Scotopic Spectral Sensitivity of the Optomotor Response in the Green Treefrog *Hyla cinerea*

**RICHARD B. KING, JOHN K. DOUGLASS, JOHN B. PHILLIPS, AND CHARLES L. BAUBE**

*Department of Biology, Indiana University, Bloomington, Indiana 47405 (R.B.K., J.B.P., C.L.B.); and Department of Biology, Yale University, New Haven, Connecticut 06511 (J.K.D.)*

**ABSTRACT**

Amphibians are unusual among vertebrates in having two spectral classes of rod photoreceptors, unique amphibian "green" rods and typical vertebrate "red" rods. Although amphibians have been the subject of extensive research on visual function, it is not known whether possession of two classes of rods is a general feature of Amphibia, nor is it clear to what behaviors each class of rods contributes. The Hylidae comprise one of the largest families within Amphibia but have been little studied with respect to visual function. Here, we demonstrate the presence of green and red rods in *Hyla cinerea* by microspectrophotometry and provide evidence for the contribution of green rods to one visually based behavior, the optomotor response. In addition, we discuss the role of green and red rods in visually based behavior in light of apparently conflicting demands resulting from the need to maximize absolute sensitivity, visual acuity, and color sensitivity.

Received June 24, 1992; revision accepted April 15, 1993. Address reprint requests to Richard B. King at his present address, Department of Biological Sciences, Northern Illinois University, DeKalb, IL 60115. J.K. Douglass' current address is ARL Division of Neurobiology, 611 Gould-Simpson Building, University of Arizona, Tucson, AZ 85719.

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METHODS

Study animals

Adult Hyla cinerea were collected in Escambia Co., Florida and Baldwin Co., Alabama from July 7 to 31, 1989. Animals used in behavioral tests were maintained in plastic terraria (40 cm × 27 cm × 16 cm) in groups of 5–6. Fresh water was available continuously and food (adult houseflies, Musca domestica) was provided every 2–3 days. Animals were kept in a room maintained at 24–27°C, with a 12:12 LD photocycle. Animals used in microspectrophotometry were maintained under similar conditions but without a strictly controlled photocycle.

Microspectrophotometry

Visual pigment absorption spectra and dichroism were measured in isolated rod outer segments (ROSs) by microspectrophotometry. Animals were dark-adapted overnight or for a minimum of 2 hr. All subsequent procedures were performed under infrared or dim red illumination (Schott RG720 or Kodak No. 70 long-pass filters). Frogs were chilled, decapitated and double-pithed, and an eye was isolated and submerged in amphibian saline. The retina was carefully removed from the pigment epithelium, and small pieces of retina were sandwiched between two coverslips for placement on the microscope stage of the microspectrophotometer (MSP).

The MSP was a modified single-beam design (full details in Zeiger and Goldsmith, in press) and was used to measure ROS absorption at 5 nm intervals from 330 to 700 nm. The monochromator was calibrated with a holmium oxide filter. Each spectral scan consisted of a downward sweep from 700 to 330 nm at 10 nm intervals followed by a return sweep of the intervening wavelengths (335–695 nm). The net orientation of visual pigment chromophores within the disk membranes was exploited in order to minimize artifacts due to absorbance by unoriented nonvisual pigment molecules. ROSs were scanned with the e-vector of the measuring beam first perpendicular, then parallel to the longitudinal axis of the ROS. A dichroic difference spectrum was computed by subtracting the “parallel” scan from the “perpendicular” scan (Liebman, ’62; Harosi, ’75). ROS’s were identified as green or red rods according to the location of the absorption maximum of the dichroic difference spectrum (ca. 430–435 nm for green rods, 500–505 nm for red rods). Each difference spectrum was zeroed to the average optical density in the longwave region (550–700 nm for green rods, 625–700 nm for red rods), and normalized to 1 at the measured wavelength closest to the expected absorption maximum (435 nm for green rods, 500 nm for red rods). Finally, each difference spectrum was Fourier filtered to remove high frequency artifacts (Zeiger and Goldsmith, ’89).

Optomotor test apparatus

Our optomotor apparatus was modeled after the one used by Cronly-Dillon and Muntz (’65). It consisted of a projector with 750 W tungsten lamp, a holder for narrow-band interference filters (6–10 nm h.b.w.), a pair of graded, circular, neutral density wedges (Ealing Electro-Optics), a clear Plexiglas pattern wheel with 16 equally spaced opaque stripes radiating from the center, and an inverted cone centered over a test container (Fig. 1). The image of the pattern wheel was focused onto the inner surface of the cone using condensing lenses and a mirror. The inner surface of the cone was painted flat white (reflectance = ca. 90% between 400 and 700 nm). A metal rod projected into the cone and served as a pedestal onto which the test container, a glass cylinder 9.2 cm in diameter and 7.4 cm tall fitted with a Plexiglas lid, was placed.

Monochromatic light passed through the pattern wheel producing a rotating pattern of alternating black and monochromatic stripes on the inner surface of the cone. Direction of pattern rotation was controlled by reversing the direction of the motor turning the pattern wheel. Spectral content of the stripes was varied using interference filters. Brightness (quantal flux) of the stripes was controlled by adjusting the position of the neutral density wedges.

Fig. 1. Schematic diagram of the optomotor apparatus used in this study. L, light source; IF, interference filter; ND, neutral density wedges; PW, pattern wheel; M, mirror; VC, infrared-sensitive video camera.
using a stepping motor. Speed of pattern rotation was set at 3.3 rpm (20°/sec) as in Cronly-Dillon and Muntz ('65). The resulting pattern had a spatial frequency of 0.04 cycles/deg and a contrast ratio of 0.30–0.32.

The optomotor apparatus was enclosed in a light tight box located in a darkened room. The behavior of test animals was monitored using an infrared sensitive video camera with illumination provided by a 3 V lamp fitted with a 780 nm long-pass filter (Schott RG780). Video monitor, stepping motor controller, switch controlling the direction of pattern rotation, and digital counter indicating the position of the neutral density wedges were located in an adjoining room.

Light levels in the optomotor apparatus were determined by positioning a calibrated photodiode (United Detector Technology PIN 10DP/SB) within the inverted cone directly above the test container pedestal. The photodiode was aligned horizontally facing the inner surface of the inverted cone. The pattern wheel was stopped during calibration and positioned so that the photodiode was not shaded by one of the black stripes. Output of the photodiode was measured with a picocammeter (Keithley Electronics). The neutral density wedges were calibrated to allow the quantal flux at each wavelength to be set at approximately 0.02 log unit steps over 3.7 log units of intensity.

**Optomotor test protocol**

Prior to testing, treefrogs were dark-adapted for at least 2 hr. Following dark adaptation, damp filter paper was placed on the bottom of the test container, a treefrog was put into the container, the container was placed onto the pedestal within the test apparatus, and rotation of the pattern wheel was started. Neutral density wedges were initially positioned at maximum optical density and a randomly selected interference filter was placed in the filter holder. Treefrogs were given 5 min to acclimate to the test container during which time they would typically settle on the side of the container facing upward. After 5 min, neutral density wedges were moved to increase light intensity by about 0.2 log quanta. We recorded number of clockwise and counterclockwise body movements and position (side or floor of the container) for the next minute. The direction of pattern wheel rotation was then reversed and treefrog movements and position were recorded for another minute. Light intensity was increased at 0.2 log quanta intervals and data collection repeated at each interval until the optomotor response (described below) was exhibited.

At low light intensity, treefrogs remained motionless, moved in either direction, or pushed at the top of the test container. Reversing the direction of pattern rotation had no noticeable effect on treefrog behavior. At threshold, behavior changed dramatically. Typically, treefrogs exhibited smooth head movements (head nystagmus) in the direction of pattern rotation, descended to the floor of the test container, moved predominantly in the direction of pattern rotation, and pushed against the bottom edge of the test container. When direction of pattern rotation was reversed, treefrogs reversed their predominant direction of movement.

After reaching threshold, neutral density wedges were returned to maximum optical density and a different interference filter was selected using a random number table. After a 5 min break, testing proceeded as described above. Treefrogs were tested at additional wavelengths as long as they continued to exhibit clear optomotor responses. All tests of a given treefrog were carried out within several days of each other. Between 6 and 11 individual treefrogs were tested at each of 10 wavelengths (400, 430, 450, 480, 500, 520, 550, 580, 600, and 620 nm) (Table 1). Treefrogs were tested only once at any given wavelength.

Threshold quantal flux, the light intensity at which the optomotor response was first seen (in quanta cm⁻² sec⁻¹) was calculated for each treefrog at each wavelength using calibration data collected as described above. Statistical significance of apparent peaks in spectral sensitivity was tested by repeated-measures multivariate analysis of variance (MANOVA) with polynomial contrasts across specific wavelength intervals using SPSS/PC + version 3.2 statistical software (Norusis, '88). In these analyses, a significant linear term indicates that sensitivity increases or decreases over the interval analyzed; a significant quadratic term indicates that the relationship between sensitivity and wave-

**TABLE 1. Scotopic spectral sensitivities of the optomotor response in Hyla cinerea**

<table>
<thead>
<tr>
<th>Wavelength (nm)</th>
<th>Sample size</th>
<th>Sensitivity ± standard error (log quanta cm⁻² sec⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>400</td>
<td>6</td>
<td>7.74 ± 0.161</td>
</tr>
<tr>
<td>430</td>
<td>11</td>
<td>7.08 ± 0.086</td>
</tr>
<tr>
<td>450</td>
<td>10</td>
<td>7.33 ± 0.162</td>
</tr>
<tr>
<td>480</td>
<td>10</td>
<td>7.18 ± 0.178</td>
</tr>
<tr>
<td>500</td>
<td>11</td>
<td>6.86 ± 0.109</td>
</tr>
<tr>
<td>520</td>
<td>7</td>
<td>7.34 ± 0.138</td>
</tr>
<tr>
<td>550</td>
<td>6</td>
<td>7.62 ± 0.207</td>
</tr>
<tr>
<td>580</td>
<td>6</td>
<td>7.64 ± 0.145</td>
</tr>
<tr>
<td>600</td>
<td>6</td>
<td>8.27 ± 0.356</td>
</tr>
<tr>
<td>620</td>
<td>6</td>
<td>8.63 ± 0.203</td>
</tr>
</tbody>
</table>
spectra (solid lines) of response (filled circles) and green and red rod pigment absorbance.

Sensitivities were standardized by recalculating each as a deviation from the mean threshold at 500 nm (the wavelength at which peak sensitivity occurred). Bars represent 1 standard error on either side of means. Pigment absorbance spectra are considered results significant if \( P < 0.05 \).

RESULTS

Absorbance spectra of rod outer segments obtained by microspectrophotometry (Fig. 2) demonstrated the presence of both green and red rods. The average wavelength of maximal absorbance was 435 nm for green rods (\( n = 10 \) rods) and 503 nm for red rods (\( n = 10 \) rods). The shapes of these spectra closely matched templates for vitamin A\(_1\) based rhodopsins having the same absorption maxima (Partridge and DeGrip, '91).

The scotopic optomotor response of \( H. \) cinerea showed two peaks in sensitivity, one at 500 nm and a second at 430 nm (Table 1, Fig. 2). Repeated-measures MANOVA revealed a significant quadratic term over the interval from 400 to 450 nm (\( P = 0.04 \)) and the interval from 480 to 520 nm (\( P < 0.001 \)), indicating that sensitivity at 430 nm and at 500 nm did exceed sensitivity at adjacent wavelengths (Table 2). A shoulder in sensitivity occurred at 550–580 nm. However, the quadratic term was not significant for the 500–620 nm interval (\( P = 0.10 \)), indicating that the decline in sensitivity from 500 to 620 nm was essentially linear (Table 2).

Mean sensitivity at 500 nm corresponded to a flux of \( 10^{8} \) quanta cm\(^{-2}\) sec\(^{-1}\) as calculated from neutral density wedge and interference filter calibration data. Absolute sensitivity in \( H. \) cinerea may differ from this value because of differences in position, orientation, and acceptance angle between a treefrog's eye and the calibration photodiode.

DISCUSSION

Two classes of rod photoreceptors with visual pigment absorption peaks at 435 and 503 nm were identified in \( H. \) cinerea by microspectrophotometry (Fig. 2). These peaks are similar in position to those reported for rhodopsin-based (vitamin A\(_1\)) visual pigments found in green rods (430–433 nm) and red rods (501–504 nm) of other adult terrestrial amphibians (Crescitelli, '72; Lythgoe, '72; Harosi, '75; Sillman, '87; Witkovsky et al., '81) and presumably have a similar biochemical basis. In contrast, larval amphibians possess porphyropsin-based (vitamin A\(_2\)) visual pigments (e.g., Liebman and Entine, '68; Witkovsky et al., '81), and some aquatic and semiaquatic amphibians possess porphyropsin-based or both porphyropsin- and rhodopsin-based visual pigments as adults (e.g., Reuter et al., '71).

### TABLE 2. Repeated-measures analysis of variance to test for peaks in sensitivity at 430 and 500 nm and for a shoulder in sensitivity at 550–580 nm (see text for details)\(^1\)

<table>
<thead>
<tr>
<th>Hypothesis</th>
<th>Comparison</th>
<th>Sample size</th>
<th>Term</th>
<th>( F )</th>
<th>( P )</th>
<th>Hypothesis supported?</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak at 430 nm</td>
<td>400, 430, and 450 nm</td>
<td>6</td>
<td>Linear</td>
<td>3.20</td>
<td>0.134</td>
<td>Yes</td>
</tr>
<tr>
<td>Peak at 500 nm</td>
<td>480, 500 and 520 nm</td>
<td>7</td>
<td>Quadratic</td>
<td>7.15</td>
<td>0.044</td>
<td>Yes</td>
</tr>
<tr>
<td>Shoulder at 550</td>
<td>500, 520, 550, 550–580</td>
<td>6</td>
<td>Linear</td>
<td>32.53</td>
<td>&lt;0.001</td>
<td>Yes</td>
</tr>
<tr>
<td>Shoulder at 550</td>
<td>500, 520, 550, 550–580</td>
<td>6</td>
<td>Quadratic</td>
<td>4.96</td>
<td>&lt;0.001</td>
<td>No</td>
</tr>
</tbody>
</table>

\(^1\)Because not all treefrogs were tested at all wavelengths, sample sizes here are smaller than those shown in Table 2.
The sensitivity peak we observed at 430 nm strongly suggests that green rods contribute to the scotopic optomotor response of *H. cincta*ina. The sensitivity peak at 500 nm could represent a contribution of red rods or accessory members of double cones, both of which have visual pigment absorption peaks of about 503 nm (Lythgoe, '72; Harosi, '75; Sillman, '87; Witkovsky et al., '81). However, because we measured threshold sensitivity following extensive dark adaptation, and because sensitivity at 500 nm was even greater than at 430 nm, we feel it is likely that red rods (rather than accessory cones) are responsible for the 500 nm peak. Furthermore, the maximum sensitivity we observed (10^6.86 quanta cm^{-2} sec^{-1}) is comparable to that observed in ERG recordings from *Xenopus* red rods (about 10^7 quanta cm^{-2} sec^{-1}, Fig. 5 in Witkovsky et al., '81). Our statistical analysis suggests that single cones and principle members of double cones, both of which have visual pigment absorption peaks at about 575–580 nm (Lythgoe, '72), did not make a significant contribution to spectral sensitivity in our experiments. However, given that in our test for a shoulder at 550–580 nm, the quadratic term approached statistical significance (P = 0.10, Table 2), further investigation of cone contributions may be warranted.

The wavelengths at which we observed peak sensitivity in optomotor behavior match closely the wavelengths at which green and red rod visual pigments exhibit peak absorption (Fig. 2). However, the way in which green and red rods interact to produce the sensitivity curve we observed remains unclear. One possibility is that at threshold, optomotor spectral sensitivity is determined by whichever population of photoreceptors is most sensitive at a given wavelength. Under this interpretation, our results suggest that green rods are more sensitive at wavelengths around 430 nm and red rods are more sensitive at wavelengths around 500 nm. In contrast, ERG recordings suggest that red rods are more sensitive at all wavelengths (by 0.3–0.8 log quanta in *Xenopus*, Witkovsky et al., '81; by 2.95–4.35 log quanta in *Rana*, Frank, '70). This difference may relate to the low abundance of green rods (<15% of the total rod populations, Donner and Reuter, '76); because of their rarity, green rods may contribute relatively little to the ERG despite being more sensitive than red rods at short wavelengths.

The results of our optomotor experiment do not permit a firm conclusion about whether green and red rod responses are processed antagonistically, as would be necessary for color sensitivity. The observation that peaks in spectral sensitivity appear narrower than peaks in visual pigment absorption (Fig. 2) is consistent with antagonistic processing (Muntz, '74). However, antagonistic processing should also result in greater separation of sensitivity peaks than would be predicted from pigment absorption curves (Muntz '74). Such separation is not evident from our results although given that optomotor thresholds were determined at 20–30 nm intervals, our ability to detect displacement of sensitivity peaks is limited. Narrow peaks in sensitivity could also arise from physical characteristics of the eye or individual photoreceptors (e.g., selective spectral absorption by lens, vitreous humour, receptor oil droplets, Dartnall, '53), but this does not appear to be the case in amphibians (Muntz, '77).

Electrophysiological recordings from retinal ganglion cells do not aid in determining the nature of green and red rod contributions to the optomotor response. Green and red rods reportedly contribute both to broad-band color insensitive units (in *Salamandra*, Temple et al., '82) and to color opponent units (in *Rana*, Reuter and Virtanen, '72; Bäckström and Reuter, '74, '75). However, green rod contributions to these units apparently drop out and red rod contributions come to predominate as illumination is reduced from mesopic to scotopic levels. Though by no means conclusive, our results leave open the possibility that opponent processing of green and red rod responses provides *H. cincta*ina with rod-based color sensitivity. We plan to test this directly using stimuli consisting of combinations of two wavelengths (as in Muntz, '66; Kicliter et al., '81) and selective chromatic adaptation.

Potential rod contributions to color sensitivity in amphibians are paradoxical given the organization of visual systems in other vertebrates. This organization appears to reflect trade-offs in the resolution of three distinct visual problems: (1) the ability to function at low light levels (absolute sensitivity), (2) the ability to resolve fine detail (visual acuity), and (3) the ability to discriminate among spectrally distinct stimuli (spectral discriminability or color sensitivity) (Muntz, '74; Lythgoe, '79; Ali and Klyne, '85). These trade-offs exist because characteristics that confer high absolute sensitivity reduce visual acuity, characteristics that confer high acuity reduce absolute sensitivity, and characteristics that confer color sensitivity reduce both absolute sensitivity and visual acuity (Table 3). In most vertebrates, these trade-offs appear to be resolved through the presence of two semi-independent visual subsystems (Muntz, '74). One subsystem consists of a single class of rod photoreceptors and operates at low light levels. This subsystem provides high ab-
TABLE 3. Visual problems, typical vertebrate solutions, and their consequences (based on Muntz, 1974; Lythgoe, 1979; Ali and Klyne, 1985)

<table>
<thead>
<tr>
<th>Problem</th>
<th>Solutions</th>
<th>Consequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absolute sensitivity</td>
<td>Large photoreceptors (rods)</td>
<td>Increases absolute sensitivity by providing more pigment and a larger area for light capture</td>
</tr>
<tr>
<td></td>
<td>High degree of neural convergence</td>
<td>Reduces visual acuity by increasing &quot;grain&quot; of the retina</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Increases absolute sensitivity by providing a larger area for light capture</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reduces visual acuity by increasing &quot;grain&quot; of the retina</td>
</tr>
<tr>
<td>Visual acuity</td>
<td>Small, tightly packed photoreceptors (cones)</td>
<td>Increases absolute sensitivity because individual photoreceptors provide only a small area for light capture</td>
</tr>
<tr>
<td></td>
<td>Low degree of neural convergence</td>
<td>Increases visual acuity by minimizing &quot;grain&quot; of the retina</td>
</tr>
<tr>
<td>Color sensitivity</td>
<td>Multiple spectral classes of photoreceptors with opponent processing</td>
<td>Necessary for color sensitivity</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reduces absolute sensitivity because antagonistic inputs reduce opponent cell responses</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reduces visual acuity because integrating signals across photoreceptors increases &quot;grain&quot; of the retina</td>
</tr>
</tbody>
</table>

Solute sensitivity but has poor visual acuity and lacks color sensitivity. The other subsystem consists of two or more classes of cone photoreceptors and operates at high light levels. This subsystem provides color sensitivity and high visual acuity but has poor absolute sensitivity.

The presence of two classes of rod photoreceptors in amphibians together with electrophysiological (Reuter and Virtanen, ’72, ’76; Bäckström and Reuter, ’74, ’75; Donner and Reuter, ’76; Kicliter et al., ’81; Himstedt et al., ’81) and behavioral (Muntz, ’66; Hailman and Jaeger, ’78; Fite et al., ’78; Kicliter et al., ’81; R. King and J. B. Phillips, unpublished data) and the resulting loss of acuity presumably is of no consequence. In contrast, prey capture, in which animals orient toward and strike at moving prey-sized stimuli (Roth, ’88), should require relatively high visual acuity and might be expected to be color-insensitive at low light levels (Table 3). We plan a series of experiments utilizing behaviors such as these to further clarify the role of green and red rods in amphibian vision. A systematic comparison of the visual bases of these behaviors will contribute to a better understanding of the adaptive trade-offs that underlie the functional organization of the amphibian visual system.

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LITERATURE CITED


