is probably between the 70th and 90th days of gestation. This is an especially interesting period in the development of the fetal lamb since it is during this period that it is developing immunological responsiveness. Ovine fetuses are able to reject skin homografts after the 77th day of gestation, but not before; it should be remembered, however, that the time of onset of the ability of the lamb to respond immunologically varies with the antigenic stimulus (*5*).

Similarly, the observations on mice, and especially those obtained when low-passage virus was used, indicate that during the interval from 1 to 10 days after birth the response of the nervous system changes from one that is mainly a necrotizing lesion to one that is mainly a nonsuppurative meningoencephalitis. During this period of 1 to 10 days after birth, there is considerable development of the ability of mice to respond immunologically (*6*).

The time at which bluetongue virus is first able to provoke an immunological response in sheep and mice is not known. However, it is an attractive hypothesis that the nervous systems of immunologically immature sheep and mice respond to bluetongue virus by developing congenital anomalies or a very necrotizing lesion that may lead to such defects, whereas the nervous system of animals that have considerable immunological maturity responds with an inflammatory lesion—a nonsuppurative meningoencephalitis. It has been suggested recently that viral infections of the nervous system can produce lesions ranging from those that are largely neuronal damage to those that are largely inflammatory and that the latter seems to be associated with partial immunity (*7*). To some extent our observations support these suggestions, not in terms of acquired antibodies, but in terms of the development of the immune responsive state.

While of uncertain significance, it is interesting that the cerebral anomalies or acute necrotizing encephalitis occurred before myelination, which begins in the forebrain of the fetal lamb at 96 days (*8*) and in mice at 10 to 15 days after birth (*9*).

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Natural Free-Running Period in Vertebrate Animal Populations

Abstract. *Regression analysis (analysis of covariance) is contrasted with the conventional "mean period length" for estimating the length of period of the spontaneous activity frequency (free-running period) in population samples of Gila monsters* (Heloderma suspectum) *and kangaroo rats* (Dipodomys merriami) *in the Sonoran Desert. The mean period length in each population does not differ significantly from 24:00 hours* ($P > .05$) *and it does not differ significantly* ($P > .05$) *between the species studied; the probability that the free-running period (in constant dark) in natural populations of Gila monsters and kangaroo rats is different from 24:00.0 is less than 1 in 1000* ($P < .001$). *The so-called "mean period length" is of little or no use for precise determination of the period and phase relationships in circadian rhythms; moreover, it is entirely without value for statistical testing of differences either within or between populations.*

It is assumed that the data on circadian rhythms represent evolutionary adaptations through genetic mechanisms involving natural selection operating in natural populations, regardless of whether ultimate control of the clock is endogenous, exogenous, or both. It is surprising, therefore, that relatively little attention has been given to sampling the nature of circadian rhythms in *natural* populations of vertebrate animals, and that sophisticated statistical treatment of time series in the circadian activity cycles of samples of animals in general is virtually wanting.

During the Cold Spring Harbor symposium on biological clocks in 1960, Sollberger (*1*) suggested that appropriate statistical methods might be developed for analyzing "such typically evolutive time series as biological rhythms." Of course, powerful statistical methods have been available for analyzing some aspects of circadian rhythms, especially for the data reduction and analysis of measurements of the spontaneous free-running period of animals that lend themselves well to accurate recording of precise cyclic events. But, unfortunately, here as elsewhere in the study of biological rhythms these often necessary methods have remained essentially unused.

Perhaps the hesitance of some authors to use sophisticated statistical measures to describe and test group characteristics of activity rhythms is a continuing reaction to early misuse of statistical procedures by some workers who subjected relatively raw data to excessive manipulation in attempts to demonstrate the mere existence of certain rhythms. This flagrant misuse prompted one worker to describe a mythical rhythm in the unicorn, based on data taken from a table of random numbers (*2*). While we are aware of the dangers of overmanipulation so dramatically pointed out by Cole's article, these dangers should not be permitted to frighten workers away from using the highly legitimate and powerful statistical procedures described in this paper. We refer to regression analysis and to the concepts and techniques of the analysis of variance applied to problems in linear regression, namely, the analysis of covariance (*3–5*).

Regression methods for calculating and comparing free-running periods should replace the inaccurate "mean period length" (*6*) that is currently conventional for analysis in investigation of circadian rhythms (*7*). The value for "mean period length" is dependent only on the first and last points of a series. To illustrate, if t_1, t_2, t_3, $\cdots$ t_n are the time of initiation of activity on successive days, then the "mean period length" is usually calculated by taking

$$t = \frac{(t_2 - t_1) + (t_3 - t_2) + \cdots (t_n - t_{n-1})}{n - 1}$$

which of course reduces simply to $(t_n - t_1)/(n - 1)$. Thus the intermediate points (t_2 through t_{n-1}), that is, the majority of days, are not even included much less evaluated in the analysis. While simple in application, this method gives values that are erroneous to a

greater or lesser magnitude depending on the degree of scatter of the unaccounted points (days) of initiation or cessation of activity between day 1 and day *n*. Accordingly, except for exceptionally good time-keepers, "mean period length" is of little or no use for precise calculation of the period and phase relationships in circadian and other activity cycles; moreover, it is entirely without value for statistical testing of differences within or between populations, since statistically useful estimates of the variances of the mean period length or their differences are not available.

DeCoursey's (*8*) excellent recordings of activity cycles in the flying squirrel *Glaucomys volans* and Rawson's (*9*) equally elegant data for the deer mouse *Peromyscus leucopus* are representative of many similar investigations that provide valuable raw data but in which application of appropriate quantitative methods for reduction and analysis are lacking, as is an experimental design for testing the actual situation in the natural population from which the experimental animals were drawn. The desirability of appropriate regression analysis in such studies is self-evident (Fig. 1). And in the absence of the probability values, wholly needless confusion may ensue, such as argument over whether a particular type of animal is characterized by a circadian rhythm of less than or greater than 24 hours when the difference from 24:00 is not significant.

Table 1. Data reductions (regression analysis) for circadian activity cycles of Gila monsters and kangaroo rats determined experimentally directly after their removal from natural populations in the Sonoran Desert. Range, mean, and standard deviation of the slope, and 95 percent confidence interval of the free-running period (hours). DD, constant dark; LL, constant light.

Light regime	Animals (*N*)	Periods (*N*)	Range (hours)	Mean and S.D. of slope (hours)	95% confidence interval (hours)
			Gila monster (Heloderma suspectum)		
DD	22	126	22:50.4 to 25:30.0	23:57.5 ± 07.7	23:43.3 to 24:12.7
LL	16	82	23:32.5 to 25:34.5	24:28.9 ± 11.6	24:05.8 to 24:52.0
			Kangaroo rat (Dipodomys merriami)		
DD	63	358	23:38.9 to 24:36.2	23:59.5 ± 00.8	23:58.0 to 24:01.0

Some appropriate data reductions, and inferences based on the derived probability (*P*) values, are given here for the spontaneous activity frequency (= natural free-running period) in natural populations of kangaroo rats and Gila monsters in the Sonoran Desert.

Kangaroo rats. The data in Table 1 and Figs. 2 and 3 for the nocturnal kangaroo rat *Dipodomys merriami* at Tucson, Arizona (lat. 32°15′N, long. 110°55′W), collected randomly throughout the year (no seasonal difference), reveal the free-running period (NP) in constant dark (DD) to vary in *individuals* ($N = 63$) in the natural population from 23:38.9 to 24:36.2; $N = 27$ with NP < 24:00, $N = 36$ with NP > 24:00 (*10*). It is clear that at the *population* level, the mean natural period of *Dipodomys merriami* is not significantly different from 24:00; the probability that it is different from 24:00 is less than 1 in 1000 ($P < .001$).

Some earlier estimates for the mean natural period of *Dipodomys merriami* in the same population were both less than and greater than 24 hours; they were based on small samples of individuals selected for good timekeeping, and then seemed to indicate a tendency for NP to be greater than 24:00 (in contrast to the condition reported by DeCoursey and others as "normal" for nocturnal animals). However with regression analysis of the larger ($N = 63$) and natural sample (unbiased for timekeeping and so forth) that was drawn from the same local population, it is clear that the genetic clock variation in the population is dispersed directly around the parameter 24:00 hours.

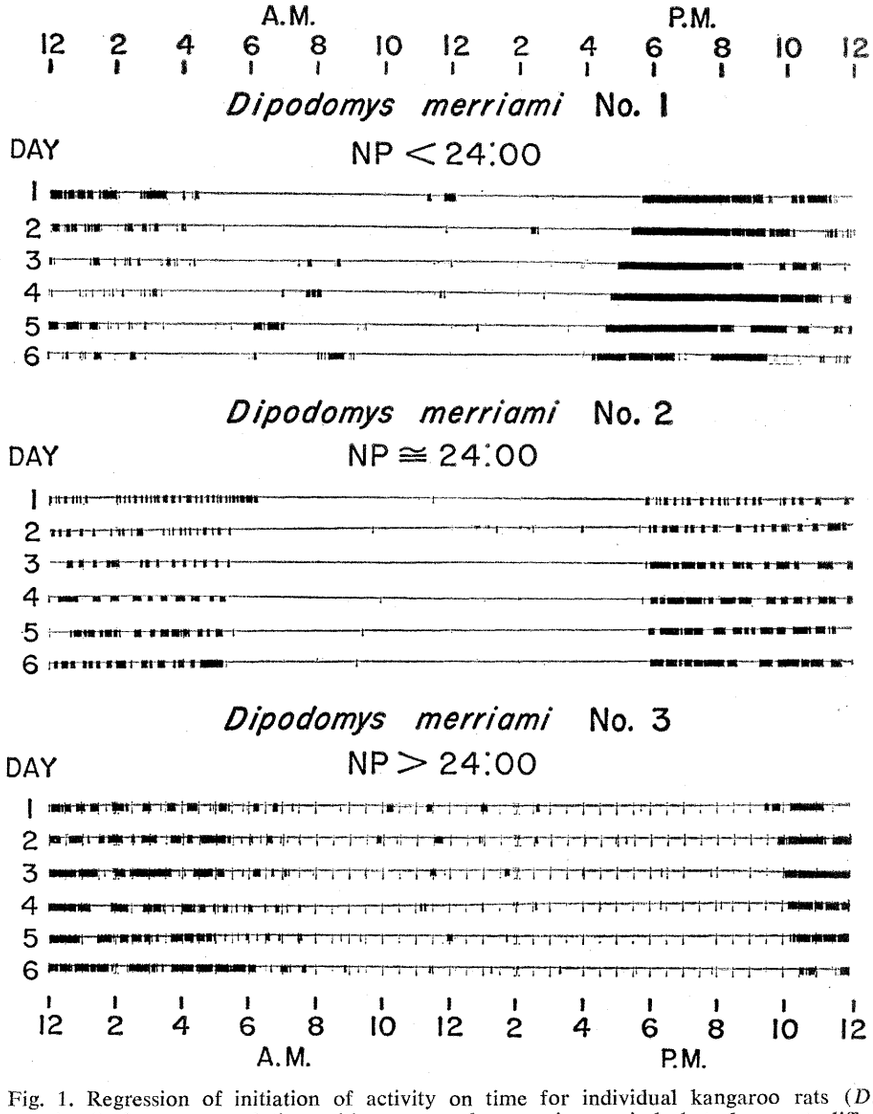

Fig. 1. Regression of initiation of activity on time for individual kangaroo rats (*D. merriami*) from a population with a mean free-running period that does not differ significantly from 24:00 hours (Table 1). The automatic record is from an Esterline-Angus strip chart recorder triggered magnetically by an activity wheel (*10*).

The values in Table 1 were calculated by the total regression method (*4*). By use of the common regression method (*4*) for the same data, the 95 percent interval becomes 23:59.0 to 24:01.0, with a mean of 24:00.0.

It is important to record here that, to estimate the natural (field) population parameter by calculating the mean natural period of the sample by regression methods, the 63 individuals constituting the population sample were brought to the experimental activity wheels during the same 24-hour period in which they were taken out of their natural desert habitat (live-trapped at night). The time of initiation of activity, under constant dark conditions, on each of the first 6 full days provided the initial points for calculation of the regression (activity time on day) for each of the individuals (Fig. 1, data in Table 1 for both kangaroo rats and Gila monsters). Data for longer periods of activity (to 20 days) were also reduced, yielding a mean natural period length of 24:00.4 ($N = 63$, same animals).

Calculation of the least-squares slope (regression coefficient) was standardized by coding both the days and the times of initiation of activity. Zero time for negative slopes (period less than 24 hours) represented the latest time of initiation in the series; for positive slopes (period greater than 24 hours), zero time represented the earliest time of initiation (Fig. 1).

Gila monsters. The data for *Heloderma suspectum* in constant dark (DD) are consistent wiht the hypothesis that the spontaneous free-running period is also not significantly different from 24:00 hours in this species (Table 1). The data are for a random sample of 22 individuals that was collected in the desert in the vicinity of Tucson, Arizona, throughout the active season (March–October). The statistical inference is the same whether the analysis of covariance is by total regression (95 percent confidence interval 23:43.3 to 24:12.7, Table 1), or by common regression where the 95 percent confidence limits are 23:47.1 and 24:07.1.

Under constant light (LL) equivalent in intensity to that during natural crepuscular activity in the habitat [10 ft-ca (110 lu/m^2)], the spontaneous free-running period of the Gila monster is clearly shifted to greater than 24 hours ($P < .05$, $N = 16$). While the Gila monster is primarily a light-active animal, and the seasonal light intensities encountered in the natural habitat are both relatively high (spring and autumn, diurnal) and relatively low (summer, crepuscular), it is clear that under constant light of low intensity the change in natural period (> 24 hours) is consistent with Aschoff's Rule (*11*) for nocturnal animals.

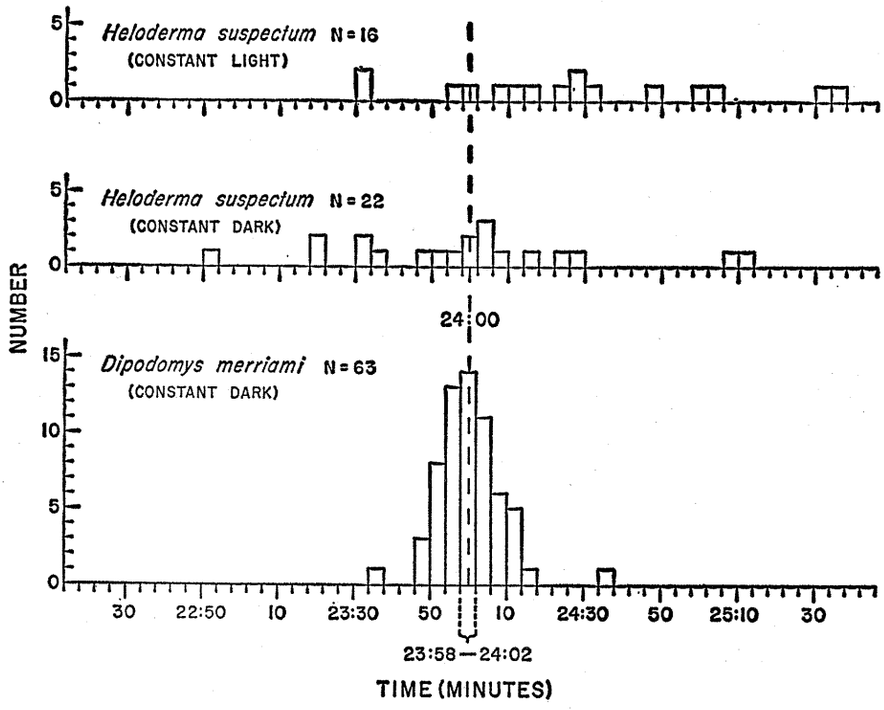

Fig. 2. Frequency distributions of the natural periods of Gila monsters and kangaroo rats under constant dark (DD) and constant light (LL) conditions as determined directly after their removal from natural populations. Data reductions in Table 1.

All of the Gila monsters (ectotherms) and kangaroo rats (endotherms) used in this work (Table 1) were collected in the same area of the Sonoran Desert. The difference between the means for period length, as determined by analysis of covariance (*3*, *4*), in these two vertebrate populations is not significantly different ($F = 0.173$, $P > .05$), and, as discussed above, neither period is significantly different from 24:00 hours (DD).

Gila monsters would be called "poor time-keepers" in a comparison with kangaroo rats for timekeeping ability (Fig. 1). In fact, the "best" *Heloderma* time-keepers are ordinarily worse (at both initiation and cessation) than the poorest *Dipodomys* time-keepers.

Thus when there is scatter about the regression line, and there usually is, the data should be properly reduced by appropriate regression methods and especially by an analysis of covariance. This becomes more important when the sample size is fairly large, as it should be in the study of population rate-functions, and when the sample also properly includes the "poor time-keepers" as well as "good time-keepers," as in our random samples of kangaroo rats and Gila monsters. Thus far in the study of spontaneous circadian activity periods, the free-running

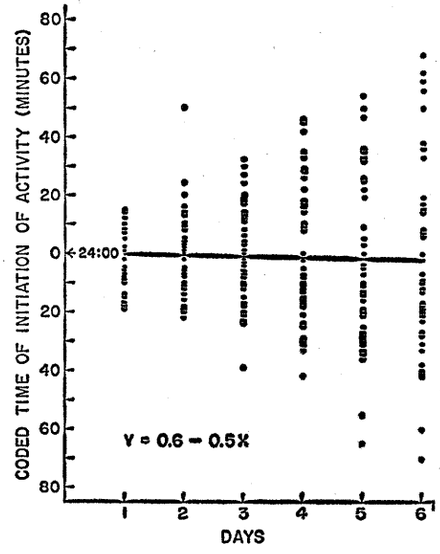

Fig. 3. Regression line ($b_{yx} = -0.5$) by analysis of covariance for 63 *Dipodomys merriami* in constant dark (DD); any point may represent more than one coded time of activity on days (1 to 6). Mean natural period is 23:59.5 ± 00.8 hours; the difference from 24:00 hours is not significant. Reduced data, with 95 percent confidence interval, are in Table 1.

period (*12*), it has not been comforting to observe inferences too frequently based on little or no actual quantitative analysis of a meaningful kind.

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6. The method of "mean period length," as termed here, was described by both DeCoursey (*8*) and Rawson (*9*).
7. See, for example, *Cold Spring Harbor Symp. Quant. Biol.* **25**, (1960).
8. P. J. DeCoursey, *Science* **131**, 33 (1960); see also, for example, *Cold Spring Harbor Symp. Quant. Biol.* **25**, 49 (1960).
9. See, for example, K. S. Rawson, *Cold Spring Harbor Symp. Quant. Biol.* **25**, 105 (1960).
10. The equipment in our laboratory is described in Sage *et al.* (*3*).
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Selective Forces in Papilio glaucus

Burns (*1*) presents data on selective mating bearing on the problem of mimetic polymorphism in *Papilio glaucus*. His data suggest the possibility that the females of the dark, mimetic form are less successful in mating than are those of the nonmimetic form, and he concludes that this selective disadvantage of the dark form is in some way balanced by the selective advantage of the dark form due to its mimicry of *Battus philenor*. He states, "Reproductive advantage [of the light form] and mimetic advantage [of the dark form] are opposed forces that may sustain female dimorphism. . . ."

It appears that Burns is suggesting that opposing selection in two different fitness components within one generation will result in a stable polymorphism. In fact, the fate of a gene in future generations is governed, as always, by the net fitness, which is the product of all fitness components within each generation. It is highly unlikely that the two fitness components in this case (survival and mating) just cancel so as to render the dark form neutral with respect to the light form. It would appear, therefore, that additional reasons must be sought for the evidently stable polymorphism in *P. glaucus* in the regions in which it is sympatric with its model, *B. philenor*.

Possible reasons are the continual migration of genes for light color into such sympatric regions from monomorphic light populations, or the often suggested hypothesis for mimetic polymorphisms of frequency-dependent selection in which the mimetic form is assumed to lose its former advantage when it gains sufficient abundance relative to its model, or both.

Burns's data on selective mating do play a role in this latter hypothesis since they explain the selective disadvantage of the mimic relative to the nonmimic which will become more important as the mimic loses its mimetic advantage (net) due to its increased frequency.

Perhaps it is this sort of scheme which Burns meant to indicate by his phrase "opposing forces." Even so, it would appear worthwhile to point out to his readers that a system of opposing fitness components cannot, by itself, produce a stable polymorphism.

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Prout suggests that I am concerned with opposed selective forces of reproductive advantage and mimetic advantage in *Papilio glaucus* (*1*) without sufficient regard for certain phenomena that are generally believed to accompany mimetic polymorphism. It seemed unnecessary in my short report to review the elements of mimicry theory, which date from the last century. That the relative numbers of model and mimic should affect the selective advantage of mimetic resemblance is self-evident.

I had thought this background involving frequency-dependent selection was satisfactorily indicated by sentence three of the abstract, paragraph two of the main text (particularly the statement that "The occurrence of the mimic . . . is correlated with the distribution and abundance of the model . . ."), and Table 1, which quantitatively documents major geographic variation in the relative frequencies of mimetic and nonmimetic morphs. I have explicitly discussed frequency-dependent selection in mimicry in an earlier paper (*2*).

Prout may be overstating the "stability" of the dimorphism, the "balance point" of which certainly shifts in space, probably fluctuates locally, and, in view of our nearly complete lack of quantitative data collected at the same place at different times, may be shifting unidirectionally in time. Contrary to widespread opinion, which I endorsed in stating (*1*) that "the dark morph . . . apparently . . . nowhere totally replaces the light morph . . . ," females of *P. glaucus* now appear to be virtually or quite monomorphic for dark in some, at least, of the southwestern part of the species range. It would not be surprising to find the mating preference evolving and hence varying geographically, with dark females becoming increasingly acceptable where they have come to make up most of the female population.

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