Rapid and Parallel Adaptive Evolution of the Visual System of Neotropical Midas Cichlid Fishes

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Abstract

Midas cichlid fish are a Central American species flock containing 13 described species that has been dated to only a few thousand years old, a historical timescale infrequently associated with speciation. Their radiation involved the colonization of several clear water crater lakes from two turbid great lakes. Therefore, Midas cichlids have been subjected to widely varying photic conditions during their radiation. Being a primary signal relay for information from the environment to the organism, the visual system is under continuing selective pressure and a prime organ system for accumulating adaptive changes during speciation, particularly in the case of dramatic shifts in photic conditions. Here, we characterize the full visual system of Midas cichlids at organismal and genetic levels, to determine what types of adaptive changes evolved within the short time span of their radiation. We show that Midas cichlids have a diverse visual system with unexpectedly high intra- and interspecific variation in color vision sensitivity and lens transmittance. Midas cichlid populations in the clear crater lakes have convergently evolved visual sensitivities shifted toward shorter wavelengths compared with the ancestral populations from the turbid great lakes. This divergence in sensitivity is driven by changes in chromophore usage, differential opsin expression, opsin coexpression, and to a lesser degree by opsin coding sequence variation. The visual system of Midas cichlids has the evolutionary capacity to rapidly integrate multiple adaptations to changing light environments. Our data may indicate that, in early stages of divergence, changes in opsin regulation could precede changes in opsin coding sequence evolution.

Key words: **Amphilophus, cichlid, crater lake, opsin, vision, visual sensitivity.**

Article

Introduction

Understanding the mechanisms underlying adaptive phenotypic divergence is one of the main challenges of molecular evolutionary biology. The visual system of animals provides an excellent model for approaching this issue for a number of reasons: it is highly diverse across organisms; the molecular mechanisms underlying its diversity are relatively well known; and there is a clear link between changes at the molecular level and their phenotypic consequences (Loew and Lythgoe 1978; Chang et al. 1995; Yokoyama and Yokoyama 1996; Yokoyama 2000; Ebrey and Koutalos 2001; Chinen et al. 2003; Hofmann and Carleton 2009; Carleton 2014; Enright et al. 2015; Dalton et al. 2017). Moreover, strong selection for tuning the visual system to the light environment is expected given the crucial sensory role of vision for different activities including foraging, predator avoidance, and mate choice. Particularly interesting are animals inhabiting aquatic environments, especially freshwater habitats, given that these are among the most spectrally diverse light environments due to the wavelength-specific absorption properties of water combined with dissolved organic matter, suspended particles, and plankton scattering light at various wavelengths (Cronin et al. 2014). Indeed, fishes have the most variation in spectral sensitivities among all vertebrates, showing a strong correlation between visual sensitivities and light environment (Loew and Lythgoe 1978; Levine and MacNichol 1979; Lythgoe 1984; Cummings and Partridge 2001; Marshall et al. 2003; Bowmaker 2008; Cronin et al. 2014; Marshall et al. 2015).

Cichlid fishes are an interesting model system to the study of visual ecology and evolution (Carleton 2009; Carleton et al. 2016), since they are one of the most species rich and colorful lineages of vertebrates (Kocher 2004; Brawand et al. 2014; Henning and Meyer 2014). These fish have undergone impressive phenotypic divergence, including visual sensitivity (Kocher 2004; Salzburger 2009; Henning and Meyer 2014; Carleton et al. 2016). The visual system of African cichlids is highly diverse, spanning most of the variation known from all fishes, and

there is compelling evidence that selection has shaped this diversity (Sugawara et al. 2002, 2005; Terai et al. 2002; Carleton 2014, Carleton et al. 2005, 2016; Hofmann et al. 2009).

Vision is mediated by visual pigments, which are composed of an opsin protein and a light absorbing retinal chromophore. These components are covalently bound, and variation in either of them results in shifts of spectral sensitivity (Wald 1968; Yokoyama 2000). Eight opsin genes, one rod-opsin that functions under dim-light conditions and seven cone opsin genes involved in color vision, have been described from cichlids, which collectively have sensitivities that span from the ultra-violet to the red part of the light spectrum (Carleton 2009; Escobar-Camacho et al. 2017). Of these eight, five are hypothesized to have been present in the common ancestor of vertebrates: the rod-opsin that functions under dim-light conditions (*RH1*) and four cone opsin genes that are involved in color vision (*SWS1*, *SWS2*, *RH2*, *LWS*; Yokoyama and Yokoyama 1996; Terakita 2005). Two additional cone opsin gene duplications (*SWS2a*–*SWS2b* and *RH2A*–*RH2B*) increased the opsin repertoire in acanthopterygians (Carleton and Kocher 2001; Parry et al. 2005). A subsequent duplication of *RH2A* (*RH2A*α–*RH2A*β) occurred in cichlids (Parry et al. 2005).

Extensive research in the visual system of African cichlids has shown that multiple mechanisms affect vision of these fish, including opsin gene expression and coexpression, opsin coding sequence differences, chromophore usage, and ocular media transmittance (Carleton et al. 2016). Cichlid retinas are highly structured, with single cones expressing one of the short-wavelength sensitive opsins (*SWS1*, *SWS2b*, or *SWS2a*) and double cones expressing one opsin in each of the two cell members, either two mid-wavelength sensitive (*RH2B*, *RH2A*α, or *RH2A*β) or one mid-wavelength and the long-wavelength opsin (LWS; Carleton and Kocher 2001; Spady et al. 2006; Carleton et al. 2008; Hofmann et al. 2009; O'Quin et al. 2010). Thus, African cichlids commonly express a combination of three cone opsins (Carleton et al. 2016; but see Parry et al. 2005; Dalton et al. 2014, 2017), resulting in large differences in spectral sensitivity among species expressing different subsets (Carleton and Kocher 2001; Spady et al. 2006; Carleton et al. 2008; Carleton 2009; Hofmann et al. 2009). This tuning mechanism underlies much of the variation observed among cichlid species from Lake Malawi (Hofmann et al. 2009). In contrast, fine-tuning of visual sensitivity is mostly achieved by amino acid substitution in the opsin protein, mainly in sites directed into the chromophore-binding pocket (Carleton et al. 2005). This has been shown to be an important tuning mechanism for the dim-light sensitive *RH1* (Sugawara et al. 2005) and for *SWS1* and *LWS* that have sensitivities at opposite extremes of the visible spectrum (Terai et al. 2002, 2006; Seehausen et al. 2008; Hofmann et al. 2009; O'Quin et al. 2010; Miyagi et al. 2012).

Visual sensitivity can also be tuned by changing chromophore type, and this mechanism is known to underlie some of the phenotypic variation between African cichlids that inhabit turbid vs. clear waters (Sugawara et al. 2005; Terai et al. 2006; Carleton et al. 2008; Miyagi et al. 2012). Two types of chromophores can be found in fish, 11-*cis* retinal derived from vitamin A1 and 3,4-didehydroretinal derived from vitamin A2. Switching from A1- to A2-derived chromophores results in sensitivities shifting toward longer wavelengths (Wald 1961; Hárosi 1994; Cronin et al. 2014). Another way to alter sensitivity is to filter light passing the cornea and lens before reaching the retina; and African cichlids are known to vary strongly in the clearness of the lenses (Hofmann et al. 2010; O'Quin et al. 2010).

The study of the visual system of African cichlids has furthered our understanding of the mechanisms involved in adaptive divergence (reviewed in Carleton et al. 2016). Yet, there remain numerous unanswered questions regarding how this diversity has evolved that might be difficult to address without exploring younger cichlid radiations (Carleton et al. 2016). One such question concerns the likelihood of different mechanisms driving early stages of differentiation. Is early divergence characterized by structural changes of opsin genes or by modifications in the pattern of opsin expression? Does one tuning mechanism or the interaction of multiple mechanisms underlie early spectral sensitivity divergence? The Midas cichlid fishes from Nicaragua (*Amphilophus* cf. *citrinellus*) provide an excellent system to address these questions, as they have recently colonized new visual environments from known source populations and are ecologically divergent in parallel along the benthic–limnetic axis within crater lakes (Elmer et al. 2014; Kautt, Machado-Schiaffino, and Meyer 2016).

Nicaragua has a rich diversity of freshwater environments including the largest lakes in Central America and a series of young (<24,000 years) and completely isolated crater lakes that are part of the Central American Volcanic Arc (Kutterolf et al. 2007). Midas cichlid populations of the great lakes Managua and Nicaragua have recently (<2,000 generations ago) and independently colonized multiple crater lakes (Barluenga et al. 2006; Barluenga and Meyer 2010; Elmer et al. 2010, 2014; Kautt et al. 2012, Kautt, Machado-Schiaffino, and Meyer 2016; Kautt, Machado-Schiaffino, Torres-Dowdall, et al. 2016). The newly colonized crater lakes differ in many aspects from the great lakes, including a drastic difference in the light environment. The great lakes are very turbid due to a high level of suspended particles whereas crater lakes tend to have clearer waters (Cole 1976; Elmer et al. 2010). This is particularly true for two of the oldest and deepest crater lakes, Apoyo and Xiloá. These crater lakes harbor small Midas cichlid radiations along a benthic–limnetic axis of divergence (4–6 endemic species each; Kautt, Machado-Schiaffino, and Meyer 2016). Benthic and limnetic Midas cichlids might experience different light conditions. Limnetic Midas cichlids forage in open water, a relatively homogenous light environment with a broad spectral bandwidth (Sabbah et al. 2011). Benthic Midas cichlids forage in the littoral zone where the light environment is likely shifted toward longer wavelength and with a narrower spectral bandwidth (Sabbah et al. 2011). Thus, Midas cichlids are an excellent system to study the evolution of sensitivities after the very recent colonization of, and speciation in a new light environment.

So far, relatively little is known about the visual system of Neotropical cichlids. Early microspectrophotometry (MSP)

studies suggested that Neotropical cichlids have long wavelength shifted spectral sensitivities (Muntz 1973; Loew and Lythgoe 1978; Levine and MacNichol 1979; Kröger et al. 1999; Weadick et al. 2012). Opsin gene expression in Neotropical cichlids supports these findings as these fish express a long wavelength sensitive palette of opsins (i.e., *SWS2a*, *RH2A*, and *LWS*, Escobar-Camacho et al. 2017). Interestingly, the most short-wavelength shifted opsins in single cones (i.e., *SWS1*) and double cones (i.e., *RH2B*) were suggested to be lost or to have become pseudogenized (Weadick et al. 2012; Fisher et al. 2015, Escobar-Camacho et al. 2017). Measures of lens transmittance in Neotropical cichlids show that the UV and violet parts of the visible spectrum are often filtered out before reaching the retina (Muntz 1973). Finally, usage of the A2-derived chromophore producing long-wavelength shifted sensitivities appears to be common in Neotropical cichlids (Loew and Lythgoe 1978; Levine and MacNichol 1979; Weadick et al. 2012). In combination, those results suggested that Neotropical cichlids might have a reduced diversity in their visual system and the potential for adaptation to new light environments with short-wavelength shifted spectra might be limited (Weadick et al. 2012).

Based on our knowledge of the evolutionary history of the Midas cichlid species complex, we aimed to understand the phenotypic and molecular consequences of colonization of new light environments. First, we compared light irradiances between great and crater lakes to better predict the expected phenotypic divergence in spectral sensitivities. Second, we used MSP to compare visual pigment sensitivity and lens spectral transmittance measurements between different Midas cichlid species inhabiting great and crater lakes and between benthic and limnetic ecomorphs within crater lakes. Finally, we explored the molecular mechanisms underlying divergence in the visual sensitivity of Midas cichlids by studying the evolution of opsin amino acid sequences, opsin gene expression, and chromophore usage.

Results

Variation in the Visual Environment in Nicaraguan Lakes

To determine the different photic environments experienced by Midas cichlids we took underwater light measurements in a turbid great lake (Lake Managua) and two clear crater lakes (Lakes Apoyo and Xiloá). These lakes differed in many aspects of their underwater light environment. Spectral irradiance measurements in the turbid great lake showed that light attenuation was dramatically higher than in the crater lakes, as expected due to their differences in turbidity (fig. 1). Therefore, the photic environment was restricted to shallower waters in the turbid great lake, but it expanded into deeper waters in the clear crater lakes. Moreover, light spectra differ among lakes. While long-wavelengths were attenuated with depth similarly in crater lakes and the great lake, short-wavelength light was better transmitted in crater lakes, resulting in a blue-shifted light spectrum compared with that of the great lake (fig. 1). A useful measure to compare the light environments of different lakes is λP_{50}, the wavelength at which the total number of photons is divided in two equal parts (McFarland and Munz 1975). Higher λP_{50} values suggest a light spectrum shifted toward longer wavelengths, whereas lower λP_{50} values indicate short-wavelength shifted light environments. In the turbid great lake, λP_{50} was 529 nm, but in the crater lakes it was shifted toward shorter wavelengths (Apoyo $\lambda P_{50} = 504–511$; Xiloá $\lambda P_{50} = 505–523$). Thus, the underwater photic environment of the crater lakes is richer both in bandwidth and intensity compared with the great lake, providing a source of strong divergent selection on the visual system of aquatic animals.

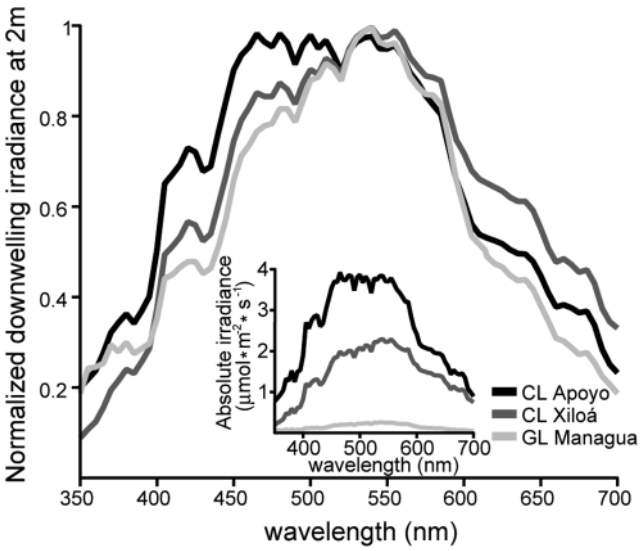

Fig. 1. Difference in the photic environment of a great lake and two crater lakes. Normalized downwelling irradiance is narrower at two meters deep in the turbid great lake in comparison to the clear crater lakes. Hence, a higher proportion of light in the blue and red part of the spectrum penetrates in the crater lakes compared with the great lake. The insert shows the absolute downwelling irradiance at 2 m in each lake, showing the differences among lakes in light extinction with depth.

Phenotypic Diversity in the Visual System of Midas Cichlids

Spectral Sensitivities of Visual Pigments

To determine if the colonization of clear water crater lakes (i.e., a new photic environment) resulted in adaptive phenotypic divergence in the visual system of Midas cichlids, we conducted MSP analyses on retinas of specimens from a turbid great lake (Lake Nicaragua) and two clear crater lakes (Lakes Apoyo and Xiloá). In addition, to explore the divergence between benthic and limnetic species within crater lakes, both ecomorphs were studied from the same two crater lakes (there are no limnetic species in the great lakes; supplementary table S1, Supplementary Material online). The peaks of maximum absorbance (λ_{max}) as well as estimates of A1/A2 chromophore ratios of rod and cone photoreceptors were determined. Analysis repeated with fish reared under common light conditions provided qualitatively similar results (supplementary fig. S1, Supplementary Material

online). Thus, we infer that the patterns described below have a genetic basis.

Rod Photoreceptors. Retina rod photoreceptor cells are particularly tuned to dim light conditions, which in aquatic environments are characteristic of deep and murky waters (Bowmaker 1995, 2008). In Midas cichlids, peaks of maximum sensitivity (λ_{max}) from 101 rod cells (number of specimens $N_{Nicaragua} = 2$, number of cells: $n_{Nicaragua} = 9$; $N_{Apoyo} = 8$, $n_{Apoyo} = 35$; $N_{Xiloá} = 11$, $n_{Xiloá} = 57$) ranged from 495 to 525 nm (fig. 2). All these were assigned to one spectral class based on the estimated pure-A1 visual pigment ($\lambda_{A1} = 497 \pm 1$ nm, mean ± SD; fig. 2), suggesting various A1/A2 chromophore ratios. No clear pattern of variation of rod photoreceptor sensitivity with lake of origin was found (supplementary fig. S2, Supplementary Material online). However, λ_{max} values in the limnetic species of both clear crater lakes were less variable (Bartlett's $\kappa^2 = 21.065$, df = 4, $P < 0.001$) and with mean λ_{max} shifted toward shorter wavelengths than sympatric benthic species (Kruskal–Wallis $\chi^2 = 22.370$, df = 1, $P < 0.001$; fig. 2).

Single-Cone Photoreceptors. Single-cone cells are one of the two types of photoreceptors involved in color vision, and their sensitivity peaks at wavelengths between 350 and 460 nm (UV to blue part of the spectrum; Bowmaker 2008). MSP analysis on 42 single cones of Midas cichlids ($N_{Nicaragua} = 2$, $n_{Nicaragua} = 12$; $N_{Apoyo} = 6$, $n_{Apoyo} = 18$; $N_{Xiloá} = 4$, $n_{Xiloá} = 12$) identified two spectral classes based on the predicted λ_{A1}, one most sensitive in the violet (431 ± 4 nm) and one in the blue (450 ± 4 nm; fig. 2) part of the light spectrum. All single cones from turbid great lake specimens were assigned to the blue spectral class. In contrast, specimens within clear crater lakes Apoyo and Xiloá had single cones assigned to the blue as well as the violet spectral classes (fig. 2). The range of λ_{max} values for the blue spectral class varied among lakes ($F = 6.190$, df = 2,6, $P = 0.035$; fig. 2), as in specimens from crater lake Apoyo the sensitivity of cones assigned to this class appeared to be shifted toward shorter wavelengths (λ_{max}: 443–457 nm) compared with those seen in crater lake Xiloá (λ_{max}: 448–467 nm) and the great lake (λ_{max}: 449–465 nm). No differences for the blue or in the violet spectral class (Apoyo λ_{max}: 431–442 nm; Xiloá λ_{max}: 425–439 nm; fig. 2) were observed between morphs within crater lakes.

Double-Cone Photoreceptors. Double cones are the second type of photoreceptor involved in color vision, consisting of two cones fused together (Cronin et al. 2014). These have peaks of sensitivities in the mid and long parts of the visible light spectrum (blue–green to red; Bowmaker 1995, 2008). We obtained 610 MSP readings of double cones from Midas cichlids' retinas identifying four spectral classes based on the predicted λ_{A1}: a red ($\lambda_{A1} = 559 \pm 2$ nm; $N_{Nicaragua} = 3$, $n_{Nicaragua} = 20$; $N_{Apoyo} = 5$, $n_{Apoyo} = 41$; $N_{Xiloá} = 9$, $n_{Xiloá} = 37$), a long-green ($\lambda_{A1} = 528 \pm 2$ nm; $N_{Nicaragua} = 3$, $n_{Nicaragua} = 49$; $N_{Apoyo} = 10$, $n_{Apoyo} = 142$; $N_{Xiloá} = 11$, $n_{Xiloá} = 136$), a short-green ($\lambda_{A1} = 509 \pm 1$ nm; $N_{Nicaragua} = 3$, $n_{Nicaragua} = 13$; $N_{Apoyo} = 9$, $n_{Apoyo} = 76$; $N_{Xiloá} = 11$, $n_{Xiloá} = 63$), and a blue–green spectral class ($\lambda_{A1} = 476 \pm 4$ nm; $N_{Nicaragua} = 2$, $n_{Nicaragua} = 4$; $N_{Apoyo} = 5$, $n_{Apoyo} = 12$; $N_{Xiloá} = 5$, $n_{Xiloá} = 17$). In Midas cichlids' red spectral class, both the λ_{max} (Kruskal–Wallis $\chi^2 = 22.295$, df = 4, $P < 0.001$) and its associated variance (Bartlett's $\kappa^2 = 86.324$, df = 4, $P < 0.001$; fig. 2) differed among species. Variance was higher in specimens from the turbid great lake as these had extremely long-wavelength shifted cones (λ_{max}: 558–623 nm) that were not observed in the clear crater lakes (fig. 2). No significant differences were observed between ecomorphs in each crater lake.

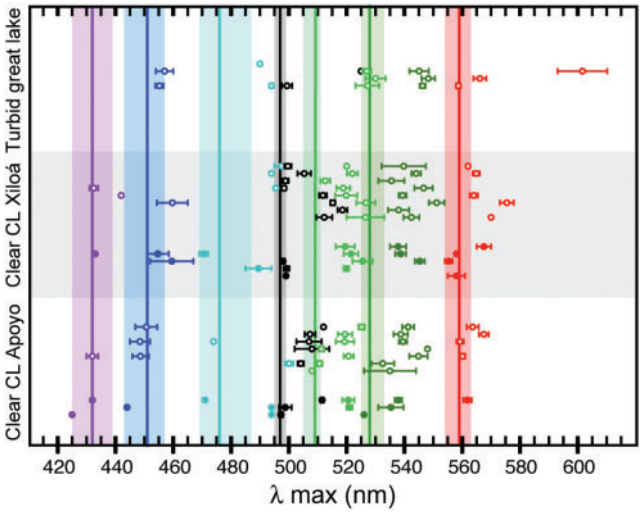

FIG. 2. Individual level peaks of maximum absorbance ($\lambda_{max} \pm$ SE) of visual pigments determined by MSP from wild-caught Midas cichlids from a turbid great lake and two clear crater lakes. Unfilled symbols correspond to specimens of the benthic ecomorph within each lake, whereas filled symbols correspond to limnetic specimens. Visual pigments were assigned to different spectral classes (indicated by the vertical lines of different colors) based on their estimated pure A1 peak of maximum absorbance (λ_{A1}). The ranges of estimated λ_{A1} are shown as shaded areas of the same color. From left to right, these spectral classes correspond to the violet, blue, blue–green, rod, green (short), green (long), and red previously identified in African cichlids (Carleton 2009). The gray shading separates samples from the different lakes.

Two spectral classes with sensitivities in the green part of the light spectrum (510–560 nm) were identified based on predicted λ_{A1} values, a short-green and a long-green (fig. 2). Interestingly, both of these were detected for most specimens examined. Within each of these two spectral classes, specimens from the crater lakes had sensitivities shifted toward shorter wavelengths than specimens from the turbid great lake (short-green: $F = 5.800$, df = 2,20, $P = 0.010$; long-green: $F = 6.500$, df = 2,21, $P = 0.006$; fig. 2). No differences were detected when comparing the limnetic and benthic species within the crater lakes.

A few double cones had visual pigments with sensitivities in the blue–green spectral class (fig. 2). These had an extremely wide range of variation in λ_{max} (469–505 nm), particularly in the crater lakes (Bartlett's $\kappa^2 = 6.283$, df = 2, $P = 0.043$; fig. 2). Very few of these cones were observed in turbid great lake specimens, and these had long wavelength-shifted sensitivities. Cones of this class were more commonly

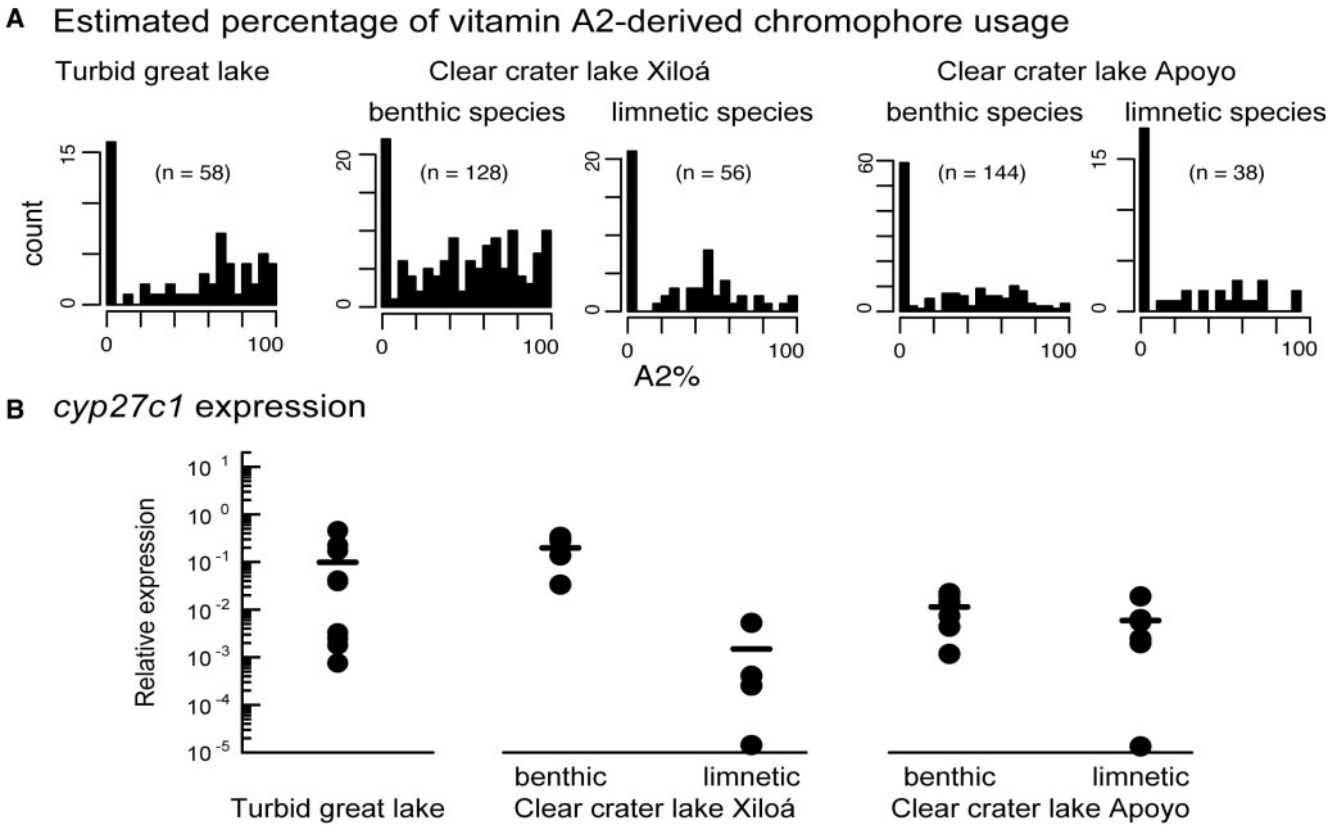

Fig. 3. Distribution of estimated proportion of vitamin A2-derived chromophore usage in Midas cichlids from the turbid great lake Nicaragua and two clear water crater lakes (*a*). Estimates of A2-derived usage for the long-green spectral class were not included due to the confounded effect of opsin coexpression. *cyp27c1* expression relative to the geometric mean of two housekeeping genes (*ldh2* and *inp2*) in the same crater lakes and the turbid great lake Managua (*b*). Horizontal lines are the mean for each group. Bonferroni corrected pair-wised comparisons are reported in supplementary table S2, Supplementary Material online.

seen in fish from the crater lakes, and these had both, long and extremely short wavelength-shifted λ_{max} values (fig. 2).

Collectively across lakes and species, the cones of adult Midas cichlids had six different spectral classes that coincide with the expected λ_{max} ranges of *SWS2b* (violet), *SWS2a* (blue), *RH2B* (blue–green), *RH2A* (short- and long-green), and *LWS* (red). We found no evidence of single-cones with maximum absorbance in the UV part of the spectrum (i.e., <400 nm, *SWS1*). Remarkably, in addition to the short-wavelength sensitive visual pigment of single cones at least three different spectral classes were detected for double cones of single Midas cichlid specimens. The presence of four different spectral channels confers these fish the potential for tetrachromatic color vision, yet functional validation would be required to determine the roles of these channels in chromatic or achromatic vision. Spectral sensitivities of the different photoreceptors were either short wavelength shifted (blue, short–green, long-green, and red spectral classes) or had reduced variability (rod and blue–green) in the clear crater lakes compared with the turbid great lake. Little divergence was observed among ecomorphs within the crater lakes.

A1/A2-Derived Chromophore Usage in Midas Cichlids

Absorbance spectra from MSP measures of photoreceptor outer segments showed significant variation in A1/A2 chromophore ratios across Midas cichlid species (Kruskal–Wallis $\chi^2 = 32.167$, $df = 4$, $P < 0.001$; fig. 3*a*). Consistent with short wavelength shifted sensitivity and less variation in sensitivities, Bonferroni corrected pairwise comparisons suggested that Midas cichlids from the clear crater lakes use relatively less vitamin A2-derived chromophores than fish from the turbid great lake. However, the benthic species from crater lake Xiloá showed vitamin A2-derived chromophore usage not significantly different from those seen in specimens from the great lake (fig. 3*a*; supplementary table S2, Supplementary Material online).

Lens Transmittance

Ocular media, and in particular lenses, can selectively limit the wavelength of light reaching the retina, thus affecting visual sensitivity (Losey et al. 2003). A large amount of variation was found in lens transmittance (as T50, the wavelength of 50% transmission) for first generation laboratory born individuals of five Midas cichlid species reared under common light conditions. However, lens transmittance cut-offs were not continuously distributed but formed discrete groups (Hartigans' dip test for unimodality $D = 0.099$, $P = 0.003$; fig. 4). One group was composed exclusively by Midas cichlids from the turbid great lake Nicaragua, having lenses blocking UV light and part of the violet light of the spectrum (T50 = 421.6,

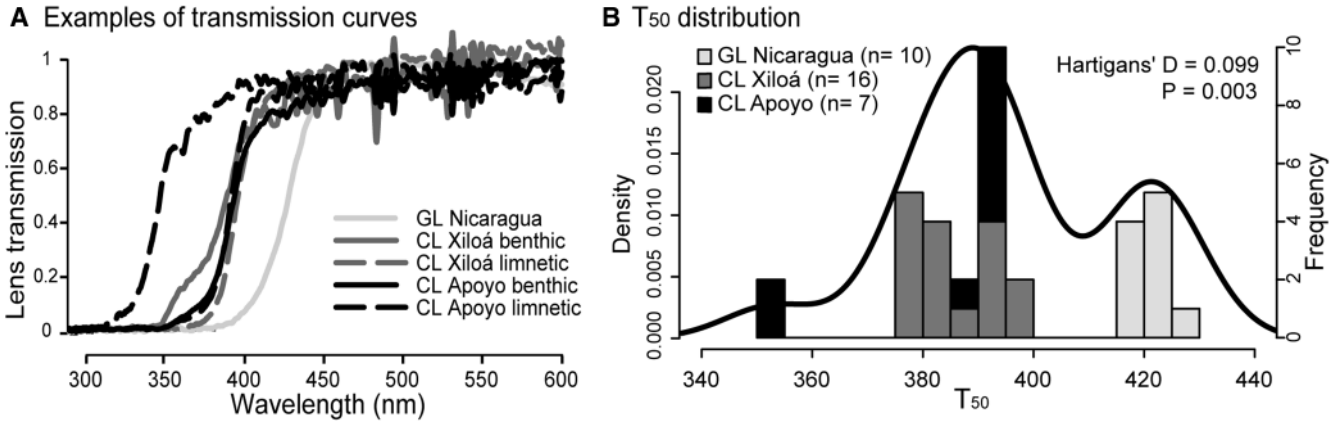

FIG. 4. Lens transmittance grouped into different categories. Example of these are shown in (*a*). Histogram depicting the frequency of lens transmittance cut-offs (T_{50}) of lab-reared Midas cichlids (*b*). Over-imposed is a density kernel showing the bimodal distribution of T_{50}.

SD = 2.9, $n = 10$). A second group had UV-blocking but violet-light-transmitting lenses and was composed of most of the fish from the clear crater lakes, including both the limnetic (Apoyo T50 = 392.9, SD = 1.8, $n = 4$ and Xiloá T50 = 393.9, SD = 2.8, $n = 7$) and benthic species from both lakes (Apoyo T50 = 389.9, SD = 0.4, $n = 3$ and Xiloá T50 = 380.4, SD = 3.0, $n = 9$). Interestingly, we found a third group including only two specimens of the limnetic species from crater lake Apoyo that had UV-transmitting lenses (T50 = 352.3 and 353.6). Thus, Midas cichlids from the clear crater lakes have shifted the lens transmittance toward shorter wavelengths compared with the ancestral species from the turbid great lake.

Mechanisms of Divergence in the Visual System of Midas Cichlids

Coding Sequence Variation of Midas Cichlid Opsin Genes

To determine the contribution of structural changes in opsin proteins to the phenotypic variation observed in photoreceptors' sensitivities (see Spectral Sensitivities of Visual Pigments section), we sequenced rhodopsin and the seven cone opsins from 64 specimens of the Midas cichlid species complex. Specimens from two species of Midas cichlids (*A. citrinellus* and *A. labiatus*) from the two turbid great lakes (Nicaragua and Managua), and individuals from one benthic and one limnetic species from the clear crater lakes Apoyo and Xiloá (*A. astorquii* and *A. zaliosus* in the former, and *A. xiloaensis* and *A. sagittae* in the latter) were included. Opsin genes and their inferred amino acid sequences were found to be highly similar across the analyzed species. Collectively across rhodopsin and all seven cone opsins, we identified a total of 16 variable nucleotide sites of which 8 resulted in amino acid substitutions (table 1). In none of these cases were different alleles fixed in different species, but rather the alleles were segregating in one or more of the Midas cichlid species.

The eight nonsynonymous substitutions found were not homogeneously distributed across opsin genes. Only one substitution was found in *RH2Aβ* and *LWS*, two in *SWS1*, and four in *RH2Aα* (none was found in *RH1*, *SWS2a*, *SWS2b*, and *RH2B*; table 1). Seven of these occurred in transmembrane regions, but only one occurred in a site directed into the retinal-binding pocket: A164S in *LWS*. We determined the frequency of alanine and serine at this position by genotyping a larger number of *A. citrinellus* (great lake Nicaragua, $n = 63$), *A. zaliosus* (crater lake Apoyo, $n = 24$) and *A. xiloaensis* (crater lake Xiloá, $n = 24$) individuals using a PCR–RFLP approach since the polymorphism generates cutting sites for different restriction enzymes (Ala = SatI, Ser = Fnu4HI). This confirmed our previous result, finding that *LWS* segregates for these two alleles only in the turbid great lake Nicaragua, but not in the species from the crater lakes.

Overall, given that no fixed differences across species were found, coding sequence variation appears to have a minor impact on the divergence of Midas cichlids' visual system. The only possible exception is *LWS*, where the A164S substitution could explain some variation seen in the great lake but not in the crater lakes. For other variable sites, mutagenesis experiments will be needed to determine their contribution to divergence in visual sensitivity.

Cone Opsin Expression in Midas Cichlids

Using quantitative real-time PCR (qRT-PCR), we quantified opsin expression in retinas of 25 wild-caught individuals of Midas cichlids, including specimens from the turbid great lake Managua and of a limnetic and a benthic species from clear crater lakes Xiloá and Apoyo. The proportion of the total cone opsin gene expression (T_{all}) comprised by each of the seven cone opsins (T_i; Carleton and Kocher 2001; Fuller et al. 2004) is reported (fig. 5).

Significant differences were found in the expression of wild-caught fish from different lakes ($A_{MRPP} = 0.43$, $P = 0.001$). In the species from the turbid great lake *LWS* constituted more than 60% of the total cone opsin expressed whereas *RH2Aβ* represented almost 24% of total opsin expression. *SWS2a* was the only single cone opsin expressed (~15% of total expression; fig. 5*a*). This pattern of opsin expression reflects the results of the MSP analyses showing that Midas cichlids in the turbid great lake have visual sensitivities shifted toward longer wavelengths.

Table 1. Nonsynonymous Nucleotide Substitution Observed in the Midas Cichlid Species Complex.

	Gene			SWS1		RH2Aα				RH2Aβ	LWS
	Nucleotide Position			138	322	190	205	343	604	649	529
	Consensus[b]			c	g	g	g	g	c	t	g
Midas Cichlid Species	Lake	*N*	Habitat								
Great Lakes											
A. citrinellus	Managua	8	Benthic	s	r	·	·	s	·	·	·
A. labiatus		8	Benthic	s	·	·	·	s	m	·	·
A. citrinellus	Nicaragua	8	Benthic	·	·	·		s	·	·	k
A. labiatus		8	Benthic	s	·	·	·	s	·	·	k
Crater Lakes											
A. astorquii	Apoyo	8	Benthic	·	·	r	·	s	m	·	·
A. zaliosus		8	Limnetic	·	·	·	·	·	·	s	·
A. xiloensis	Xiloá	8	Benthic	·	·	·	r	s	m	·	·
A. sagittae		8	Limnetic	·	·	r	·	s	m	·	·
Amino acid substitution[a]				P53R	A115T	G56S	V61I	A107P	L194M	V209L	A164S
Location[a]				TM1	TM2	TM1	TM1	TM3	E-2	TM5	TM4

NOTE.—Amino acid replacement and location for each nonsynonymous substitution are indicated at the bottom of the table.
[a]Amino acid positions, the transmembrane helices (TM 1–5) and the extracellular interhelical loop (E-2) are defined and numbered based on the bovine crystal structure of rhodopsin (Palczewski et al. 2000).
[b]In all cases we observed different alleles segregating in the corresponding population. A IUPAC/IUB single-letter amino acid code (Leonard 2003) is used to denote the nucleotides segregating at each position in the corresponding species (r: either a or g; s: either c or g; m: either a or c; k: either g or t). A dot (·) implies no departure from the consensus.

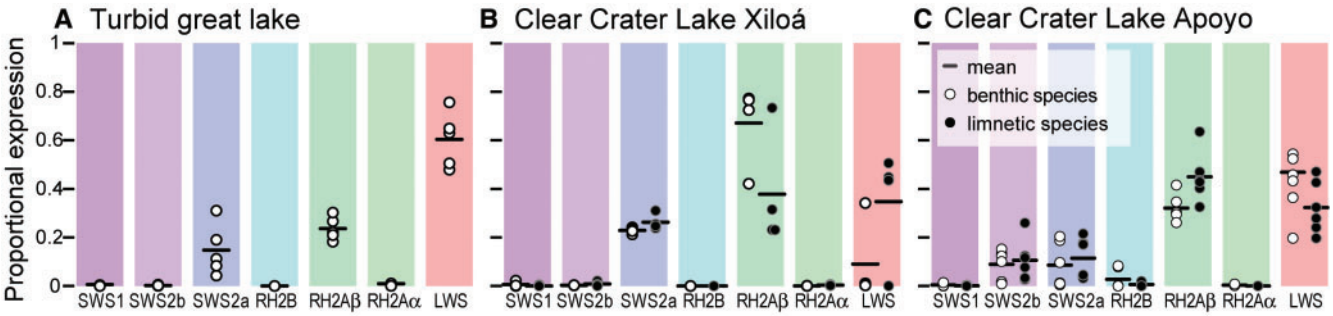

Fig. 5. Proportion of the total opsin expression comprised by each of the different opsin genes in wild-caught Midas cichlids from a turbid great lake (*a*), and two clear crater lakes (*b*, *c*). Means are shown as horizontal bars. Black circles represent expression in specimens of the limnetic ecomorph, white circles denote expression in specimens of the benthic species.

Opsin expression of clear crater lake Midas cichlids differed from that in the great lake in two aspects. First, in the crater lakes fish expressed proportionally less *LWS* and more *RH2Aβ* than those in the great lake (fig. 5*b* and *c*). Second, in crater lake Apoyo some individuals expressed the blue–green sensitive *RH2B* and the violet sensitive *SWS2b* gene (fig. 5*c*). Individuals expressing *SWS2b* expressed only traces of *SWS2a* and *vice versa*, suggesting a trade-off between single cone opsins (supplementary fig. S3, Supplementary Material online). No variation was evident between species within each crater lake.

In summary, opsin expression differences suggest shift in sensitivity toward shorter wavelengths in fish from the clear crater lakes compared with a turbid great lake. This is achieved by changes in the relative proportion of *LWS* and *RH2Aβ* expressed, and by the novel expression of *SWS2b* and *RH2B*. These patterns of opsin expression were maintained in fish reared under common light conditions (supplementary fig. S4, Supplementary Material online), suggesting a genetic basis for the divergence between species.

Opsin Coexpression in Midas Cichlids

To better understand the phenotypic consequences of differential opsin gene expression, we performed triple fluorescent *in situ* hybridization (FISH) in laboratory reared Midas cichlids from a great lake (*A. citrinellus*, Lake Nicaragua) and a clear crater lake representative species (*A. astorquii*, Lake Apoyo), with a focus in double cones (8,265 double-cone members counted). The retina of Midas cichlids from the turbid great lake was dominated by double cones expressing *LWS* (>75% of double cones consistently across the retina), including multiple twin cones (fig. 6*a–e*). Most of the rest of double cone members expressed *RH2Aβ* (17–24%; fig 6*e*). Two specimens coexpressed *LWS*

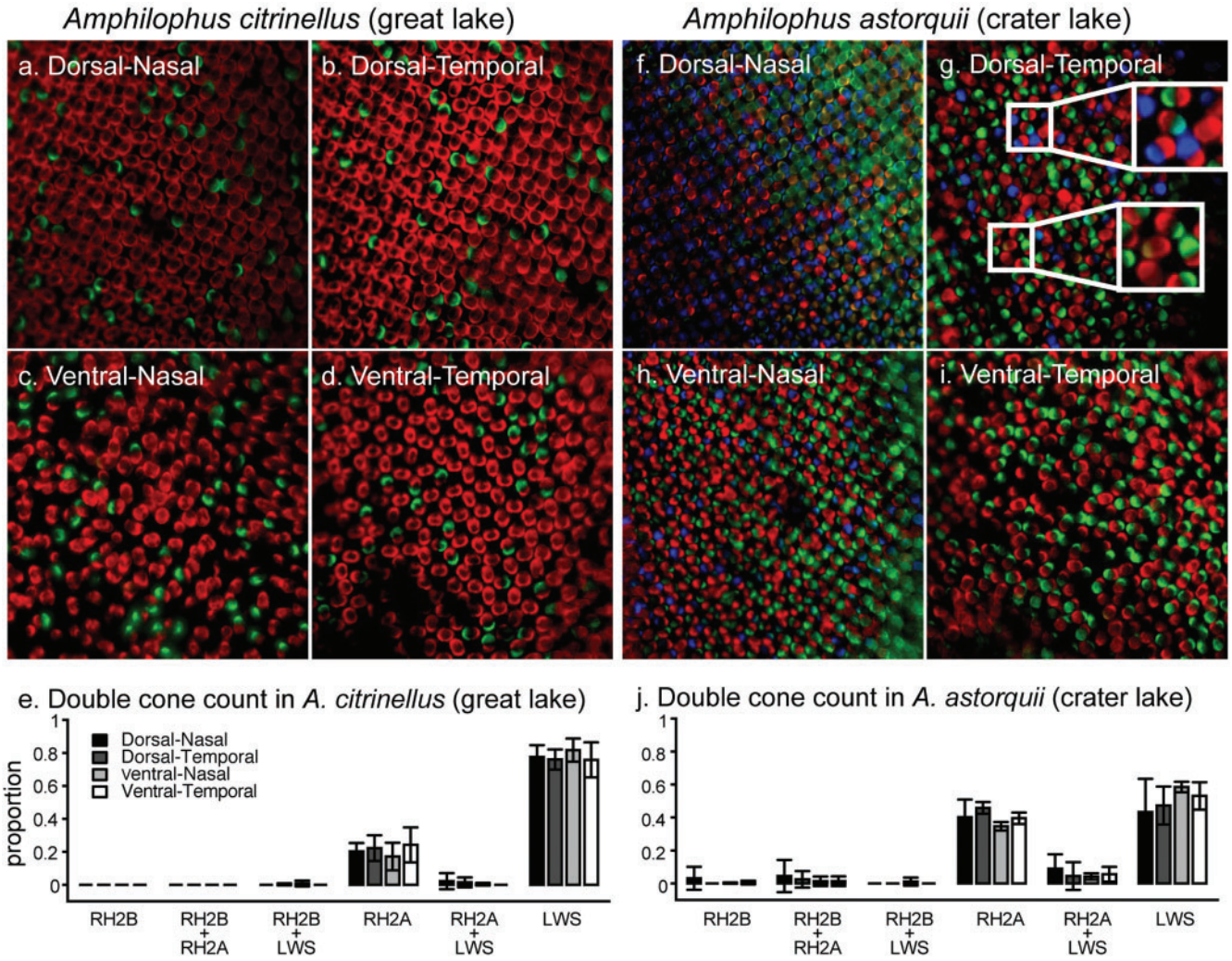

Fig. 6. Triple FISH staining of the retinas of two Midas cichlids species, one from a turbid great lake (*a*–*e*) and one from a clear crater lake (*f*–*j*) across four quadrants of the retina. Coexpression is common in specimens from both lakes, but the frequency is higher in specimens from the clear crater lake (*f*–*j*). Details in (*g*) show examples of coexpression of *LWS* and *RH2Aβ* (lower box) and *RH2B* and *RH2Aβ* (upper box).

and *RH2Aβ* in the dorsal part of the retina and one of these also coexpressed *RH2B* and *LWS* (fig 6*e*).

Retinas of *A. astorquii* differed in many aspects from those in *A. citrinellus*, and overall were more variable (fig. 6*j*). Contrary to *A. citrinellus*, in *A. astorquii* most double cones had one member expressing *LWS* and the second member expressing *RH2Aβ* (fig. 6*f*–*j*). Also, across all individuals and retinal regions there was an average of 6% of cones coexpressing *LWS* and *RH2Aβ* (in some cases representing up to 20% of the cones). Three specimens (all of them females) expressed *RH2B*, either by itself or in combination with *RH2Aβ* or *LWS* (fig. 6*f* and *j*), which could explain some of the variation seem in the blue-green spectral class (fig. 2).

Sources of A1/A2-Derived Chromophore Variation in Midas Cichlids

The enzyme Cyp27c1 mediates the conversion of vitamin A1 into vitamin A2 in the retinal pigment epithelium and the level of vitamin A2 is strongly correlated with the expression of *cyp27c1* (Enright et al. 2015). *cyp27c1* expression in retinas of Midas cichlids is in agreement with the A2 proportions estimated in the MSP experiment. Those species showing higher levels of A2-derived chromophore usage (fig. 3*a*) also had higher levels of *cyp27c1* expression (fig. 3*b*; supplementary fig. S5*a*, Supplementary Material online). In addition to significant differences in *cyp27c1* expression level (Kruskal–Wallis $\chi^2 = 17.513$, df = 4, $P = 0.002$), Midas cichlid species also differed in their variance in expression (Bartlett's $\kappa^2 = 11.337$, df = 4, $P = 0.023$), with Midas cichlids from the turbid great lake being significantly more variable than all other analyzed species (fig. 3*b*). Bonferroni corrected pairwise comparisons suggested that *cyp27c1* expression in the limnetic species from both crater lakes was significantly lower than those seen in Midas cichlids from the great lake (supplementary table S2, Supplementary Material online). All other pairwise comparisons were not significant after Bonferroni correction. Similar results in laboratory-reared specimens suggest a genetic basis for the observed pattern of variation (supplementary fig. S5b, Supplementary Material online). When comparing *cyp27c1* coding sequence among species showing high (i.e., *A. citrinellus*

from great lake Nicaragua) and low (i.e., *A. sagittae* from crater lake Xiloá and *A. astorquii* from crater lake Apoyo) levels of expression of this gene, we found almost no variation. The exception was *A. astorquii*, in which two alleles (V540 and E540) were found.

Discussion

Our results suggest rapid and parallel adaptive evolution of Midas cichlid vision in response to the colonization of a new light environment that occurred by taking advantage of different molecular mechanisms (fig. 7). Midas cichlids have colonized crater lakes Apoyo and Xiloá from the great lakes Nicaragua and Managua, respectively, <2,000 generations ago (Kautt, Machado-Schiaffino, and Meyer 2016). This event resulted in Midas cichlids experiencing a novel light environment in the crater lakes. The most important differences found between the ancestral and derived environments are that in the crater lakes light attenuation is lower, the light spectrum is broader and the overall visual environment is shifted toward shorter wavelengths compared with the great lake (fig. 1). Given the differences in the visual environments occupied by Midas cichlid species, we predicted phenotypic divergence in visual sensitivity between fish from the great lakes and the crater lakes. We found Midas cichlids to have a highly diverse visual system, both within and across species, with particularly high levels of intraspecific variation in turbid great lake Midas cichlids. Importantly, Midas cichlids from both crater lakes were found to have an overall shift in their visual sensitivities toward shorter wavelengths when compared with the source populations from the great lakes in agreement to the change observed in the photic environment (supplementary fig. S6, Supplementary Material online). This shift could not be explained by a single mechanism, but involved an integrated change that includes changes in lens transmittance, differential opsin gene expression, opsin coexpression, and the use of various A1/A2 chromophore mixes. Because most of the observed differences between species are maintained in laboratory-reared specimens (supplementary figs. S1, S4, and S5, Supplementary Material online), these traits appear to have a heritable basis.

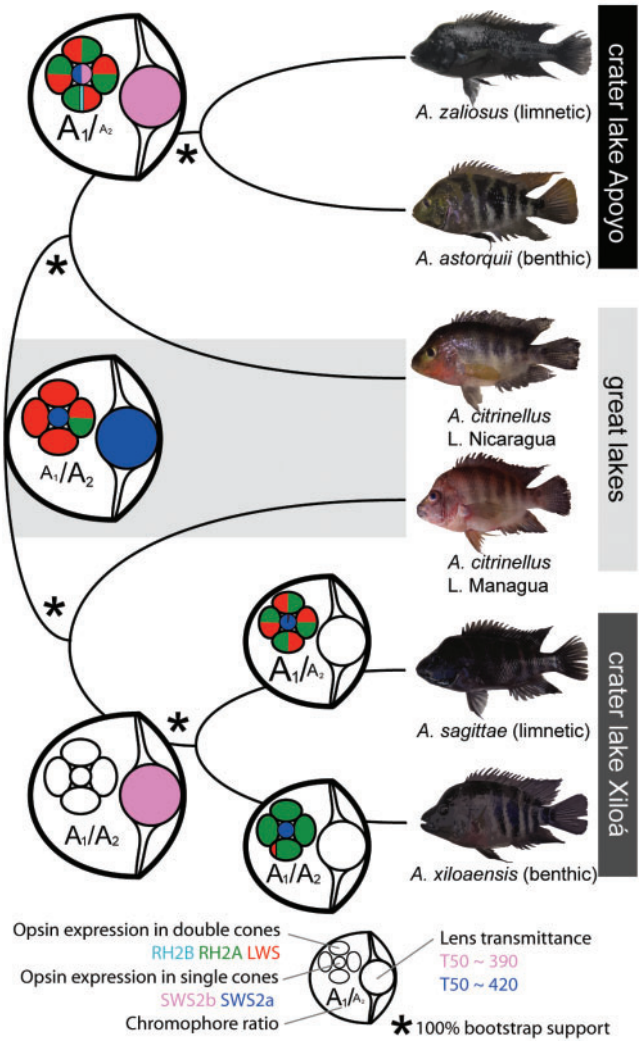

Fig. 7. The visual system of Midas cichlids evolved in parallel in the two clear crater lakes Apoyo and Xiloá after their colonization from the turbid great lakes Nicaragua and Managua, respectively. Phenotypic changes in opsin expression, chromophore usage, and lens transmittance are mapped on a phylogenetic reconstruction modified from Kautt, Machado-Schiaffino, and Meyer (2016). In fish from both crater lakes, lenses became more transmissive and expression of RH2A increased and that of LWS decreased. In all crater lake species, except for *A. xiloaensis*, chromophore usage changed from mainly A2 to mainly A1. (Fish photos by Andreas Kautt)

The Visual System of Midas Cichlids from the Great Lakes

Midas cichlids from the turbid great lake Nicaragua (*A. citrinellus*) have lenses blocking UV and partially violet light. In addition, the MSP experiment showed that Midas cichlids from this great lake have peaks of maximum sensitivity in the blue, the green and the red parts of the light spectrum (fig. 2) that correspond with the observed expression of *SWS2a*, *RH2A*, and *LWS* seen in fish from the great lake Managua (fig. 5). Interestingly, the retinas of fish from the turbid lake Nicaragua are dominated by double cones expressing *LWS* in both members (fig. 6). This dominance of the long sensitive cones might be an adaptation to the dim-light conditions experienced in the turbid great lake (fig. 1), as the long sensitive cones could be used for achromatic vision (Chiao et al. 2000; Cronin et al. 2014). The low genetic differentiation between Midas cichlids from the two great lakes ($F_{st} = 0.05$; Kautt, Machado-Schiaffino, and Meyer 2016) and the congruence of the measures taken from specimens of both lakes (i.e., opsin expression and opsin sequences from Lake Managua; and MSP, opsin expression, coexpression, opsin sequence, and lens transmittance from Lake Nicaragua) suggest that these two populations share a common phenotype.

The lens transmittance and photopigment sensitivities of Midas cichlids from the great lake Nicaragua are in agreement with what is known for Neotropical cichlids. So far, there have been few attempts to characterize lens transmittance and visual sensitivities in Neotropical cichlids (e.g., Muntz 1973; Loew and Lythgoe 1978; Levine and MacNichol 1979; Kröger

et al. 1999; Weadick et al. 2012, Escobar-Camacho et al. 2017). Yet, a clear picture emerges suggesting that in the neotropics, cichlids tend to have lenses blocking UV and partially violet light, and blue sensitive single cones, green, and red sensitive double cones representing a long wavelength sensitive palette of opsins (*sensu* Carleton et al. 2016). Thus, Midas cichlids from the great lakes have a visual system similar to that seen in South American cichlids, but given the age of these lakes (i.e., Early Pleistocene; Kutterolf et al. 2007) this species likely had enough evolutionary time to fine tune its visual system to the particular light conditions of the lakes. However, Midas cichlids depart from the general pattern in two interesting ways: by showing a large degree of intraspecific variation in visual sensitivity and by having at least four functionally visual pigments in cone cells.

Intraspecific Variation in Visual Sensitivity in Midas Cichlid from the Turbid Great Lakes

Freshwater animals inhabiting turbid environments can adaptively shift their visual sensitivities toward longer wavelengths without changing the opsin protein by using chromophores derived from vitamin A2 rather than from vitamin A1 in their photopigments (Wald 1961; Hárosi 1994; Cronin et al. 2014). Midas cichlids from the turbid great lakes use this mechanism to adjust their visual sensitivity, although there was a great degree of variation among specimens (see fig. 3*a*). A similar pattern was previously reported by Levine and MacNichol (1979), who analyzed 10 Midas cichlid individuals finding 2 discrete groups, 1 with mean λ_{max} at 454, 532, and 562 nm and the second at 463, 543, and 607 nm (for the single cones and the two members of double cones, respectively). Although the origin of fish used by Levine and MacNichol (1979) is unclear, we confirmed this variation among individuals from great lake Nicaragua (fig. 2). Since a similar variation in the blue Acara (*Aequidens pulcher*) was found (Kröger et al. 1999), it suggests that this high variability in A1/A2 might be a common pattern in Neotropical cichlids.

It has been recently shown that the enzyme Cyp27c1 is responsible for the conversion of vitamin A1 into vitamin A2 in the retinal pigment epithelium (Enright et al. 2015). In zebrafish (*Danio rerio*) the ratio of A1- to A2-derived chromophore covaries with the expression level of *cyp27c1* and knocking down this gene result in an inability of individuals to shift sensitivities toward longer wavelengths by means of differential chromophore usage (Enright et al. 2015). Midas cichlids from the turbid great lake show high intraspecific variation in the expression levels of *cyp27c1* (fig. 3*b*), providing a likely molecular mechanism for the observed variation in A1/A2 chromophore usage.

Coding sequence variation could also explain some of the intraspecific variation seen in turbid great lake Midas cichlid visual sensitivity. A164S in LWS was the only variable site directed into the retinal binding pocket identified in this study (table 1). These allelic variants of *LWS* have been found in several other organisms (e.g., Terai et al. 2006; Hofmann et al. 2009; Sandkam et al. 2015) and also as divergence among *LWS* paralogs (e.g., Asenjo et al. 1994; Ward et al. 2008; Phillips et al. 2016). The replacement of alanine with serine at this site is known to result in λ_{max} shift toward longer wavelengths (+7 nm; Asenjo et al. 1994). Measurements of absorption spectra on reconstituted LWS proteins of African cichlids showed that this substitution produced the expected λ_{max} shifts only if combined with an A2-derived chromophore (Terai et al. 2006). Interestingly, the 164A–164S allelic variants solely occur in the great lakes, where fish varied in chromophore usage. The combination of 164S and A2-derived retinal in red-sensitive pigments is proposed to be an adaptation to visual environments with a red-shifted light spectrum (Terai et al. 2006), as those experienced by fish in the great lakes. Yet, 164S is not fixed in Lake Nicaragua but it is segregating in the population. It is possible that photic environment variation across the lake favors the maintenance of the polymorphism (e.g., Terai et al. 2006).

Four Functional Spectral Classes in Midas Cichlid Cone Cells

Functional analyses with MSP suggested that Midas cichlids have four different spectral classes in their cone cells. Most examined individuals had double cones corresponding to three different spectral classes (a red, a long green, and a short green; fig. 2), that, in combination with the spectral class of single cones confer them the potential for tetrachromatic color vision. Remarkably, the fourth spectral class identified in Midas cichlid retinas appears to be the result of the coexpression of *RH2Aβ* and *LWS* on the same double cone member, rather than the expression of a different opsin gene. Combining the MSP, qPCR, and FISH experiments, we inferred that the red spectral class with λ_{max} from 560 to 623 nm corresponds to photoreceptors using LWS as the protein component of their visual pigments, and the short-green spectral class ranging from 517 to 539 nm corresponds to photoreceptors using RH2Aβ. The observed range of sensitivities within these two spectral groups is explained by variations in A1/A2 chromophore proportions in visual pigments having predicted pure-A1 sensitivities at ~560 and ~510 nm, respectively. Yet, there are several double cone members with λ_{max} values between these two groups that could not be assigned to either spectral class by just adjusting A1/A2 proportions. These cones have to be classified into a new spectral class, the long-green, and FISH staining suggests that this spectral class is the product of *RH2Aβ* and *LWS* coexpression in double cone cells.

That the long-green spectral class is the product of coexpression begs the question, why Midas cichlids do not use *RH2Aα* based visual pigments as African cichlids do? This is intriguing given that the predicted protein coded by *RH2Aα* appears to be functional. Although speculative, it is possible that gene conversion between *RH2A* paralogs (Escobar-Camacho et al. 2017) plays a role, either because both paralogs are functionally very similar or because the regulatory machinery has been affected by gene conversion. In addition, visual sensitivity curves deriving from coexpression would be significantly different (wider) compared to their pure *RH2Aα* counterpart, effectively changing the sensitivity bandwidth of this color channel and, by varying coexpression proportions,

maintaining a flexible mechanism for spectral tuning. The function of coexpression in Midas cichlids is unclear, but it may be related to increased contrast detection (Dalton et al. 2017).

Departures from trichromacy have previously been proposed for African cichlids based on measurements of maximum sensitivity by MSP (e.g., Parry et al. 2005; Dalton et al. 2014) or electroretinography (e.g., Sabbah et al. 2010), and by determining gene expression using qPCR (e.g., Hofmann et al. 2009) and *in situ* hybridization (e.g., Dalton et al. 2014). Recently, Dalton et al. (2017) showed that in the African cichlid *Metriaclima zebra* extensive regions of the retina could have very high levels of coexpression, with an incidence of more than 90%. This would imply that cones expressing only one opsin got almost completely replaced by cones showing coexpression. Midas cichlids differ from this in that cones coexpressing two opsin are distributed in low number across the retina, not replacing cones with only one opsin expressed, but coexisting with those. Thus, this extensive coexpression pattern appears to be novel to Midas cichlids. It is not clear if this is common in other Neotropical cichlids. It would be interesting to explore this issue in the neotropical pike cichlid (*Crenicichlia frenata*) given that it was reported to have a very long-shifted green sensitive double cone member (~547 nm; Weadick et al. 2012).

Adaptive Changes in the Visual Sensitivity of Crater Lakes Midas Cichlids

Midas cichlids from the crater lakes have a visual system that departs in several aspects from that seen in fish from the great lakes, resulting in an overall shift in sensitivity toward shorter wavelengths (fig. 7). The mechanisms underlying this shift include more transmissive ocular media, and changes in the chromophore and the protein component of visual pigments. Although this was apparent in species from both crater lakes, the biggest differences were observed in the species from crater lake Apoyo. This was expected given that this crater lake is the oldest (Elmer et al. 2010), has been occupied by Midas cichlids the longest (Kautt, Machado-Schiaffino, and Meyer 2016), and it differs the most in terms of photic environment from the great lakes (fig.1).

Ocular Media Transmittance in Midas Cichlids from the Crater Lakes

Eye lenses have become clearer in the crater lakes showing no overlap with the transmitting values seen in fish from the great lakes. This includes two extreme cases of UV-transmitting lenses in *A. zaliosus*, the limnetic species from crater lake Apoyo (fig. 4). Vertebrate lenses are formed by concentric layers of translucent proteins called crystallins, belonging to three large protein families (Fernald 2006). Crystallin proteins differ in their refractive indexes, so changes in crystallin usage across populations or developmental stages can result in variation in lens transmittance (Sabbah et al. 2012; Wages et al. 2013; Mahendiran et al. 2014). In addition, Neotropical cichlids tend to deposit pigments in their lenses that work as filters for short-wavelength light (Muntz 1973). Whereas having UV- and violet-blocking lenses might help reduce the loss of contrast detection due to the scattering of short-wavelength light; bearing clearer lenses in blue-shifted light environments could be adaptive, since it would allow fish to better utilize the whole available light spectrum (Muntz 1982). This is supported by a positive correlation between lens transmission and single cone's sensitivity in African cichlids (Hofmann et al. 2010). Thus, more short-wavelength transmitting lenses might be an adaptation to the light environment of clear water crater lakes. Given that these differences are observed in laboratory-born specimens reared under common conditions, we suggest that the use of different crystallin proteins or the deposition of pigments in lenses resulting in the observed cut-offs does not strictly depend on diet or light conditions, but also has a genetic component.

Cone Opsin Expression in Midas Cichlids from the Crater Lakes

There is evidence for genetically based differential opsin gene expression between Midas cichlids from the ancestral population of the great lakes and the derived populations from crater lakes that appears to be adaptive to the visual environment they experience (see figs. 1 and 5; supplementary fig. S6, Supplementary Material online). Moreover, this variation in opsin expression is consistent with the phenotypic variation determined by MSP (see figs. 2 and 5). One way in which crater lake Midas cichlids differ from great lake fish is in the proportional expression of different opsin. Whereas *LWS* represents >60% of the total expression in fish from the great lakes, it is consistently below 50% in the crater lakes. The opposite pattern is seen when comparing the expression of *RH2Aβ*. In Midas cichlids, this differential expression is translated into a higher proportion of green sensitive cones (both, RH2Aβ-based and LWS-RH2Aβ coexpression-based cones; fig. 6). It is apparent that one of the mechanisms used by Midas cichlids to improve vision in the shorter wavelength shifted light environment of the crater lakes is to increase the number of green-sensitive cones at the cost of fewer red-sensitive ones. Similar patterns of change in the proportional expression of cone opsins have been found in other cichlids suggesting it as a common mechanism of visual tuning (e.g., Carleton and Kocher 2001).

Also, Midas cichlids from the clear crater lakes have expanded their sensitivities toward the shorter part of the spectrum. Violet sensitive single cones have not been reported before for Neotropical cichlids, although they are commonly seen in African cichlids and correspond to a change from SWS2a to SWS2b as the protein component of photopigments (Carleton and Kocher 2001; Hofmann et al. 2009; O'Quin et al. 2010). Midas cichlids from the great lakes express exclusively *SWS2a*, *RH2Aβ*, and *LWS*. In the crater lakes, more distinctly in crater lake Apoyo some specimens expressed the violet sensitive *SWS2b* instead of *SWS2a* in single cones, and expressed the green-blue sensitive *RH2B* in combination with other double cone opsins (fig. 6). The

expression of *SWS2b* and *RH2B* was coupled at the individual level (supplementary fig. S3, Supplementary Material online), suggesting a general change in the pattern of expression.

To summarize, differential opsin expression is an important molecular mechanism in adaptive phenotypic divergence of Midas cichlids visual system. By changing the relative proportion of the different opsins expressed and by expressing other opsins (e.g., *SWS2b* and *RH2B*), crater lake Midas cichlids have diverged from the ancestral population in the great lake in the direction predicted based on the light environment differences (supplementary fig. S6, Supplementary Material online).

Chromophore Usage in Midas Cichlids from the Crater Lakes

In the spectral classes common to Midas cichlids from the great lakes and the crater lakes, we observed divergence in mean λ_{max} and the associated variance (fig. 2). This is most evidently in red-sensitive receptors where λ_{max} estimates were limited to the yellow in fish from the crater lakes, but expanding into the red part of the spectrum in the great lake specimens. In other spectral classes the differences are subtler, but there is still a clear trend for crater lake Midas cichlids to have λ_{max} shifted toward shorter wavelengths. This shift could be the result of structural changes in the opsin protein (Yokoyama et al. 2008) or due to differential chromophore usage (Wald 1961; Hárosi 1994). Given that only one amino acid substitution was identified in sites directed into the binding pocket across all Midas cichlid opsins (see table 1), different usage of A1- and A2-derived chromophores is the most plausible mechanism behind the observed variation in photoreceptor sensitivity. This conclusion is supported by the significant decrease in A2-derived chromophore usage seen in clear crater lake Midas cichlids compared with fish from the turbid great lakes. The down-regulation of *cyp27c1* expression in Midas cichlids from the crater lakes is the most likely mechanism underlying the changes in chromophore usage (Enright et al. 2015; supplementary fig. S5*a*, Supplementary Material online). Moreover, this variation is interpreted to have a genetic basis given that the differences in *cyp27c1* expression between species were maintained under laboratory conditions (supplementary fig. S5*b*, Supplementary Material online).

An interesting exception to this general pattern was the benthic species from crater lake Xiloá (*A. xiloaensis*) that showed high proportions of vitamin A2-derived chromophore usage and high levels of *cyp27c1* expression similar to the ancestral phenotype seen in great lake Midas cichlids. This could be adaptive in Xiloá, as this lake departs less in the photic condition from great lake Managua than crater lake Apoyo does. However, the down-regulation of *LWS* in *A. xiloaensis* strongly departs from the ancestral phenotype. Thus, this species might be using a different strategy to tune sensitivity to the new environment, but further studies are necessary to clarify this issue. Nonetheless, we did not observe this in laboratory-reared individuals of *A. xiloaensis*, suggesting that this phenotype might be plastic (supplementary figs. S4 and S5, Supplementary Material online). This highlights the multitude of mechanism that this extremely closely related set of species is capable of using during repeated adaptation to the crater lake environments.

Mechanisms of Adaptation to Divergent Visual Environments in Midas Cichlids

There is much debate about the relative importance of changes in coding sequence and of gene expression as the molecular mechanisms underlying phenotypic diversification (Hoekstra and Coyne 2007; Carroll 2008; Stern and Orgogozo 2008; Elmer and Meyer 2011; Rosenblum et al. 2014). Evidence supporting the importance of amino acid substitutions for phenotypic evolution has steadily accumulated for many decades, establishing it as an important mechanism of diversification (Hoekstra and Coyne 2007; Stern and Orgogozo 2008). On the other hand, the importance of regulatory processes for phenotypic divergence has become strongly supported more recently, as new molecular techniques resulted in the accumulation of new evidence (Wray 2007; Carroll 2008; Stern and Orgogozo 2008; Kratochwil and Meyer 2015). Changes in expression of cone opsins and *cyp27c1*, the gene responsible for changes in chromophore usage, seem to contribute the most to the observed variation in visual sensitivity. In contrast, structural changes might play only a limited role in vision tuning of Midas cichlids. Surely, amino acid substitutions are not unimportant for the phenotypic evolution of vision, as there is compelling evidence for its role in divergence in sensitivity, both, among paralogs (e.g., Yokoyama 2000) and among homologs when comparing different populations or species (e.g. Terai et al. 2002, 2006; Sugawara et al. 2005; Miyagi et al. 2012; Torres-Dowdall et al. 2015). Yet, in Midas cichlids structural changes might become more relevant in later stages of diversification as genetic variation in coding sequence would be expected to take time to appear by *de novo* mutations in young and initially small populations.

We presented evidence that the visual system of Midas cichlids has rapidly and adaptively evolved since the colonization of crater lakes, a few thousand generations ago (Kautt, Machado-Schiaffino, and Meyer 2016; fig. 7). The observed changes in visual sensitivity are the result of a combination of different mechanisms including changes in the ocular media and in both, the opsin protein and the light absorbing chromophore components of photopigments. Previous research has shown that all these mechanisms can independently tune visual sensitivity in African cichlids (reviewed in Carleton 2009; Carleton et al. 2016). Here, we showed that all these underlying mechanisms respond extremely rapidly and in an integrated way to adapt these fishes to changed light conditions that their ancestors experienced due to the colonization of the clear water crater lakes. Despite the divergence in visual sensitivity of crater lake Midas cichlids compared with the great lake ancestral populations, we did not find striking differences in sensitivity within the small radiations in each crater lake. Yet, in the limnetic species from Apoyo we observed a trend to have sensitivities shifted toward shorter wavelengths compared to the benthic species that suggests that differences might be accumulating.

The Midas cichlid species complex is only one of the many fish species that colonized Nicaraguan crater lakes from the source populations in the great lakes Managua and Nicaragua (Elmer et al. 2010; Kautt, Machado-Schiaffino, and Meyer 2016). Yet, it is clearly the most abundant species in these lakes (Dittmann et al. 2012) and the only lineage that has radiated in the crater lakes, resulting in a species complex composed of at least 13 species (Barluenga et al. 2006; Barluenga and Meyer 2010; Elmer et al. 2010; Recknagel et al. 2013; Kautt, Machado-Schiaffino, and Meyer 2016). The reasons why this species has become dominant in terms of biomass and has diversified but other species that colonized the crater lakes have not, remain largely unclear (Franchini et al. 2017). Uncovering the molecular mechanisms contributing to the adaptation of Midas cichlids to the novel conditions experienced in the crater lakes, such as a short-wavelength shifted light environment, is fundamental to progress in our understanding of this system.

Materials and Methods

Underwater Light Measurements

Underwater light measurements were taken at one site in Lake Managua, four sites in Lake Xiloá, and seven sites in Lake Apoyo, characterized by different bottom structure (rocky outcrops, boulders covered in algal material, *Chara* beds, sandy bottoms). Underwater spectral irradiance was measured with an Ocean Optics USB2000 connected to a 15-m UV–VIS optical fiber fitted with a cosine corrector, just under the surface and at 2-m depth, orienting the probe upwards (for downwelling light) and toward four orthogonal directions horizontally (sidewelling light). The four horizontal measurements were averaged to derive a single measurement of side-welling light at depth. Downwelling irradiance is presented in the main text; sidewelling irradiance is presented in supplementary fig. S7, Supplementary Material online. We calculated the total quantal flux for each irradiance integrating each spectral measurement in the range (350–700 nm) relevant to cichlid vision. Following McFarland and Munz (1975), we derived λP50, i.e. the wavelength that halves the total number of photons in the selected range of visible spectrum and that identifies the spectral region with the highest abundance of quanta.

Retinal MSP Measurements

We conducted MSP in wild-caught Midas cichlids from great lake Nicaragua ($n = 5$), crater lake Apoyo ($n = 10$), and crater lake Xiloá ($n = 12$; species identities, number of rods and cones analyzed per species, mean peak of maximum absorption, and A1% are noted in supplementary table S1, Supplementary Material online) and in laboratory reared Midas cichlids from great lake Nicaragua ($n = 2$), crater lake Apoyo ($n = 4$), and crater lake Xiloá ($n = 8$). Analyses followed standard methods (Loew 1994; Fuller et al. 2003; Losey et al. 2003). Before conducting MSP, fish were maintained under dark conditions for a minimum of 4 h and then euthanized with an overdose of MS-222 followed by cervical dislocation. The eyes were rapidly enucleated under dim red light, and the retinas removed and maintained in phosphate-buffered saline (pH 7.2) with 6% sucrose. Small pieces of the retina were placed on a cover slide, fragmented to isolate individual photoreceptors, and sealed with a second cover slide and Corning High Vacuum grease. We used a single-beam, computer-controlled MSP, with a 100-W quartz iodine lamp that allowed for accurate absorption measurements down to 340 nm (Loew 1994; Losey et al. 2003). Peak of maximum absorption (λ_{max}) of photoreceptors was obtained by fitting A1- or A2 templates to the smoothed, normalized absorbance spectra (Lipetz and Cronin 1988; Govardovskii et al. 2000). We used the criteria for data inclusion into the analysis of λ_{max} described in Loew (1994) and Losey et al. (2003).

We conducted statistical comparisons at two levels. First, to test for the effect of colonization of clear water crater lakes on the visual system of Midas cichlids, we considered lake of origin as explanatory variable, ignoring species or ecomorphs within crater lakes. Second, to test for the effect of microhabitat (i.e. limnetic vs. benthic) we only used data from the crater lakes, where both ecomorphs are found, and included lake of origin and ecomorph as explanatory variables in the statistical model. In both cases, we first conducted a Bartlett's κ^2 test of homoscedasticity within each spectral class to determine if there were differences in variance among groups. This was interpreted as a test for variation in A1- to A2-derived chromophore usage as we found little structural variation in opsin proteins that could explain variation within spectral class (see Coding Sequence Variation of Midas Cichlid Opsin Genes section above). If the Bartlett's κ^2 test did not reject homoscedasticity, we conducted a linear mixed model using λ_{max} values for individual photoreceptors within each spectral class as response variable, lake of origin as explanatory variable, and specimen as a random variable. When testing for the effect of microhabitat, ecomorph and its interaction with lake of origin were also included as explanatory variables. If the Bartlett's κ^2 test suggested heteroscedasticity, we used a nonparametric Kruskal–Wallis test. All analyses were conducted in R (R Core Team 2014). Significant results are reported in the main text, nonsignificant tests are reported in supplementary table S3, Supplementary Material online.

Ocular Media Transmission

We measured ocular media transmission in laboratory-reared individuals of *A. citrinellus* from great lake Nicaragua ($n = 10$), the limnetic *A. zaliosus* ($n = 6$) and the benthic *A. astorquii* ($n = 3$) from crater lake Apoyo, and the limnetic *A. sagittae* ($n = 7$) and the benthic *A. amarillo* ($n = 9$) from crater lake Xiloá. All fish were euthanized using an overdose of MS-222 and subsequent cervical dislocation. The eyes were enucleated, carefully hemisected, and the corneas and lenses were placed on a black paper with a small hole. A pulsed xenon lamp (PX-2, Ocean Optics) was directed through the hole and transmission was measured with an USB2000+UV-VIS-ES spectrometer (Ocean Optics). For each specimen, three measures of transmission were obtained from each of the two eye ocular media. As previously reported for cichlids (Hofmann

et al. 2010; O'Quin et al. 2010), we found that the lenses are the limiting ocular media, so we subsequently measured only lens transmission. We calculated lens transmission (T50) following Hofmann et al. (2010), measuring the wavelength of maximum slope (i.e., inflection point in the sigmoid curve) within the range of 300–700 nm. This method was shown to be less sensitive to departures from perfect sigmoid shape than methods that determine T50 as the halfway point between the minimum transmission and that of maximum transmission, and both are highly correlated (Hofmann et al. 2010). Using this last method did not produce a qualitative difference in our results.

Opsin Coding Regions Amplification and Sequencing

Genomic DNA was isolated using standard phenol–chloroform extractions from a total of 64 specimens of Midas cichlids, including representatives of two species from each of the great lakes Managua and Nicaragua, and two species from each of the crater lakes Apoyo and Xiloá (table 1). Genomic sequences of all opsin genes were obtained by polymerase chain reaction (PCR) using standard protocols. Primers were designed in PRIMER 3 (Rozen and Skaletsky 2000) using the *A. citrinellus* draft genome as a template (Elmer et al. 2014; primer list and PCR conditions in supplementary table S4, Supplementary Material online). Samples were sequenced bi-directionally and using internal primers on a 3130xl Genetic Analyzer. Sequence editing and assembly was performed using SeqMan II (DNAstar).

Analyses of Opsin and cyp27c1 Gene Expression

We measured cone opsin and *cyp27c1* expression in wild-caught (WC) and laboratory-reared (LR) individuals of *A. citrinellus* ($n_{WC} = 6$ from Lake Managua; $n_{LR} = 8$ from Lake Nicaragua), the limnetic *A. zaliosus* ($n_{WC} = 6$; $n_{LR} = 4$) and the benthic *A. astorquii* ($n_{WC} = 6$; $n_{LR} = 4$) from crater lake Apoyo, and the limnetic *A. sagittae* ($n_{WC} = 4$; $n_{LR} = 4$) and the benthic *A. xiloaensis* ($n_{WC} = 4$; $n_{LR} = 4$) from crater lake Xiloá. All fish were killed using an overdose of MS-222 and subsequent cervical dislocation. The eyes were rapidly enucleated and the retinas removed and stored in RNAlater (Sigma-Aldrich, USA) until RNA extraction. RNA was extracted using a commercial kit (RNeasy Mini Kit, Qiagen) and RNA concentrations were measured using the Colibri Microvolume Spectrometer, (Titertek Berthold, Germany). Total RNA was reverse transcribed with the first-strand cDNA synthesis kit (GoScriptTM Reverse Transcription System, Promega, Madison, WI, USA).

Gene expression levels were quantified using Quantitative Real-Time PCR (qPCR). Real-Time reactions were run in a CFX96^{TM} Real-Time System (Bio-Rad Laboratories, Hercules, CA, USA) using specifically designed primers (supplementary table S4, Supplementary Material online). Amplification efficiencies were determined for each primer pair. Standard PCR and Sanger sequencing of PCR products were performed for each opsin gene to check for specificity of amplification. Expression levels of genes were quantified with three technical replicates and mean C_t values were used for further analyses. Quantitative Real-Time PCR was performed under standard conditions following the manufacturer's protocol (GoTaq qPCR Master Mix, Promega, Madison, WI, USA). Proportional opsin expression was determined for each specimen by calculating the proportion of each opsin (T_i) relative to the total opsin expression (T_{all}) after Fuller et al. (2004) using the following equation:

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

where E_i represents the primer efficiency for primer i and Ct_i is the critical cycle number for gene i (the proportional expression values of the seven cone opsins add up to 1 for each specimen). *cyp27c1* expression was normalized using the geometric mean of two selected housekeeping genes (*ldh2* and *imp2*) using the following equation:

$$RQ_i = E_i^{(Ct_{HKG} - Ct_i)}$$

Nonparametric Multi-Response Permutation Procedures (MRPP) tests (Mielke et al. 1981) were used to compare cone opsin expression among species and between wild-caught and laboratory-reared specimens. Pairwise comparisons between wild-caught and laboratory-reared specimens within each species were also conducted and significant differences were found only for the benthic species of crater lake Xiloá (*A. xiloaensis*; supplementary table S5, Supplementary Material online). Kruskal–Wallis tests were used to compare expression of *cyp27c1* among species and between wild-caught and laboratory-reared specimens. As with opsin gene expression, using pairwise comparisons we only found differences in *cyp27c1* due to rearing condition for *A. xiloaensis* (supplementary table S2, Supplementary Material online).

Analyses of Opsin Gene Coexpression

We performed triple FISH (fluorescent *in situ* hybridization) in five laboratory-reared individuals per species of a Midas cichlid from a turbid great lake (*A. citrinellus*) and one from a clear crater lake (*A. astorquii*). All samples were probed for all three cone opsin genes. Probes for RH2B, RH2A, and LWS were cloned into the pGEMT or pGEMTE vector systems (Promega #A3600 and #A3610) using primers: RH2B-FW ATGGCATGGGATGGAGGACTTG; RH2B-RV GAAACAGA GGAGACTTCTGTC; RH2A-FW TGGGTTGGGAAGGAGG AATTG; RH2A-RV ACAGAGGACACCTCTGTCTTG; LWS-FW ATGGCAGAAGAGTGGGGAAA; LWS-RV TGCAGGA GCCACAGAGGAGAC.

The FISH was performed as described (Woltering et al. 2009) with modifications enabling triple fluorescent instead of single colorimetric detection. In brief, eyes were rapidly enucleated and retinas fixed in 4% PFA in PBS overnight and stored in methanol at −20°C until further use. Duration of tissue bleaching in 1.5% H_2O_2 in methanol and Proteinase K treatment were decreased to 3 min each. Probes with three different detection labels were synthesized using DIG-labeling mix (Roche #11277073910), Fluorescein labeling mix (Roche #116855619910), custom made DNP

labeling mix 10× [DNP-11-UTP (Perkin Elmer #NEL555001EA) 3.5 mM combined with UTP 6.5 mM, CTP 10 mM, GTP 10 mM, ATP 10 mM (ThermoFischer #R0481)]. Antibody incubation was performed overnight at 4°C using anti-Fluorescein-POD (Roche #11426346910), anti-DIG-POD (Roche #11207733910) and anti-DNP-HRP (Perkin Elmer #FP1129). To amplify fluorescent signal, we used tyramide signal amplification (TSA) for each of the different labels; TSA plus-Fluorescein (Perkin Elmer #NEL753001KT), TSA plus-Cyanine 3 (Perkin Elmer #NEL753001KT), and TSA plus-Cyanine 5 (Perkin Elmer #NEL745001KT). Antibody incubation and corresponding signal amplification were performed sequentially. Prior to incubation with the next antibody, POD activity of the previous one was deactivated in 100 mM glycine solution (pH 2.0) for 15 min followed by 15 washes for 10 min each in TBS-T and once overnight. Before mounting, retinas were cleared in 70% glycerol overnight at 4°C.

Expression levels were quantified in four quadrants of the retina divided as dorsal-nasal, dorsal-temporal, ventral-nasal, and ventral-temporal. Per retinal region, five sampling areas were randomly chosen and in each all the cones in a frame of 55 × 55 μm were examined for RH2B, RH2A, and LWS expression and for coexpression genes within one member of a double cone. This assured that more than 200 double cone members were characterized in each region for each fish.

Supplementary Material

Supplementary data are available at *Molecular Biology and Evolution* online.

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