Vision Research 206 (2023) 108185

Contents lists available at ScienceDirect

Vision Research

journal homepage: www.elsevier.com/locate/visres

Nutrition-induced macular-degeneration-like photoreceptor damage in jumping spider eyes

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ARTICLE INFO

Keywords:
Model system
Neuronal health
Neurodegeneration
Visual systems
Photoreceptor health
Invertebrates

ABSTRACT

Age-related macular degeneration (AMD) is a leading cause of vision loss in humans. Despite its prevalence and medical significance, many aspects of AMD remain elusive and treatment options are limited. Here, we present data that suggest jumping spiders offer a unique opportunity for understanding the fundamentals underlying retinal degeneration, thereby shedding light on a process that impacts millions of people globally. Using a micro-ophthalmoscope and histological evidence, we demonstrate that significant photoreceptor damage can occur during development in the image-forming anterior lateral eyes of the jumping spider *Phidippus audax*. Furthermore, we find that this photoreceptor degeneration is exacerbated by inadequate nutrition and is most prevalent in the high-density region of the retina, like AMD in humans. This suggests that similar to those in vertebrates, the retinas in *P. audax* are challenged to meet high-energy cellular demands.

1. Introduction

Vision loss in humans is commonly caused by age-related macular degeneration (AMD). A 2014 study estimated that 8.7% of the world-wide population has some form of AMD (Wong et al., 2014). Globally, visual impairment due to AMD was estimated to be responsible for $255 billion in direct health care costs in 2010 (Gordois et al., 2010). AMD is divided into two types: a dry form (85%–90% of AMD) and a wet form (10%–15% of AMD). The dry form is characterized by photoreceptor loss and retinal pigment epithelium (RPE) dysfunction, while the wet form is characterized by neovascularization, hemorrhage, and RPE detachment. These forms can co-occur in patients, subsequently leading to retinal degeneration and vision loss (Schultz et al., 2021; Bandello et al., 2017). Utilizing vertebrate animal models including rodents, rabbits, nonhuman primates (Pennesi et al., 2012), and zebrafish (Angueyra & Kindt, 2018), pathways including oxidative stress, immune-related inflammation, and lipid metabolism dysfunction have been identified to play a role in the development of AMD (Rho et al., 2021, Abokyi et al., 2020, Bandello et al., 2017). While great advancements have been made in the treatment of wet AMD using anti-VEGF biologics (Ammar et al., 2020), there remains no FDA-approved treatment for dry AMD, which accounts for the majority of cases. Research aimed at better understanding the pathophysiology and mechanisms associated with this visually devastating disease is imperative to develop a treatment for dry AMD (Bandello et al., 2017). To achieve these long-term goals, the availability of additional animal models could be useful.

To investigate the molecular pathways of vision, invertebrate models such as *Drosophila melanogaster* have proven fruitful due to the highly conserved biochemical processes of photoreceptor function and the shared neuro-visual circuity between fly and vertebrate organisms (Sanes & Zipursky, 2010). For example, a recent study in *D. melanogaster* showed that vitamin A deprivation substantially affects gene expression and, as in humans, causes retinal damage (Dewett et al., 2021). However, using *D. melanogaster* to model complex diseases such as AMD has limitations. Fly eyes are compound eyes and hence do not share some of the key structural principles indicated in AMD, most notably an image-forming eye with high- and low-density photoreceptor areas. Among other invertebrates, eyes with such key features are found in jumping spiders. Unlike *Drosophila*, the anterior lateral eyes (ALEs) of jumping spiders are image-forming eyes that use a single lens and feature

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https://doi.org/10.1016/j.visres.2023.108185
Received 5 October 2022; Received in revised form 13 January 2023; Accepted 22 January 2023
Available online 7 February 2023

variability in photoreceptor density, with a high-density region (HDR) located centrally in the retina, similar to the human macula. Furthermore, the number of photoreceptors and retinal organization remain constant throughout spider development (Goté et al., 2019). The development of these spiders typically takes 3–5 months, and the relationship to size and the developmental timing of the 6th–8th (male) or 7th–9th (female) instars has previously been mapped out (see Supplemental Fig. A.1 in Goté et al., 2019). This affords an opportunity to study the integrity of the retina longitudinally across their lifespan.

Considering the limitations of other invertebrate models, the question remains: Can macular-degeneration-type damage be modeled in a jumping spider retina? After noticing that some older spiders exhibit photoreceptor damage concentrated in the HDR of their retinas, we considered the possibility that the ALE photoreceptors in the HDR may face challenges similar to those of the macula in vertebrates. In humans, successful advancements to address the risk factors of AMD have focused on interventions modifying lifestyle risk factors (alcohol and smoking) and optimizing certain nutrients through dietary supplementation to slow disease progression (AREDS, 2000; AREDS2, 2013). Micronutrients are thought to reduce the AMD risk factors related to oxidative stress, metabolism, and the availability of nutrients within the highly packed photoreceptor array. Driven by the body of evidence in humans surrounding nutrition in combination with our anecdotal observations indicating retinal damage in jumping spiders, we investigated whether nutrient deprivation could initiate or exacerbate macular-degeneration-type damage in the retina of the jumping spider *Phidippus audax*. To accomplish this task, we reared spiders under nutrient-deprived and nutrient-rich conditions. Once spiders reached their 5th instar stage, a customized ophthalmoscope was used to examine the integrity of the entire retina (Stowasser et al., 2017), with particular emphasis on five areas (the HDR and four surrounding low-density areas). We further evaluated damaged and intact retinas at the ultrastructural level using electron microscopy.

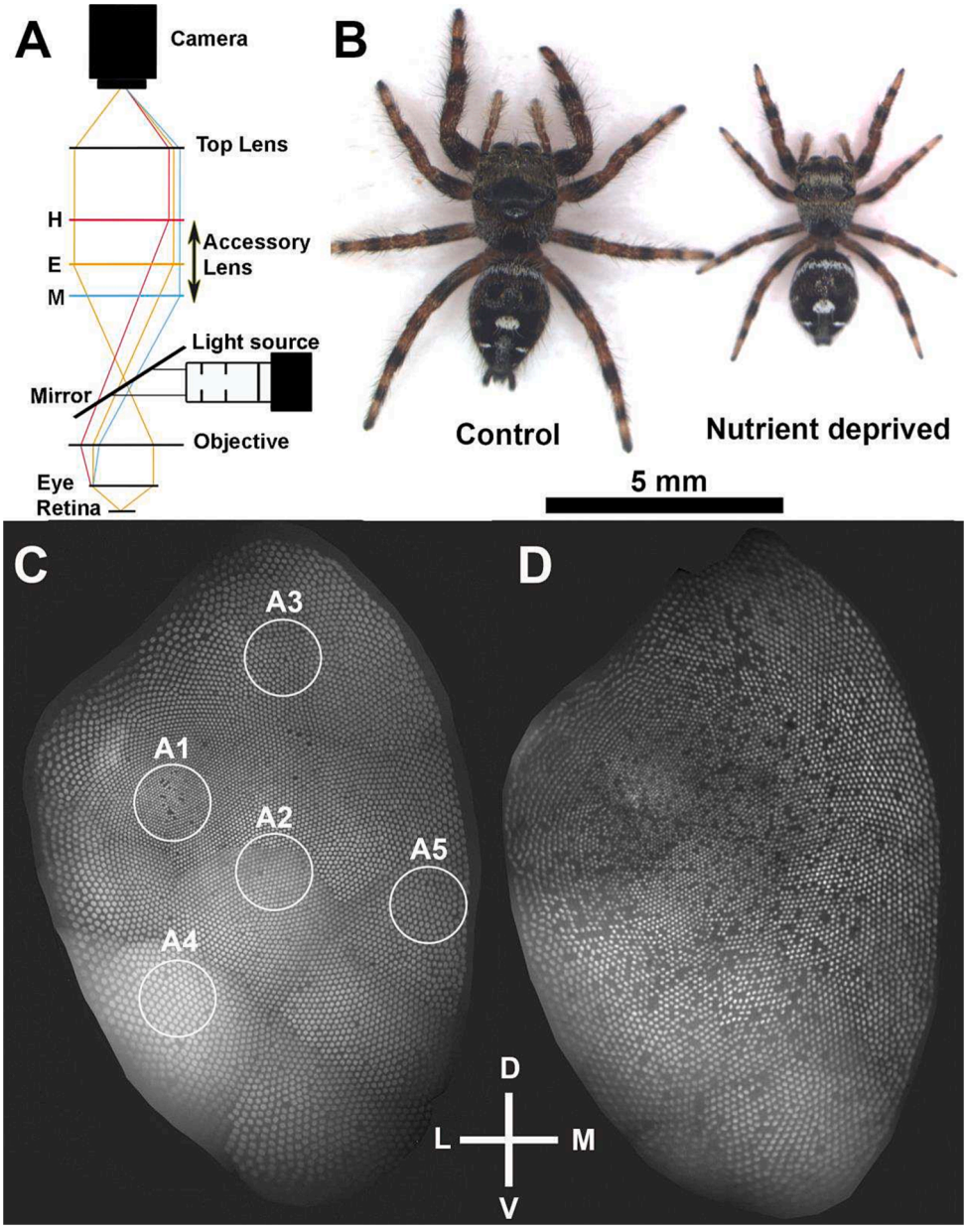

Fig. 1. *Phidippus audax* ALE retina comparison between nutrient-deprived and control individuals. A. Schematic of the customized ophthalmoscope used to image the retina by moving the accessory lens into the position that resulted into the best-focused image (which depends on the optical error of the eye; H: hyperopia; E: emmetropia; M: myopia; see Stowasser et al., 2017). B. Example of 5th instar sibling spiders, with the nutrient-deprived individual being clearly smaller. C and D. Composite ALE retinal maps from ophthalmoscopic imaging. C. Representative retinal map of a control spider showing a nearly completely intact regular matrix of fluorescent photoreceptor rhabdoms. D. Retinal map of a nutrient-deprived spider, showing major rhabdom degeneration.

2. Materials and methods

2.1. Animals

Spiderlings were the offspring of field-caught adult spiders that laid egg clutches shortly after being transferred to the laboratory. As soon as they emerged from the egg sack (typically at the 2nd or 3rd instar stage (Goté et al., 2019)), spiderlings were isolated and reared separately in small plastic cups with unlimited water and the following food. The smallest spiders received a diet of two *D. melanogaster* 2× per week until their next molt, after which the spiders from each clutch were divided into two groups. Nutrient-control and nutrient-deprived spiderlings received the same diet, except that the nutrient-control individuals were fed 2× per week and the nutrient-deprived individuals only 1× per week. For each feeding, spiders initially received four *D. melanogaster* and then six *D. melanogaster* after a further molt. All animals were reared at ~25 °C with a 14 h day–10 h night light cycle and measured after they had molted 3× post hatching, which corresponds to the 5th or 6th instar stage. As the spiders were still juveniles, we were unable to determine their sex.

2.2. Fluorescence imaging and data analysis

The retinas of the ALEs of the spiders were imaged using the custom-built ophthalmoscope and methods described by Stowasser et al. (2017). Briefly, the fluorescence of the retina was imaged using a Texas red filter after bringing the retina into focus with the accessory lens (Fig. 1). Each image captured a relatively small portion of the retina, so to construct retinal maps detailing the entire retina of the ALE, we took a series of images as the spiders were rotated in a custom Euler cradle. Subsequently, these images were carefully assembled in Photoshop using the Auto-Align function (Adobe, San Jose, CA, USA).

Completed retinal maps were checked for proper alignment and image stitching to ensure that all regions of interest were adequately represented. To define the five retinal areas for counting fluorescent photoreceptors, two template lines were determined as follows: a line passing through both apexes of the dorsal and ventral periphery curves of the retina (L1), a line passing through the midpoint of L1 and the apex of the lateral periphery curve (L2), a line passing through the center of the acute zone and the apex of the dorsal periphery (L3), and a line passing through the apexes of the lateral and ventral periphery curves of the retina (L4). A diagram of this methodology is shown in Fig. 2B. Five general regions in the ALE were chosen: the acute zone (A1, the area capturing the smallest rhabdom diameters), the central retina (A2, centered on the midway point of L1), the dorsal periphery (A3, falling on the dorsal edge of L1), the ventrolateral periphery (A4, centered on the midway point of L4), and the ventromedial periphery (A5, falling on the medial edge of L2, filling the full space with photoreceptors). Once these five regions of interest were located on the composite map, five raw ophthalmoscope image files were identified by selecting the best single image layer that captured each area of interest within the composite map. A white circle (diameter of 225 pixels) was aligned and overlaid on each corresponding raw image. Once the five retinal images with the outlined areas of interest were identified for each spider, image analysis was conducted in ImageJ (version 1.51n, Abràmoff et al., 2004).

To count the rhabdoms in each area, we used ImageJ to collect the xy coordinates of the centers of all discernible rhabdoms more than half contained within the bounds of the region. Additionally, 10 inter-rhabdomal points were randomly selected in each region to find the background fluorescence and to correct for differences in exposure between images. Using a customized MATLAB program and the xy coordinates of the rhabdoms, we then found the grayscale values of all identified rhabdoms and counted all rhabdoms that had a higher grayscale value than the average grayscale value of the 10 interrhabdomal points.

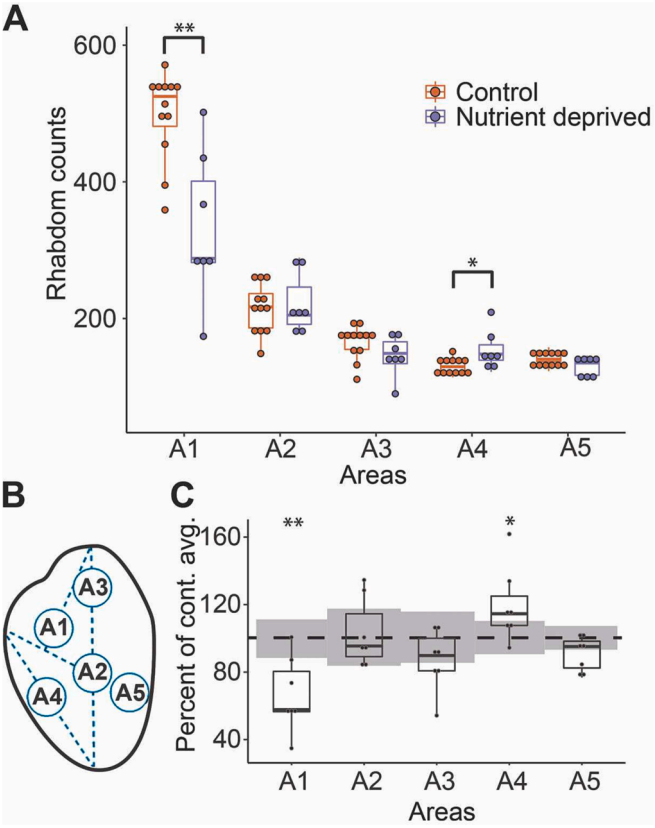

Fig. 2. Comparison of fluorescent rhabdoms in five retinal areas (A1–A5). A. Number of fluorescent rhabdoms in A1–A5 in nutrient-deprived and control spiders. A1 had significantly fewer fluorescent rhabdoms in nutrient-deprived spiders than in control spiders (Wilcoxon's rank sum test; $n_{control} = 12$; $n_{nutrient\ deprived} = 9$; $p = 0.004$). B. Schematic of the location of the five areas. A1 is the area of the retina with the highest photoreceptor density. A2 is in the center of the retina. A3–A5 are peripheral areas surrounding A1 and A2. C. Number of fluorescent photoreceptors in A1–A5 in nutrient-deprived spiders represented as a percent of the nutrient-control spider average (percent of cont. avg.). The gray boxes indicate the standard deviation of the nutrient-control spiders. The box and whisker plots indicate the data spread, with the median indicated by the dark line and the mean by the dot. The percentage count of fluorescent photoreceptors in A1 was significantly reduced in nutrient-deprived spiders compared to control spiders ($p = 0.0040$, Wilcoxon's rank sum test). We also noted a weak statistical significance in A4, which is adjacent to the HDR ($p = 0.04$, Wilcoxon's rank sum test). However, when the individual with a somewhat unusually high rhabdom count in A4 was excluded, the difference was no longer significant.

2.3. Transmission electron microscopy (TEM)

To confirm that a lack of fluorescence is a valid assessment for photoreceptor degeneration, we first imaged adult spiders with intact retinas or massive retinal degeneration in the ophthalmoscope and then investigated the retinal ultrastructure with TEM for comparison ($n = 2$ for controls and tests). The tissue for TEM imaging was processed as described in Wolff (2011) with a few modifications. In brief, spiders were anesthetized on ice, then the cephalothorax was removed, opened posteriorly, and fixed with EM fixative composed of 4% paraformaldehyde, 3.5% glutaraldehyde, and 1% tannic acid in Sorensen's buffer solution for ~14 h at 4 °C. After fixation, the tissue was washed in phosphate buffer and placed in 2% osmium tetroxide on ice for 1 h and then at room temperature for 1 h. The tissue then was washed and placed in uranyl acetate for 16–20 h at room temperature. After washing again, the tissue was dehydrated in a series of increasingly concentrated ethanol solutions and then a 1:1 mixture of acetone and pure ethanol

followed by pure acetone. Subsequently, the tissue was placed in a 1:1 mixture of acetone and Spurr Low-Viscosity Embedding mixture (Polysciences, Warrington, PA, USA) for 16–20 h and then in pure embedding medium for 16–20 h before being transferred into embedding molds and cured for 12–24 h at 60 °C. Finally, the blocks were sectioned with a rotary microtome at 8 μm until the ALE retina was exposed. Semi- and ultrathin sections of the resin-embedded samples were then obtained using a Reichert Ultracut E ultramicrotome. The ultrathin sections were collected on grids and visualized using a JEOL JEM1230 transmission electron microscope with an AMT Advantage Plus 2 k × 2 k digital camera.

3. Results and discussion

To assess retinal damage in the ALEs of nutrient-deprived spiders, we measured 12 nutrient-control spiders and 9 nutrient-deprived spiders. Using a customized ophthalmoscope (Stowasser et al., 2017), we created ALE retinal maps of 5th instar spiders (Fig. 1A). From these maps, we counted the fluorescent rhabdoms in five distinct areas (A1–A5). The retinal areas were chosen based on the photoreceptor density distribution, where A1 is in the highest density area and A2–A5 are representative surrounding low-density areas (Goté et al., 2019).

Overall, we noted that the nutrient-deprived spiders were considerably smaller in body size than the nutrient-control spiders (example shown in Fig. 1B), indicating that the nutrient deprivation affected the spiders more generally, although this was not formally assessed using objective measures. On the level of the ALE, the retinal maps (Stowasser et al., 2017; Goté et al., 2019) of the nutrient-control spiders showed uniform matrices of fluorescent rhabdoms, as expected (Fig. 2C). In stark contrast, the matrices of the nutrient-deprived spiders were irregular and degenerate (example shown in Fig. 2D). Furthermore, nutrient-deprived spiders appeared to have high variability in the autofluorescence of the retina. Some rhabdoms seemed to show reduced autofluorescence, while the autofluorescence of others seemed to have increased. This is similar to the autofluorescence changes associated with AMD in humans, where both an increase and a loss of autofluorescence have been described (Schmitz-Valckenberg et al., 2009; Schmitz-Valckenberg et al., 2021; Guymer & Wu, 2020; Hammer et al., 2021).

It is important to note that in humans, changes in autofluorescence, as assessed using fundus photography, are a byproduct of RPE alteration rather than changes in individual photoreceptors, as evident in the spider retinas (Fig. 2). In humans, enhanced autofluorescence is thought to occur during the early stages of retinal damage, associated with the accumulation of lipofuscin and extracellular debris, called drusen, under the RPE, likely due to oxidative stress and immune dysregulation. On the other hand, a loss of autofluorescence may indicate RPE atrophy (Schmitz-Valckenberg et al., 2009; Schmitz-Valckenberg et al., 2021; Guymer & Wu, 2020; Hammer et al., 2021). As demonstrated in flies, autofluorescence in the rhabdomeric photoreceptors of arthropods is due to metarhodopsin (Pichaud & Desplan, 2001; Franceschini et al., 1981), and changes in the level of fluorescence are also related to photoreceptor health and possibly oxidative stress. Therefore, even though the associated fluorophore species in spiders likely differ from those in humans, the similarity in autofluorescence changes suggests that autofluorescence imaging can be used as a diagnostic tool for the *in vivo* evaluation of retinal health in spiders, just like in humans (Ly et al., 2017).

Our data suggest that late stages of retinal damage can be detected as a loss of autofluorescence and that early stages of damage are likely indicated by increased autofluorescence in spider retinas. However, due to significant challenges presented when accounting for absolute brightness values, as discussed in the analysis of fundus fluorescence imaging in humans (Schmitz-Valckenberg et al., 2009), we could not analyze specific differences in fluorescence. In addition, since we cannot reliably attest that a gap in the retinal mosaic is indicative of a dead photoreceptor (i.e., these could indicate abnormalities in the retinal matrix independent of photoreceptor death), we instead quantitatively analyzed the number of rhabdoms that remained fluorescent.

To assess how many rhabdoms might have been lost, we analyzed the number of fluorescent rhabdoms by comparing the remaining rhabdoms in the five retinal areas (Fig. 2A&B) and as the percentage of the rhabdom count in the nutrient-deprived spiders relative to the average rhabdom count in the nutrient-control spiders for each area (Fig. 2C). The rhabdom count revealed that the density in A1 was much higher than that in any of the sampled surrounding areas (ANOVA with post-hoc TUKEY test; controls, $p < 0.0000001$; experimental, $p < 0.007$), as expected (Goté et al., 2019). The loss of fluorescent rhabdoms was most prevalent in the high-density area (Fig. 2). Specifically, A1 in the nutrient-deprived spiders had a significantly decreased rhabdom count compared to that in the nutrient-control spiders (Wilcoxon's rank sum test; $n_{control} = 12$; $n_{nutrient\ deprived} = 9$; $p = 0.004$; Fig. 2A). In addition, A4 appeared to show a significant difference in rhabdom count between the nutrient-control and nutrient-deprived spiders ($p = 0.04$, Fig. 2A). However, when one individual with a somewhat unusually high rhabdom count in this area was excluded, the difference was no longer significant. The same trend was observed when analyzing our data as a percentage count. Only A1 had a significantly decreased percentage count relative to the nutrient-control spiders ($p = 0.0040$, Wilcoxon's rank sum test, Fig. 2C), indicating that the greatest loss of rhabdom fluorescence occurred in the high-density area, while low-density areas were only mildly affected, if at all.

To investigate if and how changes in rhabdom fluorescence relate to retinal morphology, we collected TEM images of rhabdoms within the ALE retinas of spiders with and without signs of degeneration. The TEM images of spiders with intact rhabdom fluorescence in the retinal matrix showed the expected morphological organization of an ALE retina, consisting of a regular assembly of segments, as previously described by Eakin and Brandenburger (1971). Each segment contains one photoreceptor cell at its center, forming two adjacent rhabdomeres. Each rhabdomere is in direct contact with a support cell, and these three cells are surrounded by pigment cells (Fig. 3A–C). In comparison, the TEM images of spiders with degenerated fluorescence matrices displayed an irregular organization (Fig. 3D&E). The imaged retinal units showed various stages of retinal degeneration, such as partially degenerated rhabdoms (arrow in Fig. 3G) or portions of rhabdoms that had disintegrated into large vacuoles (arrows in Fig. 3G&H). We also noted cases where vesicles had entirely replaced rhabdomeres (arrows in Fig. 3I), where photoreceptor cells were swollen and rhabdoms were displaced (arrows in Fig. 3J), and where entire retinal units appeared necrotic (NC in Fig. 3J).

Taken together, our findings suggest that autofluorescence changes in micro-ophthalmologic imaging indicate damage in photoreceptors at the ultrastructural level. The observation that photoreceptor damage in the nutrient-deprived spiders mostly occurs in the HDR (A1) suggests that, as is the case for macular degeneration, the challenge of maintaining a functioning retina is exacerbated in tight spaces, where it is particularly difficult to meet nutrient requirements. These findings not only illuminate interesting parallels in limits between vertebrate and invertebrate eyes but also align with the principle put forth by Niven & Laughlin that the maintenance of sensory systems may be limited by the availability of energy (Niven & Laughlin, 2008). This is thought to be of particular importance for the retina due to its high energy demands (Niven & Laughlin, 2008).

Perhaps most noteworthy is the parallel between our findings and those of the AREDS studies in humans. The AREDS studies investigated the effects of nutrient supplements on the progression of AMD in humans and found that certain dietary supplements can slow disease progression (AREDS, 2000; AREDS2, 2013). Similarly, our results show that nutrient deprivation exacerbates retinal degeneration in spiders. Important questions remain regarding which critical micronutrients are targetable as therapeutics and the uncertainty of the critical time frame for meeting

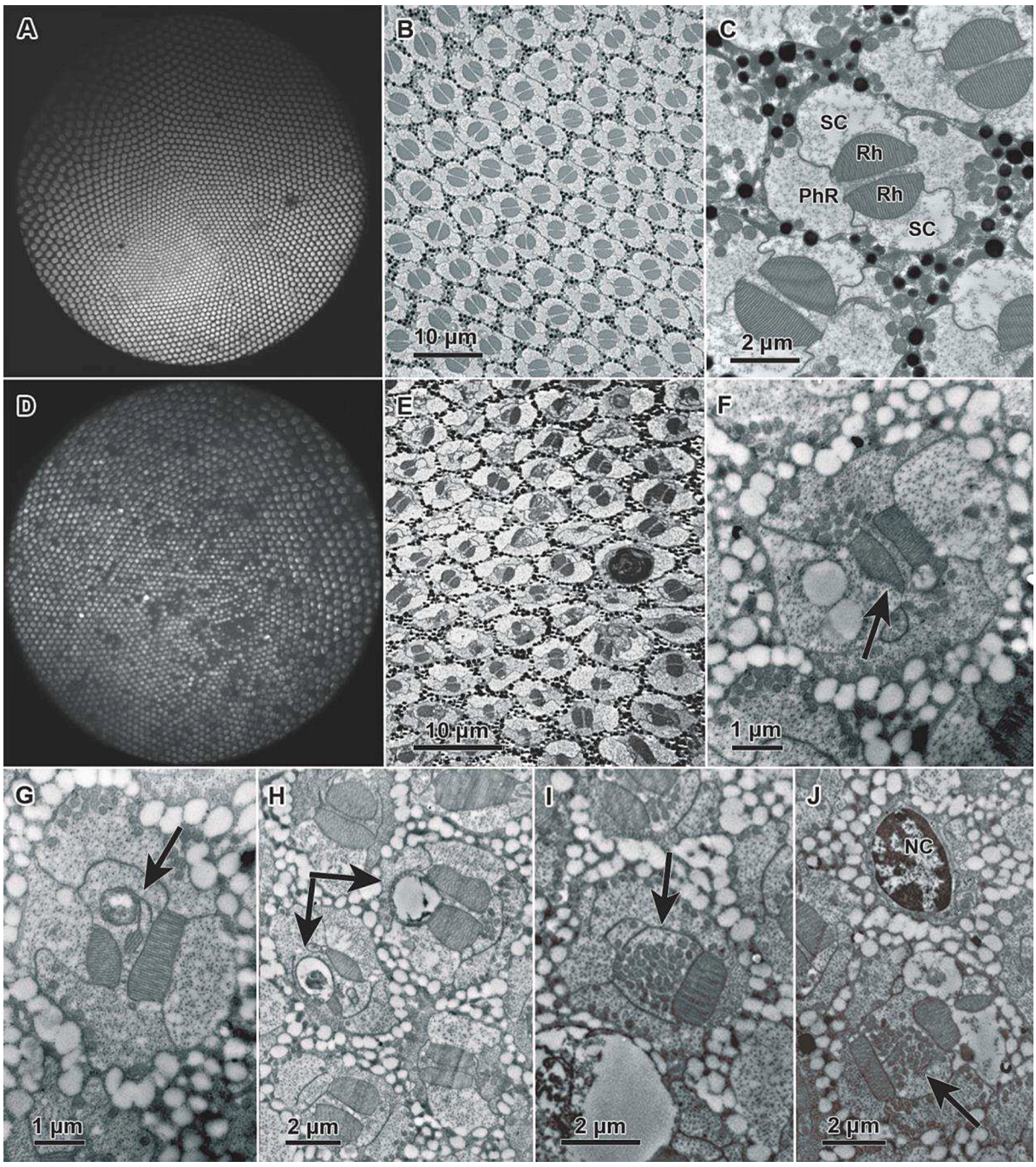

Fig. 3. Ophthalmoscope and TEM images of the ALE. A. Representative ophthalmoscope image of the retina of a spider with seemingly intact photoreceptors. The photoreceptor matrix is regular, and the fluorescence of the photoreceptors is relatively homogenous. B and C. TEM images of the retina of the spider imaged in A, confirming its integrity. The photoreceptor matrix is very regular (B) and the retina units are morphologically intact (C), as indicated by each unit having a complete and well-shaped photoreceptor cell (PhR) with two rhabdomeres (Rh) and two support cells (SC). D. Ophthalmoscope image of the retina of a spider that shows major defects in the fluorescence of the photoreceptors. The photoreceptor matrix appears irregular, with several photoreceptors either lacking fluorescence or appearing unusually bright. E–J. TEM images of the retina of the spider imaged in D, illustrating major damage in the photoreceptor array. The matrix of the retinal units appears irregular (E), and the retinal units show a variety of morphological defects, including somewhat degenerated rhabdoms (F, arrow) and parts of rhabdoms disintegrated into large vacuoles (G and H, arrows). There are also cases where vesicles are present instead of rhabdomeres (I, arrow), photoreceptor cells are swollen and rhabdoms are displaced (J, arrow), or the entire retinal unit shows necrosis (J, NC).

nutritional needs to slow or even prevent retinal degeneration. Our results suggest that the effect of nutrient depletion may precede actual cellular degeneration, raising key follow-up questions such as: When should additional nutrients be supplemented to maximize their therapeutic effect in slowing or even reversing damage? In humans, AREDS-formulated micronutrients benefited patients with intermediate or late AMD by delaying further progression. Importantly, supplementation did not reverse retinal damage. While diet formulation has proven quite effective, it continues to be evaluated with possible further improvements (AREDS, 2001, AREDS2, 2022); however, due to the effectiveness of the treatment, this is limited in humans for ethical reasons.

As in vertebrates, invertebrate photoreceptors, including those of spiders, are neurons that are supported by adjacent glia-like support cells (Blest & Maples, 1979). Therefore, spider eyes are a good model for

investigating aspects of neurodegeneration more broadly. In this context, it has been shown that micronutrient supplements have a positive effect on various neurogenerative diseases such as Parkinson's disease, Huntington's disease, and Alzheimer's disease (Rahimmi et al., 2022; Kumar et al., 2021). This alleviation is in part expected to be mediated by improving glia-neuron metabolic support. The observation that spiders, just like humans, tend to suffer from degeneration in areas with tight packing (the HDR) opens the door to broader explorations of the effects of nutrients on neuronal health. The spider system with ophthalmoscope-based 'health checks' is particularly powerful, as the retina is already completely differentiated in 1st instars (Goté et al., 2019). Therefore future studies could focus on investigating spider health longitudinally as a function of age. This is possible because these spiders have a long life span (in our anecdotal experience, exceeding 1 year under laboratory conditions) and these methods allow retinal health to be monitored in a relatively noninvasive manner. Nonetheless, it must be quantified whether light exposure during ophthalmoscopic measurements affects retinal health. Similarly, questions about how nutrient demand changes when specific stressors are applied (such as exposure to bright light or toxins) and the contribution of genetic predispositions should be addressed. Recent advances on the molecular front provide new possibilities here. For example it has become clear that the molecular machinery underlying phototransduction in spiders shares many elements with that in *D. melanogaster* (Morehouse et al., 2017). It would be interesting to determine which genes are impacted by nutrition-induced degeneration, especially considering the finding that molecular mechanisms associated with photoreceptor degeneration can be specific to environmental stressors (Dewett et al., 2021). Finally, our findings also have major implications for the ecology of jumping spiders, as it raises the possibility that the nutrient supply of high-resolution arthropod eyes can be challenged. This study therefore identifies an important potential limit for the evolution of eye diversity.

Author contributions: AS and EKB contributed to the study conception and design. Material preparation, data collection, and analysis were developed and performed by MB, SR, JTG, NIM, and AS. The first draft of the manuscript was written by AS, SR, JTG, and EKB. All authors edited the manuscript.

Funding: This research was supported by National Science Foundation grants IOS-1734291 and IOS-1831767 to NIM and IOS-1456757 and IOS-1856241 to EKB.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgement

The authors would like to thank Patrick Butler for his crucial support in spider collection and extensive assistance in image analysis, Madeline Owens and Isaiah Giordullo for their contributions to data analysis, and Tamara Pace for her editorial feedback.

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