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Articles

Changes in the colour of light cue circadian activity

Michael J. Pauers*, James A. Kuchenbecker, Maureen Neitz, Jay Neitz*

Department of Ophthalmology, University of Washington Medical School, Seattle, WA, U.S.A.

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The discovery of melanopsin, the nonvisual opsin present in intrinsically photosensitive retinal ganglion cells (ipRGCs), has created great excitement in the field of circadian biology. Now, researchers have emphasized melanopsin as the main photopigment governing circadian activity in vertebrates. Circadian biologists have tested this idea under standard 12:12 h light:dark cycles in the laboratory that lack the dramatic daily colour changes of natural skylight. Here we used a stimulus paradigm in which the colour of the illumination changed throughout the day, thus mimicking natural skylight, but luminance, sensed intrinsically by melanopsin containing ganglion cells, was kept constant. We show in two species of cichlid, *Aequidens pulcher* and *Labeotropheus fuelleborni*, that changes in light colour, not intensity, are the primary determinants of natural circadian activity. Moreover, opponent-cone photoreceptor inputs to ipRGCs mediate the sensation of wavelength change, and not the intrinsic photopigment, melanopsin. These results have implications for understanding the evolutionary biology of nonvisual photosensory pathways and answer long-standing questions about the nature and distribution of photopigments in organisms, including providing a solution to the mystery of why nocturnal animals routinely have mutations that interrupt the function of their short-wavelength-sensitive photopigment gene.

For several decades, cycles of alternating white light and darkness have been the standard stimuli used in laboratory studies of circadian activity. These stimuli have been used to investigate circadian behavioural patterns in organisms ranging from bacteria to primates (Tang et al. 1999; Morgan 2004). In nature, however, dramatic diel changes in skylight colour are associated with concomitant changes in luminance (Krull et al. 1985; Endler 1993). Although the role such chromatic changes might play in the setting of circadian activity patterns has been largely ignored, two recent studies on captive redheaded bunting, *Emberiza bruniceps*, showed that wavelength is crucial in the regulation of circadian activity (Rani & Kumar 2000; Malik et al. 2004).

A careful consideration of the role chromatic changes might play in circadian activity patterns via cone opponent neural pathways has the potential to resolve the unexplained associations between cone photoreceptor complement (i.e. both the sensitivity and relative numbers of photoreceptors) and circadian activity. Arrhythmic and strongly nocturnal organisms lack functioning short-wavelength-sensitive photopigments (Peichl & Moutairou 1998; Peichl 2005). Furthermore, with the exception of some strongly nocturnal species, sensory systems involved in the entrainment of daily activity rhythms include spectrally opponent components that could detect chromatic time-of-day information in the natural world (Neitz & Neitz 2011). Well-characterized examples of such sensory systems include the simple intracellular photoreceptor complex of *Halobacterium salinarium* (Kokoeva et al. 2002), the parietal eye of *Uta stansburiana* (Su et al. 2006), and the retinal ganglion cells of *Macaca* (Dacey et al. 2005).

Retinoid-based photopigments evolved in the most ancient organisms, the archeans, which lack the visuoneural capacity for object discrimination. Furthermore, photoreceptors are thought to have evolved only once throughout the history of life and were co-opted into the various types of eyes that evolved much later within the animals (Land & Nilsson 2002). Evidence from the aforementioned marine archean *H. salinarium* demonstrates that its spectrally opponent photoreceptor complex, with opposing actions produced by peak sensitivities to orange and ultraviolet wavelengths, acts as a solar clock to confer an essentially crepuscular activity pattern. Phototaxis towards the ocean surface is exhibited at dawn and dusk when long-wavelength light used in photosynthesis is prevalent, and migration away from the surface is exhibited at midday when lethal ultraviolet light is most intense (Dundas & Larsen 1962).

The universal presence of colour-opponent inputs to circadian systems in extant animals suggests that they would retain the ability to compare the relative stimulation among spectral subtypes of photoreceptors in order to extract time-of-day information from the natural world, much like *H. salinarium* (Land & Nilsson 2002;

* Correspondence and present address: M. J. Pauers, Milwaukee Public Museum, Division of Vertebrate Zoology, 800 W. Wells Street, Milwaukee, WI 53233, U.S.A.; Jay Neitz, Department of Ophthalmology, University of Washington Medical School, 1959 NE Pacific Street, Seattle, WA 98195, U.S.A.
E-mail addresses: mjpauers@gmail.com (M. J. Pauers), jneitz@uw.edu (J. Neitz).

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Arendt 2003). Furthermore, the key component of such a system is the presence and spectral positioning of a short-wavelength-sensitive photoreceptor (Gehring & Rosbash 2003). We hypothesize that colour opponency initially evolved to regulate activity patterns in response to changes in sunlight colour that accompany the approximately 24 h rotation of the earth, and that this colour-opponent input remains the most important source for time-of-day information under natural conditions. A prediction of this hypothesis, then, is that if cone photoreceptors influence circadian activity patterns, some wavelengths of light should suppress activity. This is counter to the prevailing notion that light, in general, stimulates activity (Tang et al. 1999; Morgan 2004).

Rod, cone and nonrod/noncone photopigments all have inputs into circuitry that provide information about illumination to systems that control daily activity patterns. Since fishes have a wide variety of photoreceptors, including several nonvisual receptors found in the pineal area and in the retinal ganglion cells (e.g. Forsell et al. 2001; Foster & Hankins 2002; Falcon et al. 2003; Davies et al. 2011), and since, except for highly specialized species, they also lack eyelids, we believe that fish are an ideal and overlooked organism to use in the determination of the roles of the different photopigment inputs to circadian behaviour under natural, as opposed to laboratory, conditions.

METHODS

The experimental arena was an 80-litre aquarium with lighting provided by a custom-engineered light-emitting diode (LED) system. We arranged 28 groupings of three computer-controlled LEDs (manufacturer specified wavelengths of 440 nm, 505 nm and 630 nm, respectively; obtained from LEDSupply.com) across a circuit board. Four infrared emitter/detector packages monitored fish activity patterns, and programmable logic devices controlled the output of the infrared detectors. The entire circuit board was connected via parallel port to a remote computer, which recorded the activity counts from the infrared detectors. The circuit board was held 10.16 cm above the aquarium, and a diffuser was placed underneath the LEDs. The aquarium, with circuit board and detectors, was placed in a light-tight enclosure, in which the fish only received light from the LEDs.

LED Calibration

Crucial to these experiments was both the accuracy and consistency of the LEDs. One non-ideal property of LEDs is that peak wavelength can vary with current. This was eliminated by implementing a pulse-width modulation (PWM) paradigm to control intensity. This produces an all-or-nothing current through the LED for a varying length of time (duty cycle) within a fixed period. The period of the switching frequency is much faster than the integration time of the photoreceptor, and, therefore, duty cycle sets the LED intensity. The linearity of the PWM method was verified as follows: Each LED type was set to intensity gains of 0.25, 0.50, 0.75 and 1 times their maximum power. Spectroradiometric measurements in W/m^2/wavelength was measured (Fig. 1a), converted to quanta, integrated across wavelength, and then normalized by the maximum value. Those quotients were then plotted against the theoretical set points of 0.25, 0.5, 0.75 and 1 (Fig. 1b, c, d). A linear trend line fitted to the data with X intercept fixed to zero yielded R^2 values for the 440 nm, 505 nm and 630 nm LEDs of 0.976, 0.996 and 1, respectively. Additionally, the dominant wavelength (as measured by the spectroradiometer) shifted by only 1 nm over the intensity ranges of all three LEDs.

A diffuser was placed between the tank and the LED array to integrate the light output from the LED triads. A UV-Visible Spectrometer (StellarNet EPP2000C-50) placed inside the empty tank was used to measure LED reflection off of a calibrated (250–2000 nm) Spectralon white diffuse reflectance standard

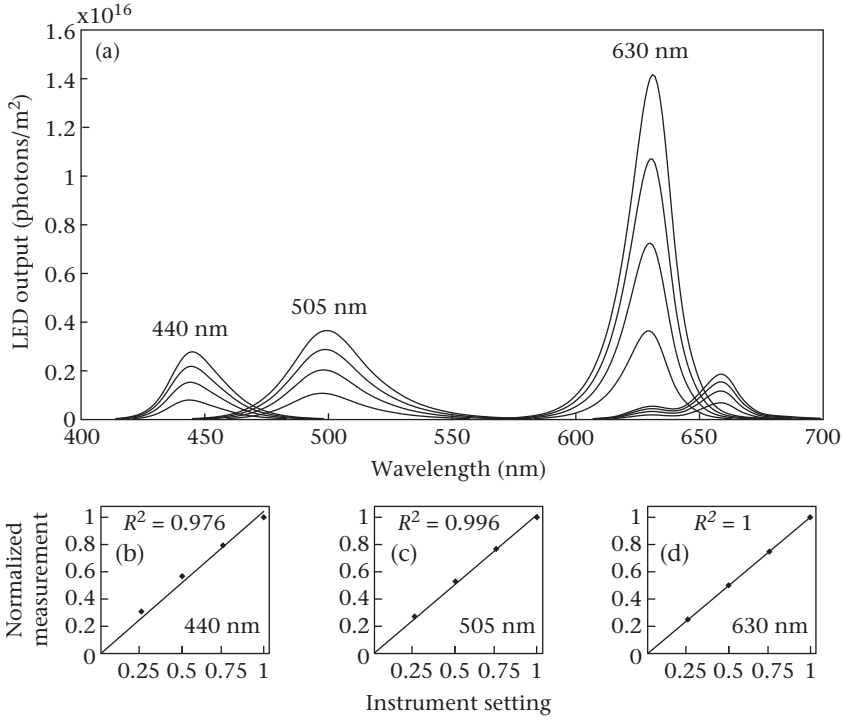

Figure 1. Verification of light-emitting diode (LED) peaks and pulse-width modulation (PWM) of LED output. (a) LED peak wavelengths for each of the three LED types used. Upper trace: output at 100% power; next lowest trace: output at 75% power; next lowest trace: output at 50% power; lowest trace: output at 25% power. (b) Linearity of the violet LED. (c) Linearity of aqua LED. (d) Linearity of red LED.