Title

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Journal

Biology Letters, 15(10)

ISSN

1744-9561

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Publication Date

2019-10-01

DOI

10.1098/rsbl.2019.0554

Peer reviewed

BIOLOGY LETTERS

royalsocietypublishing.org/journal/rsbl

Research

Cite this article: McGowan KL, Passow CN, Arias-Rodriguez L, Tobler M, Kelley JL. 2019 Expression analyses of cave mollies (*Poecilia mexicana*) reveal key genes involved in the early evolution of eye regression. *Biol. Lett.* **15**: 20190554.
http://dx.doi.org/10.1098/rsbl.2019.0554

Received: 26 July 2019
Accepted: 2 October 2019

Subject Areas:
bioinformatics, evolution, molecular biology

Keywords:
cave-dwelling, Poeciliidae, regressive evolution, RNA-sequencing, vision

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Electronic supplementary material is available online at https://doi.org/10.6084/m9.figshare.c.4696403.

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Expression analyses of cave mollies (*Poecilia mexicana*) reveal key genes involved in the early evolution of eye regression

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Eye regression occurs across cave-dwelling populations of many species and is often coupled with a decrease or loss in eye function. Teleost fishes are among the few vertebrates to undergo widespread colonization of caves and often exhibit eye regression with blindness. Cave populations of the poeciliid fish *Poecilia mexicana* (cave molly) exhibit reduced—albeit functional—eyes, offering the opportunity to investigate partial eye regression. We sequenced eye transcriptomes of cave and surface populations of *P. mexicana* to identify differentially expressed genes that potentially underlie eye regression in cave mollies. We identified 28 significantly differentially expressed genes, 20 of which were directly related to light sensitivity, eye structure and visual signaling. Twenty-six of these genes were downregulated in cave compared to surface populations. Functional enrichment analysis revealed eye-related gene ontologies that were under-represented in cave mollies. In addition, a set of co-expressed genes related to vision and circadian rhythm was correlated with habitat type (cave versus surface). Our study suggests that differential gene expression plays a key role in the beginning evolutionary stages of eye regression in *P. mexicana*, shedding further light on regressive evolution in cavefish.

1. Background

Regressive evolution is common in cave-dwelling populations compared to their surface relatives [1]. In teleost fishes, cave-dwelling phenotypes are often similar across species and include reductions in eye size and function [2–4] and loss of skin pigmentation [5]. The genetic and evolutionary processes underlying regressive evolution, however, are still not fully understood. Prior studies suggest that regressive phenotypes can evolve through mutation [6], differential gene expression [7], or possibly both mechanisms. Moreover, both selection [8,9] and genetic drift [3] have been implicated in the evolution of regressive phenotypes.

The Atlantic molly (*Poecilia mexicana*, Poeciliidae) offers the opportunity to examine eye regression in a vertebrate system with reduced eyes in cave populations. In the Tacotalpa drainage in southern Mexico, this species has colonized three extreme environments on a small spatial scale, including a surface stream (El Azufre) with high levels of toxic hydrogen sulfide (H_2S), a H_2S-rich cave (Cueva del Azufre) and a non-toxic freshwater cave (Cueva Luna Azufre) (electronic supplementary material, figure S1). Previous comparisons of these extremophile populations with *P. mexicana* from an ancestral non-sulfidic surface population

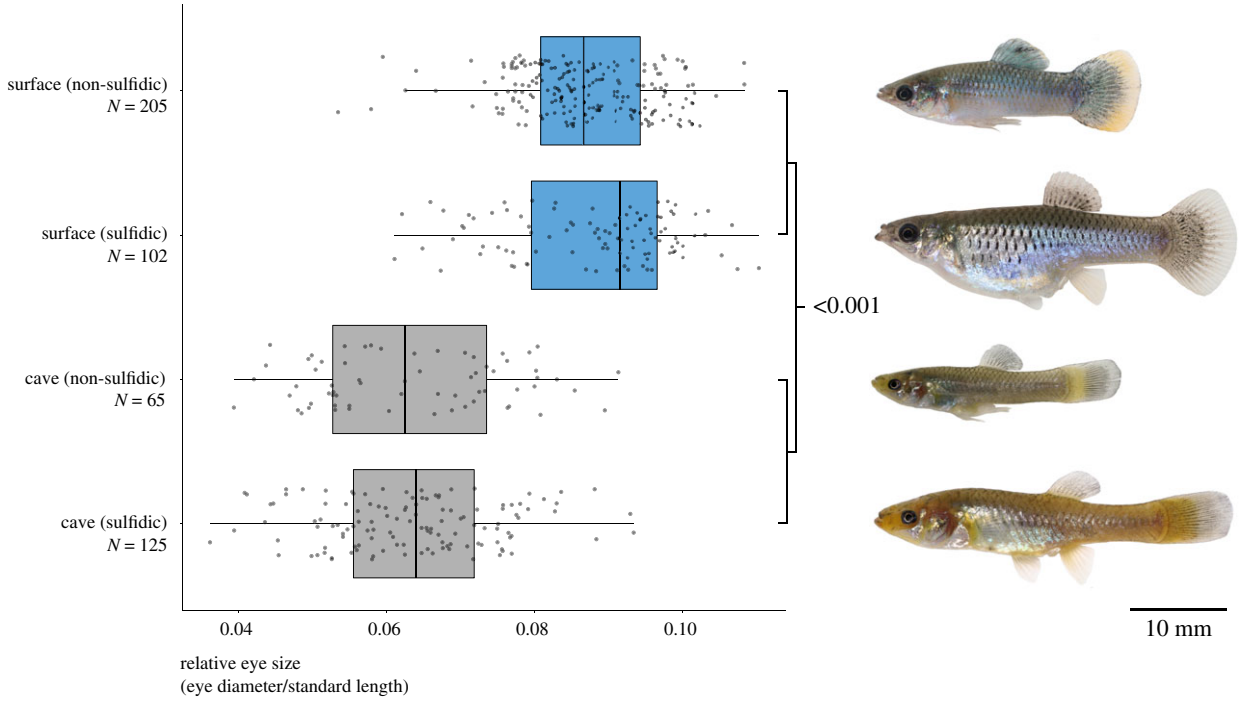

Figure 1. Relative eye size across populations of *Poecilia mexicana*. Box-plots: centre line, median; box limits, upper and lower quartiles; whiskers, 1.5× interquartile range; points, raw data. (Online version in colour.)

revealed local adaptation to both cave and sulfidic environments, strong genetic differentiation by habitat type, low gene flow and ongoing parapatric speciation [10]. Populations of *P. mexicana* in the Tacotalpa drainage colonized sulfidic habitats approximately 10 000 years ago [11]. The two cave populations (Cueva del Azufre and Cueva Luna Azufre) likely originated from an ancestral sulfidic population after the initial divergence [12]. Therefore, we estimate that the divergence between cave and surface populations in this system is recent (less than 10 000 years).

Cave populations in the Cueva del Azufre system are characterized by partial eye regression. Eyes of the cave mollies remain functional, as these fishes are still photophilic [13]. However, cavefish exhibit a heritable reduction in eye size compared to surface populations (figure 1) [14–17] and exhibit reduced opsin gene expression in retinal tissue [18]. Here, we compared transcriptome-wide gene expression in the eyes between two cave and two surface *P. mexicana* populations. We hypothesized that genes related to eye regression would be differentially expressed in cave populations compared with surface populations. We predicted that genes involved in visual sensitivity, eye structure, or eye signaling would be downregulated in cave mollies compared to surface fish. We also aimed to identify genes that were differentially expressed in sulfidic compared with non-sulfidic populations (i.e. the second major axis of environmental variation in this system), because eyes are in direct contact with the water. We hypothesized that genes involved in H_2S detoxification and metabolism would be upregulated in the sulfidic populations, as observed in other tissues [12].

2. Methods

(a) Relative eye size of cave mollies compared to surface fish

Male and female *P. mexicana* were collected from a non-sulfidic surface, sulfidic surface, non-sulfidic cave and sulfidic cave habitats (electronic supplementary material, figure S1; [10]). Eye diameter and standard length were measured with callipers to the closest 0.1 mm. Relative eye size was calculated by dividing eye diameter by standard length. Owing to unequal variance across the four populations, a Kruskal–Wallis test was used to determine if the average relative eye size was significantly different. Pairwise population comparisons were performed using a Wilcoxon rank-sum test with a Benjamini–Hochberg correction for multiple testing.

(b) RNA-sequencing

Two whole eye samples ($N = 4$ individuals per population; total $N = 16$; see electronic supplementary material, table S1 for further details) were taken from adult females from sites described above. Sample collection, RNA extraction, library preparation and sequencing are described in the electronic supplementary material. Trimmed reads were mapped to the *P. mexicana* reference genome (GenBank: LMXC00000000.1) with an appended mitochondrial genome (GenBank: KC992991) using BWA-mem [16]. A gene counts matrix was produced from the mapped reads using StringTie and the associated Python script (prepDE.py) [19]. In StringTie, mitochondrial genes were not included in the analyses.

(c) Identifying differentially expressed genes in the eyes of cave mollies compared to surface fish

Differentially expressed genes were identified using EdgeR and the *limma* package in R ([19,20]; see electronic supplementary material). The two surface populations were compared with the two cave populations, irrespective of the presence or absence of H_2S, in one model using a quasi-likelihood *F*-test with contrasts [21] to determine which genes were significantly differentially expressed between the two habitat types (false discovery rate, FDR < 0.05). The same approach was used to compare the two sulfidic populations with the two non-sulfidic populations, irrespective of cave or surface, to test whether H_2S also impacted eye transcriptomes. Annotations for differentially expressed genes were obtained from the annotated general feature format (GFF) file of the *P. mexicana* reference genome (GenBank: GCF_001443325.1).

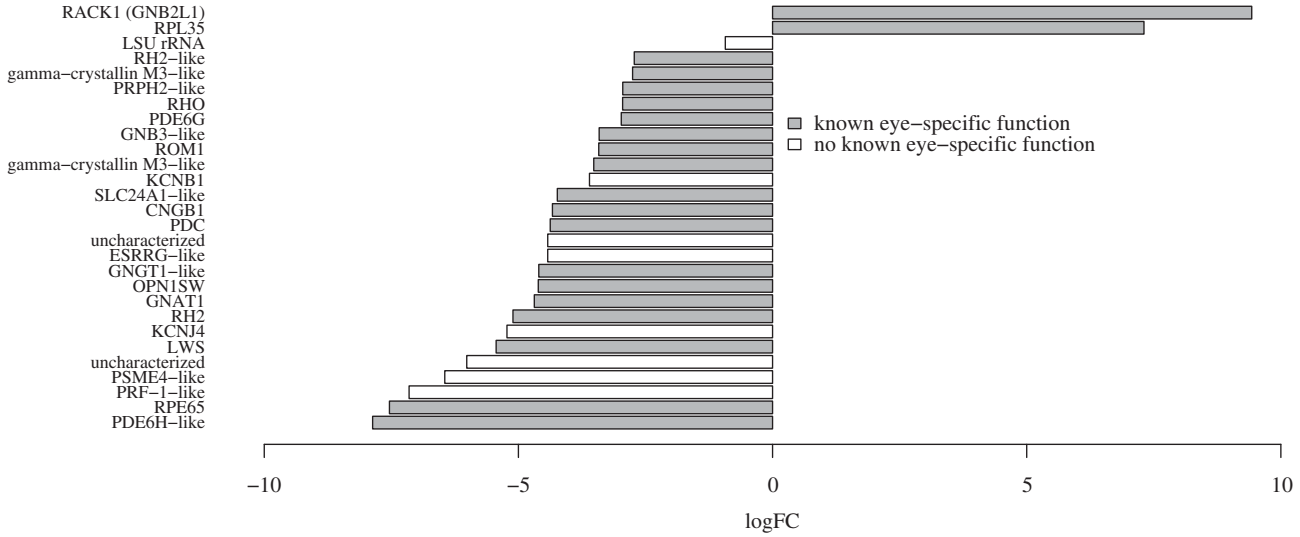

Figure 2. Log_2-fold change (logFC) for significantly differentially expressed genes between cave and surface populations. Positive values indicate upregulation, whereas negative values indicate downregulation in cave compared to surface populations.

(d) Functional annotation of gene expression based on the environment

A gene set enrichment analysis (GSEA) was conducted using GSEA v.3.0 [22]. Genes with human homologues identified by SwissProt ID were ranked by their log_2-fold change in expression comparing cave to surface populations. The ranked list was input into GSEAPreranked to determine whether specific biological process gene ontology (GO) terms were over- or under-represented (see electronic supplementary material for details).

(e) Identifying co-expressed gene modules correlated with cave adaptation

An unsigned weighted gene co-expression network analysis (WGCNA) was performed using the R package WGCNA [23] on normalized (log_2-counts per million) gene expression data to determine whether modules of co-expressed genes were significantly correlated with cave versus surface or sulfidic versus non-sulfidic environment. Only genes that were expressed in two or more samples were retained for analysis (see electronic supplementary material for details).

3. Results

Relative eye size was significantly different across the four populations of *P. mexicana* ($\chi^2 = 266.2$, d.f. = 3, $p < 0.001$; figure 1). The relative eye size of both cave populations was significantly smaller than that of both surface populations ($p < 0.001$ for each pairwise comparison).

We obtained more than 153 million paired-end raw reads from sequencing 16 eye samples (electronic supplementary material, table S2). Over 139 million reads remained after trimming (electronic supplementary material, table S2). An average of 2.4 ± 0.4 million trimmed reads (equivalent to 27.15 ± 3.51%) per sample mapped to the *P. mexicana* reference genome (electronic supplementary material, table S3). Variation in gene expression related to library size ($R^2 = 0.74$, $p < 0.0001$; first multidimensional scaling axis (MDS1) in electronic supplementary material, figure S2). Samples were partitioned by cave versus surface habitat along the second MDS axis.

After filtering out genes with zero counts across all samples, 23 419 of 31 805 genes remained in the gene counts matrix. Of these, 28 genes were significantly differentially expressed between cave and surface populations, irrespective of the presence or absence of H_2S (FDR < 0.05; table 1). All but two of these genes (encoding RACK1 and RPL35) were downregulated in cave compared to surface populations, indicated by a negative log_2-fold change (figure 2 and electronic supplementary material, figure S3). Twenty of the 28 differentially expressed genes had known eye-related functions (table 1), including five genes associated with light sensitivity, six with eye structure and nine with signaling. Gene expression differences were almost absent comparing sulfidic to non-sulfidic populations, irrespective of cave or surface, with LSU rRNA as the only downregulated gene (FDR < 0.05; electronic supplementary material, table S4).

We found a negative correlation between habitat type (cave versus surface) and 19 eye-related gene ontologies involving visual sensitivity, eye development and signaling using GSEA (electronic supplementary material, table S5). The top GO term associated with gene expression was the *detection of light stimulus* (GO:0009583). One module of 156 co-expressed genes was significantly correlated with cave versus surface habitat in the WGCNA ($r = -0.7$, $p = 0.003$; electronic supplementary material, figure S4). The module contained many eye- and circadian rhythm-related genes (electronic supplementary material, table S6). The circadian rhythm-related genes included those encoding circadian locomotor output cycles protein kaput (CLOCK), circadian-associated transcriptional repressor (CIART), period circadian protein homologue 2 (PER2), aryl hydrocarbon receptor (AHR), mitogen-activated protein kinase 10 (MK10), nuclear receptor ROR-beta (RORB) and several opsins including melanopsin (OPN4), among others. These findings suggest that changes in circadian genes were associated with the colonization of and adaptation to cave environments. No WGCNA modules were significantly correlated with the presence or absence of H_2S.

4. Discussion

Eye regression is common in cave-dwelling populations and has evolved convergently in multiple phyla [1]. Determining the mechanisms that drive phenotypic eye regression can help us understand regressive evolution in caves. Based on our finding

Table 1. Significantly differentially expressed genes in eye tissues from cave populations of *Poecilia mexicana* compared to surface populations. Functions are from UniProt/GeneCards. LogFC, $\log_2$-fold change; FDR, false discovery rate.

						eye-related function		
gene ID	protein/RNA	full protein name	function	logFC	FDR	sensitivity	structural	signaling
106925399	PDE6H-like	retinal cone rhodopsin-sensitive cGMP 3′,5′-cyclic phosphodiesterase subunit gamma-like	inhibitory subunit of cone-specific cGMP-phosphodiesterase in retina	−7.87	4.80×10^{-3}			■
106930879	RPE65	retinoid isomerohydrolase	composes the retinal pigment epithelium that supports the retina, converts all-trans-retinal to 11-cis retinal	−7.54	1.84×10^{-2}		■	
106928172	PRF-1-like	perforin-1-like	functions in the immune response by promoting cytolysis of target cells	−7.15	3.92×10^{-2}			
106915159	PSME4-like	proteasome activator complex subunit 4B-like	promotes the degradation of histones	−6.45	2.97×10^{-2}			
106911668	uncharacterized	unknown	unknown	−6.02	4.48×10^{-4}			
106904761	LWS	red-sensitive opsin	visual pigment	−5.44	4.48×10^{-4}	■		
106915481	KCNJ4	potassium voltage-gated channel subfamily J member 4	inward rectifier potassium channel, involved in nervous system signaling	−5.23	1.46×10^{-4}			
106918408	RH2	green-sensitive opsin	visual pigment	−5.11	4.71×10^{-4}	■		
106916330	GNAT1	guanine nucleotide-binding protein G(T) subunit alpha-1	triggers the coupling of rhodopsin and cGMP-phosphodiesterase	−4.69	2.41×10^{-9}			■
106931761	OPN1SW	blue-sensitive opsin	visual pigment	−4.61	1.85×10^{-3}	■		
106923979	GNGT1-like	guanine nucleotide-binding protein G(T) subunit gamma-T1-like	subunit of transducin, a G protein in rod outer segments	−4.60	1.50×10^{-2}			■
106910731	uncharacterized	unknown	unknown	−4.42	2.75×10^{-2}			
106910433	ESRRG-like	oestrogen-related receptor gamma-like	orphan receptor	−4.42	4.80×10^{-3}			
106913888	PDC	phosducin	phosphoprotein in the rod cells of the retina, may be involved in the regulation of visual phototransduction or photoreceptor metabolism	−4.37	2.63×10^{-2}			■
106908783	CNGB1	cyclic nucleotide-gated channel beta-1	regulates ion flow into the outer segment of the rod photoreceptor	−4.33	1.46×10^{-4}			■
106924058	SLC24A1-like	sodium/potassium/calcium exchanger 1-like	potassium-dependent sodium/calcium exchanger protein in rod and cone photoreceptors	−4.23	1.56×10^{-2}			■
106931054	KCNB1	potassium voltage-gated channel subfamily B member 1	voltage-gated potassium channel, primarily functions in brain	−3.60	1.82×10^{-2}			

(*Continued*.)

Table 1. (*Continued.*)

gene ID	protein/RNA	full protein name	function	logFC	FDR	eye-related function		
						sensitivity	structural	signaling
106933243	gamma-crystallin M3-like	gamma-crystallin M3-like	component of eye lens	−3.52	1.36×10^{-4}			
106930234	ROM1	retinal outer segment membrane protein 1	involved in optic disk development and stability, maintains rim curvature, paralog of PRPH2	−3.42	9.06×10^{-5}			
106919898	GNB3-like	guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-3-like	involved in transmembrane signaling, including retinal development and rhodopsin mediated signaling pathway	−3.41	8.93×10^{-3}			
106922414	PDE6G	retinal rod rhodopsin-sensitive cGMP 3′,5′-cyclic phosphodiesterase subunit gamma	effector molecule that binds to G proteins involved in phototransduction in rods and cones	−2.98	1.44×10^{-2}			
106905024	RHO	rhodopsin	pigment in rod cells, required for scotopic vision	−2.95	8.89×10^{-24}			
106922592	PRPH2-like	perpherin-2-like	involved in optic disk development and stability, maintains rim curvature, paralog of ROM1	−2.95	1.40×10^{-2}			
106933251	gamma-crystallin M3-like	gamma-crystallin M3-like	component of eye lens	−2.75	8.93×10^{-3}			
106918407	RH2-like	green-sensitive opsin-like	visual pigment	−2.72	4.47×10^{-2}			
106910732	LSU rRNA	large subunit ribosomal RNA	component of 60S subunit	−0.93	3.40×10^{-9}			
106914319	RPL35	ribosomal protein L35	component of 60S subunit	7.31	4.90×10^{-2}			
106934004	RACK1 (GNB2L1)	receptor for activated C kinase 1	scaffolding protein, involved in melanopsin signaling	9.43	1.60×10^{-5}			

that more genes were differentially expressed between cave and surface populations when compared with sulfidic and non-sulfidic populations of *P. mexicana*, we concluded that the presence or absence of light—not presence or absence of H_2S—was the primary environmental driver of gene expression variation in the eye. While genes involved in H_2S detoxification and metabolism are upregulated in other organs in sulfidic populations [12], eye tissues do not appear to be involved in the maintenance of H_2S homeostasis. Furthermore, the downregulation of opsin gene expression documented here supports previous findings based on qPCR, where medium- and long-wavelength-sensitive opsins were downregulated in individuals of *P. mexicana* from deep cave chambers of the Cueva del Azufre compared to those exposed to partial or full light [18].

Many eye-related genes from our analyses were also downregulated in other species of cavefish [7,24], suggesting possible parallel mechanisms underlying eye regression. Genes encoding RHO, GNAT1, GNB3-like, GNGT1-like and OPN1SW were downregulated in adult cave mollies as well as in the adult eyeless golden-line fish, *Sinocyclocheilus anophthalmus* [7]. There were also similarities in gene expression associated with visual sensitivity and signaling in the Mexican tetra (*Astyanax mexicanus*), which has undergone several independent cave colonizations [25] and is a model system in cavefish biology [26]. For example, the expression of genes coding for ROM1 and GNAT1 was completely suppressed in blind adult *A. mexicanus* cavefish (Pachón population [24]) and significantly reduced in the eye tissues of adult *P. mexicana*. Similarly, genes encoding PDE6H-like, PDE6G, PRPH2-like and gamma-crystallin M3-like were significantly downregulated in both adult *A. mexicanus* [24] and adult *P. mexicana* cavefish. Interestingly, a reduced eye phenotype also exists in several cave lineages of adult *A. mexicanus* [27] and is often present after introgression with the eyed surface morph [28–30]. However, the reduced eye in adult *P. mexicana* cave mollies is not the result of introgression with surface populations [10]. Consequently, these *P. mexicana* populations provide an opportunity to examine the beginning evolutionary stages of eye regression in cave mollies that is not the result of admixture [31] but owing to recent divergence from surface relatives [11,12].

Gene expression differences between cave and surface populations were not only related to eye function. The WGCNA also uncovered correlations with the expression of genes associated with circadian rhythm. The circadian clock typically relies on a light/dark cycle, so this correlation—while unsurprising—offers interesting insights into which circadian-related genes are affected by cave adaptation in *P. mexicana*. Studying circadian rhythm is difficult in fishes because, unlike mammals, they do not have a master clock regulating circadian function [32]. However, genes encoding CIART, CLOCK, AHR, MK10, PER2 and RORB, which were significantly correlated with habitat in this study, are all involved in cyclical feedback loops that control circadian rhythm ([33] and GeneCards). Melanopsin (OPN4) also correlated with cave versus surface habitat in *P. mexicana* and has been suggested as a key clock regulator in *Phreatichthys andruzzii*, a Somalian cavefish whose circadian rhythm depends on food availability [34].

Collectively, our gene expression analyses suggest that regressive evolution (reduced eyes) is correlated with a general downregulation of genes that have key functions in the eyes of *P. mexicana*. This finding leads to broader questions about the proximate and ultimate causes driving expression differences in those genes. The evidence for the downregulation of eye-related genes in *P. mexicana* and the parallels with other cavefish may suggest strong directional selection; however, it is also possible that the promoter regions of eye-related genes neutrally accumulate mutations because of a reduction in purifying selection, which leads to reduced expression in cave environments. Further work is needed to determine if these changes in gene expression are owing to selection, genetic drift, or a combination of these mechanisms.

Ethics. Procedures for all experiments were approved by the Institutional Animal Care and Use Committee at Kansas State University (Protocol no. 3418). Fieldwork was approved by the Mexican government (Fieldwork Permit DGOPA.00093.120110.-0018).

Data accessibility. NCBI Accession: PRJNA484191 for eye transcriptomes. Relative eye size data: electronic supplementary material, table S7. Code: github.com/jokelley/pmex_eye_expression.

Authors' contributions. C.N.P., M.T. and L.A.-R. conducted fieldwork. C.N.P. performed sequencing work. K.L.M. and J.L.K. conducted analyses. M.T. contributed to the statistical design. K.L.M. and J.L.K. wrote the manuscript; all authors contributed to revisions. All authors approved the final version. All authors agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Competing interests. We declare we have no competing interests.

Funding. This work was funded by grants from the National Science Foundation (grant nos IOS-1121832, IOS-1463720, IOS-1557860 and IOS-1557795) and the U.S. Army Research Office (W911NF-15-1-0175) to M.T. and J.L.K.

Acknowledgements. This research used resources from the Center for Institutional Research Computing at WSU. Thank you to Anthony P. Brown, Muh-Ching Yee, Alexandra Sockell, Manfred Schartl, Wesley C. Warren and Carlos Bustamante for their input.

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