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Visual receptor cycle in normal and *period* mutant *Drosophila*: Microspectrophotometry, electrophysiology, and ultrastructural morphometry

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Abstract

Visual pigment, sensitivity, and rhabdomere size were measured throughout a 12-h light/12-h dark cycle in *Drosophila*. Visual pigment and sensitivity were measured during subsequent constant darkness [dark/dark (D/D)]. MSP (microspectrophotometry) and the ERG (electroretinogram) revealed a cycling of visual pigment and sensitivity, respectively. A visual pigment decrease of 40% was noted at 4 h after light onset that recovered 2–4 h later in white-eyed (otherwise wild-type, $w\,per^{+}$) flies. The ERG sensitivity [in $w\,per^{+}$ flies in light/dark (L/D)] decreased by 75% at 4 h after light onset, more than expected if mediated by visual pigment (MSP) changes alone. ERG sensitivity begins decreasing 8 h before light onset while decreases in visual pigment begin 2 h after light onset. These cycles continue in constant darkness (D/D), suggesting a circadian rhythm. White-eyed *period (per)* mutants show similar cycles of visual pigment level and sensitivity in L/D; *per's* alterations, if any on the D/D cycles were subtle. The cross-sectional areas of rhabdomeres in $w\,per^{+}$ were measured using electron micrographic (EM) morphometry. Area changed little through the L/D cycle.

Keywords: Rhodopsin, Electroretinogram, Sensitivity, Microspectrophotometry, Morphometry, Turnover

Introduction

Turnover of visual membranes functions to maintain the health of visual cells (White, 1964, 1968; Young, 1970). Although membrane turnover was emphasized in our previous studies (Sapp et al., 1991; Stark, et al., 1988), here we concentrated on visual pigment, the keystone protein of visual membranes, and the sensitivity mediated by visual pigment levels. When flies were maintained on a L/D cycle, a visual pigment decrease had been noted within several hours after light onset (Stark et al.,1988; Zinkl et al., 1990). This decrease could be due to the direct effects of light, a biological rhythm, or both. Testing these alternatives was a primary objective. *Drosophila* visual pigment levels can be easily determined in the R1-6 rhabdomeres of live, white-eyed flies using MSP of the deep pseudopupil (Stark & Johnson, 1980). We have also correlated our MSP measurements with data on ERG sensitivity and rhabdomere size.

One consistent finding of visual membrane cycling is its diurnal rhythm. In general, vertebrate rods shed their discs at dawn while cones shed at dusk (LaVail, 1980; Young, 1978). In *Limulus*, the rhabdom is degraded after light onset, then reconstructed (Barlow et al., 1987; Chamberlain & Barlow, 1979). In crabs, rhabdom membrane is internalized into the photoreceptor at dawn, and new membrane is prepared for export to the rhabdom at dusk (Stowe, 1980). Despite this level of interest there is a paucity of information on visual rhythms in flies.

In *Drosophila*, circadian as well as ultradian rhythms are affected by the *per* mutants (Hall & Rosbash, 1987, 1988): per^{L} has long cycles; per^{s} has short cycles; and per^{01} lacks normal wild-type rhythmicity. Considerable attention has been devoted to this gene's molecular biology (Rosbash & Hall, 1989). Especially pertinent to the present study is the finding that the compound eye is a major site of *per* gene transcription (Liu et al., 1988; Saez & Young, 1988) and translation (Siwicki et al., 1988). Also, *per* gene product actually feeds back to control *per* mRNA in a circadian manner (Hardin et al., 1990). For instance, using an anti-*per* antibody, immunocytochemistry reveals *per* gene product near photoreceptor nuclei at night but not in the day (Siwicki et al., 1988; Zerr et al., 1990). Furthermore, flies continue to show this difference in distribution or

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level for several days in D/D. These findings suggested the possibility that a visual pigment cycle could be entrained and that *per* mutants might affect this cycle.

Williams (1982) proposed that membrane turnover is continuous in the sheep blowfly, *Lucilia*; however, visual pigment turnover in *Drosophila* is not (Stark et al., 1988). Our long-term goal is to relate turnover at membrane, visual pigment, and sensitivity levels. We integrate MSP, ERG, and EM morphometry to relate the various aspects of daily turnover in *Drosophila* visual receptors. Abstracts of some of these findings have been published (Chen et al., 1990; Stark et al., 1989*a*).

Abbreviations

D/D	dark/dark
EM	electron microscopy
ERG	electroretinogram
L/D	light/dark
MSP	microspectrophotometry
MVB	multivesicular body
norpA	no receptor potential
UV	ultraviolet

Gene mutations

per	*period*
per^+	non-*period* controls
per^L	long-cycle *period*
per^s	short-cycle *period*
per^{01}	lacks normal wide-type rhythmicity
cn, bw	cinnabar brown (white-eyed)
w	white-eyed flies

Materials and methods

Animals

Drosophila melanogaster were raised on medium adequate for visual development (Stark et al., 1985). Yellow cornmeal probably provides sufficient carotenoids, but, to provide a margin of safety, our medium was also supplemented with β-carotene (0.125 mg/ml). This amount is the minimum needed to mediate maximal sensitivity in flies raised in otherwise carotenoid-deprived medium (Stark et al., 1977; Stark et al., 1990). Flies were reared at room temperature (23 ± 0.3 °C).

All flies were white-eyed (*w*). This eliminated the special effects of eye color pigments. Flies carrying *w*, but which were otherwise wild type, were used as non-*per* [per^+] controls. Mitchell S. Dushay of Jeffrey C. Hall's laboratory at Brandeis University constructed the white-eyed *per* stocks. He used the second chromosomal mutants *cn* and *bw* to block the syntheses of the two respective classes of eye color pigments. The per^L, per^s, and per^{01} alleles are on the first, X chromosome. These mutants were made homozygous for *cn bw* by simple Mendelian genetics. The procedures described by Hamblen et al. (1986) were implemented to confirm the *per* mutant genotypes, in the *cn bw* genetic background, by monitoring the locomotor activity of 5–10 flies of each stock.

Light/dark conditions

Flies were reared in transparent stoppered vials under a 12-h light/12-h dark (L/D) cycle of laboratory fluorescent (Phillips 30-W Cool White) lighting at an intensity of 75–80 lux (Lutron LX 101 digital lux meter). Use of this diel photoperiod of 12-h light/12-h dark is arbitrary. Temperature did not vary significantly as a function of time on the L/D cycle. In one test, temperature was recorded for a 22-day period: during 60% of the days, temperature differences for L *vs.* D were not observed. When variation was observed, it was only about a 1°C difference (except for one day of 2°C variation). In summary, the L/D cycle was probably, although not certainly, the only zeitgeber.

Vials were cleared of adults one day, and flies that emerged in the ensuing 24 h were isolated in new vials. These adults were maintained in the L/D cycle for 4 days, then either prepared for study or placed in containers for D/D studies (during the scotophase of the 4th day). Flies placed in D/D were exposed to light only at the beginning of the experiment.

In $w\,per^+$ visual pigment does not decrease until several hours after light onset (Stark et al., 1988; Zinkl et al., 1990), and since light is unavoidably used in preparation and measurement, we confined ourselves to a short measurement period once flies were removed from the L/D chamber of the D/D containers. Also, because visual pigment measurements expose the flies to light, a "between subjects" design was used, with each measurement from a different fly. A "within subjects" design, with repeated measurements from individual preparations, would not be appropriate because the lights used in preparation and measurement might interfere after some time.

Deep pseudopupil microspectrophotometry (MSP)

We used well-established techniques to measure the visual pigment levels from R1-6 visual receptors of live white-eyed *Drosophila* (e.g. Stark & Johnson, 1980; Stark et al., 1988; Stark et al., 1990). After lightly etherizing the flies, their heads were glued to a microscope slide with clear nail polish in such a way as to expose the dorsal portion of a compound eye. Bodies were then immobilized with dental wax (Scutan, Premier, ESPE, Norristown, PA). The deep pseudopupil was viewed with yellow light (579 nm), which is near the metarhodopsin's absorbance maximum. This pseudopupil image was aligned with the closely fitting aperture of a photomultiplier. The image was then optimized with condenser and fine focus controls. The 579-nm measuring beam was dimmed to a level that would not alter the visual pigment during the 2-s measuring pulse. Rhodopsin was maximized by applying a 620-nm (red) stimulus for 10 s at 16.0 log quanta/cm^2·s. Transmission through the deep pseudopupil was then measured with the 579-nm beam. Transmission was again determined after 10 s of 450-nm (blue) light at 15.3 log quanta/cm^2·s, which maximized metarhodopsin. The log of the ratio of the transmissions after red *vs.* after blue is the absorbance difference.

The high values of our absorbance difference measurements (cf. Stark et al., 1988) indicate the effectiveness of our present refinements. One refinement involved fitting the measuring aperture more closely around the deep pseudopupil, increasing the signal-to-noise ratio and thus the absorbance difference. Also, the pseudopupil was viewed more posteriorly where the rhabdomeres are longer, making our values of absorbance difference higher than previously reported.

We pooled data from many animals ($n = 25$) for each data point. The midpoint of the data collection time was used for placing the absorbance difference on the time axis. When several groups of animals were pooled to achieve $n = 25$, data were obtained at similar times and a weighted average of median time points located the absorbance difference on the time axis. Data

are plotted with 0 = light onset of the last L/D cycle before D/D.

Electrophysiology

ERGs were obtained using standard techniques (Harris et al., 1976; Stark, 1977*a*,*b*; Stark et al., 1990; Stark et al., 1977; Stark & Johnson, 1980; Stark et al., 1990; Stark et al., 1985). A 150-W xenon arc lamp (Hanovia 901C, Newark, NJ) provided a light source. A Bausch and Lomb (Rochester, NY) 500-mm monochromator provided monochromatic stimuli at 12 wavelengths from 350–625 nm. At 600 and 625 nm, a UV blocking filter (Wratten #3, Kodak, Rochester, NY) blocked short-wavelength light from the monochromator's harmonic spectrum. At 350 and 370 nm, a UV transmitting visible blocking filter (Corning, Corning, NY) was added, to block significant white-light leakage. Inconnel on glass neutral density filters, calibrated on a spectrophotometer, were used to attenuate the light. Lenses and a 10× achromat microscope objective focused the image of an iris onto the eye for ERG recording and onto a photodiode for optical calibrations.

ERGs were recorded with subcorneal NaCl glass microelectrodes pulled from inner filament glass with a Narashige puller. Signals were amplified with a Getting (Model 5, Iowa City, IA) amplifier and fed into a Tektronix oscilloscope (5100 series, Portland, OR). A glass microelectrode was inserted into the retinula cell layer. In this layer the waveform and amplitude of ERG recordings are nearly invariant. The ERG was recorded using bright 625-nm light (average intensity = 15.7 log quanta/$cm^2 \cdot s$). This tested whether the eye was healthy enough to provide valid data. A normal fly's ERG, in the retinula cell layer, shows both the positive ON-transient and the negative OFF-transient, in addition to the negative sustained potential. The ON-transient elicited by the 625-nm stimulus had to be more than 2 mV for the fly to be considered healthy enough for use. The eye was located at the focal plane of the stimulus using long-wavelength light (625 nm with an average intensity of about 15.7 log quanta/$cm^2 \cdot s$). The electrode was inserted, and the fly was dark adapted for 40 min in the light-proof Faraday cage (average temperature = 24.6°C). The preparation time was about 10 min after removal from the L/D chamber or D/D vial.

A 2-mV criterion response was obtained or was interpolated from flanking stimuli that differed in intensity by about 0.3 log units. We used the total ERG from the peak of the cornea positive ON-transient to the peak of the negative OFF-transient (including the negative sustained receptor potential). The peak-to-peak ERG yields data closer to absolute threshold than the receptor wave only. In a separate experiment, five flies were used to show that peak-to-peak sensitivity is 1.4 log units higher than if the negative wave alone had been analyzed. These ERGs are dominated by R1-6 since the transients are from R1-6 synapses and the near threshold receptor wave is mostly from R1-6 (e.g. Shaw, 1981).

For *w per*$^+$, throughout the 24 h of L/D, we obtained dark-adaptation functions (Fig. 5) to compare their shapes and to construct the graph of sensitivity as a function of time (Fig. 6). Because this analysis of data was time consuming, and because dark-adaptation functions were the same shape (see Results) for all further time points (*w per*$^+$ 24–72 h, Fig. 6, *per*^{L};*cn bw*, Fig. 8A, and *per*^{s};*cn bw*, Fig. 8B), criterion 2-mV peak-to-peak ERGs were obtained after a 30-min dark-adaptation period to determine the ERG sensitivity cycles.

Analysis of MSP and ERG data

The MSP and ERG (470 nm only) data were entered into the University of Missouri's IBM 3090-170J mainframe computer. Statistical Analysis System (SAS) version 5.0 was used to perform a nonlinear regression analysis. The output was used to generate the best-fitting cosine waveforms. These were plotted to overlay the original data for convenient comparison (Figs. 10 and 11). The waveforms were generated with the equation: $y = A \cos[(2\pi/\tau)T + \phi] + B$; where A = amplitude, T = time, τ = period, ϕ = phase, and B = d.c. offset. To estimate a goodness of fit, we calculated an F value using the equation F = [(Corrected sum of squares due to regression)/(Degrees of freedom)/(Sum of squares residual)/(n-degrees of freedom)]. This estimate of the goodness of fit is presented along with the parameters estimated from the curve fitting in Table 1.

Optical calibrations

Sensitivity determinations and the adjustment of appropriate actinic stimuli for ERG and MSP require accurate optical calibrations. Over the years, we have developed and modified procedures to measure light intensity (Stark et al., 1985). We calibrated our instruments with a photodiode (EG&G HUV 4000B, Salem, MA) which had been cross calibrated to other calibrated devices. The focused light spots in the ERG and MSP setups were passed through a measured pinhole in front of the diode for accurate area determinations. Calibrated neutral density filters were used to keep the diode's stimulation in its linear range. Quantum flux was calculated by taking into account the neutral density filters used in calibrations and experiments.

Morphometry

We fixed fly heads for EM morphometry to determine rhabdomere size. Flies (*w per*$^+$) were fixed at 1-h intervals in the photophase and at 1.5-h intervals throughout the scotophase. The aldehyde prefix and osmium postfix procedures have been described elsewhere (Stark & Clark, 1973; Stark & Sapp, 1988; Stark et al., 1989b,c; Stark et al., 1988). Although xylene is commonly used to expand sections while they are floating in the boat of the diamond knife, this step was omitted to avoid variability. We photographed sections at 8000× on a Siemens 101 Transmission EM. Because rhabdomeres taper, they were always photographed at a plane of section in the distal–proximal extent at the R1-6 nuclear layer. For further control, we only looked at well cross-sectioned rhabdomeres near the equator. Although these controls were important, they restricted the number of rhabdomeres we could examine.

Images were fed into an AIC (Analytical Imaging Concepts, Roswell, GA) model IM morphometric and densitometric system operating on a Northgate 286 computer. EM negatives were loaded into memory by placing them on a color control light box and viewing them through an RCA Hi Pot SA video camera (with a Fujicon HF35A-2 lens). To measure rhabdomeres, the organelle was selected for measurement on the basis of grey level, or the perimeter was manually drawn. The number of multivesicular bodies (MVBs) in the retinula cell cytoplasm were also counted from these same negatives for *w per*$^+$ during the photophase. To determine density, i.e. MVB counts per unit area, the area of the cytoplasm was determined on the computer by outlining the perimeter.

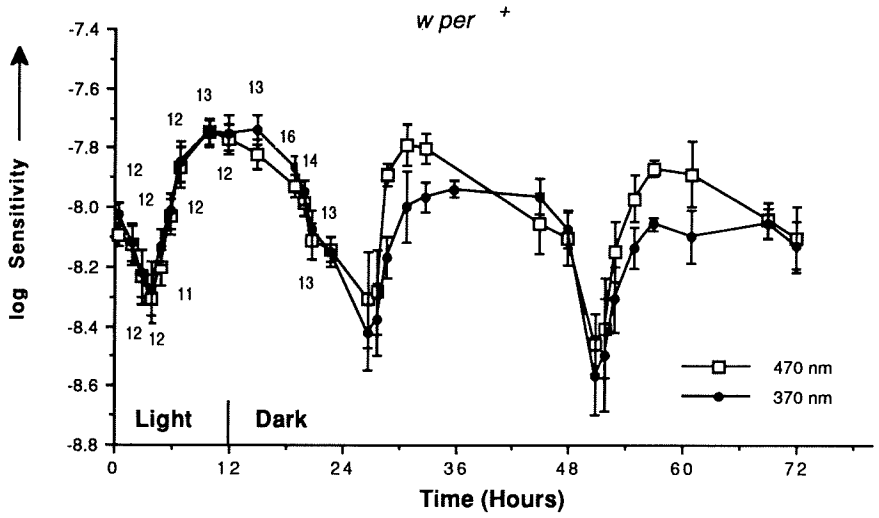

Fig. 6. Sensitivity (plotted as inverse, i.e. negative, $\log_{10}$ threshold intensity for a criterion 2-mV peak-to-peak ERG, also in Figs. 8, 9, and 11) as a function of time in *w per*$^+$ *Drosophila* on a 12-h light/12-h dark cycle as constructed by using the levels of dark-adaptation functions from 10–60 min. Sensitivity for the D/D period was determined for $n = 5$, except where noted—without the dark-adaptation experiment. Standard errors are shown.

The ERG sensitivities of *per*^{L};*cn bw* and *per*^{s};*cn bw* (Fig. 8) show a daily rhythm which persists for 2 days in constant darkness (24–72 h). The *per*^{L};*cn bw* and *per*^{s};*cn bw* data are much like those for *w per*$^+$ (Fig. 6); sensitivity decreases to a minimum at about 3.75 h for *per*^{L};*cn bw* (Fig. 8A) and 2.75 h for *per*^{s};*cn bw* (Fig. 8B), respectively. Sensitivity gradually increases to a maximum at 9.75 h after light onset in *per*^{s};*cn bw* and 12.75 h after light onset in *per*^{L};*cn bw*. The difference between maximum and minimum sensitivity during the first 24 h is 0.6–0.65 log units. Every sensitivity cycle (three strains tested, each at two wavelengths) persists in constant darkness. Consistent with its low visual pigment *per*^{s};*cn bw* has the lowest overall sensitivity.

UV (370 nm) and blue (470 nm) sensitivity varied in parallel in L/D cycles and subsequently in D/D (Figs. 6 and 8). Fig. 9 expands on this finding by presenting sensitivities measured across the entire spectrum at different times: 2.75–3.75 h, 8.75 h, and 69.75–71.75 h. The shapes of spectra are the same at these time points. On this basis, we discount any importance in 370–470 nm cycle differences. There are small differences of sensitivity between *w per*$^+$ and the *per* mutants. The largest difference is at 2.75–3.75 h after light onset, but this sensitivity difference decreases to near zero by 69.75–71.75 h.

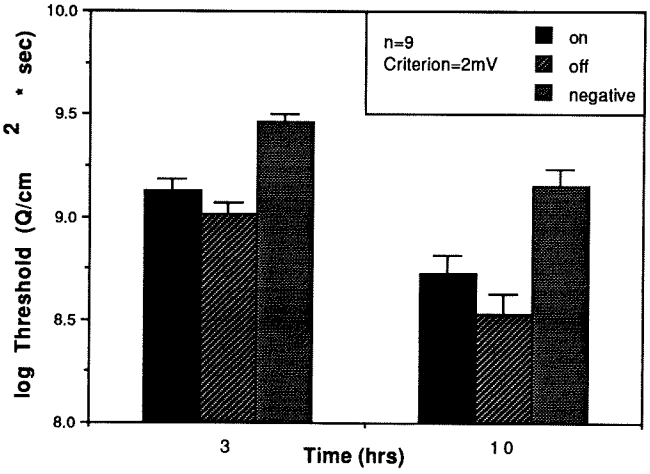

Fig. 7. Threshold for 2 mV ON-transient, OFF-transient, and negative sustained potential ERG measured at 3 h and 10 h. $n = 9$ for each component.

Curve fitting for MSP and ERG data

Although periodicity analyses are customary in the field of chronobiology, these data are particularly problematic. First, it is risky to conclude rhythmicity from 1 cycle of L/D followed by 2 cycles of D/D; the best analyses would come from more extended time spans. Another problem is the irregularly spaced data points (see Methods). As a compromise solution, Mr. Tie Su, of the University of Missouri's Statistics Department, fitted a cosine to our data using least squares.

The parameters from the computer analyses of MSP and ERG (470 nm only) data are presented in Table 1. The statistic {see Methods, $F = [$(Corrected sum of squares due to regression/degrees of freedom)/(Sum of squares residual)/(n-degrees of freedom)]} shows that the cosine fits the data well (significance beyond the 1% level in each case as shown); F reflects the proportion of the total variance accounted for by the fitted curve. For instance, for the best-fitting MSP curve (*w per*$^+$), as judged by F, 40% of the variance is accounted for by the cosine function, while, for the worst-fitting MSP curve (*per*^{L};*cn bw*), the figure is 34%. The corresponding percentage for the best-fitting ERG (for *w per*$^+$) is 70%, while the percentage for the curve with the lowest F (*per*^{s};*cn bw*) is 45%. Figs. 10 and 11 show the cosine functions generated from these data plotted to overlie the original MSP and ERG data, respectively. Regression for the MSP data shows *w per*$^+$'s period to equal 24.34 h, *per*^{L};*cn bw*s period to equal 25.68 h, *per*^{s};*cn bw*s period to equal 16.21 h, and *per*^{01};*cn bw*s period to equal 25.60 h (Table 1 and Fig. 10). Unfortunately, these values are not entirely consistent with the ERG data. For the ERG data, the regression shows *w per*$^+$s period to equal 24.63 h, *per*^{L};*cn bw*s period to equal 23.38 h, and *per*^{s};*cn bw*s period to equal 22.83 h (Table 1 and Fig. 11).

Morphometry

Williams (1982) reported that rhabdomere size remains constant as a function of time of day in the sheep blowfly *Lucilia*, a mus-

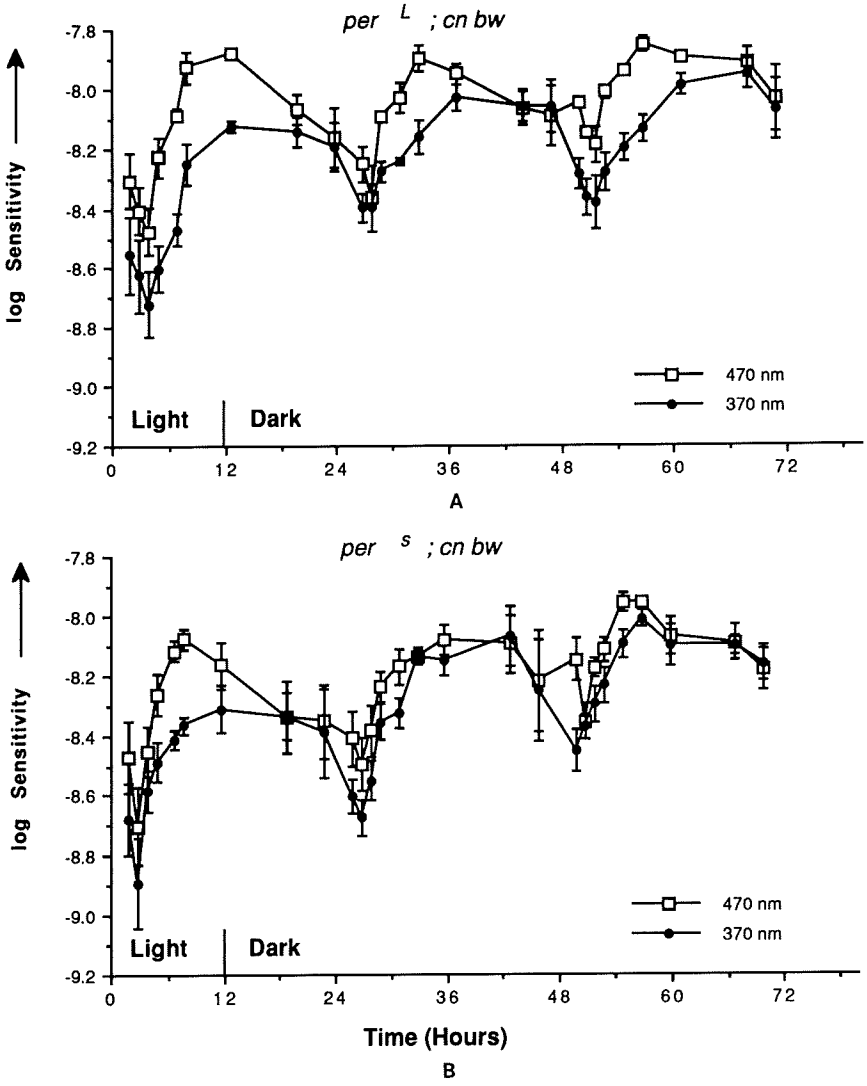

Fig. 8. Sensitivity cycles as a function of time in per^L;*cn bw* (A) and per^s;*cn bw* (B) *Drosophila* on 12-h light/12-h dark L/D (0–24 h) and D/D (24–72 h). Test stimuli were 470 nm and 370 nm as marked. For each time point $n = 5$ flies. Error bars show S.E.'s.

Table 1. *Results of SAS analysis of MSP and ERG (470 nm) data*

	MSP				ERG		
	w per$^+$	per^L;*cn bw*	per^s;*cn bw*	per^{01};*cn bw*	*w per*$^+$	per^L;*cn bw*	per^s;*cn bw*
Period	24.346	25.686	16.213	25.600	24.635	23.383	22.836
Amplitude	−0.016	−0.016	−0.017	−0.017	0.258	0.185	0.166
Phase	5.355	5.955	5.814	6.261	−0.094	−0.638	−0.462
D.C. offset	0.198	0.195	0.160	0.212	7.975	8.024	8.190
F	7.557	5.505	6.248	7.167	11.799	7.418	4.371
Regression DF	4	4	4	4	4	4	4
Residual DF	49	46	40	40	28	25	26
Sig. level	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01

coid. However, he presented only two time points (light onset and light offset) pooled from only four rhabdomeres. Our finding that visual pigment and sensitivity do change in *Drosophila* prompted re-examination of Williams's finding. Data for *w per*$^+$ (control) *Drosophila* entrained under L/D conditions for 4 days and fixed for morphometry are presented in Fig. 12. We saw no significant change in rhabdomere diameter throughout the L/D cycle, and we hope that such area invariance is mediated by an invariance in volume, beyond the scope of our measurements. We used the same micrographs to count MVBs during the first 12 h of light (after 4 days entrainment). We normalized the data to represent the number of MVBs/μm^2 of

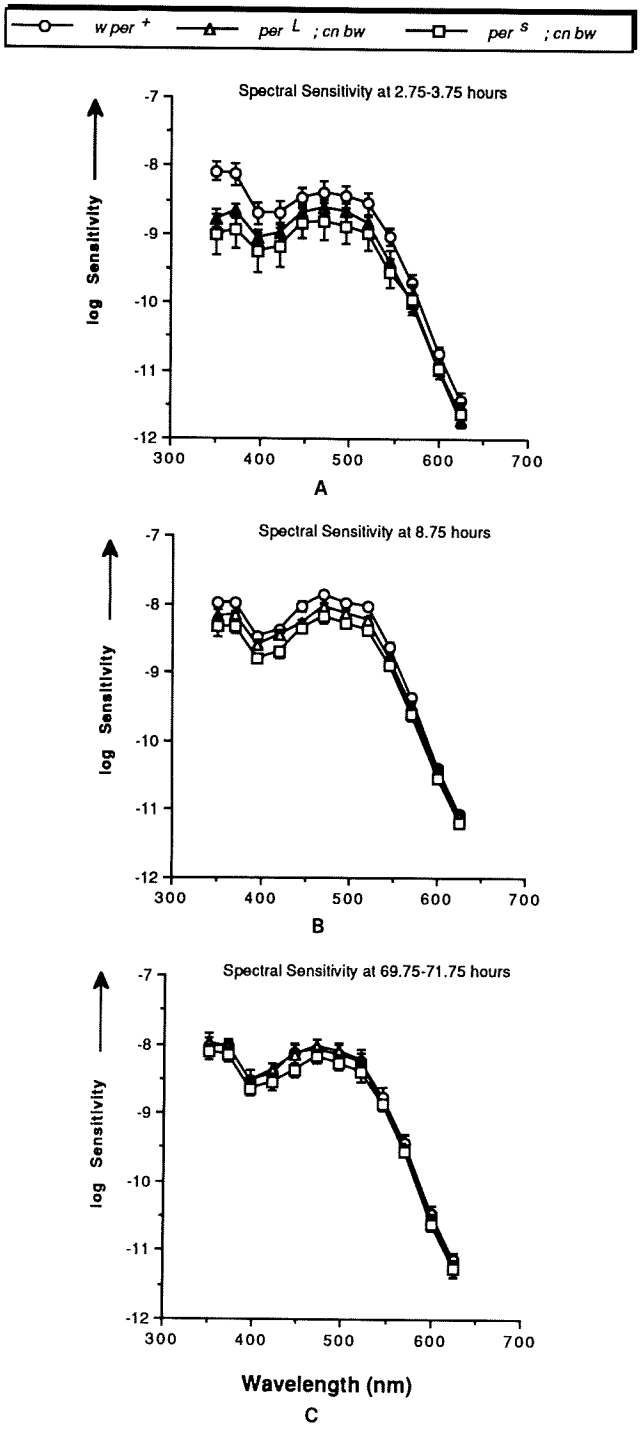

Fig. 9. Spectral sensitivity at different time point in L/D and in D/D. See text for details.

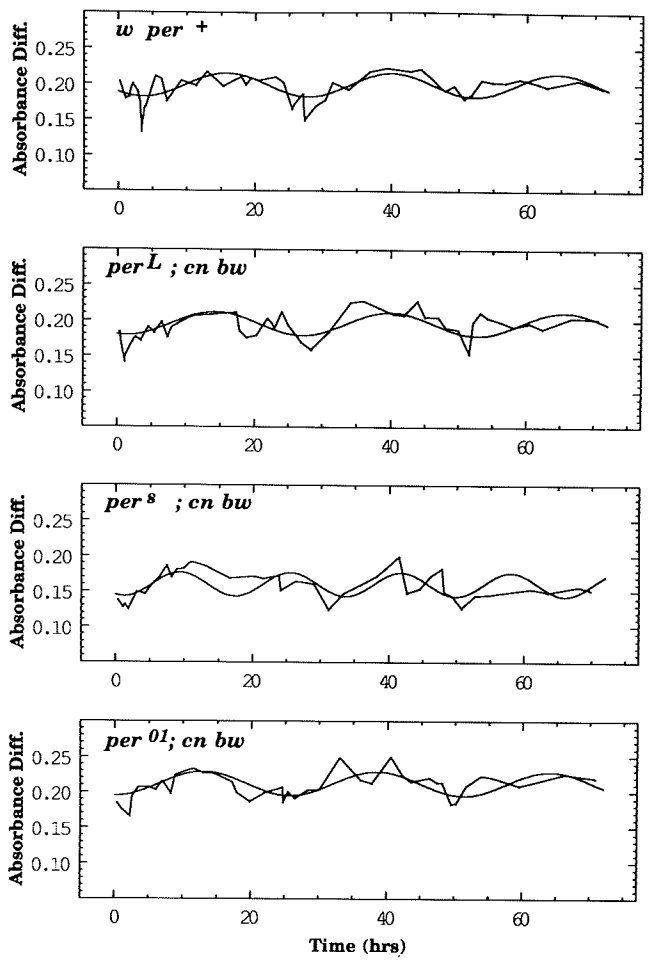

Fig. 10. Plots of the cosine waves generated from computer analysis of the absorbance differences for white-eyed per^{+}, per^{L}, per^{s}, per^{01}. The cosine waves are plotted over the original data for comparison.

retinula cell cytoplasmic area. Our data are presented in Fig. 13. Again we saw no significant change in the number of MVBs during the first 12 h of light.

Discussion

A priori, one might have expected that (1) *Drosophila*'s visual pigment and sensitivity would change substantially in L/D and D/D since membrane (e.g. White, 1968) and sensitivity (e.g. Barlow et al., 1987) change in virtually all other studies using different species; and (2) per^{L}, per^{s}, and per^{01} would alter the rhythms, at least in D/D, to 29 h, 19 h, and arhythmicity, respectively. Our major conclusion is that these hypotheses were proved to be largely doubtful. The subjective impression is that there are consistent dips associated with a 24-h rhythm and that *per* may have subtle effects on this rhythm [per^{s}s lower absorbance difference and sensitivity, and the decrease in goodness of fit (*F* value, cf. Methods and Results)] for all *per* strains (Table 1 and Figs. 10 and 11).

One of the reasons for the relative weakness of the rhythmicity is that the visual pigment decrease in *Drosophila* is transient and might not be well fit by a trigonometric function. The visual pigment decreases after light onset (Fig. 1), but recovery soon follows, while the lights remain on. In this respect, the cycle parallels the membrane cycle in crayfish (G.S. Hafner, personal communication) and *Limulus* (Barlow et al., 1987). It differs from the membrane cycle of the crab (Stowe, 1980), which has rhabdom loss at dawn and buildup at dusk. The pulse shape of the visual pigment's decrease at 3–4 h after light onset (Figs. 1–4) suggests that light onset may initiate the mechanisms of both decrease and recovery of visual pigment during

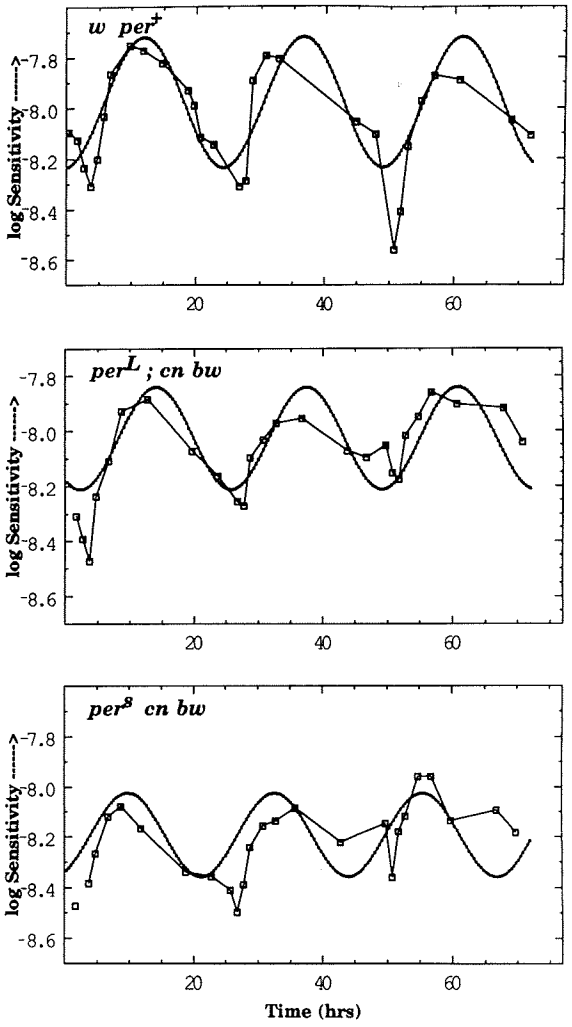

Fig. 11. Plots of the cosine waves generated from computer analysis of the sensitivity at 470 nm for *w per*$^+$, *per*^{L};*cn bw*, and *per*^{S};*cn bw*. The cosine waves are plotted over the original data for comparison.

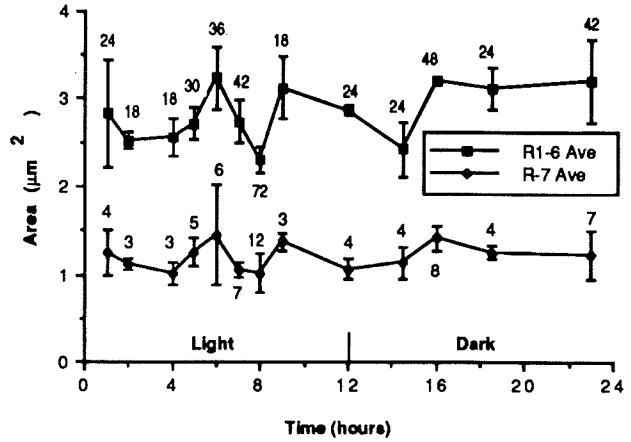

Fig. 12. Rhabdomere cross-sectional area as a function of time in the L/D cycle for *w per*$^+$ *Drosophila*. Each data point represents about three ommatidia per head at the R1-6 nuclear layer near the equator from one or two heads. The numbers of rhabdomeres measured are marked next to each data point. Although other data in this report (Figs. 1–5) pertain to R1-6 specifically, we present the areas for R7 as well as for R1-6 in this analysis for the sake of completion. Standard deviations are shown.

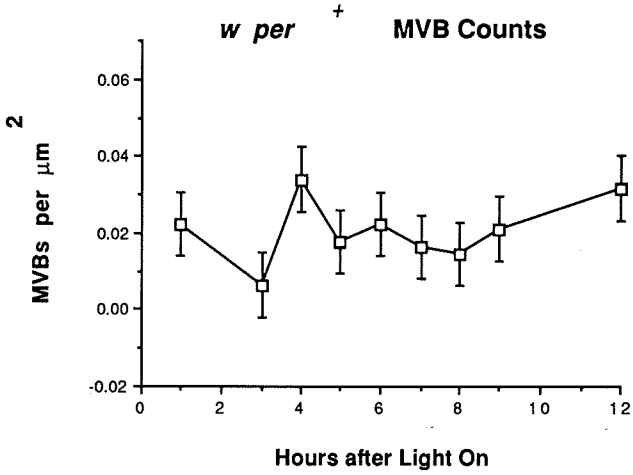

Fig. 13. Counts of MVBs/μm^2 as a function of time in the first 12 h of the L/D cycle, after 4 days entrainment, in *w per*$^+$ *Drosophila*. Each data point represents 4–5 ommatidia from one fly in each condition. No significant difference was observed.

entrainment. Also, although visual pigment and sensitivity cycles persist in D/D, they become less pronounced (Figs. 1–4, 6, and 8).

The demonstration of a small but measurable visual pigment cycle is unique because most studies of photoreceptor daily cycles focus on membrane turnover (e.g. Stowe, 1980). The deep pseudopupil technique facilitates quantification of visual pigment in living white-eyed *Drosophila* to supplement electrophysiology and histology. Photochemistry can be accomplished on a robust sample size: this study reports MSP from 4000 animals (Fig. 1–4). In a related approach, Arikawa et al. (1988) and Isono et al. (1986) monitored the visual pigment chromophore using high-pressure liquid chromatography (HPLC) in the crab *Hemigrapsus sanguineus* and the locust *Locusta migratoria*, respectively, and compared these data with measurements of rhabdom size. Crabs and locusts show parallel changes of chromophore (retinal) and rhabdom in D/D, suggesting that these animals also have circadian rhythms of visual pigment. Our measurements in *Drosophila* suggest a circadian rhythm. That *per* mutants exhibit periods similar to those of controls provided no evidence that *per* influences the visual pigment cycle. This is remarkable considering the fact that *per* product cycles in the retina (see Introduction).

That the visual pigment cycle (Fig. 1) finds no correlated rhabdomere cycle (Fig. 12) suggests that visual pigment and membrane turnovers are unrelated. Light induces an endocytosis of certain materials into the photoreceptor cells (Wilcox & Franceschini, 1984; Zinkl et al., 1990). Furthermore, coated vesicle formation, the first step of rhabdomere degradation (autophagy), was previously suggested to mediate this uptake (Stark et al., 1988). However, we now suggest that visual pigment may not be removed stoichiometrically with membrane quantity. In ocelli of the wasp *Paravespala germanica*, light ad-

aptation decreases P-face particles (Pabst & Kral, 1989) suggesting that visual pigment can vary independently of membrane area. Do coated vesicles internalize visual pigment? Does light accelerate this membrane uptake? Pilot results argue against such a direct relationship of light and coated pits, since there is no rhodopsin cycle in the *norpA* mutant (Zinkl et al., 1990), even though autophagic bodies abound in *norpA* (Stark & Sapp, 1989; Stark et al., 1989*b*).

How are sensitivity and visual pigment changes related? Low points in visual pigment and in ERG sensitivity coincide at about 4 h after light onset (compare Figs. 1 and 8). This is expected since decreasing visual pigment should decrease sensitivity. The change in ERG sensitivity is, however, much greater than would be predicted by the photopigment change. A sensitivity change of 0.6 log units suggests a change of 4 times; whereas the decrease in absorbance difference of 40% suggests a change of less than 2 times, which is contrary to sensitivity and visual pigment levels being linearly related with a slope of 1 (Boschek & Hamdorf, 1976; Harris et al., 1977; Stark & Johnson, 1980). Furthermore, ERG sensitivity starts to drop at 16 h, i.e. 4 h after light offset (Fig. 6), while visual pigment drops, at least in *w* (otherwise wild-type), only after light onset (Fig. 1). In *Limulus*, three factors mediate higher sensitivity at night: (1) increased quantum catch; (2) increased gain; and (3) decreased noise (Barlow et al., 1987). Alterations in visual pigment would fall into the first category, while post-photopigment aspects of transduction would go beyond quantum catch. In summary, we have evidence for sensitivity changes beyond those resulting from visual pigment changes alone.

Might any variables other than visual pigment content influence our MSP determinations? Ockham's razor suggests that this would not be the case since our methodology is geared to determine visual pigment. If intraretinular eye color pigment granules were present, they could migrate as a function of light or time. However, we can unequivocally state that *w* and *cn bw* lack even unpigmented granules within the retinula cells on the basis of numerous studies (e.g. Stark & Sapp, 1987, 1988, 1989; Stark et al., 1989*b*; Stark et al., 1988). Furthermore, transmissions were measured immediately after maximal adaptation with 620- or 450-nm light, so that even if granules were present and optically functional they would always be in the fully light adapted position. Some aspect of the optics of the rhabdomere, such as index of refraction or rhabdomere size (Stavenga, 1975), might possibly change as a function of time, confounding our measurements, Determining changes in index of refraction changes is difficult since differing amounts of visual pigment in the rhabdomeres might affect this phenomenon. Could differing cross-sectional areas underlie alterations in amount of visual pigment? We measured rhabdomere dimensions as a function of time in the L/D cycle in *Drosophila* using morphometry and EM and found no cycle (Fig. 12). We also did not find any cycle in the MVB counts (Fig. 13). MSP excluded the contributions from any opsin without chromophore or any visual pigment in cytoplasmic compartments, if present. Other alternative approaches, such as freeze fracture and immunocytochemistry, would not measure rhabdomeric rhodopsin unequivocally for the following reasons: (1) opsin can exist without chromophore, at least in the vitamin A deprived rat (Katz et al., 1991); and (2) vitamin A deprivation in *Drosophila* does not completely eliminate P-face particles (Harris et al., 1977) suggesting that proteins other than visual pigment (e.g. opsin without chromophore, structural molecules or other transduction molecules) may reside in this organelle.

The mechanisms of the two sensitivity peaks in R1-6 photoreceptors has stimulated much interest. For this reason, the ERG at 370 and 470 nm was measured (Figs. 5–6 and 8) as well as spectral sensitivities (Fig. 9). There is reason to believe that a sensitizing pigment mediates the UV sensitivity peak (Kirschfeld, 1986; Stark & Tan, 1982). The fact that sensitivities at UV and blue wavelengths change in parallel shows that there is no preferential alteration of the UV sensitizing pigment. Thus, rhodopsin, the final common pathway of both visible and UV sensitivities, mediates changes in both the visible wavelength sensitivity and the UV sensitivity.

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