

Exploring visual plasticity: dietary carotenoids can change color vision in guppies (*Poecilia reticulata*)

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Abstract Differences in color vision can play a key role in an organism's ability to perceive and interact with the environment across a broad range of taxa. Recently, species have been shown to vary in color vision across populations as a result of differences in regulatory sequence and/or plasticity of opsin gene expression. For decades, biologists have been intrigued by among-population variation in color-based mate preferences of female Trinidadian guppies. We proposed that some of this variation results from variation in color vision caused by plasticity in opsin expression. Specifically, we asked about the role of dietary carotenoid availability, because carotenoids (1) are the precursors for vitamin A, which is essential for the creation of photopigments and (2) have been linked to variation in female mate choice. We raised guppies on different carotenoid-level diets and measured opsin expression. Guppies raised on high-carotenoid diets expressed higher levels of long wavelength sensitive opsin (LWS) opsins than those raised on lower levels of carotenoids. These results suggest that dietary effects on opsin expression represent

a previously unaccounted for mechanism by which ecological differences across populations could lead to mate choice differences.

Keywords Opsins · LWS · Mate choice · Population divergence · Visual ecology

Abbreviations

SWS1	Short wavelength sensitive 1 opsin
SWS2	Short wavelength sensitive 2 opsin
RH1	Rhodopsin
RH2	Rhodopsin-like opsin
LWS	Long wavelength sensitive opsin

Introduction

Navigating one's environment requires information from a broad range of stimuli including obstacles, predators, food, and conspecifics. Differences in the sensory systems responsible for detecting and discriminating among these stimuli, such as color vision, can have far-reaching effects on an organism's behavior. Traditionally, color vision was thought to only vary across species, but recent work in several taxa has found that color vision can also vary across populations [e.g., bluefin killifish (Fuller et al. 2004), cichlids (Carleton et al. 2005), stickleback (Flamarique et al. 2013), guppies and close relatives (Sandkam et al. 2015a, b)] which can be due to both genetic variation and plasticity (Fuller et al. 2005; O'Quin et al. 2012; Ehlman et al. 2015; reviewed in Carleton et al. 2016). Several studies have recently shown that visual systems are plastic with respect to developmental stage, time of day, and lighting environment (Fuller and Claricoates 2011; Johnson et al. 2013; Flamarique et al. 2013; Dalton et al. 2015; Ehlman et al. 2015; Sakai et al. 2016).

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Surprisingly, however, ecological links between color vision and diet are not well studied, despite the fact that ingestion of vitamin A is necessary for functional photoreceptors. In one of the few studies testing for the effects of diet, Knott et al. (2010) found that diet affects color vision in birds through changes in the filtering properties of oil droplets.

Guppies (*Poecilia reticulata*) provide an outstanding model to investigate the implications of variation in color vision and the factors underlying plasticity in color vision, as color vision varies across populations both on the island of Trinidad (Sandkam et al. 2015a) and on mainland South America (Sandkam et al. 2015b). Variation in color vision has important implications for mate choice behavior. Mate choice and color vision have recently been shown to co-vary across populations of guppies, making vision a likely factor involved in mate choice divergence across populations (Endler and Houde 1995, Sandkam et al. 2015a). Recently, Ehlman et al. (2015) used a split-brood design in the laboratory to show that guppy color vision also has a component of developmental plasticity. Identifying the forces that shape differences in vision would allow for specific predictions regarding the strength and direction of signal evolution and direct comparisons to other taxa.

Color vision is accomplished by comparing signals from the cone cells in the retina that are stimulated by different wavelengths of light (Sharpe and Gegenfurtner 1999). The wavelengths by which a cone cell is stimulated is largely determined by which opsin protein is bound to either an A1 or A2 chromophore (which chromophore is used can also change wavelength sensitivity) (Hofmann and Carleton 2009). Guppies possess nine opsin genes which are classified according to the wavelength of light they maximally detect; short wavelength sensitive 1 opsin (SWS1—detect ultraviolet), short wavelength sensitive 2 opsin (SWS2A and SWS2B—detect blues and purples), rhodopsin-like 2 opsin (RH2-1 and RH2-2—detect greens), and long wavelength sensitive opsins (LWS-1, LWS-2, LWS-3 and LWS-R—detect reds/oranges) (Ward et al. 2008; Watson et al. 2010, 2011; Sandkam et al. 2012; Tezuka et al. 2014). Proportional measures of total retina opsin gene expression have been shown to provide a good proxy for cone cell abundance across a range of taxa including humans (Hagstrom et al. 2000), killifish (Fuller et al. 2005), black bream (Shand et al. 2008), and cichlids (Carleton et al. 2008). Thereby, differences in opsin gene expression reveal differences in color vision. Once expressed, opsin proteins are bound to retinal, a derivative of carotenoids, which is obtained from the diet (Hofmann and Carleton 2009). For guppies, the main source of carotenoids in nature is attached unicellular algae (Grether et al. 1999, 2001). Variation among sites in canopy cover leads to variation in photosynthetically active light; this, in turn, is correlated with variation in algal carotenoid availability and carotenoid deposition in

the orange spots of males (Grether et al. 1999). As a result, guppy populations differ dramatically in the abundance of carotenoids in their diet (Grether 2000). The strength of female mate preference for orange (carotenoid-based) color spots on male guppies is negatively affected by carotenoid intake in the laboratory (Grether et al. 2005). This suggests that female preferences for orange coloration are expressed more strongly at sites with lower carotenoid availability in the wild. Sandkam et al. (2015a) recently found that the strength of female preferences for orange color spots on males also co-varies with differences in opsin expression. This raises the possibility that changes in carotenoid content of the diet are driving changes in the visual system and impacting female preferences across populations. In other systems, carotenoids can influence various properties of the retina, such as opsin levels, visual/spectral sensitivity, and photoreceptor cell death (Bennett and White 1989, 1991; Maier and Bowmaker 1993; Sommerburg et al. 1999; Rapp et al. 2000; Landrum and Bone 2001; Thomson et al. 2002; Billsten et al. 2003; Goyret et al. 2009).

We predicted that if dietary carotenoids are capable of driving changes in color vision, then guppies raised on diets with different levels of carotenoids should have different opsin expression profiles. To test this prediction, we raised male and female guppies on diets containing three different carotenoid levels (trace, low, high) and compared opsin expression profiles. If carotenoid levels in the diet do affect vision, then this could be a factor contributing to across-population variation in mate choice preferences.

Materials and methods

Individuals used in this study were F1 individuals from seven crosses between laboratory populations originally established from low-predation populations in the Marianne and Quare drainages (four pairs with a Marianne female and Quare male; three pairs with a Quare female and Marianne male). For each cross, one male and one virgin female were housed in a mating tank and fed a trace-carotenoid diet. Tanks were checked daily for the presence of offspring, which were immediately distributed among 2-L plastic tanks with a maximum of 6 fish per tank and were assigned to one of three diets varying in carotenoid level: trace, low, or high-carotenoid following the methods described in Kolluru et al. (2006). These treatments provide an adequate level of vitamin A to ensure proper growth ($\geq 17,600$ IU/kg of food = 10.56 ppm beta-carotene equivalents (M. Lamon, pers. comm.)) yet differ dramatically in the amount of carotenoids. The trace-carotenoid diet contained ≤ 0.2 $\mu\text{g/g}$ carotenoids, the low-carotenoid diet contained about 5.05 $\mu\text{g/g}$ total carotenoids (1.99 ppm beta-carotene), and the high-carotenoid diet contained

about 1339.30 $\mu\text{g/g}$ total carotenoids (522.30 ppm beta-carotene) (Kolluru et al. 2006). While it is likely that the fish on the high-carotenoid diet had higher levels of vitamin A (because carotenoids can be synthesized into vitamin A), even the fish on trace-carotenoid diets had vitamin A levels 2–3 times higher than that required for optimal guppy growth (Shim and Tan 1990). Individuals were raised on these carotenoid diets until males were fully pigmented (approximately 6–8 months) at which time all males and females were euthanized with an overdose of MS-222. Both eyes from each individual were quickly removed and frozen in RNAlater[®] buffer (Ambion, Inc., Austin, TX) until RNA extractions were performed.

RNA extraction, cDNA synthesis, and real-time PCR

Eyes were homogenized in 500 μL Trizol and added to a phase lock tube with an additional 500 μL Trizol and 10 μL of 1 mg/mL glycogen. The RNA was extracted with chloroform, precipitated with isopropanol, washed with ethanol, resuspended in 20 μL RNase-free water, and stored at -80°C until quantified. The cDNA was synthesized by reverse transcription using 0.5 ng of total RNA in the presence of superscript II and oligo (dT) primers.

qPCR assays

The qPCR used in this study was conducted using SYBR Green assays to measure expression of five opsins across all four of the cone opsin classes (SWS1, SWS2A, SWS2B, RH2-1, and LWS) and one housekeeping gene (elongation factor 1-alpha) of individuals from the F1 cross described above. Each fish had all five 10- μL reactions run in triplicate on the same plate which consisted of: 2 μL cDNA, 1.5 μL each of the forward and reverse primers (3 μM), and 5 μL of SYBR Green qPCR master mix (Applied Biosystems). These assays used primers designed for bluefin killifish (*Lucania goodei*) (Fuller et al. 2005). The primer sequence for the reporter gene (elongation factor 1- α) was taken from the work on *Fundulus heteroclitus* (Scott and Schulte 2005; Scott et al. 2005, 2006). These fishes all occur in the order Cyprinodontiformes (Nelson 2006). These experiments were initiated in 2006, and, at that time, we did not have sequence data for guppy RH2-2 or the four, separate LWS loci.

All qPCR reactions were run on an Applied Biosystems 7900HT. The PCR efficiencies for each assay were determined by making a 1000-fold serial dilution of four randomly chosen individuals. qPCR reactions for each assay were run in triplicate on each concentration, and then, absolute efficiency was calculated using:

$$\text{slope}(C_i) = -\ln(1 + E)$$

where $\text{slope}(C_i)$ is the slope of the critical threshold for an assay plotted across the dilution series, and E is the absolute efficiency for that assay (primer sequences and efficiencies are presented in Supplementary Table S1).

While using assays designed originally for other species is not ideal, the results presented here can still provide valuable insight to the question being addressed in this study: are dietary carotenoids capable of driving differences in color vision? Furthermore, primer efficiencies were found to be high when calculated using guppies in this study (Supplementary Table S1). Our assays should be informative concerning differences across treatments and, therefore, for testing the hypothesis that dietary carotenoids are capable of driving differences in color vision. However, it should be noted that these assays do not provide us with an absolute description of an individual's color vision as this requires accurate knowledge of expression levels for all opsins.

Opsin expression was measured in two ways: relative_(hk) and proportional. Relative_(hk) expression measures the amount of opsin relative to a housekeeping gene, in this case, elongation factor 1-alpha. Measures of relative_(hk) opsin expression are independent of other opsins and reflect alterations in the expression of each gene (Fuller and Claricoates 2011). Relative_(hk) expression measures were made following Fuller and Claricoates (2011) using the equation:

$$\frac{T_i}{T_{ef}} = \frac{(1/(1 + E_i)^{C_i})}{(1/(1 + E_{ef})^{C_{ef}})}$$

where T_i/T_{ef} is the expression of each individual opsin i relative to the expression of elongation factor 1-alpha, E_i is the PCR efficiency for each set of probes, E_{ef} is the PCR efficiency of elongation factor 1-alpha, C_i is the average critical cycle number for each gene run, and C_{ef} is the average critical cycle number for elongation factor 1-alpha.

Proportional opsin expression gives insight to differences in color vision, because it acts as a proxy for relative cone cell abundance in the retina (Hagstrom et al. 2000; Fuller et al. 2003, 2004; Cheng and Flamarique 2004; Fuller and Claricoates 2011). With proportional expression, the expression of one opsin can theoretically affect the measures of expression of the other opsins. Hence, proportional opsin expression may not necessarily reflect differences in the transcription of the individual opsins but does reflect differences in color vision. Proportional expression measures followed Fuller et al. (2004) and were made using the equation:

$$\frac{T_i}{T_{all}} = \frac{1/(1 + E_i)^{C_i}}{\sum (1/(1 + E_i)^{C_i})}$$

where T_i/T_{all} is the expression of each individual opsin i relative to total expression, E_i is the PCR efficiency for each set of probes, and Ct_i is the average critical cycle number for each gene run.

Analyses

To determine whether or not diet and sex impacted opsin expression, we ran a MANOVA on relative_(hk) measures. Given significant effects for overall opsin expression, we also ran individual ANOVAs for each opsin (for both proportional and relative_(hk) measures) to identify the effects of diet and sex on each opsin separately. All analyses were performed in R v3.0.2 (R Development Core Team 2014). Data and R code file of analyses from this experiment are available in the Dryad Digital Repository at <http://dx.doi.org/10.5061/dryad.n01rh>.

Results

A MANOVA using relative_(hk) measures revealed that diet and sex both had significant effects on overall opsin expression profiles (Diet: $F_{2,68} = 2.736$, $P = 0.004$; Sex: $F_{1,68} = 2.659$, $P = 0.030$); however, there was no significant diet*sex interaction ($F_{2,68} = 0.986$, $P = 0.459$). The significant effect of diet indicates that overall opsin expression profiles vary among the dietary (carotenoid) treatments.

Across all treatments, the most highly expressed opsins were LWS and RH2-1 (Fig. 1). To understand the direction and extent of the effects of diet and sex on individual opsins, we ran ANOVAs on both relative_(hk) and proportional measures. Diet had significant effects on relative_(hk) measures for LWS and on proportional measures for LWS and RH2-1 (Table 1). Sex had significant effects for relative_(hk) measures, such that males had higher expression than females for both SWS2A and RH2-1 (Table 1). Sex also had significant effects for proportional measures of SWS2A (males higher), RH2-1 (males higher), and LWS (females higher) (Table 1).

Discussion

We proposed that differences in the amount of dietary carotenoids ingested across guppy populations could provide a mechanistic explanation for differences in color vision. We found LWS opsin expression relative to housekeeping genes to be lowest in diets containing trace amounts of carotenoids. Our results show that dietary carotenoid intake can influence opsin expression, and thereby, ecological differences in diet can alter color vision. Below we discuss these results and the potential implications for our

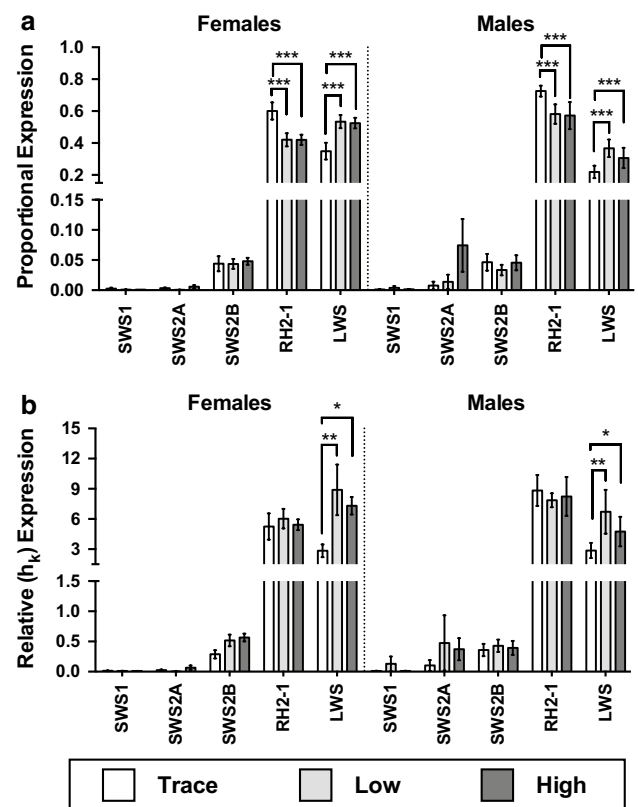


Fig. 1 Proportional (a) and relative_(hk) (b) measures of opsin expression by sex. Note difference in scale and range of upper and lower section of Y axis within each graph. Sample sizes of each treatment/sex: trace (female $N = 9$, male $N = 11$), low (female $N = 13$, male $N = 9$), and high (female $N = 22$, male $N = 10$). Stars denote significant main effects across dietary treatments (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$)

understanding of the well-studied behavioral differences in mate choice across populations.

Dietary carotenoids impact color vision

Opsin expression profiles of homogenized retinas have been shown to provide robust approximations for cone cell abundance in humans (Hagstrom et al. 2000) and fish [including bluefin killifish (Fuller et al. 2004); black bream (Shand et al. 2008); and cichlids (Carleton et al. 2008)]. Thereby, differences in opsin expression profiles can reveal differences in color vision across individuals. Here, we tested the hypothesis that manipulating carotenoid diet will change opsin expression. We found support for this hypothesis in the analyses of both overall opsin expression profiles (MANOVA) and expression of the individual opsins (ANOVAs). This directly supports the conclusion that carotenoid intake influences opsin expression and, thereby, changes color vision.

The ability of dietary carotenoids to alter opsin expression is perhaps not surprising as carotenoids can be used to

Table 1 Results of ANOVAs run on relative_(hk) and proportional measures for each opsin

		Diet		Sex		Diet * Sex	
		<i>F</i> _{2,68}	<i>P</i>	<i>F</i> _{1,68}	<i>P</i>	<i>F</i> _{2,68}	<i>P</i>
Relative _(hk)	SWS1	1.16	0.319	1.51	0.223	1.62	0.205
	SWS2A	0.33	0.723	5.02	0.028	0.69	0.504
	SWS2B	2.22	0.116	0.98	0.325	0.85	0.434
	RH2-1	0.35	0.708	8.43	0.005	0.26	0.772
	LWS	5.09	0.009	1.70	0.196	0.37	0.696
Proportional	SWS1	0.64	0.530	0.66	0.418	1.62	0.206
	SWS2A	1.46	0.240	6.61	0.012	2.64	0.079
	SWS2B	0.36	0.701	0.17	0.680	0.18	0.838
	RH2-1	9.54	<0.001	12.70	<0.001	0.06	0.939
	LWS	9.78	<0.001	21.45	<0.001	0.47	0.630

Bold denotes *P* < 0.05

synthesize retinoic acid (Napoli and Race 1988). Zebrafish treated with retinoic acid during development has been shown to switch which LWS opsin is expressed in the cone cells (Mitchell et al. 2015). Fujieda et al. (2009) found expression of the LWS opsins in mice to be controlled by a retinoic acid receptor upstream of the LWS loci resulting in a positive correlation between the amount of retinoic acid and LWS opsin expression. This receptor is conserved across a broad range of taxa and, importantly, is present and functional in the guppy (Tam et al. 2011). Thereby, synthesis of retinoic acid from carotenoids provides a potential mechanism to explain our findings that dietary carotenoids can change color vision in guppies.

Mate choice and dietary carotenoids

The fact that we found that LWS expression differed across dietary carotenoid treatments in both relative_(hk) and proportional measures is noteworthy as LWS opsins play a large role in detecting red/orange coloration and, for female guppies in most populations, orange coloration plays a primary role in female mate choice decisions (Endler and Houde 1995; reviewed in Houde 1997). Color discrimination is accomplished by comparing the relative excitation of cone cells with different wavelength sensitivities. By increasing the relative signal received by cones expressing LWS opsins compared to those expressing RH2 opsins (the next shortest class), the red/orange colors may be perceived as brighter. Indeed, Sakai et al. (2016) recently showed that guppies with higher LWS expression have a greater sensitivity to orange light. Furthermore, expression of LWS opsins has been shown to co-vary with mate preferences for orange male coloration across natural populations in Trinidad (Sandkam et al. 2015a). Female guppy preference for orange male coloration has also been shown to vary with dietary carotenoids, such that females raised on lower

carotenoid diets express stronger preferences for orange (Grether et al. 2005). As dietary carotenoids clearly have the ability to affect both opsin expression (this study) and female color preferences (Grether et al. 2005), differences across populations in the availability of dietary carotenoids could provide a mechanism to explain population differences in mate choice.

Interestingly, the direction of change in expression presented here is opposite of what might be predicted. High-predation populations generally have a more open canopy and, thus, higher light levels that result in more resources and higher availability of carotenoids than corresponding low-predation populations within the same watershed (Reznick et al. 2001; Grether et al. 1999). Therefore, based on the results presented here, one might predict to see higher levels of LWS expression in high-predation populations. However, Sandkam et al. (2015a) found high-predation populations to have lower levels of LWS opsin expression than corresponding low-predation populations. While our results clearly show that differences in the abundance of dietary carotenoids can influence LWS opsin expression, there are likely additional factors involved in tuning opsin expression that could help explain this discrepancy, such as light environment.

Ehlman et al. (2015) recently found that lower abundance of available light resulted in higher expression of the LWS opsins. We found that low levels of dietary carotenoids decrease LWS opsin expression. In nature, low-predation populations have lower light abundance (Reznick et al. 2001) but also lower levels of dietary carotenoids (Grether et al. 1999). Further study is needed to determine the interplay between carotenoids, light, and potential other factors influencing LWS opsin expression. Such examination of the variation in opsin expression among guppy populations and factors contributing to that variation will be an exciting avenue of research for discovering what has led to

the remarkable diversity in mate choice preferences among populations of the Trinidadian guppy.

Historically, color vision has been assumed to vary only across species, however, a growing body of work is emerging that shows dramatic differences across populations within species [e.g., Bluefin killifish (Fuller et al. 2004), cichlids (Smith et al. 2011; reviewed in Carleton et al. 2016), stickleback (Flamarique et al. 2013), sand goby (Larmuseau et al. 2009), pied flycatchers (Lehtonen et al. 2011), guppies and close relatives (Sandkam et al. 2015a, b)]. Several factors of the lighting environment have been identified that impact opsin expression, and here, we add diet as a further factor influencing opsin expression. Future work is needed that directly addresses the interaction between such factors before we can conclusively explain the direction and strength of sensory system evolution, a longstanding goal of visual ecology.

Sex differences in opsin expression

The sex differences we found in opsin expression are interesting, because sexual dimorphism in opsin expression has been reported in laboratory populations of guppies (Laver and Taylor 2011; Ehlman et al. 2015) but was not found for wild populations on Trinidad (Sandkam et al. 2015a) or mainland South America (Sandkam et al. 2015b). This difference could result from differences in carotenoid uptake if wild populations are somewhat carotenoid-limited and one sex does a better job of compensating for low carotenoids. Additional studies using qPCR assays designed specifically for guppies are needed to clarify the ubiquity of sexual dimorphism in guppy opsin expression.

Conclusions

Our results indicate that differences in carotenoid intake can change opsin expression, which in turn could change color perception. As such, dietary effects on color vision present a previously unaccounted for mechanism by which ecological differences across populations could lead to mate choice differences and drive population divergence.

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Compliance with ethical standards

Ethical approval All procedures performed involving animals were approved and in accordance with the ethical standards of the University of California, Los Angeles Animal Research Committee (protocol #1999-197-23).

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