scientific reports

Check for updates

OPEN

Trichromacy and ultraviolet vision in a nocturnal marsupial

Leah M. Nielsen[1], Harald Beck[1]✉, Christopher Oufiero[1], Robert J. Johnston Jr.[2], Jesse S. Handler[3] & Joanna F. D. Hagen[2]

Color vision among mammals is diverse and complex, with many physiological and genetic factors affecting spectral sensitivity, the ability to perceive different wavelengths of light. In this study, the color vision of the sugar glider (*Petaurus breviceps*), a nocturnal, gliding mammal, was examined through a series of behavioral tests, genetic analyses, and immunohistochemistry. This is the first study to classify the color vision capabilities of this species. Sugar gliders demonstrated trichromacy and ultraviolet (UV) sensitivity, the latter of which was further supported by genetic analysis. Visualization of the sugar glider retina exhibited a rod-dominant retina that expresses rhodopsin, short-wavelength sensitive 1 opsin, and long/medium-wavelength sensitive opsin. Diurnal primates were thought to be the only mammals able to visualize trichromatically, however the results of this examination and evidence from a few other marsupial studies provide support for nocturnal trichromacy in Metatheria. Intriguingly, the genetic basis for the medium-wavelength sensitivity in marsupials has yet to be discovered. Our results are evidence of a fourth Australian marsupial that is UV-trichromatic, supporting complex spectral sensitivity and UV vision as benefits to survival in nocturnal environments. Given that Rh1 sensitivity at 501 nm explains the green sensitivity behaviorally, question arises how many other nocturnal 'dichromatic' species use rods for trichromatic vision in mesopic light.

Keywords Color, Vision, Mammal, Marsupial, Trichromatic, Ultraviolet

Visual spectral sensitivity or the ability of an animal to perceive different ranges of wavelengths of light has been studied for decades in mammals, and the visual capabilities of diurnal, eutherian mammals, including humans, has been of particular interest. Color vision was previously thought to be a trait only utilized by diurnal species in their photo-abundant environments[1]. The low-light environments of nighttime were thought to have too little light available to sense color, with lunar illuminance being 0.05–0.25 lx (one lumen/m^2) depending on lunar phase, compared to the possible 100,000 lx of solar illuminance[2–4]. However, in the last twenty years, several studies have found evidence that nocturnal species are physiologically capable of dim light color vision[5–14]. Furthermore, species have been found to have nocturnal dichromacy, or the ability to perceive colors in two distinctive ranges of wavelengths, and even trichromacy, the ability to visualize in three ranges of light[8,9,11,15–19]. While there is evidence of some nocturnal species and aquatic species losing color vision functionality[12,13,20], some have retained the genes for color vision over significant evolutionary time in nocturnality[9,10]. Color vision is costly in that it competes for energy and retinal space against achromatic vision, producing a tradeoff between high spectral sensitivity and spatial resolution[19,21]. So why is color vision in nocturnal mammals selectively retained? Correlations have been found between color vision gene retention in nocturnal mammals and foraging technique, roosting behavior, and amount of forest canopy cover[12,13,18]. While color vision in diurnal, eutherian mammals, has been thoroughly investigated, the body of knowledge regarding Australian marsupial visual sensitivity is limited. Examining the spectral sensitivities of nocturnal marsupials would provide insights into the evolutionary history of color vision in mammals, and the long-term genetic conservation of color vision observed despite nocturnal environments.

In vertebrates, photosensitivity is facilitated by transmembrane proteins expressed in the photoreceptor cells of the retina, known as ciliary opsins[22–25]. These opsins have evolved into multiple subtypes that are sensitive to different wavelengths of light, forming the physiological basis of color vision. While there is debate surrounding how the opsin subtypes have diversified, it is agreed five opsin subtypes evolved from the ancestral opsin and were likely present in the ancestor of all vertebrates: Rhodopsin (RH1: rod opsin, sensitive to green), Rhodopsin 2 (RH2: cone opsin, sensitive to green), Short-wavelength Sensitive 1 (SWS1: cone opsin, sensitive to

[1]Department of Biology, Towson University, Baltimore, MD 21252, USA. [2]Johns Hopkins University, Baltimore, MD 21218, USA. [3]The Johns Hopkins University School of Medicine, Baltimore, MD 21205, USA. ✉email: hbeck@towson.edu

ultraviolet), Short-wavelength Sensitive 2 (SWS2: cone opsin, sensitive to blue), and Long-wavelength Sensitive (LWS: cone opsin, sensitive to red)[26–29]. Major evolutionary events have facilitated the loss of opsin (including Rh2) functionality in mammals from the five-opsin retina of the ancestral vertebrate. SWS1 has been lost in monotremes, and SWS2 has been lost in marsupial and eutherian mammals[6,30].

Trichromacy has been observed in marsupials but is different from that of eutherian mammals in that the peak spectral sensitivities are in the ultraviolet (UV), green, and orange ranges (UV-trichromatic) rather than the violet, green, and red ranges respectively[5,31,32]. Trichromacy is thought to have been lost during a period of nocturnality in the mammal lineage and re-evolved in some diurnal primates through a duplication event of the gene responsible for the long-wavelength sensitive opsin[6,33–35]. The short-wavelength sensitive 1 (SWS1) and long/medium-wavelength sensitive opsin genes are conserved in marsupials[17,36,37] The third cone type, which makes trichromacy possible in certain marsupials, does not contain a genetically duplicated, downshifted long-wavelength sensitive opsin on the X chromosome as seen in primates[17].

Three hypotheses have been proposed for the medium-wavelength sensitivity in marsupials. First, it is thought that green sensitivity could be attributed to an opsin gene derived from a duplicated LWS gene, which neofunctionalized to be sensitive to lower wavelengths, similar to some primates[5,38]. Second, medium-wavelength sensitivity may be attributed to retention of the Rh2 gene, a gene that has evidence of having been lost in eutherian mammals and monotremes[5,30]. These hypotheses remain without evidence as no orthologous medium-wavelength sensitive or Rh2 genes were found in the genomes of two marsupials, the stripe-faced dunnart (*Sminthopsis macroaura*) and the fat-tailed dunnart (*Sminthopsis crassicaudata*)[37], the latter of which presented trichromacy in a microspectrophotometric study and a behavioral study[5,31]. Lastly, medium wavelength sensitivity may be derived from duplication of the Rh1 gene and subsequent expression in cone cells, leading to green sensitivity[17,39]. Co-opting of rods into cone cells has been proposed in crocodilians, and the fat-tailed dunnart was found to have two copies of the Rh1 gene[39,40]. However, this duplicated rod opsin does not appear to be expressed in cones in the dunnart[17]. That being said, there is evidence for an unidentified medium-wavelength sensitive opsin. Previous studies observed a considerable proportion of cones that were not labeled by either SWS- or L/M-opsin antibody, suggesting the presence of a cone type with a third opsin[17]. Sufficient evidence has not yet been provided to confirm any of the three hypotheses.

Spectral tuning is an adaptation by which opsins have modified sensitivity, providing a range in spectral sensitivity for each opsin subtype across species. Spectral tuning towards distinct light wavelengths arises from molecular changes in the opsin gene sequence. These changes can affect the opsin's interaction with retinal photopigment and therefore the absorbance required to initiate the phototransduction cascade[41]. Spectral tuning is thought to be driven by environmental pressures such as foraging and activity patterns[13,23]. Key spectral tuning sites have been established for the opsin subtypes, and of particular interest is site 86 in the SWS1 gene. Phenylalanine at position 86 in the SWS1 gene indicates sensitivity in the UV ranges, rather than Tyrosine, which produces violet sensitivity. This single amino acid switch has been detected in multiple species[42–44]. Tuning of the SWS1 opsin from sensitivity in the violet range towards UV sensitivity is often associated with pollinators such as bees and birds[45–48], and nocturnal eutherian mammals including rodents and bats[49–51]. Additionally, UV sensitivity has been found in Australian marsupials of different families[52] and recently, UV biofluorescence was discovered in the pelage of mammals spanning all three major branches: monotremes, marsupials, and placental mammals[53]. The ecological implications behind biofluorescence are yet to be discovered and there are possible links between ultraviolet vision, nocturnal environments, and biofluorescence in mammals.

The goals of this study were to investigate nocturnal color vision, trichromacy, and UV sensitivity in marsupials by examining the spectral sensitivity of the sugar glider (*Petaurus breviceps*). The sugar glider is a small (95 –160 g), Australo-Papuan, nocturnal marsupial that exhibits certain behaviors that invoke interesting links between color vision and their ecology. The first unique behavior is the diet of the sugar glider, as they are omnivorous, eating tree exudates, nectar, and arthropods[54–56]. They have even been observed eating smaller vertebrates such as lizards and birds[57,58]. Floral nectar and pollen consumption has been linked to complex visual capabilities in hummingbirds and bees and dichromacy in nocturnal mammals[18,48,59]. The consumption of arthropods is also noteworthy because sugar gliders likely benefit from acute visual contrast to obtain this prey at night[60,61]. Sugar gliders are adept predators, catching insects on tree trunks and out of mid-air[55]. This species' titular behavior of gliding could also be heavily reliant on visual contrast, whether that be color contrast or rod-based contrast. Sugar gliders have convergently evolved gliding behavior, where they use a fold of skin between their forelimbs and hindlimbs known as the patagium, to perform controlled descents in the forest canopy[62,63]. They can glide over 45 m through various forest environments such as old growth eucalyptus and dense rainforest[54,56,64]. This distance could surpass their use of chemical or olfactory cues, indicating a heavy reliance on vision as they navigate through complex, three-dimensional forest habitat in low-light environments[54,56,62].

To determine the range of visual spectral sensitivity in the sugar glider, we used a combination of behavioral, physiological, and genetic analyses on captive-bred animals. This powerful combination of classical genetic and physiological studies alongside behavioral experiments can unequivocally establish color discrimination function in a species[31,65,66]. By determining the opsin genes present, visualizing opsin expressions in retinas, and performing behavioral experiments, we will define the spectral sensitivities of the sugar glider, adding to the knowledge of marsupial, nocturnal color vision.

Results

Behavioral evidence for UV sensitivity and trichromatic color vision in sugar gliders

To determine the spectral sensitivity of the sugar glider, we performed single color discrimination and dual color discrimination tests, employing a Y-maze apparatus (Supplemental Fig. S1). The sugar gliders had freedom to choose one of the two maze ends. For single color discrimination, the sugar gliders were trained to choose the maze end with the colored light stimulus versus the end without a stimulus. Performing binomial tests on

individual performance, our results indicate strong UV sensitivity, with all gliders choosing the UV light more than by chance (Fig. 1). Glider 1 chose the UV light 37 out of 45 trials with a corrected p value of $6.16e^{-5}$, Glider 2 ($p = 2.17e^{-6}$), Glider 3 ($p = 3.46e^{-9}$), Glider 4 ($p = 1.25e^{-5}$). Evaluating group performances with random effects models with a binomial link, we found similar UV sensitivity between sexes (male performance ($p = 3.81e^{-08}$), female performance ($p = 1.21e^{-9}$) and for all gliders as a whole group ($p < 6.00e^{-16}$).

In the more rigorous UV fluorescent test, two gliders chose the UV light more than by chance in a maze that appeared entirely dark for the researchers ("Ultraviolet fluorescent" in Fig. 1). Glider 2 ($p = 3.70e^{-5}$) and Glider 4 ($p = 0.0264$) had significant results. Glider 1 and Glider 3 chose the UV fluorescent light 30 out of 45 trials ($p = 0.143$), which is not significant with Bonferroni correction. When evaluating whole group performance with a random effects model, the gliders were sensitive to this light ($p = 6.06e^{-8}$). UV sensitivity does not appear to be sexually dimorphic, as both male performance ($p = 1.33e^{-3}$) and female performance ($p = 7.08e^{-4}$) were significant. Together these results suggest that sugar gliders do visualize in ultraviolet, whether male or female.

In addition to UV light, two gliders showed evidence of yellow light sensitivity (Glider 2 ($p = 1.25e^{-5}$), Glider 4 ($p = 3.15e^{-7}$), Fig. 1). Glider 3's performance was not significant ($p = 0.0644$). The sensitivity was significant in both sexes male ($p = 1.46e^{-8}$), female ($p = 3.72e^{-4}$) and in the group performance ($p = 1.74e^{-8}$). Glider 1 passed away before the completion of the testing period, completing 39 of 45 trials. Of the 39 trials Glider 1 chose yellow 33 times, demonstrating yellow sensitivity ($p = 1.29e^{-6}$).

Similarly, we found some evidence for red-sensitivity in the sugar gliders, with two of the animals choosing red light more than by chance, Glider 3 ($p = 0.0264$) and Glider 4 ($p = 3.15e^{-5}$). Glider 1 ($p = 0.290$) and Glider 2 ($p = 0.0644$) had non-significant performances. Sensitivity was present in both sexes when grouped male ($p = 0.0435$), females ($p = 6.90e^{-4}$) and they were sensitive as a whole group ($p = 1.29e^{-4}$).

To test the gliders' ability to distinguish between spectrums of light, we performed dual color discrimination tests. We first trained gliders to distinguish between two relatively distant colors on the color spectrum: blue and yellow. Since Glider 1 passed prior to this training, only Gliders 3 and 4 were successfully trained to distinguish between yellow and blue light by the 12th day of training. During the testing phase, both Gliders 3 and 4 were able to distinguish yellow from blue (Glider 3 ($p = 7.76e^{-8}$); Glider 4 ($p = 2.78e^{-4}$) and yellow from green (Glider 3 ($p = 5.16e^{-5}$); Glider 4 ($p = 4.32e^{-9}$) (Fig. 2). Neither glider preferentially chose a specific side in the yellow vs. yellow control (Fig. 2). Unfortunately, Glider 2 only achieved a significant performance twice within the 17 days of training, non-consecutively. This untrained glider's performance for yellow vs. green and yellow vs. blue was like that of the control, yellow vs. yellow, and insignificant (Fig. 2).

Genetic evidence for UV vision in the sugar glider

To genetically verify the presence of opsin subtypes and predict their spectral sensitivities within the sugar glider, we performed molecular analysis of opsin genes within the glider genome[63]. Rhodopsin (RHO) and short-wavelength sensitive 1 (SWS1) genes were identified in the annotation provided by Feigin et al. 2023[63]. We detected single copies for RHO, LWS, and SWS1 genes within the glider genome. The queries for RHO, LWS, and SWS1 produced multiple regions with significant alignment separated by intervening genomic regions presumably representing exons and introns, respectively. Each of the candidate sugar glider LWS, RHO, and SWS

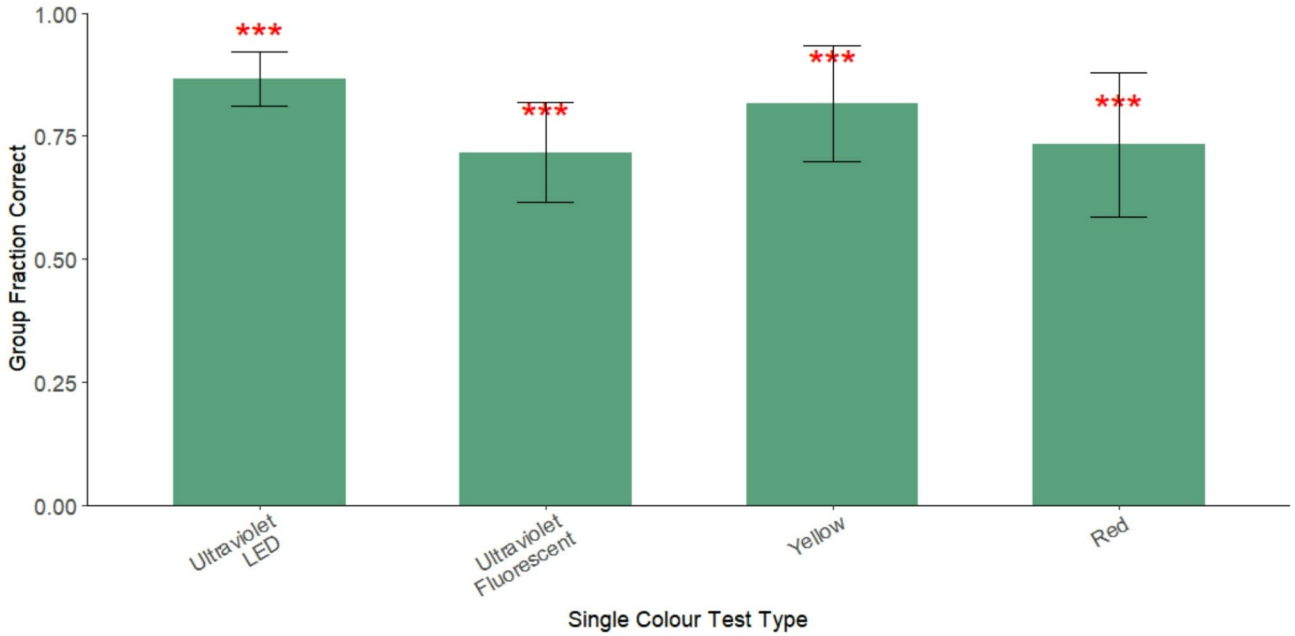

Fig. 1. Fraction of "correct" choices in single color tests. The x-axis shows each single-color test. Total group significance is in red (*** = $p < 0.001$). Yellow testing had fewer trials due to Glider 1 passing and completing 39 of the standard 45 trials. Relative standard deviation error bars are shown.

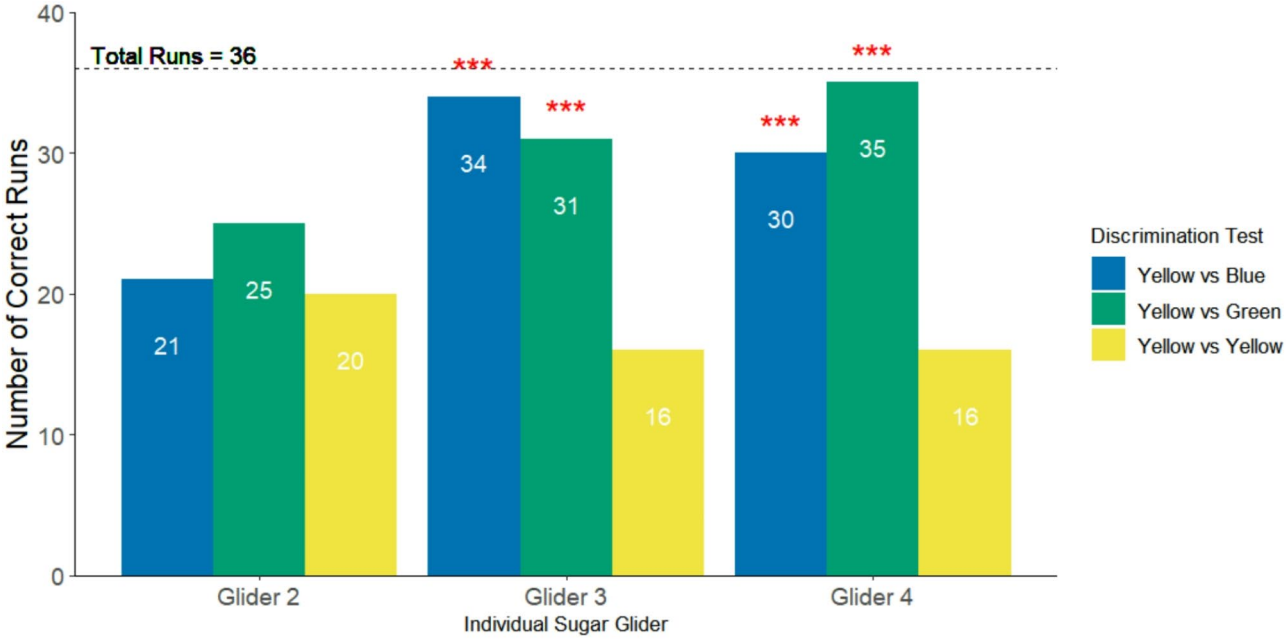

Fig. 2. Individual performances in the dual color discrimination tests. Counts show the number of times each glider chose yellow in the yellow vs. blue and yellow vs. green tests and how many times each glider chose the randomly determined rewarded yellow in the yellow vs. yellow test. Asterisks represent the level of significance. Dashed line represents the total runs completed in each test.

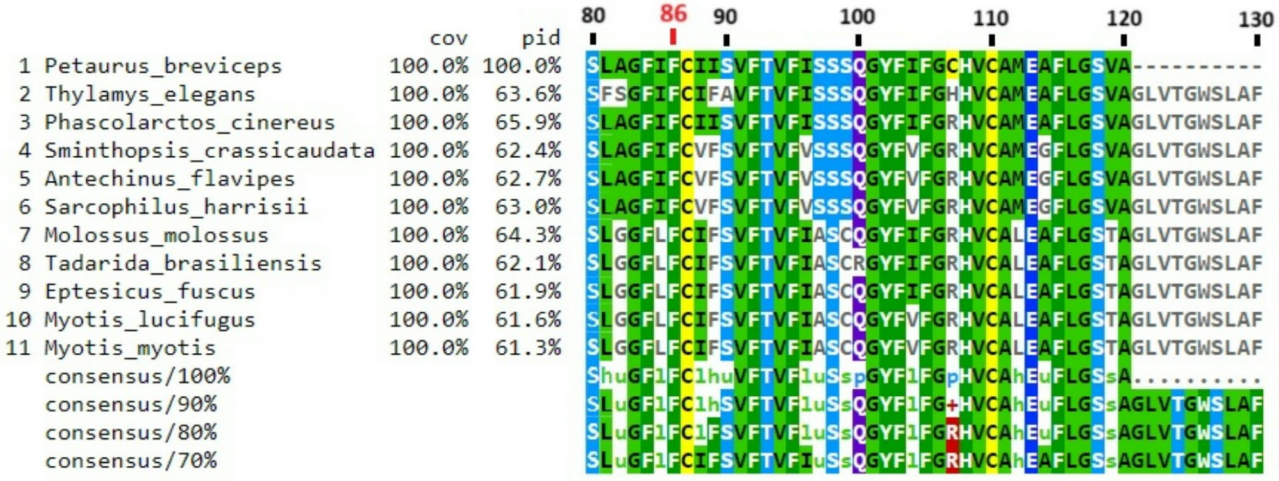

Fig. 3. Candidate Sugar Glider SWS Gene Encodes Phenylalanine at Position 86.
The amino acid sequence for a region of the candidate SWS protein in the sugar glider (*Petaurus breviceps*) and the ten closest genetic matches in other species. Position 86 is emphasized along the top axis in red. A gap in the candidate sugar glider sequence, which we believe is due to inadequate sequencing coverage, can be seen starting at position 121.

translated proteins had gaps of between 5 and 136 amino acids relative to the most closely related proteins in the non-redundant protein sequences database (Supplementary material, Genetic Queries and Sequences). This is most likely related to inadequate sequencing coverage in those regions rather than those regions being truly absent from the sugar glider genome. In the SWS gene protein sequence, Phenylalanine is present at position 86 rather than tyrosine, providing evidence for UV sensitivity (Fig. 3)[42–44].

In contrast to RHO, LWS, and SWS1, we were unable to identify Rh2 within the glider genome. Local alignment search against the glider genome with zebra finch Rh2 sequence produced no significant hits. Similarly, local alignment search against the glider genome with an SWS2 complete coding sequence from the

platypus produced multiple short fragment matches (longest 144 base pairs) throughout the genome, but no convincing SWS2 gene was detected.

To determine evolutionary relationships of the identified opsins, we generated cladograms of RHO, LWS, and SWS1 with the ten most closely related opsin genes of other sequenced species (Supplementary Fig. S3). For RHO, the most recent common ancestor is shared with the koala (*Phascolarctos cinereus*). The other RHO related species span mammalian clades including the polar bear (*Ursus maritimus*), the domestic cow (*Bos taurus*), and the south-central black rhinoceros (*Diceros bicornis minor*). This phylogeny indicates that as expected, RHO is a highly conserved gene across mammals[23,67]. The SWS1 phylogenetic tree contains a few other Australian marsupials including the koala, the fat-tailed dunnart, and the Tasmanian devil (*Sarcophilus harrisii*), but the prominent outgroup contains four different bat species. Many bat species are known to have SWS1 opsins that are UV sensitive, and this is likely the reason for their genetic similarity to the SWS1 gene in the sugar glider[12]. The LWS gene phylogenetic tree is the most specialized, only containing Australian marsupials. This is indicative of a gene that is not highly conserved across mammals and likely diverged from the LWS gene in the common ancestor shared with eutherians.

Opsin expression in the retina of the sugar glider

To visualize opsin expression within glider retinas, we performed immunohistochemistry of rhodopsin, long/medium- wavelength (LM) and SWS1 opsin protein (Supplementary Fig. S2). We detected the presence of rhodopsin and LM opsin. It remains inconclusive whether SWS1 opsin was present, which may be due to lack of conservation in the SWS1 protein or issues in our tissue preparation. Rods appear to be the most abundant throughout the retina (Fig. S2). This rod-dominant retina is consistent with observations of many nocturnal species[1,68–70].

Higher densities of both rods and LM cones were detected in the peripheral region of the retina compared to the central region (Fig. S2). However, large regions of the retina remain unlabeled by any opsin staining. We hypothesize this lack of signal is due to tissue damage, incompatibility of opsin antibody labeling due to lack of sequence conservation, or, similar to the fat-tailed dunnart, these unstained regions could contain cones unlabeled by the three antibodies, and instead express an unidentified medium-wavelength sensitive opsin[17].

Discussion

Using a combination of molecular, behavioral, and immunohistochemical analyses, we improved our understanding of nocturnal marsupial color vision. The sugar glider was found to be a UV-trichromatic species with three distinct spectral range sensitivities, including sensitivity in the UV range. Our behavioral evidence suggests an ability to differentiate yellow (590 nm) from both blue (476 nm) and green (512 nm), which is indicative of trichromacy. This is the first behavioral experiment to examine spectral sensitivity in the sugar glider. These tests were performed with stimuli at three different light levels, meaning performance was not affected by perceived brightness, and the gliders were truly choosing based on color.

Single copies of RHO, SWS1, and LWS gene were found within the sugar glider genome. However, the subtype of opsin responsible for glider green sensitivity remains unclear. It has been widely debated whether Rh2 (green sensitivity) has been retained in some non-placental mammals, though we did not find molecular evidence of this in our study[5,6]. Duplication and spectral downshifting of the LWS gene allowed for trichromacy in primates, however no duplicated LWS gene has been found in marsupials[17,33–35], nor in our study. The presence of a third cone expressing Rh2 or a divergent LWS opsin remains to be confirmed immunohistochemically, though could be present within the retinal areas not positive for rhodopsin, SWS1, or LM opsin staining. To detect SWS1 opsin we should have fixed the eye tissues first in formalin prior to freezing[17,32].

The sugar gliders demonstrated trichromacy with green sensitivity, however, genetically they appeared dichromatic with two potential cone opsin genes and one rhodopsin gene. Anomalous trichromacy has been found in other species. Jacobs[71] was the first to find evidence of trichromacy in owl monkeys who are medium/long wavelength monochromats. Oppermann et al.[72] similarly found trichromacy in genetically monochromatic harbor seals. Both suggest the interaction and joint contribution of cones and rods at differing light levels could contribute to color vision in these species. Other researchers have found evidence of rhodopsin or rhodopsin-like genes being expressed in cone structures[73,74]. Clear cone versus rod structures could not be identified in our retinal sample, therefore we are not able to determine whether the rhodopsin is expressed within cones and/or rods. If rhodopsin was expressed within or interacting with cones in the sugar glider retina, with the estimated photosensitivity of the sugar glider Rh1 gene as ~ 501 nm, green sensitivity in this species would be accounted for.

Although we lack physiological evidence for green sensitivity, our behavioral analyses support trichromacy in the sugar glider. This is consistent with findings in the fat-tailed dunnart[5,17,31,39] and multiple hypotheses, previously outlined, have arisen attempting to explain this. Replicative studies should be conducted to corroborate the results found here, specifically on wild-caught individuals to further connect spectral sensitivity to ecological functions. The sugar glider is now the fifth Australian marsupial to demonstrate trichromacy joined by the quokka, the quenda (*Isoodon obesulus*), the honey possum, and the fat-tailed dunnart[17]. Whatever the mechanism, evidence suggests Australian marsupials re-evolved trichromacy in a different way than eutherian mammals. This is a striking example of convergent evolution, indicating the importance of trichromacy as an evolutionary advantage in multiple geographic and temporal environments and throughout multiple evolutionary lineages.

Sugar gliders demonstrated UV sensitivity behaviorally and have Phenylalanine at position 86 in the SWS1 gene, further supporting the connection between this locus and UV sensitive opsins in nocturnal mammals[42–44]. One possible reason for UV vision in this species is improved visualization at twilight and improved ability to see visual cues. One of the major predators of sugar gliders are owl species, and UV sensitivity has been

noted to improve the detection of aerial predators against the UV-rich background of the sky[75,76]. Sugar gliders are a nocturnal species, yet they are active at twilight where illumination is enriched with short-wavelengths including UV-A[4]. In an environment with relatively high ambient UV light levels compared to daytime, there is the potential for species that see UV to have an entirely different visualization of the environment than animals without UV sensitivity.

The sugar glider presents evidence of being a UV-trichromatic species with middle-wavelength sensitivity yet to be conclusively identified. The spectral sensitivities of this species demonstrate convergent evolution to trichromatic eutherian mammals, emphasizing the importance of middle-wavelength sensitivity to survival in multiple environments, including those that are nocturnal. Additionally, our understanding of UV sensitivity in marsupials and mammals is limited, and classifying UV sensitivity in this species can help define the ecological relationships that make this characteristic important in nocturnal environments.

Materials and methods

Sugar glider husbandry

Four sugar gliders, two neutered males and two females were used for behavioral assays. The sugar gliders were acquired from domestic breeders in November of 2017, making them five years of age at the start of this study. Gliders were kept in a communal housing enclosure measuring 80 cm x 155 cm x 180 cm with various, regularly changed enrichment activities including a soft fabric pouch, running wheels, climbing ropes, and platforms. The housing room was kept near 24°C and 45% humidity, and they had a photo period of 01:00–13:00. Starting at 13:00, when the overhead lights went off, the gliders were provided with a small amount of light (measured at 0.05 lx at the front of the enclosure) from an Exo Terra reptile night light[77]. They were fed a diet of New World primate biscuits[78]; high-protein, nutrient-fortified sugar glider dry food[79]; various fresh vegetables and fruit including bell peppers, carrots, apples, bananas, blueberries, peas, and corn; and limited treats including candied pineapple, yogurt drops, and nectar pods[80]. They were always provided with two sources of water that were changed daily. It should be noted that the sugar gliders in this study are part of the U.S. pet population, which originates from West Papua, Indonesia[81] All methods, animal care, and animal use approved by Towson University's IACUC #1,703,000,185 and were in accordance with the ARRIVE guidelines (https://arriveguidelines.org). Furthermore, all methods were designed and carried out within the guidelines of the Scientific Reports. During our behavioral experiments no animals were harmed and we followed all regulations of the Basel Declarations, including the 3R principles. After completion of this study, no animal was euthanized and remain in our facility following USDA and IACUC regulations.

Behavioral experiments

To determine the spectral sensitivity of sugar gliders, we performed single and dual color discrimination tests using a Y-maze, which has been used previously to examine the visual behavior of various taxa[31,51,59,66,82,83]. The Y-maze[84] is an enclosed structure made of opaque, black, 0.5 cm-thick, acrylic #2025 plastic with three equal-length arms of 50 cm and widths of 10 cm (Supplementary Fig. S1). All color discrimination tests were performed in this apparatus. Specifically, we assessed the spectral sensitivity of the gliders to LEDs with peak wavelengths per manufacturer of: UV (365 nm)[85], Blue (476 nm)[86], Green (512 nm)[87], Yellow (590 nm)[88], Red (660 nm)[89]. This span of wavelengths allowed us to determine the upper and lower bounds of the glider's spectral sensitivity and their potential for mid-range wavelength sensitivity.

During each test, a researcher placed one randomly selected sugar glider in the entrance chamber, a space with the area 10 cm x 15 cm x 30 cm designated by a liftable divider within the maze (Fig. S1). Another researcher stood poised in between the two Y-maze arm ends with a sealed bag of equal, pre-portioned yogurt treats[80] for positive encouragement. The sugar glider remained in total darkness in the entrance chamber for a period of three minutes before the beginning of each day of testing to acclimate their vision to a low-light environment. After the dark acclimation period, the divider between the entrance chamber and the Y-maze was lifted, and the sugar glider was free to move through the maze. Because the Y-maze was covered, dark, and opaque, two FlexiForce A201 25lb pressure sensors[90] were placed within the Y-maze at either arm end to detect the animal's choice of arm via body weight. A 10 cm x 10 cm dark gray, plastic plate with a 0.5 cm diameter "puck" centered beneath it was placed on top of each pressure sensor[91]. This concentrated the glider's body weight to a central point, giving more accurate pressure readings.

Outside of the experimental room, a third researcher monitored the pressure sensors live with the software ELF 4.3.3.0[92]. Once the animal reached either end of the Y-maze, it was detected by the live-feed pressure sensors, and the outside researcher notified the researcher between the Y-maze arms if the desired arm was chosen and whether to give a positive reward or not. The sugar glider was given three minutes to choose an arm end, and if the animal did not choose in that time, it was removed from the maze and returned to the entrance chamber for the next run without a reward. If the desired side was chosen, the researcher between the arms would immediately open a Ziploc bag of pre-portioned yogurt treats and drop a piece through a 1.25 cm diameter hole in the top of the Y-maze arm end. The researchers allowed several seconds for the animal to complete consumption of the treat, opened the Y-maze, retrieved the animal, and placed it back in the entrance chamber for the next run. At no point during the tests was a treat present within the Y-maze. To minimize olfactory cues, the Y-maze was sanitized with diluted hydrogen peroxide between each run of each test. The maze was also fully wiped down with diluted hydrogen peroxide between each glider testing. Furthermore, the treats were kept in a sealed bag equidistant from either arm end to dissuade olfactory preference for either arm. In between runs, researchers used dim, red-light head lamps to orient themselves and place the gliders back in the entrance chamber. All runs were done in total darkness and silence.

For the single-color discrimination tests, a plate with LED lights (365, 476, 512, 590, and 660 nm) was placed above either arm's end. When on, the LED light shines down into the Y-maze arm end, illuminating only that

arm. The colors could be manually changed between runs. The colors were all projected by single, through-hole, 5 mm LEDs that were placed in a line, minimizing spatial differences in where the light would shine down. All the human visual color LEDs had luminous intensities of 1500 millicandela. During a single-color test, the color stimulus was randomized using Random.org[93] to either arm end. The other arm did not have any light stimulus present and was covered with a blackout plate. To prevent preference behavior for one arm or the other, the color stimulus appeared in one arm consecutively no more than three times. The order of the sugar gliders ($n=4$) testing within a day was also randomized with Random.org with each sugar glider representing a number (Glider 1 to 4). During training and testing, the sugar glider was given a treat when it chose the arm with the color stimulus.

Following single color UV LED testing, it was noted that the UV LED gave off a faint, blue glow to the researcher's eye. Therefore, it was decided to complete more rigorous UV testing involving a UV fluorescent bulb emitting a peak wavelength of 368 nm, suspended (15 cm) above either arm of the maze[94]. A blackout cover was used on the arm without a stimulus, and the arm with the UV light had a cover with a 1.25 cm diameter hole directly below the light. The lux measurements were taken directly underneath the light source on the floor of the Y-maze The UV LED stimulus was 2450 lx and for the UV fluorescent light was 1890 lx. Therefore, this second UV single color test provided a dimmer, more conservative and less-blue UV stimulus. The Y-maze appeared entirely dark to the researchers.

UV LED, UV Fluorescent, Red, and Yellow single-color testing was carried out in this order to provide a lower and upper limit to the animal's visual sensitivity. Each testing day the sugar gliders completed no more than 15 runs or 30 min of testing per glider. All testing, including the dual color discrimination testing, was completed between the hours of 14:00 and 18:00, with 14:00 being one hour after their photocycle changed to darkness, giving them time to awake and hydrate. Following testing, sugar gliders were returned to their communal housing enclosure located in a separate room, with free access to water and food.

Towards the end of yellow single-color testing, Glider 1, one of the male sugar gliders, passed away. This death was determined, via necropsy performed by the IACUC veterinarian, to not be associated with our research.

For dual color discrimination testing, two LED light stimuli lit each arm of the Y-maze, allowing for a comparison. Sugar gliders ($n=3$) were given a food reward when they chose the yellow light. To determine the sugar glider's ability to discriminate colors relatively distant on the visual spectrum, a training period of yellow versus blue light was implemented. Just as before, the arm with the yellow light was randomized with yellow appearing in the same arm no more than three times in a row. A glider was considered trained when they chose the yellow light 80% of the time five testing days in a row. To determine that the color discrimination observed was due to color and not perceived brightness of the light, a second three-day training period was implemented that introduced three different brightness levels to the yellow and blue light, similar to Gutierrez et al.[66] and Young et al.[83]. The importance of including brightness cues in behavioral vision testing was made clear by Jacobs[95]. The different brightness levels in this test were achieved by placing a photo slide with neutral density filter in front of the LED lights. There were three different levels of neutral density filter: ND3 which filters one stop of light or 50% of the light, ND6 which filters two stops of light or 75% of the light, and ND9 which filters three stops of light or 87.5% of the light[96]. Each pairing of yellow at a certain brightness level and blue at a certain brightness level was presented once and randomized via a random number generator in R Statistical Software 4.3.0[97], providing a total of nine runs per training day. Thus, the same brightness levels were paired but so were "mismatched" brightness levels. The Blue at the brightest and Yellow at the brightest were paired four times. But all the mismatched brightnesses were also paired four times e.g. Blue at the darkest and Yellow at the second brightest. The gliders continued to receive a treat when they chose the randomized yellow arm.

Following the two training periods, the testing phase included 12 total days of testing where the sugar gliders were presented with each possible pairing of color and brightness level four times. The test provided the sugar glider with an "easy" discrimination for dichromats and trichromats (yellow vs. blue at all brightness levels), a "difficult" discrimination for dichromats and "easy" discrimination for trichromats (yellow vs. green at all brightness levels), and an "impossible" discrimination as a control (yellow vs. yellow at all brightness levels with the rewarded side randomized in each run). With this test we wanted to determine if sugar gliders have sensitivity in the middle wavelengths, represented by green, which would demonstrate trichromacy in this species.

A binomial test was conducted on each glider's individual performance for each test using R Statistical Software 4.3.0[97]. All p values were Bonferroni corrected for each test ($n=4$). Additionally male, female, and whole group performances were evaluated using random effects models with a binomial link. This allowed for analysis of the group performances while capturing the connection of each individual glider to their trials. All p values were Bonferroni corrected for the random effects models as well ($n=3$).

Genetic analyses

The genome of the sugar glider was sequenced by Feigin et al.[63] and annotated by incorporating transcript and protein evidence against the koala (*Phascolarctos cinereus*) to identify gene models. Blastn from the blast 2.13.0 package[98] was used to search for significant alignments between fat-tailed dunnart (*Sminthropsis cassicaudata*) LWS, RHO, and SWS1 complete coding sequences (cds) and the sugar glider genome (JAMXIF010000546.1 Petaurus sp. PSGF00001 85516, whole genome shotgun sequence[99]. Furthermore, the sugar glider genome was examined for significant alignment with RH2 from the zebra finch (*Taeniopygia guttata*) and SWS2 from the platypus (*Ornithorhynchus anatinus*). These examinations were performed from the Unix command line on the Rockfish high performance computing cluster at Johns Hopkins University. Query sequences and code are found in the supplemental file.

Using SnapGene software (version 6.0.2), regions found with significant alignment were assembled and manually adjusted to produce the longest open reading frames possible (i.e., in some cases one or two nucleotides

were removed from the 5' or 3' ends of the individual regions). Open reading frames were then translated into candidate protein sequences.

Blastp was used to search for significant alignments between candidate sugar glider LWS, RHO, and SWS1 proteins and sequences in the NCBI's non-redundant protein sequences database using the blastp suite web interface[100]. For each protein of interest, the candidate protein as well as the top ten most closely related proteins in the non-redundant protein sequences database were aligned using Clustal Omega[101] via EMBL-EBI's web interface[102,103] using default settings. Alignments were visualized using MView and phylogenetic trees generated using Simple Phylogeny again with default settings and via EMBL-EBI's web interface.

Opsin expression analysis within sugar glider retinal tissue

Two sugar gliders that passed from natural causes, one male (Glider 1) and one female (Glider 5), were obtained and enucleated. Glider 1 was a participant in the behavioral study, a standard gray glider, five years of age. Cause of death was determined to be a urethral blockage. Glider 5 was a privately-owned, female, mystery red glider, just under 3 years of age and not used in the behavioral tests of this study. The cause of death was determined to be cancer of the liver and spleen. Upon discovery, both Glider 1 and Glider 5 were stored in a standard refrigerator until enucleation occurred less than 24 h later. Whole eye globes were placed into liquid nitrogen for several minutes and then stored at -80 °C. There was no apparent damage to the eyes nor cloudiness in the cornea of either specimen's eyes prior to freezing.

To classify and visualize the opsins present in the sugar glider retina, antibody labeling of the retina was performed. Each frozen eye was fully submerged in 1x Phosphate Buffered Saline (PBS) for a few minutes to thaw. The iris was then removed, and the sample was fixed in 10% formalin overnight at 4 °C. The retinal tissue was dissected from the globe and several radial cuts were made to flatten the tissue onto a slide. The retinal tissue was blocked for 48 h in 0.3% Triton X-100 and 4% donkey serum at 4 °C. Each sample was incubated in primary antibodies diluted in blocking solution for 48 h at 4 °C. Primary antibodies and dilutions were as follows: chick anti-S opsin (1:200, provided by Dr. Jeremy Nathans), rabbit anti-L/M opsin (1:200, AB5405)[104], and mouse monoclonal anti-Rhodopsin (1:200, MA5-11741)[105]. Samples were then washed three times in 1xPBS for 15 min each time. They were then incubated with secondary antibodies in blocking solution overnight at 4 °C. The secondary antibodies were all Alexa Fluor-conjugated (1:400) and were as follows: donkey anti-mouse IgG (A31570)[106], donkey anti-rabbit IgG (A31573)[107], and donkey anti-chicken IgY (703-545-155)[108]. The retinal samples were again washed in 1xPBS three times for 10 min each. Nuclei were labelled by incubating in Hoechst 33,342 (Biotium 40046 #10H0212, diluted 1:2000 in 1xPBS) for 10 min and then washed three more times in 1xPBS for 15 min each time. Samples were then mounted in SlowFade™ Gold (S36940)[109]. The slide was stored at 4 °C prior to imaging.

Samples were imaged using a Zeiss LSM980 laser scanning confocal microscope at x20 and x63 magnification. Tile regions were stitched together using ZEN processing software, and maximum intensity projections of z-stacks were rendered in the same program[110].

Data availability

The data and code used in this study are deposited at https://github.com/Sugar-Glider-Lab/Trichromacy-UV-Sugar-Gliders. Sequencing data were obtained from Feigin, C. Y., et al. (2023) and are deposit at Figshare.com, available at the following URL: https://figshare.com/s/d6c585fbae0c1f22e8df.

Received: 24 October 2024; Accepted: 25 February 2025
Published online: 04 March 2025

References

1. Ahnelt, P. K. & Kolb, H. The mammalian photoreceptor mosaic-adaptive design. *Prog Retin Eye Res.* **19**, 711–777 (2000).
2. Breitler, J. C. et al. Full moonlight-induced circadian clock entrainment in Coffea arabica. *BMC Plant. Biol.* **20**, 1–11 (2020).
3. Kyba, C. C. M., Mohar, A. & Posch, T. How bright is moonlight? *Astronomy Geophys.* **58**, 131–132 (2017).
4. Spitschan, M., Aguirre, G. K., Brainard, D. H. & Sweeney, A. M. Variation of outdoor illumination as a function of solar elevation and light pollution. *Sci. Rep.* **6**, 1–14 (2016).
5. Arrese, C. A., Hart, N. S., Thomas, N., Beazley, L. D. & Shand, J. Trichromacy in Australian marsupials. *Curr. Biol.* **12**, 657–660 (2002).
6. Jacobs, G. H. Losses of functional Opsin genes, short-wavelength cone photopigments, and color vision - A significant trend in the evolution of mammalian vision. *Vis. Neurosci.* **30**, 39–53 (2013).
7. Kelber, A., Yovanovich, C. & Olsson, P. Thresholds and noise limitations of colour vision in dim light. *Philos. Trans. R. Soc. B: Biol. Sci.* **372**, (2017).
8. Kelber, A. & Roth, L. S. V. Nocturnal colour vision - Not as rare as we might think. *J. Exp. Biol.* **209**, 781–788 (2006).
9. Moritz, G. L., Lim, N. T. L., Neitz, M., Peichl, L. & Dominy, N. J. Expression and evolution of short wavelength sensitive Opsins in Colugos: A nocturnal lineage that informs debate on primate origins. *Evol. Biol.* **40**, 542–553 (2013).
10. Perry, G. H., Martin, R. D. & Verrelli, B. C. Signatures of functional constraint at aye-aye Opsin genes: the potential of adaptive color vision in a nocturnal primate. *Mol. Biol. Evol.* **24**, 1963–1970 (2007).
11. Robertson, J. M., Bell, R. C. & Loew, E. R. Vision in dim light and the evolution of color pattern in a crepuscular/nocturnal frog. *Evol. Ecol.* **36**, 355–371 (2022).
12. Simões, B. F. et al. As blind as a Bat? Opsin phylogenetics illuminates the evolution of color vision in bats. *Mol. Biol. Evol.* **36**, 54–68 (2019).
13. Veilleux, C. C., Louis, E. E. & Bolnick, D. A. Nocturnal light environments influence color vision and signatures of selection on the OPN1SW Opsin gene in nocturnal lemurs. *Mol. Biol. Evol.* **30**, 1420–1437 (2013).
14. Warrant, E. & Somanathan, H. Colour vision in nocturnal insects. *Philos. Trans. R. Soc. B: Biol. Sci.* **377**, (2022).
15. Roth, L., V, S. & Kelber, A. Nocturnal colour vision in geckos. *Proc. Royal Soc. B: Biol. Sci.* **271**, 485–487 (2004).
16. Johnsen, S. et al. Crepuscular and nocturnal illumination and its effects on color perception by the nocturnal Hawkmoth Deilephila elpenor. *J. Exp. Biol.* **209**, 789–800 (2006).

17. Ebeling, W., Natoli, R. C. & Hemmi, J. M. Diversity of color vision: not all Australian marsupials are trichromatic. *PLoS One*. **5**, e14231 (2010).
18. Veilleux, C. C. & Cummings, M. E. Nocturnal light environments and species ecology: implications for nocturnal color vision in forests. *J. Exp. Biol.* **215**, 4085–4096 (2012).
19. Kelber, A. & Jacobs, G. H. Evolution of color vision. *Hum. Color. Vis.* 317–354. https://doi.org/10.1007/978-3-319-44978-4 (2016).
20. McGowen, M. R. et al. Positive selection and inactivation in the vision and hearing genes of cetaceans. *Mol. Biol. Evol.* **37**, 2069–2083 (2020).
21. Gerl, E. J. & Morris, M. R. The causes and consequences of color vision. *Evolution: Educ. Outreach.* **1**, 476–486 (2008).
22. Kolb, H. *Photoreceptors*. (2005). https://webvision.med.utah.edu/book/part-ii-anatomy-and-physiology-of-the-retina/photoreceptors/.
23. Hagen, J. F. D., Roberts, N. S. & Johnston, R. J. The evolutionary history and spectral tuning of vertebrate visual Opsins. *Dev. Biol.* **493**, 40–66 (2023).
24. Okada, T., Ernst, O. P., Palczewski, K. & Hofmann, K. P. Activation of rhodopsin: new insights from structural and biochemical studies. *Trends Biochem. Sci.* **26**, 318–324 (2001).
25. Terakita, A. The Opsins. *Genome Biol.* **6**, 1–9 (2005).
26. Bowmaker, J. K. & Hunt, D. M. Evolution of vertebrate visual pigments. *Curr. Biol.* **16**, 484–489 (2006).
27. Collin, S. P., Davies, W. L., Hart, N. S. & Hunt, D. M. The evolution of early vertebrate photoreceptors. *Philosophical Trans. Royal Soc. B: Biol. Sci.* **364**, 2925–2940 (2009).
28. Lagman, D. et al. The vertebrate ancestral repertoire of visual Opsins, transducin alpha subunits and Oxytocin/vasopressin receptors was established by duplication of their shared genomic region in the two rounds of early vertebrate genome duplications. *BMC Evol. Biol.* **13**, 1–21 (2013).
29. Yokoyama, S. Molecular bases of color vision in vertebrates. *Genes Genet. Syst.* **74**, 189–199 (1999).
30. Wakefield, M. J. et al. Cone visual pigments of monotremes: filling the phylogenetic gap. *Vis. Neurosci.* **25**, 257–264 (2008).
31. Arrese, C. A., Beazley, L. D. & Neumeyer, C. Behavioural evidence for marsupial trichromacy. *Curr. Biol.* **16**, 193–194 (2006).
32. Arrese, C. A. et al. Cone topography and spectral sensitivity in two potentially trichromatic marsupials, the Quokka (Setonix brachyurus) and Quenda (Isoodon obesulus). *Proc. Royal Soc. B: Biol. Sci.* **272**, 791–796 (2005).
33. Dulai, K. S., Von Dornum, M., Mollon, J. D. & Hunt, D. M. The evolution of trichromatic color vision by Opsin gene duplication in new world and old world primates. *Genome Res.* **9**, 629–638 (1999).
34. Surridge, A. K., Osorio, D. & Mundy, N. I. Evolution and selection of trichromatic vision in primates. *Trends Ecol. Evol.* **18**, 198–205 (2003).
35. Veilleux, C. C. & Bolnick, D. A. Opsin gene polymorphism predicts trichromacy in a cathemeral lemur. *Am. J. Primatol.* **71**, 86–90 (2009).
36. Hunt, D. M. et al. The rod Opsin pigments from two marsupial species, the South American bare-tailed woolly opossum and the Australian fat-tailed Dunnart. *Gene* **323**, 157–162 (2003).
37. Strachan, J., Chang, L. Y. E., Wakefield, M. J., Graves, J. A. M. & Deeb, S. S. Cone visual pigments of the Australian marsupials, the stripe-faced and fat-tailed Dunnarts: sequence and inferred spectral properties. *Vis. Neurosci.* **21**, 223–229 (2004).
38. Sumner, P., Arrese, C. A. & Partridge, J. C. The ecology of visual pigment tuning in an Australian marsupial: the honey possum Tarsipes rostratus. *J. Exp. Biol.* **208**, 1803–1815 (2005).
39. Cowing, J. A., Arrese, C. A., Davies, W. L., Beazley, L. D. & Hunt, D. M. Cone visual pigments in two marsupial species: the fat-tailed Dunnart (Sminthopsis crassicaudata) and the honey possum (Tarsipes rostratus). *Proc. Royal Soc. B: Biol. Sci.* **275**, 1491–1499 (2008).
40. Emerling, C. A. Archelosaurian color vision, parietal eye loss, and the crocodylian nocturnal bottleneck. *Mol. Biol. Evol.* **34**, 666–676 (2017).
41. Sekharan, S., Katayama, K., Kandori, H. & Morokuma, K. Color vision: 'OH-site' rule for seeing red and green. *J. Am. Chem. Soc.* **134**, 10706–10712 (2012).
42. Shi, Y., Radlwimmer, F. B. & Yokoyama, S. Molecular genetics and the evolution of ultraviolet vision in vertebrates. *Proc. Natl. Acad. Sci. U S A.* **98**, 11731–11736 (2001).
43. Cowing, J. A. et al. The molecular mechanism for the spectral shifts between vertebrate ultraviolet- and violet-sensitive cone visual pigments. *Biochem. J.* **367**, 129–135 (2002).
44. Deeb, S. S. et al. The cone visual pigments of an Australian marsupial, the Tammar Wallaby (Macropus eugenii): sequence, spectral tuning, and evolution. *Mol. Biol. Evol.* **20**, 1642–1649 (2003).
45. Ödeen, A. & Håstad, O. *The Phylogenetic Distribution of Ultraviolet Sensitivity in Birds*. http://www.biomedcentral.com/1471-2148/13/36 (2013).
46. Cuthill, I. C. et al. Ultraviolet vision in birds. *Adv. Study Behav.* **29**, 159–214 (2000).
47. Chittka, L. & Wells, H. Color Vision in Bees: Mechanisms, Ecology, and Evolution. *Complex Worlds from Simpler Nervous Systems* Preprint at (2004). https://doi.org/10.7551/mitpress/1994.003.0014.
48. Chittka, L. & Vorobyev, M. Bee colour vision–the optimal system for the discrimination of flower colours with three spectral photoreceptor types. *Sensory Systems of Arthropods* 211–218 Preprint at (1993). http://chittkalab.sbcs.qmul.ac.uk/1993/5Chittka_etal_1993.PDF.
49. Jacobs, G. H., Fenwick, J. A. & Williams, G. A. Cone-based vision of rats for ultraviolet and visible lights. *J. Exp. Biol.* **204**, 2439–2446 (2001).
50. Jacobs, G. H. & Williams, G. A. Contributions of the mouse UV photopigment to the ERG and to vision. *Doc. Ophthalmol.* **115**, 137–144 (2007).
51. Marcos Gorresen, P., Cryan, P. M., Dalton, D. C., Wolf, S. & Bonaccorso, F. J. Ultraviolet vision May be widespread in bats. *Acta Chiropt.* **17**, 193–198 (2015).
52. Travouillon, K. J., Cooper, C., Bouzin, J. T., Umbrello, L. S. & Lewis, S. W. All-a-glow: spectral characteristics confirm widespread fluorescence for mammals. *R Soc. Open. Sci.* **10**, (2023).
53. Anich, P. S. et al. Biofluorescence in the platypus (Ornithorhynchus anatinus). *Mammalia* **85**, 179–181 (2021).
54. Smith, M. J. Petaurus breviceps. *Am. Soc. Mammalogists*. **30**, 1–5 (1973).
55. Smith, A. P. Diet and feeding strategies of the marsupial sugar glider in temperate Australia. *J. Anim. Ecol.* **51**, 149–166 (1982).
56. Waterhouse Smithsonian Institutional Press, Washington D.C.,. Sugar Glider: Petaurus breviceps. In *Mammals of Australia* (ed. Strahan, R.) 229–231 (1995).
57. Gunn, R. C. On the introduction and naturalization of Petaurus sciureus in Tasmania. *Royal Soc. Tasmania* 253–255 (1851).
58. Stojanovic, D., Webb, M. H., Alderman, R., Porfirio, L. L. & Heinsohn, R. Discovery of a novel predator reveals extreme but highly variable mortality for an endangered migratory bird. *Divers. Distrib.* **20**, 1200–1207 (2014).
59. Stoddard, M. C. et al. Wild hummingbirds discriminate nonspectral colors. *Proc. Natl. Acad. Sci. U S A.* **117**, 15112–15122 (2020).
60. Stoddart, D. M. Olfactory biology, social behavior and ecophysiology: an integrated marsupial study. *Adv. Biosci.* **93**, 201–210 (1994).
61. Carthew, S. M. Foraging behaviour of marsupial pollinators in a population of Banksia spinulosa. *Oikos* **69**, 133–139 (1994).
62. Stoddart, D. M. Olfactory biology, social behavior and ecophysiology: an integrated marsupial study. *Adv. Biosci.* **93**, 201–210 (1994).

63. Feigin, C. Y. et al. Convergent deployment of ancestral programs during the evolution of mammalian flight membranes. *Sci. Adv.* **9 7511**, 1–16 (2023).
64. Jackson, S. M. Population dynamics and life history of the Mahogany glider, Petaurus gracilis, and the sugar glider, Petaurus breviceps in North Queensland. *Wildl. Res.* **27**, 21–37 (2000).
65. Kelber, A., Vorobyev, M. & Osorio, D. Animal colour vision - Behavioural tests and physiological concepts. *Biol. Rev. Camb. Philos. Soc.* **78**, 81–118 (2003).
66. Gutierrez, E., de Pegoraro, A. & Magalhães-Castro, B. M. Pessoa, V. F. Behavioural evidence of dichromacy in a species of South American marsupial. *Anim. Behav.* **81**, 1049–1054 (2011).
67. Yokoyama, S. Molecular Evolution of Vertebrate Visual Pigments. *Prog. Retinal Eye Res.* **19** (2000).
68. Jacobs, G. H. Evolution of colour vision in mammals. *Philos. Trans. R. Soc. B: Biol. Sci.* **364**, 2957–2967 (2009).
69. Peichl, L. Diversity of mammalian photoreceptor properties: adaptations to habitat and lifestyle? *Anat. Record - Part. Discov. Mol. Cell. Evol. Biol.*. **287**, 1001–1012 (2005).
70. Kim, J. W. et al. Recruitment of rod photoreceptors from Short-Wavelength-Sensitive cones during the evolution of nocturnal vision in mammals. *Dev. Cell.* **37**, 520–532 (2016).
71. Jacobs, G. H. Visual capacities of the Owl monkey (Aotus trivirgatus)-II. Spatial contrast sensitivity. *Vis. Res.* **17**, 821–825 (1977).
72. Oppermann, D., Schramme, J. & Neumeyer, C. Rod-cone based color vision in seals under photopic conditions. *Vis. Res.* **125**, 30–40 (2016).
73. Raymond, P. A., Barthel, L. K., Rounsifer, M. E., Sullivan, S. A. & Knight+, J. K. Expression of Rod and Cone Visual Pigments in Goldfish and Zebrafish: A Rhodopsin-like Gene Is Expressed in Cones. *Neuron* **10** (1993).
74. Bhattacharyya, N., Darren, B., Schott, R. K., Tropepe, V. & Chang, B. S. W. Cone-like rhodopsin expressed in the all-cone retina of the colubrid pine snake as a potential adaptation to diurnality. *J. Exp. Biol.* **220**, 2418–2425 (2017).
75. Cronin, T. W. & Bok, M. J. Photoreception and vision in the ultraviolet. *J. Exp. Biol.* **219**, 2790–2801. https://doi.org/10.1242/jeb.128769 (2016).
76. Blythman, M. D., Sansom, J. L. & Lohr, M. T. Use of ultraviolet light to help age nightjars, owlet-nightjars, frogmouths and owls. *Corella* **40**, 98–99 (2016).
77. Exo Terra Full Moon Reptile Terrarium Night Light. *Exo Terra* Preprint at https://exo-terra.com/products/lighting/specialty-lights/full-moon/.
78. Mazuri® New World Primate Biscuit. *Mazuri® Exotic Animal Nutrition* Preprint at https://mazuri.com/products/maz-new-world-primate.
79. Vitakraft VitaSmart. High-Protein Blend Nutrient-Fortified with Essential Vitamins & Minerals Sugar Glider Food. *Vitakraft* Preprint at https://www.vitakraft.us/products/.
80. Sugar Glider Treat Variety Pack and Nectar Pods Variety Pack. *Exotic Nutrition Pet Supply Company* Preprint at https://www.exoticnutrition.com/.
81. Campbell, C. D., Pecon-Slattery, J., Pollak, R., Joseph, L. & Holleley, C. E. The origin of exotic pet sugar gliders (Petaurus breviceps) kept in the United States of America. *PeerJ* 1–15 (2019).
82. Anderson, C. et al. Natural variation in stochastic photoreceptor specification and color preference in Drosophila. *Elife* **6**, 1–20 (2017).
83. Young, M., Salmon, M. & Forward, R. Visual wavelength discrimination by the loggerhead turtle, Caretta caretta. *Biol. Bull.* **222**, 46–55 (2012).
84. Custom, Y-M. *Noldus Information Technology, Inc.* Preprint at (2021). https://www.noldus.com/.
85. Manufacturer #MTE3661N1-UV. *Marktech Optoelectronics, Inc.* Preprint at https://www.mouser.com/ProductDetail/Marktech-Optoelectronics/MTE3661N1-UV?qs=DRkmTr78QATPRjZ1g%252BipRA%3D%3D.
86. Manufacturer #LTL17KTBS3KS. *Light-On* Preprint at https://www.mouser.com/ProductDetail/Lite-On/LTL17KTBS3KS?qs=plZGoh4IuyF1ttdBDGeOaw%3D%3D.
87. Manufacturer #SSL-LX5093UEGC. *Lumex* Preprint at https://www.mouser.com/ProductDetail/Lumex/SSL-LX5093UEGC?qs=icbinUxoJoc%2FxWOWIAugMQ%3D%3D.
88. Manufacturer #151053YS04500. *Wurth Elektronik* Preprint at https://www.mouser.com/ProductDetail/Wurth-Elektronik/151053YS04500?qs=LlUlMxKIyB1om6aP3%252B2orA%3D%3D.
89. Manufacturer #SSL-LX5093SRC/DV. *Lumex* Preprint at https://www.mouser.com/ProductDetail/Lumex/SSL-LX5093SRC-DV?qs=5y%252B7s3ShyV5GF8wwMxgcig%3D%3D.
90. FlexiForce A201 25lb Sensors. *Tekscan, Inc.* Preprint at https://www.tekscan.com/products-solutions/force-sensors/a201.
91. FlexiForce, L. C. *Tekscan, Inc.* Preprint at https://www.tekscan.com/products-solutions/force-sensors/flxlc-flxlc-b.
92. Economical Load and Force (ELF). Preprint at https://tekscan.com/ (2015).
93. Random.org. True Random Number Generator. Preprint at (2023). https://www.random.org/.
94. GE F15T8-BLB UVA Bulb. General Electric Company Preprint at. (2006).
95. Jacobs, G. H. Prospects for trichromatic color vision in male Cebus monkeys. *Behav. Brain. Res.* **101**, 109–112 (1999).
96. Lighting Neutral Density Gels Filter Sheet. 16 x 20 inches Kit, ND3, ND6, ND9 for Photo Studio Video Flashlight Led Light. *Renian*. Preprint at https://www.amazon.com/dp/B08PFW95RN?psc=1&ref=ppx_yo2ov_dt_b_product_details.
97. R Core Team. R: A Language and Environment for Statistical Computing. Preprint at (2023). https://www.R-project.org/.
98. Zhang, Z., Schwartz, S., Wagner, L. & Miller, W. *A Greedy Algorithm for Aligning DNA Sequences. J. Comput. Biol.* **7** https://www.liebertpub.com (2000).
99. Petaurus Whole Genome Shotgun Sequence.
100. Blastp Web Interface. Preprint at https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins.
101. Sievers, F., Higgins, D. G. & Clustal Omega Accurate alignment of very large numbers of sequences. *Methods Mol. Biol.* **1079**, 105–116 (2014).
102. Madeira, F. et al. Search and sequence analysis tools services from EMBL-EBI in 2022. *Nucleic Acids Res.* **50**, W276–W279 (2022).
103. EMBL-EBI web interface. Preprint at https://www.ebi.ac.uk/jdispatcher/msa/clustalo.
104. Millipore, A. O. & Antibody Red/Green in IH (P). Catalog # AB5405. Lot # 2387835. Preprint at https://www.emdmillipore.com/US/en/product/Anti-Opsin-Antibody-Red-Green,MM_NF-AB5405.
105. Thermo Fisher Scientific. Rhodopsin Monoclonal Antibody (RET-P1) in IHC (P). Catalog # MA5-11741. Preprint at https://www.thermofisher.com/antibody/product/Rhodopsin-Antibody-clone-RET-P1-Monoclonal/MA5-11741.
106. Invitrogen Donkey anti-Mouse IgG (H + L) Highly Cross-Absorbed Secondary Antibody, Alexa Fluor™ 555. Catalog # A31570. Preprint at https://www.thermofisher.com/antibody/product/Donkey-anti-Mouse-IgG-H-L-Highly-Cross-Adsorbed-Secondary-Antibody-Polyclonal/A-31570.
107. Invitrogen Donkey anti-Rabbit IgG (H + L) Highly Cross-Absorbed Secondary Antibody, Alexa Fluor™ 647. Catalog # A31573. Preprint at https://www.thermofisher.com/antibody/product/Donkey-anti-Rabbit-IgG-H-L-Highly-Cross-Adsorbed-Secondary-Antibody-Polyclonal/A-31573.
108. Alexa Fluor™ 488 AffiniPure Donkey Anti-Chicken IgY(IgG) (H + L). Catalog # 703-545-155. *Jackson ImmunoResearch Laboratories Inc.* Preprint at https://www.jacksonimmuno.com/catalog/products/703-545-155.
109. Thermo Fisher Scientific. SlowFade™ Gold Antifade Mountant. Catalog # S36940. Preprint at https://www.thermofisher.com/order/catalog/product/S36940
110. Zeiss, Z. E. N., Microscopy & Software Preprint at https://www.zeiss.com/microscopy/en/products/software/zeiss-zen.html.

Acknowledgements
Thank you to Jeffrey Klupt for designing and creating the LED light plates. Thank you to our undergraduate researchers: Karlina Messer, Veronica Tayviah, N'Deye Walton, Morgan Hawkins, Sereena Cao, Kate Dingman, Kyla Ghirardello, and Diana Barlow, without which this project would not have been possible.

Author contributions
L.M.N.: Experimental design, data collection, analyses, laboratory management, and writing. H.B.: Supervisor, experimental design, writing, and editing. C.E.O.: Experimental design, writing, and editing. R.J.J.: Experimental design, editing. J.F.D.H.: Retinal visualization, methods, and writing. J.S.H.: Genetic analysis, methods, and writing.

Funding
Funding provided by the Towson University Foundation, FCSM Endowment Grants, Kim Fabian, Ian Mutton, and The Towson University Graduate Student Association without whom this project would not be possible. J.F.D.H. was supported by EMBO ALTF 318-2021 and NIH K99EY035756. R.J.J. was supported by the National Eye Institute R01EY030872, the BrightFocus foundation G2019300, and MSCRF 140841.

Declarations

Competing interests
The authors declare no competing interests.

Additional information
Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-025-92039-z.

Correspondence and requests for materials should be addressed to H.B.

Reprints and permissions information is available at www.nature.com/reprints.

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.