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BRIEF COMMUNICATION

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Knockdowns of *red Malphigian tubules* reveal pigmentation roles in the milkweed bug

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Abstract

Classical *Drosophila* eye color mutations have unearthed a toolkit of genes that have permitted candidate gene studies of the outstanding diversity of coloration patterns in other insects. The gene underlying the eye color phenotypes of the *red Malphigian tubules* (*red*) fly mutant was mapped to a LysM domain gene of unknown molecular function. Here, we used RNAi to test the role of a *red* ortholog in the pigmentation of the milkweed bug *Oncopeltus fasciatus*, and contrast its effect with the ommochrome biosynthetic pathway gene *vermilion* (*ver*). Pigmentation was reduced in the cuticle of embryonic legs and first instar abdomens following parental RNAi against *red*, but not against *ver*, likely reflecting an effect on pterin biogenesis. Nymphal RNAi of *red* and *ver* both resulted in adult eye depigmentation, consistent with an effect on ommochrome content. These results suggest *red* loss-of-function impacts biochemically distinct types of pigments, and we discuss its putative role in the biogenesis of lysosome-related organelles such as ommochromasomes and pterinosomes.

KEYWORDS
milkweed bug, ommochromes, pigmentation, pterins, *red Malpighian tubules*

1 | INTRODUCTION

Pigmentation has long been a focus of molecular genetics research in *Drosophila*, due to the straightforward scoring of eye color phenotypes, ease of maintenance of mutant lines, and utility as genetic markers. As a result, the biosynthesis of pigments found in *Drosophila* eyes has been described extensively, first with the isolation, generation, and mapping of many mutant lines (Futahashi & Osanai-Futahashi, 2021). The red-brown eye color of wild-type *Drosophila* is due to two classes of pigments, pteridines (red), and ommochromes (brown). Ommochromes are tryptophan metabolites used as visual pigments in the eyes of arthropods and have repeatedly been co-opted to form body pigmentation (Futahashi & Osanai-Futahashi, 2021; Orteu & Jiggins, 2020; Reed et al., 2008). Pterins are guanosine triphosphate (GTP) metabolites commonly used to create yellow, orange, and red pigmentation in insects (Andrade & Carneiro, 2021; Futahashi & Osanai-Futahashi, 2021). They were first described as the pigments in butterfly wings (Hopkins, 1895; Wijnen et al., 2007), but are also common across the tree of life as biosynthetic precursors (Andrade & Carneiro, 2021; Ayling et al., 2012; Feirer & Fuqua, 2017). Studies in water striders and butterflies suggest that pterin pathway components have repeatedly been co-opted from eyes to wing and body tissues to drive the evolution of aposematic red (Vargas-Lowman et al., 2019; Wee & Monteiro, 2017). The two biosynthetic pathways are independent from each other, but several eye color genes involved in the transport of precursors as well as in vesicular trafficking can affect both pigment types (Futahashi & Osanai-Futahashi, 2021; Lloyd et al., 1998). Genes from the "granule group" of fly eye-color mutations in particular are essential for the function of lysosome-related organelles such as the ommochromasomes and pterinosomes, the intracellular vesicles where pigments are formed and stored (Figon & Casas, 2019; Figon et al., 2021; Grant et al., 2016; Lloyd et al., 1998; Reaume et al., 1991).

The *Drosophila red M. tubules (red)* eye color mutations were first described in the 1950s (Aslaksen & Hadorn, 1957; Oster, 1954), and have been used as a marker gene (Breen & Harte, 1991; Henikoff, 1979; Paton & Sullivan, 1978). Mutant flies show darker eyes and abnormally

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pigmented Malpighian tubules, with an overall decreased content of ommochromes and pterins, except for a strong accumulation of colorless biopterin in eyes, and of the ommochromes xanthommatin and ommin A in Malpighian tubules (Ferré et al., 1986; Ruiz-Vázquez et al., 1996; Silva et al., 1991; Wessing & Bonse, 1966). *Red* was recently mapped to a LysM-domain containing protein in *Drosophila* (Grant et al., 2016). Here we used RNA interference (RNAi) to test a role for an homolog of *red* (*Of_red*) in pterin and ommochrome pigmentation of the heteropteran insect and evo-devo model *O. fasciatus* (milkweed bug) (Chipman, 2017). Importantly, previous work has shown that the ommochrome pathway is only important for the pigmentation of the *Oncopeltus* adult eye, while the pterin pathway appears to be the major determinant of red-orange coloration in the embryonic and nymphal cuticle as well as in the adult elytra (Forrest et al., 1966; Liu, 2016; Smissman & Orme, 1969). Knockdowns of *vermilion* (*Of_ver*) provide ommochrome-specific phenotypes in the milkweed bug, and we thus contrasted *Of_red* RNAi with both *Of_ver* RNAi and uninjected controls to tease apart the contribution of ommochrome and non-ommochrome pigment classes at various stages.

2 | RESULTS

2.1 | *Of_red* knockdowns impact pigmentation in embryos and first instar nymphs

We searched a range of annotated arthropod genome sequences using TBLASTN and found a single ortholog of the *Drosophila melanogaster* gene *red* per species across this radiation (Figure 1a). Next, we used parental RNAi in *O. fasciatus* to test for a function of the *Of_red* homolog in 96 h embryos and first instar nymphs, which are characterized by pterin pigments responsible for yellow and red cuticular pigmentation (Forrest & Smith, 2019; Forrest et al., 1966; Liu, 2016; Smissman & Orme, 1969). *Of_ver* and uninjected controls were used to control for the wild-type phenotype (Liu, 2016). Parental RNAi of *Of_red* and *Of_ver* resulted in a reduction of the targeted transcripts in hatchlings, suggesting knockdown effects persisted throughout embryonic development (Figure 1b). In terms of phenotypic effect at these early stages, *Of_red* RNAi resulted in marked decreases in red pigmentation of the embryonic cuticle, while *Of_ver* did not produce visible effects (Figure 1c–e), in accordance with previous knockdowns of three ommochrome pathway genes (Liu, 2016). Likewise, parental RNAi yielded a visible reduction of red pigmentation after *Of_red* but not *Of_ver* knockdowns in the first instar nymphs (Figure 1f–h). To quantify this effect, we extracted the color profile of 273 abdomen images in L*a*b* space, plotted the distribution of pixels according to their a* ranks (Figure 1i–k), an uniform measure of red chromaticity on a green-to-red axis, and found a significant effect of *Of_red* RNAi on mean a* ranks (Figure 1l, pairwise Wilcoxon rank-sum test: $p < 10^{-8}$), while *Of_ver* RNAi did not show any detectable effect ($p = 0.069$). *Drosophila* MTs normally accumulate two fluorescent metabolites: the 3-hydroxykynurenine ommochrome precursor, a tryptophan derivative that is converted to a red ommochrome in *red* mutants (Sullivan et al., 1980; Wessing & Bonse, 1966; Yagi & Ogawa, 1996), as well as riboflavin, a purine metabolite (like pterins) that is commonly observed in insect MTs (Nickla, 1972; Sullivan et al., 1979; Weber & Roberts, 1967; Zhang et al., 2018). Here we did not detect accumulation of ommochrome pigment or fluorescent metabolites in the MTs of freshly hatched first instar nymphs following *Of_red* depletion, but further investigation will be needed to test if this holds true at later stages (see *Discussion*). In summary, we found that *Of_red* but not *Of_ver* knockdowns result in a reduction of red contrast in the cuticle of embryos and nymphs, likely reflecting a decrease in pterin content at these stages (Forrest et al., 1966; Liu, 2016).

2.2 | *Of_red* knockdown affects ommochrome content in the adult eye

Next, we performed dsRNA injection in the last instar nymphs and monitored color changes in adults. While this method was previously found to induce visible discoloration of the orange cuticle of adult elytra following knockdown of the pterin pathways genes *Of_purple* and *Of_punch* (Liu, 2016), we did not observe a visible effect of *Of_red* knockdowns in adult body or wing cuticle color. Instead, depletion of *Of_red* showed a slight depigmentation of the adult eye crescent similar to the one observed in the *Of_ver* knockdown (Figure 2a–c), as well as for other ommochrome pathway gene knockdowns (Liu, 2016). These results provide evidence that impairing *red* can disrupt ommochrome content in *Oncopeltus* eyes, the only external tissue where ommochromes are predominant in this insect (Liu, 2016).

3 | DISCUSSION

Ommochrome and pterin pigments are found in animal groups as diverse as birds and insects, where along with other pigment families they are capable of producing yellow, orange, red, and brown colors. The biosynthetic pathway for pterins and ommochromes have been dissected in great detail from classic *Drosophila* eye pigmentation mutants, though some hypothetical components of both pathways are yet to be described. The historical lack of a genomic position for *red* in *Drosophila* has prevented the identity of the gene from proliferating into annotations of recently sequenced insect genomes, meaning it has been excluded from candidate gene studies of pigmentation. Here we found that similar to its dual effect on eye pterin and ommochrome content in *Drosophila*, knockdowns of *Of_red* in *Oncopeltus* lead to a decrease in body pterin pigmentation, detectable in the external cuticle of embryos and first instar nymph abdomens, as well as a decrease in adult eye ommochrome pigmentation. Future transcriptomic analyzes or candidate gene studies should include this gene as a potential modulator of insect pigmentation. It will also be interesting to further characterize how ommochrome and pterin precursors (tryptophan and purine derivatives, respectively) are metabolized in these ecologically divergent insects: for instance, *Drosophila* relies on MTs to excrete a large amount of purine metabolites as meconium, while *Oncopeltus* can instead store these compounds in its tissues (Pant, 1988; Harmsen, 1966). We suggest that CRISPR somatic

knock-out approaches (Reding & Pick, 2020), by generating null clones rather than transient knockdowns, may generally be more amenable to the study of pigment genes of the "granule group" in *Oncopeltus* in a wider variety of stages and tissues, including epithelia, MTs, and fat.

Of note, the effect on pterin RNAi phenotypes in embryos and hatchlings was here subtle. This is especially true when compared to knock-outs and knockdowns of *Of_white*, which trigger strong depigmentation phenotypes at these stages as well as in adult eyes (Liu, 2016; Reding & Pick, 2020), consistent with a conserved and dual role in both pterin and ommochrome precursor uptake. Likewise, RNAi depletion of the pterin pathway enzymes Purple, Punch, and Rosy result in the marked loss of yellow and red pigments in embryos of milkweed bugs and water striders (Liu, 2016; Vargas-Lowman et al., 2019), suggesting a shared mechanism in Heteroptera. Thus, unlike these genes, *Of_red* knockdowns do not block pterin synthesis, and may result instead in complex changes in colorless precursors and pigment compounds that will deserve further study. Importantly, the intracellular transport and maturation of pigment pathway metabolites occur in ommochromasomes and pterinosomes, intracellular compartments that are endomembrane derivatives known as the lysosome-related organelles (Figon & Casas, 2019; Figon et al., 2021; Grant et al., 2016; Reaume et al., 1991). Key factors for vesicular trafficking and lysosomal function have been discovered as *Drosophila* eye pigmentation mutants due to their visible effects on ommochrome and pterin content (Figon & Casas, 2019; Futahashi & Osanai-Futahashi, 2021; Lloyd et al., 1998), while other components have come from vertebrate melanosomes (another lysosome-related organelle) for similar reasons (Delevoye et al., 2019; Marks et al., 2013). Here we note that the mutant effects of the fly

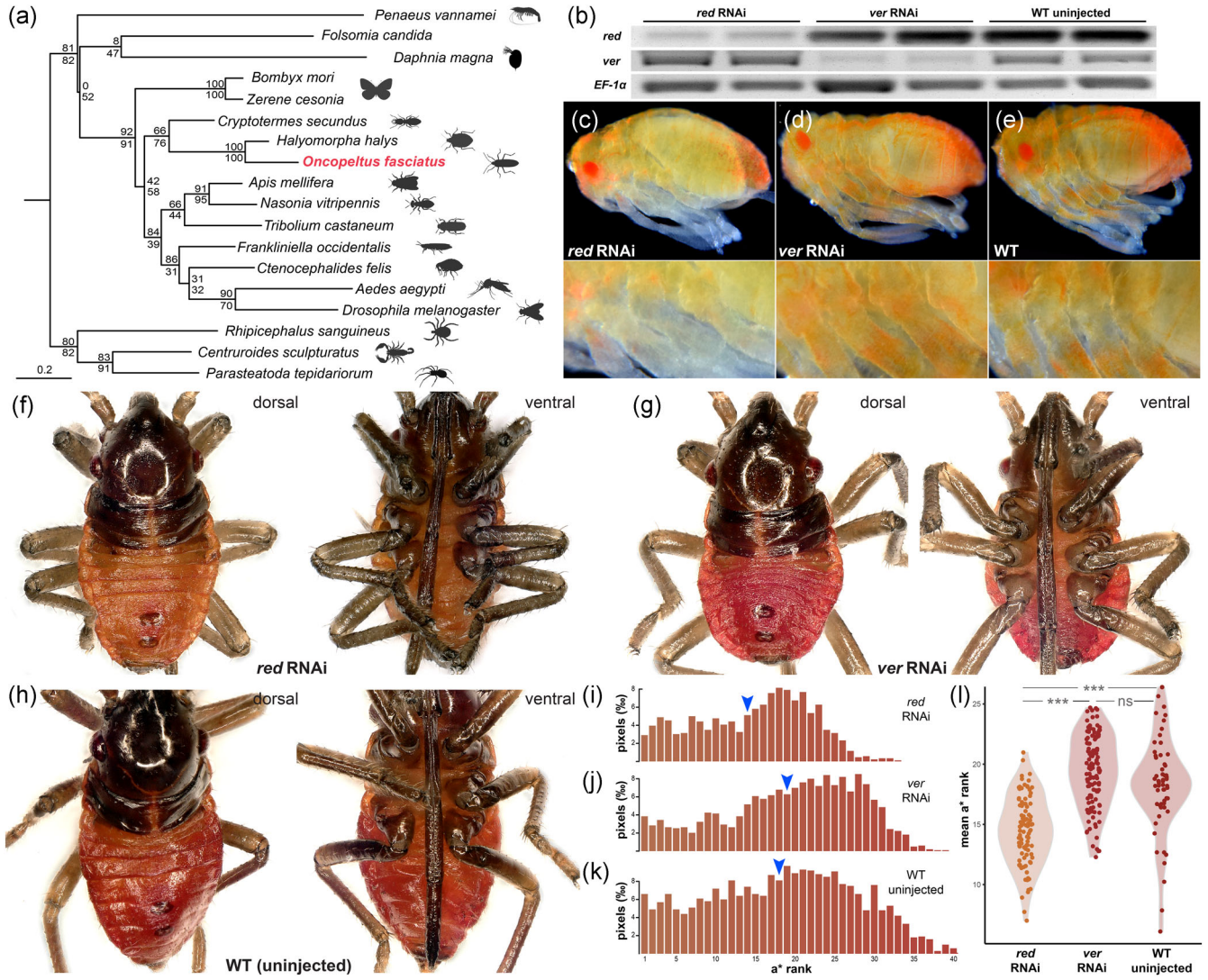

FIGURE 1 Parental RNAi knockdowns of *Of_red* in embryos and first instar nymphs. (a) Maximum likelihood reconstruction of arthropod Red/Lys-M domain homologs. Node support values are shown as approximate likelihood ratio test (top) and ultrafast bootstrap (bottom) percentages. (b) RT-PCR analysis of *Of_red*, *Of_ver* expression in hatchlings following parental RNAi. (c–e) Effect of *Of_red* RNAi on abdominal pigmentation of first instar nymphs. (f–h) Effect of *Of_red* RNAi on abdominal pigmentation in first instar nymphs. (i–k) Examples of histogram distribution of pixel a* ranks (red contrast indicator) following parental RNAi. Each plot corresponds to 1000 random pixels from the dorsal abdomen of a knockdown or control individual representative of its treatment group. Arrowheads: mean a* rank as plotted in panel L. (l) Violin plots of mean a* ranks among 273 knockdowns and control first instar nymph abdomens. Wilcoxon rank-sum tests, ***: $p < 10^{-8}$; *n.s.* (nonsignificant): $p = 0.069$

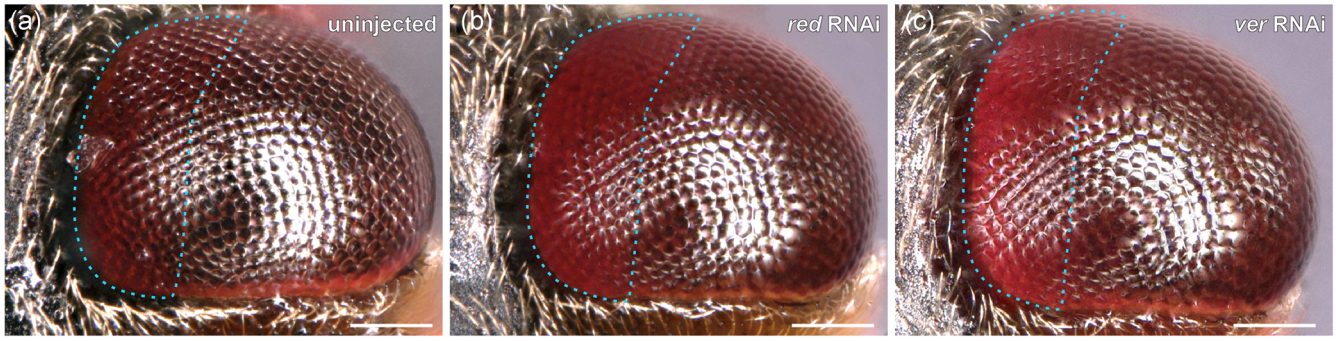

FIGURE 2 Nymphal RNAi knockdowns of *Of_red* and *Of_ver* in adult eyes. (a–c) The medial third of imago eyes consists of adult tissue developed during the last nymphal instar (dotted lines). Adult ommatidia show a decrease in the inclusion of brown pigments following *Of_red* and *Of_ver* dsRNA injection in the last nymphal instar, consistent with an alteration of ommochrome content (Liu, 2016). *Of_red* nymphal RNAi resulted in eye phenotypes in four out of five adults, *Of_ver* nymphal RNAi in seven out of eight adults. Scale bars = 100 μm

LysM-domain gene *red* are similar to those observed in *chocolate* (*cho*) mutants of the vacuolar ATPase gene *VhaAC39-1*, as these two mutants affect both ommochrome and pterin content and are the only two known genes with pigment phenotypes in both eyes and MTs (Grant et al., 2016; Tearle, 1991). These concordant effects suggest that like *cho*, *red* may regulate the activity of lysosome-related organelles via the vacuolar ATPase complex, possibly by mediating the pH acidification that is essential for lysosomal function (Futai et al., 2019; Luzio et al., 2014). In mammals, several LysM-domain genes interact with the vacuolar ATPase complex in mouse neurons and kidney cells, at least one of which is essential for lysosomal function (Castroflorio et al., 2021; Eaton et al., 2021). This precedent leads us to speculate that *red* and *cho* may indeed work in unison in the regulation of lysosome-related organelles, and that their mutant phenotypes are explained by a failure of ommochromasomes and pterinosomes to properly acidify, similarly to the effect of pH misregulation in vertebrate melanosomes (Grant et al., 2016; Le et al., 2020; Sirés-Campos et al., 2021). Further work will be needed to test this model in various insects.

4 | MATERIALS AND METHODS

4.1 | Phylogenetic analysis of *red*

Amino-acid sequences from putative arthropod *red* homolog (File S1) were aligned using MAFFT (Katoh & Standley, 2013), curated using GUIDANCE2 (Sela et al., 2015) and used for maximum likelihood tree reconstruction using W-IQ-TREE (Trifinopoulos et al., 2016). A GUIDANCE2 threshold alignment score of >0.707 resulted in the most robust tree for this data set.

4.2 | dsRNA synthesis

RNAi reagents were prepared following a published method with minor modifications (Reding et al., 2019). Total RNAs from pools of three first and second instar nymphs were extracted from the dissected abdomen using the Qiagen RNeasy Kit following bead-shaking homogenization on a Retsch Mixer Mill 400 instrument. First-strand cDNA synthesis was performed using the ThermoFisher SuperScript III kit and oligo$(dT)_{20}$ primers, followed by touchdown PCR. Primers bearing 5′ T7 promoters to amplify a 967 nt *Of_ver* template as previously described (Liu, 2016), and a 567 nt *Of_red* template (T7-F: 5′-[TAATACGACTCACTATAGGG]GGAAGACTTGATTAGTTTGGA; T7-R: 5′-[TAATACGACTCACTATAGGG]AATAATTCATCACTCTCTTTCTCT). The PCR product was then purified using the Invitrogen Purelink PCR Purification Kit for removal of background amplification products <300 bp, followed by a second PCR reaction of 50 μl and column-based purification. For dsRNA synthesis, 250–800 ng of the resulting PCR template was transcribed in 22 μl reactions at 37°C for 16 h using the ThermoFisher MEGAshortSCRIPT T7 kit, followed by LiCl precipitation, ethanol 70% wash, and resuspension in RNAse-free H_2O. This method generated 245–730 μg of dsRNA per reaction.

4.3 | Parental and nymphal RNAi knockdowns

For parental RNAi, virgin females were injected following a published procedure (Chesebro et al., 2009; Liu et al., 2014) after CO_2 narcosis using a Drummond Nanoject III instrument, delivering 2 μl of 4 μg/μl dsRNA. Embryos that were laid at 28°C, were dissected following a rapid heptane dechorionation method (Reding et al., 2019), mounted under coverslips in glycerol 60%, and imaged on a Nikon AZ100 stereoscope equipped with a Nikon D5100 camera. First instar nymphs were frozen after hatching. Malpighian tubules (MTs) were dissected using Dumont Tweezers #5 with Biology Tips, mounted in glycerol 60%, and examined under various light and green epifluorescence settings on an Olympus BX53 microscope. For nymphal RNAi (Liu et al., 2014; Liu, 2016), fourth instar nymphs were injected with 2 μl of 4 μg/μl dsRNA, and melanized adults were frozen until observation. First instar nymphs and adult eyes were imaged on a Keyence VHX-5000 digital microscope mounted with a VH-Z100T lens.

4.4 | Reverse transcription polymerase chain reaction (RT-PCR)

Batches of 100–200 mg of first instar nymphs were stored in TRI reagent solution immediately after hatching, and homogenized for total RNA extraction and cDNA first-strand synthesis. The *Of_EF-1alpha* expression reference gene (Meinzer et al., 2019) was amplified using custom primers (*F_438nt*: 5′-CCAGGCTGACTGTGCTGTGC; *R_438nt:* 5′-ACATCCTGCAGGGGGAGCCT) for 28 cycles with a primer annealing temperature 62°C. *Of_red* (*F_388nt:* 5′-GCAAGAAGCTTGAAAAAGTATGGT; R_388nt: 5′-GCCTCCATTGGGTCTGATGG; 47°C annealing) and *Of_ver* (primers as above, 53°C annealing) transcripts were amplified for 32 cycles without reaching saturation. Final amplicons were electrophoresed on the same gel.

4.5 | Color analysis

For color analysis, five random first instar nymphs per clutch were frozen within 12 h after hatching, yielding 96 *Of_red*, 121 *Of_ver*, and 57 control individuals. Dorsal abdomens of newly hatched nymphs were imaged under constant conditions on a Keyence VHX-5000 digital microscope mounted with a VH-Z00T lens. Photographs were then analyzed with the R package *colordistance* (Weller & Westneat, 2019). Briefly, the L*a*b* color coordinates of 1000 pixels were sampled for each individual, with filters retaining values between 0 and 80 of L, 15 and 50 of a*, and 10 and 140 of b*. Individual distributions of pixel density were plotted as histograms using the *getImageHist* command, with extracted pixels binned ranks of a* ranging from 1 to 50 (low to high red contrast). Differences in mean a* ranks across treatments were tested using pairwise Wilcoxon rank-sum tests in R.

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CONFLICT OF INTERESTS

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Supporting data files are available online in the Supplementary Material section of this article.

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SUPPORTING INFORMATION

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