect the function of the major components and cause a period change. However, since the ancillary components in these mutants are still normal, they act to partially 'stabilize' the clock phenotype. Thus, the loss of these ancillary components in the *so–per* mutants further amplifies the mutant phenotypes and results in the enhancement of the defects in the temperature compensation mechanism. A corollary of this model is that it should be possible to isolate clock period mutants whose defects are in the ancillary components in the optic lobes. The removal of the optic lobes by the *so* mutation in these mutants should *remedy* the defective clock phenotypes.

The results from the complementation studies indicate that, at least in *Drosophila* where a relatively more precise estimate of period length can be made (compared to the other two clock genetic systems), the concept of recessiveness/dominance is not a useful one. No clock mutant allele is yet found that is totally recessive to the corresponding wild-type allele or to any other mutant

allele at the same locus. Even a 'null' allele as per^0 (ref.2-1) asserts a lengthening effect when present in a heterozygous mutant. Whether this is a general feature of clock genes remains to be seen.

There are two observations on the findings presented in this chapter that attract much attention by the author. Both observations are derived from a source of information that has not been emphasized in traditional circadian research , viz. the variability of the expressions of clock phenotype between individuals of nearly identical genotype, which extends into the following two sub-cases: (1) the range of period lengths attained by different circadian clocks in genetically very similar animals under a uniform environment. (2) the fraction of animals of a single genotype that fail to show overt circadian rhythmicity under a uniform environment. The first observation is that while, with the exception of *And* , the precision of individual activity rhythms of the clock mutants is about the same as that of the wild type, the inter-individual variability of period lengths tends to increase with the average period length of a strain in a much more than linear fashion. The second observation is that, if we look at the expression of clock phenotypes of mutants at the *per* locus, we find an apparent gradient in the order of per^s, per^{l1}, per^{l2}, and per^0. Thus, the mutant per^s , being the shortest in period, expresses the least variability in period distributions and provides no cases of arrhythmicity. The mutant per^{l1} , being much longer in period, expresses much larger variability in period distributions but still provides no cases of arrhythmicity. The mutant per^{l2}, slightly longer in average period length than per^{l1}, not only expresses yet larger variability in period distributions but the range of variability extends to include a significant number of cases of arrhythmicity. Finally, in per^0, arrhythmicity is expressed in all cases. Of particular interest is that in per^{l2}, not only does the instability of clock phenotypes include the realm of arrhythmicity, but the complementation results, shown in Tables 2-3 and 2-4 , clearly suggest this mutation is 'arrhythmic-like'. Put

together, these observations suggest a general classification of the groups of genetically identical clocks studied in this chapter into a sequence : 'short and tight among the group' --- 'long and loose among the group' -- 'very long, very loose among the group and maybe arrythmic' -- 'always arrhythmic among the group'. Could these observations be purely co-incidental side-effects of the mutations involved? Or, could these correlations be revealing some features inherent in the ways the *Drosophila* clock is affected by these mutations? One is tempted to speculate that, whatever mechanisms these mutations use to lengthen the period of the clock, the result is achieved through a process which *decreases* the *precision* with which the clocking apparatus can be reproduced from animal to animal and yet preserving the *accuracy* of the individual clock thus produced. On the other hand, the mechanisms which shorten the clock period in the mutants seem to preserve, or even *increase*, such precision of clock 'reproductions'. One way that these can happen -- so continues the speculation -- is to demand that the speed of the circadian clock be proportional to some quantity, be it the number of neurons in a certain nucleus, or the number of a particular type of synapses, or the number of a certain neuro-chemical vesicles, and so on. The point is : to slow the clock, one needs to lower the value of such a quantity, and the lower the value of such a quantity, the more susceptible is the system to inter-individual variability that is introduced through the inherently stochastic processes encountered in its ontological development. And, finally , when the value of such a quantity decreases below a certain point, the circadian oscillation stops all together.

Chapter 2 References

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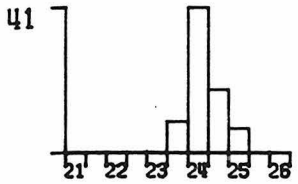
Chapter 2 Figures

Figure 2-1. *Distribution of periods of a wild-type and five mutant clocks as a function of temperature.* Animals are reared in 22°C. and under ambient light level of about 200 lux and transferred to continuous darkness at 17C., 22C., or 25°C. at the age of 1 to 3 days. Only males are used. Digital activity data are binned at either 15 min. or 30 min. intervals. Period estimates are derived from periodogram analysis of a segment 4 to 6 cycles long, starting from the second cycle in darkness. The resulting periods are rounded to the next higher half hour bin so that a period of 23.75 hour, for example, will be added to the '24 hour' bin, and so on.

DISTRIBUTION OF TAU

W-TYPE 17C N= 75
MEAN=24.15 S.D.=0.40



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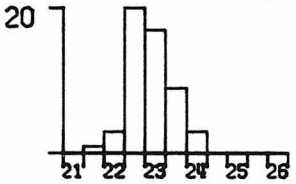
MEAN=22.87 S.D.=0.52



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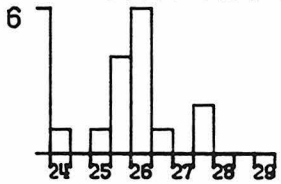

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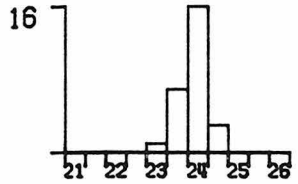

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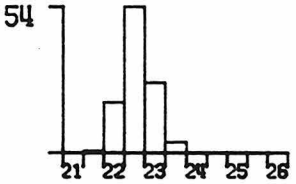

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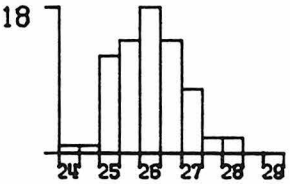

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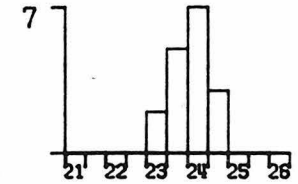

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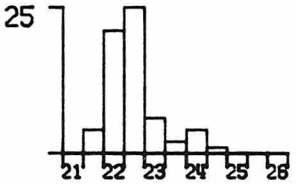

'AND' 25C N= 21
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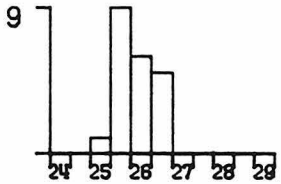

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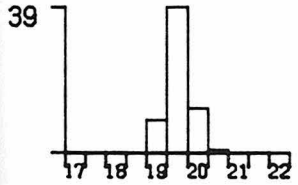
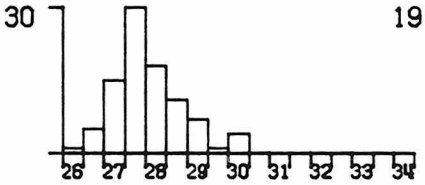
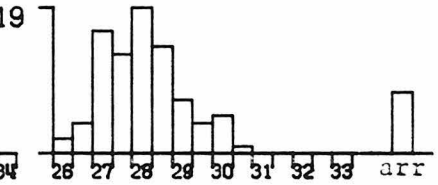
MEAN=19.54 S.D.=0.32



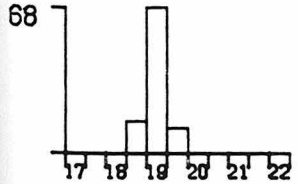
PER-L1 17C N= 92
MEAN=27.81 S.D.=0.82



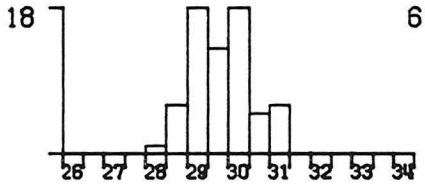
PER-L2 17C N= 93
MEAN=28.00 S.D.=0.98



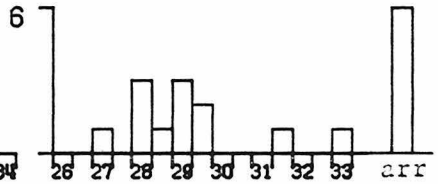
PER-S 22C N= 95
MEAN=18.98 S.D.=0.27



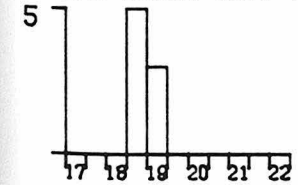
PER-L1 22C N= 67
MEAN=29.60 S.D.=0.72



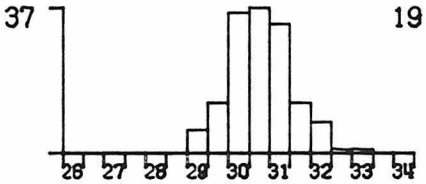
PER-L2 22C N= 18
MEAN=29.17 S.D.=1.57



PER-S 25C N= 8
MEAN=18.69 S.D.=0.24



PER-L1 25C N=148
MEAN=30.54 S.D.=0.76



PER-L2 25C N= 27
MEAN=30.31 S.D.=2.29

Figure 2-2. ***Relationship between the standard deviation of the period distribution of a strain and the average period of the same strain.*** Abbreviations used: W=wild-type, S=per^s, 6=Clk^{KO6}, A=*And*, L1=per^{l1}, L2=per^{l2}. Subscripts denote temperatures at which measurements are made.

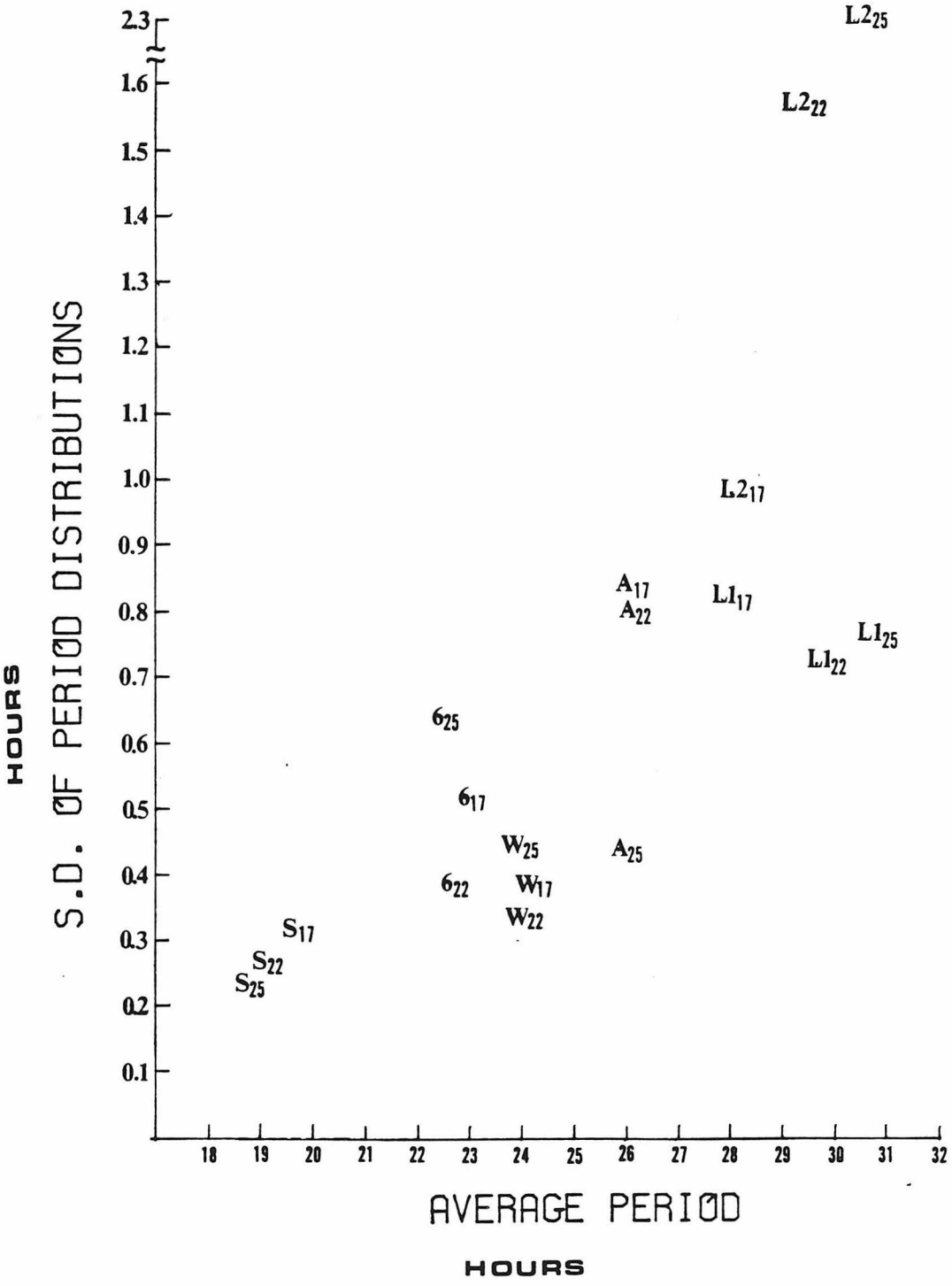
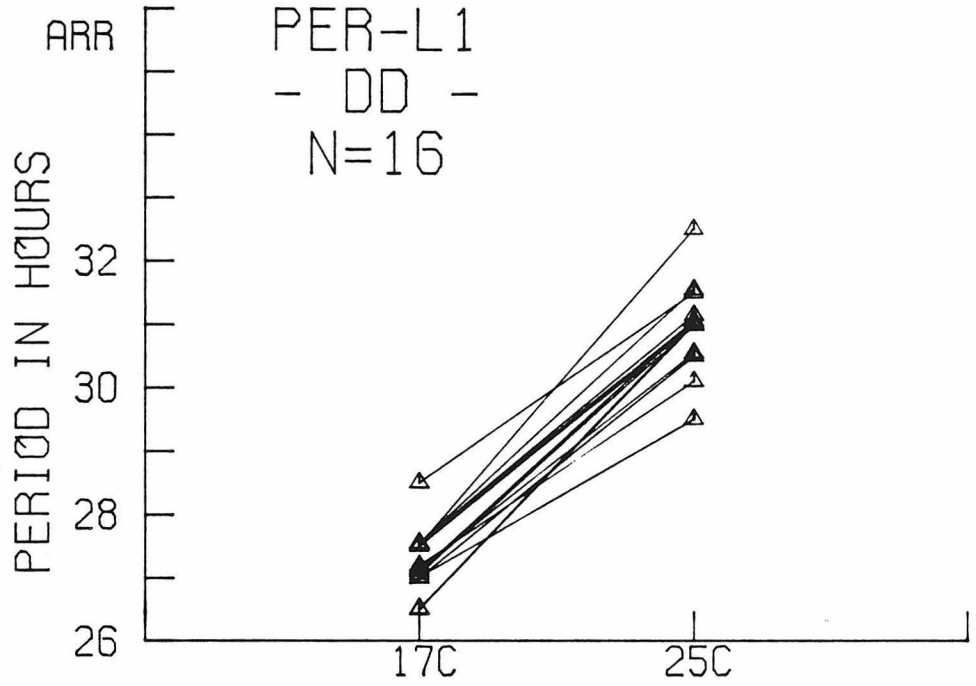

S.D. OF PERIOD DISTRIBUTIONS
HOURS
2.3
1.6
1.5
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
L2$_{25}$
L2$_{22}$
L2$_{17}$
A$_{17}$
A$_{22}$
L1$_{17}$
L1$_{25}$
L1$_{22}$
6$_{25}$
6$_{17}$
W$_{25}$
A$_{25}$
6$_{22}$
W$_{17}$
W$_{22}$
S$_{17}$
S$_{22}$
S$_{25}$
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
AVERAGE PERIOD
HOURS

Figure 2-3. *Behavior of individual per^{l1} and per^{l2} clocks at two different temperatures*. Animals are reared in 22°C. at about 200 lux and released into constant darkness(DD) at 25°C. for 10 days, the temperature is then lowered to 17°C. for another 10 days with the animals still in DD. Locomotive activity is monitored. Only males are used. Counts are binned at 30 min. intervals. Period is estimated by periodogram analysis. Each triangle represents the period of one animal at one temperature. A line is drawn between two triangles representing periods of the same animal. Some triangles are overlapped because of the overlapped values in periods.

LACK OF TEMP. COMPENSATION IN PER-L1 AND PER-L2 INDIVIDUALS



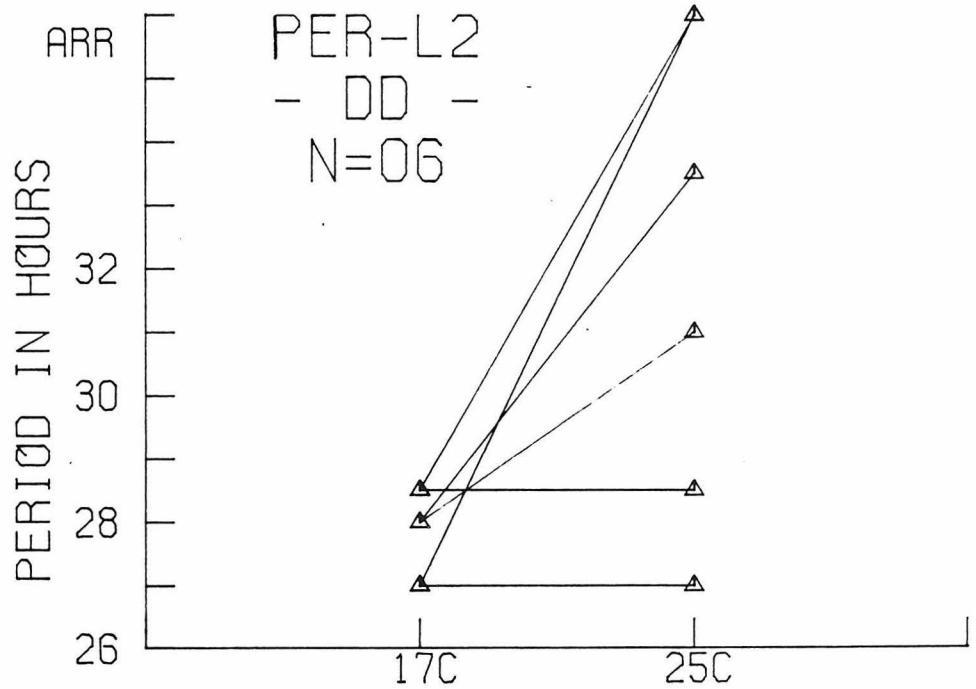
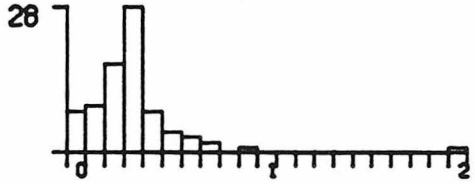


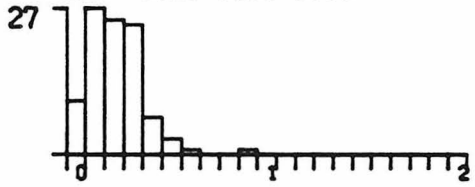
Figure 2-4. *Precision of activity rhythm at 22°C.* The precision of activity rhythm is defined as the standard deviation of a linear regression fit on 5 consecutive offsets (defined as the phase point where 95% of activity counts of a particular cycle have occurred) of an animal's free-run in DD. These values are shown in histograms with 0.1 hour bin.

PRECISION OF ACTIVITY RHYTHM AT 22 C

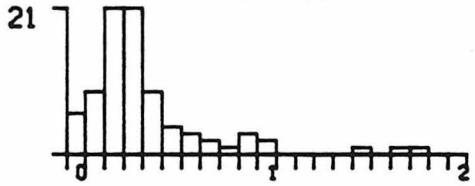
W-TYPE N= 81
MEAN= 0.33 S.D.=0.31



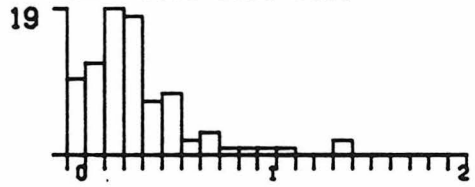
PER-S N= 98
MEAN= 0.25 S.D.=0.15



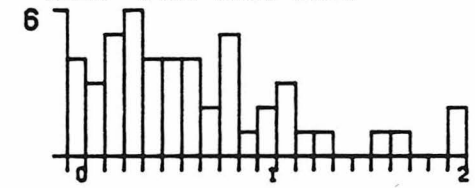
'NO.6' N= 84
MEAN= 0.39 S.D.=0.34



PER-L1/PER-S N= 85
MEAN= 0.36 S.D.=0.29



AND N= 49
MEAN= 0.67 S.D.=0.54



PER-L1 N= 36
MEAN= 0.36 S.D.=0.23

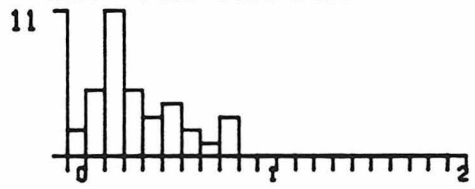
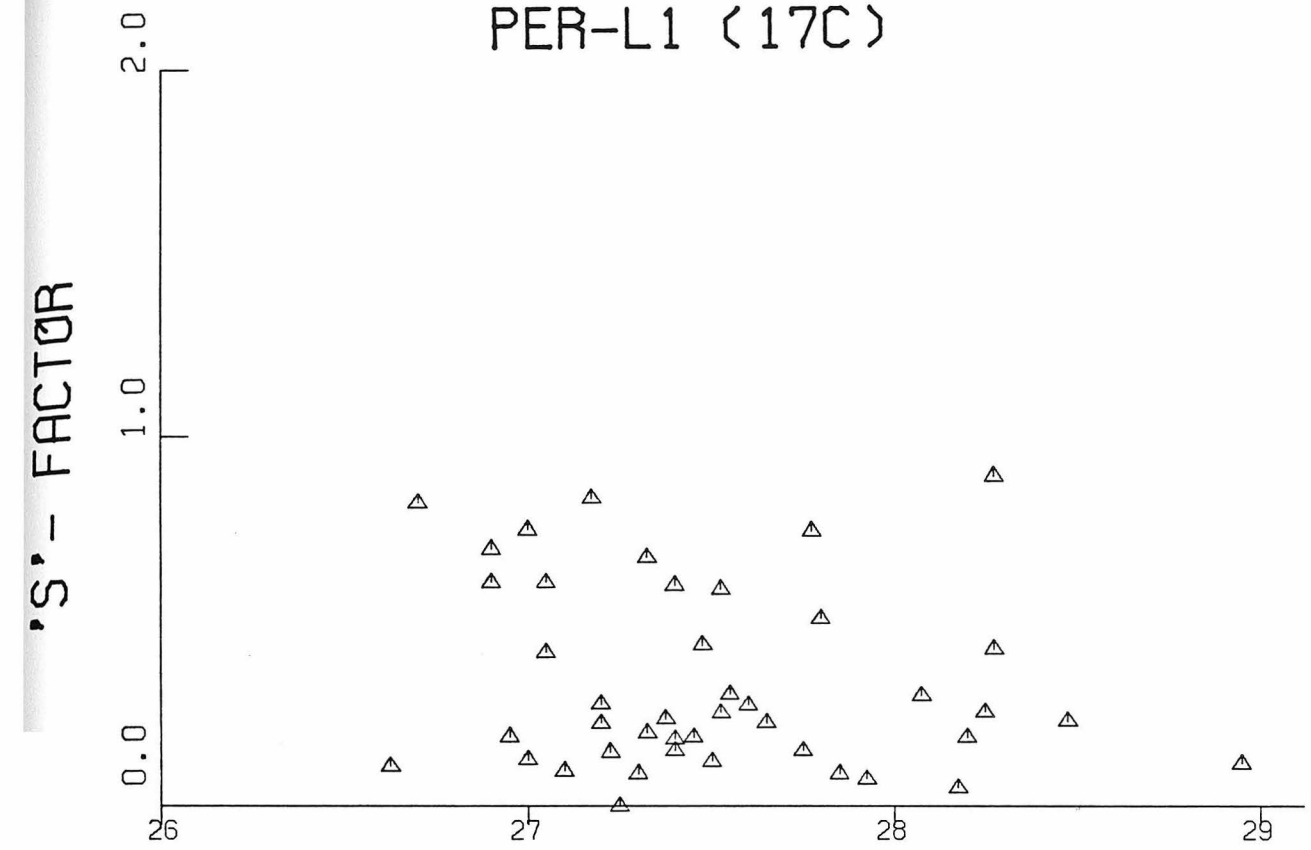


Figure 2-5. *Lack of correlation of precision of rhythm and clock period in* per^{l1}. The 'S'-factor is a measure of precision of the activity rhythm, as described in the caption in Fig.2-4. Periods are given by the slope of the linear regression line of the 5 offsets taken to calculate the 'S'-factor. Calibrations in both axes are in hours.


PER-L1 (17C)
'S'- FACTOR
2.0
1.0
0.0
26
27
28
29

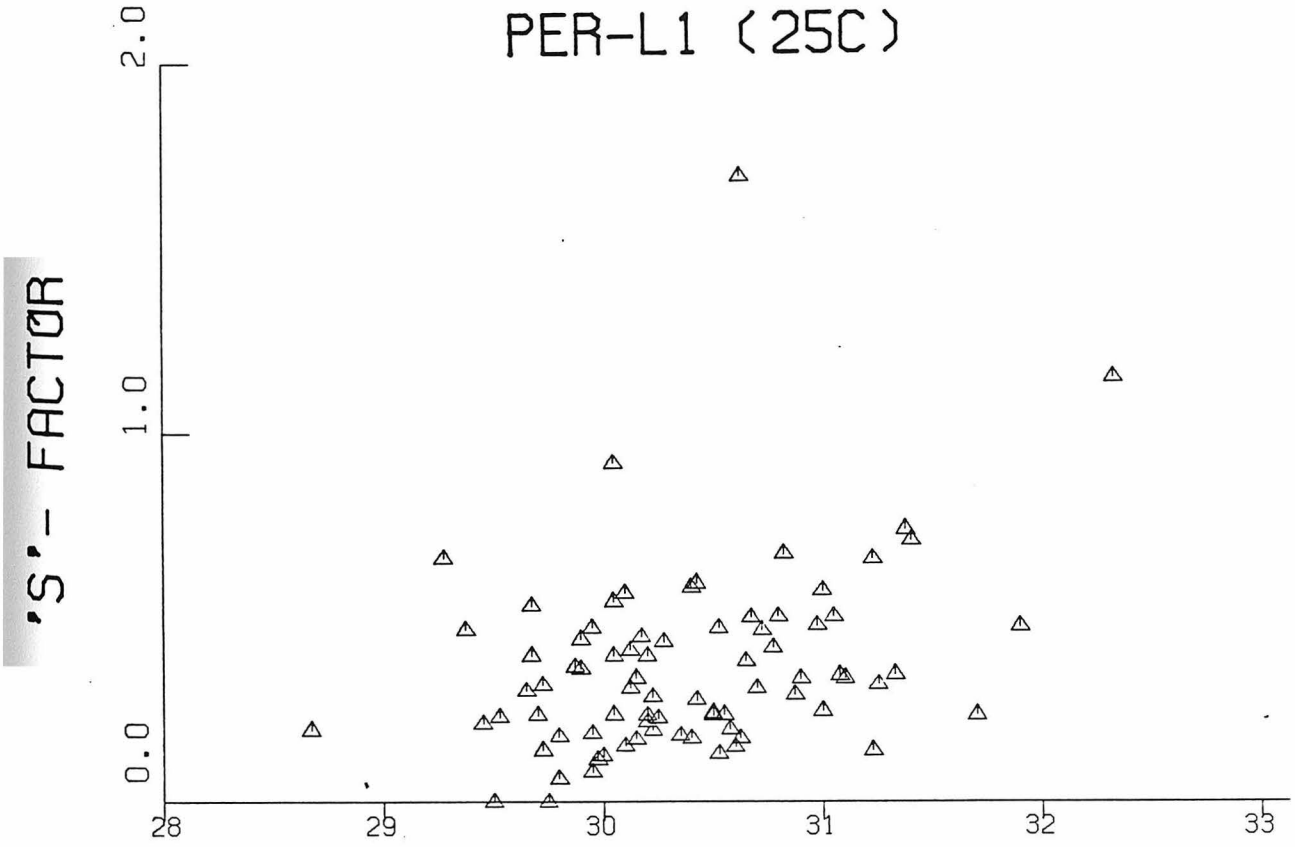
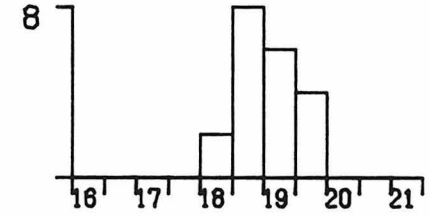

PER-L1 (25C)
'S'- FACTOR
2.0
1.0
0.0
28
29
30
31
32
33
PERIOD IN HOURS

Figure 2-6. *Effects of optic lobes on the period of per^s at 22°C.* Locomotive activity counts from 5 to 8 cycles of DD-freerun are collected as 30 min. bins and analysed by periodograms. Photos on the right show examples of the phenotypes of the animals whose periods appear in the corresponding histograms on the left. The flies in (a) belong to a *per^s* strain which is derived from the genetic crosses that give rise to the flies in (b),(c), (d),and (e) and is used here as a control. The flies in (b),(c),(d),and (e) are genetically identical and differ only in the expressions of 'eyelessness'. Both males and females are used, and the results are combined.

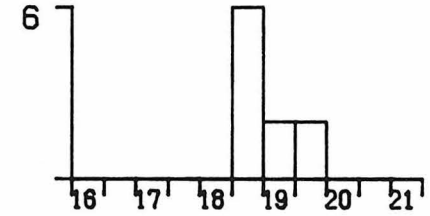
EFFECTS OF OPTIC LOBES ON PERIOD OF PER-S

SO/SM5 (+/+) N= 20
MEAN=18.80 S.D.=0.46



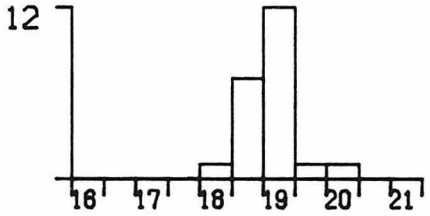
A

SO/SO (+/+) N= 10
MEAN=18.80 S.D.=0.40



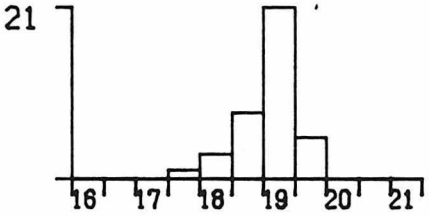
B

SO/SO (+/-) N= 22
MEAN=18.86 S.D.=0.45

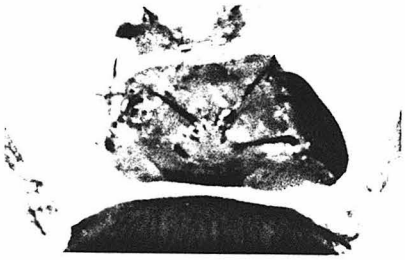


C

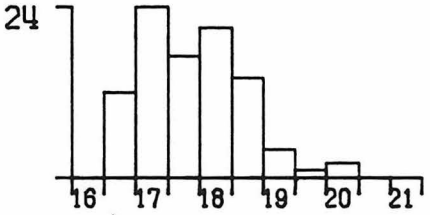
SO/SO (-/+) N= 38
MEAN=18.84 S.D.=0.45



D



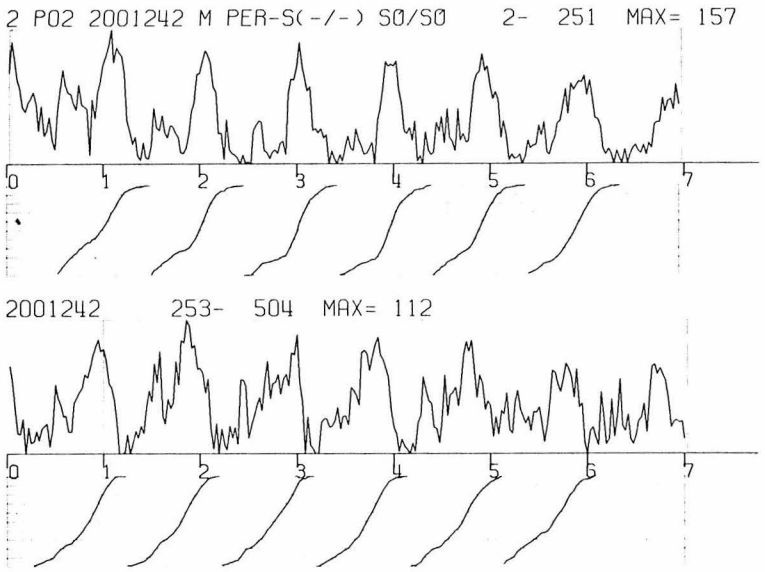
SO/SO (-/-) N= 95
MEAN=17.64 S.D.=0.80



E

Figure 2-7. *The activity rhythm of a per^{s},s.o./s.o. animal* The animal has full expression of the *s.o.* genes and therefore has no ocelli and eyes and has drastically reduced optic lobes. It is reared at 22°C. and at about 200 lux, and transferred to constant darkness at 22°C. at the point where the activity record shown begins. The records are artificially broken into two segments, of about 150 hours each. Data are collected in 30 mins bin. (a) Normalized counts of activity are plotted against time. Tick marks represent cycles of 18 hours. (b) Form estimates of the activity rhythm, also referred to as the activity profile in this study, is simply the normalized average activity counts over a certain number of cycles. The duration of the interval that is averaged is boxed in on the figure. (c) Periodogram analysis of the activity counts is done for the same interval as in (b). See ref.6-1 for details on the method of periodogram analysis. (d) Simulated actogram plot of the activity rhythm is accomplished through a computer program that plot out each count of digital record as a tick mark in a fashion similar to the 'Estaline-Angus' plots that are used by earlier investigators in the field. Whereas, in the 'Estaline-Angus' plot, each tick registers the exact time at which a count is recorded, in the simulated plot such precision is lost for counts within a bin --- such counts are represented as ticks uniformly spread out within the intervals of a bin, creating a slightly sharper contrast of activity and rest that a 'real' actogram would have provided.

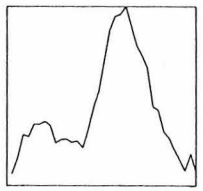
ACTIVITY PLOT



FORM ESTIMATE

AT 17.5 HR.

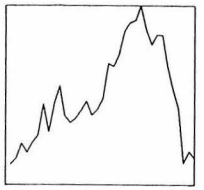
2001242 2- 251



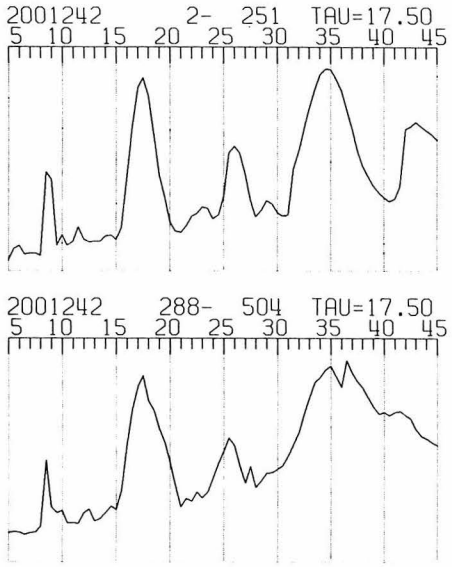
FORM ESTIMATE

AT 17.5 HR.

2001242 288- 504



PERIODOGRAM



SIMULATED ACTOGRAM PLOT

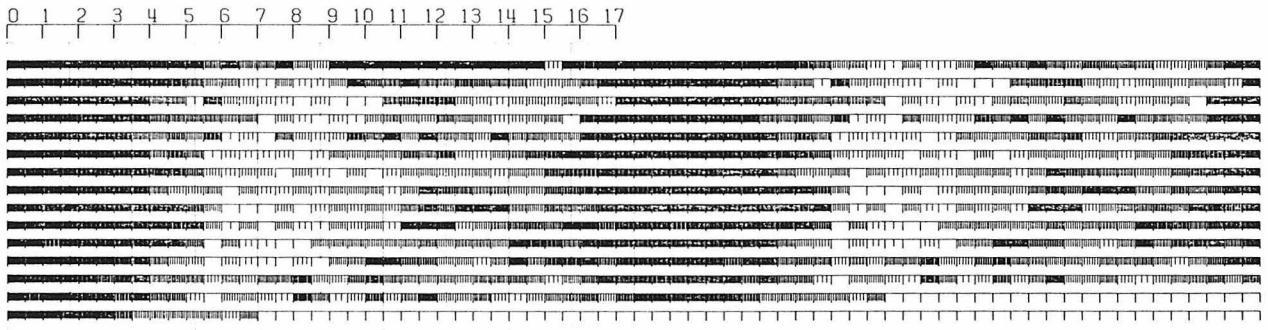


Figure 2-8. *Periods of individual per^s;so/so* animals at 17°C/22°C/25°C The animals whose periods are demonstrated in (a) lack eyes and optic lobes on both sides while those in (b) lack eyes and optic lobes on only one side. Each of these animals is reared in constant light of about 200 lux and at ambient temperature of about 22°C. Each is then transferred to darkness at 17°C. The temperature is then raised to 22°C. and then 25°C., with the duration of each constant temperature period being about 5 days. Data are collected in 30 min. bins. Periods are estimated by periodogram analysis. Each period value is represented by a triangle at the temperature of the measurement. Triangles representing the same animal are joined by straight lines.

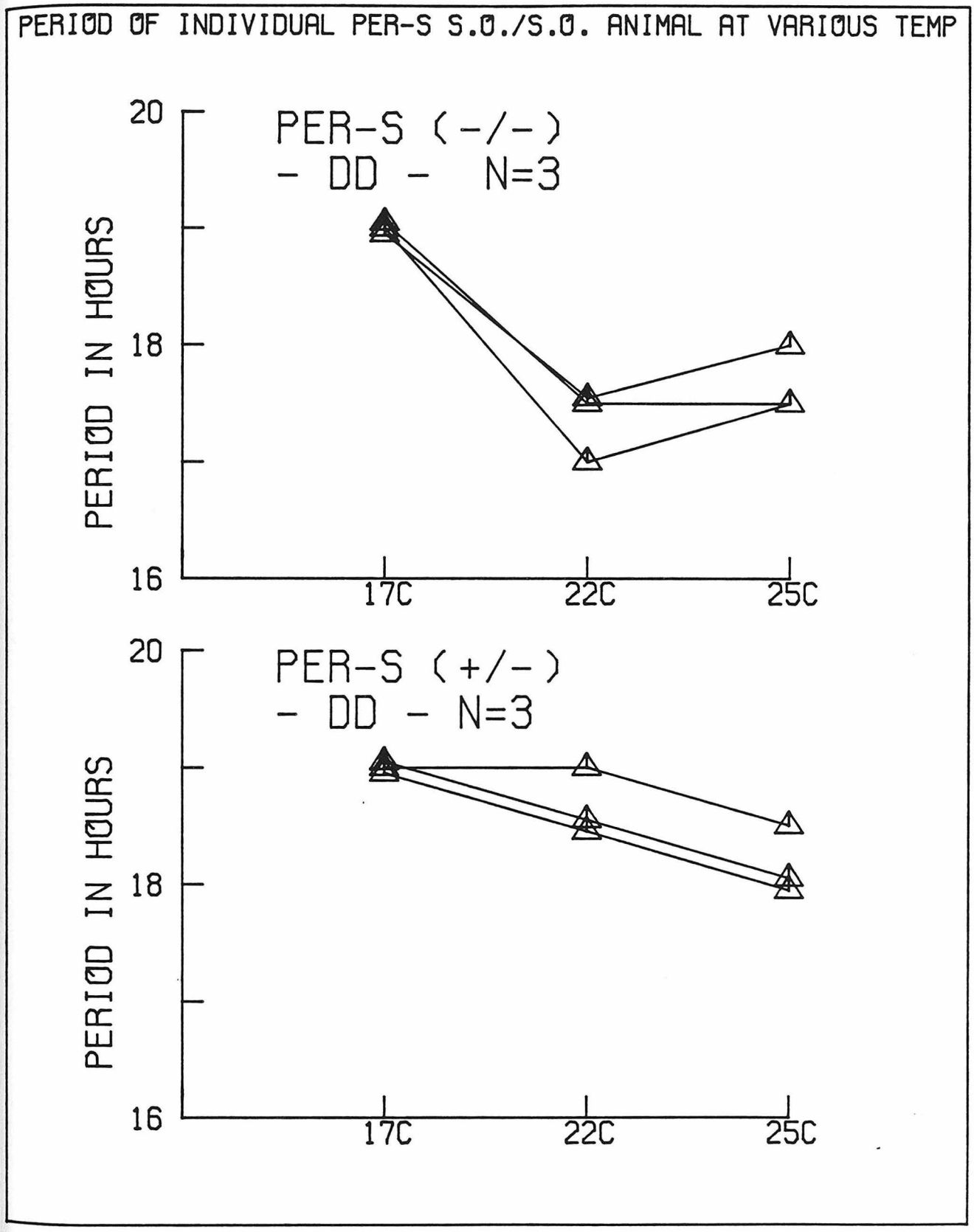
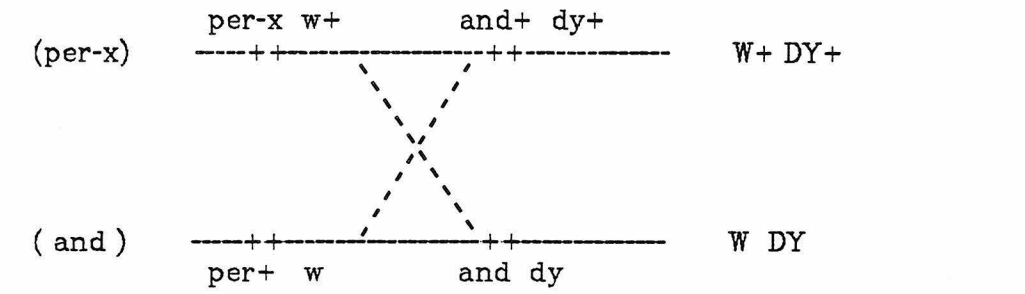

PERIOD OF INDIVIDUAL PER-S S.O./S.O. ANIMAL AT VARIOUS TEMP
PER-S (-/-)
- DD - N=3
PERIOD IN HOURS
20
18
16
17C
22C
25C
PER-S (+/-)
- DD - N=3
PERIOD IN HOURS
20
18
16
17C
22C
25C

Figure 2-9. *Interaction of the And and the per loci at 22°C.* Histograms contain periods of the siblings from two identical crosses. The crosses are as follows:

$per^{z}\ w^{+}\ And^{+}\ dy^{+}/\ Y$ X $per^{+}\ w\ And\ dy\ /\ per^{+}\ w\ And\ dy$

$per^{z}\ w^{+}\ And^{+}\ dy^{+}\ /\ per^{+}\ w\ And\ dy$ X wild-type males.

The clock and physical phenotypes associated with the X-chromosome on the $F1$ females are shown as follows:



where per^{z} represents per^{s}, per^{l1} or per^{l2}. Data are collected at 30 mins. bin. Periods are estimated by periodograms.

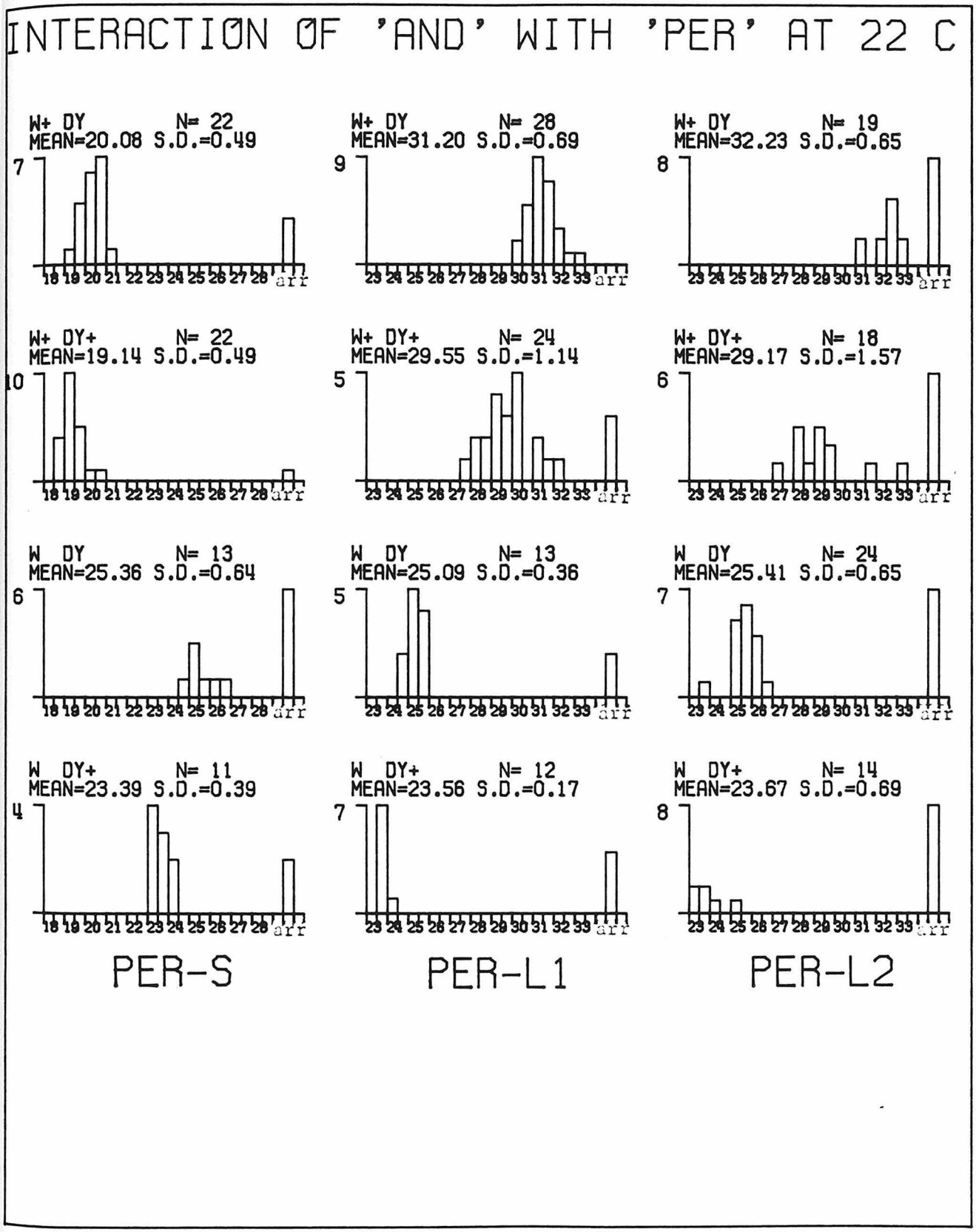

INTERACTION OF 'AND' WITH 'PER' AT 22 C
W+ DY N= 22
MEAN=20.08 S.D.=0.49
W+ DY N= 28
MEAN=31.20 S.D.=0.69
W+ DY N= 19
MEAN=32.23 S.D.=0.65
W+ DY+ N= 22
MEAN=19.14 S.D.=0.49
W+ DY+ N= 24
MEAN=29.55 S.D.=1.14
W+ DY+ N= 18
MEAN=29.17 S.D.=1.57
W DY N= 13
MEAN=25.36 S.D.=0.64
W DY N= 13
MEAN=25.09 S.D.=0.36
W DY N= 24
MEAN=25.41 S.D.=0.65
W DY+ N= 11
MEAN=23.39 S.D.=0.39
W DY+ N= 12
MEAN=23.56 S.D.=0.17
W DY+ N= 14
MEAN=23.67 S.D.=0.69
arr
PER-S
PER-L1
PER-L2

Table 2-1. Effects of optic lobes on temperature compensation in *per*^{s}

			Temperature (°C)					
			17°		22°		25°	
*	Genotype	Phenotype	Period ± SD	*n*	Period ± SD	*n*	Period ± SD	*n*
a	*per*^{s}	2 eyes	19.5 ± 0.3	61	19.0 ± 0.3	95	18.7 ± 0.2	8
b,c	*per*^{s}; *so/so*	2 eyes	--		18.8 ± 0.4	10	--	
d		1 eye	19.1 ± 0.5	13	18.8 ± 0.5	62	18.1 ± 0.5	14
e		no eyes	18.7 ± 0.2	14	17.6 ± 0.8	95	17.5 ± 0.5	13

* These labels correspond to the labels in fig. 2-6

Table 2-2. Effects of optic lobes on temperature compensation in *per*^{l1}

			Temperature (°C)					
			17°		22°		25°	
	Genotype	Phenotype	Period ± SD	*n*	Period ± SD	*n*	Period ± SD	*n*
a	*per*^{l1}; *so/so*	2 eyes	--		29.1 ± 0.6	8	29.6 ± 0.6	9
			--		arrhythmic	2	arrhythmic	4
b		no eyes	27.6 ± 0.9	7	28.9 ± 0.7 [1]	11	31.6 ± 1.9 [2]	16
			--		arrhythmic	2	arrhythmic	1

1. P=0.54 , Student's t-distribution test
2. P< 0.01 , Student's t-distribution test

Table 2-3. Interaction of per^0 with other *per* locus mutants

		Temperature (°C)					
		17°		22°		25°	
	Genotype	Period ± SD	*n*	Period ± SD	*n*	Period ± SD	*n*
a	per^s/Y	19.5 ± 0.3	61	19.0 ± 0.3	95	18.7 ± 0.2	8
b	per^s/per^0	20.8 ± 0.5	8	20.4 ± 0.5	13	19.7 ± 0.2	6
		arrhythmic	4	arrhythmic	2	--	
c	per^{l1}/Y	27.8 ± 0.8	92	29.6 ± 0.7	67	30.5 ± 0.8	148
d	per^{l1}/per^0	27.1 ± 0.7	7	30.6 ± 0.9	5	31.7 ± 1.2	9
		arrhythmic	5	--		arrhythmic	4
e	per^{l2}/Y	28.0 ± 1.0	84	29.2 ± 1.6	12	30.3 ± 2.3	8
		arrhythmic	9	arrhythmic	6	arrhythmic	19
f	per^{l2}/per^0	29.9 ± 3.5	7	31.5	1	34.8 ± 1.3	2
		arrhythmic	10	arrhythmic	10	arrhythmic	16
g	per^+/Y	24.2 ± 0.4	75	23.9 ± 0.3	27	23.8 ± 0.5	17
h	per^+/per^0	24.8 ± 0.6	4	25.3 ± 0.4	11	24.7 ± 0.4	17
i	$per^+/Df(1)64f1$	24.9 ± 0.8	12	--		25.2 ± 0.4	8

Table 2-4. Interaction of per^{l2} with other *per* locus mutants

		Temperature (°C)					
		17°		22°		25°	
	Genotype	Period ± SD	*n*	Period ± SD	*n*	Period ± SD	*n*
a	per^{+}/Y	24.1 ± 0.4	75	23.9 ± 0.3	27	23.8 ± 0.5	17
b	per^{+}/per^{l1}	25.0 ± 0.2	19	26.1 ± 0.2	6	25.7 ± 0.3	14
c	per^{+}/per^{l2}	24.3 ± 0.8 arrhythmic	5 3	24.7 ± 0.4 arrhythmic	12 2	24.7 ± 0.3 arrhythmic	9 3
d	per^{+}/per^{o}	24.8 ± 0.6	4	25.3 ± 0.4	11	24.7 ± 0.4	17
e	per^{s}/Y	19.5 ± 0.3	61	19.0 ± 0.3	95	18.7 ± 0.2	8
f	per^{s}/per^{l1}	22.8 ± 0.4	32	22.9 ± 0.4	142	23.6 ± 0.5	26
g	per^{s}/per^{l2}	20.5 ± 0.5 arrhythmic	11 1	20.5 ± 0.4 arrhythmic	10 1	19.9 ± 0.2 --	9
h	per^{s}/per^{o}	20.8 ± 0.5 arrhythmic	8 4	20.4 ± 0.5 arrhythmic	13 2	19.7 ± 0.2 --	6
i	per^{l1}/per^{l2}	26.9 ± 0.8	12	30.8 ± 0.5	7	31.3 ± 0.9	11
j	per^{l1}/per^{o}	27.1 ± 0.7 arrhythmic	7 5	30.6 ± 0.9 --	5	31.7 ± 1.2 arrhythmic	9 4

Table 2-5. Interaction between *And* and per^{s}, per^{l1}, per^{l2}, and Clk^{K06}

		Temperature (°C)					
		17°		22°		25°	
	Genotype	Period ± SD	*n*	Period ± SD	*n*	Period ± SD	*n*
a	per^{s} *And*	20.8 ± 0.6 arrhythmic	7 9	20.1 ± 0.5 --	19	19.7 ± 0.3 --	12
b	if additive:	21.2		21.1		20.8	
c	Δ period*:	+0.4		+1.0		+1.1	
d	per^{l1} *And*	29.9 ± 1.1	7	31.2 ± 0.7	28	32.8 ± 0.9	21
e	if additive:	29.5		31.7		32.6	
f	Δ period*:	-0.4		-0.5		-0.2	
g	per^{l2} *And*	30.5 ± 0.4 arrhythmic	4 9	32.2 ± 0.7 arrhythmic	11 6	34.0 ± 0.7 arrhythmic	2 19
h	if additive:	29.7		31.3		32.4	
i	Δ period*:	-0.8		-0.9		-1.6	
j	Clk^{K06} *And*	--		23.7 ± 0.3	12	24.2 ± 0.3	8
k	if additive:	24.4		24.3		24.2	
l	Δ period*:	--		+0.6		0.	

* Δ = expected - observed

Table 2-6. Semi-dominance of the mutant *Clk*^{KO6}

		Temperature (°C)					
		17°		22°		25°	
	Genotype	Period ± SD	*n*	Period ± SD	*n*	Period ± SD	*n*
a	wild-type	24.2 ± 0.4	75	23.9 ± 0.3	27	23.8 ± 0.5	17
b	*Clk*^{KO6} / +	23.4 ± 0.5	18	--		23.2 ± 0.3	19
c	*Clk*^{KO6} / *Clk*^{KO6}	22.9 ± 0.5	53	22.6 ± 0.4	104	22.5 ± 0.6	63

Chapter 3

Temporal Structure of the Drosophila Circadian Clock as revealed by 'Phase Response Curves' Due to Light Pulses

Summary

(1) The method used for constructing a Phase Response Curve (PRC) of the adult Drosophila circadian clock using the activity rhythm as an assay is presented (Figs. 3-1,3-2). The results from 785 out of 2500 experimental animals were used in such studies.

(2) A comparison of the PRC's for per^s, per^{l1} and wild-type at 17 ° C. suggests that the mutations per^s and per^{l1} change the period of the circadian clock by differentially shortening and lengthening, respectively, the duration of the 'subjective day' phase of the oscillation (Fig.3-3).

(3) Comparisons between the PRC's for per^s at 17°C, 22°C, and 25°C. and comparison between the wild-type PRC's at 17°C and 22°C. do *not* reveal major changes in the temporal structure of these two circadian clocks over the stated temperature ranges (Fig.3-4).

(4) Ten 'Phase-Plane Plots' obtained with saturating light pulses for six strains of flies at various temperatures are presented. (Fig.3-7 a-i) The results indicate that: (*i*) All ten cases exhibit basically 'type-1' (weak) resetting behavior. (*ii*) All ten cases show a bigger range for phase-delays than for phase-advances. (*iii*) Even though the period lengths represented in these ten cases range from 19 to 30 hours, the duration of the phase at which significant phase-delays can be elicited is similar (about 6 hours) in all cases where such measurements can be made.

(5) Examples are presented to show that rather strong 'after-effects' can be caused by the perturbing light pulses -- in the forms of change in activity profile, change

in period lengths and loss of rhythmicity (Figs. 3-8, 3-9, 3-10, 3-11, 3-12).

(6) Discussions on the above findings and on the limitation of the PRC approach are presented.

3.1 Introduction

One of the goals that most circadian physiologists try to attain is to be able to delineate the temporal structure of the clock cycle in terms of a sequence of physiological events or groups of such events and to understand the interrelationships between them. This approach is often compared to a similar analysis undertaken in the study of the cell cycle (ref.3-1,ref.3-2). Yet, a major difference between the two systems is often ignored. Thus, researchers of the cell cycle have at their disposal a wealth of anatomical, physiological and molecular descriptions (at least in some cells) on events of the cycle -- e.g. bud emergence, nuclear migration, DNA synthesis, cell separation, etc. These events can usefully serve as phase reference points on the cycle and the study of the temporal structure of that clock can be formulated by asking what causal relationship exists or does not exist between any of these events (ref.3-3). The circadian physiologist is less fortunate in this respect, because, even though discrete circadian oscillators have been successfully isolated (refs.3-14, 3-15, 3-16), the decomposition of a circadian cycle into functionally, or even phenomenologically, discrete units has not been available. Instead, most of the rhythmic phenomena available for circadian analysis provide the researcher with only one phase reference point (e.g. the time at which a pupal insect ecloses, the onset or offset of the locomotor activity of an animal, the time at which the average firing rate of a neuron peaks, etc.). To remedy this scarcity of phase reference points and to try to get at the state of the driving oscillator directly, Pittendrigh developed in 1967 (ref.3-4) an operational distinction between the overt rhythmicity and the driving oscillation of a circadian system. In this protocol, one makes use of the fact that practically all free-running circadian rhythm can be phase-shifted, to a more or less degree, by single brief light pulses. In most of the cases examined so far (ref.3-6), the magnitude and signs of the phase shifts thus induced are dependent on the phase[1] at which the light perturbations are introduced. A plot of the

phase of the cycle at which the light pulse is given *vs.* the phase shift elicited by the perturbation is called the phase response curve (PRC) due to the light pulses. The PRC is a simple and yet powerful tool to study the circadian clock because it gives us information that reflects, in the most direct way available so far, the state of the clock. In recent years, many investigators have extended this protocol to the application of other kinds of perturbation of the clock using a wide variety of stimuli other than light. For example, for the circadian rhythm in the eye of *Aplysia* -- one of the most well studied models of circadian oscillators -- PRC's have been obtained using the following stimuli: protein synthesis inhibitor (ref.3-20), metabolic inhibitors (ref.3-21), high potassium solution (ref.3-22), serotonin (ref.3-23) and temperature pulses (ref.3-24). While the use of these different agents as phase-shifting stimuli have brought useful insights to the understanding of the *mechanism* underlying circadian oscillators (such as the establishment of the role of protein synthesis in the generation and maintenance of circadian rhythms as alluded to in Chapter 1), they all share the same drawback of needing a relatively prolonged duration of application (usually 4 to 6 hours) in order to exert a significant phase-shifting effect so that the resulting resolution is generally not enough to answer any question on the *temporal fine structure* of the system. To this date, light remains the only environmental agent known that can offer the resolution that one needs for such a purpose.

In this chapter, we describe PRC studies on the clock period mutants of *Drosophila* , using brief light pulses as the perturbations. The basic questions that we ask are : When a clock mutation changes the frequency of the circadian clock, how does it affect the temporal structure of the cycle ? Specifically, can clock period mutations be classified in terms of what *phase* of the clock cycle they particularly affect?

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1. The phase of the cycle is defined in reference to a fixed point in the overt rhythm or to the environmental (most often light or temperature) regime to which the circadian oscillator was exposed before being released into the free-running condition.

3.2 Construction of the *per*^{s} PRC at 22° C

Fig.3-1 shows simulated actogram plots for three *per*^{s} animals free running in darkness at 22° C. The free-running periods for the three animals in the segment of record before the light pulse are 18.8 hours, 18.7 hours, and 18.5 hours respectively. The time, T_0, at which the phase reference point of the circadian cycle (*viz.* the projected *zeroth* offset of the activity rhythm) is assumed to start is 1.5 hours, 1.2 hours, and 1.8 hours after the $LL \rightarrow DD$ transition for the three animals respectively.[2] At 99.5 hours after the animals have been released into darkness, they are simultaneously exposed to a 60 second light pulse of about 2000 lux in intensity. Because of the slight differences in the period lengths and in the phase reference points of the circadian cycle with respect to the $LL \rightarrow DD$ transition, the light pulse, even though given to the animals at the same point in time, falls on slightly different phases of the individual clock cycles of these three animals. Thus, on a scale in which the period of an individual clock is normalized to the value 1.0, the first animal receives the pulse at phase point 0.22, the second at 0.26 and the third at 0.28 . The results of these slight differences in phase points at which the light perturbations occur are rather dramatic. As indicated in the actograms, the light pulse causes a substantial phase *delay* (4.4 hours) in the first animal, causes no significant change in phase in the second, and causes a substantial phase *advance* (2.8 hours) in the third animal.[3]

A summary of the responses of 98 *per*^{s} animals which are perturbed at various phases of the circadian cycle with light pulses of 2000 lux but with various durations is presented in fig.3-2(a). Two features are to be noted in the figure. First, even though the responses vary to some extent from one animal to another, the general

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2. See caption to fig.3-1 for definition of T_0. Distribution of T_0's for all the strains studied can be found in figs. 3-6 and 3-7.

3. Note that, as these three examples illustrate, there are no significant transients observed in phase-shifting the locomotor rhythm in *D. melanogaster*, which is quite unlike most other systems studied.

shape of the PRC resembles the 'typical' PRC of other circadian clocks very well (ref.3-6) -- with a phase-delay region in the 'early subjective night', a phase-advance region in the 'late subjective night', and a relatively light-insensitive region ('dead-zone') in the 'subjective day'. Secondly, we find no significant difference in the responses of the animals receiving the pulses of various durations ranging from 6 seconds to 80 minutes, suggesting that the sensitivity of the clock photoreceptive processes have been saturated at below the 6-second & 2000-lux limit. In the following PRC experiments to be described, unless otherwise stated, 10-minute & 2000-lux light pulses are used to ensure a saturating effect.

Fig.3-2(b) shows a mean PRC for the strain per^s at 22 ° C. as derived from the individual responses described in fig.3-2(a). In arriving at the mean value for a particular phase point[4] , we weight each phase-shift with the factor $\frac{1}{\sigma_i^2}$, where σ_i is the uncertainty associated with that particular phase-shift measurement.[5] We use this weighting method because we believe that the uncertainty associated with each phase-shift measurement is mainly due to the cycle-to-cycle variability of the overt rhythm and the cause for this may differ from animal to animal.

4. Phase points are quantized into 1-hour bins
5. See description in the captions to fig.3-1 and fig3-2.

3.3 Mutations at the *per* locus affect length of the subjective day

Fig.3-3 shows the average PRC's for wild-type, per^s and per^{l1} at 17°C. The three PRC's are lined up such that the phase point 0 in each plot represents the point in time the *zeroth* activity offset occurs.The calibrations in both axes represent one hour of *real* time. We note that the portion of the cycle in which significant phase-delays can be invoked in the three PRC's are all about 6-7 hours long; likewise, the portion of the cycle in which significant phase-delays can be invoked is also about 6-7 hours long. On the other hand, the portion of the cycle that is relatively insensitive to light seems to vary rather much in length in the three strains, being about 5 hours in per^s, about 10 hours in the wild-type, and about 13-14 hours in per^{l1}.

We proceed to compare two extreme possibilities : (1) Period changes in the *per* mutants are caused by an overall contraction or expansion of the cycle; and, (2) Period changes in the *per* mutants are caused by a differential contraction or expansion of the 'subjective day' part of the cycle only. To this end, two hypothetical PRC's 19 hours in period are constructed from the raw *wild-type* data. The first PRC is obtained by averaging the results in bins of 24/19 hours rather than 1 hour, therefore simulating a uniform contraction of the 24 hour cycle. The second PRC is obtained by averaging the results in hourly bins as usual but arbitrarily 'truncating' a segment of 5 hours from the subjective day (the time segment of hours 14 through 18 is taken out). A test of which of these two PRC's is more similar to the actual per^s PRC is performed by calculating the coefficient of linear correlation between each of the sequences of points making up these two PRC's and the sequence of points making up the actual per^s PRC. The resulting coefficients are 0.71 for the PRC representing uniform contraction of the cycle and 0.95 for the PRC representing the differential contraction of the subjective day portion. Similarly, two hypothetical PRC's are obtained for the per^{l1} mutant by (1) averaging the raw data in bins of 27/24 hours, simulating a contraction of the supposedly uniformly

expanded cycle back to 24 hours; and (2) by arbitrarily truncating a segment of 3 hours in the subjective day of the 27 hour cycle (the segment containing hours 17 through 19 is chosen) . Calculations of the linear correlation coefficients of these two sequences of points with the sequence representing the actual wild-type PRC give values of 0.78 for the case of uniform expansion and 0.87 for the case of differential expansion of the subjective day. Thus, the above calculations favor the hypothesis of differential contraction/expansion of the subjective day over the hypothesis of uniform contraction/expansion of the whole cycle in the *per* mutants examined.

3.4 Near point-to-point temperature compensation in the wild-type and *per*^{s} PRC's

In the introduction to section 2.2 in this thesis, we discussed the question of whether the periods of circadian systems are temperature *independent* or temperature *compensated*, and presented arguments that favor the latter case. Insofar as the dynamics of the circadian clock can be analysed in terms of a PRC, models on the mechanisms of temperature compensation can generally be separated into two classes. First, the rate of whatever mechanism makes up the circadian oscillation could be compensated for temperature variation at each phase point in time. The second alternative is that the circadian cycle can be made up of composite (sequential) processes that have complementary temperature coefficients in such a way that the deferential 'shrinking' and 'stretching' of the durations of these processes may balance out to attain the overall temperature compensation of the period of oscillation.

Fig.3-4 (a) and (b) show comparisons of the wild-type PRC's at 17°C. and 22°C. and *per*^{s} PRC's for 17°C., 22°C., and 25°C., respectively. In both cases, the overall shapes of the PRC's at the different temperatures resemble one another very well. However, two differences are observed. First, the amplitude of the phase-advance region of the wild-type PRC at 22°C. seems to be reduced compared to the PRC at 17°C. Secondly, the phase-delay to phase-advance transition point for the *per*^{s} PRC at 22°C. occurs between 4 and 5 hours after activity offsets, an hour earlier than the corresponding points in the PRC's at 17°C. and 25°C.[6] Fig.3-5 shows the 'correlation profiles' between the various PRC's at the various temperature. It shows that the highest value of the linear correlation coefficient attained between the *per*^{s} PRC's at 17°C. and 22°C. is 0.95; similar values for the 22°C./25°C. and the 17°C./25°C. pairs

6. The fact that all animals are reared at about 22°c. means that those animals used in the 17°C. and 25°C. measurements receive a temperature step-down and step-up, respectively, concurrent with the light/dark transition. Whether these conditions are responsible for the 1-hour 'slip' in the position of the phase reference point is not known.

are both 0.90; and, for the two wild-type PRC's, it is 0.92. These calculations suggest that there is no *major* change of waveform of the driving oscillator in both the wild-type and the per^s circadian system.

3.5 'Phase Plane Plots' of wild-type and clock mutant D. melanogaster

So far we have presented phase-shifting data in terms of PRC's, in which the phase-shift inflicted on the circadian system by a perturbing stimulus is plotted against the time (phase point) at which the stimulus is introduced. An alternate way to look at the same data is to plot, instead of the phase shifts, the new phase to which the clock is reset. By systematically treating the phase-resetting data in the circadian literature in this later way, Winfree (ref.3-7) discovered two interesting features in the resetting behavior of circadian clocks in general. First, the average slopes in the 'old-phase *vs.* new phase' plane for all the resetting curves known -- to the resolution offered by the relevant data, which in many cases are fairly poor -- fall around two discrete values, *viz.* 0 and 1. Hence, resetting curves are generally classified by being 'type-0' or 'type-1'. 'Type-0' resetting behavior is generally associated with 'strong' responses of a system to a stimulus, and 'type-1' resetting behavior is generally associated with 'weak' responses of a system to the stimulus. We note that even though, in the limit of an extremely weak stimulus, all resetting behavior is 'type-1', yet 'type-1' behavior does not necessarily mean weak responses. This point is illustrated in fig.3-6 .

The second interesting feature discovered by Winfree concerns the behavior of certain 'type-0' systems as the magnitude of the perturbing stimulus is decreased (and thus forcing the system into 'type-1' behavior). It is found that, in the realm of the 'type-0' to 'type-1' transition, a stimulus of the appropriate strength and duration can cause arrhythmia.[7] Furthermore, since the circadian clock controlling the pupal eclosion rhythm of per^s was observed to be of 'type-0' (ref.3-8), Winfree and Gordon (ref.3-10) proceeded to find and succeeded in finding such a 'singularity' in

7. One hastens to mention that in all the systems in which such phenomenon has been observed, only rhythms of a population of organisms (e.g. the *D. pseudobscura* eclosion rhythm) or of a population of cells within an organism (e.g. the CAP rhythm in the *Aplysia* eye) are monitored, and the question on whether the arrhythmia observed reflects true arrhythmia at the level of individual organisms or desynchronization of a population of individually rhythmic members has so far not been satisfactorily settled.

the clock of this mutant, even though the wild-type *D. melanogaster* clock, possessing 'type-1' resetting behavior (ref.3-8), would not allow the existence of such a singular phenomenon.

In this section, we present 10 of such phase-plane plots (figs.3-7 (a)-(j)) for six strains of *D.melanogaster* to seek for generalities that may emerge to shed light on the 'defects' of these mutants in the clock mechanisms. (Some of the data have appeared in an average form in the PRC's in the previous sections.) The first observation on these phase-plane plots is that, even though we have shown above that saturating stimuli are used, eight out of the ten cases show strictly weak,'type-1' resetting behavior. The two exceptional cases, per^s / per^{l1} (fig.3-7h) and Clk^{KO6} (fig.3-7i), show strong 'type-0' resetting in the early subjective night (the 'phase-delay' portion) but weak, 'type-1' behavior in the late subjective night (the 'phase-advance' portion). Since the over all average slopes for these two graphs are still around 1, we have classify them as belonging to 'type-1' also. These two cases are in fact extreme forms of our second observation, which is a general trend, observed in all cases, which shows that (1) the average magnitude of phase advances are smaller than that for phase-delays, and, (2) the distributions of phase-advances are much tighter than that for phase-delays. (See insets in figs.3-8 (a) through (j).) These facts are reflected in the phase plane plots in that the phase-advanced points are commonly displaced upward for about 2 hours, regardless of where the stimulus is applied, while the phase-delayed points are more often displaced downward for a variable amount, dependent on the phases at which the stimuli are introduced -- in such a way that the final (new) phases tend to cluster around a limited range of about 2 to 3 hours. This is especially clear in fig.3-7 (a), (d), (f), (g), (h) and (i). The third observation we make is that even though the period lengths represented in the 10 plots range from 19 to 30 hours, the range of phases within which a light pulse can inflict a significant phase-delay (greater than 1.5 hours) seem to be invariably

around 6 hours.

3.6 Strong 'After-Effects' of light pulses in *D.melanogaster*

In the previous sections, we present data that intend to give us a 'peek' into the state of the circadian clock as abstracted from the behavior of a phase reference point in a rhythm it drives. The assumption that the internal dynamics of such a complex system as a biological clock can be represented by a one-dimensional continuum of states is bound to break down at some point. In fact, the surprise is, at least to first approximation, such analyses seem to provide rather meaningful results in the *Drosophila* system. In this section we give examples of three classes of 'anomalous' behavior in the activity rhythm of *Drosophila* elicited by single, brief, but 'strong' light pulses. Beside serving the purpose of documenting the technical difficulties of employing locomotor activity as an assay of the clock in the protocol used in this chapter, these examples, we hope, could help illustrate the underlying complexity of the circadian clock.

3.6.1 Drastic change of activity profile due to a light pulse

Fig.3-7 shows the activity rhythm of a wild-type animal. The activity pattern of the DD_1 segment, as seen from the form estimate profile, consists of a major, conspicuous 'band' of activity plus a much smaller component that precedes it.[8] The phase reference point of the rhythm, which we define as the point in time at which 95% of the activity counts have occurred, is well defined throughout this segment. A 10-minute 2000-lux pulse is then given to the animal at the end of the *DD*1 segment which causes a drastic, and *lasting* change to the activity profile in that (1) the relative phase relationship of the major and minor components of activity seems to have drastically changed, and (2) the profile becomes very 'noisy'. For these two reasons, the phase reference points in the DD_1 and DD_2 segments, if the latter can be defined at all, seem to bear no obvious relationship to one another. To calculate

8. This bi-modal activity pattern is seen in about 15-20% of the wild-type animals. Such occurrences seem to be much more rare in the clock mutants studied.

phase-shift in cases similar to this is therefore meaningless. All records for animals showing such behavior are therefore discarded for the purpose of this chapter.[9]

3.6.2 Sizeable period changes induced by light pulses

Fig.3-8 gives an example of a well known "after-effect" on circadian systems after light treatments: The free-running frequency of a clock is changed, *'permanently'*, by a brief exposure to light. In fig.3-9, we show distributions of period changes of animals whose activity profiles are 'rigid' enough to pass the examination in last section and are therefore included in the PRC's presented in this chapter. We observe, firstly, that the period changes are of both signs (lengthening and shortening), and , secondly, that, even though the periods of most animals change less than an hour, there are a number of animals whose period lengths are changed by as much as 2 to 3 hours. Since in both the PRC and the 'phase-plane' analyses, tacit assumptions are made about the constancy of the parameters of the oscillating mechanisms (beside allowing a few cycles of 'transients'), one could see from such examples that these analyses are at best approximate studies. In fig.3-10, we have picked six strains of animals which show the largest amplitudes in the resetting curves and ask whether the signs of the period changes are related to whether phase advances or delays are caused by the light pulses. The results show that, in all six cases, such a relationship is lacking.

3.6.3 Arrhythmicity induced by light pulse

Fig.3-11 shows the record of a wild-type animal whose activity rhythm in DD_1 is relatively noisy. A light pulse at the end of the DD_1 segment disturbs the rhythm further so that nearly complete arrhythmicity occurs in the DD_2 segment. The

9. A total of 2500 animals have been used for the phase-resetting experiments described in this chapter. About one third of these animals did not live long enough to give 4 cycles or more after the light pulses. The results of another one third are discarded because the activity records are not clean enough for unambiguous phase point determination, or because of change of activity profile as reported here, or because the light pulses totally disrupt the rhythm as reported in a later section. The rest of the animals, 785 in total, contribute to the results shown. Thus, for experiments of this kind in *Drosophila*, the yield is about 30% (ranging from 10% in *And* to 45% in per^S.

percentage of animals with this behavior is small compared to the number of animals whose activity profiles are drastically changed. However, a fair number of cases (in the tens out of the 2500 animals) have been observed. At present we do not know whether this loss of overt rhythmicity reflects a corresponding state of the clock, and this issue is not pursued further.

3.7 Discussion

The fact that the *per*^{s} mutation does not shorten the clock cycle uniformly but rather decreases the duration of the light insensitive phase ('subjective day') was first observed by Konopka (refs. 3-8, 3-9) in the circadian clock controlling the eclosion rhythm of *Drosophila*. The results presented in section 3.3 confirm this observation in the case of the clock controlling adult locomotor activity. The additional observation that *per*^{l1} seems to affect the clock period by lengthening the same phase of the cycle raises the interesting possibility that the function of the *per* locus is predominantly involved in the control of this part of the circadian cycle. In contrast, recent studies (ref.3-11) on the PRC's of the *frq* mutants in *Neurospora* (see footnote 12 in chapter 2) have also suggested that these mutations do not alter the clock period uniformly, but instead change the duration of the *early subjective night*. Whether these results reflect basic differences in the structure of the circadian clock or simply the fact that the equivalent '*per*' locus in *Neurospora* and the equivalent '*frq*' locus in *Drosophila* have not been isolated await further mutant screening and more exhaustive, systematic exploration in these two systems.

The specific issue of whether temperature compensation of circadian period works on a point by point manner was first experimentally addressed by Zimmerman et al. (ref.3-12). These authors, using the eclosion rhythm of *D. pseudobscura* as an assay, found that, to the resolution of the experiment (about 1 hr.) point by point compensation is indeed at work in this system. Our results in section 3.4, which show that there is no major change in the form of the wild-type and *per*^{s} PRC's at various temperatures, support this conclusion in the adult *D. melanogaster* clock. Moreover, our observation on the similar behavior in *per*^{s} suggests, again within the resolution of the experiment (also about 1 hour), that this feature in the temperature compensation mechanism is preserved in this mutant.

In face of the findings both of Konopka (ref.3-8) and Winfree and Gordon (ref.3-10) that the PRC of per^s to 'strong' light stimulus is of 'type-0' nature, it is a rather unexpected finding that the PRC of the adult clock of the same strain is 'type-1'. Three obvious explanations are available at this point. First, it could be that, even though the *per* mutations are found to affect both the pupal eclosion and the adult locomotor activity rhythms, the two processes may be controlled by separate clocks -- a possibility first implicated by (the rather weak) data in *D. pseudobscura* (ref.3-13). A second possibility is that there is only one circadian clock involved in the control of the two processes and the difference observed in the resetting types reflects ontological developments in properties of the clock -- either in the dynamics of the clocking mechanisms or in the sensitivity of the photo-receptive processes -- from the pupal to the adult stage. Lastly, Winfree (refs.3-17,3-18) showed that it is possible to obtain 'type-0' resetting in the aggregate rhythm of a population of 'type-1' clocks that are incoherent in phases. Thus, the possibility of the 'type-0' resetting behavior observed in the per^s eclosion rhythm being strictly a population artifact remains open.

It is a curious fact that even though the ten resetting curves studied represent periods varying from 19 hours to 30 hours, the phase at which significant phase phase delays can be elicited is about 6 hours in all cases. We note that this portion of the cycle coincides with the oxygen-dependent phase of the *D pseudobscura* clock(ref.3-19). Whether these results indicate a crucial role of a 'charging' phase in the early subjective night of the *Drosophila* clock cycle awaits further investigations.

In conclusion, given the crude assumptions underlying the PRC analyses, the insights gained in the efforts described in this chapter are, in the opinion of the author, quite encouraging. We have seen, through the aforementioned phase resetting results, that the dynamics of the circadian clock are not simple. Nevertheless, the question that we posed at the beginning of the chapter, *viz.* whether clock

period mutations can be classified in terms of what phase of the clock cycle they affect, seems to be a valid one. To be sure, to proceed further with the 'temporal mapping', one needs to isolate many more period mutants in *Drosophila*. One of the lessons we learn after pulsing the 2500 flies in this study is that, owing to the fact that the temporal structure of the clock being revealed this way is restricted by the phase resolution of the *overt* rhythm (about 1 hour), mutants with *drastically* altered clock periods are bound to offer more insight in this kind of analysis.

Chapter 3 References

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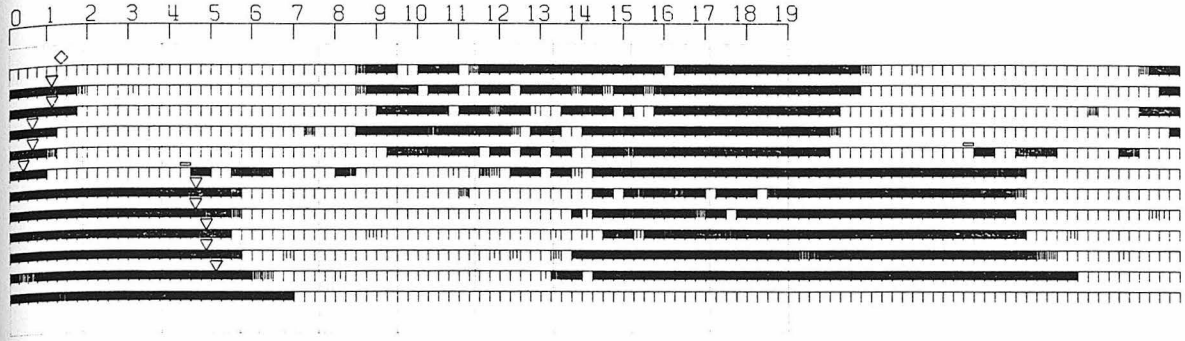
Chapter 3 Figure Captions

Figure 3-1. *Effect of a light pulse on the activity rhythm of three per^s animals.* The animals are reared in constant light of about 200 lux at 22° C and are released into DD at 3 days old. Data are collected in 15 minute bins. The offset of the locomotor activity of each cycle, as calculated by the method detailed in section A.4, is marked with an inverted triangle. The animals are exposed to a 60-second light pulse at the time marked by the small rectangular box. The diamond near the beginning of each record marks the time of the *zeroth* offset (called T_0 below), which is calculated from a linear regression fit of all the offsets observed up to the time of the light pulse. This point is taken to be the point when the rhythm starts.

In the following description of the calculation of phase-shifts, the following terminologies are used. The segment of free-run before the pulse is called DD_1 and the post-pulse free-run is called DD_2. N_1 denotes the number of cycles of activity observed in DD_1 , and N_2 denotes the similar number for DD_2. In all the experiments described in this chapter, N_1 ranges from 4 to 7 and N_2 ranges from 4 to 8.

Phase shifts are calculated by : $\Delta\varphi = \varphi_{1,DD2} - \varphi_{e,DD1}$, where $\varphi_{1,DD2}$ is the first offset of the DD_2 segment as *calculated* from linear regression of the sequence of N_2 *actual* offsets observed in the segment, and $\varphi_{e,DD1}$ is the *extrapolated* N_1+1^{th} offset calculated from the N_1 actual offsets observed in DD_1. The phase of the circadian cycle at which the animal receives the light pulse is given by : $\varphi_{pulse} = (T_{pulse} - T_0)$ *Modulo* (τ_{DD1}) ; where T_{pulse} is the time at which the light pulse is given; T_0 is the starting time of the rhythm as defined above; and, τ_{DD1} is the period of the activity rhythm in the DD_1 segment as calculated from the slope of a linear regression line through the offsets of the same segment.

2 E05 0281369 M1D PER-S II DD1 BIN 00052 , 1' 2000 LUX BIN 00449



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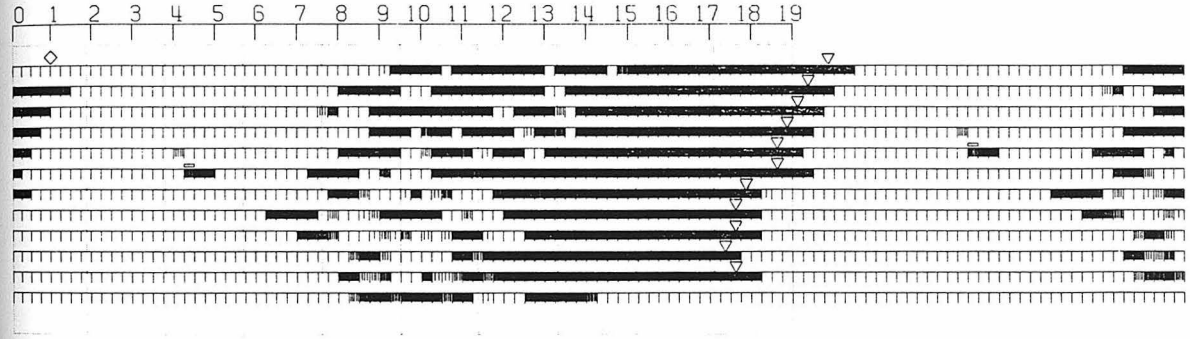

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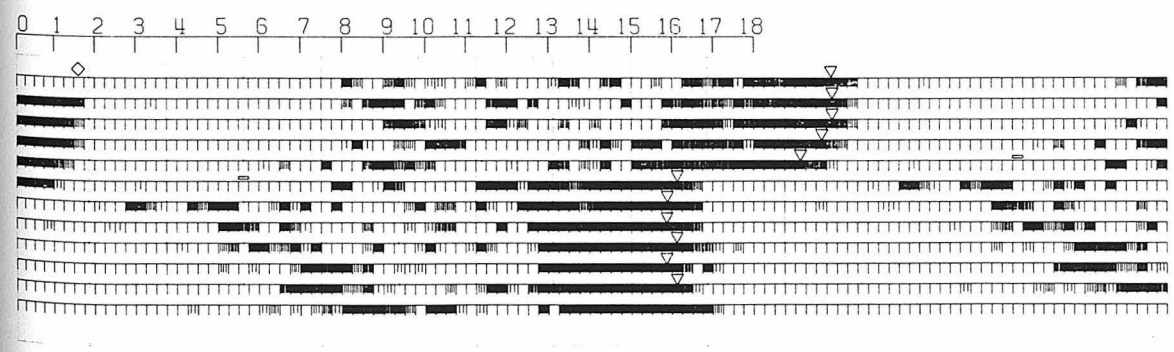
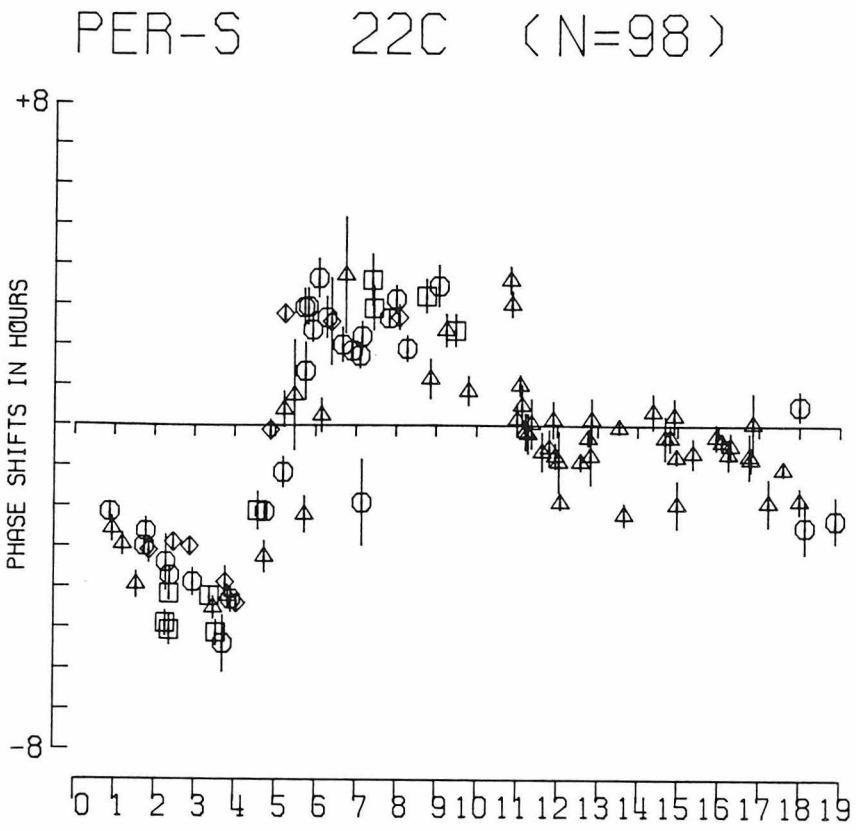


Figure 3-2. *(a) Phase-shift of per^s individual activity rhythms by light pulses of various durations and applied at different phase points.* The phase-shifts of 98 *per^s* animals are plotted against the phase points at which the light pulses are applied. Each symbol represents the result from one animal. The error bar that is associated with each symbol represents the uncertainty in estimating the phase shifts, which is given by $\sigma=\sqrt{\sigma_1+\sigma_2}$; where σ_1 is the standard deviation of the linear regression fit of the DD_1 offsets, and σ_2 is the similar value for the DD_2 segment. (See caption to fig.3-1.) Meaning of the symbols : *Octagons* = 6-second pulses ; *Diamonds* = 60-second pulses ; *Triangles* = 10-minute pulses ; *Squares* = 80-minute pulses.

(b) Average Phase Response Curve for per^s as calculated from the individuals' responses presented in (a). The results in (a) are divided into 1-hour bins. The average phase-shift for each bin is calculated by $\overline{\Delta\varphi}=\frac{1}{\sum_i^n \frac{1}{\sigma_i^2}}\sum_i^n \frac{\Delta\varphi_i}{\sigma_i^2}$, and the uncertainty of the mean of a particular bin, σ_μ, is given by : $\sigma_\mu=\frac{1}{(\sum_i^n \frac{1}{\sigma_i^2})^{1/2}}$, where n is the number of observation in that bin. In the case shown here, σ_μ turns out to be smaller than the size of the symbols used in most bins. The above methods are used to calculate the mean and uncertainty of the mean because we take the view that most of the uncertainty involved in each phase-shift measurement is due to the uncertainty of the driven rhythm itself. (ref.3-5)


PER-S 22C (N=98)
+8
-8
PHASE SHIFTS IN HOURS
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19

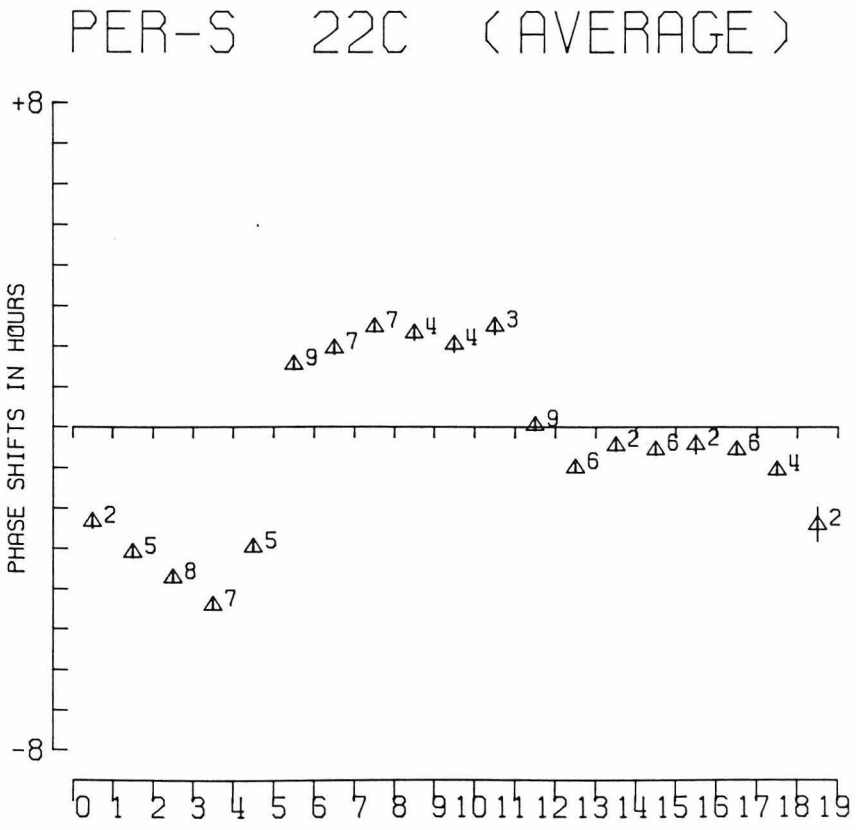
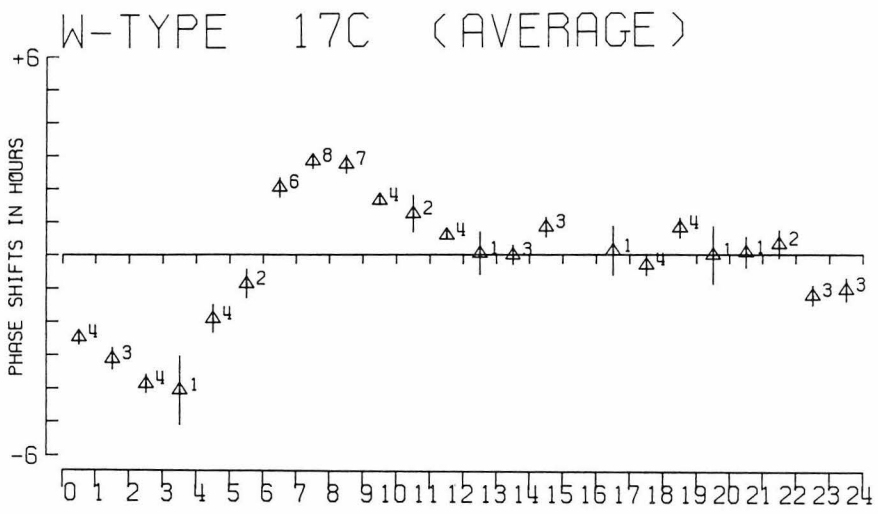
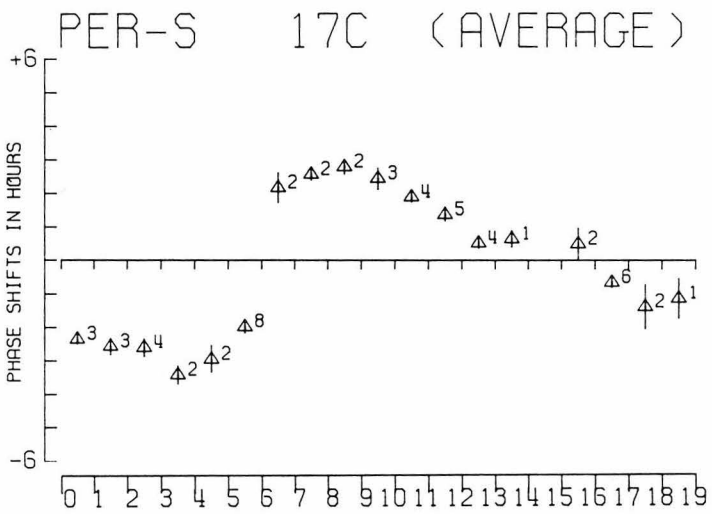
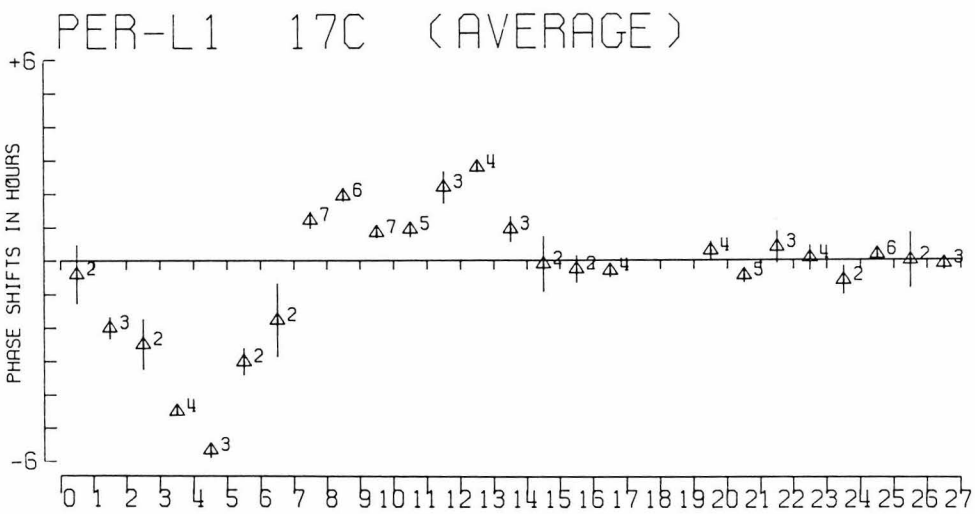

PER-S 22C (AVERAGE)
+8
-8
PHASE SHIFTS IN HOURS
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
PHASE IN HOURS, RELATIVE TO ACTIVITY OFFSETS

Figure 3-3. *Comparison of the PRC's for wild-type, per^s and per^{l1} at 17° C.* Average PRC's are obtained for the three strains using the methods described in figs. 3-1 and 3-2. The number next to the symbol represents the number of animals whose data go into that bin. 10 minute, 2000 lux pulses are used as the perturbing stimuli.



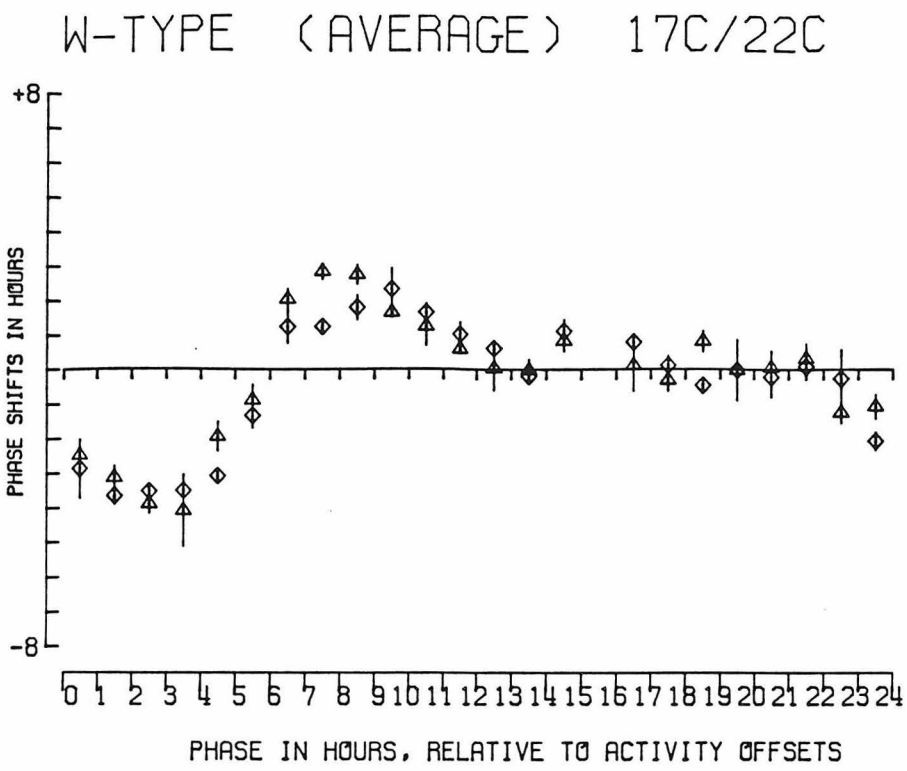




PHASE IN HOURS, RELATIVE TO ACTIVITY OFFSETS

Figure 3-4. *(a) Comparison of the wild-type PRC's obtained at 17 ° and 22 ° C.* Average PRC's are obtained using the methods explained in figs. 3-1 and 3-2. The PRC's for per^s are normalized to period length of 19 hours. Symbols used: *Triangles* = 17° C. ; *Diamonds* = 22° C.

(b) Comparison of the per^s PRC's obtained at 17 ° and 22 ° and 25 ° C. Symbols used: *Triangles* = 17° C. ; *Diamonds* = 22° C.; *Octagons* = 25° C.


W-TYPE (AVERAGE) 17C/22C
+8
-8
PHASE SHIFTS IN HOURS
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
PHASE IN HOURS, RELATIVE TO ACTIVITY OFFSETS

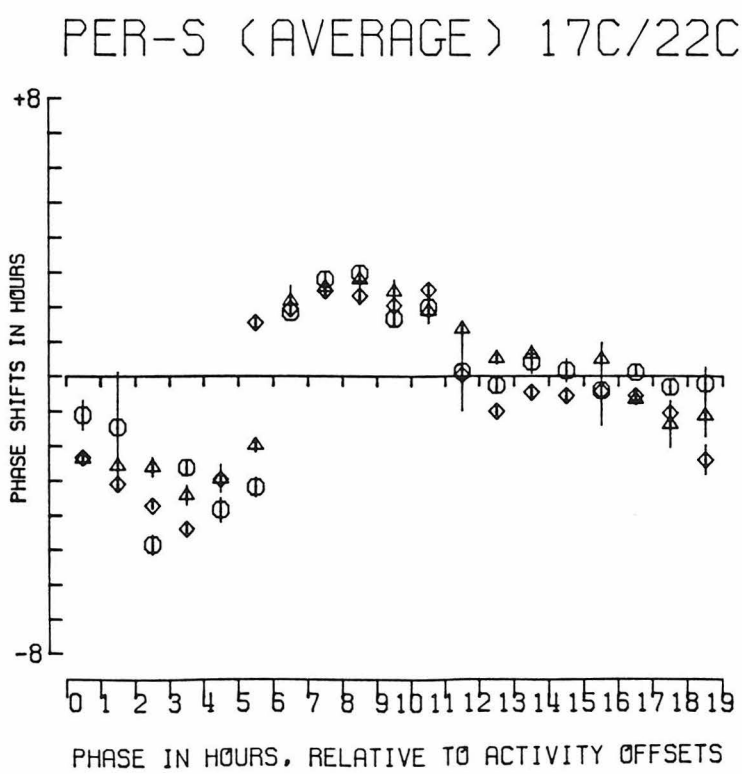

PER-S (AVERAGE) 17C/22C/25C
+8
-8
PHASE SHIFTS IN HOURS
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
PHASE IN HOURS, RELATIVE TO ACTIVITY OFFSETS

Figure 3-5. *'Correlation Profiles' between the PRC's in fig.3-4.* Linear-correlation coefficient (See p.121, ref.3-5) is calculated for the two sequences of data points making up the two PRC's under test, giving the value at relative phase = 0. One of the two sequences is then 'slided' against the other sequence to generate a 'correlation profile'. This 'sliding' is done to cover the possibility of two PRC's being very similar in shape but have different phase relationships with respect to the overt rhythmic event or to the $LL \rightarrow DD$ transition. *Cases*: (*a*) Correlation of the per^s PRC's at 17° C.and 22° C. ; (*b*) Correlation of the per^s PRC's at 17° C.and 25° C. ; (*c*) Correlation of the per^s PRC's at 22° C.and 25° C. ; (*d*) Correlation of the *wild-type* PRC's at 17° C.and 22° C.

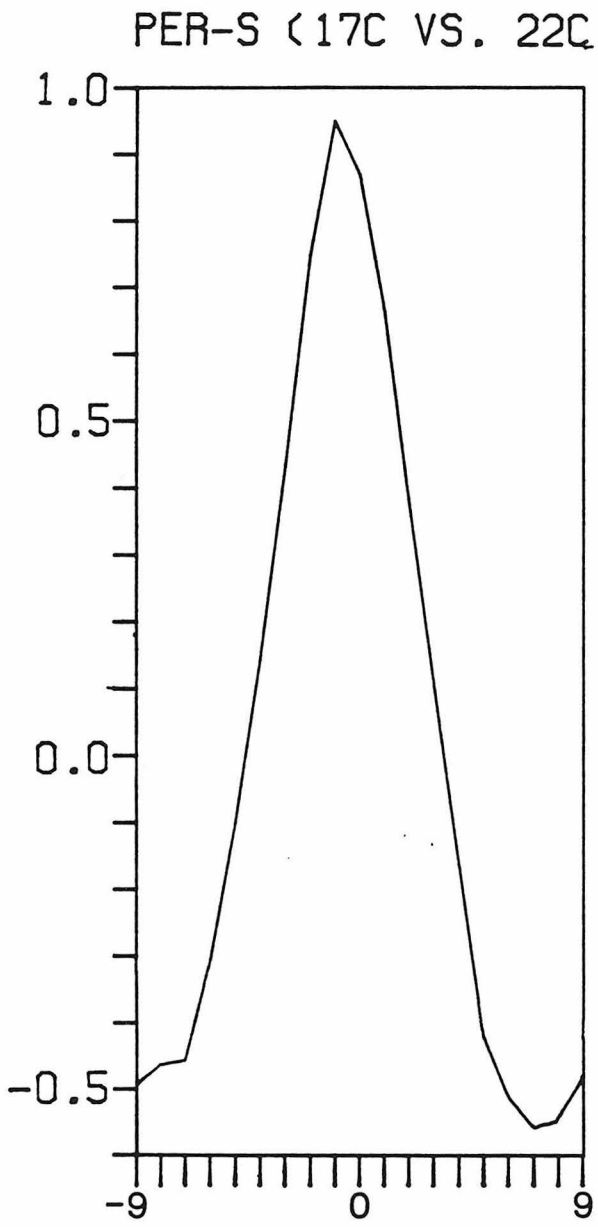


Fig. 3-5 (a)

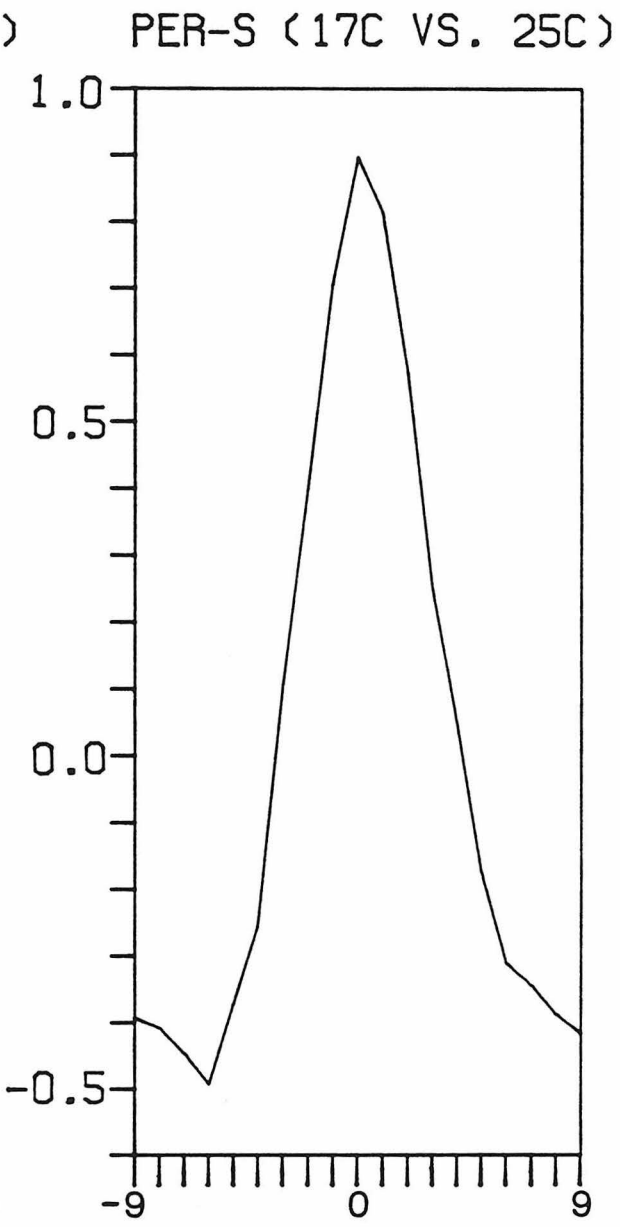


Fig. 3-5 (b)

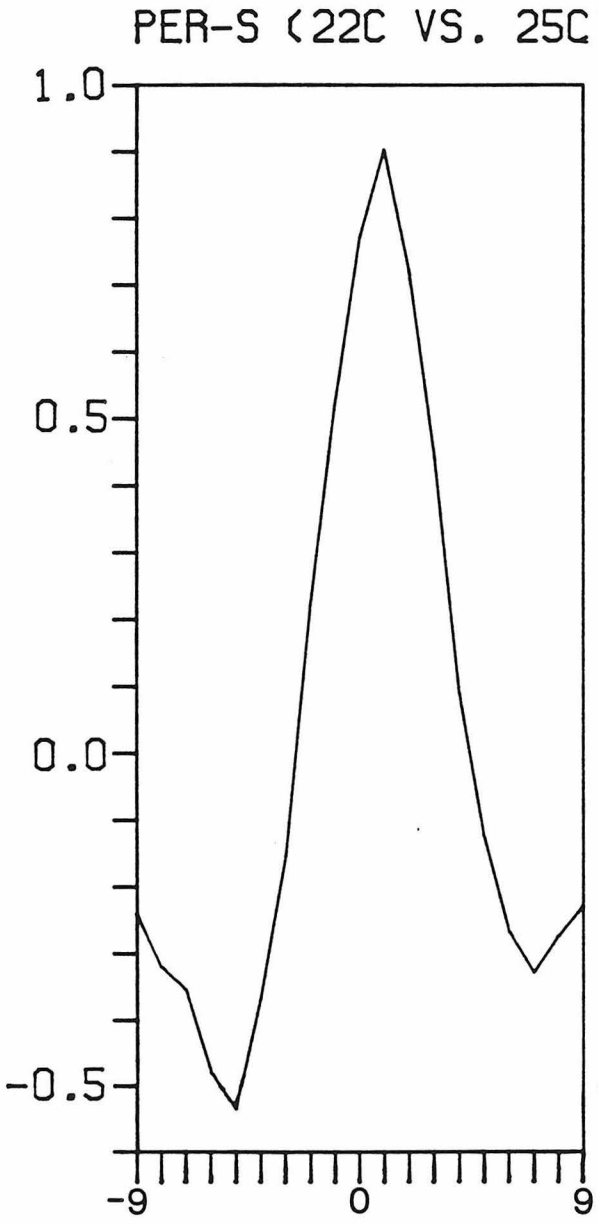


Fig. 3-5 (c)

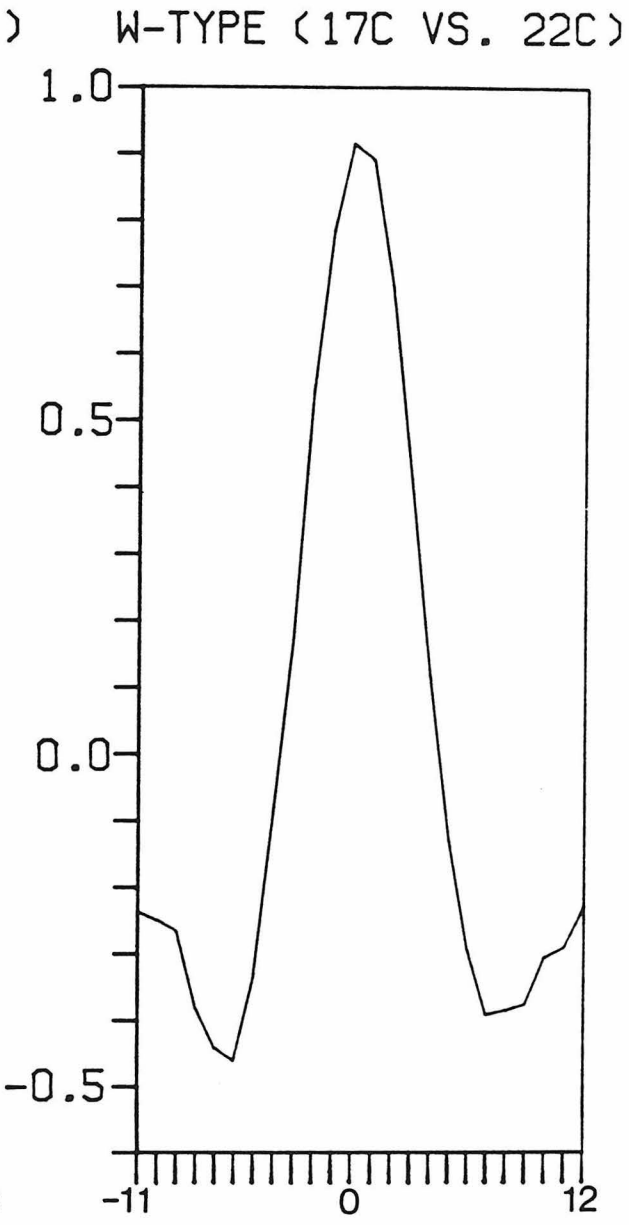


Fig. 3-5 (d)

Figure 3-6 *Illustration on 'type-0' and 'type-1' phase-resetting*. Old phase vs.new phase plots are given for three extreme cases.

Graph A : No response for all phases. (Type-1)

Graph B : Strong response, identically at all phases. (Still type-1)

Graph C : Reset to *same* final phase at all phases. (Type-0)

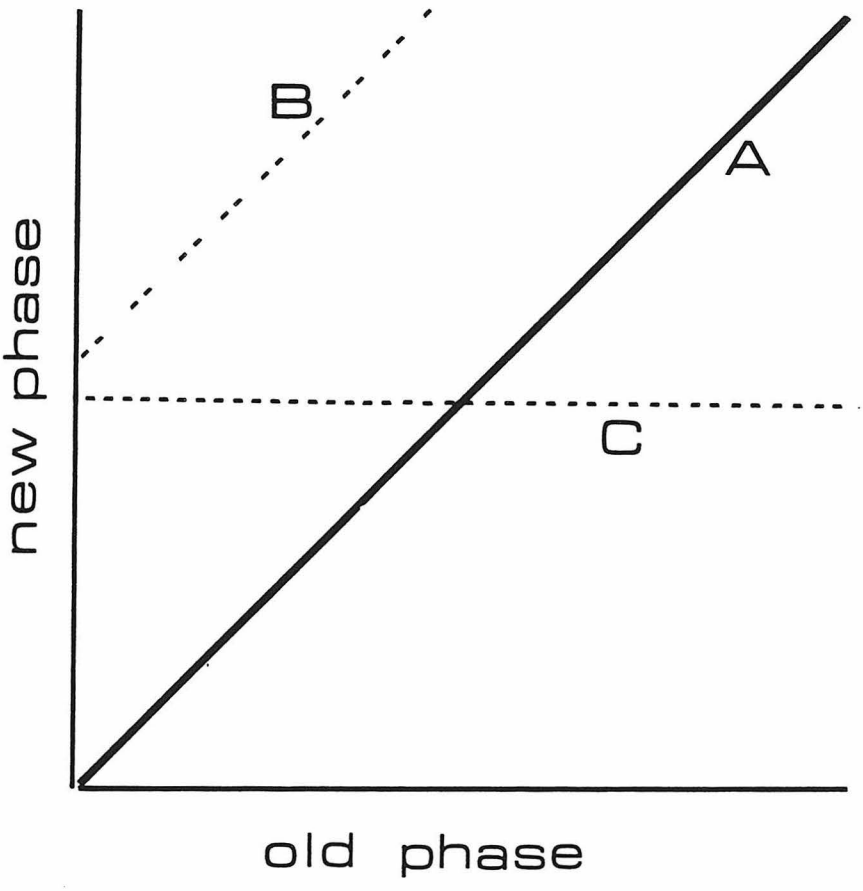
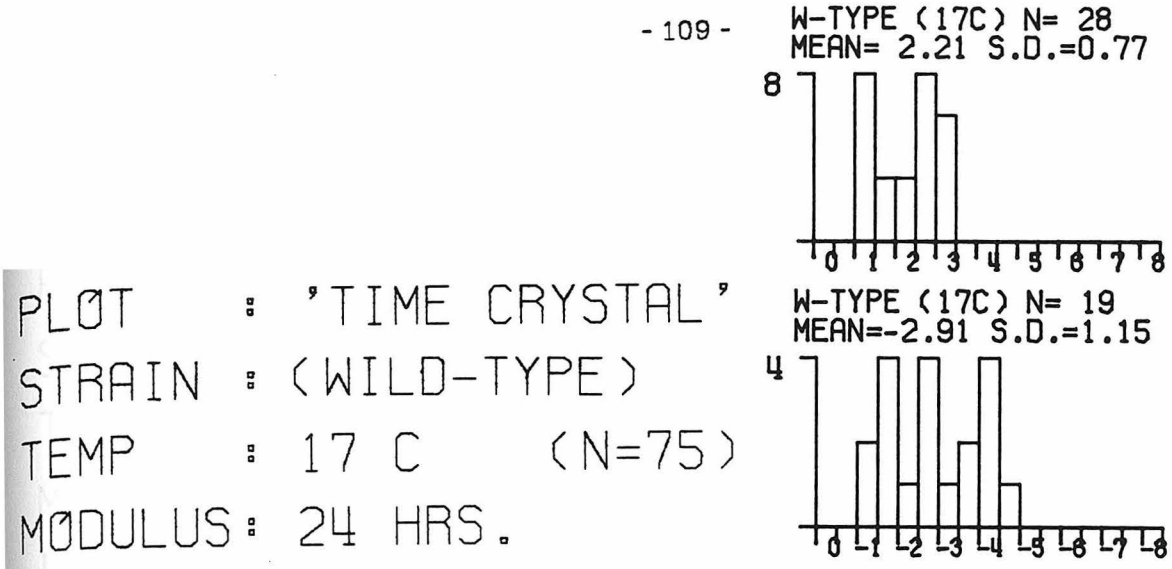

B
A
C
new phase
old phase

Figure 3-7. *Phase Resetting Curves ('Time-Crystals') for six strains of Drosophila at various temperatures.* The 'old' phase is the phase point at which the perturbing light pulse is given. The 'new' phase is simply the 'old' phase plus the phase-shift, where the phase-shift is calculated as described in figs.3-1 and 3-2.

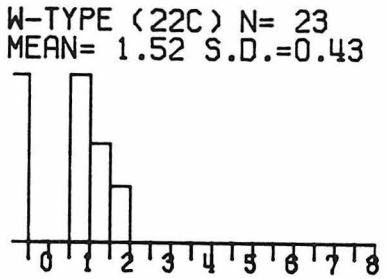
Symbols : *Octogon* = 6-second pulses ; *Diamonds* = 60-second pulses ; *Triangles* = 10-minute pulses ; *Squares* = 80-minute pulses. Each symbol represent the result from one animal and the error bar associated with each symbol represent the uncertainty in the new phase determination as explained in the caption to fig.3-2(a).

Insets: The top histogram represents the distribution of all *phase-advances* of magnitude greater than or equal to one hour that are observed in a strain. The lower histogram represents the distribution of all *phase-delays* of magnitude greater than or equal to one hour observed in the same strain.

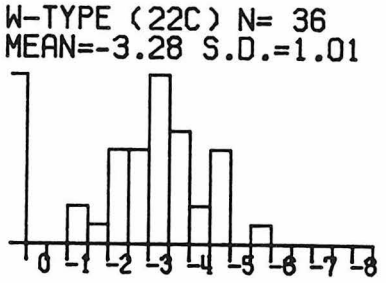
Cases: (*a*) *wild-type* at 17° C. (*b*) *wild-type* at 22° C. (*c*) per^s at 17° C. (*d*) per^s at 22° C. (*e*) per^s at 25° C. (*f*) per^{l1} at 17° C. (*g*) per^{l1} at 25° C. (*h*) per^s / per^{l1} at 22° C. (*i*) Clk^{KO6} at 22° C. (*j*) *And* at 22° C.

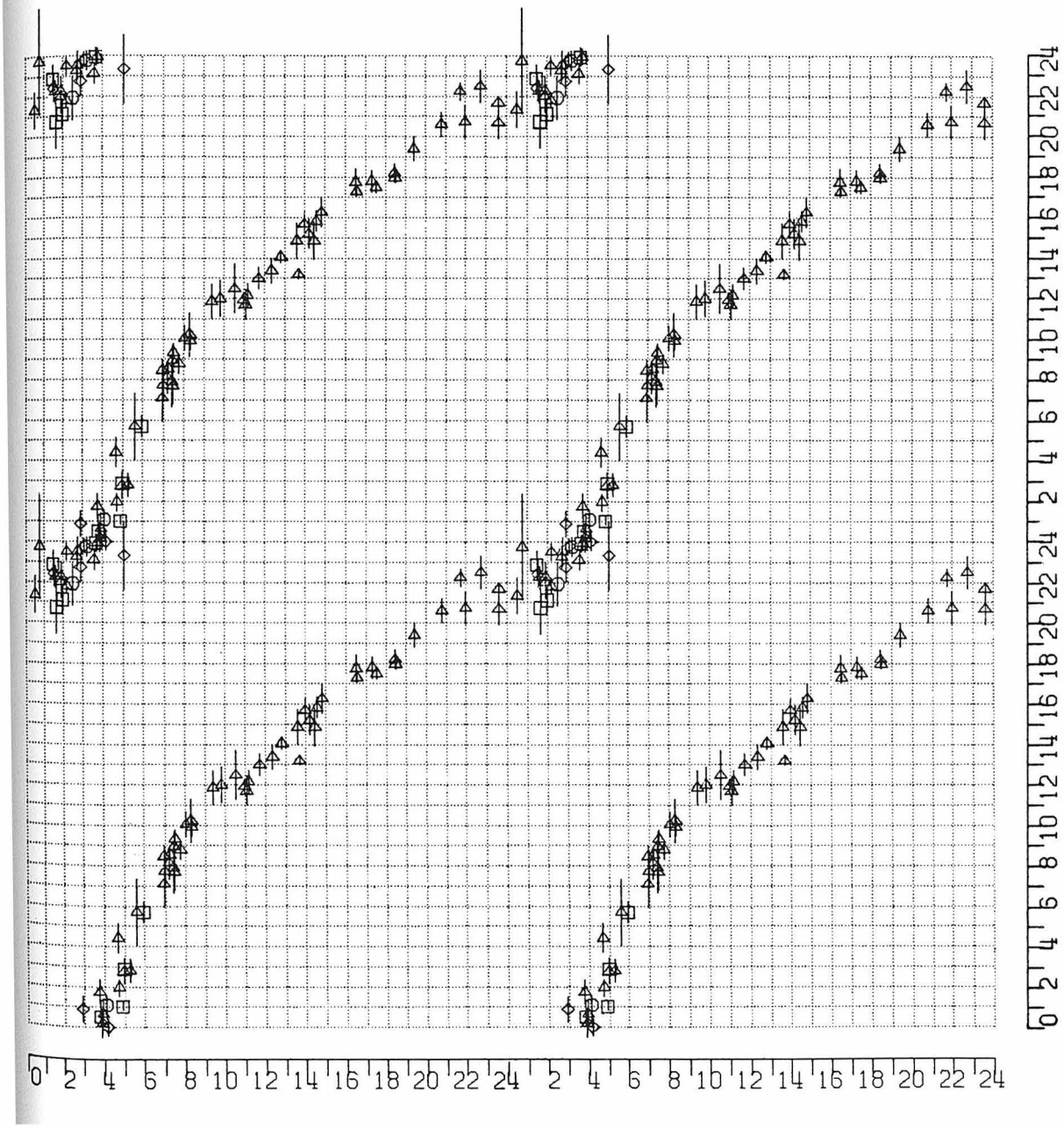

W-TYPE (17C) N= 28
MEAN= 2.21 S.D.=0.77
8
0 1 2 3 4 5 6 7 8
PLØT : 'TIME CRYSTAL'
STRAIN : (WILD-TYPE)
TEMP : 17 C (N=75)
MØDULUS: 24 HRS.
W-TYPE (17C) N= 19
MEAN=-2.91 S.D.=1.15
4
0 -1 -2 -3 -4 -5 -6 -7 -8
0 2 4 6 8 10 12 14 16 18 20 22 24 2 4 6 8 10 12 14 16 18 20 22 24
0 2 4 6 8 10 12 14 16 18 20 22 24
NEW PHASE
ØLD PHASE

A



PLOT : 'TIME CRYSTAL'
STRAIN : (WILD-TYPE)
TEMP : 22 C (N=81)
MODULUS: 24 HRS.

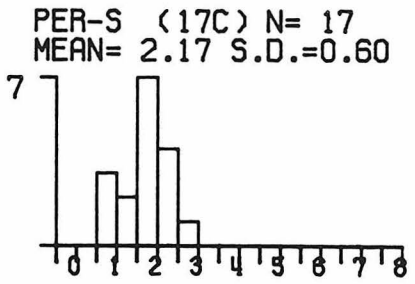




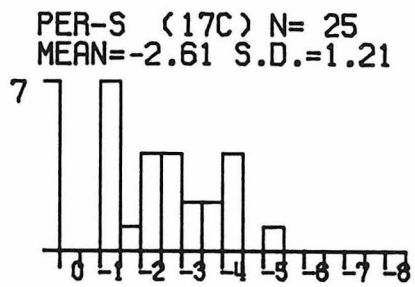
OLD PHASE

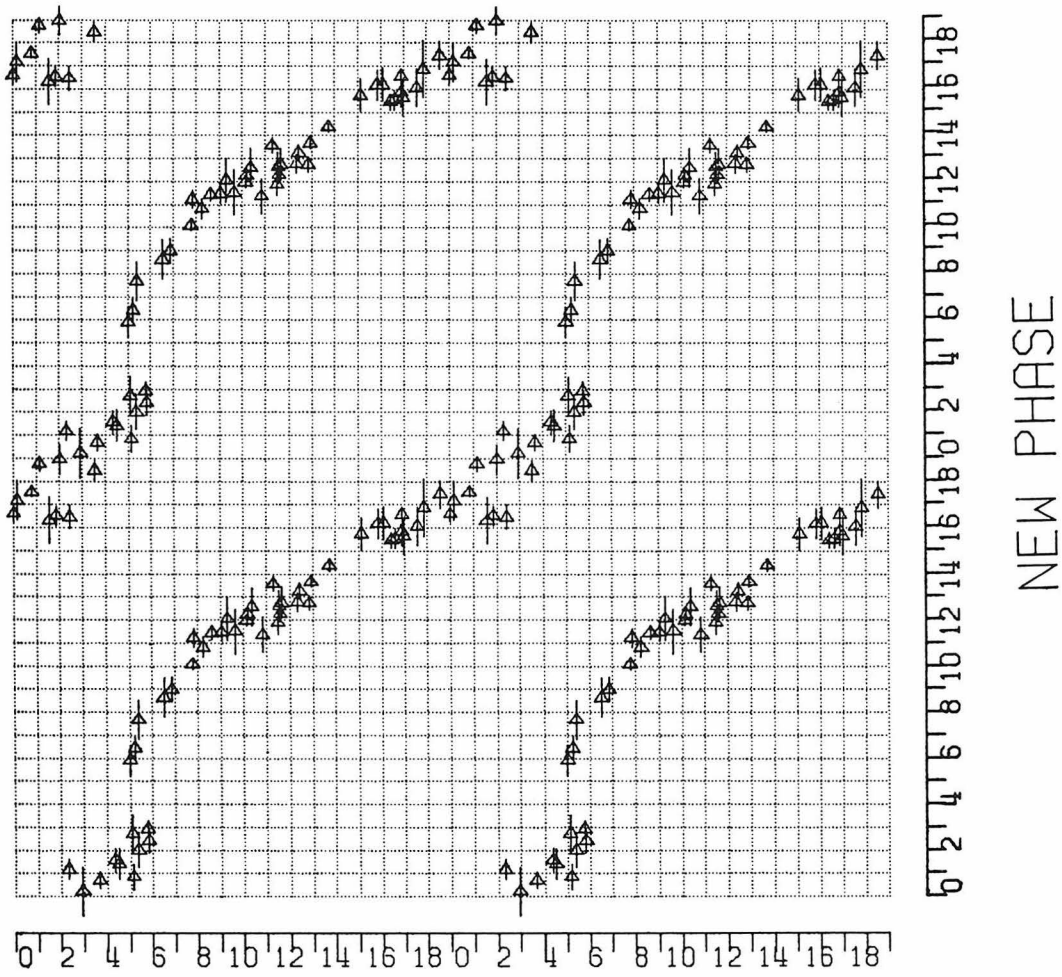
NEW PHASE

B



PLØT : 'TIME CRYSTAL'
STRAIN : PER-S
TEMP : 17 C (N=56)
MØDULUS: 19 HRS.



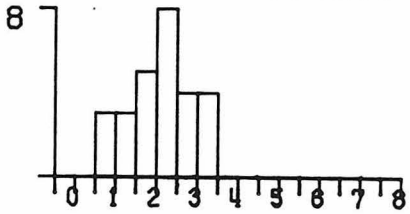


ØLD PHASE

NEW PHASE

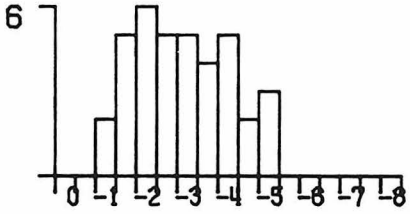
C

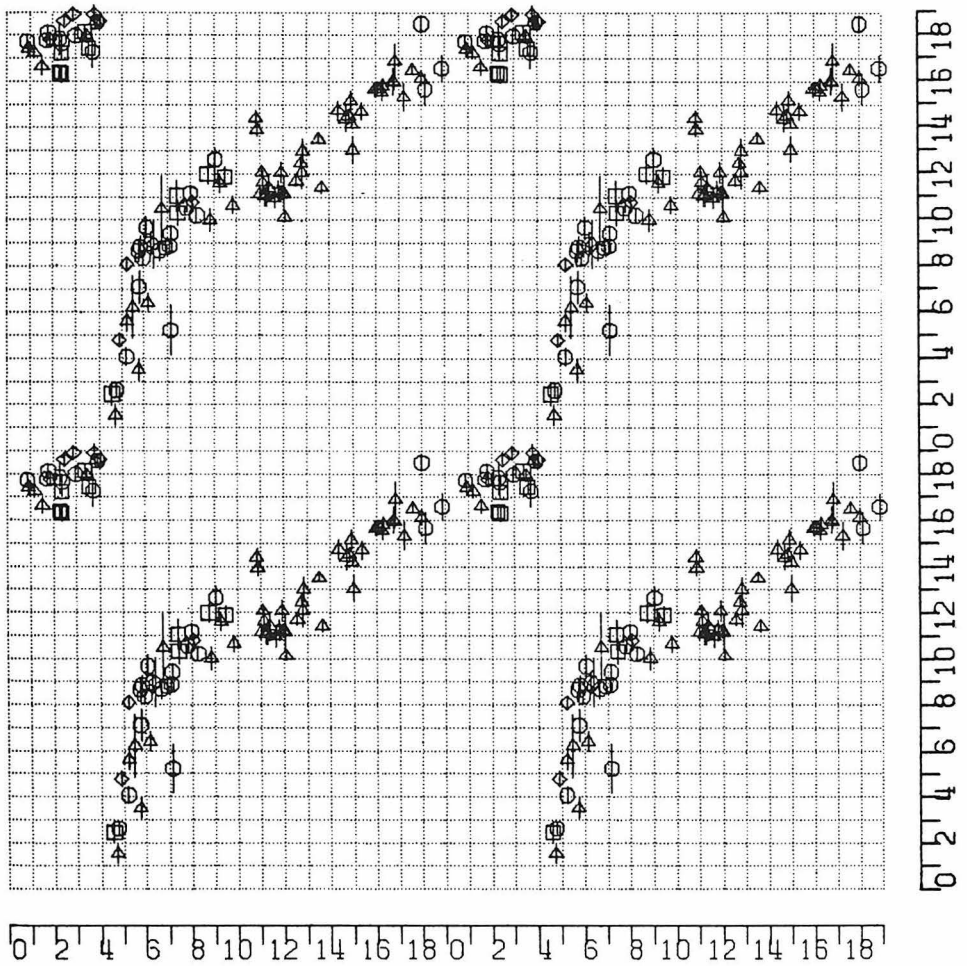
PER-S (22C) N= 27
MEAN= 2.58 S.D.=0.74



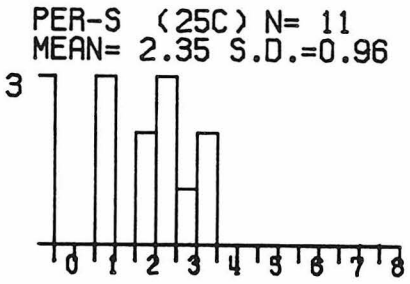
PLOT : 'TIME CRYSTAL'
STRAIN : PER-S
TEMP : 22 C (N=98)
MODULUS: 19 HRS.

PER-S (22C) N= 37
MEAN=-3.13 S.D.=1.15

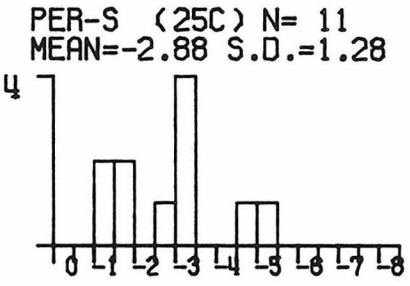


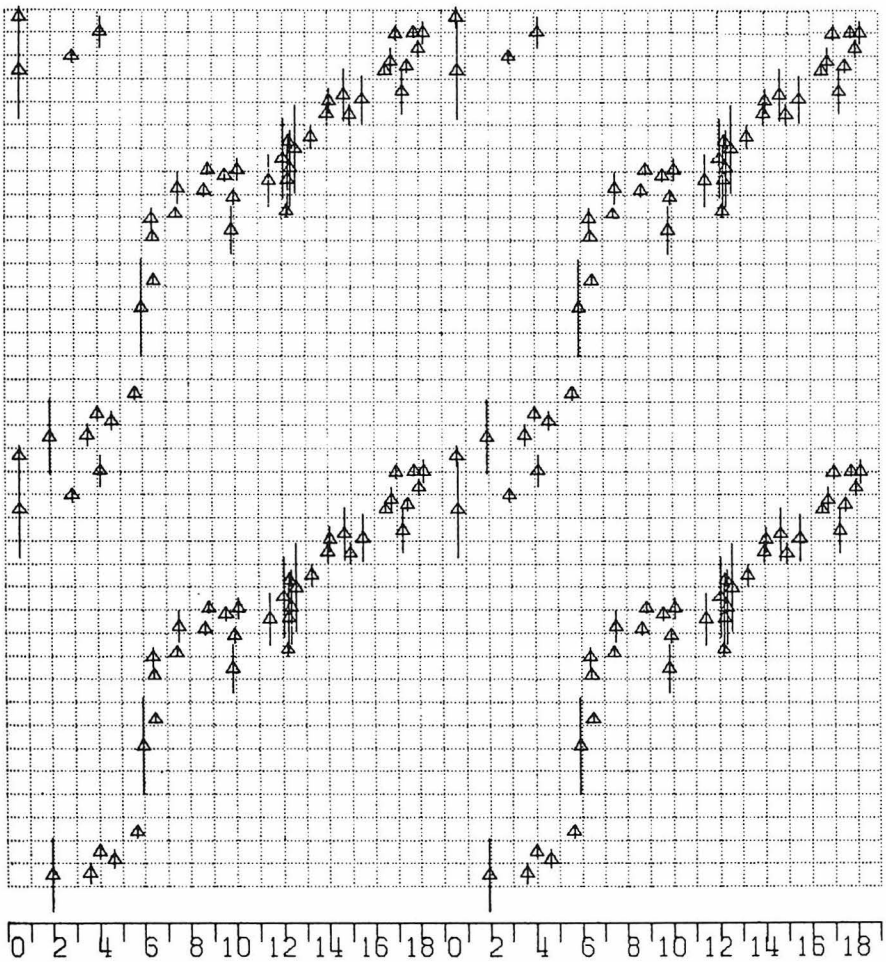


D



PLØT : 'TIME CRYSTAL'
STRAIN : PER-S
TEMP : 25 C (N=42)
MØDULUS: 19 HRS.



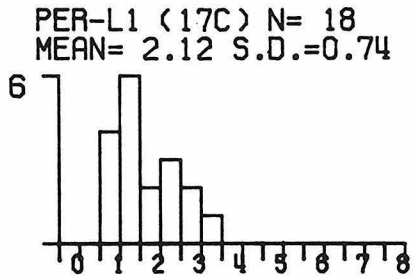


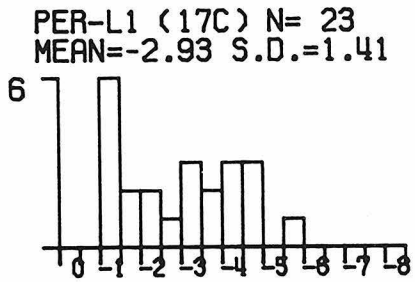
ØLD PHASE

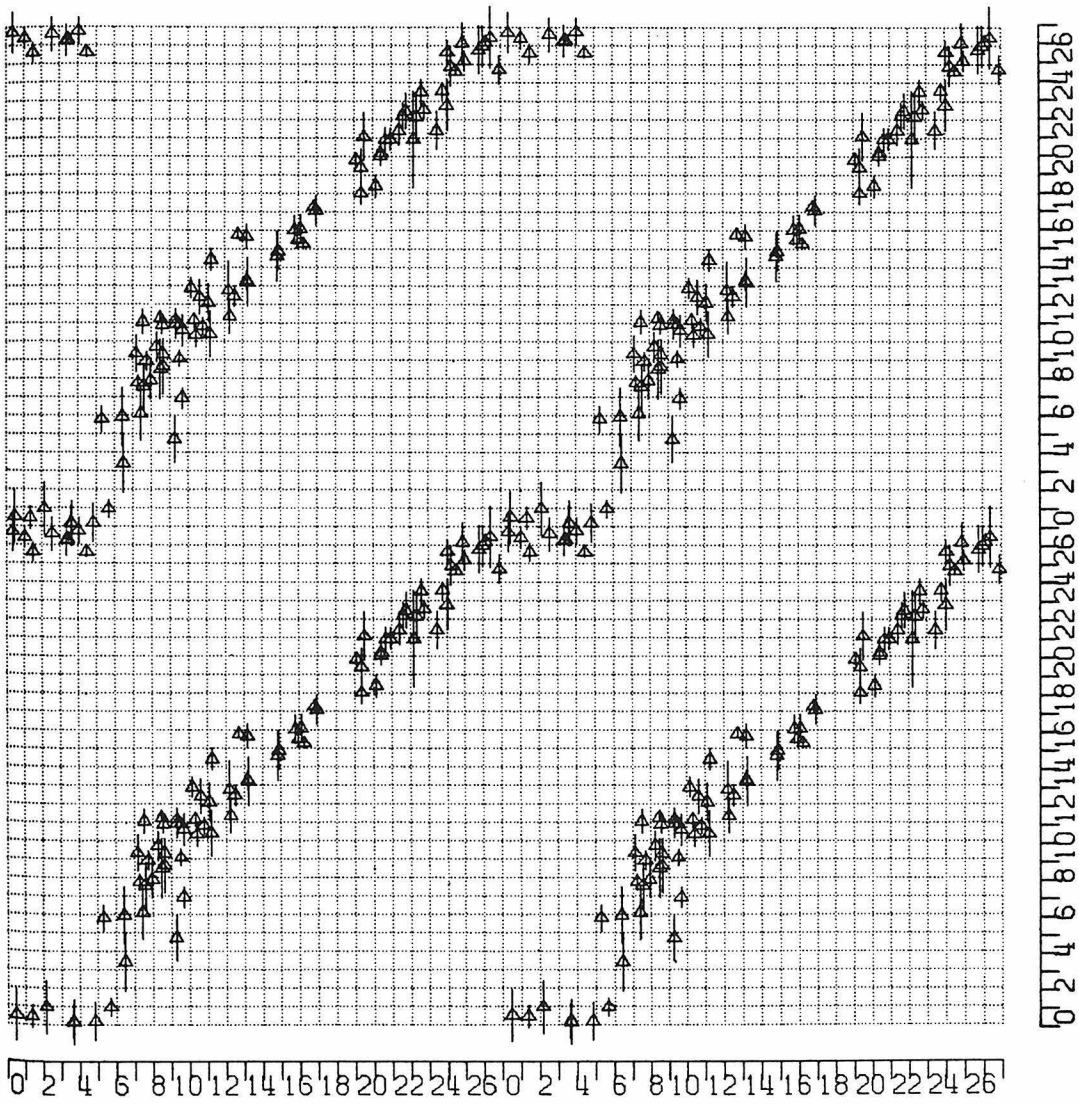
NEW PHASE

E

PLØT : 'TIME CRYSTAL'
STRAIN : PER-L1
TEMP : 17 C (N=90)
MØDULUS: 27 HRS.

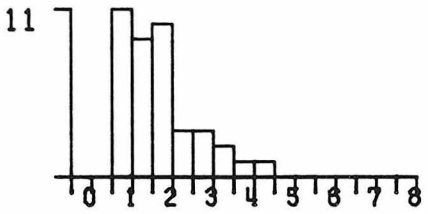






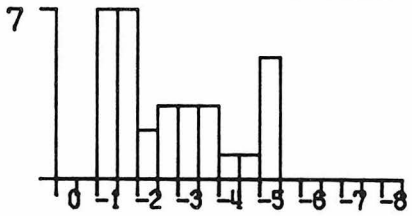
F

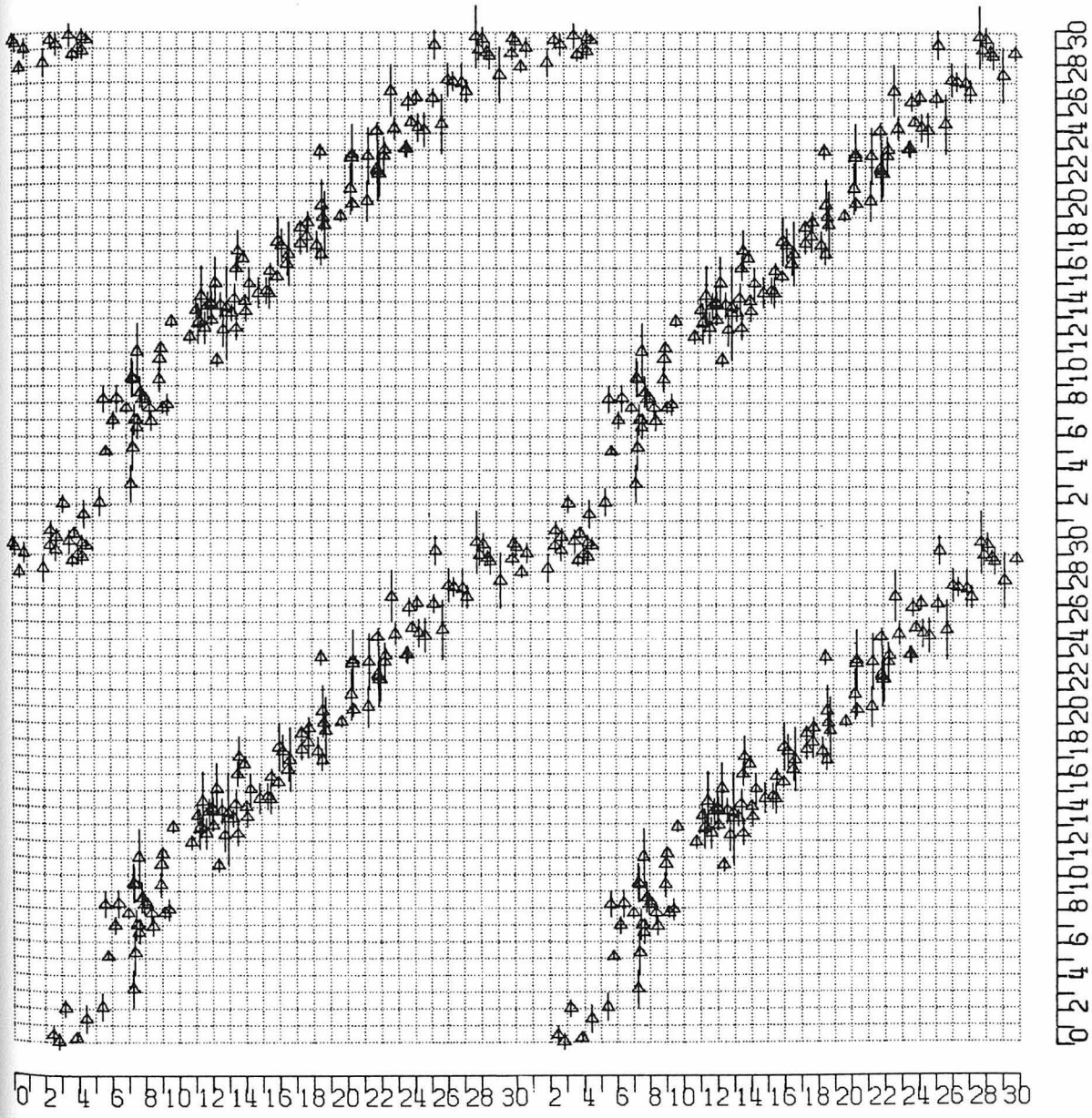
PER-L1 (25C) N= 40
MEAN= 2.10 S.D.=0.90



PLØT : 'TIME CRYSTAL'
STRAIN : PER-L1
TEMP : 25 C (N=125)
MØDULUS: 30 HRS.

PER-L1 (25C) N= 32
MEAN=-2.73 S.D.=1.43



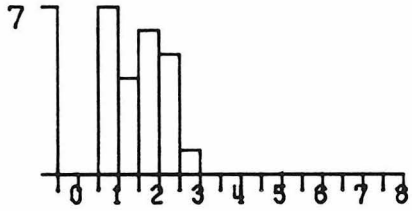


ØLD PHASE

NEW PHASE

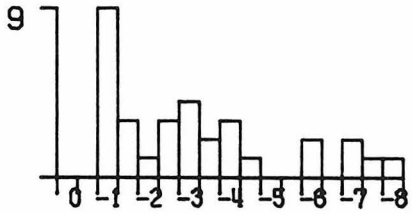
G

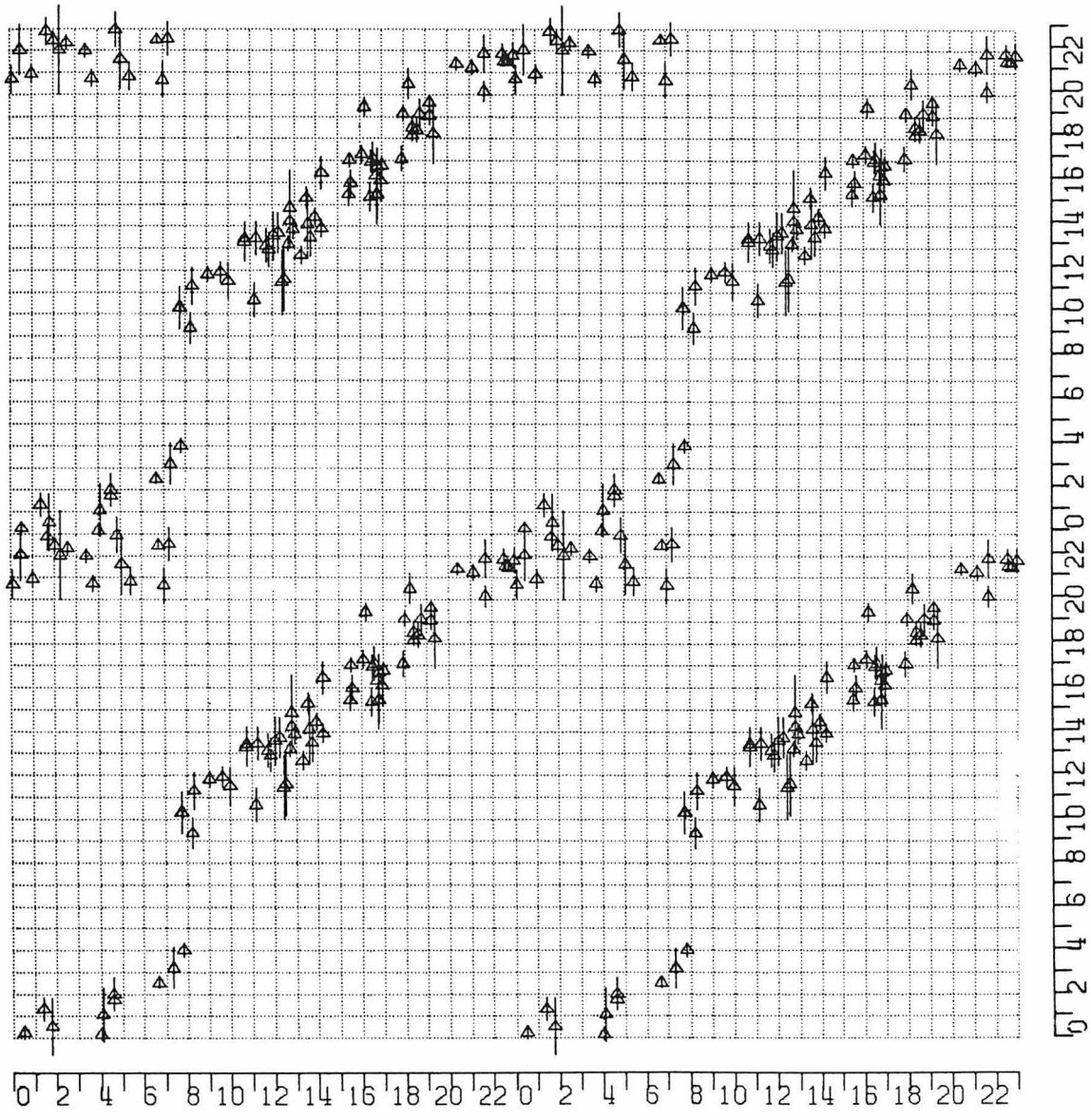
S/L1 (22C) N= 23
MEAN= 1.97 S.D.=0.65



PLØT : 'TIME CRYSTAL'
STRAIN : PER-S/PER-L1
TEMP : 22 C (N=85)
MØDULUS: 23 HRS.

S/L1 (22C) N= 32
MEAN=-3.35 S.D.=2.25



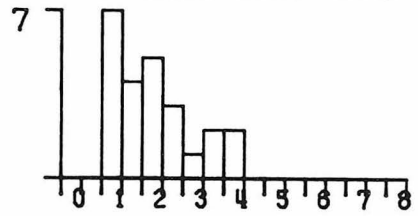


NEW PHASE

ØLD PHASE

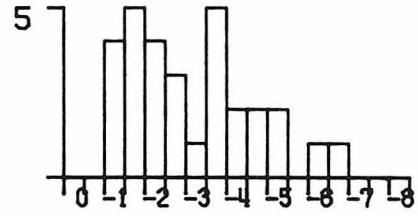
H

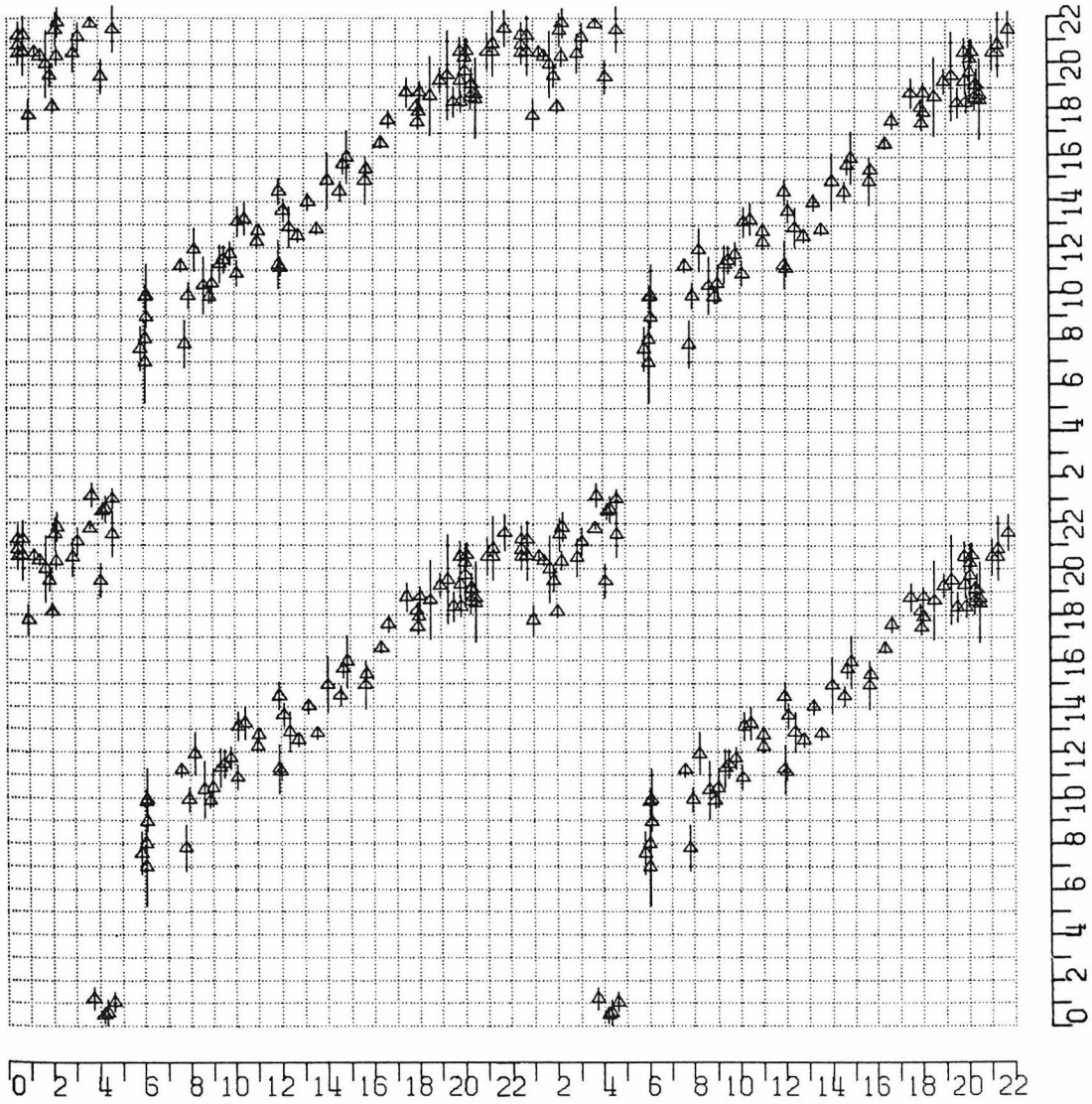
'NØ.6' (22C) N= 24
MEAN= 2.16 S.D.=0.96



PLØT : 'TIME CRYSTAL'
STRAIN : 'NØ.6'
TEMP : 22 C (N=84)
MØDULUS: 22 HRS.

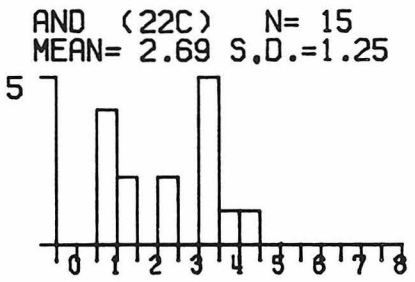
'NØ.6' (22C) N= 30
MEAN=-3.08 S.D.=1.50

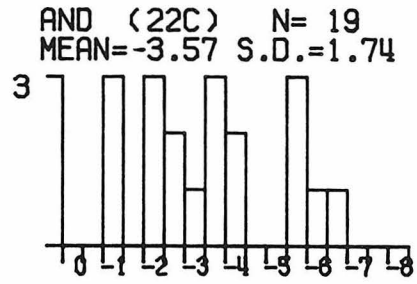




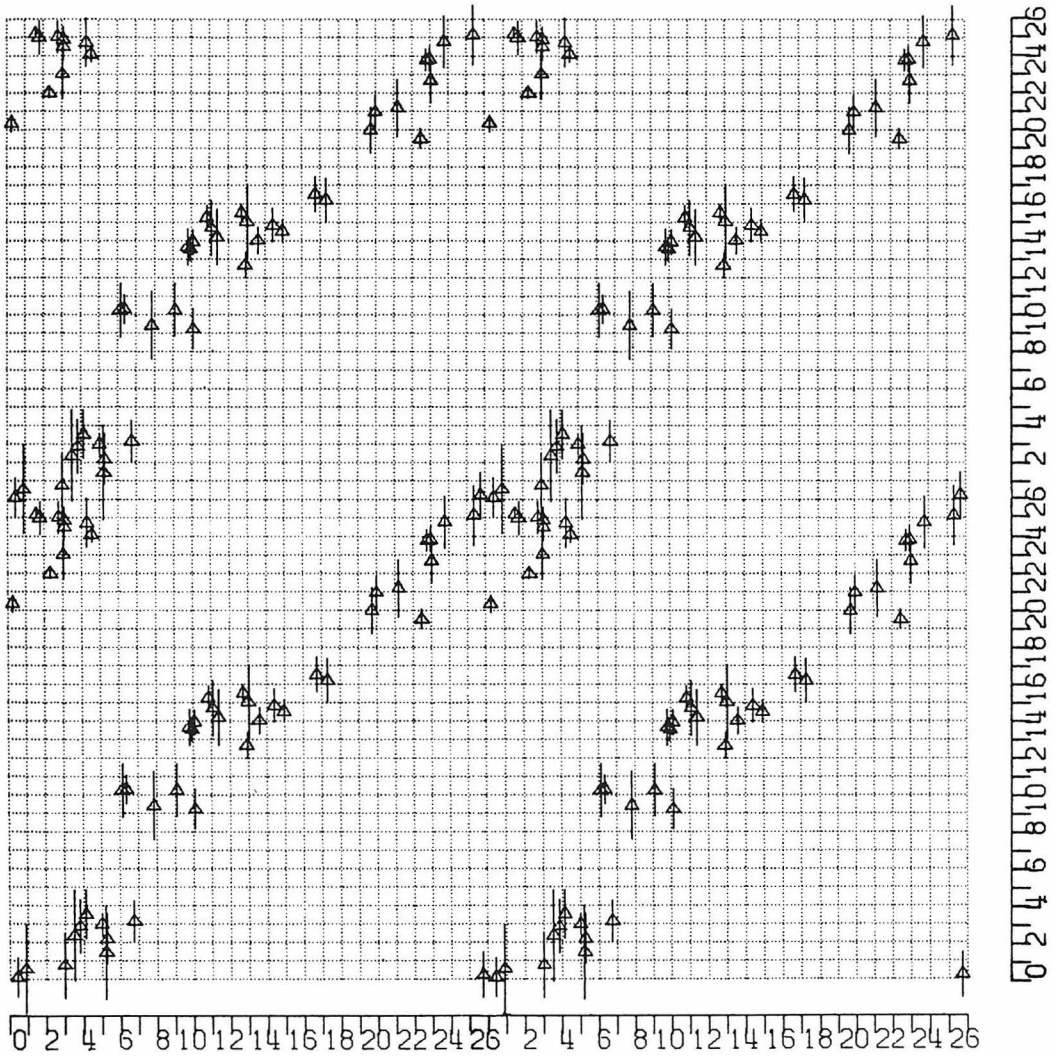
NEW PHASE

ØLD PHASE





PLØT : 'TIME CRYSTAL'
STRAIN : AND
TEMP : 22 C (N=49)
MØDULUS: 26 HRS.



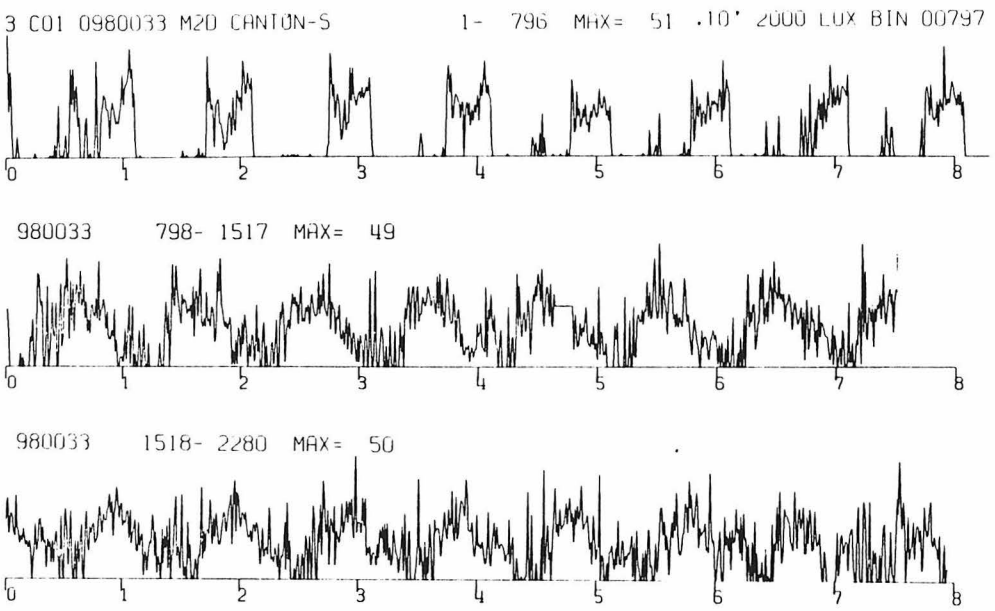
ØLD PHASE

NEW PHASE

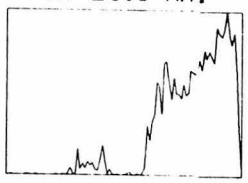
J

Figure 3-8. ***A light pulse induces drastic change in activity profile in a wild-type animal.*** The animal is reared at 22 C. and at about 200 lux, and transferred to constant darkness at 17 C. at the point where the activity record shown begins. Data are collected in 15 mins bin. (a) Normalized counts of activity are plotted against time. Tick marks represent cycles of 24 hours. The records are artificially broken into three segments (DD_1, DD_2, and DD_3), with the first break at the time of the 10-minute light pulse and the second break at 7.5 days after the light pulse. (b) Form estimates of the activity rhythm in DD_1, DD_2,and DD_3 (c) Periodogram analysis of the activity counts is done for the same intervals as in (a) & (b). See ref.A-1 for details on the method of periodogram analysis. (d) Simulated actogram plot of the activity rhythm of the whole run. (See caption to fig.A-2 for details about the actogram simulation)

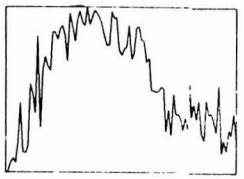
ACTIVITY PLOT



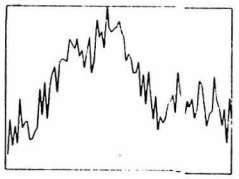
FORM ESTIMATE AT 24.0 HR.



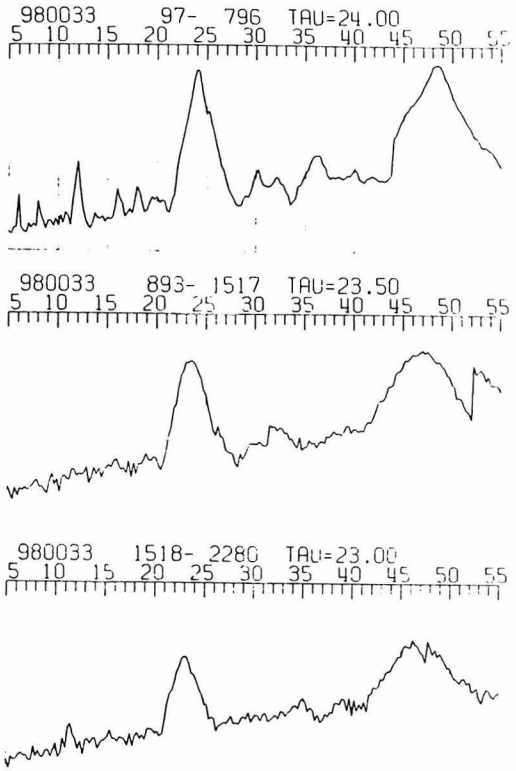
FORM ESTIMATE AT 23.5 HR.



FORM ESTIMATE AT 23.0 HR.



PERIODOGRAM



SIMULATED ACTOGRAM PLOT

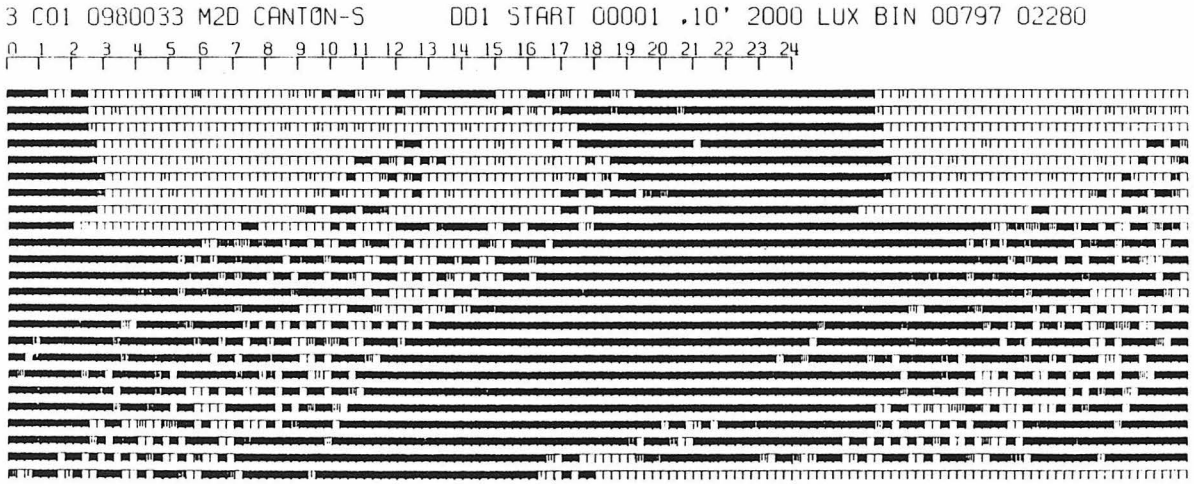


Figure 3-9. ***A light pulse induces change in period length in the activity rhythm of a wild-type animal.*** The animal is reared at 22 C. and at about 200 lux, and transferred to constant darkness at 17 C. at the point where the activity record begins. Data are collected in 15 mins bin. The simulated actogram is plotted at modulus = 24.5 hours. The animal is exposed to a 10-min 2000 lux light pulse at the point indicated by the small rectangle during the 8^{th} cycle. The free-running period of this animal is 24.5 hours for the seven cycles before the light pulse, but it shortens to 23.5 hours after the light pulse. The shortening persists throughout the 16 cycles of post-pulse observation.

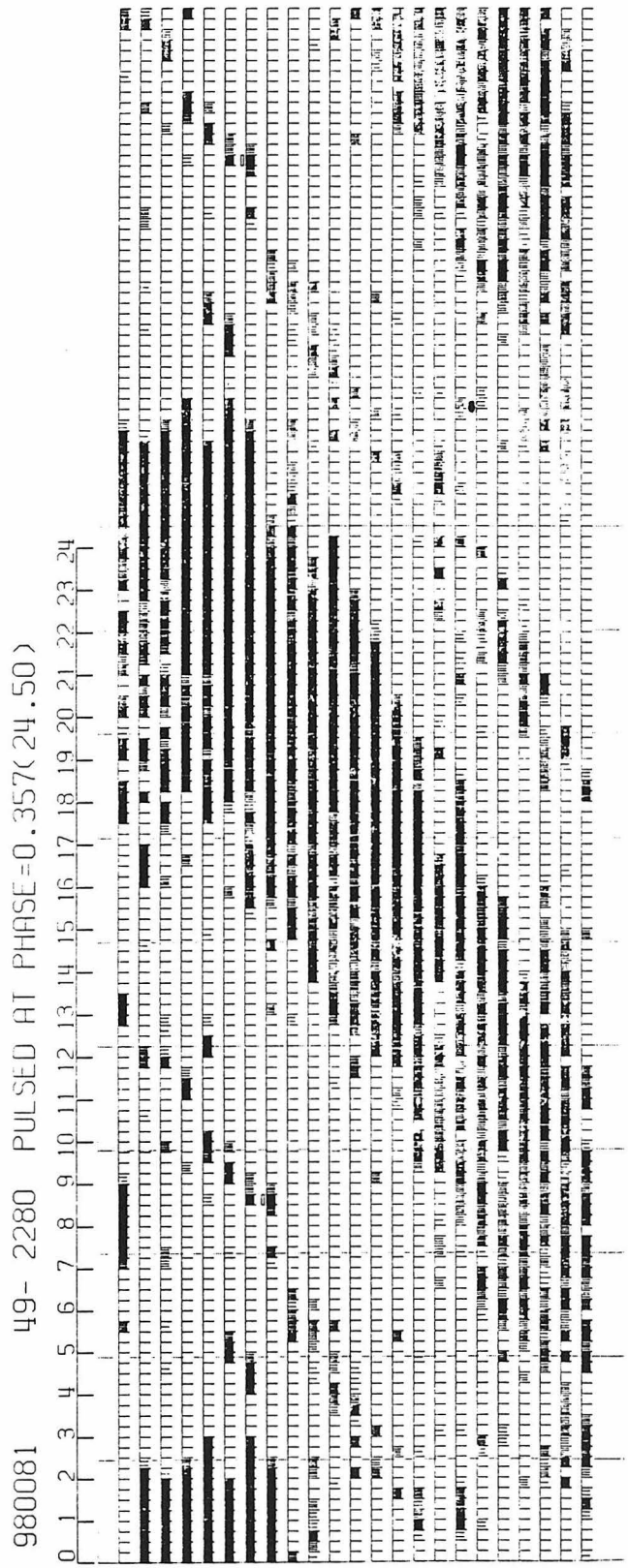

980081 49- 2280 PULSED AT PHASE=0.357(24.50)
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24

Figure 3-10. ***Distribution of light pulse induced period changes (I).*** The histograms show the difference in post-pulse and pre-pulse periods ($\tau_{DD2}-\tau_{DD1}$) for various strains at various temperatures. Periods are estimated by the slopes of the linear regression lines fitted to the pre-pulse and post-pulse activity offsets in the manner described in the caption to fig.3-1.

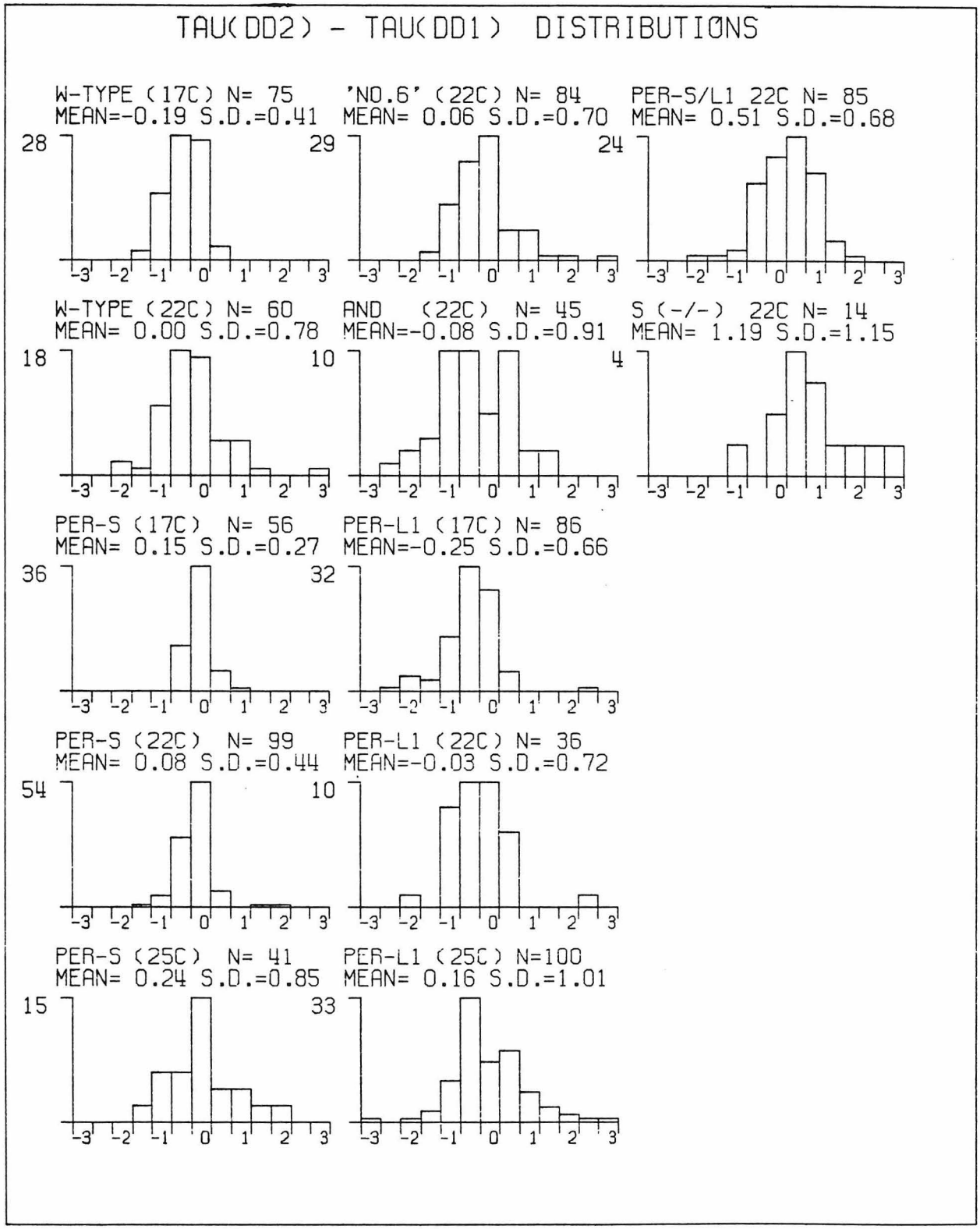

TAU(DD2) - TAU(DD1) DISTRIBUTIONS
W-TYPE (17C) N= 75
MEAN=-0.19 S.D.=0.41
'NO.6' (22C) N= 84
MEAN= 0.06 S.D.=0.70
PER-S/L1 22C N= 85
MEAN= 0.51 S.D.=0.68
W-TYPE (22C) N= 60
MEAN= 0.00 S.D.=0.78
AND (22C) N= 45
MEAN=-0.08 S.D.=0.91
S (-/-) 22C N= 14
MEAN= 1.19 S.D.=1.15
PER-S (17C) N= 56
MEAN= 0.15 S.D.=0.27
PER-L1 (17C) N= 86
MEAN=-0.25 S.D.=0.66
PER-S (22C) N= 99
MEAN= 0.08 S.D.=0.44
PER-L1 (22C) N= 36
MEAN=-0.03 S.D.=0.72
PER-S (25C) N= 41
MEAN= 0.24 S.D.=0.85
PER-L1 (25C) N=100
MEAN= 0.16 S.D.=1.01

Figure 3-11. *Distribution of light pulse induced period changes (II).* The histograms show distributions of $\tau_{DD2}-\tau_{DD1}$ as in fig.3-9, except that the results are decomposed into two categories. The upper histogram shows period changes for animals with phase-advances and the lower shows period changes for those with phase-delays. No significant difference is observed between the two groups in all six cases.

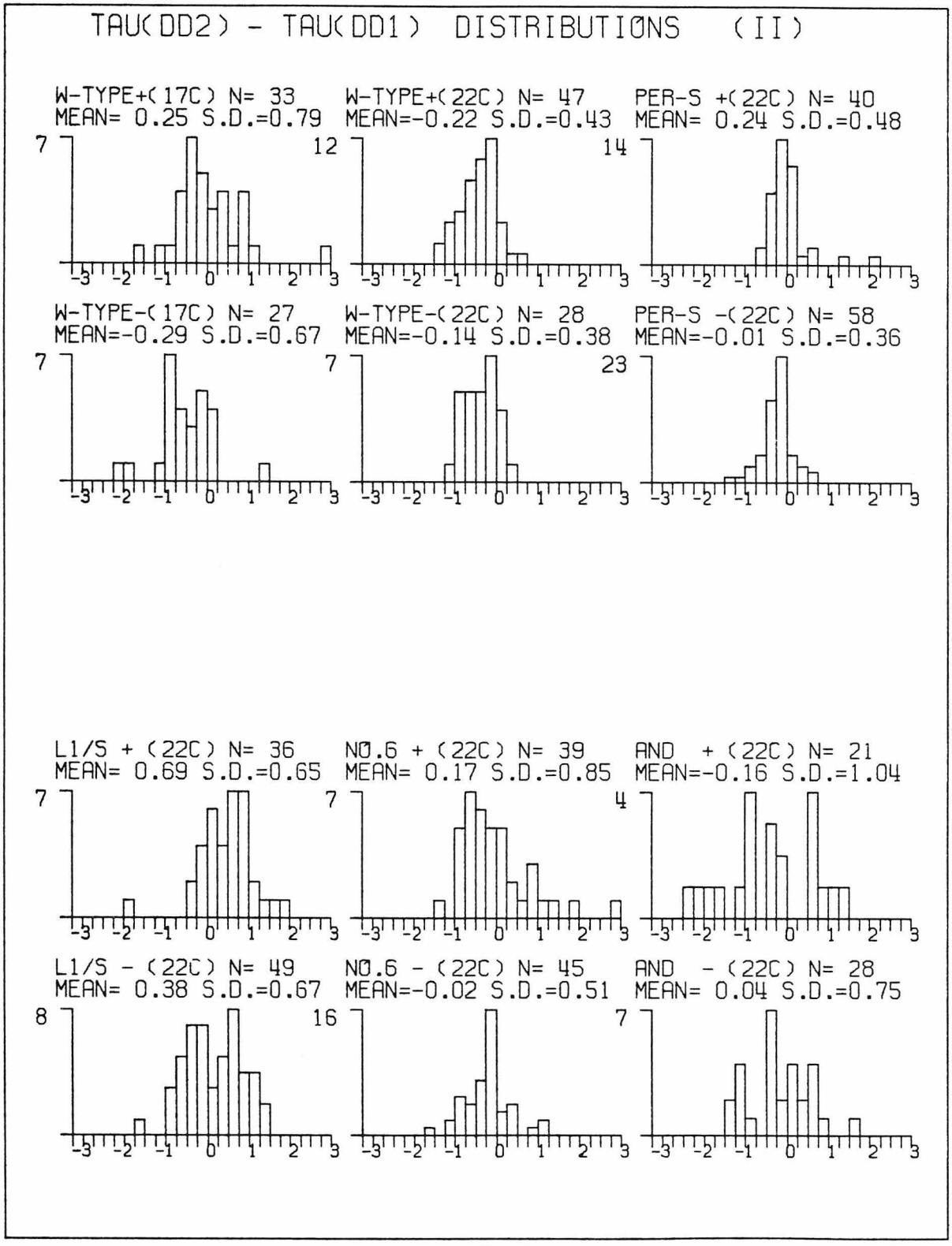
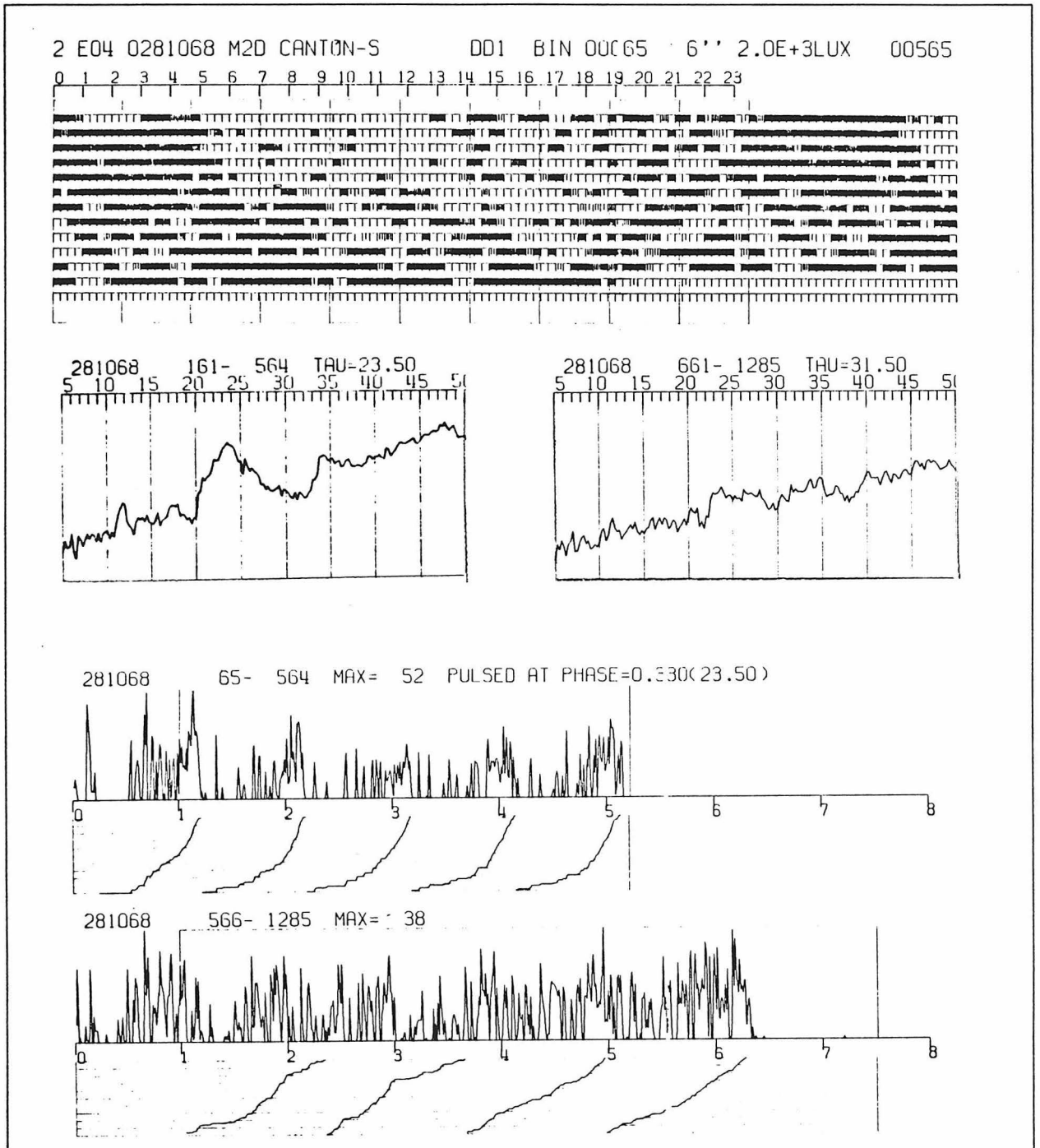

TAU(DD2) - TAU(DD1) DISTRIBUTIONS (II)
W-TYPE+(17C) N= 33
MEAN= 0.25 S.D.=0.79
W-TYPE+(22C) N= 47
MEAN=-0.22 S.D.=0.43
PER-S +(22C) N= 40
MEAN= 0.24 S.D.=0.48
W-TYPE-(17C) N= 27
MEAN=-0.29 S.D.=0.67
W-TYPE-(22C) N= 28
MEAN=-0.14 S.D.=0.38
PER-S -(22C) N= 58
MEAN=-0.01 S.D.=0.36
L1/S + (22C) N= 36
MEAN= 0.69 S.D.=0.65
NØ.6 + (22C) N= 39
MEAN= 0.17 S.D.=0.85
AND + (22C) N= 21
MEAN=-0.16 S.D.=1.04
L1/S - (22C) N= 49
MEAN= 0.38 S.D.=0.67
NØ.6 - (22C) N= 45
MEAN=-0.02 S.D.=0.51
AND - (22C) N= 28
MEAN= 0.04 S.D.=0.75

Figure 3-12. ***A light pulse induces arrhythmicity in the locomotor activity of a wild-type animal*** Actogram, periodogram, and activity plots of an animal whose activity pattern starts out 'spiky' in DD_1 but is nevertheless rhythmic as shown by periodogram on the left. A 10-minute 2000 lux light pulse given at the subjective night of the sixth cycle disrupts the rhythm rather drastically, as shown in the activity plot and the periodogram for the DD_2 segment of the record.


2 E04 0281068 M2D CANTON-S DD1 BIN 00C65 6'' 2.0E+3LUX 00565
281068 161- 564 TAU=23.50
281068 661- 1285 TAU=31.50
281068 65- 564 MAX= 52 PULSED AT PHASE=0.330(23.50)
281068 566- 1285 MAX= 38

Chapter 4

The Effect of Tonic Illumination on Wild-type and Mutant *Drosophila* Clocks

Summary

(1) The responses of one wild-type and five mutant circadian clocks to tonic dim light of the range 5 X 10^{-4} lux to 50 lux are studied (figs.4-3, 4-6). Six classes of responses are described (fig.4-2).

(2) In each of the strains studied, a critical 'window' of light intensity is found within which a variety of unstable clock features are observed (figs.4-2, 4-3, 4-4, 4-5). Such features include : greatly increased slopes of period vs. light intensity curves; drastically lengthened (up to 50 hours in period) rhythm with increased cycle-to-cycle variability; spontaneous phase-shifts; apparent sub-circadian components; and, loss of rhythmicity. The light intensity at which this critical window occurs in each of the mutants is 5 to 10 times lower than that in the wild-type. This is the most substantial difference in a particular circadian physiological variable between wild type and the clock mutants in *D. melanogaster*.

(3) The dim light response curves in this chapter, together with the phase response curves (PRC) described in chapter 3, are found to be incompatible with a particular model of the Velocity Response Curve (VRC) concept to inter-relate the tonic to phasic effects of light (section 4.3).

(4) The dim light responses of an ERG-defective mutant (*norpA*) are found to be qualitatively, but not quantitatively, similar to those of the wild-type (fig.4-7). The dim light responses of an eyeless and ocelli-less mutant (*sine oculus*) indicate that both period changes and arrhythmicity can be elicited by light in the absence of the compound eyes and ocelli but the sharp dependence of the occurrences of these phenomena on light intensity is lost in this mutant(fig.4-8).

(5) Possible relationships between the effects of dim light and the effects of clock mutations in their actions on period changes are explored. Arguments are presented to suggest that none of the four mutations -- *And* , Clk^{KO6}, per^{s}, and per^{ll} -- cause changes of period by an action similar to light.

4.1 Introduction

In chapter 2, we sought to gain insight into the action of the clock mutations in *Drosophila* by studying their effects on crucial clock properties such as temperature compensation and the stability of periods and by studying the interactions among the various mutant alleles. In chapter 3, we approached the same question of how do these mutant genes act on the clock period through observations of the dynamics of the wild-type and mutant circadian systems in their responses to phasic light perturbation. Yet another approach to the understanding of the mechanism of action of these mutant genes is to study the interaction between effects induced on the clock (particularly on its period) by such mutations and those that are affected by some known external agents. One of the most generally known agents that can affect the behavior of circadian clocks is light. However, the actions of light on circadian clocks are numerous; viz. it can start a circadian rhythm, it can stop it (cause arrhythmia), it can phase shift it, entrain it, and, also, it can change its period. Each of these is an individually a very well known phenomenon in the circadian literature (ref.4-1); yet, except for some mathematical modeling approaches (ref.4-2, ref.4-3) , attempts to inter-relate these phenomena are very few, and a definitive demonstration of such an inter-relation is lacking.

In this chapter, we describe the behavior of one wild type and five mutant circadian clocks under tonic light levels of various intensities, again using the activity rhythm of the individual adult fly as an assay. We focus mainly on the following questions : (1) Are there hints on whether any of the above mentioned actions of light on circadian systems *are* or *are not* related? (2) Are there any hints that a common mechanism underlies the period changes due to ambient light levels and the period changes due to the various mutations under study? (3) Whether the effects of tonic light on the behavior of the *D. melanogaster* circadian clock are mediated through the eyes, the ocelli, or some extraretinal photo-

receptors in the brain ?

4.2 Behavior of activity rhythm in dim light

4.2.1 Classification of clock responses into six classes

In this section, we describe the responses of the clocks from one wild-type and four mutant strains of flies to tonic dim light. The protocol used and relevant terminologies are described in fig.4-1 and its caption. Of the 181 animals studied which survived the first dimlight period (LL_1) for five or more cycles, we find it possible to define six classes of changes in the activity rhythm in LL_1, as follows :

(1) Animals that continue to maintain stable rhythmic behavior throughout LL_1. This class includes 69% (124 of 181) the animals and includes cases where there is slight shortening in period (up tp 1.5 hours), to no significant changes in periods, to where there is period lengthening of up to more than 10 hours. An example is given in fig.4- 2a. Animals that fall into this class are represented by triangles in fig.4-3.

(2) Animals that are apparently rhythmic (as shown in periodogram) during the whole LL_1 period; but their activity records are quite a bit 'noisier' in LL_1 than the corresponding DD_1 segment, and the activity peak has a trend to spread out more and more. Seven of the 181 animals fall under this class and are represented by diamonds in fig. 4-3 . An example is given in fig. 4-2b.

(3) Animals that are rhythmic for the first 3-4 cycles in LL_1 and then become arrhythmic afterwards. The six animals that fall into this class are represented by five-pointed stars at the level of the rhythmic LL_1 portion in figure 3. An example is given in fig.4-2c. It could be the case that animals in class (2) and in class (3) represent basically the same behavior and that class(2) animals just take longer to become arrhythmic. Since, for technical reasons, our LL_1 observations are mostly limited to 10 cycles or less, it is not possible for us to further investigate this possibility.

(4) Animals that become arrhythmic almost immediately in LL_1. The 35 animals

that fall under this class are represented by asterisks in fig. 4-3 . An example is given in fig. 4-2d.

(5) Animals whose LL_1 activity rhythm clearly show two major peaks, separated by a few hours, in the periodogram . In the records of the four animals that show such a twin-peak, there are no evidence of discrete rhythms of two periods running co-existently with one another. Indeed, fig. 4-2e shows a per^s animal whose twin-peak in the LL_1 periodogram is caused by a single phase shift at 130 hours into the LL_1 situation. On the other hand , for the *And* animal in fig. 4-2f, the twin-peak seems to be a result of cycle to cycle variation in period lengths during the LL_1 duration. Since a phase-shift is equivalent to period instability of *one* single cycle, we can categorize animals in this class as having occasional or constant cycle-to-cycle period-length instability. That such instabilities are internally rather than environmentally induced is suggested by the fact that, in our experimental set-up, animals could be as close as 1 cm. to one another[1] and any changes in the environment (like temperature or illumination level) can hardly affect a single animal without also influencing adjacent ones. Animals in this class are represented by crosses in fig. 4-3.

(6) Animals that seem to show a sub-circadian rhythm. In five of the animals studied, the activity rhythms in LL_1 are dispersed into bursty occurrences, usually on a noisy background. The interpretation of the periodograms of such segments is ambiguous (see fig.4-2g) because a sub-circadian 'harmonic', which normally either exists in minor form or does not exist at all, becomes in these cases a major component in the range of 9 to 13 hours. Because of this ambiguity, animals in this class are not represented in the summary diagram in fig. 4-3. However, the following three points deserve to be mentioned: First, such behavior is so far observed only in the two short period mutants. Secondly, all observed cases occur at light intensities that

1. The activity tubes are placed in grooves in solid black plexiglass plates and the animals are therefore visually isolated from one another.

are high enough for most other animals to become arrhythmic. (See next section for discussion on threshold of arrhythmia.) Thirdly, such sub-circadian behavior is seen much more frequently in our pilot study for this project, in which animals were directly transferred from LL to LL_1, without dark adaptation in the DD_1 segment.[2] This is reminiscent of the situation of dim-light induction of split rhythms in rodents (ref.4-4) , where the direction of the light level shift is crucial in inducing splitting or not.

4.2.2 Diverse responses in the various strains.

A summary of the behavior of the rhythms of individual animals of the one wild-type and four mutant strains of flies classified in the last section under various LL_1 light levels is shown in fig. 4-3. Since the distribution of DD_1 free-running periods are widely varied between strains-- and, for the cases of per^{l1} and *And* , even among individuals of the same strain (ref. fig.2-1 in this thesis) -- we find it most convenient to present the results as the difference between the free-running periods of an individual in LL_1 and the corresponding period in DD_1 (the first dark period), plotted against the intensity of the light level in LL_1.

In wild-type flies, we observe that there seems to be no significant response in period changes for light intensity of less than .05 lux, but in between about .05 to 5 lux , the period of the rhythm steadily increases- in fact, seemingly in a linear fashion - with the logarithm of the light intensity. Yet between 5 to 10 lux or so is a region where (a) some flies become arrhythmic (class(4)) , (b) some stay rhythmic, but only transiently so (class (3)) , and (c) those that stay rhythmic (classes (1) & (2))do so with periods that vary greatly between individuals. Furthermore, this 'critical region' exists in all but one of the strains examined in this study.

In per^s, per^{l1} & *And* , the threshold light intensity at which this 'critical'

2. Because of this difference in protocol and also of the lack of precise light level measurement in the pilot study, these interesting cases are not included in this chapter.

phenomenon occurs is lowered an order of magnitude to the window from 0.5 to 1.0 lux. Again, arrhythmia occurs toward the upper edge of the window. In all four of the mutants, a linear region similar to the wide-type behavior between .1 and 5 lux seems to be either missing or drastically reduced. We note here that the change in period of per^s and per^{l1} flies to light level dimmer than about 0.1 lux are reciprocal. This point will be further addressed in the discussion. We also note that both *And* and Clk^{KO6} do not show significant period changes at light intensity dimmer than .05 lux, and are, in this respect, similar to wild-type animals. It is of interest that , for Clk^{KO6}, no animals have been observed that show period lengthening of more than 4 hours. However, we do not know whether this is due to the sample size at the relevant light interval not being large enough (See next section).

4.2.3 A 'Critical' window of light intensity

Results presented in the previous section demonstrate the existence of a window of light intensity within which a wide range of behavior of the clock – from almost no period change to lengthening of up to 50% of DD values and even arrhythmia in some cases --is seen. The question we raise is whether such diverse behavior is due to some inherent instability of the clock under a certain level of ambient illumination or it is due to some kind of inter-animal differences (e.g. in clock photo-sensitivity) affecting an inherently clean-cut process. Simply put, is this a region of steep slopes or a region of instability? Two approaches to answer this question are presented in the following.

Fig. 4-4 shows the periodograms of two per^s animals which were exposed to light through the following sequence - - DD_1, LL_1, DD_2 , LL_2; with the same light intensity in LL_1 as in LL_2. The intensity used for one animal is 0.17 lux, which is near the bottom of the 'critical' window; and the intensity used for the other is 0.75 lux, which is just at the top of it. The figure shows that the rhythm of the animal at

the low end of the window gets lengthened by 4 hours in LL_1, and by a similar amount in LL_2, although the periodogram in LL_2 is a bit more dispersed. On the other hand, the other animal becomes arrhythmic at LL_1, and , after restoring its rhythm in DD_2, becomes arrhythmic again in LL_2. These results, plus similar results from two other pairs of animals treated in an identical way, suggest that the behavior of an individual clock in a given light level within this critical window is consistent: there is no gross instability.

The second approach used is to bring individual animals through many closely spaced levels of light intensity, following the protocol of fig.1, and try to see whether there is any 'abnormal' behavior within a single animal. The results, shown in fig. 4-5 , indicate that, although there are inter-animal differences in responses (especially in wild-type and in per^{l1}), yet within the same animal, the response is clean-cut and follows the general pattern of the group summary shown before in fig. 4-3. Each animal seems to have its 'critical' region, in which an extra bit of light makes a drastic change in period or even causes arrhythmia. That is to say, there is indeed a region of steep slope for each animal.

4.3 Relationship of tonic to phasic effects of light

In section 4.1, we mentioned that there had been some mathematical modeling work in the literature which attempted to interrelate the different effects of light on circadian systems. One such attempt is a theoretical construct called 'Velocity Response Curve' (VRC) proposed by Swade in 1969 (ref.4-3) in an effort to explain the entrainment of rodent circadian rhythms to a non-discrete light schedule (as in nature). Briefly, the 'VRC' approach to explain entrainment implies that at some phases of the circadian cycle, light (*continuously*) increases the angular velocity of the oscillation compared to what the velocity would have been if in darkness, and, at other phases of the cycle, it decreases the angular velocity. A major difficulty with this theory is that a method for the experimental measurement of a 'VRC' has yet to be invented! In 1976, Daan and Pittendrigh (ref.4-5) proposed an explicit version of Swade's concept in their work on rodent clock behavior in tonic light. Basically, this specific version of the 'VRC' concept assumes that the effect of tonic light input to the clock is simply a summation of the effects of a contiguous series of single light pulses, taking into account adaptation. Thus if a PRC to single light pulses of certain species is known, the response in dim light is predicted by integrating the PRC with respect to time with a 'transformation' factor that represents the adaptation of the system. Since, in this interpretation, the portion of the cycle where light pulses normally induce phase delays will be expanded while the part of the cycle where light pulses induce phase advances will be contracted, the periodicity of the system in dim light will depend *more strongly* on the structure of the *phase –delay* portion than the rest of the clock cycle. Specifically, if the assumptions in this approach are right, then a PRC with a large phase-delay section and relatively small phase advance section would produce large over-all lengthening of the clock period in constant light, and a PRC with the reverse ratio would produce less or even no lengthening of the clock period. Since our previous study (see chapter 3) showed that there

were no drastic differences in the ratio of the phase-delay and phase advance portions of the circadian cycles among the different strains -- with the exception of per^{s}/per^{l1} -- we expect the various clocks to behave similarly in tonic dim light. However, as has been described in section 4.2, the various strains behave very differently indeed. Thus, while the PRC of the 'Clk^{KO6}' strain shows a delay/advance ratio that is comparable to the wild-type, the ability of its clock to be lengthened in dim light seems to be *reduced*. On the contrary, the large range of periods to which per^{l1} clocks can be lengthened compared to the other strains would imply a much bigger delay/advance ratio in this strain, which we do not observe. Finally, the strongest test on this model is to be found in the heterozygous mutant per^{s}/per^{l1}, which provides a PRC with a distinctly larger amplitude in the delay portion (fig.3-7 (h)) than the other strains and should therefore show the biggest lengthening under tonic light condition. However, the results for this mutant, shown in fig.4-6, do not indicate drastic lengthening at the light levels measured when compared to the results for per^{s} and per^{l1}.

4.4 The role of eyes and optic lobes in the tonic 'dim light' effect

The literature on the photoreceptors for circadian rhythm [3] is rather extensive (see ref.4-7 for review). The research efforts can be generally divided into two main classes: (1) Attempts to identify the photoreceptor pigments involved through spectral analysis; this approach being particularly popular in the study of clocks in plants and unicellular organism. (2) Attempts to anatomically localize the site of photoreception in an organism; this approach being more popular in the study of clocks in higher animals.

Results from the spectral analysis studies have shown rather convincingly that the clock photoreceptors in lower plants use blue-absorbing pigments like flavoproteins and/or carotenoids while those of the higher plants employ mainly chlorophyll and phytochrome (ref.4-7). In the fruitfly *D. pseudobscura*, Frank and Zimmerman (ref.4-8) first found that action spectra for phase advance and delay in the middle of the subjective night are consistent with the possibility that the photoreceptor is a carotenoid or a flavoprotein. This result was confirmed in a more detailed measurement by Klemm and Ninnemann (ref.4-9), who showed an action spectrum that is rather sharply peaked at 440-460 *nm*.

On the other hand, in the approach of anatomical localization, McMillan et al.(ref.4-10,4-11,4-12) found that, in the house sparrow (*Passer domesticus*), both retinal and extraretinal brain photoreceptors contribute to the entrainment of locomotive activity by light ,the change of period in tonic dim light, and the arrhythmia resulting in bright light. Even though the retinal input seems to contribute to all three responses, it is *required* only in the third. Thus in the house sparrow, it appears that the retinal and extra-retinal pathways couple to the circadian oscilla-

3. In this literature, there is generally no distinction made between the various effects of light on the circadian clock. The phenomenon dealt with by most researchers conceals the entrainment of a rhythm by light regimes of one sort or another; however, the term 'photoreceptor for the circadian clock' is taken to mean the receptor for *any* light effects.

tor in a parallel, but not identical fashion. Attempts to localize the clock photoreceptor in insects have been plentiful. The results fall in two groups, with insects in the first group using the compound eyes and insects in the second group using extraretinal photoreceptor for clock input. To the first group belong the cockroach and the cricket: Both the entrainment of the locomotive activity of the cockroaches, *Leucophoea maderiae* and *Periplaneta americana*,(ref.4-13,4-14), and the entrainment of the stridulatory rhythm of the cricket, *Teleogryllus commodus* (ref.4-15), have been shown to be via the compound eyes only. To the second group belong the grasshoppers, the giant silkworm and the fruitfly. The oviposition rhythm of the grasshopper,*Chorthipus curtipennis*, was shown to be entrainable by light after removal of both ocelli and compound eyes(ref.4-16). The eclosion rhythm of the silkmoth, *Hyalophora cercropia*, was shown to be entrainable by light falling on tranplanted brains in brainless host regardless of whether the transplants are to the head or the abdominal cavity (ref.4-17). Finally, the eclosion rhythm of the fruitfly *Drosophila melanogaster* was demonstrated to be entrainable by light in an eyeless and ocelli-less mutant (ref.4-18).

In this section, we seek to establish the site of the photoreceptor in *D. melanogaster* which mediates the effects of dim light that are discussed in section 2.2 . To this end, two mutations that affect the external photoreceptor of the fly are used. The first mutation used, *norpA*, is a mutation which severely affects the production of the receptor potential in the compound eye, apparently due to the defects in the generation of 'quantum bumps' at the receptor cell level (ref.4-19). The second mutation used, *sine oculus*, which causes absence of both the compound eyes and the ocelli, is the same mutant that is used in chapter 2 of this thesis and in ref.4-18 .

Fig.4-7(c) shows the activity records, plus periodograms, of the DD_1 and LL_1 segments of a *norpA* mutant. We observe in this animal that the period of the

freerunning rhythm is changed from 24.5 hours in the DD_1 segment to 30 hours in the LL_1 segment of 2.2 lux. The lengthened rhythm is sustained stably throughout the seven cycles of observation. Fig.4-7(d) shows the electro-retinogram (ERG) of the *same* animal whose activity record is shown in (c), indicating a complete lack of response in a light intensity 200 times stronger than it is exposed to during the LL_1 exposure. The responses of 41 such mutant animals are summarized in fig.4-7(a) in the same format as is in fig.3. We note that both the responses of period change and arrhythmicity are observed, suggesting the compound eyes are not needed for the mediation of these effects. However, two features of the responses in this mutant - (*i*) the slope of the major period change segment and (*ii*) the threshold to arrhythmicity -- seem to resemble the same features in per^s more than the wild-type (see fig.4-3). Fig.4-7(b) shows a similar summary for the per^s *norpA* mutant,[4] showing that such independence of the dim light effects on the compound eyes is preserved in the clock mutant. On the other hand, quite different effects are observed in the mutant, per^s ;*so*/*so* . As shown in fig.4-8, the responses of this eyeless and ocelli-less mutant are very variable: In the range of light intensity of 10^{-2} to 10^{-1} lux, the periods of three animal are shortened, those of another four are lengthened, and five animals become arrhythmic; while within the range of light intensity of 10^{-1} lux to 1 lux, the periods of five animals are lengthened and nine animals become arrhythmic. Fig.4-9 shows two series of periodograms for two such mutants which were brought through the sequence DD_1, LL_1, DD_2, LL_2 with $I_{LL_1}=I_{LL_2}=0.4\ lux$ for animal #2101153 and $I_{LL_1}=I_{LL_2}=0.02\ lux$ for animal #2101238. These periodogram series confirm that the arrhythmia observed in the LL_1 segment is truly due to the change of ambient light levels and not due to spontaneous loss of rhythmicity due to some other 'side effects'.

4. This mutant also has a *white* mutation on the same X-chromosome as the other two mutations of interest. The mutation *white* mutation, which causes the absence of all the red screening pigments in the compound eyes, normally enhances the ERG response to blue light of over 100-fold. However, even with the *white* mutation, this mutant does not show significant ERG responses.

4.5 Discussion

4.5.1 Possible interactions of the various effects of light

It is a commonly known fact among circadian clock researchers that, under extremely high light intensities, most circadian rhythms fail to be expressed (ref.4-1). It is not known -- and from a behavioral point of view, probably unknowable -- whether this arrhythmia truly reflects stopping of the circadian clock or simply manifests a masking effect of light on the expression of the rhythm. [5]

On the assumption that the arrhythmia induced by light reflects true arrhythmia of the driving circadian oscillator, Aschoff (ref.4-1) raised the interesting question of whether such arrhythmia occurs because the circadian system has been driven towards too long or too short periods or whether a certain threshold in the illumination is reached (to trigger a new mechanism into action). One of the motives for our experiments described in section 4.2 was to address this question . A novel feature that comes out of this study is the discovery of a 'critical window' of light intensity, as described in section 4.2, in between the 'dim' light region which causes period changes and the 'bright' light region which causes arrhythmia. Within this window of light intensity, the slope of the period *vs.* light intensity curve becomes very steep, and this is followed by the appearance of various unstable phenomena of the clock, which include exceedingly long, but unstable, periodicities, apparent sub-circadian components, very 'noisy' and dispersing rhythms, and spontaneous changes in cycle length in a single or many consecutive cycles. Finally, with the exception of the eyeless per^s mutants, this 'critical window' is invariably associated with a threshold of light intensity over which arrhythmia occurs. Taken together, we feel that the above data suggest, but do not prove, that arrhythmia is

5. It is the opinion of the present author that what is observed is probably true arrhythmia because, in all animals that become arrhythmic in LL_1 in this study, the rhythm in the subsequent DD_2 segment always restarts from the same phase point as in the LL to DD1 transition -- regardless of the duration in LL1 in which the animal has been placed. However, this result can also be explained by a masking effect of light in LL_1 **and** a resetting of the rhythm by the LL_1 to DD_2 transition(ref.4-6).

caused by light through 'over-stretching' the period length of the *Drosophila* clock. It is curious to note that, at least in *Drosophila*, lengthening the period of the clock -- regardless of whether through genetic or environmental means -- always tends to be associated with causing arrhythmia (see also results in chapter 2), while shortening the period of the clock never seems to cause such an effect and, in fact, tends to 'tighten' the rhythm in most cases. In this connection, it would be fruitful to carry out a similar study on circadian clocks that are speeded up by light such as in the diurnal birds.

A second relation between the various effects of light that we seek is the possible connection of the phasic to the tonic effects of light. The availability of the relatively large number of PRC's as well as dim light response curves in the various strains of *D. melanogaster* presented in this thesis provide us with an opportunity to test the consequences of Daan and Pittendrigh's version of Swade's VRC concept, which demanded a strong positive correlation between the delay/advance ratio of the PRC of a strain and the degree to which light could lengthen the period of the clock in the same strain. Our results do not show this kind of correlations and therefore do not lend support to such a hypothesis.

4.5.2 Possible interactions between light and clock mutations

Our original question for the studies in this chapter was to inquire into the possibility of common mechanism(s) underlying the effects of light and of the clock mutations in their action on changing the clock period. In this section, we depict three different possibilities and compare them with the results actually observed. Our model for this discussion involves the following sequence of events: "light --> photoreceptor --> oscillating mechanism --> period changes".

The first possibility we consider is that the light effect and the clock mutations use completely different and non-interacting mechanism(s) in changing the period

of the clock. Fig.4-10 (a), graph W, represents an 'idealized' plot of the wild-type response in which we have a horizontal segment on the left for the region of low light intensity (< 0.07 lux) where the changes in period are small, and a slanted straight line in the region where we find linear increase in period length against the logarithm of light intensity, and finally an asterisk represents where instability of oscillation occurs.[6] Graph L and graph S in the same figure represent the hypothetical responses of a long and a short period mutant, respectively. Because of the assumed lack of interaction between the two mechanisms, the graphs are simply vertical displacements from the wild-type graph. That is to say, the results are additive.

The second possibility we consider is that a clock mutation can cause a period change because it affects some part of the photoreception system such that its output signal to the oscillating mechanism is changed. A concrete example of this possibility would be that the frequency of the clock is dependent upon a tonic discharge level of the photoreceptor output in such a way that an *increase* in this discharge level, normally caused by tonic illumination, *slows* the oscillating process. In such a scheme, a mutation that increases the tonic 'dark' discharge level would then produce a long period clock phenotype; and, likewise, a mutation that *decreases* this dark discharge would produce a short period clock phenotype.[7] The resulting responses of such a long period mutant which is 'seeing light in the dark' and a short mutant which is 'extra-blind in the dark' are depicted graphically in fig.4-10(c). The salient points are: Graph L is horizontally displaced to the left, with a *lowered* threshold at which significant period lengthening starts to occur; and graph

6. In figs.4-10 (a) and (b), we assume that clock instabilities occur at a ***fixed light intensity*** for all three strains, while in fig.4-10 (c), we assume that clock instabilities occur at a ***fixed period length***. Insofar as the relationship of the period lengthening effect and arrhythmia causing effect of light has not been definitively demonstrated (see previous section in this discussion), this choice is totally arbitrary, and using different assumptions in these different cases is just to increase the variety of illustrations (at the risk of causing confusion!).

7. This scheme would be equally valid, of course, with all the 'signs' of the interactions reversed.

S is horizontally displaced to the right, with an *increased* threshold.

The third possibility we consider is that a clock mutation may leave the photoreceptor system intact but causes a period change by affecting the 'bias' and/or the 'gain' of the interaction between the photoreceptor system and that part of the oscillating process that couples to it. Pursuing the concrete example developed in the previous example further, we envisage, in the current hypothesis, an identical 'dark discharge' from the photoreceptor system in the mutant and in the wild-type but this same discharge is causing a different effect on the oscillation in the mutant because of the changed interaction. Since the mode of this interaction is totally unknown at this point, models arising from these assumptions are necessarily more vague. One such model is given in fig.4-10(b), in which we show the hypothetical responses of a long period mutant which results from an increase in 'bias' and a decrease in 'gain' and a short period mutant which results from a decrease in 'bias' and an increase in gain.

The actual results for the five strains of flies, in summary form, are presented in part (d) of fig.4-10. We observe that, for the mutants *And* and Clk^{KO6}, the results suggest a simple addition of the effects of light and mutations, with one peculiarity, as noted before, that the threshold at which clock instability occurs in the mutants is decreased five- to ten- fold when compared to the wild type.

The responses of the mutants per^{s} and per^{l1} are more complex. They are different from the wild-type responses in three ways: (1) The threshold for clock instability is (again) decreased five- to ten- fold compared to the wild-type. (2) Within the region of light intensities where major (> 1 hour) period changes occur, the slope of period change vs. light intensity increases significantly over that of the wild-type. (3) As shown by the histograms in fig.4-11, at light intensity below 0.07 lux, the sign of the responses of wild-type animals is variable: the periods of some clocks are

shortened, some are lengthened, and some are unchanged. However, at these light intensities, the periods of all per^s clocks are either unchanged or *shortened* while those of the per^{l1} clocks are either unchanged or *lengthened*. (Also shown in fig.4-11 are responses for the heterozygous mutant per^s/per^{l1}, which follow the pattern of per^{l1} and those for Clk^{KO6}, which show no significant changes .) Our conclusion from these observations is that (1) neither of the two *per* mutants affect the period of the clock through the photoreceptor system, as depicted in fig.2-10(c), and (2) whatever mechanism is used by these mutants, it is not totally independent from the light effect, as depicted in fig.4-10(a). The fact that, at intensities above 0.07 lux, the two *per* mutants respond to light in a similar fashion but, at intensities below 0.07 lux, they respond in a reciprocal fashion suggest that light has a dual action on these two mutant oscillators.

In summary, none of the four mutations examined seems to affect the period of the clock by simply changing the 'apparent' light level to the oscillator proper. However, whatever part(s) of the oscillating processes these mutations seem to affect, they are sensitive to light to some degree because, in all four cases, the threshold of light intensity at which clock instabilities occur are lowered about five- to ten- fold compared to that for the wild-type. Furthermore, a subtle response of the clock at very dim light levels (below 0.07 lux) is uncovered in the two per^s and per^{l1} which suggests probably more than one component of the circadian oscillator is sensitive to the tonic light.

4.5.3 Photoreceptor for the tonic 'dim light' effects in Drosophila

The responses to dim light of the ERG-defective mutants, per^+ *norpA* and per^s *w norpA*, and of the eyeless and ocelli-less mutant, per^s; *so/so* , strongly suggest that the 'dim light' effects, like the entraining and phase-shifting effects of light, are mediated - at least predominantly - through extraretinal receptors. That

the compound eyes and ocelli are completely uninvolved cannot be established at this point because of two quantitative observations. First, even though the responses of the *norpA* mutant include all features of the wild-type, the detailed shape of the response curve is found to be more similar to the per^s curve than the wild-type curve. Secondly, even though period shortening, period lengthening and arrhythmicity can be caused by light in the eyeless and ocelli-less per^s mutant, the sharp dependence of the occurrences of these phenomena on light intensity, as observed in per^s, is lost.

Preliminary experiments on a second wild-type strain, 'Oregon-R', indicate that the response pattern of this strain follows that of the wild-type strain used in this study ('Canton-S') in both the slope of the major period change vs. intensity curve and the threshold to arrhythmicity. It thus appears that the changed response observed in *norpA* might not be simply a genetic background effect. Further investigations into this problem would likely provide insight on the mechanism of action of light on both the wild-type and mutant circadian oscillators.

The large inter-individual differences in the responses of the per^s; *so/so* mutant further substantiate the observations on the increase of instability of such clock features as the period (fig.2-6) and the phases (fig.A-7) when the eyes and major parts of the optic lobes are absent.

Chapter 4 References

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Chapter 4 Figures

Figure 4-1. *Experimental Protocol.* All animals are reared in 22°C. and about 200 lux. To avoid 'after effects' problems that may interfere with inter-animal comparisons (ref.4-1), animals are put into darkness for about 5 cycles (the range is 4 to 20) before exposure to any levels of ambient light intensities. Those that survive DD_1 and show good rhythm are released into LL_1. Flies that survive LL_1 are transferred to DD2; and so on. (Before a second light level is introduced, an animal is always re-introduced to a *DD* period of about 5 cycles in order to start the clock in each *LL* run in as similar a state as possible.) The intensities of LL_1, LL_2, LL_3... may not be in any ascending or descending series. The protocol as well as the terminologies are illustrated in the figure. In about 20% of the cases in wild-type and in a few cases in per^s & per^{l1} animals -- in the early stages of the experiment and before we arrived at a most suitable form of nutrients for the animals--, a food(tube) change is needed during the DD_1-> LL_1 transitions. Also, most (but not all)of the LL_1-> DD_2, LL_2-> DD_3, LL_3-> DD_4 transitions are accompanied by food change. During each food change, the animal is exposed to ambient light (less than 100 lux) for 20-60 seconds. In most of the cases in this study, whenever an animal is shown to be arrhythmic in a certain situation, a subsequent run in DD is carried out - provided the animal survives - to assure that rhythmicity resumes. In the few cases where rhythmicity does not resume, it is assumed that the expression of the activity rhythm is disrupted by some unknown physiological causes , and the records are discarded. All experiments described in this chapter follow the above protocol.

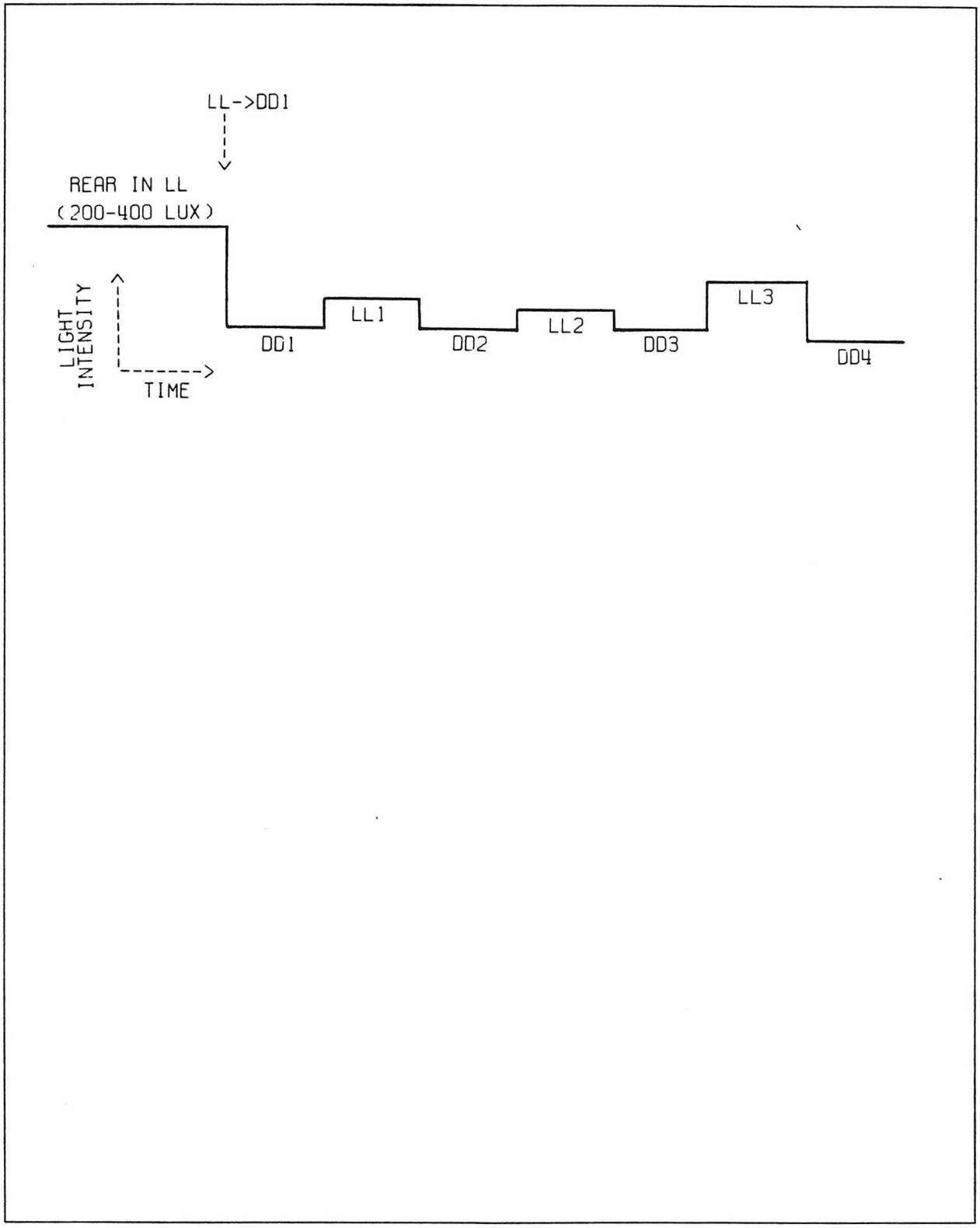

LL->DD1
REAR IN LL
(200-400 LUX)
LIGHT INTENSITY
TIME
DD1
LL1
DD2
LL2
DD3
LL3
DD4

Figure 4-2. *Classification of clock behavior in LL_1 into six classes.* Examples are drawn from the five strains of *D. Melanogster* to illustrate the salient features in the different categories. The continuous record of each animal is broken up into the respective DD_1 and LL_1 (and DD_2) segments; the periodogram corresponding to each segment is placed on the right. The duration of observation in LL_1 for the majority of the animals is between 7 and 10 cycles long, with the longest durations exceeding 30 cycles.

(a) *Class 1.* A wild-type fly is shown free-running with period of 30 hours in LL_1. The lengthened period persists steadily for 10 cycles.

(b) *Class 2.* A per^{l1} fly free-runs in LL_1 with much lengthened period, but the rhythm tends to disperse after the third cycle.

(c) *Class 3.* A wild-type fly free-runs for 5 cycles in LL_1 with *drastically* lengthened period and then abruptly becomes arrhythmic.

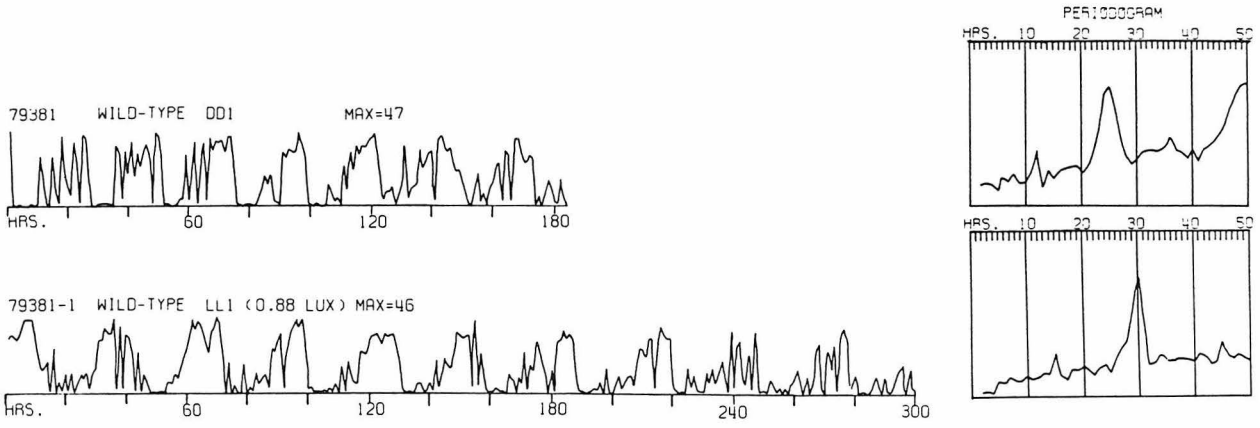
(d) *Class 4.* A wild-type fly goes into arrhythmia immediately after introduction into LL_1. The rhythm is subsequently restored when the animal is transferred to DD2.

(e) *Class 5.* A split-peak is shown in a periodogram that corresponds to a long LL_1 record of a per^s fly. A major 'spontaneous' phase shift is observed at around the 120th hour into LL_1. Periodograms on the first 120 hours and the last 180 hours of this LL_1 segment both show oscillations of single frequencies, suggesting that the split-peak in this LL_1 segment is an artifact of the 'spontaneous' phase shift.

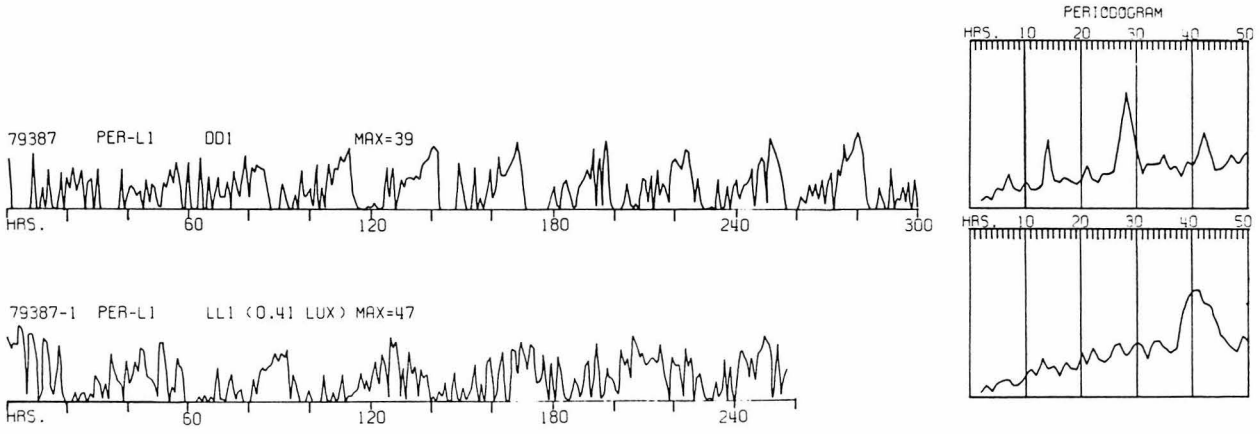
(f) *Class 5.* A major split-peak is observed in the periodogram of an LL_1 segment of an *And* fly. No obvious phase shift is seen or definable for this animal as the period length varies greatly from cycle to cycle. No concurrently existing oscillations of different frequencies are apparent.

(g) *Class 6.* After 3 days of apparent arrhythmia in relatively 'bright' LL_1, the

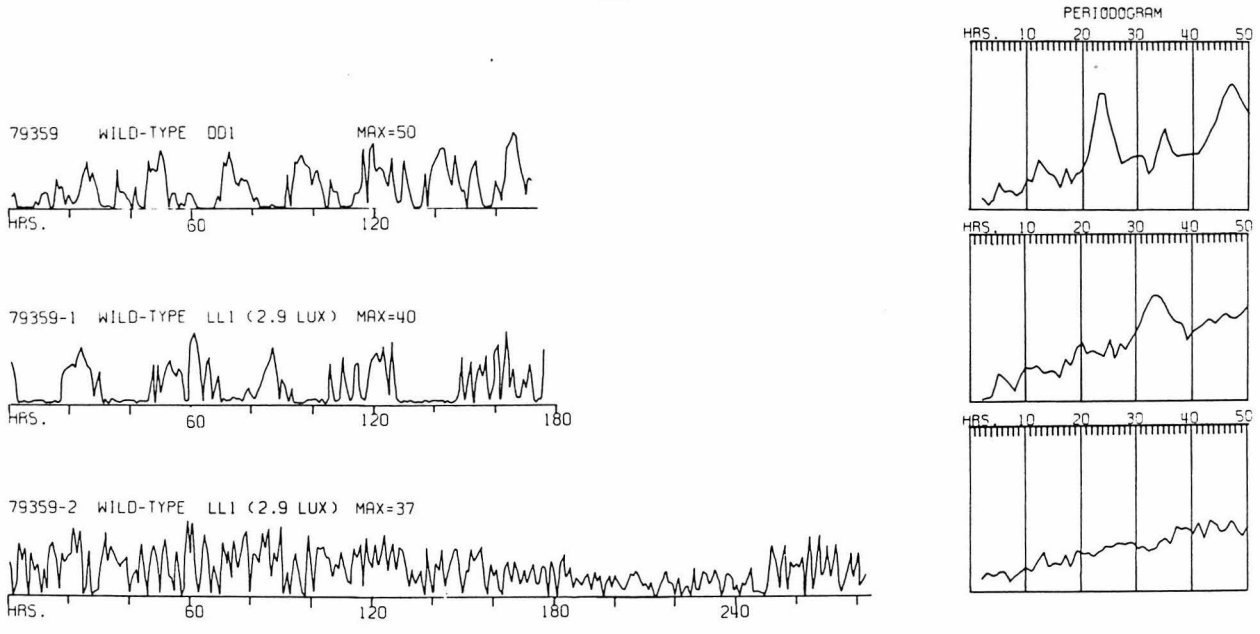
activity of a '$Clk^{KD6'}$ fly breaks up into bursts that produce a major peak at 11.5 hour in the periodogram. On transferring to DD2, the activity pattern becomes normal again. The periodograms in this figure correspond not to the whole segments to the left, but only to the regions inside the dashed boxes.


79381 WILD-TYPE DD1 MAX=47
HRS. 60 120 180
79381-1 WILD-TYPE LL1 (0.88 LUX) MAX=46
HRS. 60 120 180 240 300
PERIODOGRAM
HRS. 10 20 30 40 50
HRS. 10 20 30 40 50

(a)


79387 PER-L1 DD1 MAX=39
HRS. 60 120 180 240 300
79387-1 PER-L1 LL1 (0.41 LUX) MAX=47
HRS. 60 120 180 240
PERIODOGRAM
HRS. 10 20 30 40 50
HRS. 10 20 30 40 50

(b)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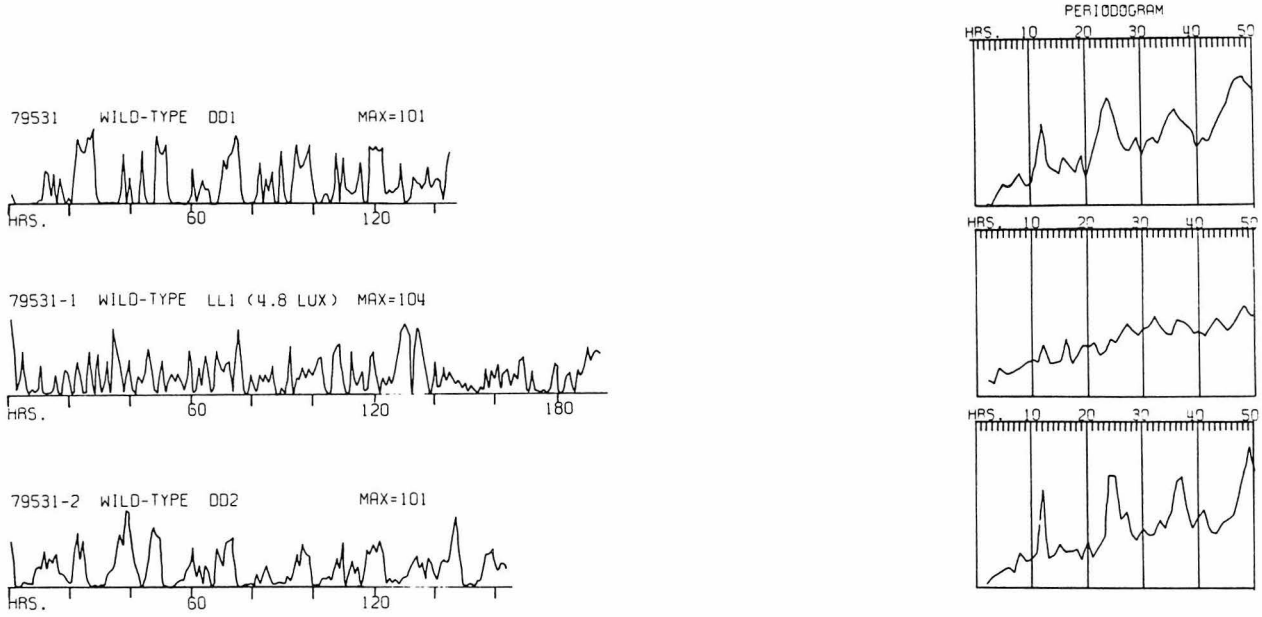
HRS. 60 120
79359-1 WILD-TYPE LL1 (2.9 LUX) MAX=40
HRS. 60 120 180
79359-2 WILD-TYPE LL1 (2.9 LUX) MAX=37
HRS. 60 120 180 240
PERIODOGRAM
HRS. 10 20 30 40 50
HRS. 10 20 30 40 50
HRS. 10 20 30 40 50

(c)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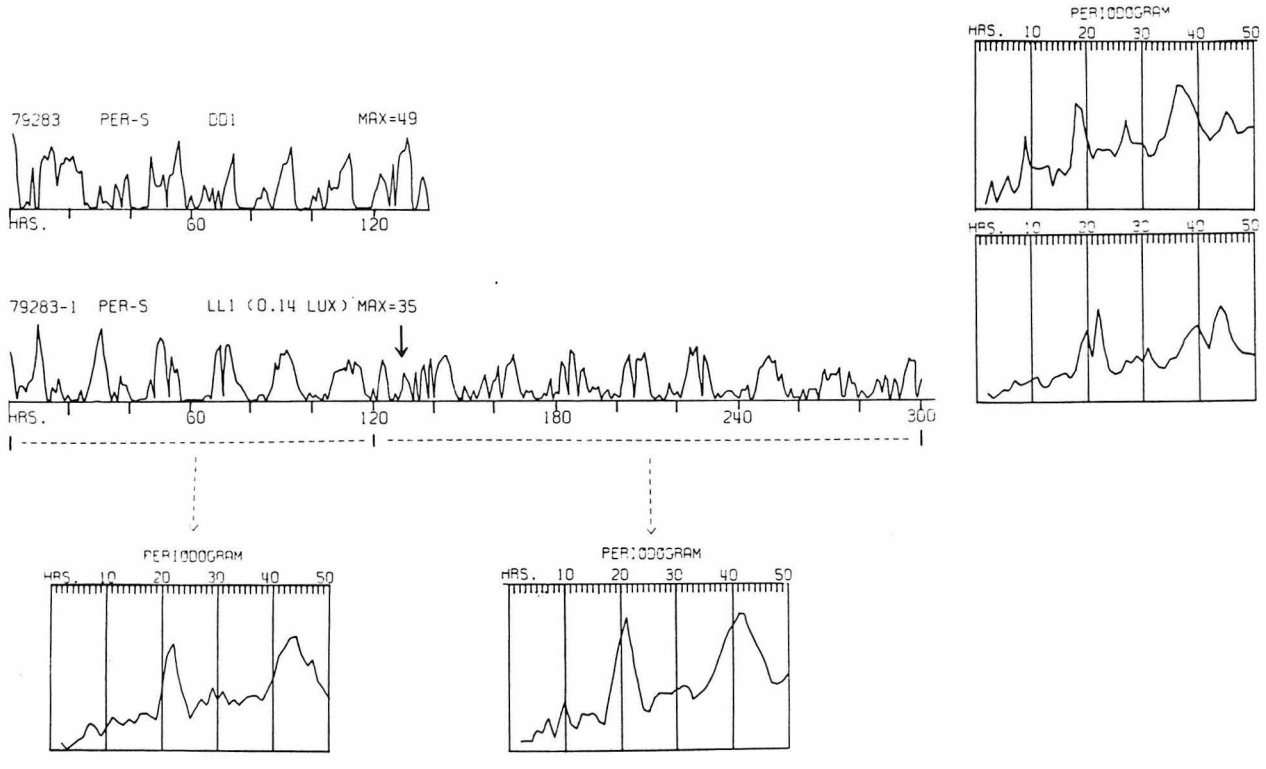
HRS. 60 120
79531-1 WILD-TYPE LL1 (4.8 LUX) MAX=104
HRS. 60 120 180
79531-2 WILD-TYPE DD2 MAX=101
HRS. 60 120
PERIODOGRAM
HRS. 10 20 30 40 50
HRS. 10 20 30 40 50
HRS. 10 20 30 40 50

(d)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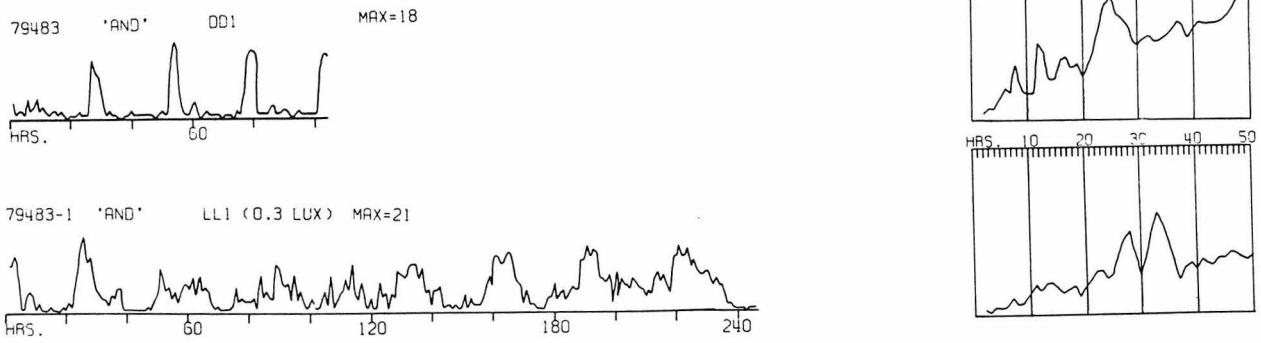
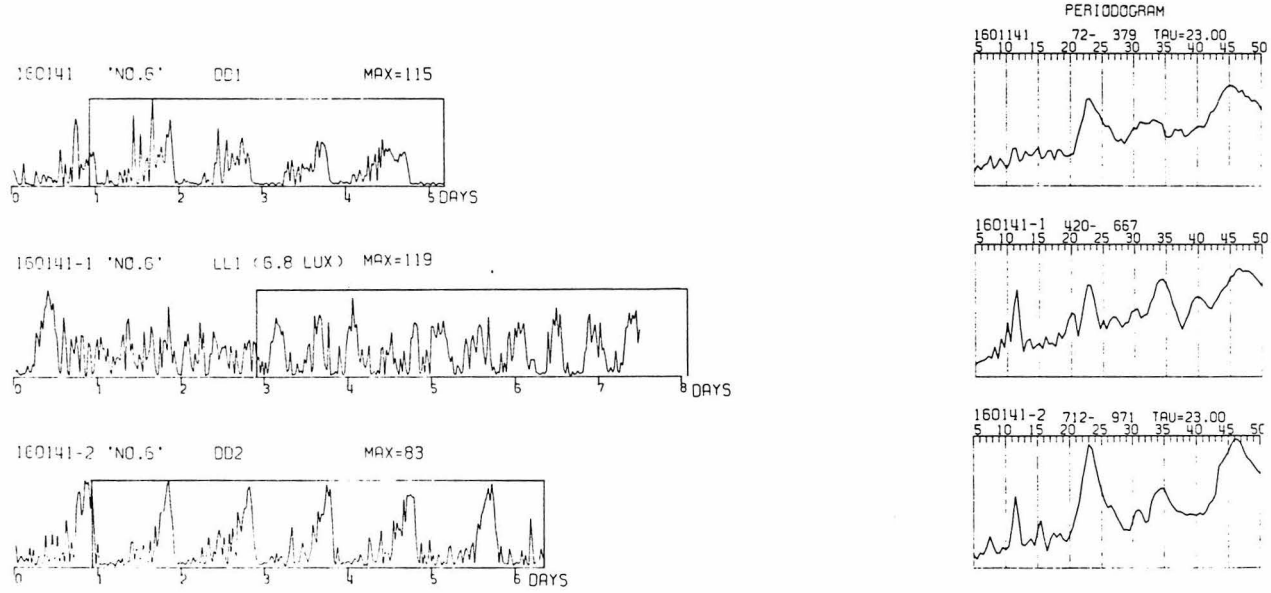
HRS. 60 120
79283-1 PER-S LL1 (0.14 LUX) MAX=35
HRS. 60 120 180 240 300
PERIODOGRAM
HRS. 10 20 30 40 50
HRS. 10 20 30 40 50
PERIODOGRAM
HRS. 10 20 30 40 50
PERIODOGRAM
HRS. 10 20 30 40 50

(e)


79483 'AND' DD1 MAX=18
HRS. 60
79483-1 'AND' LL1 (0.3 LUX) MAX=21
HRS. 60 120 180 240
PERIODOGRAM
HRS. 10 20 30 40 50
HRS. 10 20 30 40 50

(f)


160141 'NO.6' DD1 MAX=115
0 1 2 3 4 5 DAYS
160141-1 'NO.6' LL1 (6.8 LUX) MAX=119
0 1 2 3 4 5 6 7 8 DAYS
160141-2 'NO.6' DD2 MAX=83
0 1 2 3 4 5 6 DAYS
PERIODOGRAM
160141 72- 379 TAU=23.00
5 10 15 20 25 30 35 40 45 50
160141-1 420- 667
5 10 15 20 25 30 35 40 45 50
160141-2 712- 971 TAU=23.00
5 10 15 20 25 30 35 40 45 50

(g)

Figure 4-3. ***Summary for clock behavior in LL_1 for wild-type and four mutant D. melanogaster strains.*** The difference of the LL_1 period and the DD_1 period of the activity rhythm of a fly is plotted against the LL_1 light level. Each symbol represents the result from a single fly. The different symbols represent different classes of behavior which are explained in detail in fig. 2 and in the text. Animals whose activity become arrhythmic immediately upon entry into LL_1 (fig. 2d)are represented by asterisks placed at the 'ARR' level. Animals whose rhythms sustain for at least a few cycles (fig. 2c) are represented by five-pointed stars at the level corresponding to the period of the initial LL_1 rhythm. Animals whose LL_1 periodograms show split-peaks are represented by a cross at the level of the major peak. Diamonds represent animals whose rhythms become less sharp as the duration of LL_1 progresses (fig. 2b).Triangles represent flies which remain stably rhythmic during the whole LL_1 period (fig.2a). Difference in LL_1 and DD_1 periods , rather than absolute LL_1-period, is used here to facilitate inter-individual and inter- strain comparison of the results; since the DD_1 periods of per^{l1} flies are very different (see fig.2-1).

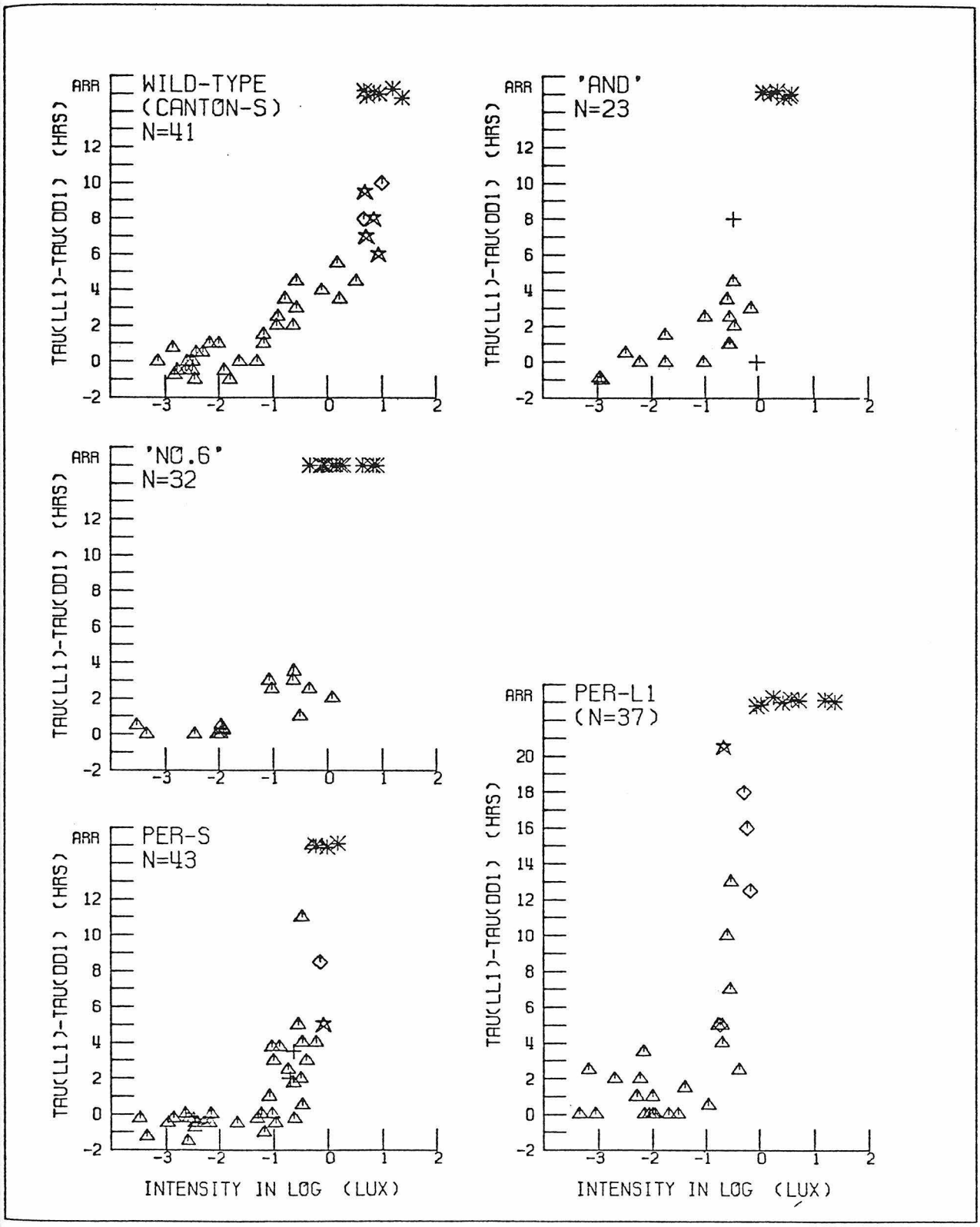

WILD-TYPE
(CANTON-S)
N=41
'AND'
N=23
'NO.6'
N=32
PER-S
N=43
PER-L1
(N=37)
ARR
TAU(LL1)-TAU(DD1) (HRS)
INTENSITY IN LOG (LUX)

Figure 4-4. *Testing for stability of individual clock around the 'critical' light intensities.* Periodograms are shown for two per^S flies which are brought into LL_1 at two separate light intensities that bracket the critical region for this strain. Each animal is introduced to the same intensity twice, after dark-adapting in 5 cycles of DD each time. The period of the fly which is exposed at the lower intensity (animal #281485) is lengthened by about 4 hours during both *LL* intervals, whereas the fly which is exposed to the higher light intensity (animal #281490) becomes arrhythmic during both *LL* duration. The result suggests that there is no instability in the response of a single animal to various levels of ambient light intensities around the 'critical' region.

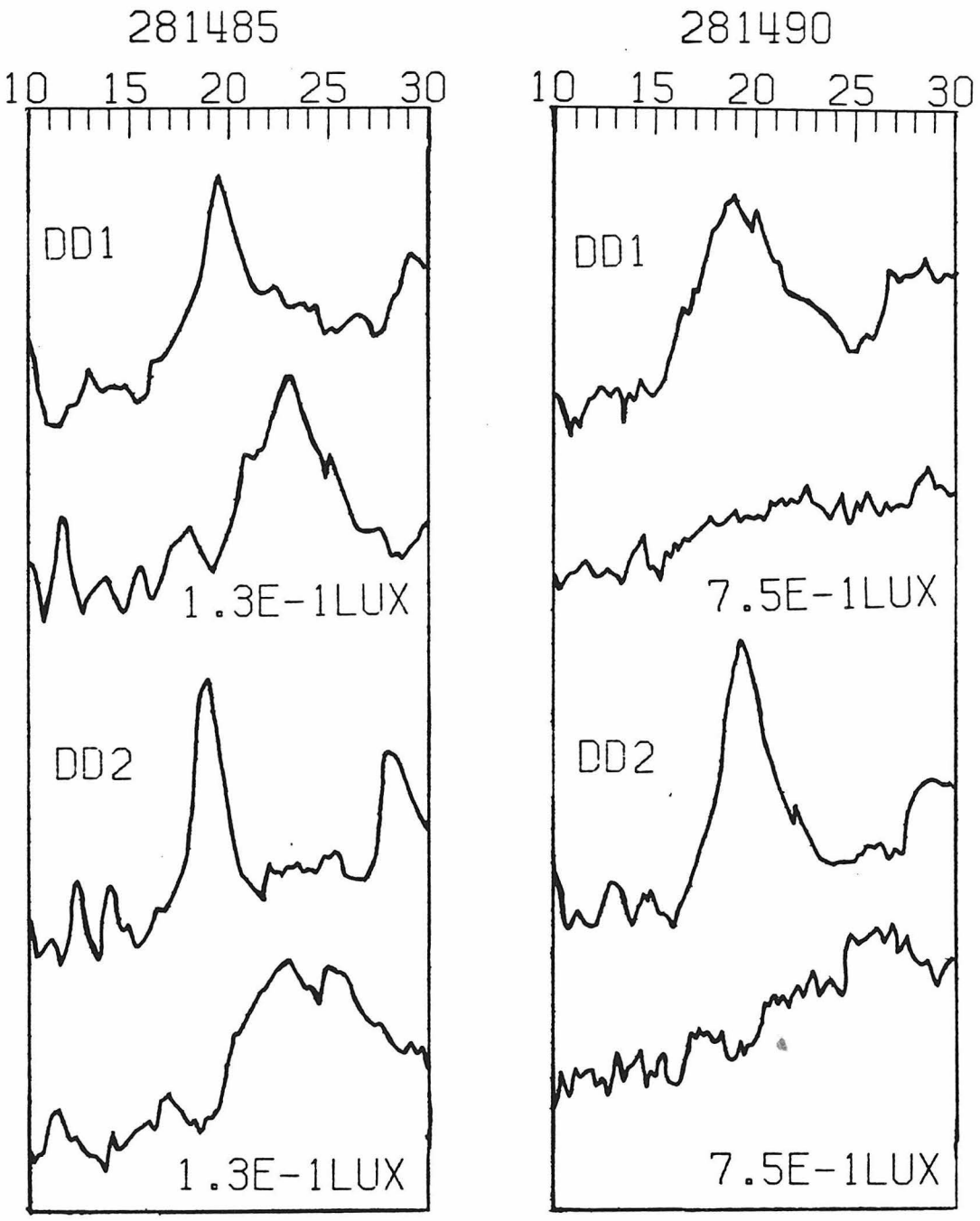

281485
10 15 20 25 30
DD1
1.3E-1LUX
DD2
1.3E-1LUX
281490
10 15 20 25 30
DD1
7.5E-1LUX
DD2
7.5E-1LUX

Figure 4-5. *Behavior of individual clock in four different strains at various light levels.* Each animal is exposed to a series of illumination levels according to the protocol presented in fig.1. Absolute values of periods of each individual at various light intensities are plotted against the latter. For clarity of presentation, DD periods - with the exception of DD_1 - are not plotted.

Cases: (*a*) Wild-type (*b*) per^s (*c*) *And* (*d*) per^{l1}

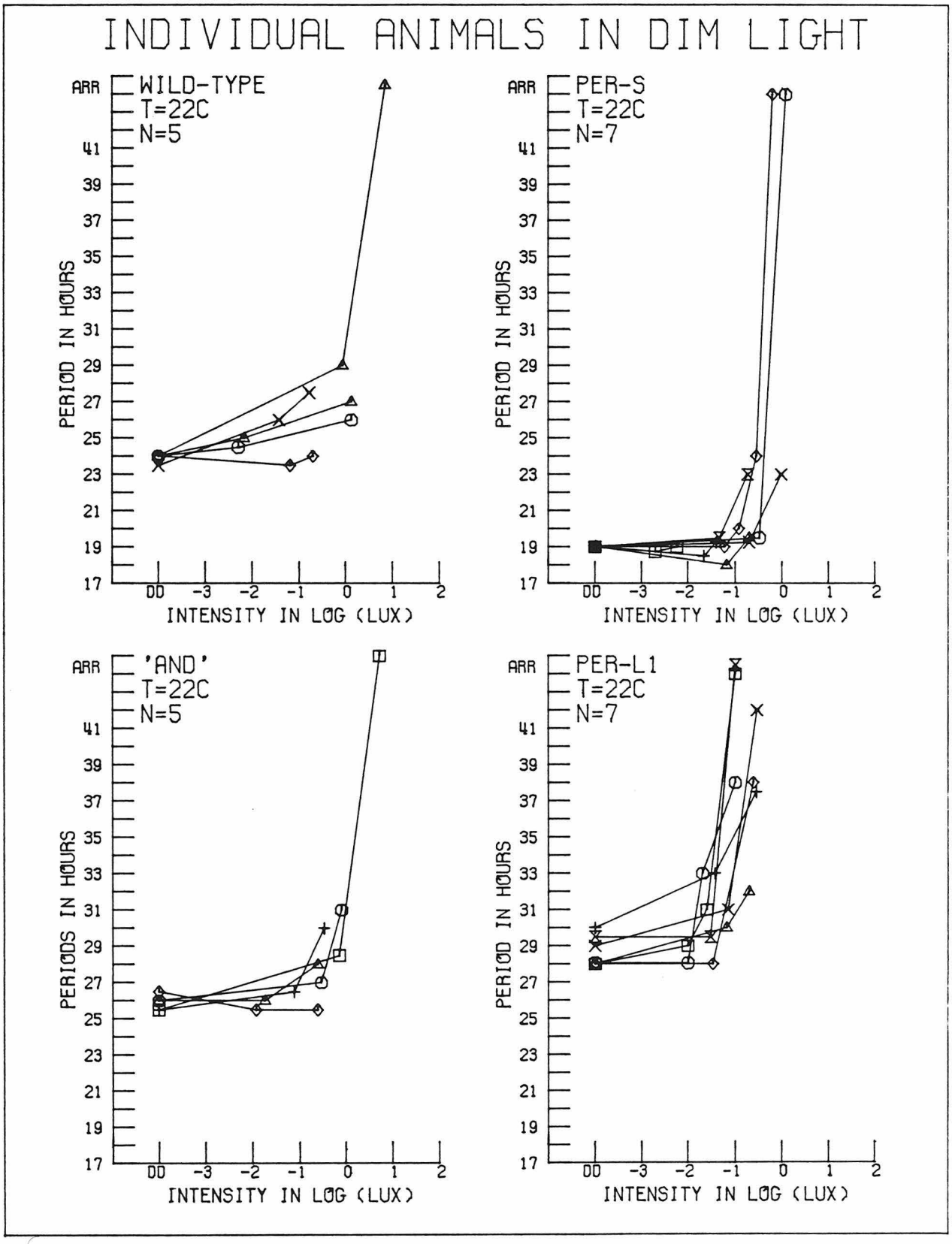

INDIVIDUAL ANIMALS IN DIM LIGHT
ARR
WILD-TYPE
T=22C
N=5
PERIOD IN HOURS
INTENSITY IN LOG (LUX)
ARR
PER-S
T=22C
N=7
PERIOD IN HOURS
INTENSITY IN LOG (LUX)
ARR
'AND'
T=22C
N=5
PERIODS IN HOURS
INTENSITY IN LOG (LUX)
ARR
PER-L1
T=22C
N=7
PERIOD IN HOURS
INTENSITY IN LOG (LUX)

Figure 4-6. *Summary for clock behavior in LL_1 for per^s /per^{l1}* at 22°C. The difference of the LL_1 period and the DD_1 period of the activity rhythm of a fly is plotted against the LL_1 light level. Symbols are same as in fig.4-3.

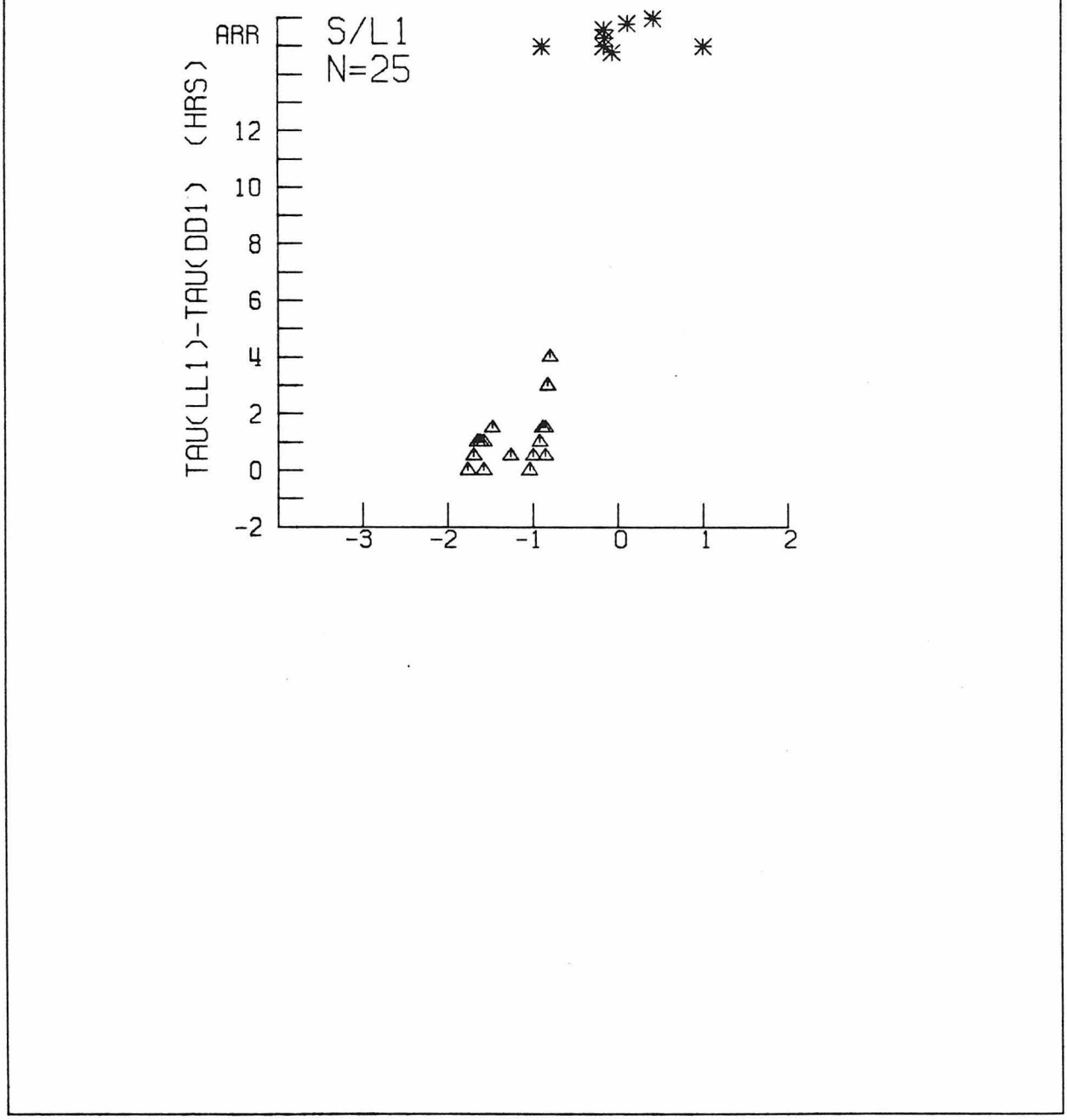

S/L1
N=25
ARR
TAU(LL1)-TAU(DD1) (HRS)
12
10
8
6
4
2
0
-2
-3
-2
-1
0
1
2

Figure 4-7. *Clock response to tonic dim light in wild-type and per^s animals with defective ERG*

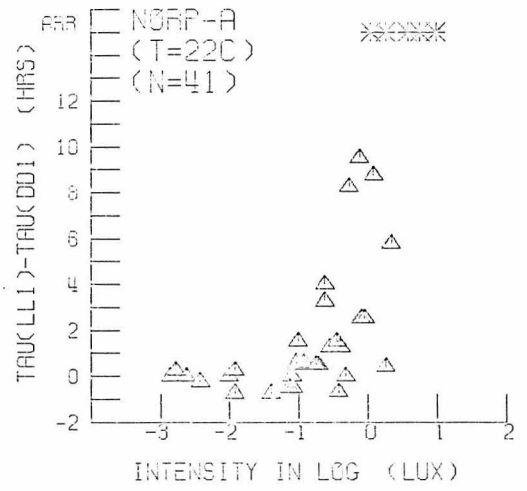
(*a*) Summary for clock behavior in LL_1 for the ERG-defective mutant *norpA*

(*b*) Summary for clock behavior in LL_1 for the ERG-defective mutant per^s *w norpA*

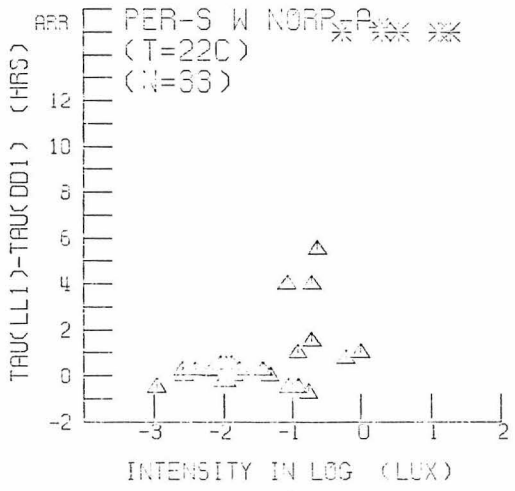
(*c*) Activity plot and periodogram of the DD_1 and LL_1 segments of a *norpA* animal, showing lengthening of period from 24.5 hour in DD_1 to 30 hours in LL_1 of 2.2 lux.

(*d*) ERG record of the *same norpA* animal whose activity record is shown in (c). Stimulus strength is about 500 lux. Calibrations of grids on the strip chart are : *vertical* = 4 mV/div ; *horizontal* = 2.5 sec/div.

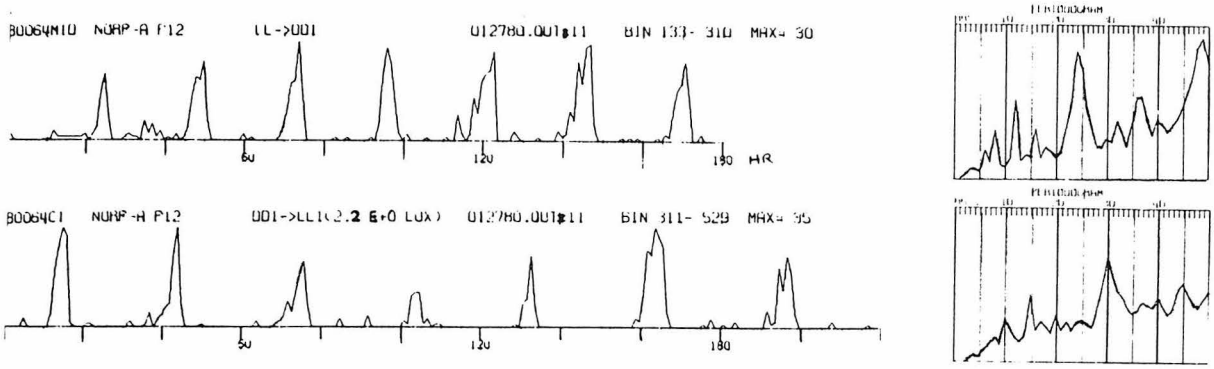
(*e*) ERG record of a wild-type *Dorsophila* using same stimulus as is used in (*d*).


NORP-A
(T=22C)
(N=41)
TAU(LL1)-TAU(DD1) (HRS)
INTENSITY IN LOG (LUX)

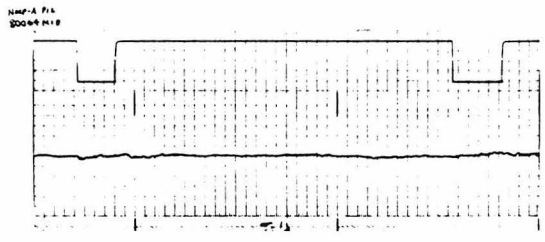
a


PER-S W NORP-A
(T=22C)
(N=33)
TAU(LL1)-TAU(DD1) (HRS)
INTENSITY IN LOG (LUX)

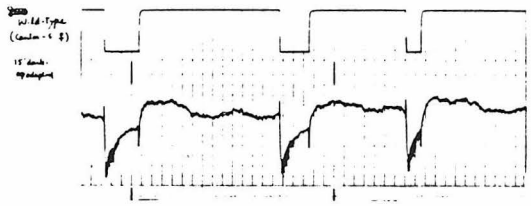
b


NORP-A
HR

c



d



e

Figure 4-8. *Summary for clock behavior in LL_1 for per^s ; so/so* at 22°C. The difference of the LL_1 period and the DD_1 period of the activity rhythm of a fly is plotted against the LL_1 light level. Only flies which completely lack ommatidium on both sides of the head are used. Symbols are same as in fig.4-3.

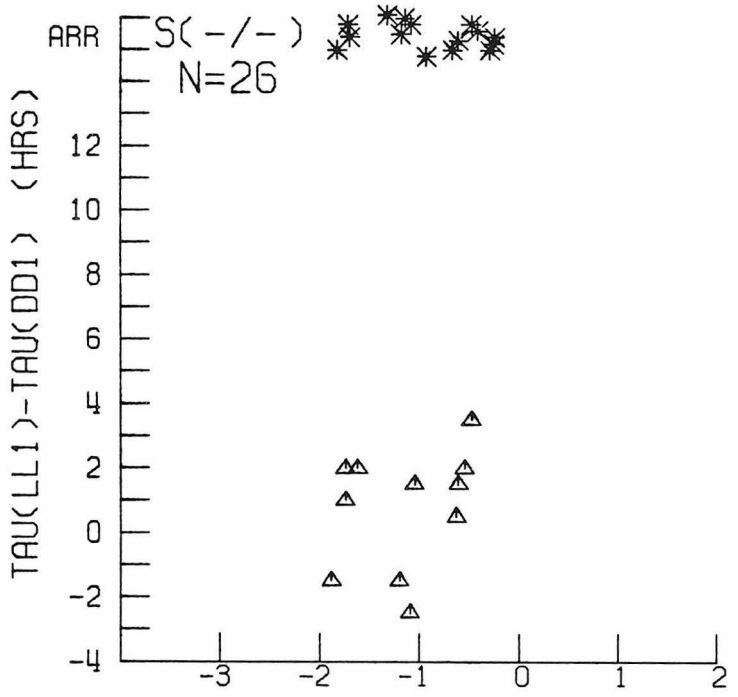

ARR
S(-/-)
N=26
TAU(LL1)-TAU(DD1) (HRS)
12
10
8
6
4
2
0
-2
-4
-3
-2
-1
0
1
2

Figure 4-9. *Tonic light causes arrhythmia in eyeless per^s animals* Periodograms are shown for two per^s ; *so/so* (totally eyeless) animals which are brought into LL_1 at two separate light intensities (0.4 lux and 0.02 lux, respectively). Each animal is introduced to the same intensity twice, going through the series DD_1(81 hours) --> LL_1(168hours) --> DD_2(120 hours) --> LL_2(187 hours). Except for the changes of ambient light levels, all factors in the environment are kept constant throughout the experiment. (Specifically, there were *no* food changes or temperature changes.)

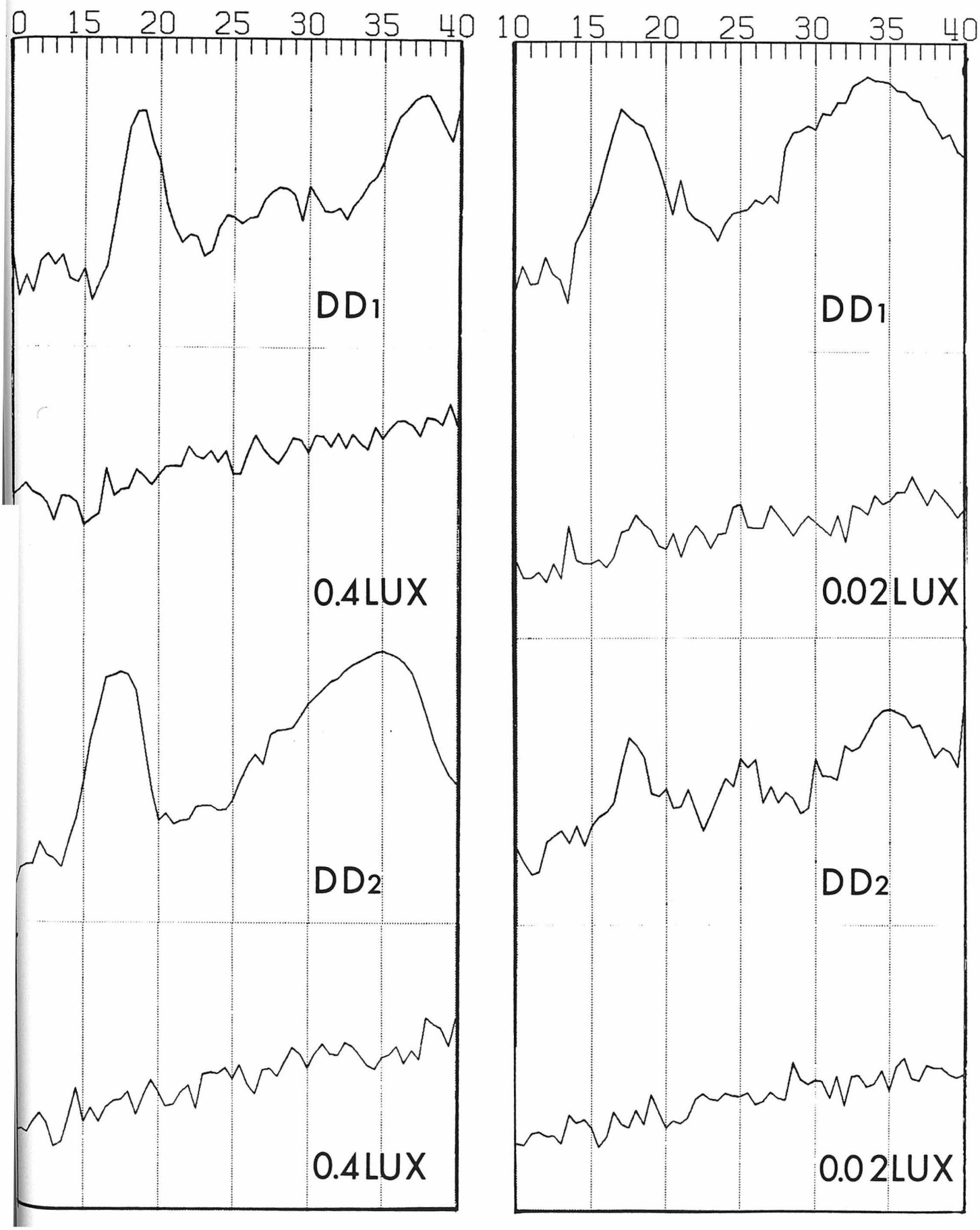

2101153
0 15 20 25 30 35 40
DD1
0.4LUX
DD2
0.4LUX
2001238
10 15 20 25 30 35 40
DD1
0.02LUX
DD2
0.02LUX

Figure 4-10. *Comparison of the period vs. light intensity relationship of the five strains of D. Melanogaster studied against three different models of clock photophysiology.*

(a) Behavior of a long-period mutant (L) and a short-period mutant (S), compared to a wild-type strain (W), derived from the assumption that the genetic effects and the light effects act completely independently in changing the periodicity of the clock.

(b) Behavior of long and short period mutants under the assumption that the mutations affect the part of the oscillating process which is coupled to the photoreceptor in a way which is detailed in the text.

(c) Behavior of long and short period mutants under the assumption that the mutations affect the photoreceptor system in such a way that its output to the oscillating mechanism is changed.

(d) Idealized summaries of the actual behavior of the five different clocks studied in this paper. (The asterisks in all graphs represent the point at which unstable to arrhythmic behavior is observed.) *Symbols*: 6=Clk^{KO6}; A= *And* ; L1=per^{l1}; S=per^{s}; W=Wild-type

POSSIBLE INTERACTIONS OF LIGHT & CLOCK MUTATIONS

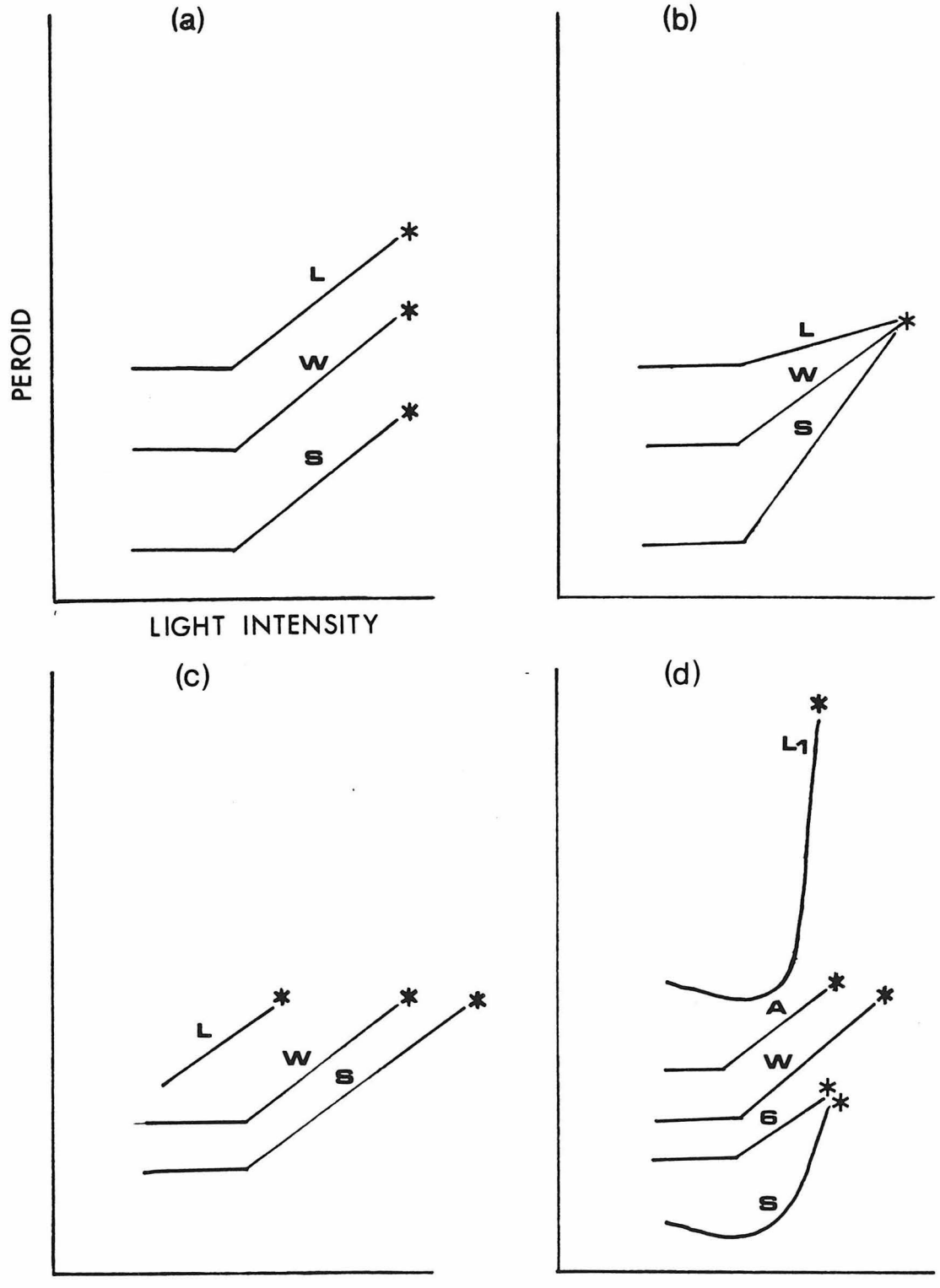
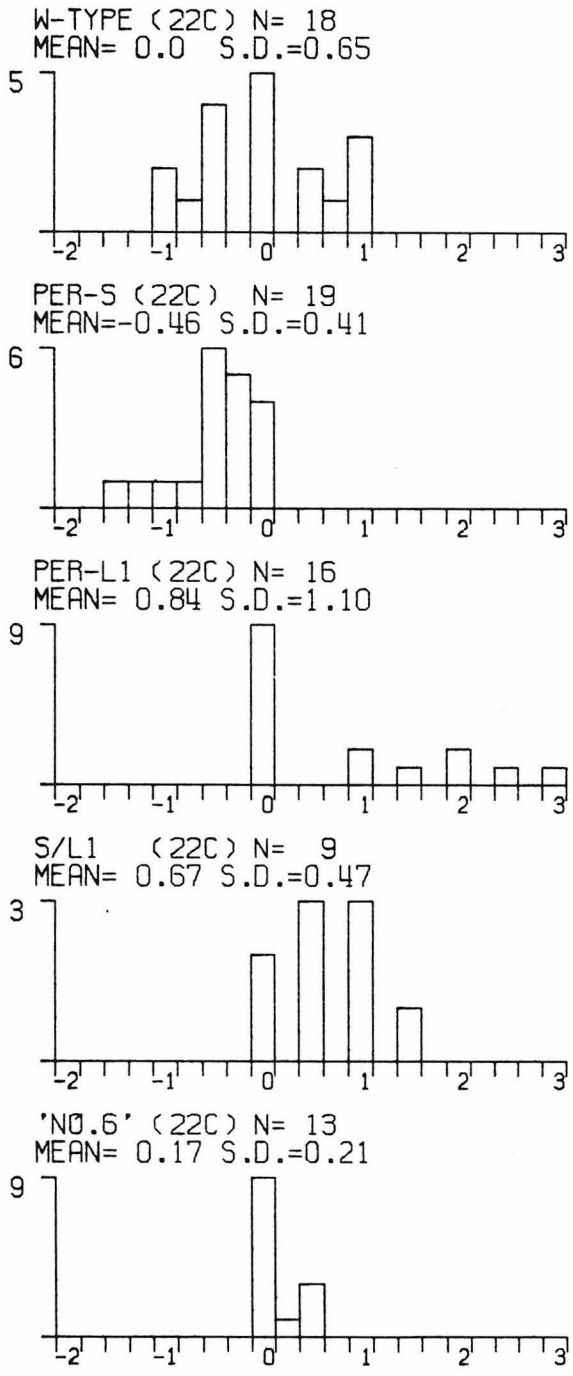


Figure 4-11. ***Reciprocal behavior of per^s and per^{l1} in very dim light.*** Periods are estimated by periodogram and binned at 15 minute intervals. Data are taken from those animals in fig.4-3 and fig.4-10 which are exposed to LL_1 levels of 0.07 lux and below.

CHANGE ØF PERIØD AT LL1 LEVEL UNDER 0.07 LUX



Chapter 5

Concluding remarks

We mentioned in chapter 1 that one goal of the behavioral geneticist in his/er approach to the study of circadian clocks is to be able to isolate mutants that affect different *discrete functional units* of the clock so that, by studying the relationships of such mutations and by tracing the anatomical and physiological alternation underlying the changed behavior, one hopes to be able to 'dissect' circadian systems into subsystems that are more amenable to rigorous analysis with conventional biochemical and biophysical techniques. The job of the behavior geneticist is therefore three-fold: (1) creating mutants of relevant phenotypes; (2) obtaining hints at a behavioral level as to whether the observed mutant phenotype reflects alternation of certain components of the concerned behavior , or simply the results of some general, pleiotropic effects; and, (3) searching for the anatomical and physiological correlates for such altered behavior. The studies reported in this thesis attempted to carry out such an exercise with six clock mutations, located at three loci, on the X-chromosome of *D. melanogaster*.

Throughout the work of this thesis, we have one major question in mind : How does each of these clock mutants *not* work ? And, we seek answers to this question through four lines of observations; viz. how does a particular mutant clock behave (with comparison to the behavior of the normal clock, whenever appropriate) in its interaction with temperature, with light, with other clock mutations, and with mutations that have well established effects on the anatomy and physiology of the nervous system of the fruitfly.

In chapter 2, we found out that clock period can be changed either with or without the impairment of the temperature compensation mechanism. Because of the limited number of mutants studied, one cannot yet make the generalization of whether temperature compensation fails whenever a certain locus is mutated, or whenever the period length is changed to outside a certain limit. This is an important question that *can* be answered when more mutants are isolated. In chapter 3,

we find that both in wild-type *D. melanogaster* and in the clock mutant per^s, the mechanism of temperature compensation works on a point by point basis (to the resolution of the experiment) throughout the circadian cycle. What is still unknown is whether (1) the *same* mechanism is involved throughout the cycle, or (2) the cycle consists of different sequential processes, each of which -- in its own way -- provides temperature compensation in a point by point manner. The observations that the average period of the mutant per^{l1} changes from 27 hours at 17 ° to to 30 hours at 25 ° and yet the phase-delay portion of the phase-resetting curve of the same mutant remains at about 6 hours at the two temperatures suggest that the second possibility is the case. Unfortunately, the resolution of our experimental protocol, plus the vast inter-individual variations in per^{l1}, prevents us from establishing, for this mutant, whether the phase at which temperature compensation is impaired is the same phase which is found to be differentially lengthened when compared to wild-type. The resolution of this issue awaits new experimental designs, which should include finding more phase reference points for the *Drosophila* clock.

Interactions of the mutant effects and light are sought for in two forms: brief, bright light perturbations and tonic, dim illumination. Phase response curves from the brief light pulse studies indicate the function of the *per* locus may be predominantly involved in the 'subjective day' of the fruitfly -- a suggestion that these mutations are indeed affecting a *functional unit* of the clock. A surprising result that comes out of this study is the 'type-1' nature of the per^s locomotive rhythm PRC even though the corresponding PRC for the eclosion rhythm has been shown twice before (ref.5-1,ref.5-2) to be of 'type-0'. Whether this disparity reflects different properties of the pupal and adult clocks or a population artifact in the eclosion rhythm assay can be distinguished by monitoring the activity rhythm of adult individuals which were transferred into free run and received the perturbing light pulse *in their pupal stage*. [1] The dim light studies indicate that all of the four

mutants studied, *And*, Clk^{KO6}, per^{s}, and per^{l1}, do *not* change the period of the clock by mimicking the effect of light through changes of the clock photoreceptor output. But there is no reason that some future mutants should not fall into this group. Besides testing for the dim light response of clock mutants isolated in darkness, one can also start screening for mutants with abnormal response in dim light. The use of such mutations, in conjunction with mutations that cause known effects on the nervous system of the fruitfly, would be a useful tool to 'dissect' the circadian clock in this animal.

The genetic interaction studies show that, while both the interaction between the *And* locus and the *per* locus and that between the *And* locus and the Clk^{KO6} locus are either very weak or non-existent , the interactions between the different alleles of the *per* locus are very strong. Based on observations on these interactions and on the pattern of inter- and intra- individual clock stabilities, we have proposed to arrange the phenotypes of the *per* mutants into a sequence that seems to reveal a certain stochastic feature of the circadian clock. Further isolation of mutants at this locus to consolidate or invalidate this sequence should provide useful insights for understanding the basic clock mechanism in *Drosophila*.

While our use of the eyeless and ocelli-less mutant (*sine oculus*) and ERG-defective mutant (*norpA*) indicates that extraretinal clock photoreceptor in the fruitfly is sufficient to mediate all the dim light responses, the combination of the *sine oculus* mutation with the per^{s} and per^{l1} mutations, respectively, reveals that when certain element(s) in the optic lobes of these two mutants are missing, the stability of the clock is clearly decreased -- both in terms of inter-individual variability of period lengths and in terms of the severity of impairment in the temperature

1. This experiment would require a 'parallel' protocol, in which the phase of control animals are compared with experimental animals, rather than the 'serial' protocol used throughout this thesis, in which the phase of an animal before experimental treatment is compared with the phase of the *same* animal after the treatment. In adult *D. melanogaster*, the 'parallel' protocol requires up to more than five times the number of animals to provide equivalent resolution as the 'serial' protocol. Yet, this experiment is entirely feasible with our current experimental set-up.

compensation mechanism. An anatomical investigation into this effect seems, at this point, both possible and justified. Another mutation that affects the nervous system of *Drosophila* and may prove helpful in understanding the circadian clock is nap^{ts} (ref.5-3). This mutation causes temperature-dependent, reversible blocking of nerve impulses. We have performed pilot experiments that indicate it is possible to use this mutant to ask whether action potentials are needed for light to phase shift the wild-type as well as mutant clocks. The use of these physiological and anatomical mutations, coupled with the techniques of generating histochemical mosiacs in the nervous system (ref.5-4) -- plus the hands and eyes of a good insect neuroanatomist -- will remain, in the opinion of the author, one of the most powerful tools available for the localization of the *Drosophila* clock.

In the research described in this thesis, we have employed the tools currently available to a behavioral neurogeneticist working with *Drosophila* to confront some very basic questions posed in the literature of circadian clock research. The answers we get, though far from complete, are encouraging in two ways. First, they show that the questions we posed are legitimate ones in the framework of the adult *Drosophila* activity rhythm. Secondly, they indicate that the tools we have are sufficient to answer many, if not all, of our questions. To do so, however, will require the availability of *many* more clock mutants than are currently available.

Chapter 5 References

5-1.Konopka,R.J. (1979) Fed. Proc. 38(12) : 2602 - 2605

5-2.Winfree A.T. & Gordon, H. (1977) J. Comp. Physiol. 122 : 87 - 109

5-3.Wu C.F.,Ganetzky B.,Jan L.Y.,Jan Y.N. and Benzer S. (1978) P.N.A.S. 75(8) 4047 - 4051

5-4.Kankel D.R. and Hall J.C. (1975) Dev. Biol. 48 : 1 - 24

6. Appendices

6.1 Animals

Wild-type. The activity rhythm of the following strains of wild-type flies have been studied : Canton-S, Leuserne-S, Oregon-R, Swedish-C. The rhythms of Canton-S and Leuserne-S are found to be most 'noise-free'. The control animals used throughout the experiments described in this thesis are, with no exception, Canton-S.

per^s. The original, no-marker stock from R. Konopka is used. Flies are kept as a homozygous stock.

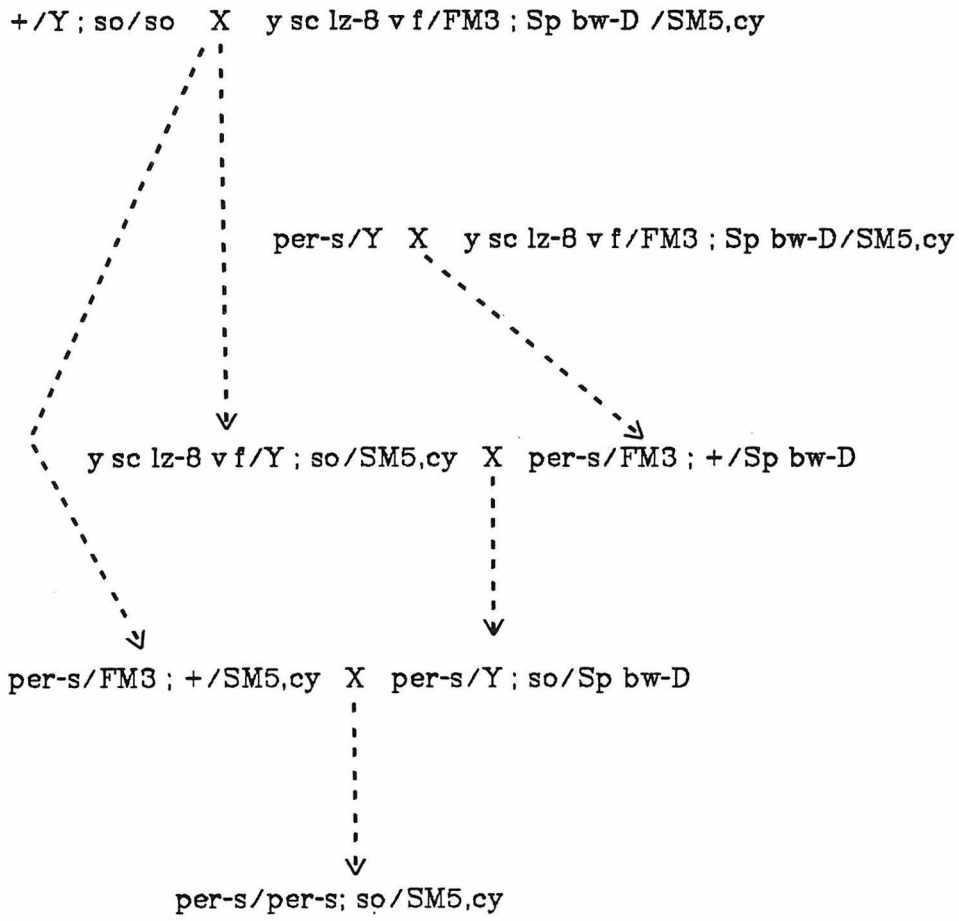
per^{l1}. The original, no-marker stock from R. Konopka is used. Male mutants are kept over *yellow forked attached*-*X* females.

per^{l2}. The original, no-marker stock from R. Konopka is used. Flies are kept as a homozygous stock.

Clk^{KO6}. Two different strains of this mutant, $Clk^{KO6}e$ and $Clk^{KO6}c$, have been used. Males used are taken mainly from the $Clk^{KO6}e$ strain while females (used in crosses) are from the $Clk^{KO6}c$ strain. $Clk^{KO6}e$ males are kept over *yellow forked attached*-*X* females while $Clk^{KO6}c$ is a homozygous stock.

And . This strain has been supplied by R. Smith and is the 'standard' no-marker, homozygous strain kept in the Konopka lab.

Eyeless and *Ocelli–less per^{s} stock.* The following scheme and stocks are kindly supplied by Steven Green.



where -X = superscript X

6.2 Intrumentation and Data Collection

Flies are placed inside transparent glass tubes (approximately 3" in length and 0.2" in inner diameter) filled at one end with sterilized and hydrated Catalina-424 fly food medium and the other end with a piece of adhesive tape (3M). One fly is placed in each tube. The tube is placed between an infrared source (from a MONSANTO ME-61 LED) and a FAIRCHILD FPT120 phototransistor placed behind Kodak Wratten gelatin (infra-red) filter (no. 87 C). 256 of such assemblies, each one called an 'activity channel', are maintained in two incubators (G.E. Model 806) at constant temperature. Thermometers are placed in various shelves of the incubators, which normally indicate that there are fluctuations of about one degree Centigrade at different parts of the incubators and over different times of the day. When a fly crosses the LED-phototransistor pair, the infrared light beam is interrupted and a count is registered. Channels are built to have a "dead" time of about one second to filter out spurious counts such as those due to repetitive flipping of wings. These counts are latched by electronic gates which are sampled and cleared every second by a laboratory micro-computer (Rockwell AIM-65). These accumulated counts are stored onto magnetic tapes at the end of every time bin, which are either 15, 30, or 60 minutes depending on the needs of the experiment. At the end of each experiment, data are entered into the campus IBM370/3032 computer for numerical and graphic analysis.

6.3 Light intensity level calibration

A wide range of light levels is obtained by exposing all the channels in rather uniform illumination from sets of G.E. cool- white fluorescent lamps inside the incubators and then covering each channel with black plastic lids with windows made of KODAK NO.96 Wratten gelatin filter of various N.D. values. Before or after each fly is run in a particular channel with a particular filter, light level inside of the plexiglass or glass tube is measured (with the infrared LED off)using a Pin-3DP (UNITED DETECTOR TECHNOLOGY INC.) photodiode with an active measuring area of 0.03 sq.cm. It is found that ,due to the geometrical relationship of the tube, the food, the window, and the not perfectly uniform fluorescent light source, there are some variations of light intensities at different positions of the tube. In a few extreme cases, intensity can vary up to a factor of 2 depending on whether a fly is in the shady or the bright part; but in the majority of cases such variations are well within 20%. Measurement from the Pin-3DP is then readily converted into watt/sq.cm. Since the conventional unit in the circadian literature is the 'lux', the Pin-3DP is calibrated against a Gossen Luna-Pro photographic system exposure meter operating in the 'incident' mode under various levels of illumination from a cool-white fluorescent lamp with SCR control. The conversion factor obtained under this system of measurements is : 1 lux = 30 nWatt/sq.cm.

6.4 Analysis of the locomotor activity of adult *D. Menlanogaster*

6.4.1 Activity Profiles

Fig.6-1 illustrates the essential problem encountered in the quantitative analysis of locomotor activity in *D. melanogaster* – that of the definition of phase reference points. The three records shown are examples of some extreme cases. The top record represents an activity profile (from an *And* mutant) that has sharp onsets but relatively ill-defined offsets. The middle record shows an animal (per^s/per^{l1}) with both sharp onsets and offsets. The bottom record shows a profile which is very representative of the per^{l1} mutants, which consists of bursts of activity spread throughout the subjective day followed by an intensely active duration of a few hours before a sharp cutoff of activity. This 'heavy band of activity followed by sharp offset' feature is observed in most (over 80%) of the animals used in this study, except that the 'bursty' nature of the activity profile is much more often observed in the per^{l1} and per^{l2} mutants.

6.4.2 Definition of Phase Reference Points

Fig.6-2 gives a graphical description of how phase reference points are defined. The animal involved, a per^s mutant, had been reared at 22°C. and at about 200 lux, and was transferred into freerun in constant darkness at 22°C. at the point where the activity record shown begins. The records are artificially broken into two segments at a point in time when the animal was given a light pulse of 1 minute (2000 lux). Data are collected in 30 mins bin.

In figure (a), normalized counts of activity are plotted against time. Tick marks represent cycles of 19 hours. The slanted, wiggly lines below each cycle of activity is a normalized plot of cumulative counts of that cycle. The set of 11 parallel dotted lines are spaced 0.1 unit apart, starting from 0.0 and ending at 1.0 and is used to help visualize phase reference points. For example, the intersection of one of the

slanted, wiggly lines and the 6th dotted line will give the time at which the median of the activity counts of that cycle (labelled as 'M' in the figure), and the intersection between the wiggly line and a imaginary line drawn half-way between the 10th and 11th lines would give the time at which 95% of the activity of that cycle has occurred. These latter points (labelled as 'O' in the figure) define the term 'activity offsets' in this study.

In figure (d) is a simulated actogram plot of the activity rhythm. This is accomplished through a computer program that plots out each count of digital record as a tick mark in a fashion similar to the 'Estaline-Angus' plots that are used by earlier investigators in the field. Whereas, in the 'Estaline-Angus' plot, each tick registers the exact time at which a count is recorded, in the simulated plot such precision is lost for counts within a bin --- such counts are represented as ticks uniformly spread out within the intervals of a bin, creating a slightly sharper contrast of activity and rest than a 'real' actogram would have provided. The inverted triangles shown on the actogram are the same time points that are labelled as 'O' in figure (a), while the circles shown are the same time points that are labelled as 'M' in figure (a). To evaluate which phase points give better cycle to cycle precision, a linear regression line is fitted to five consecutive phase points (either from the 2^{nd} to the 6^{th} cycles or from the 3^{rd} to the 7^{th} cycles) and the standard deviation of the fit, called the 'S' factor for that particular set of phase reference points, is taken as a measure of precision. For the record shown, the fit through the 'offsets' gives a deviation of about 5 minutes while the fit through the 'medians' gives a deviation of about 20 minutes. In figure (e), a relation between this value of standard deviation as a function of phase points fitted is given for this particular animal. A general inclination is observed in that the closer one approaches the offset, the better seems the fit. This observation is upheld by the results shown in figs. 6-3, 6-4, 6-5. Based on these results, we decided to choose the offsets of the activity rhythm as the most

precise phase reference points.

6.4.3 Estimation of Period Lengths

Two methods are used for the estimation of period in this study. The first is the method of periodogram (see ref.6-1 for explanation of this widely used method). Very briefly, the series of binned data are arranged in order in rows with N elements, where N is the trial period (in unit of the bin width). A mean value is calculated for each of the N columns so formed and a standard deviation calculated for the column means. A normalized plot of such standard deviations as a function of N (where N in our cases varies from about 5 to 200) constitute a periodogram. For experiments in which the time bin is 15 or 30 mins., the period measurement is made with 15 or 30 min. resolution, respectively. For cases where the time bin is 1 hour, the resolution of measurement is kept to 30 min. by reference to the "second harmonic" peak in the periodogram; e.g. a rhythm that has a period of 19.5 hrs. will show approximately equal amplitudes at 19 and 20 hrs. but a clear peak at 37 hrs. When resolution of more than the minimum bin width of 15 minutes is required, as in the construction of the PRC's in Chapter 3, period is estimated by the slope of a linear regression line through 5 consecutive offsets.

6.4.4 Phase Distributions

Fig. 6-6 gives the phase (as defined by the projected *zeroth* offset) of the activity rhythm with respect to the constant light to constant dark transition for six strains at different temperatures. We note that maximum inter-individual differences vary from about 5 hours for per^s to about 10 hours for per^{l1}. It is not clear at this point how much of these variations reflect phase differences at the level of the circadian oscillators or of the driven rhythms. The fact that we are able to obtain rather precise PRC's by assuming that the overt phase differences reflect the state of the clock (see Chapter 3) indicates that the latter possibility is the case. Figure 6-7 illustrates

that these phase distributions can be drastically affected by the absence of the eyes, ocelli, and major parts of both optic lobes in the mutant *sine oculus per*^{s}.

Appendices References

6-1.Enright J.T. (1965) J. Theoret. Biol. 8 : 426 - 468

Figures for the Appendices

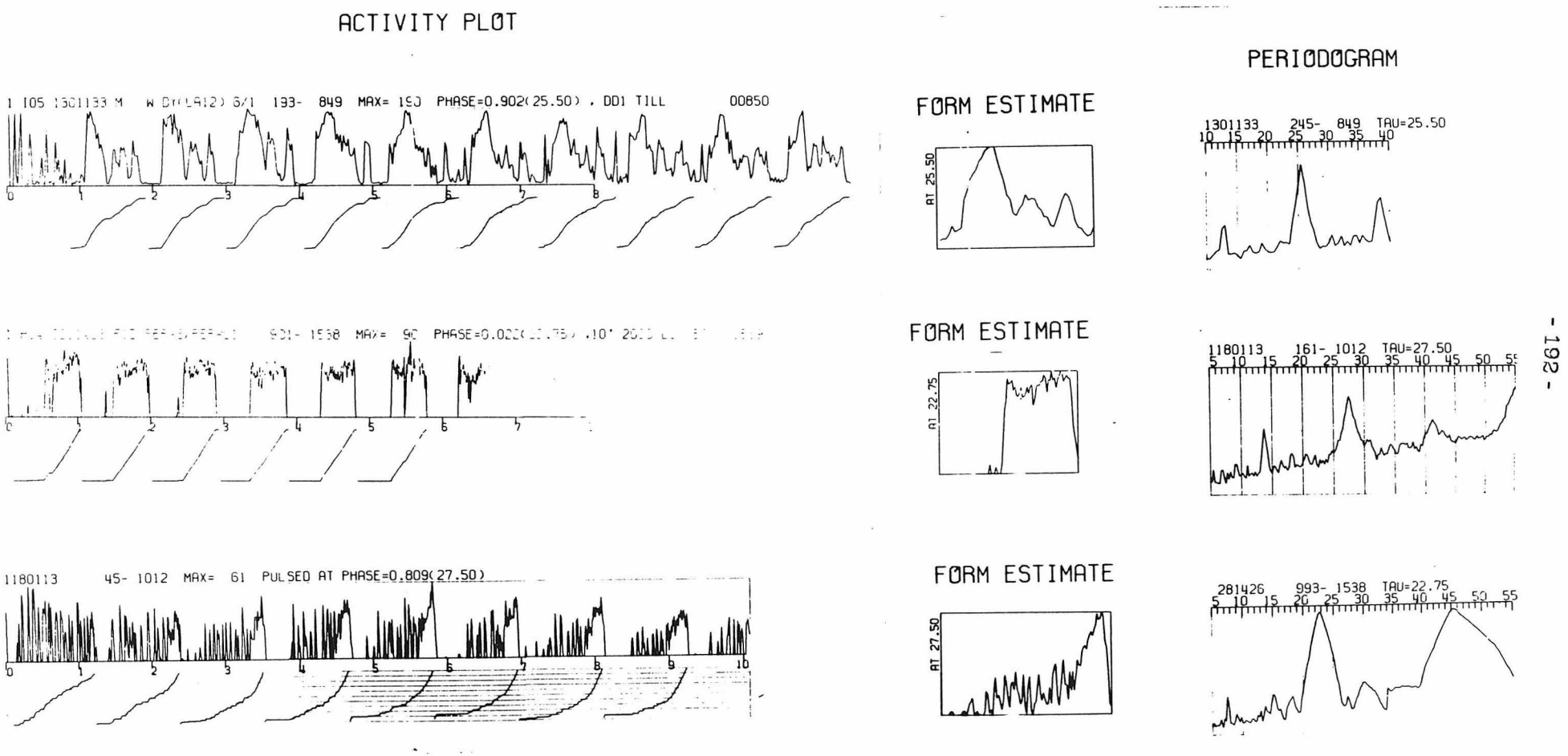
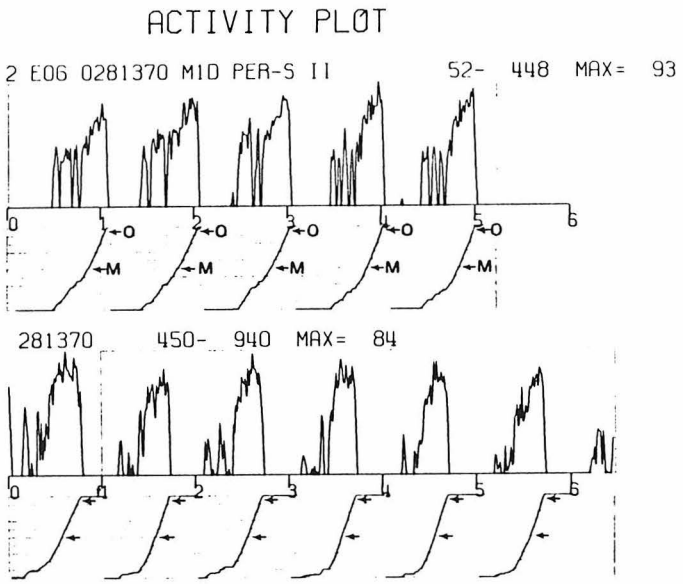
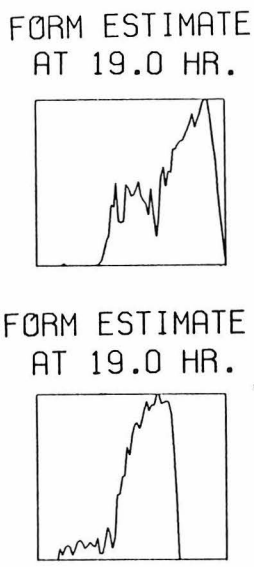
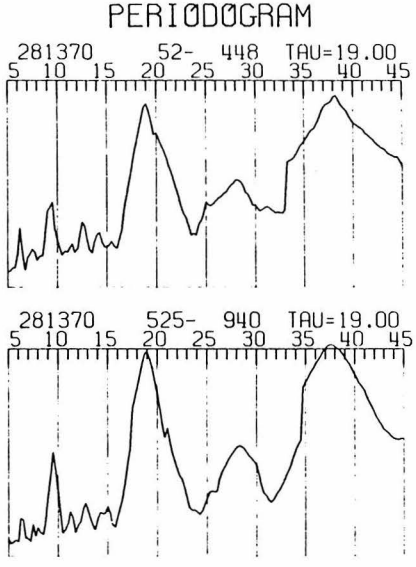
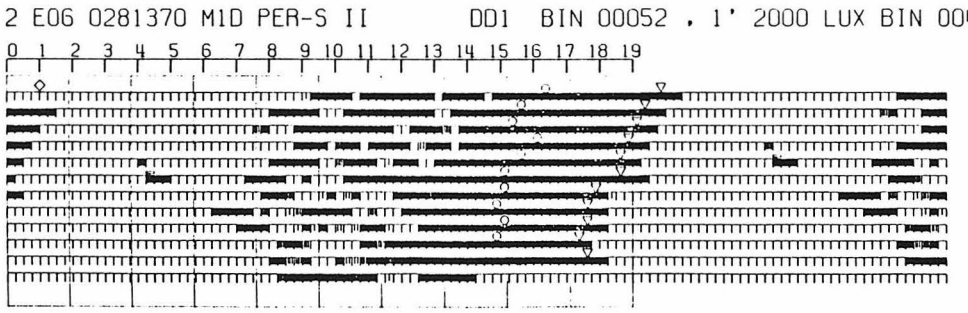

ACTIVITY PLOT
FORM ESTIMATE
PERIODOGRAM
1 105 1301133 M W DY(LR12) G/1 193- 849 MAX= 193 PHASE=0.902(25.50) , DD1 TILL 00850
AT 25.50
1301133 245- 849 TAU=25.50
10 15 20 25 30 35 40
FORM ESTIMATE
AT 22.75
1180113 161- 1012 TAU=27.50
5 10 15 20 25 30 35 40 45 50 55
1180113 45- 1012 MAX= 61 PULSED AT PHASE=0.809(27.50)
FORM ESTIMATE
AT 27.50
281426 993- 1538 TAU=22.75
5 10 15 20 25 30 35 40 45 50 55

Fig.6-1


ACTIVITY PLOT
2 E06 0281370 M1D PER-S II
52- 448 MAX= 93
281370
450- 940 MAX= 84


FORM ESTIMATE
AT 19.0 HR.
FORM ESTIMATE
AT 19.0 HR.


PERIODOGRAM
281370 52- 448 TAU=19.00
281370 525- 940 TAU=19.00


SIMULATED ACTOGRAM PLOT
2 E06 0281370 M1D PER-S II DD1 BIN 00052 . 1' 2000 LUX BIN 00449

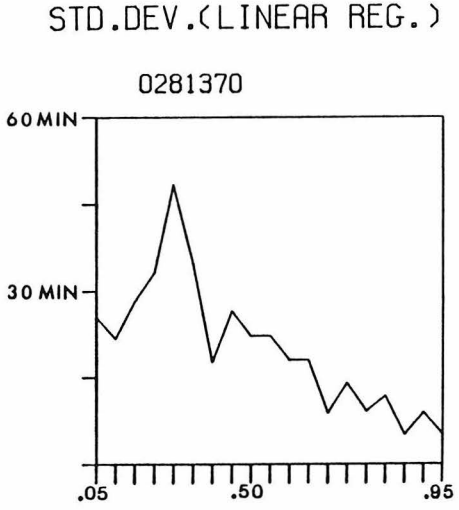

STD.DEV.(LINEAR REG.)
0281370
60 MIN
30 MIN
.05
.50
.95

Fig.6-2

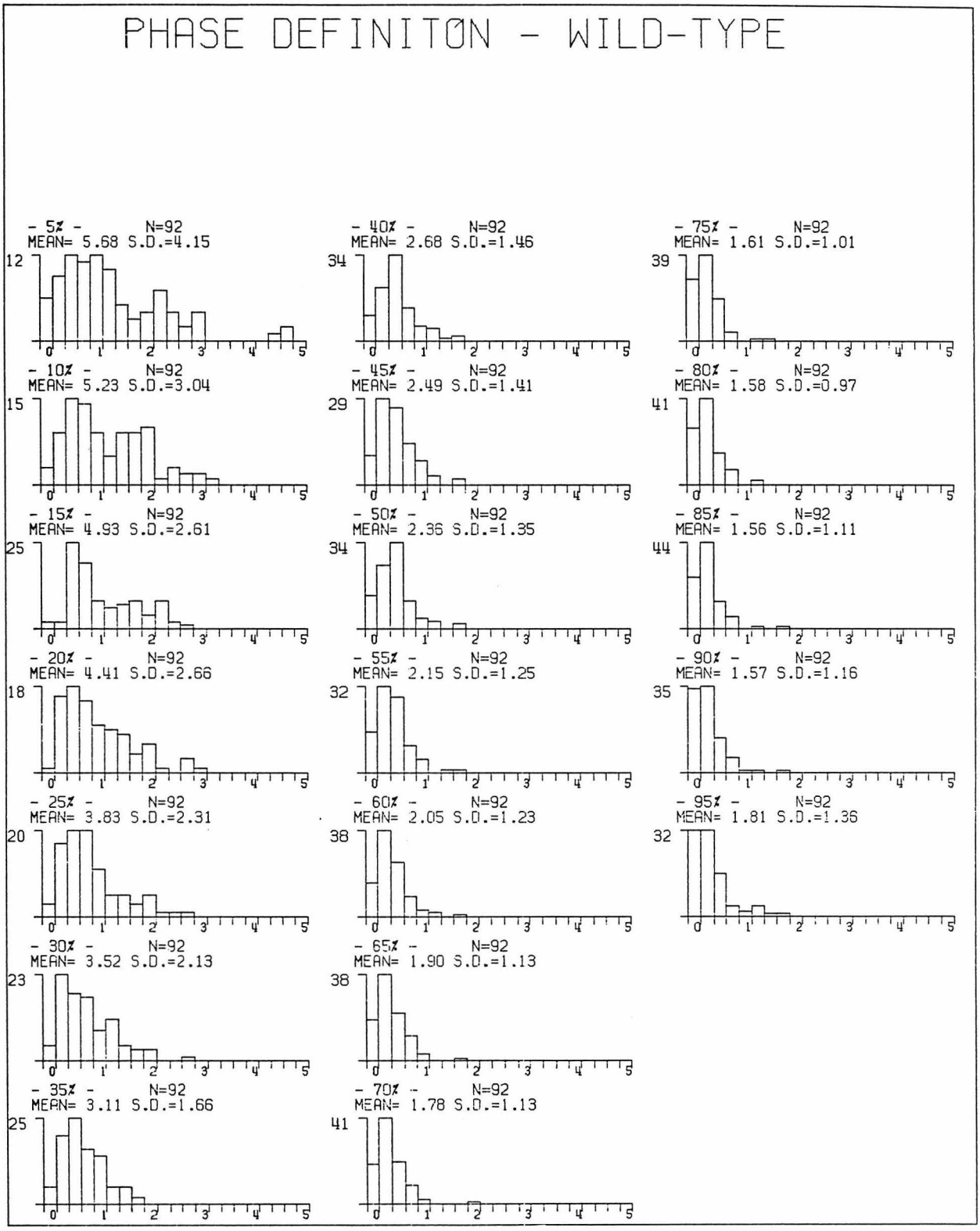

PHASE DEFINITØN - WILD-TYPE
- 5% - N=92
MEAN= 5.68 S.D.=4.15
12
- 10% - N=92
MEAN= 5.23 S.D.=3.04
15
- 15% - N=92
MEAN= 4.93 S.D.=2.61
25
- 20% - N=92
MEAN= 4.41 S.D.=2.66
18
- 25% - N=92
MEAN= 3.83 S.D.=2.31
20
- 30% - N=92
MEAN= 3.52 S.D.=2.13
23
- 35% - N=92
MEAN= 3.11 S.D.=1.66
25
- 40% - N=92
MEAN= 2.68 S.D.=1.46
34
- 45% - N=92
MEAN= 2.49 S.D.=1.41
29
- 50% - N=92
MEAN= 2.36 S.D.=1.35
34
- 55% - N=92
MEAN= 2.15 S.D.=1.25
32
- 60% - N=92
MEAN= 2.05 S.D.=1.23
38
- 65% - N=92
MEAN= 1.90 S.D.=1.13
38
- 70% - N=92
MEAN= 1.78 S.D.=1.13
41
- 75% - N=92
MEAN= 1.61 S.D.=1.01
39
- 80% - N=92
MEAN= 1.58 S.D.=0.97
41
- 85% - N=92
MEAN= 1.56 S.D.=1.11
44
- 90% - N=92
MEAN= 1.57 S.D.=1.16
35
- 95% - N=92
MEAN= 1.81 S.D.=1.36
32
0 1 2 3 4 5

Fig.6-3

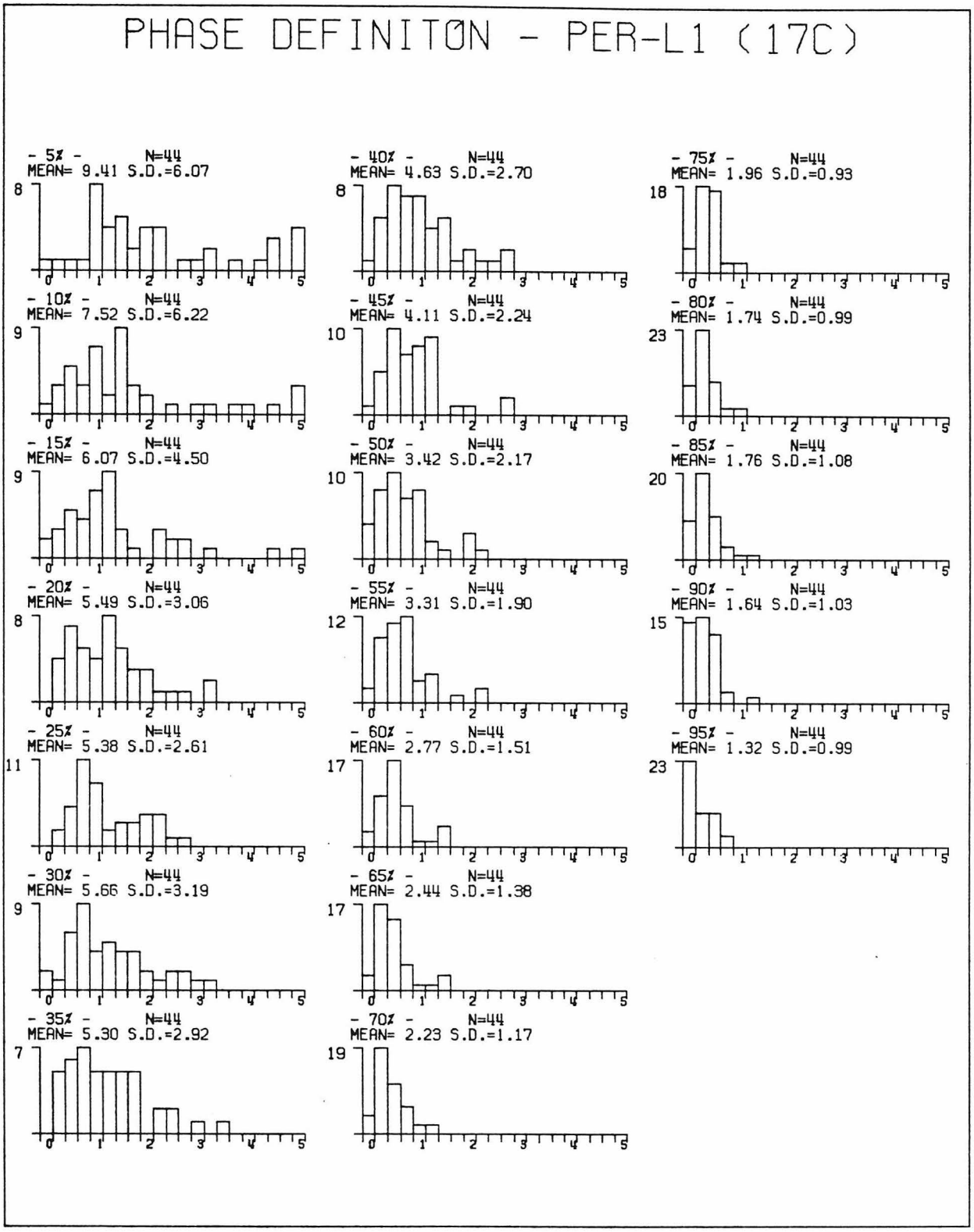
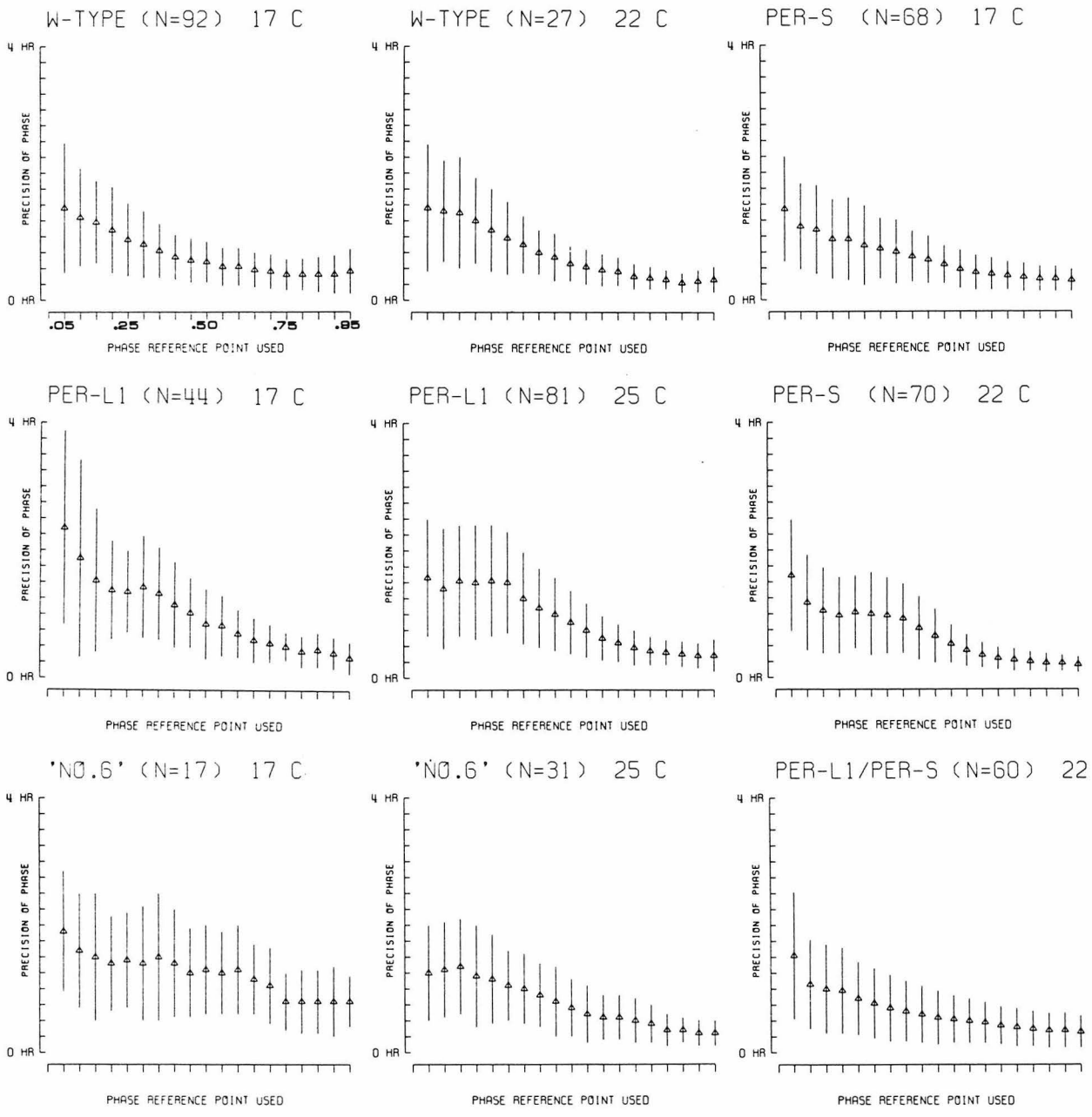

PHASE DEFINITON - PER-L1 (17C)
- 5% - N=44
MEAN= 9.41 S.D.=6.07
- 10% - N=44
MEAN= 7.52 S.D.=6.22
- 15% - N=44
MEAN= 6.07 S.D.=4.50
- 20% - N=44
MEAN= 5.49 S.D.=3.06
- 25% - N=44
MEAN= 5.38 S.D.=2.61
- 30% - N=44
MEAN= 5.66 S.D.=3.19
- 35% - N=44
MEAN= 5.30 S.D.=2.92
- 40% - N=44
MEAN= 4.63 S.D.=2.70
- 45% - N=44
MEAN= 4.11 S.D.=2.24
- 50% - N=44
MEAN= 3.42 S.D.=2.17
- 55% - N=44
MEAN= 3.31 S.D.=1.90
- 60% - N=44
MEAN= 2.77 S.D.=1.51
- 65% - N=44
MEAN= 2.44 S.D.=1.38
- 70% - N=44
MEAN= 2.23 S.D.=1.17
- 75% - N=44
MEAN= 1.96 S.D.=0.93
- 80% - N=44
MEAN= 1.74 S.D.=0.99
- 85% - N=44
MEAN= 1.76 S.D.=1.08
- 90% - N=44
MEAN= 1.64 S.D.=1.03
- 95% - N=44
MEAN= 1.32 S.D.=0.99

Fig.6-4


W-TYPE (N=92) 17 C
W-TYPE (N=27) 22 C
PER-S (N=68) 17 C
PER-L1 (N=44) 17 C
PER-L1 (N=81) 25 C
PER-S (N=70) 22 C
'NØ.6' (N=17) 17 C
'NØ.6' (N=31) 25 C
PER-L1/PER-S (N=60) 22
4 HR
0 HR
PRECISION OF PHASE
.05
.25
.50
.75
.95
PHASE REFERENCE POINT USED

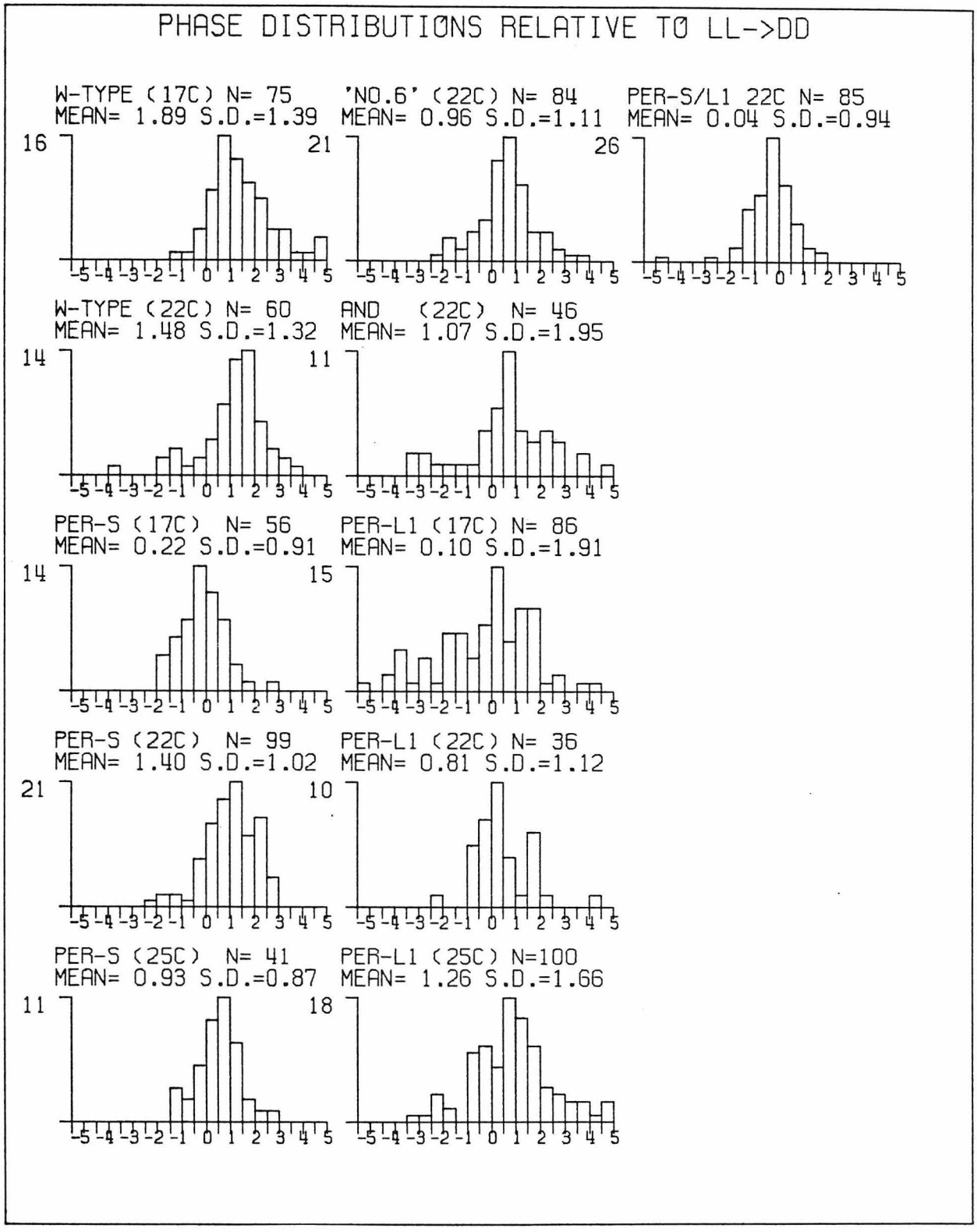

PHASE DISTRIBUTIONS RELATIVE TO LL->DD
W-TYPE (17C) N= 75
MEAN= 1.89 S.D.=1.39
'NO.6' (22C) N= 84
MEAN= 0.96 S.D.=1.11
PER-S/L1 22C N= 85
MEAN= 0.04 S.D.=0.94
W-TYPE (22C) N= 60
MEAN= 1.48 S.D.=1.32
AND (22C) N= 46
MEAN= 1.07 S.D.=1.95
PER-S (17C) N= 56
MEAN= 0.22 S.D.=0.91
PER-L1 (17C) N= 86
MEAN= 0.10 S.D.=1.91
PER-S (22C) N= 99
MEAN= 1.40 S.D.=1.02
PER-L1 (22C) N= 36
MEAN= 0.81 S.D.=1.12
PER-S (25C) N= 41
MEAN= 0.93 S.D.=0.87
PER-L1 (25C) N=100
MEAN= 1.26 S.D.=1.66
-5 -4 -3 -2 -1 0 1 2 3 4 5

Fig.6-6

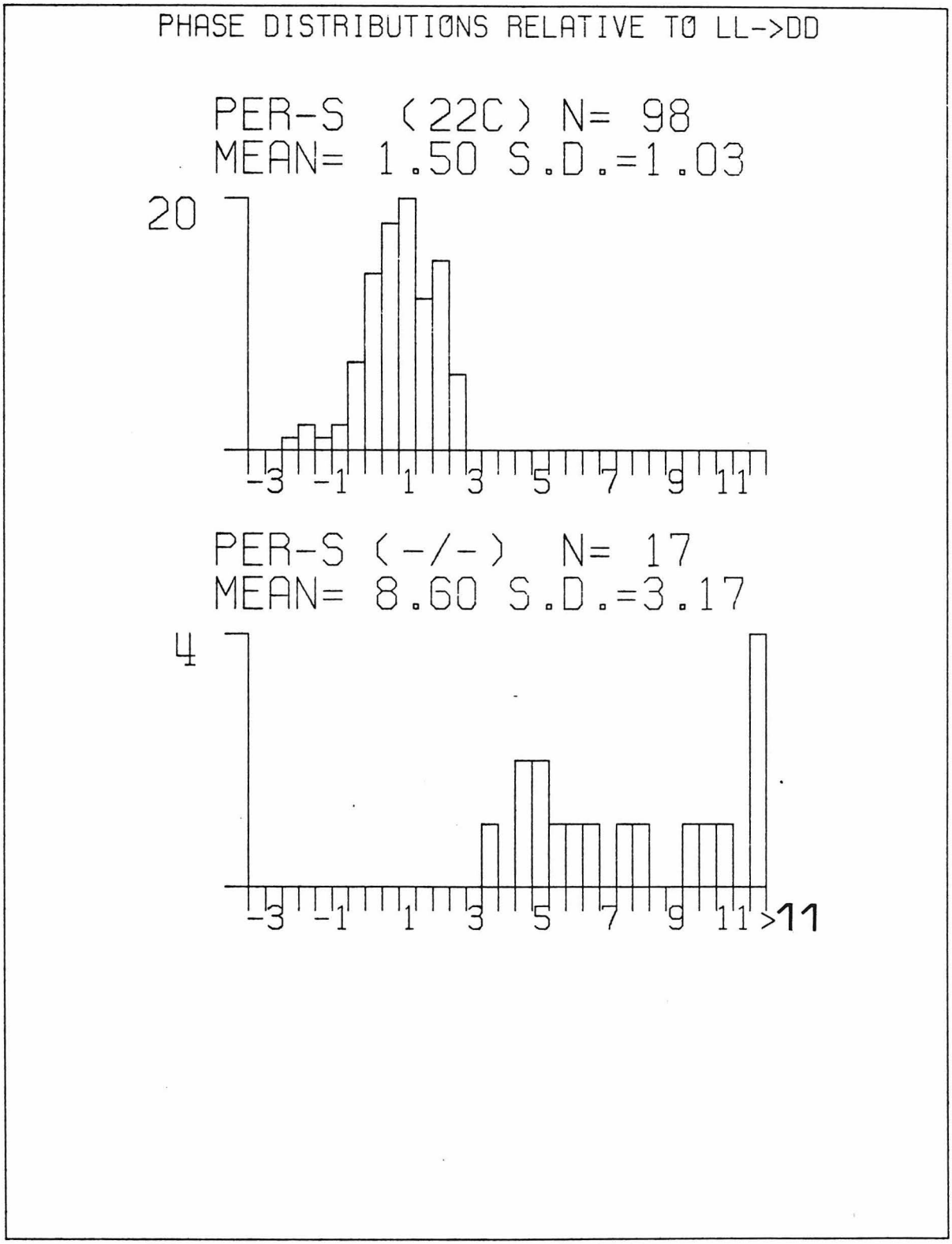

PHASE DISTRIBUTIONS RELATIVE TO LL->DD
PER-S (22C) N= 98
MEAN= 1.50 S.D.=1.03
20
-3
-1
1
3
5
7
9
11
PER-S (-/-) N= 17
MEAN= 8.60 S.D.=3.17
4
-3
-1
1
3
5
7
9
11
>11

Fig.6-7