Circadian Modulation of Temporal Properties of the Rod Pathway in Larval *Xenopus*

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Submitted 2 April 2004; accepted in final form 10 June 2004

Solessio, Eduardo, David Scheraga, Gustav A. Engbretson, Barry E. Knox, and Robert B. Barlow. Circadian modulation of temporal properties of the rod pathway in larval *Xenopus*. *J Neurophysiol* 92:2672–2684, 2004; 10.1152/jn.00344.2004. Circadian clocks are integral components of visual systems. They help adjust an animal’s vision to diurnal changes in ambient illumination. To understand how circadian clocks may adapt visual sensitivity, we investigated the spatial and temporal properties of optomotor responses of young *Xenopus laevis* tadpoles (Nieuwkoop and Faber, developmental stage 48) using a modified 2-alternative preferential-viewing method. We maintained animals in constant darkness and measured temporal sensitivity during their subjective day and night. We found that their behavioral responses can be explained in terms of 2 mechanisms with different temporal properties. The more sensitive mechanism operates at low temporal frequencies and intermediate wavelengths (λmax = 520 nm), properties consistent with rod signals. Threshold for this mechanism is approximately 0.04 photoisomerizations rod−1 s−1, consistent with single-photon detection. A less-sensitive mechanism responds to higher temporal frequencies (cutoff = 12 Hz) and has broad spectral sensitivity (370–720 nm), properties consistent with cone signals. This cone mechanism does not change, but the cutoff frequency of the more sensitive rod mechanism shifts from 0.35 Hz at night to 1.1 Hz during the subjective day, thereby enhancing the animal’s sensitivity to dim rapidly changing stimuli. This day–night shift in rod temporal cutoff frequency cycles in complete darkness, characteristic of an endogenous circadian rhythm. The temporal properties of the behaviorally measured rod mechanism correspond closely with those of the electrophysiologically measured retinal response, indicating that the rod signals are modulated at the level of the outer retina.

INTRODUCTION

Many animals, vertebrates and invertebrates, anticipate the natural diurnal changes in ambient lighting, enabling them to adjust visual sensitivity for the approaching bright conditions of daytime and darkness of night (Barlow 2001). Diverse mechanisms at many levels underlie the daily modulation of visual sensitivity by endogenous circadian oscillators (Anderson and Green 2000; Green and Besharse 2004).

In vertebrate retinas, several cellular features are directly or indirectly under circadian control; a few examples have included: activity of serotonin N-acetyltransferase (Besharse and Iuvone 1983), level of dopamine (Doyle et al. 2002; Kolbinger et al. 1990; Manglapus et al. 1999), retinomotor movements (Burnside 2001), synaptic gain (Krizaj and Witkovsky 1993; Ribelayga et al. 2002; Wang and Mangel 1996), horizontal cell spinule formation (Wagner et al. 1992), gene transcription (Green and Besharse 1994; Pierce et al. 1993), and photoreceptor disk shedding (LaVail and Ward 1978). At the electrophysiological level, the amplitude of the ERG b-wave exhibits a circadian rhythm, although the phase of the cycle differs among species (Brandenburg et al. 1983; Li and Dowling 1998; Manglapus et al. 1998; Shaw et al. 1993). These examples are drawn from species of many vertebrate taxa and suggest that circadian modulation in the retina is a common theme. Ultimately, it is the concerted action of the multiple cellular mechanisms under circadian control that determines the sensitivity of the visual system, but the interactions of these mechanisms are largely unknown. Similarly, how circadian changes at the cellular level affect the capacity of an animal to process visual information is not known.

To better understand how circadian rhythms affect vision we measured the optomotor responses of young *Xenopus* tadpoles at different times of subjective day and night. We selected *Xenopus* because circadian rhythms have been detected in its photoreceptors (Cahill and Besharse 1993) and because the animal exhibits robust optomotor responses (Burgers 1952; Cronyl-Dillon and Muntz 1965). We designed experiments to measure the temporal sensitivity of *Xenopus* vision using optomotor responses. Sensitivity was determined with temporal transfer functions (TTFs) that describe the temporal output properties of a system in response to modulated input. They are analogous to contrast sensitivity functions (CSFs) that have been valuable in describing the ability of visual systems to process spatiotemporal properties of the visual image at near-threshold levels of illumination (Kelly 1979; Robson 1966). They differ in that TTFs are measured with stimuli of 100% visual contrast, whereas CSFs are typically measured with variable-contrast stimuli. Both TTFs and CSFs have band-pass properties attributed to the limited sensitivity of visual systems to high and low spatiotemporal frequencies. The precise shapes of the functions depend on several other factors including retinal eccentricity (Regan and Beverley 1983), mean luminance level, and temporal modulation of the grating (Bilotta and Powers 1991; De Valois et al. 1974; Kelly 1979; Robson 1966; van Nes et al. 1967).

Our strategy is to measure TTFs during an animal’s subjective day and night with the hope of determining what stage(s) of the visual system shapes those functions and whether they...
are modulated by circadian oscillators. In electrophysiological experiments on cats, Campbell and Robson (1968) analyzed the CSFs of ganglion cells in terms of spatial and temporal properties of receptive fields. Kelly (1971), Rovamo et al. (1999), and Jarvis et al. (2003) analyzed CSFs of human vision in terms of temporal response properties of the outer retina. In this study of *Xenopus* tadpoles we analyze TTFs in terms of temporal response properties of the rod pathway and conclude that they are under circadian control and that the site of circadian modulation is in the outer retina.

**Methods**

**Husbandry**

*Xenopus laevis* tadpoles were generated by in vitro fertilization (Sive et al. 2000), raised in 2- to 5-liter tanks in tadpole water [0.5 g/L sea salt (Instant Ocean, Aquarium Systems, Mentor, OH) and 4 mM NaHPO₄], and fed a diet of nettle powder (Wundich-Dez, Short Hills, NJ) and tadpole powder (Xenopus Express, Plant City, FL). Tadpoles were kept under normal Syracuse, NY, natural lighting to allow entrainment of their circadian rhythms to the local time. Animals were kept in darkness 24 h before the experiment and at times between tests. In view of the yearly variation in the photoperiod, experiments were conducted at hours away from the L–D or D–L transitions. Thereby, our results indicate changes in sensitivity observable during subjective day and nighttimes. Room temperature was 22°C. The developmental stage of each animal was determined according to standardized morphological characteristics (Nieuwkoop and Faber 1994). All procedures were approved by the SUNY Upstate Medical University IACUC and were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (National Academy of Sciences, Washington, DC, 1996).

**Stimulator light source**

Light from a 150-W xenon lamp passed through a monochromator (Model 77250, Oriel, Stamford, CT) to produce a narrow-band (10 nm band-pass) beam centered at the desired wavelength. The beam was attenuated in 0.5 log increments with neutral density filters and focused onto the entrance aperture of a fiber-optics light pipe. The light pipe terminated in a ring illuminator that was centered above a dish housing the tadpole. A conical reflector dispersed the light to illuminate the inside of a rotary drum surrounding the tadpole dish (Fig. 1A).

**Optomotor response and data analysis**

We measured visual sensitivity of tadpoles by observing their optomotor response to a rotating pattern of alternating black and white vertical bars (Cronly-Dillon and Muntz 1965). The experimental paradigm was an adaptation of the 2-alternative preferential-viewing method (Bilotta and Powers 1991; Teller et al. 1974). Tadpoles were maintained in constant darkness for ≥24 h before all experiments. A dark-adapted tadpole was put into a transparent, water-filled, 60-mm-diameter dish located on a stationary pedestal at the center of a rotating drum within a light-tight box. The inner surface of the drum was lined with the black and white pattern and was approximately 10 cm high and 14 cm from the center of the tadpole container. An observer viewed the tadpole with an infrared video system and could not see the stimulus (Fig. 1A).

The task of the observer was to infer the direction of drum rotation by observing the animal’s behavior. In each trial a computer randomly chose the direction of rotation and rotated the drum for 5 s. At the end of that time the observer decided whether the drum had rotated clockwise or counterclockwise. We found that the animal’s head and tail (Fig. 1B) were the most reliable indicators of the direction of rotation and under bright levels of illumination the observer made a correct determination on nearly every trial. At low levels, the animal’s movements were more subtle, to the point where the observer achieved only chance correct responses. The observer received auditory feedback indicating either a correct or incorrect response.

At the end of 20 trials (one test) the percentage of correct responses was computed and percentage correct was plotted against the intensity of drum illumination (Fig. 2A). To minimize light adaptation, all tests began with the dimmest light intensity (4.5 log units of light attenuation). In successive tests illumination intensity was increased in steps of 0.5 log unit. We tested a minimum of 4 intensity levels per tadpole and continued until the observer scored 75% or higher. A criterion threshold was calculated by linear regression and defined as the intensity necessary for the observer to infer the direction of drum rotation at a 75% correct level. Sensitivity was defined as the inverse of the illumination intensity required to reach threshold. All thresholds measured from an animal under the various experimental conditions were normalized relative to the threshold determined in response to a “standard” grating [1.6 cycles/radian (cyc/ rad)] rotating at 0.34 rad/s (3.3 rpm) and illuminated with 520-nm light during the subjective day. These stimulus parameters were previously shown to give reliable optomotor responses under photopic conditions (Burgers 1952). All figures show normalized averages ± 1 SD obtained from 4 or more tadpoles.

We measured thresholds for patterns of alternating black and white vertical bars with 100% contrast. We define a Raleigh contrast modulation index as...
The time constant. The time to peak of the impulse response is

\[ t_p = \frac{r_i(t)}{Q_f} \]

Sensitivity factor, \( e \) is the base of the natural logarithm, and \( n \) the temporal frequency (\( f_T \)) of the rotating grating as the

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definition to rotate past a point in the animal’s field of view. Temporal frequency relates to the spatial frequency (SF) of the pattern and the speed of rotation (SR)

\[ f_T (\text{Hz}) = \frac{\text{SF} (\text{cyc/rad})}{\text{SR} (\text{rad/s})} \]

Note that the patterns consist of bars and not sinusoidal waveforms. Therefore temporal frequency is the dominant frequency of the pattern.

The visual sensitivity functions are generally defined in terms of the contrast levels (Bilotta and Powers 1991) or of the amplitudes of the stimuli (Kelly 1961) necessary to reach an arbitrary threshold. Schaefer and Neumeyer (1996) and Krauss and Neumeyer (2003) measured the spectral sensitivity of the optomotor response by determining the intensity levels necessary to reach a criterion proportion of positive responses (PPRs) to a rotating bar pattern of constant contrast. In our study we measured the sensitivity function of the optomotor response by determining the illumination levels required to reach an arbitrary number of correct responses (75%).

Because the visual sensitivity exhibited low-pass filter characteristics, we fitted the sensitivity as a function of temporal frequency with Lorentzian functions

\[ S(f) = \frac{S_0}{1 + (f/T_c)^2} \]

where \( S \) is sensitivity, \( S_0 \) is the maximal sensitivity, \( f_T \) is the temporal frequency, \( f_{TC} \) is the cutoff frequency, and \( n \) is the order of filter. A second-order filter \( (n = 2) \) was required to fit the data. The cutoff frequency for the function described by Eq. 3 is defined as the frequency at which the sensitivity decreases to 25% of the maximum.

The impulse response \( r_i(t) \) for the 2nd-order Lorentzian function is (Fuortes and Hodgkin 1964)

\[ r_i(t) = Q_i \Delta T S_i e^{-\tau} \]

where \( Q_i \) is the photon flux, \( \Delta T \) is the flash duration, \( S_i \) is the sensitivity factor, \( e \) is the base of the natural logarithm, and \( \tau \) is the time constant. The time to peak of the impulse response is

\[ t_p = \tau \]

\( \tau \) is related to the cutoff frequency \( f_{TC} \) by

\[ f_{TC} = \frac{1}{2 \pi \tau} \]

The integration time \( t_i \) for a 2nd-order Lorentzian function is

\[ t_i = \int_0^\infty r_i(t) dt = \tau \]

where \( r_{Max} \) is the peak amplitude of the impulse response. Integration time can also be estimated from the intensities of short flash (impulse) and step stimuli required to elicit the same response amplitude

\[ t_i = \frac{Q_i \Delta T}{Q_s} \]

where \( Q_i \) and \( Q_s \) are intensities (photon flux) of the short flash and step stimuli, respectively, and \( \Delta T \) is the duration of the short flash.
Electrophysiology

Electroretinograms (ERGs) were recorded in a light-proof Faraday cage from dark-adapted tadpoles anesthetized with 0.1% tricaine in 0.1 × Marc’s modified Ringers (Sive et al. 2000). The animal was placed in a small chamber and positioned so that its right eye pointed upward. Under infrared illumination a glass micropipette (tip diameter about 2–3 μm and filled with Xenopus Ringers solution) was inserted into the eye. A similar reference electrode was inserted subcutaneously in the nasal region. The signals were amplified, band-pass filtered (0.1–20 Hz), and sampled at 100 Hz using a Digidata 1200 acquisition board and pClamp7 acquisition software (Axon Instruments, Union City, CA). Light stimuli were delivered by a fiber-optics light pipe positioned above the eye and directed along its optic axis. Intensity and spectral composition were controlled with neutral density and narrow-band (10 nm) chromatic filters. We averaged 4 to 8 ERG responses to flashes 20 ms or 2 s in duration and measured the amplitude from the peak of the a-wave to the peak of the b-wave. The ERG response duration was the interval between flash onset and the recovery of the b-wave (crossing 0 μV).

Fourier analysis

We estimated power density spectra to obtain frequency representations of the flash ERGs described above. Window length was 20.48 s long, sampled at 100 Hz, for a total of 2,048 samples. No weighting window was applied to reduce edge effects. Spectra obtained from multiple retinas were averaged (n = 4 or more) and a running average window was applied to smooth the spectra at frequencies above 0.3 Hz. Data values in the plots represent means ± SE.

Model of spectral sensitivity

Absorbance spectra for vitamin A2 pigments, centered at 440 nm [αB(λ)], 520 nm [αG(λ)], and 610 nm [αR(λ)], were generated using polynomial templates (Govardovskii et al. 2000) and scaled with factors αB, αG, and αR (Cameron 2002) to fit each spectral sensitivity αT(λ) in response to low and high temporal frequencies

\[ αT(λ) = αB × αB(λ) + αG × αG(λ) + αR × αR(λ) \]

The scaling factors were normalized to the αG at low frequency.

RESULTS

Visual sensitivity changes with time of day

Figure 2A plots the percentage of correct responses as a function of light intensity for an animal tested with the standard grating during the subjective day and night. The wavelength of light was 520 nm, which is the peak of the spectral sensitivity of the principal rod in *Xenopus* (Dartnall 1954). Under these conditions, for this animal, the minimum threshold (greatest sensitivity) was measured during the subjective day and corresponded to a light intensity of \( 1.9 \times 10^{-2} \) photons μm\(^{-2}\) s\(^{-1}\) at the surface of the cornea. The average threshold for all animals during the subjective day was \( 2.6 \times 10^{-2} ± 0.73 \times 10^{-2} \) photons μm\(^{-2}\) s\(^{-1}\). This average threshold intensity is the “normalizing” intensity (0 log) used throughout this study, and is within the range of rod-dominated scotopic vision for *Xenopus* (see Discussion). In the subjective night, threshold was 1.5 log units higher, that is, for this task, the tadpole was 30 times less sensitive than during the day (Fig. 2A).

Figure 2B tracks the thresholds of 2 tadpoles maintained for 72 h in constant darkness. Stimulus conditions were the same as in Fig. 2A. The changes in sensitivity exhibited a rhythm with a period of about 24 h. Sensitivity was highest during the subjective day and peaked around noon. All tadpoles tested were less sensitive during the subjective nighttime hours, showing minimal sensitivity between midnight and 3 A.M. The amplitude of the rhythm was about 1.5 log units throughout the testing period. Three other tadpoles of the same age showed similar rhythmic changes in sensitivity. These results demonstrate a circadian control of behaviorally measured visual sensitivity in young (stage ~ 48) *Xenopus* tadpoles. The circadian changes we detected in stage 48 tadpoles were not detected in stage 56 tadpoles. Why circadian control of retinal properties changes during the course of development is not known. Perhaps it adapts the animal for changes in lifestyle.

Optomotor responses are limited by quantal fluctuations

The optomotor responses of Fig. 2, A and B were evoked by rotating patterns having 100% contrast. We next examined the dependency of contrast sensitivity on illumination by varying the contrast of the patterns. Spatial and temporal frequencies of the gratings were the same as those above (1.6 cyc/rad and 0.34 rad/s, 520 nm). Thresholds at different percentage contrast modulation levels were determined by adjusting illumination. We then plotted percentage contrast modulation (Eq. 1) as a function of threshold illumination (Fig. 2C). Higher intensities were required to evoke responses to patterns of lower contrast and we found that percentage contrast modulation varied inversely with the square root of illumination at higher contrasts. This square-root relationship follows the de Vries–Rose Law, indicative of a contrast-detection mechanism limited by quantal fluctuation (De Vries 1943; Rose 1948; van Nes et al. 1967). As pattern contrast decreases, detection departs from the square-root relationship and requires higher intensities (>0.3–0.4 hν μm\(^{-2}\) s\(^{-1}\)), indicating a transition to just-noticeable differences according to Weber’s law. During the subjective nighttime hours contrast modulation thresholds increased 2-fold, although they remained proportional to the square root of the illumination levels (our light source did not provide sufficient light to reach the transition between the de Vries–Rose and Weber-like behaviors during the night). The subjective nighttime contrast sensitivity experiments were performed between 10 P.M. and midnight when circadian modulation of threshold is submaximal. All remaining experiments in this study were performed using 100% contrast modulation and low-illumination levels where optomotor threshold is limited by quantal fluctuations.

Tadpoles sense the temporal frequency of the rotating patterns

Is it the spatial or temporal (or both) properties of the visual system that are under circadian control? To answer this question we measured the optomotor thresholds of the animals in both subjective day and night to stimuli having different rotation speeds and bar widths (Fig. 3, A and B). Thresholds were plotted as a function of temporal frequency (Eq. 2). In general, the sensitivity functions overlapped closely, indicating that the animals were equally sensitive to the three spatial frequency patterns tested (0.64, 1.6, and 6.4 cyc/rad). We
and B subjective day and nighttime hours (bars fit the Lorentzian functions night to higher frequency in the day. Cutoff frequency of the other mechanism shifts from a lower frequency at but extends to higher temporal frequencies and does not change with time of day. Concluded that the tadpoles responded to the rate at which high contrast edges moved through their fields of view.

A circadian clock modulates temporal properties of tadpole vision

At low temporal frequencies all animals showed about the same sensitivity, in subjective day and night. As the stimulus frequency increased, sensitivity decreased monotonically at about 4 log units per decade to reach a minimum plateau of about –1.5 log units (Fig. 3, A and B). At night, however, sensitivity began to fall off at a lower temporal frequency and as a result the plateau was reached at <1 Hz, as opposed to about 3 Hz, during the day.

We fitted the temporal frequency data with two 2nd-order Lorentzian functions (Eq. 3). During the subjective day, a Lorentzian with a cutoff frequency of 1.1 Hz best fits the data in the low-frequency range and a second Lorentzian with lower magnitude (–1.5 log) and a higher cutoff frequency (12 Hz) fits the data well in the higher-frequency range (Fig. 3A). To describe the subjective night data (Fig. 3B) the low-frequency daytime Lorentzian must be shifted to a lower cutoff frequency (on the order of 0.35 Hz as opposed to 1.1 Hz during the subjective day). The plateau again was about 1.5 log units below the maximum level and coincident with the Lorentzian function fitting the “low-sensitivity” mechanism during daytime hours.

Figure 3C consolidates the data gathered at 520 nm into a single plot to illustrate the subjective day–night changes. Only the Lorentzian functions are shown. The cutoff frequency of the higher sensitivity function is lower by a factor of 4 for the nighttime. This lower cutoff frequency translates into a decreased ability to follow higher frequency stimuli. To illustrate this point, we compared subjective day–night changes in kinetics of the predicted impulse response (i.e., flash response). The impulse response in the temporal domain that we use here is analogous to the line-spread function (Kelly 1979) that approximates the profile of the receptive fields in the spatial domain. Applying the Fourier transform, we derived the impulse responses (Fig. 3C, insets) from the Lorentzian functions, assuming linearity of the system. The computed daytime impulse response (Eqs. 5 and 6) has a time-to-peak of 0.15 s, 3 times faster than the impulse response predicted at night. Integration times are 0.4 and 1 s for the day and night, respectively (Eq. 7). We show below that the ERG in subjective day and night has similar temporal properties. In summary, both low- and high-sensitivity temporal mechanisms mediate the optomotor response, but the temporal properties of only the high-sensitivity mechanism are under circadian control.

Spectral sensitivity depends on the temporal properties of the stimulus

During the subjective day, spectral sensitivity was maximal at 520 nm when tadpoles were stimulated with low temporal frequency patterns, 0.22 Hz (Fig. 4A) and 0.55 Hz (Fig. 4B). The sensitivity was lower in the longer wavelength region (about 2.5 log units lower at 720 nm) than in the blue region of the spectrum (1.5 log lower at 370 nm). Higher temporal frequency patterns (2.2 Hz) significantly changed the spectral sensitivity profile (Fig. 4C). Compared with lower temporal frequencies (Fig. 4, A and B), sensitivity decreased in the middle wavelength region with the maximum decrease (10-fold) at 520 nm and smaller decreases at longer and shorter wavelengths. The selective change of sensitivity in one spectral region indicates that more than one type of photoreceptor contributes to the optomotor response.

Circadian modulation of spectral sensitivity

Figure 4A shows that the spectral sensitivity of the optomotor response does not differ during the subjective day and night.
CIRCADIAN CHANGES IN TEMPORAL SENSITIVITY

for the lowest temporal frequency stimulus (0.22 Hz). Likewise, at the highest frequency, 2.2 Hz, the spectral profiles in the subjective day and night were similar, with a slight decline in the middle wavelengths [about 3-fold lower at night compared with day at 520 nm (Fig. 4C)]. However, at night, the sensitivity to the 0.55-Hz pattern decreases dramatically (50-fold at 520 nm) in the middle wavelength region (Fig. 4B). No changes in sensitivity were detected at the long (720 nm) and short (370 nm) wavelength regions of the spectrum. Therefore we conclude that the circadian shift in spectral sensitivity is mediated by photoreceptors with \( \lambda_{\text{max}} \approx 520 \) nm, most likely the principal rods. This high-sensitivity mechanism dominates the optomotor response to 0.55-Hz stimuli during the subjective day but not at night.

To quantitatively analyze photoreceptor contributions to the optomotor response, we fitted the spectral sensitivity curves with weighted sums of the different chromatic mechanisms known to be present in tadpoles (Witkovsky et al. 1981). The subjective daytime and night spectral sensitivity to 0.22-Hz stimuli (Fig. 4A) and that of the daytime to 0.55 Hz (Fig. 4B) are dominated by the middle-wavelength component, corresponding to the principal rod (\( \lambda_{\text{max}} = 520 \) nm) (Fig. 4D). The red component, corresponding to the abundant cones (\( \lambda_{\text{max}} = 611 \) nm), plays an important role in shaping the long-wavelength end of the spectrum where the responses are least sensitive. The blue component, corresponding to either the s-cones or the blue-sensitive rod (\( \lambda_{\text{max}} = 440 \) nm), however, does not appear to contribute significantly to the overall spectral sensitivity. The weighted sum of the chromatic components matches the behavioral data well.

The spectral sensitivities to high-frequency stimuli during the subjective day and night (Fig. 4C) and during the subjective night in response to 0.55-Hz stimuli are broadly tuned (Fig. 4E). We fitted the data primarily by decreasing the scaling factor of the middle wavelength component. Because there is no evidence that the Xenopus retina possess green-sensitive cones (Witkovsky et al. 1981; Zhang et al. 1994), the change in the spectral sensitivity most likely reflects a change in the response properties of principal rods. The weighted sum in Fig. 4E matches the behavioral data well, except at the short wavelength end. Here, it appears that an additional cone component, perhaps with a \( \lambda_{\text{max}} \) in the UV is operative. We conclude that both long- and short-wavelength spectral mechanisms underlie the spectral sensitivity of the optomotor response to high-frequency stimuli, and that a circadian shift in the rod cutoff frequency (Fig. 3C) is responsible for the change in spectral sensitivity at 0.55 Hz.

Circadian modulation of the ERG

What is the mode of action of a circadian oscillator on the kinetic properties of tadpole vision? Does the clock act at the level of the brain or retina? To answer these questions we...
examined retinal sensitivity by recording the ERG from stage 48 tadpoles during subjective day and night hours. Figure 5A shows ERG responses to 20-ms (520 nm) flashes of increasing intensity. Day and night ERGs consisted of characteristic b-waves preceding a slight undershoot (we seldom observed any a-waves at the intensities used). However, b-waves at night had consistently smaller saturating voltages (47.5 ± 6.5 μV, n = 4) than during the day (118 ± 12 μV, n = 5). In addition, night responses were sluggish, with significantly longer times to peak and recovery than day responses (see following text).

To quantify the subjective day–night effects on the ERG, amplitudes of the b-waves were plotted as a function of flash intensity (Fig. 5B). The day and night sensitivities are comparable in response to dim, 520-nm flashes; however, as intensities increase, the night ERG responses saturate, whereas the amplitude of the daytime response continues to increase almost 2.5-fold before saturating. Similar observations apply to ERG responses to red (700 nm) and blue (400 nm, not shown) flashes. The data were well fit (R² > 0.95) with Michaelis–Menten functions.

Spectral sensitivities were computed at a criterion response level of 22 μV for 400, 520, and 700 nm flashes (Fig. 5C). Both subjective day and night spectral sensitivities were well fit by the absorption curve of a vitamin A2–based rhodopsin (Govardovskii et al. 2000) with maximal sensitivity at 520 nm (Dartnall 1954; Witkovsky et al. 1981) and approximated the spectral sensitivity of the optomotor responses to low temporal frequencies (dashed line, open circles).

**Circadian modulation of the temporal properties of the b-wave**

Figure 6A illustrates that ERGs evoked by dim 20-ms flashes are slower during the subjective night that during the subjective day. Daytime ERGs grow rapidly to reach maximal amplitude 0.6 s after the flash, recover (time to zero-crossing = 1.3 s), and then undershoot the baseline voltage. At night the ERG peaks 0.86 s after a similar flash and recovers more slowly (1.8 s to zero-crossing). The curvilinear functions (dashed lines) in the graph are the impulse responses estimated from the temporal optomotor sensitivity functions (insets Fig. 3C). They are scaled in amplitude and delayed 400 ms along the time axis to fit the onset of the b-waves. The fits fail to describe the undershoot phase of the responses, but they qualitatively capture the day–night changes in kinetics of the b-wave, suggesting that the mechanisms constraining the temporal response of the optomotor response and those affecting the ERG b-wave are similar (see following text).

**Temporal integration by the retina increases at night**

We next examined the capacity of the retina to integrate dim light by recording ERGs in response to longer (2-s) flashes during both subjective day and night. Two seconds is about half the period of the slowest temporal frequency that produces maximal optomotor sensitivity (Fig. 3C). The intensity–response functions of the ERG are shifted to the left along the intensity axis (Fig. 5B), suggestive of longer integration times. To estimate the integration times we first verified linearity of the responses to dim stimuli. The output of a linear system can be computed by the convolution of the input signal with the system’s impulse response. Assuming the ERG response to a 20-ms flash (Fig. 6A, continuous lines) is representative of the system’s impulse response, we convolved it with a 2-s flash input to produce the system response (Fig. 6B, continuous lines). The good match of the predicted and recorded ERG
responses to 2-s flashes indicates that the system behaves linearly at low illumination levels. We applied Eq. 8 to calculate an approximate value for the integration times of the b-waves, yielding 1 and 0.4 s for the day and night values, respectively, the same values derived from the optomotor responses using Eq. 7.

A circadian clock modulates the bandwidth of the ERG

The frequency characteristics of a linear system are described by its power spectrum. We computed the power spectra of the ERGs recorded during subjective day and night to compare their frequency characteristics with those of the behavioral responses measured during subjective day and night. The power density spectra of the ERGs have the shape of band-pass filters (Fig. 6D) that coincide in the low-frequency range and have maxima at 0.15 Hz. However, differences in bandwidth are observed, as the daytime power spectrum extends to higher frequencies. Both spectra fall off at about 4 log units/decade, the same slope obtained in the behavioral experiments.

The differences between the subjective day and night power density spectra are similar to those of the optomotor sensitivity functions. To better compare them, the sensitivity functions for the optomotor responses were scaled vertically to match the ERG spectra at the lowest frequencies. The high-frequency cutoff of the ERGs and the optomotor responses correspond closely, particularly at night; during the day, the bandwidth of the ERG is slightly narrower than that of the optomotor data. These results are compatible with the notion that the bandwidth of the optomotor response is set early in the visual process at the level of the outer retina. It also indicates that the circadian modulation of the optomotor response acts at the level of the outer retina.

![Figure 6](https://example.com/figure6.png)

**FIG. 6.** Temporal properties of the ERG b-wave are under circadian control. A: average responses (n = 5) to dim flashes recorded from a dark-adapted tadpole during subjective day have a shorter time course than those recorded in the subjective night. Flash (arrow) duration was 20 ms at 520 nm. Flash intensity was 160 photons μm⁻² s⁻¹. Error bars are SE. Dashed lines are impulse responses shown in Fig. 3C (insets) and scaled to fit the b-wave amplitudes. B: average ERG responses to 2-s flashes, 520 nm (horizontal line). Flash intensity was 5.1 photons μm⁻² s⁻¹. After a 0.5- to 0.6-s delay, subjective day and night b-waves have similar onsets, but the day response saturates after 1 s, whereas the night response continues to grow, saturating 1.7 to 2.0 s after flash onset. Continuous lines are the responses predicted by convolution of the 2-s stimulus with the ERG responses to dim (20-ms) flashes in A. C: dim light responses in A normalized relative to respective saturating voltages (20 ms, 520 nm, Fig. 5B). Onset of the b-waves overlapping closely for 0.2 s, at which time the daytime b-wave recovers, whereas the night response continues to grow. Dashed lines are the impulse–response functions from Fig. 6A normalized relative to their respective saturating voltages. NR, normalized response. D: power spectral density of the ERG responses in A. Subjective day and night have similar power levels in the low frequency range, but the day power spectrum exhibits a higher cutoff frequency. Cutoff frequencies of the day and night power density spectra correspond closely to the cutoff frequencies of the temporal sensitivity functions (dashed lines) of the optomotor response (Fig. 3). Optomotor sensitivity functions were scaled vertically to have equal power at the lowest frequency. E: power spectral density of the normalized ERG responses to brief flashes in C have a band-pass shape tuned at about 0.15 Hz. Subjective night ERGs contain more power in the low-frequency end of the spectrum but not at the higher end where both day and night power spectra fall along the same asymptote of 4 log units/decade.
changes in amplitude. Normalizing the responses in Fig. 6A relative to their respective saturating voltages (Fig. 6C), we found that b-waves recorded during subjective day and night grew steadily at the same rate for about 0.2 s. Then, the day responses diverged and began to recover, whereas the amplitude of the night response continued to grow. This behavior can be explained by a modulation in the strength of a feedback mechanism. That is, after a short delay, the feedback mechanism “kicks in” and suppresses the response. The feedback is stronger during the day, thereby reducing the time to peak and speeding up the recovery.

Changes in the strength of feedback result in a trade-off between power in the low frequencies and total bandwidth (Marc and Liu 2000; E. Solessio, unpublished observations). In Fig. 6E the power spectra for the normalized subjective day and night ERGs (Fig. 6C) have the same shapes as the power spectra in Fig. 6D but now the nighttime power spectrum is shifted vertically about 10-fold from the daytime power spectrum and both have the same high-frequency asymptote. This suggests that the low-frequency component of the ERG is under circadian control but not the high-frequency mechanism common to both day and night. Further, the trade-off between low-frequency energy and bandwidth suggests that the circadian mechanism acts through a feedback pathway.

Finally, the 2 properties of amplitude and bandwidth of the ERG may or may not be independently modulated by the circadian clock. First, the clock modulates the strength of a feedback mechanism—weakly during the night, strongly during the day—to set the bandwidth of the information relayed to the brain, and then it compensates for the loss in sensitivity that is intrinsic to feedback mechanisms by increasing the gain during the day (Fig. 5B).

**DISCUSSION**

We found that young *Xenopus* tadpoles do not see the same day and night. Further, we found that their optomotor sensitivity can be explained in terms of 2 mechanisms: one that operates in the middle range of the visible spectrum with high sensitivity to low temporal frequencies, and another less-sensitive mechanism that has broader spectral and temporal tuning. A circadian clock modulates the temporal properties of the more sensitive “rod” mechanism.

**The optomotor response**

Optomotor responses of vertebrates have been studied in both amphibia (Birukow 1949; Burgers 1952) and fish (Schae rer and Neumeyer 1996). Commonly, optomotor responses are scored using the proportion of positive responses (PPRs) or the optomotor gain (Schaerer and Neumeyer 1996). We instead used a modified version of the 2-alternative preferential-looking method to determine visual thresholds (Bilotta and Powers 1991; Teller et al. 1974). This method requires about 10 min per animal and yields thresholds that are consistent among observers. Critical to this consistency is auditory feedback, which signals both correct and incorrect decisions by the observer. In essence, tadpoles “teach” the observer their optomotor response.

Optomotor measures of visual sensitivity depend on the contrast and on the spatial and temporal properties of the rotating grating. We used gratings of 100% contrast to determine the intensity of light necessary to reach threshold. We found for *Xenopus* tadpoles, as did Schaerer and Neumeyer (1996) for goldfish, that within the range we tested, sensitivity is invariant relative to the spatial frequency of the gratings. In zebrafish, an extended range of spatial frequencies was tested and significant losses in sensitivity were observed at high frequencies (Maaswinkel and Li 2003). From optical considerations alone (Chung et al. 1975), we would not expect *Xenopus* tadpoles to exhibit optomotor responses to spatial frequencies >9 cyc/°.

**Rod and cone contributions to the optomotor response**

We found that the spectral sensitivity of dark-adapted tadpoles is greatest at about 520 nm for low temporal frequencies, during subjective day and night, and most likely reflects the activity of principal rods (Fig. 4D). Similar tuning in the middle of the spectrum was observed in goldfish optomotor responses tested under dark-adapted (scotopic) conditions (Schaerer and Neumeyer 1996). At high temporal frequencies, the spectral sensitivity curve is broader and likely reflects multiple chromatic contributions to the response (Fig. 4E). The loss of sensitivity in the center of the spectrum, where rods are maximally sensitive, suggests that the rods cannot respond effectively to the higher temporal frequencies. Therefore additional illumination is necessary to reach the threshold of cones that follow higher frequency stimuli and broaden the spectrum of the response. In an earlier study using *Xenopus* tadpoles (Cronly-Dillon and Muntz 1965), spectral sensitivity was also broad but had maximal sensitivity at longer wavelengths, about 650 nm. The different $\lambda_{\text{max}}$ in spectral sensitivity curves we find is most likely attributable to our testing of tadpoles under dark-adapted conditions rather than the light-adapted conditions used by Cronly-Dillon and Muntz. In fact, optomotor responses under photopic conditions in both goldfish (Schaerer and Neumeyer 1996) and zebrafish (Kraus and Neumeyer 2003) also had peak sensitivities in the long-wavelength end of the spectrum, coincident with the peak sensitivity of the L-cone receptors.

**Scotopic and photopic thresholds**

Our results indicate that motion detection in *Xenopus* is restricted by the variability in the quantal content of the light stimulus that governs the response thresholds. This follows from the relationship between modulation thresholds and background illumination (Fig. 2C). The relationship follows the square-root behavior expected from an ideal detector limited by quantal fluctuations (De Vries 1943; Rose 1948; van Nes et al. 1967).

How many photons do rods absorb at behavioral threshold? For low temporal frequency stimuli (0.2–0.3 Hz, 100% contrast) we found that at threshold the light intensity was $5.2 \times 10^{-2}$ photons $\mu\text{m}^{-2} \text{s}^{-1}$ incident at the surface of the cornea. The light reaching the back of the eye is proportional to the ratio of the areas of the pupil and the retina ($\approx 0.5$) (Lyubarsky and Pugh 1996), yielding $2.6 \times 10^{-2}$ photons $\mu\text{m}^{-2} \text{s}^{-1}$. However, because of losses of the ocular media, about 20% of
the incident light (5.2 × 10^{-3} photons μm^{-2} s^{-1}) reaches the retina (Barlow 1977). Next, we determined the number of photoisomerizations per rod of stage 48 tadpoles, taking into account the density of rhodopsin and quantum efficiency of photoisomerization. This calculation yields an “effective rod collecting area” of 4 μm^2 for nonpolarized transverse illumination (E. Solessio, unpublished observations) that when corrected for linear dichroic effects (Harosi 1975) yields 8 μm^2. Multiplying 5.2 × 10^{-3} photons μm^{-2} s^{-1} by the effective collecting area of 8 μm^2 indicates that about 0.04 photoisomerizations rod^{-1} s^{-1} are necessary to reach threshold. Put in other terms, at the threshold level of illumination single rods absorb on average one photon every 25 s.

This remarkable similarity is similar to the sensitivity of Bufo bufo toads that, on average, require that each rod absorb one photon every 50 s to detect and catch prey in dim illumination (Aho et al. 1988). The high visual sensitivity in Bufo correlates with the sensitivity of a particular class of ganglion cells characterized by large receptive fields (input from ~4,000 rods), high gain (~100×), and long integration times (~1.5 s) (Copenhagen et al. 1987). Xenopus ganglion cells should possess similar properties for tadpoles to detect the extremely dim moving gratings. Our ERG recordings indicate that long integration time is a feature already found in the outer retina (Fig. 6B).

Threshold for optomotor responses at 520 nm increased 30-fold for stimuli having high temporal frequency. That is, a tadpole was 30 times less sensitive to high than to low frequencies (Fig. 3). The change in spectral sensitivity (Fig. 4) indicates that cones mediate the optomotor responses to high-frequency stimuli. Cones constitute about 50% of photoreceptors in the Xenopus retina, of which 90% are red-sensitive (Zhang et al. 1994). We estimated the number of photoisomerizations per cone using the same procedure as described above for rods. The collecting area of cones (0.8 μm^2) was estimated by scaling the rod-collecting area by the ratio of the cone-to-rod volumes. Using the dimensions of red cones from Zhang et al. (1994), we determined that, at threshold, each cone absorbs one photon every 10 s. This rate is comparable to that estimated for rod thresholds, and implies that in Xenopus tadpoles, cones may have the capacity to signal the absorption of single photons. This is a controversial notion because a cone responds to a single photon with a decrease of <0.1–0.5% of its dark current (Donner et al. 1998; Perry and McNaughton 1991; Schnapf et al. 1990), which is not large enough to reliably signal single photoisomerizations. In rods, single-photon responses represent about a 3–5% change in the dark current (Baylor et al. 1979; Kefalov et al. 2003). However, we leave open the possibility that cones at larval stages may possess highly sensitive mechanisms for signaling dim light. For example, we have found significantly lower levels of GCAP proteins in stage 48 Xenopus cones than in juveniles and adults (E. Solessio, unpublished observations). GCAP proteins mediate the recovery of the light response, and their absence in GCAP−/− knock-out mice dramatically increases photoreponses (Burns et al. 2002). It is not known whether in early development lower levels of GCAP or other proteins enhance single-photon responses by cones consistent with the behavioral thresholds we observe.

**Tuning of the temporal transfer function**

The shape of the rod-sensitivity function in Xenopus is low-pass as in other species ranging from humans to goldfish (Bilotta et al. 1998; Hess and Nordby 1986). This low-pass characteristic reflects the limitations of the scotopic system to process high temporal frequencies. We cannot rule out that tests using temporal frequencies <0.2 Hz will yield sensitivity functions having band-pass characteristics (Borst and Egelhaaf 1989; Kelly 1979). Because trials were limited to 5-s duration we used temporal frequencies >0.2 Hz to ensure that animal’s retina was exposed to at least one full period of the moving pattern during each trial.

The differential assignment of rod and cone contributions to the optomotor responses at different temporal frequencies is also supported by electrophysiological responses recorded from photoreceptors (Krizaj 2000; Krizaj et al. 1998). Recordings from “gatepost” rods, principal rods that are coupled to red cones by gap junctions, yield both rod and cone responses to sinusoidal excitation. The rod responses were attenuated above 2 Hz (Fig. 7A), whereas cone responses extended to 8 Hz. These cutoff frequencies are in good agreement with the
cutoff frequencies of the high- and low-sensitivity mechanisms that we propose mediate the optomotor response.

The ERG as a measure of retinal activity at optomotor threshold levels

It has not escaped our notice that the levels of illumination we used to evoke the ERG responses were considerably higher than the threshold levels of the optomotor responses. Do the ERG recordings provide a valid measure of retinal sensitivity at threshold? Indeed, others have shown that scotopic threshold response (STR) responses arising from inner retinal activity in mammals are significantly more sensitive than a- or b-waves (Siewing et al. 1986) but we did not observe such responses in stage 48 tadpoles. However, we analyzed ERGs elicited by long (2-s) flashes and found the slopes of their intensity–response functions approached a value of 1.0 on log-log coordinates (Fig. 5B), indicating a linear relationship between stimulus intensity and response amplitude. We were able to detect ERG responses to flashes delivering 0.3 to 1 photons \( \mu m^{-2} s^{-1} \) at the cornea (Fig. 5B), which is about 10-fold higher than the illumination level required to reach threshold of the optomotor responses. We were unable to record ERG b-waves to flash intensities below 0.3 photons \( \mu m^{-2} s^{-1} \).

Temporal constraints in the outer retina limit the optomotor responses

It is well accepted that the ERG a-wave arises from the activity of photoreceptors (Brown 1968), whereas the b-wave is largely representative of ON bipolar cell responses (Gurevich and Slaughter 1993), and therefore of information transmitted to the inner retina. We found close correspondence between the time-to-peak of the b-wave (disregarding the delay after the flash) and the cutoff frequency of the optomotor response (Fig. 6), suggesting that temporal filtering properties of the outer retina of Xenopus tadpoles limit the temporal properties of the optomotor response. This notion is supported by the corresponding subjective day and night changes in cutoff frequency. In humans, limitations in flicker frequency responses are also imposed at the level of the outer retina (Kelly 1971). The notion that the slowest process along a chain of events determines the response of the system is analogous to the concept of dominant time constant applied to the study of transduction pathways in rod photoreceptors (Nikonov et al. 1998; Peperberg et al. 1992).

Bandwidth of the scotopic optomotor response and its circadian modulation

We compared the temporal transfer function of the optomotor responses with the power spectrum of dark-adapted Xenopus rod photocurrents in response to dim flashes (Fig. 7A; E. Solessio, unpublished observations). The power spectrum of the photocurrents was fitted with a 2nd-order Lorentzian function with a cutoff frequency of 0.08 Hz, considerably lower than the subjective night or daytime cutoff frequencies of the optomotor responses (0.35 and 1.0 Hz, respectively). That the bandwidth of the input stage (photocurrents) is considerably narrower than the bandwidth of the output (behavior) presents a dilemma. How does the visual system expand its frequency range? Our illumination levels are insufficient to accelerate rod and/or ganglion cell photoresponses (Donner et al. 1995) and alter the shape of the sensitivity functions. We found (Fig. 6) how circadian control of feedback mechanisms may underlie the day–night changes in the temporal properties of optomotor responses. Such feedback mechanisms may act at molecular (E. Solessio, unpublished observations), cellular (Baylor et al. 1984), and circuitry (Marc and Liu 2000) levels.

In an attempt to reconcile the differences in bandwidth between photocurrents and behavior, we explored a common characteristic of feedback systems: a trade-off between system gain and temporal bandwidth (Marc and Liu 2000). Figure 7B illustrates the subjective day and night optomotor responses (Fig. 6E) scaled to match the high frequency asymptote of the single photon photocurrent power spectrum. Increasing the strength of feedback results in an expansion of the response bandwidth. Figure 7B also illustrates how temporal characteristics of rod photovoltages (Krizaj 2000) might be explained in terms of feedback mechanisms. The comparable bandwidths of rod photovoltage responses, daytime ERGs, and optomotor responses suggest that the principal feedback loop may act at the level of the rod inner segment.

This simplistic view of the role of feedback and its intrinsic attenuation of gain may explain why synaptic transmission to bipolar and ganglion cells has high gain (Copenhagen et al. 1987); that is, they compensate in part for gain lost in the photoreceptor response. It may also explain how the dominant time constant limiting the recovery of rod photocurrents (Nikonov et al. 1998; Peperberg et al. 1992) could set the asymptote of the frequency responses for all subsequent stages involved in mediating the optomotor response.

Circadian modulation of the optomotor response

The day–night changes in the optomotor response differ from the “Purkinje shift” that is regulated in some animals by circadian oscillators (Mangel 2001). In the Purkinje shift, night vision slows down and becomes more sensitive as the system shifts from cone- to rod-dominant mechanisms. We describe a circadian modulation of the temporal properties of the rod system whereby the rod responses speed up during the subjective day and slow down at night. The location of the circadian oscillator(s) that modulates the rod system is not yet known.

ACKNOWLEDGMENTS

We thank Drs. Erik Herzog, Steven Chamberlain, Bart Farell, and Paul Witkovsky for helpful comments on the manuscript and discussions and M. Kelly, Y. S. Sohn, C. McGuinness, and V. Venkatadass for assistance.

GRANTS

This work was supported by National Institutes of Health Grants EY-11256 and EY-12975 to B. E. Knox, EY-00667 and MH-49741 to R. B. Barlow, EY-13772 to G. A. Engbretson, Research to Prevent Blindness (Unrestricted Grant to State University of New York Upstate Medical University Department of Ophthalmology and Career Development Award to E. Solessio, and The Lions Clubs of Central New York State.

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Witkovsky for helpful comments on the manuscript and discussions and M. Kelly, Y. S. Sohn, C. McGuinness, and V. Venkatadass for assistance.

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