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Evidence of Retinal Light Damage in *Rana cascadae*: A Declining Amphibian Species

KATHERINE V. FITE, ANDREW BLAUSTEIN, LYNN BENGSTON, AND HEATHER E. HEWITT

Diurnal wildlife populations that inhabit high-altitude environments may be at risk of cumulative retinal injury from increased penetration of solar ultraviolet radiation resulting from a declining concentration of stratospheric ozone. Previously, the deleterious effect of ultraviolet radiation on the eggs and hatching success has been shown in *Rana cascadae*, a high-altitude species undergoing major population declines. To assess whether the retinas of this species may be showing signs of injury resulting from cumulative exposure to solar radiation, both the histological condition and photoreceptor densities were compared in *R. cascadae*, in experimentally light-damaged *R. pipiens*, and in control *R. pipiens*. Distinctive outer-retinal abnormalities observed in the inferior retina of *R. cascadae* were similar to those observed in light-damaged *R. pipiens*. These included an abnormal distribution of retinal pigment epithelial melanin, damaged photoreceptors and the presence of large, pigment-filled macrophages. Statistically significant, negative correlations were found between extent of outer-retinal histopathology and cone photoreceptor densities both in light-damaged *R. pipiens* ($r = -0.54$) and *R. cascadae* ($r = -0.59$). Overall cone photoreceptor densities were lower in the inferior versus superior retina of *R.cascadae* and in light-damaged *R. pipiens* compared with controls. In *R. pipiens*, experimental light damage significantly reduced green-rod and miniature-cone densities in the inferior retina; both contain a short-wavelength sensitive photopigment. The lowest densities of these two photoreceptor types were found in the inferior retina of *R. cascadae* as well. Similarities between the major abnormalities observed in *R. cascadae* and in experimentally light-damaged *R. pipiens* suggest that environmental solar radiation is damaging the retinas of *R. cascadae* and perhaps other high-altitude species as well.

MAJOR population declines are occurring in a number of formerly abundant amphibian species (Wake, 1991; Blaustein et al., 1994a; Drost and Fellers, 1996). Although the global extent of these declines is not well documented (Pechmann and Wilbur, 1994), considerable evidence exists that many species are undergoing precipitous declines over a wide range of habitats and geographic locations, even in areas not overtly disturbed by anthropogenic influences. Although no single explanation can account for all reported declines, a number of variables such as habitat alteration or destruction, environmental pollution, introduced predators, pathogens, overcollection, natural population fluctuations, and increased solar ultraviolet radiation may be having direct or contributory effects on amphibian populations.

At present, amphibian population declines in the United States are extensive in the Pacific Northwest, where five of 34 species are now listed as candidates for the U.S. endangered species list (Blaustein et al., 1994b). With regard to *R. cascadae*, 22% of historical populations have disappeared in Oregon (Marshall et al., 1996), and this species has become rare over the southern portion of its range in California (Fellers and Drost, 1993). Recently, an increased penetration of solar ultraviolet (UV) radiation, particularly UV-B (280–315 nm) caused by catalytic destruction and thinning of the stratospheric ozone layer, has been implicated in the increased embryonic mortality of several high-altitude (> 1000 m) species in the Pacific Northwest. Blaustein and coworkers (1994b) have investigated embryonic mortality rates in *R. cascadae*, which deposits its eggs in open, shallow water, exposing them to prolonged periods of direct sunlight (Nussbaum et al., 1983). Results indicate that increased ambient UV-B, acting alone or in synchrony with a pathogen, is contributing to declines in *R. cascadae* and perhaps other species, as well (Kiesecker and Blaustein, 1995).

Major losses of amphibian species are occurring in other geographic areas as well. For example, as many as 14 amphibian species have disappeared in Australia in recent years (Tyler, 1991; Richards et al., 1993).

The adverse effects of solar UV-radiation on the eye and retina are well known and have been documented in humans over a wide range

of professions in which individuals are exposed to bright sunlight on a regular or daily basis (Young, 1988). Both epidemiological and experimental studies show a strong association between solar UV radiation and increased risk of damage to the cornea, lens, and retina, even at relatively low exposure levels (Taylor et al., 1988; Werner, et al., 1989; Rapp and Smith, 1992). Cone photoreceptors are particularly vulnerable to light damage since their photopigments have high photon-absorption peaks in the ultraviolet range (Sperling 1980; Sperling et al., 1980; Van Norren and Schellekens, 1990).

Ranid frogs have a large, panoramic field of vision due to the elevated, periscopic position of the eyes (Fite, 1974). Both incident and reflected light impinges upon the eyes and retina with little attenuation or absorption by the optical components of the eye. Also, frogs lack the light-shielding, protective mechanisms provided by recessed orbits, eyebrows, eyelashes, regular eye blinks, and spontaneous eye movements found in other highly visual vertebrates. Frogs are highly dependent upon vision for food acquisition and predator avoidance, and any damage to the optical and/or neural tissues of the eye may seriously impair visual function and reduce individual survival rates. Solar UV-B radiation is more prevalent at higher elevations (Blumthaler et al., 1992; Ambach et al. 1993; Aurer, 1993). High-altitude species such as *R. cascadae* that inhabit shallow water with the head and eyes above the surface for long periods of time are at particular risk of ocular light damage from prolonged exposure to direct or reflected sunlight. The transparency of the ocular media to UV has been reported to go as low as 330 nm in one ranid species (Govardovsky and Zueva, 1974).

In an effort to determine whether evidence of cumulative light damage is present in a high-altitude, amphibian species, the histological condition of the outer retina and densities of different photoreceptor types were analyzed in *R. cascadae*, a species currently undergoing major population declines in the Pacific Northwest (Drost and Fellers, 1996; D. B. Marshall, M. W. Chilcote, and H. Weeks, Oregon Dept. of Fish and Wildlife, Portland, 1996, unpubl.). The characteristic morphological changes associated with retinal light damage in the frog retina were identified in a nondeclining species, *R. pipiens*, using a light-exposure model known to produce retinal light damage in other species. Experimentally light-damaged *R. pipiens* retinas were compared with those of control *R. pipiens* using quantitative, histopathological measures developed previously (see Fite et al., 1993, 1994).

Materials and Methods

Adult *R. cascadae* were collected from a small pond in Linn County Oregon (elevation 1190 m); adult *R. pipiens* were obtained from a commercial supplier (Hazen Frog Farms, Alburg, VT). Based upon normative data for body size (snout–vent length), animals of both species were judged to be three years of age. Following anesthetization (immersion in MS222, conc. 1: 500), ophthalmoscopic examination of the intraocular lens and retina was followed by euthanasia and removal of both eyes. The posterior chamber of each eye was perfused with 3 cc of fixative (1% paraformaldehyde, 1.24% gluteraldehyde in 0.12 M phosphate buffer, pH 7.4, with 9.23 mM CaCl2 added).

The anterior segment of the eye and vitreous were removed, and four, 2-mm-diameter tissue punches containing the retina, choroid, and sclera were obtained from each eye of six adult *R. cascadae*. Tissue punches were taken from the center of each retinal quadrant in both eyes of each animal (e.g., superior-temporal, superior-nasal, inferior-temporal, and inferior-nasal quadrants). Tissue samples were dehydrated and embedded in Polybed 812, and one-micron thick, transverse sections were cut from each tissue block. The orientation of each block was adjusted during preliminary sectioning so that the final plane of section was parallel to the photoreceptors. Sections were stained with 5% thionin for light-microscopic evaluation at a magnification of ×1000.

Adult *R. pipiens* maintained on a 12:12 h light: dark cycle (ambient luminance, 28–32 lux) were experimentally light-damaged as follows. The light-exposure chamber consisted of a moist, air-cooled, glass aquarium surrounded on all sides by cool-white, "daylight" fluorescent lamps with broad, spectral-emission curves (standard illuminant C). Animals were exposed to a constant luminance of 3200 lux for 8 h, and returned to the 12:12 light:dark cycle for 48 h. [For reference, the midday sky obscured by clouds registers 500–2000 lux; clear sky on a sunny day may reach 10,000–30,000 lux. (Thorington, 1985)]. Subsequently, each animal was euthanized, both eyes removed from two light-damaged *R. pipiens*, a 1-mm-diameter punch was obtained from each of four retinal quadrants in both eyes of each animal and processed for histological analysis using procedures described above for *R. cascadae*.

Histopathological evaluation of the outer retina was based upon a semiquantitative, rating scale developed previously for assessment of experimentally light-damaged avian retinas (see

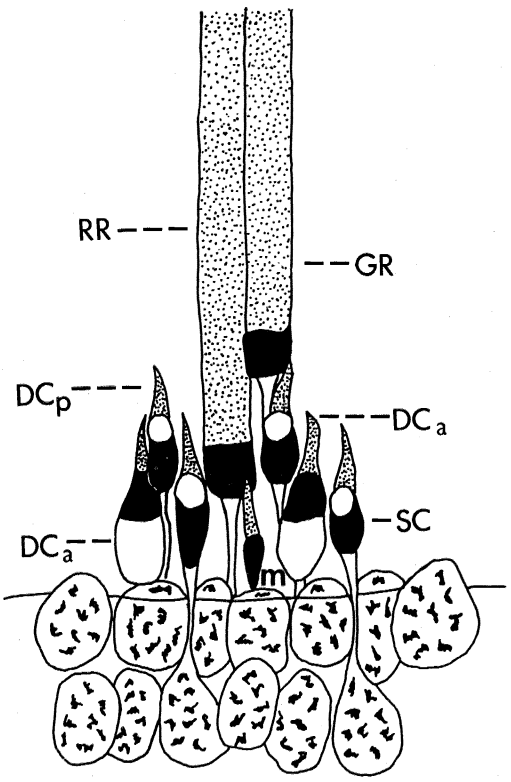

Fig. 1. Composite drawing illustrating the six types of photoreceptors found in frog retinas: RR = red rod, GR = green rod, DCp = principal cone of the double-cone pair, DCa = accessory cone of the double-cone pair, SC = single cone, m = miniature cone. Note the oil-droplet between the inner and outer segments of single cones and principal cones.

Fite et al., 1992, 1993). RPE cells and photoreceptors were analyzed using a four-point scale (0 = normal, 1 = detectable abnormality, 2 = conspicuous abnormality; 3 = severe abnormalities). Morphological features evaluated included the appearance of RPE cells, intracellular distribution of RPE melanin, presence of macrophages in the subretinal space, photoreceptors with swollen, truncated and/or disoriented outer segments, atypical staining characteristics, and abnormal intracellular vacuoles. These histopathological features are characteristic of light-damaged retinas and were rated for two sections cut from each tissue block (eight sections/eye) separated by at least 25 microns. A mean histopathology rating was calculated for each retinal quadrant.

Photoreceptor densities were quantified in each section for the following photoreceptor types illustrated in Figure 1: red (rhodopsin) rods (λ max = 502 nm), green rods (λ max = 432 nm), single cones and principal cones (λ max = 580 nm), and miniature cones, (λ max < 432 nm). A calibrated, eyepiece micrometer and oil-immersion objective (× 100) were used to obtain photoreceptor counts from eight, consecutive, 10-micron-wide intervals across the RPE/photoreceptor layer. Photoreceptor densities were obtained from sections that were evaluated for histopathology.

Initial data analysis revealed that measures from superior-nasal and superior-temporal retinal quadrants were quite similar for each animal, as were measures from inferior-nasal and inferior-temporal retinal quadrants. Therefore, histopathology ratings and photoreceptor densities measures from superior-nasal and superior-temporal sections in the superior hemiretina were combined for statistical analysis; likewise, measures obtained from inferior-nasal and inferior-temporal sections in the inferior hemiretina were combined. Tests for significant differences between mean rod densities and mean cone densities in the inferior versus superior retina were made within each of the three groups (*R. cascadae*, light-damaged *R. pipiens*, and control *R. pipiens*) using ANOVA and pairwise comparison of means. Photoreceptor densities obtained from inferior-retinal and from superior-retinal sections were compared for light-damaged *R. pipiens* versus control *R. pipiens*. Pearson correlation coefficients and probabilities were calculated for histopathology ratings versus photoreceptor densities within each group of animals.

To determine the effects of experimental light damage on specific photoreceptor populations, tests for significant differences in photoreceptor densities for light-damaged *R. pipiens* versus control *R. pipiens* were performed for red rods, green rods, cones with oil droplets, cones without oil droplets, and miniature cones. Also, photoreceptor densities of specific types were compared in the inferior versus superior retina in *R. cascadae*.

Results

In *R. cascadae*, conspicuous abnormalities were found in the RPE and photoreceptor layers, particularly in tissue samples taken from the inferior retina (Figs. 2–3). Retinal pigment epithelial (RPE) cell abnormalities included clumping and irregular distribution of melanin granules, presence of multiple, large, pigment-filled macrophages, and unusual staining of RPE intercellular junctions in regions showing alteration or disruption of photoreceptor outer segments. In rods, a disorganization of the nor-

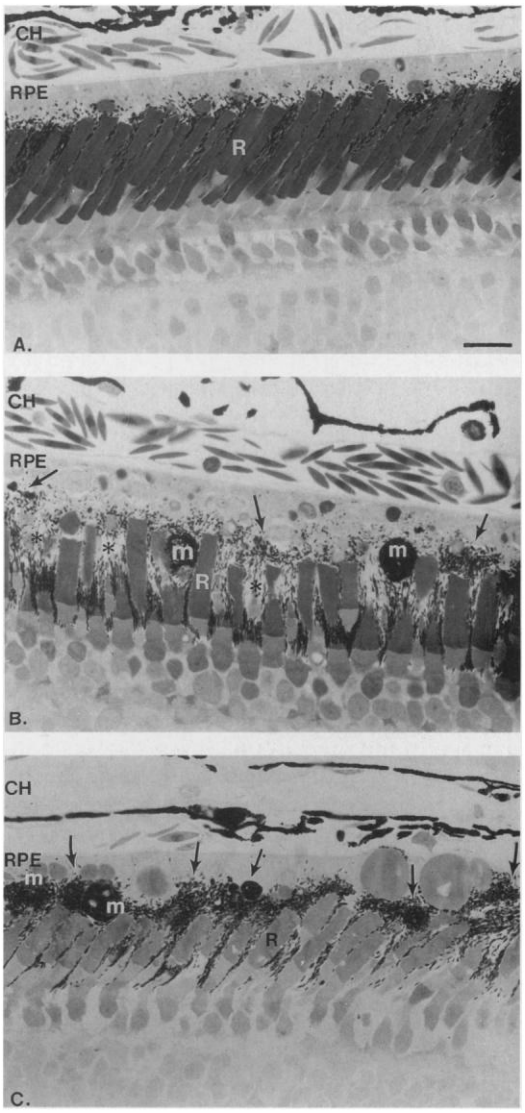

Fig. 2. (A) Transverse section from the inferior retina of a control *Rana pipiens* showing the retinal pigment epithelial cell layer (RPE) adjacent to the rod (R) outer segments (CH, choroid layer, magnification bar = 10 microns). (B) Retinal section from light-damaged *R. pipiens* showing regions of abnormal distribution of RPE melanin pigmentation (arrows), swollen and damaged rod outer segments, large, pigment-filled macrophages (m), and areas with missing or severely truncated outer segments (*) (C) Inferior retinal section from *R. cascadae* showing major abnormalities in RPE melanin pigment distribution (arrows), and large, pigment-filled macrophages (m) adjacent to abnormal rod outer segments.

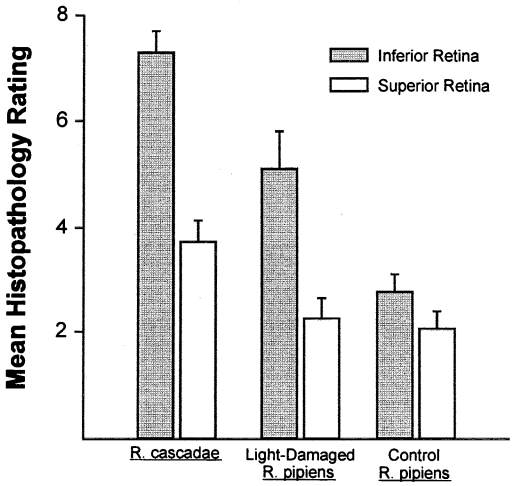

Fig. 3. Mean histopathology ratings from inferior and superior retina comparing *Rana cascadae*, light-damaged *R. pipiens*, and control *R. pipiens*.

mally regular array of outer segments as well as fragmentation and shortening of outer segments were observed. In cones, abnormal vacuoles were observed in the inner segments of cones having darkly stained, pyknotic nuclei, indicative of necrosis.

In experimentally light-damaged *R. pipiens*, a pattern of outer-retinal histopathology similar to that seen in *R. cascadae* also was observed (Figs. 2–3). Significant differences ($P < 0.01$) in histopathology ratings for the inferior versus superior retina occurred in light-damaged *R. pipiens* (Fig. 3). Significant differences in histopathology ratings also occurred in the inferior retina of light-damaged *R. pipiens* when compared with the inferior retina of control *R. pipiens* ($P < 0.01$).

With regard to photoreceptors, total rod densities were significantly reduced in the inferior retina of light-damaged *R. pipiens* ($P < 0.01$; Fig. 4A) compared with control *R. pipiens*. However, total cone densities were significantly lower in the inferior versus the superior retina of all three groups of frogs ($P < 0.01$, Fig. 4B). In light-damaged *R. pipiens*, total cone densities were 30% lower in the inferior retina and 20% lower in the superior retina when compared with control *R. pipiens*. Cone densities in *R. cascadae* were remarkably similar to those found in light-damaged *R. pipiens* (Fig. 4B). Significant negative correlations were found between total cone densities and the degree of outer-retinal histopathology in both light-damaged *R. pipiens* [$r = -0.54$ ($P < 0.01$)] and in *R. cascadae* [$r = -0.59$ ($P < 0.01$)].

Specific effects of experimental light damage

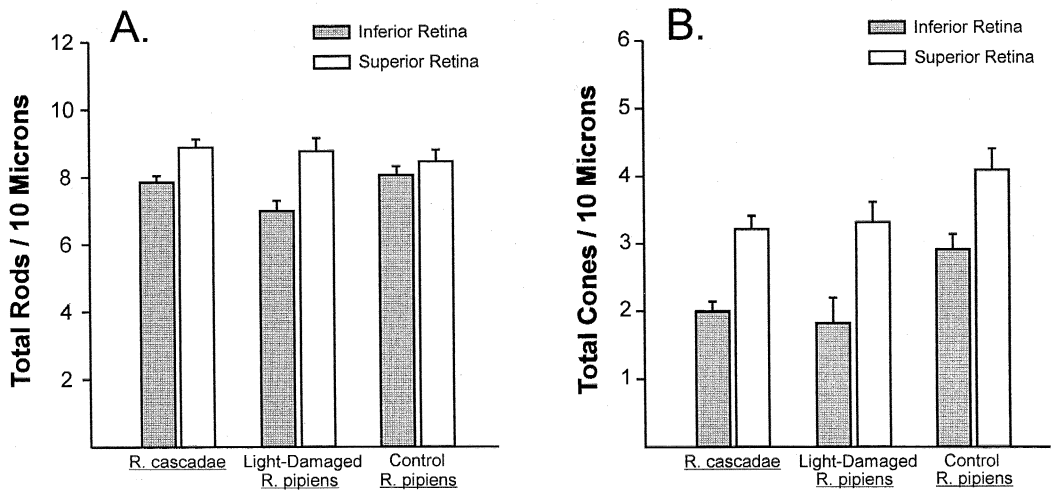

Fig. 4. (A) Total rod and (B) total cone densities in inferior versus superior retina of *R. cascadae*, experimentally light-damaged *R. pipiens*, and control *R. pipiens*.

on the densities of different photoreceptor types in the superior and inferior retina were compared in light-damaged *R. pipiens* versus control *R. pipiens*. In addition, relative densities for specific photoreceptor types were compared in light-damaged *R. pipiens* and *R. cascadae*. In the inferior retina of light-damaged *R. pipiens* versus control *R. pipiens*, green-rod densities (Fig. 5A) were reduced by 63% ($P < 0.05$), but red-rod densities were only slightly reduced (5%) compared with controls (Fig. 5B). In *R. cascadae*, green-rod densities were 24% lower in the inferior versus superior retina, whereas red-rod densities were 16% lower in the inferior versus superior retina.

Densities of cones that contain oil droplets (single cones and principal cones) were significantly reduced both in the inferior retina and superior retina of light-damaged *R.pipiens* when compared with control *R. pipiens* ($P < 0.05$; Fig. 5C). *Rana cascadae* showed the same relative densities of these cone types in the inferior versus superior retina as did light-damaged *R. pipiens*. Miniature cones showed a 60% reduction in the inferior versus superior retina of experimentally light-damaged *R. pipiens* ($P < 0.02$) compared with control *R. pipiens* (Fig. 5D). Similarly, 57% fewer miniature cones were found in the inferior versus superior retina of *R. cascadae*.

Discussion

Over the past two decades, many investigations have shown that a strong association exists between solar UV-radiation and increased risk of damage to the eye and retina from sustained or even low levels of visible, short-wavelength and UV-radiation (Rosenthal et al., 1985; Taylor, 1989; Dolin, 1994). Long-term exposure to UV radiation causes morphological changes and damage to the outer retina. Even moderate intensities of UV-B (less than 1% of incident values) can produce significant damage in the cone photoreceptor population essential for sharp vision (Sperling et al., 1980; Ham et al., 1982; Collier et al,, 1989).

The histopathological features observed in the outer retina of *R. cascadae*, particularly those in the inferior retina, were strikingly similar to those observed following experimental light damage in *R. pipiens*. Whereas the quantitative effects of an acute, eight-hour exposure to a broad-spectrum luminance of 3200 lux are not directly comparable to the cumulative effects of light exposure in the natural environment, the qualitative similarities in the overall pattern of outer-retinal histopathology and densities of specific photoreceptor types in *R. cascadae* and light-damaged *R. pipiens* strongly suggest that environmentally induced retinal light damage is occurring in *R. cascadae*, particularly in the inferior retina. Abnormal morphological features in light-damaged *R. pipiens* and in *R. cascadae* ranged from irregularities in the density and distribution of RPE melanin to major pathological signs such as the presence of large, pigment-laden macrophages, damaged outer segments, and lower overall cone densities, all of which have been documented in light-damaged vertebrate retinas (Rapp and Smith, 1992; Fite, et al., 1993).

Previously, Eckmiller and Steinberg (1981) de-

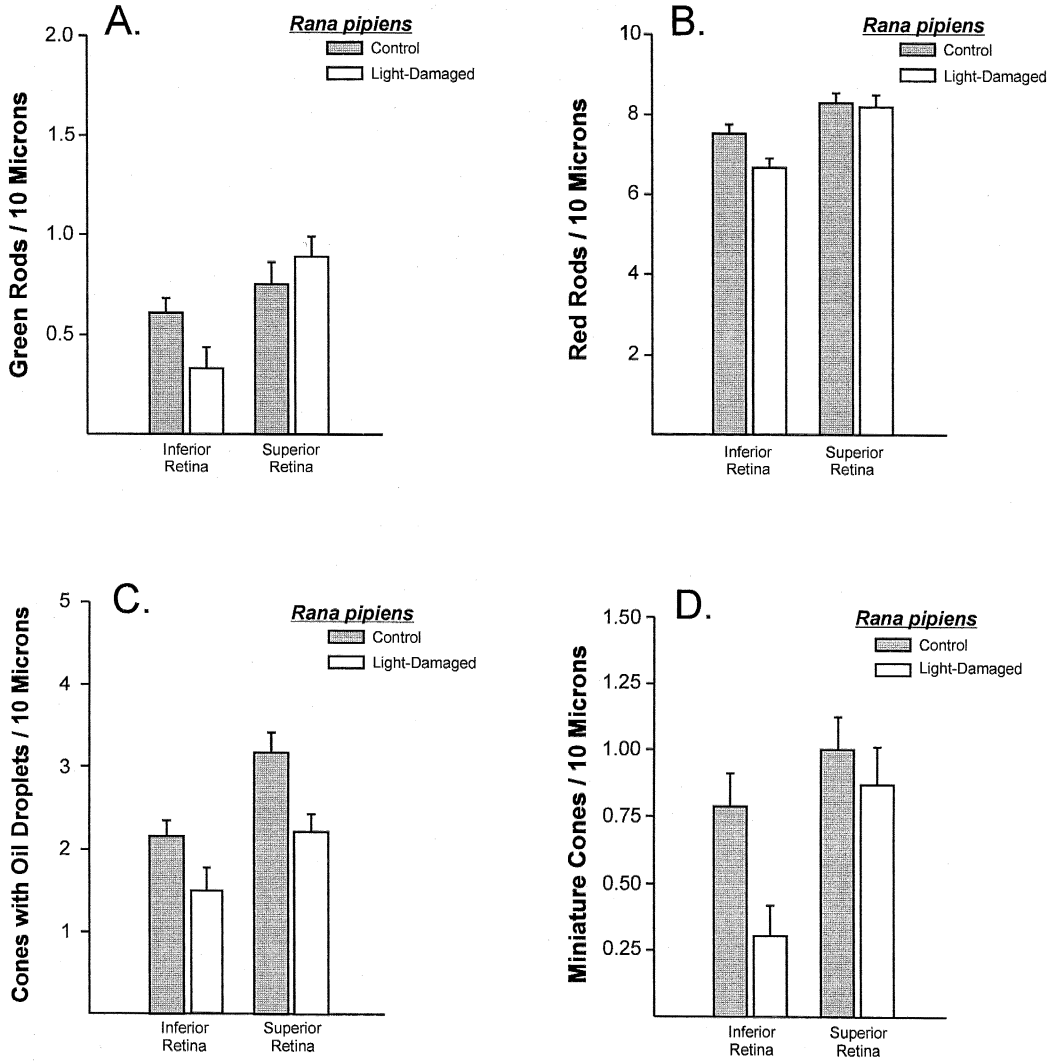

Fig. 5. Comparison of photoreceptor densities for inferior versus superior retina in control and light-damaged *Rana pipiens*. (A) Mean green-rod densities, (B) mean red-rod densities, (C) mean densities of cones with oil droplets (single cones, principle cones), and (D) mean miniature-cone densities.

scribed localized areas of abnormal RPE pigmentation, disruption of photoreceptors and large, and pigment-laden macrophages in the inferior retina of large bullfrogs (*R. catesbeiana*) but observed none of these features in smaller (presumably younger) bullfrogs. Pigment-filled macrophages were found among the tips of rod outer segments, either singly or in clusters, particularly in regions showing disruption of photoreceptors. Such macrophages have been found in the vertebrate retina under a variety of pathological conditions, including light damage (Friedman and Kuwabara, 1968). Eckmiller and Steinberg concluded that cumulative, solar radiation was the most likely cause of the abnormalities seen in the inferior retina of large bullfrogs resulting from exposure to high levels of daily illumination in their natural environment.

In general, two kinds of light damage have been described in outer retina of vertebrates. The first is caused by thermal injury due to the high-energy absorption of radiant energy by RPE melanin granules that occurs during sungazing, arc-welding injuries, laser exposure, etc. (Marshall and Weale, 1976). A second type of retinal light damage results from photochemical or "actinic" effects due to extended exposure to short wavelengths. The severity of actinic effects increases exponentially toward the short-

er wavelengths; and both rod and cone photopigments have high photon-absorption peaks in the ultraviolet range. Irreparable damage to photoreceptors may result from UV radiation, and short-wavelength sensitive cones are more easily damaged by excessive and/or cumulative exposure to short-wavelength radiation (Ham et al., 1980a, 1980b). For example, exposure to 360 nm is 50–80 times more effective than 500 nm in causing photoreceptor losses in the rat retina (Rapp and Smith, 1992), and the primate retina is approximately six times more sensitive to UV than to short-wavelength, visible light (Ham, et al., 1976, 1982; Ham, 1983). Exposure to UV causes major photoreceptor damage in other species as well (mice, Zigman and Vaughn, 1974; squirrels, Collier et al., 1989). In humans, chronic exposure to ambient, UV radiation produces a loss of cone sensitivity (Rapp and Smith, 1992) and often leads to more serious effects, including impaired visual acuity and onset of disorders such as macular degeneration.

In the present study, statistically significant, negative correlations were found between cone densities and the degree of outer-retinal histopathology, both in experimentally light-damaged *R. pipiens* and in *R. cascadae*, consistent with the greater vulnerability of cones to light damage. In light-damaged *R. pipiens*, miniature cones that lack oil droplets were more affected than cones with oil droplets. The colorless oil droplet located between the inner and outer segments may act as a filter to selectively absorb and attenuate the more energetic and damaging short wavelengths of light. Also, both miniature-cone and green-rod densities were significantly reduced in the inferior retina of light-damaged *R. pipiens* versus control *R. pipiens*. These two photoreceptor types contain photopigments with maximal absorption at shorter wavelengths (Zhang et al., 1994), placing them at greater risk of actinic light damage.

Rod densities were less affected by experimental light damage than cones in *R. pipiens*, consistent with results reported in other species. Rods have greater reparative capabilities than cones due to the rapid and continuous renewal of their outer-segment membranes (Young, 1988; Allen and Hallows, 1997). Also, in nonmammalian retinas, light stimulates a migration of melanin granules into the RPE apical processes that closely surround rod outer segments, thereby shielding rods from excessive photon absorption. At the same time, cones physically contract, increasing their exposure to light (Grigonis and Fite, 1983), which increases the risk of actinic damage.

The profile of abnormalities observed in the inferior retina of adult *R. cascadae* is consistent with damage to the retina that would be caused by extended exposure to solar radiation from overhead. Due to the dorsal and upwardly oriented eye position of ranid frogs (Fite, 1974), the inferior retina receives the greatest amount of light exposure from the sun and sky. Many species of frog remain quite motionless without blinking for long periods, and eye movements in frogs are both infrequent and of low amplitude. The cumulative effects of solar UV-radiation exposure could lead to progressive decline in visual ability and impairment of crucial visually guided behaviors, including prey-detection/capture and successful avoidance of predators, decreasing survival rates in the adult population.

UV-B exposure to the eye is substantially affected by ground reflectance and the degree of open sky (Sliney, 1986). Whether retinal abnormalities might exist in *R. cascadae* from different geographic locations and whether retinal histopathology would be seen in animals raised under controlled ambient illumination remains to be determined. Efforts to rear *R. cascadae* under controlled illumination to obtain adults of the same age as those obtained from the field have not been successful. No abnormal or pathological alterations were found in the outer retina of premetamorphic *R. cascadae* tadpoles using analyses identical to those of the present study (K. V. Fite and C. Wang, unpubl. results). Thus, the retinal histopathology observed in *R. cascadae* adults must have occurred during postmetamorphic stages and/or adulthood.

The risk of damage to the eye and retina from solar UV radiation increases, the higher the altitude at which exposure occurs. Increased penetration of solar UV-B is caused by thinning of the stratospheric ozone layer (Blumthaler et al., 1992; Gleason et al., 1992, Ambach et al., 1993); at 2000 m, 60% more ultraviolet flux occurs than at 1000 m (Gates, 1966; Brilliant et al., 1983). The World Meteorological Organization estimates that stratospheric levels of ozone-depleting chlorine and bormine will peak around 1998, with further increases in UV-B of 8–15%, depending upon latitude, and a further progression from currently impacted areas to lower latitudes is anticipated for the near future (Aurer, 1993). Presently, ozone concentrations are falling at temperate latitudes, and further depletion with increased UV penetration is anticipated for a broader geographic range over the next several decades (Madronich and De Gruijl, 1994; Maloy et al., 1997).

Increasing terrestrial levels of solar UV-radiation represent a serious environmental threat to

species across many ecosystems, including humans (Lerman, 1988; Tevini, 1993; Hader, 1997). Unlike the skin, the retina does not develop a tolerance to ultraviolet radiation but actually becomes more sensitive and vulnerable to damage with cumulative exposure. To date, no systematic studies have been conducted on the eyes of wildlife species in regions where UV-B levels are known to be increasing. The extent to which the eyes and retinas of other wildlife populations may be experiencing damage from increasing solar ultraviolet radiation urgently needs to be assessed.

Acknowledgments

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(KVF, LB, HH) NEUROSCIENCE AND BEHAVIOR PROGRAM, TOBIN HALL, UNIVERSITY OF MASSACHUSETTS, AMHERST, MASSACHUSETTS 01003; AND (AB) OREGON STATE UNIVERSITY, CORVALLIS, OREGON. E-mail: (KVF) kfite@psych.umass.edu. Send reprint requests to (KVF). Submitted: 7 Oct. 1997. Accepted: 27 May 1998. Section editor: R. G. Bowker.