Accelerated Evolution and Functional Divergence of the Dim Light Visual Pigment Accompanies Cichlid Colonization of Central America

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Abstract

Cichlids encompass one of the most diverse groups of fishes in South and Central America, and show extensive variation in life history, morphology, and colouration. While studies of visual system evolution in cichlids have focussed largely on the African rift lake species flocks, Neotropical cichlids offer a unique opportunity to investigate visual system evolution at broader temporal and geographic scales. South American cichlid colonization of Central America has likely promoted accelerated rates of morphological evolution in Central American lineages as they encountered reduced competition, renewed ecological opportunity, and novel aquatic habitats. To investigate whether such transitions have influenced molecular evolution of vision in Central American cichlids, we sequenced the dim-light rhodopsin gene in 101 Neotropical cichlid species, spanning the diversity of the clade. We find strong evidence for increased rates of evolution in Central American cichlid rhodopsin relative to South American lineages, and identify several sites under positive selection in rhodopsin that likely contribute to adaptation to different photic environments. We expressed a Neotropical cichlid rhodopsin protein in vitro for the first time, and found that while its spectral tuning properties were characteristic of typical vertebrate rhodopsin pigments, the rate of decay of its active signalling form was much slower, consistent with dim light adaptation in other vertebrate rhodopsins. Using site-directed mutagenesis combined with spectroscopic assays, we found that a key amino acid substitution present in some Central American cichlids accelerates the rate of decay of active rhodopsin, which may mediate adaptation to clear water habitats.

Key words: **clade models of evolution, evolution of the visual system, visual ecology, site-directed mutagenesis, in vitro protein expression.**

Article

Introduction

Colonization of novel habitats and subsequent diversification provides important insight into the ecological drivers of phenotypic diversity and molecular adaptation. Neotropical cichlid fishes (subfamily Cichlinae) have emerged as a compelling group in which to study shifts in phenotypic evolution and parallel adaptations in morphology during a continent-wide adaptive radiation (López-Fernández et al. 2013; Arbour and López-Fernández 2014; Astudillo-Clavijo et al. 2015). These cichlids are among the most species-rich fish families in the Neotropics, and comprise over 600 species predominantly distributed across three major tribes (Geophagini, Cichlasomatini, Heroini). Neotropical cichlids differ from their African relatives in that their diversification probably took place primarily in rivers, rather than lakes, and occurred over a much longer temporal scale (López-Fernández et al. 2010, 2013). In contrast to the restricted geographic region over which much of African cichlid diversity is concentrated, the Neotropical cichlid radiation offers an opportunity to investigate the evolution of this group across a wide range of riverine (and occasionally lacustrine) environments, and by extension, visual ecologies. Much of Neotropical cichlid diversification took place within South America, with additional diversification in Central America, the Antilles, and Mexico (hereafter referred to collectively as Central America) following several major colonization events (Hulsey et al. 2010; Tagliacollo et al. 2015). South American rivers likely impose unique constraints on the visual system, due to the various distinct water types, including white (turbid), black (translucent but stained with tannins), and clear (Sioli 1984, Winemiller et al. 2008; Costa et al. 2012). Past work on Amazonian tributaries has shown that the chemical properties of these waters (and their associated optical and

ecological characteristics) may influence distributions of fishes more strongly than tributary structure or geography alone (e.g., Goulding et al. 1988; Cooke et al. 2012). Central America is characterized by extensive geological upheaval (e.g., glaciation, volcanic uplift) relative to South America, resulting in a complex hydrogeological history (Albert and Reis 2011; Bagley and Johnson 2014). Central American freshwater habitats comprise both riverine and lacustrine environments that may be turbid, stained, or clear. Together, the predominantly riverine diversification of Neotropical cichlids and their widespread distribution across the varied environments of South and Central America presents a compelling opportunity to investigate visual system evolution in a macroevolutionary context.

Studies of the visual system, specifically the visual opsin proteins that mediate the first step in vision, have been particularly useful for investigating the effects of ecology, biogeography, and other evolutionary forces on molecular evolutionary rates and visual pigment protein functional properties. Visual pigments are seven-transmembrane proteins belonging to the G protein-coupled receptor (GPCR) superfamily, and consist of an opsin protein moiety covalently bound to a retinal chromophore, 11-*cis* retinal (Palczewski et al. 2000). Upon absorption of a photon, the chromophore isomerizes to its all-*trans* form, triggering a conformational change in the opsin into its active state metarhodopsin II (meta II), initiating the phototransduction cascade (Lamb and Pugh 2004). Eventually, all-*trans* retinal is released from the pigment and the opsin regains sensitivity upon binding of a new 11-*cis* retinal. Shifts in the rates of these different steps can have substantial effects on visual sensitivity and dark adaptation (recovery of rod photoreceptors from photobleaching).

Variation in cone opsin genes (which function in bright light to mediate colour vision) that reflects disparate visual ecologies has been investigated in a variety of animals including mammals (Emerling et al. 2015; Melin et al. 2016), fishes (Stieb et al. 2017), reptiles (Emerling 2016), primates (Veilleux et al. 2013), and fireflies (Sander and Hall 2015). The dim-light visual pigment rhodopsin (RH1), while typically highly conserved across vertebrates (e.g., Hauser et al. 2016), also shows variation associated with differences in visual environments (Niemiller et al. 2012; Van Nynatten et al. 2015; Dungan et al. 2016; Castiglione et al. 2017). Shifts in visual pigment function mediated by mutations at key amino acid sites have been experimentally demonstrated in many vertebrates, such as fishes (Hunt et al. 1996; Sugawara et al. 2010), birds (Odeen et al. 2012; van Hazel et al. 2016), and mammals (Bickelmann et al. 2015; Dungan et al. 2016). In cichlid fishes, cone opsins have received substantial attention due to their role in the African rift lake cichlid radiation (the "sensory drive" hypothesis) (Carleton and Kocher 2001; Seehausen et al. 2008 reviewed in Carleton et al. 2016). Different sets of cone opsins are also differentially expressed (referred to as an opsin palette) in cichlid retinas to optimally tune visual sensitivity in diverse spectral environments, and across ontogeny (Spady et al. 2006; Hofmann et al. 2010; Carleton et al. 2016). RH1 variation is also instrumental in tuning visual adaptations in cichlids. In African cichlids, several key amino acid substitutions have been associated with enhanced visual sensitivity in deep waters (Sugawara et al. 2005, 2010). Recent work investigating evolution of RH1 across the cichlid family has suggested that ecological differences between lakes and rivers may drive the divergence of this opsin more strongly than phylogenetic history or geography (Schott et al. 2014; Torres-Dowdall et al. 2015).

Although the visual system of Neotropical cichlids has been less extensively characterized compared with African cichlids, recent studies focussed on South American cichlids have suggested that they have a visual system particularly suited for red-shifted or light limited visual environments, such as black or white waters, respectively (e.g., Costa et al. 2012). First, the pike cichlid (*Crenicichla frenata*) was shown to have a reduced set of opsin genes relative to African cichlids, including loss of the ultraviolet-sensitive opsin (SWS1) and pseudogenization of the violet opsin (SWS2b) (Weadick et al. 2012). Transcriptome and genomic analyses of three additional South American cichlid species have also revealed that rhodopsin is the most highly expressed opsin in the retina, and a long-wavelength sensitive cone opsin palette (i.e., predominant expression of blue-, green-, and orange/red-sensitive cone opsin classes) was consistently expressed (Escobar-Camacho et al. 2017). Amazonian cichlids have also been reported to have yellow lenses and corneas, which would serve as cutoff filters for shorter wavelengths of light entering the eye (Muntz 1973). Therefore, although a comprehensive account of the diversity of Neotropical cichlid visual systems is still emerging, this recent research on South American cichlid opsins suggests their visual system may be particularly suited to dim-light environments where long wavelengths are more prevalent. Additional work on the visual opsins in South American cichlid lineages evolving in clear water rivers would shed further light on the breadth of South American cichlid visual repertoire. By contrast, little is known about opsin evolution and visual sensitivity in Central American cichlids, with the exception of the lake-dwelling Midas cichlid species flock (*Amphilophus*). In this group, visual sensitivity may be rapidly modulated by differential opsin gene expression, rather than amino acid variation (Torres-Dowdall et al. 2017), so the extent of opsin sequence variation in Central American cichlid groups remains unknown. More generally, colonization of Central America represented an important opportunity for South American cichlids for a number of reasons: First, prior to Central American invasion, cichlids were competing intensely for resources alongside other dominant South American fish lineages (e.g., Characiformes and Siluriformes). In Central America, despite a comparatively restricted geographic area, cichlids encountered relatively little competition, and together with poeciliids became the principal fish fauna assemblage in the region (Tagliacollo et al. 2015; Arbour and López-Fernández 2016). This renewed opportunity in Central America likely promoted accelerated phenotypic diversification and adaptive divergence, expanding the ecological repertoire of cichlids into a variety of novel niches (Hulsey et al. 2010; López-Fernández et al. 2013; Arbour and López-Fernández 2014, 2016). Second, while scarce in South America, lacustrine

environments, most notably crater lakes, are more common in Central America and may offer additional opportunity for divergence from riverine ancestors, and for expansion into additional niches (e.g., via differences in depth) (Malinsky et al. 2015). On a macroevolutionary scale, it is currently unknown whether a transition from South to Central America and the associated ecological opportunity may have influenced visual system evolution in cichlids.

Although the three main Neotropical cichlid tribes largely inhabit South American rivers, the Heroini lineages that invaded and colonized the riverine and lacustrine habitats in Central America underwent significant diversification (>100 species) despite a comparatively restricted geographic area (Hulsey et al. 2010). Because past work investigating Neotropical cichlid rhodopsin has focussed on South American lineages of Geophagini and select lake-dwelling members of Heroini (Schott et al. 2014; Torres-Dowdall et al. 2015), the extent to which diversification within Central America may have influenced the evolution of this pigment remains unclear. Given the substantial increase in phenotypic evolutionary rates in Neotropical cichlids following their colonization of novel habitats in Central America (Arbour and Lopez-Fernandez, 2016), as well as the remarkable diversity of ecomorphological phenotypes in extant Central American cichlids, we hypothesized that this major macroevolutionary and ecological transition may have also driven diversifying selection on RH1 in Neotropical cichlids. To test this hypothesis, we used cross-species targeted exon capture to sequence the rhodopsin gene across Neotropical cichlid species spanning a wide range of life histories, morphologies, and habitats, and used codon-based likelihood models to test for both positive and divergent selection. We found evidence for positive selection across all Neotropical cichlids; however, clade model analyses isolating Central American lineages revealed significant acceleration and divergence in rhodopsin evolutionary rates relative to South American lineages. To examine potential functional differences between South and Central American cichlid rhodopsins, we used in vitro expression and site-directed mutagenesis approaches to experimentally investigate Neotropical cichlid RH1, and the effects of mutating site 83, a site which has been hypothesized to be important for modulating visual sensitivity in diverse photic environments, and was found to be under positive selection in Central American cichlids in our study. Spectral and kinetic assays on the mutant rhodopsin pigment revealed a shift in rhodopsin function consistent with possible adaptation to clear water environments encountered by several lineages in Central America.

Results

Capture Performance and Sequence Accuracy

Full-length RH1 sequences were obtained via targeted sequence capture from 101 species of Neotropical cichlid, spanning the diversity of the clade. Given the possibility of artificial variation introduced into captured sequences via the capture or assembly method, we compared a data set of captured RH1 against previously published Sanger sequenced data sets of the tribe Geophagini (Schott et al. 2014), and lake-dwelling members of the tribe Heroini (Torres-Dowdall et al. 2015). Pairwise comparisons between the captured and Sanger sequenced genes showed 99.7% average similarity at the amino acid level. All rhodopsin sequences obtained in this study are deposited in GenBank (Accession IDs supplementary table S1, Supplementary Material online).

Rhodopsin Gene Tree Does Not Resolve Species Relationships

The maximum likelihood RH1 gene tree for all Neotropical cichlids did not resolve monophyly of the major Neotropical cichlid clades (supplementary fig. S5, Supplementary Material online). While the Geophagini tribe was resolved as monophyletic, the Heroini and Cichlasomatini tribes were not. South American heroine cichlids were placed outside of other South American-dwelling geophagines, and several South American cichlasomatines grouped within heroine cichlids. The RH1 gene tree of South American cichlids also did not resolve monophyly of Heroini; rather, some were grouped within Cichlasomatini, while other heroine species fell outside of all three major clades (supplementary fig. S6, Supplementary Material online). The Central American RH1 gene tree was also inconsistent with established species relationships (supplementary fig. S7, Supplementary Material online). Given the poor resolution of cichlids relationships recovered with the RH1 gene tree (likely because RH1 is under positive selection), and that monophyly and divergence of the various Neotropical cichlid tribes is well established (e.g., Matschiner et al. 2017; Ilves et al. 2017), clade model analyses were conducted on a topology representing species relationships (López-Fernández et al. 2010; Říčan et al. 2016; Ilves et al. 2017; fig. 1; supplementary fig. S1, Supplementary Material online) to ensure spurious results were not introduced with the use of a gene tree.

Positive Selection in Neotropical Cichlid Rhodopsin

Random sites analyses on the RH1 alignment and species tree revealed that as a group, Neotropical cichlids show significant evidence for pervasive positive selection in rhodopsin (4.0% of sites with ω of 5.4; table 1). These results were consistent when the RH1 gene tree was used (supplementary table S3, Supplementary Material online). Comparing South and Central American cichlids yielded the most disparate results; while South American cichlids were found to be under levels of positive selection comparable to those found previously (5.6% of sites with ω of 3.8) (Schott et al. 2014), Central American cichlid rhodopsin was under much stronger positive selection ($\omega = 12.0$) at a similar number of sites (4.5%; table 1); higher than any individual cichlid tribe (supplementary table S2, Supplementary Material online). When gene trees of both South and Central American cichlids were used for random sites analyses, Central American cichlids still showed evidence for higher positive selection in rhodopsin (supplementary table S3, Supplementary Material online). Both the proportion and magnitude of sites under positive selection in Central American cichlids are comparable to those found in their rapidly radiating African rift lake relatives (Spady et al. 2005; Schott et al. 2014; Torres-Dowdall et al. 2015).

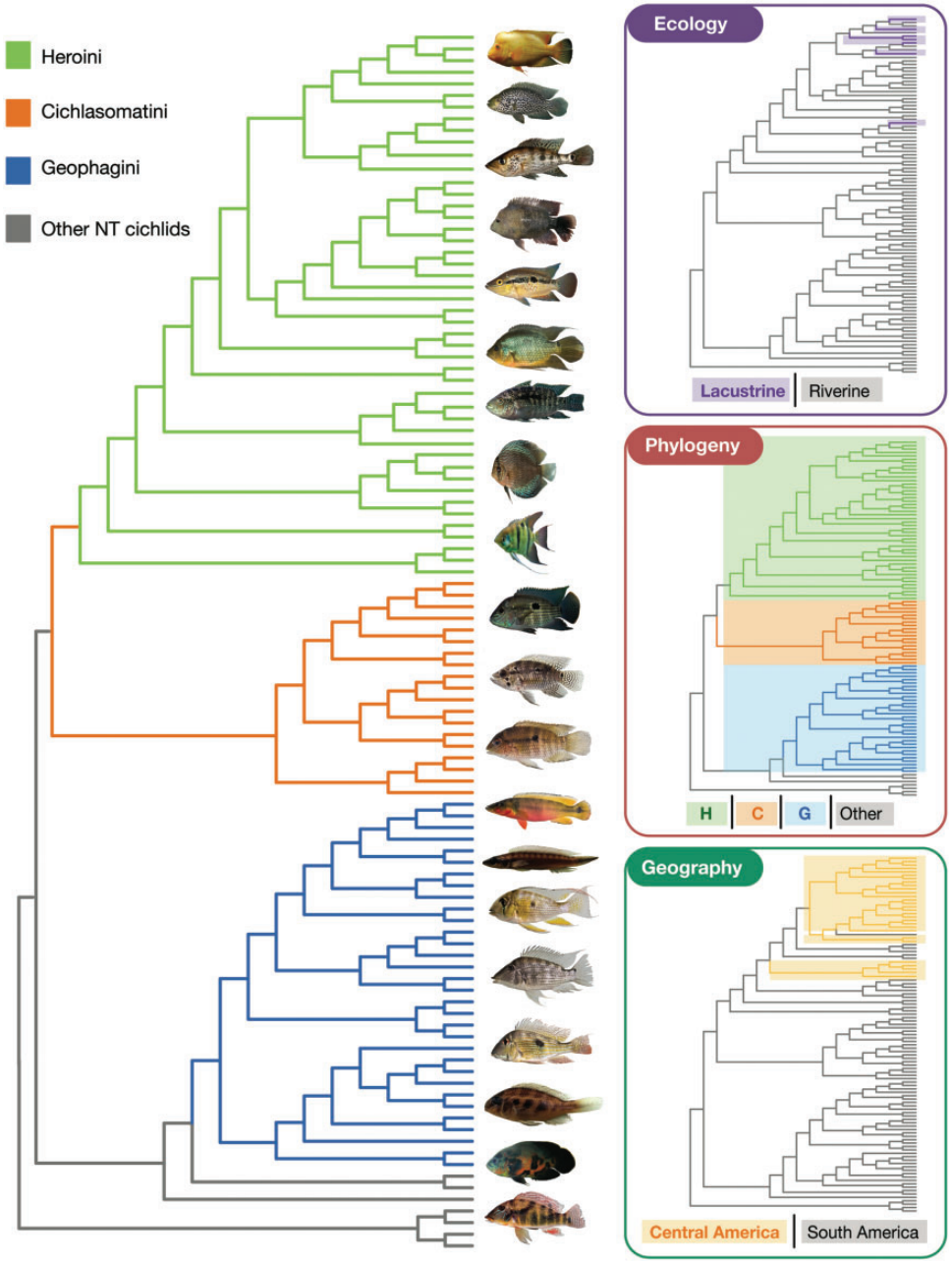

Fig. 1. Schematic of Neotropical cichlid relationships and examples of clade model partitions. Cichlid species tree used for all subsequent analyses, with Neotropical tribes highlighted. To the right of the species tree are three different clade model partitions implemented in the study, representing either ecological, phylogenetic, or geographic hypotheses of rhodopsin divergence. The additional partition representing the background lineages is shown in grey in each case. All tested partitions are listed in table 2 and highlighted on phylogenies in supplementary figure S3, Supplementary Material online. Species included in each partition are listed on the phylogeny in supplementary figure S1 and table S1, Supplementary Material online. Photographs depict selected taxa illustrating phenotypic diversity across the Neotropical cichlid clade. Image credits: Jessica Arbour and Hernán López-Fernández.

Divergent Positive Selection in Central American Cichlid Rhodopsin

To test the hypothesis that ecological, phylogenetic, or geographic factors may be driving accelerated molecular evolutionary rates in Neotropical cichlid rhodopsin, we used PAML's Clade Model C (CMC; Bielawski and Yang 2004), which permits a class of codon sites to evolve differently along the phylogeny (Baker et al. 2016), in order to test for a shift in the level of selection (i.e., divergent selection) among major Neotropical cichlid clades, as well as ecological and biogeographical partitioning schemes, using the species topology. First, we examined whether lineage-specific factors influenced rhodopsin evolution by isolating each major tribe (Heroini, Cichlasomatini, Geophagini) as a foreground clade and found that Heroini and Geophagini were supported to be under divergent selection relative to the null M2a_rel model, which does not allow for divergence (but the ω value remains unconstrained; Weadick and Chang 2012). Second, an

Table 1. Results of RH1 Random Sites (PAML) Analyses on All Neotropical Cichlids, South American Cichlids, and Central American Cichlids.

Data Set[a]	Model	np	*ln*L	K	Parameters[b]			Null	LRT	df	*P**
					ω_0/p	ω_1/q	ω_2/ω_p				
All Neo	M0	207	−6494.59	3.09		0.27		n/a			
	M1a	208	−6034.93	2.59	0.01 (87.4%)	1 (12.5%)		M0	919.3	1	0.0000
	M2a	210	−5909.86	3.12	0.01 (86.8%)	1 (9.1%)	5.5 (4.1%)	M1a	250.1	2	0.0000
	M2a_rel	210	−5909.85	3.12	0.01 (86.8%)	1 (9.1%)	5.5 (4.1%)	M1a	250.2	2	0.0000
	M3	211	−5909.67	3.14	0.01 (87.1%)	1.1 (9.0%)	5.7 (3.9%)	M0	1169.8	4	0.0000
	M7	208	−6064.58	2.66	0.01	0.02		n/a			
	M8a	209	−6033.58	2.54	0.035	1.30	1 (10.2%)				
	M8	210	−5908.77	3.10	0.01	0.045	5.4 (4.0%)	M7	311.6	2	0.0000
								M8a	249.6	1	0.0000
South Am.	M0	149	−5622.85	2.83		0.24		n/a			
	M1a	150	−5264.18	2.41	0.01 (88.0%)	1 (12.0%)		M0	717.4	1	0.0000
	M2a	152	−5192.21	2.95	0 (87.8%)	1 (7.1%)	4.10 (5.0%)	M1a	143.9	2	0.0000
	M3	153	−5189.79	2.73	0.02 (90.0%)	1.92 (8.8%)	9.02 (1.3%)	M0	866.1	4	0.0000
	M7	150	−5271.29	2.50	0.01	0.26		n/a			
	M8a	151	−5241.21	2.39	0.01	0.116	1 (12.4%)				
	M8	152	−5192.31	2.84	0.04	0.49	3.81 (5.6%)	M7	158.0	2	0.0000
								M8a	137.8	1	0.0269
Central Am.	M0	57	−2291.29	5.00		0.44		n/a			
	M1a	68	−2211.90	3.60	0 (91.1%)	1 (8.9%)		M0	158.8	1	0.0000
	M2a	60	−2143.08	5.42	0 (93.0%)	1 (3.2%)	12.75 (3.9%)	M1a	137.6	2	0.0000
	M2a_rel	60	−2144.70	5.42	0 (93.0%)	1 (3.2%)	13.25 (3.8%)	M1a	134.4	2	0.0000
	M3	61	−2140.38	5.35	0.00 (94.6%)	4.63 (3.3%)	17.6 (2.5%)	M0	301.8	4	0.0000
	M7	58	−2210.80	3.65	0.01	0.05		n/a			
	M8a	59	−2208.19	3.64	0.005	46.9	1 (0.1%)				
	M8	60	−2145.39	3.80	0.01	0.25	12.02 (4.5%)	M7	130.8	2	0.0000
								M8a	125.6	1	0.0000

NOTE.—np, number of parameters; lnL, ln likelihood; K, transition/transversion ratio; df, degrees of freedom; n/a, not applicable. Additional subsets are listed in supplementary table S2, Supplementary Material online.

[a]The tree and alignment were pruned to contain only Central American cichlids (Central Am.) or South American cichlids (South Am.)

[b]ω values of each site class are shown for models M0–M3 (ω0 – ω2) with the proportion of each site class in parentheses. For M7–M8, the shape parameters, p and q, which describe the beta distribution are listed. In addition, the ω value for the positively selected site class (ωp, with the proportion of sites in parentheses) is shown for M8a (where ωp is constrained to equal one) and M8.

*Indicates statistical significance ($p < 0.05$).

ecologically based partition testing for differences between lake and riverine cichlids did not identify lake-dwelling Heroine cichlids as under divergent selection, when compared against M2a_rel. Our geography-based partitioning scheme contrasting Central versus South American cichlids yielded a superior fit relative to all other partitions tested, including a simplified partition that included embedded South American lineages in the Central American group ("Central America clade;" supplementary fig. S3, Supplementary Material online; table 2), suggesting that this transition and diversification has had a substantial influence on rhodopsin evolution in Neotropical cichlids. Using CmC, we also explicitly tested for the presence of positive selection in the divergent site class by comparing the best-fitting CmC partition to a nested CmC model where the divergent site class is constrained to an omega of one (Schott et al. 2014), and found that the model allowing for positive selection was a significantly better fit (supplementary table S3, Supplementary Material online).

To investigate whether acceleration in rhodopsin evolutionary rates could be due in part to small population size or genome-wide elevated molecular evolutionary rates in Central American cichlids, we also tested for divergence in two phylogenetic markers (ENC1 and GPR85) and found both genes to be highly conserved, with no evidence for either positive or divergent selection in Central American species (fig. 2B, supplementary table S7, Supplementary Material online). However, future investigations contrasting a wider array of protein coding genes with RH1 evolutionary rates would lend additional support to these results (e.g., Havird et al. 2017).

Recent work examining African and Neotropical cichlid rhodopsin together has found evidence for significant divergent selection in rhodopsin likely mediated by ecological differences between lake and riverine environments, as well as substantially higher rates of positive selection in lake-dwelling cichlid lineages (Schott et al. 2014; Torres-Dowdall et al. 2015). While the majority of African cichlid diversity is found in lakes, South and Central American cichlids are largely riverine; however, select lineages have colonized crater lake environments (e.g., the Midas cichlid *Amphilophus* in Nicaragua). We tested for divergent selection on rhodopsin in Neotropical lacustrine versus Neotropical riverine cichlids using CmC, but found that this partition was not a significantly better fit relative to the null M2a_rel model (table 2). This is likely due in part to the limited number of lake species (7) compared with riverine species (97) in our Neotropical cichlid data set; any signal of divergent selection in these lake lineages may be overwhelmed by the primarily riverine cichlid sampling. To

Table 2. Results of Clade Model C (CMC) (PAML) Analyses on the Neotropical Cichlid Rhodopsin Data Set.

Model and Partitions[a]	np	*ln*L	K	Parameters[b]			ΔAIC[c]	Null	LRT	df	*P*
				ω_0	ω_1	ω_2/ω_d					
M1a	208	−6034.93	2.59	0.01 (87.5%)	1 (12.5%)		263.6	n/a			
M2a	210	−5909.86	3.12	0.01 (86.8%)	1 (9.1%)	5.31 (4.1%)	17.5	M1a	250.14	2	0.0000*
M2a_rel	210	−5909.85	3.13	0.01 (86.8%)	1 (9.1%)	5.47 (4.1%)	17.5	M1a	250.16	2	0.0000*
CmC: Cichlasomatini	211	−5909.84	3.12	0.01 (86.8%)	1 (9.1%)	5.43 (4.0%) C: 5.71	19.4	M2a_rel	0.020	1	0.8875
CmC: Heroini	211	−5906.21	3.09	0.01 (86.8%)	1 (9.5%)	4.63 (3.7%) H: 8.0	12.2	M2a_rel	5.9	1	0.0151*
CmC: Geophagini	211	−5907.70	3.10	0.01 (86.8%)	1 (9.4%)	6.5 (3.8%) G: 4.30	15.2	M2a_rel	4.11	1	0.0426*
CmC: Central America (clade)	211	−5900.12	3.08	0.01 (86.8%)	1 (9.7%)	4.47 (3.4%) CA (clade): 11.7	7.3	M2a_rel	14.82	1	0.0001*
CmC: Central America	211	−5896.45	3.07	0.01 (86.8%)	1 (9.7%)	4.47 (3.4%) CA: 14.8	0	M2a_rel	23.67	1	0.0000*
CmC: Lake	211	−5909.02	3.13	0.01 (86.8%)	1 (9.2%)	5.41 (4.0%) Lake: 10.25	25.1	M2a_rel	0.170	1	0.6801
CmC: CA_river/CA_lake	212	−5895.80	3.13	0.01(86.8%)	1(9.7%)	4.5 (3.5%)	0.7	M2a_rel	27.2	2	0.0000*
						CA river:15.7		CA	0.38	1	0.5376
						CA lake:11.3					
CmC: Cichlasomatini/Heroini/ Geophagini	213	−5904.67	2.44	0.01 (86.8%)	1 (9.7%)	3.98 (3.4%)	13.1	M2a_rel	7.776	3	0.0157*
						C: 6.36					
						H: 8.71					
						G: 4.10					

NOTE.—np, number of parameters; lnL, ln likelihood; K, transition/transversion ratio; df, degrees of freedom; n/a, not applicable.
[a]Partitions listed are explained in figure 1 and supplementary figure S3 and table S1, Supplementary Material online. In all cases, an additional partition exists that contains the remaining taxa (e.g., outgroups).
[b]ω values of each site class are shown with the proportion of each site class in parentheses. ωd is divergent site class that has a separate value for each partition.
[c]The difference in AIC values was calculated compared with the overall best-fitting model, Central America, with an AIC of 12214.9.
*Indicates statistical significance ($p < 0.05$).

mitigate this, we isolated the Central American cichlid lineages (29 species total) and tested for divergent selection between lake and riverine species, but still did not find statistical support for divergent selection in partitions isolating lake versus riverine cichlids (supplementary table S6, Supplementary Material online). Random sites analyses also suggest no appreciable differences in positive selection on rhodopsin between lake and riverine cichlids ($\omega_{CAriverine} = 12.85$ at 4.1% of sites; $\omega_{CAlake} = 11.22$ at 3.8% of sites; supplementary table S7, Supplementary Material online).

Unique Positively Selected Sites in South and Central American Cichlid Rhodopsin

Several positively selected sites are shared between Central and South American cichlid rhodopsin; however, in general, positively selected Central American cichlid rhodopsin sites had much higher d_N/d_S estimates relative to South American species (fig. 2C; supplementary fig. S3, Supplementary Material online). Rhodopsin site 166 is under positive selection in both South and Central American cichlids, and divergently selected in Central American cichlids. This site likely mediates spectral tuning based on recent in vitro work in the African cichlid *Astatotilapia calliptera* (Malinsky et al. 2015). Rhodopsin in shallow water-dwelling African cichlid ecomorphs was found to have A166 with a λ_{max} of 506 nm while the benthic ecomorph predominantly had S166, contributing to a blue shifted λ_{max} (503 nm), suggesting adaptation to deeper waters. This variation is paralleled across Neotropical cichlids, with South American cichlids possessing either S166 or T166, while several Central American lineages have transitioned to A166; however, whether such variation reflects differences in light availability or depth requires additional ecological information for these species.

Sites under positive selection identified by both PAML BEB and FUBAR that were not shared between South and Central America have been shown to mediate both spectral and kinetic properties of rhodopsin (fig. 2C; supplementary table S7, Supplementary Material online). Rhodopsin site 299 is under positive selection in South American cichlids, and divergent selection between South and Central American cichlids. While most Central American cichlids have S299, transitions to A299 occur in several South American lineages (similar to what has been observed in African cichlids). Site-directed mutagenesis studies at this site indicate it can affect spectral tuning (S299A produces a 2 nm blue shift) in the rhodopsin pigments of fishes and aquatic mammals (Hunt et al. 2001; Bischoff et al. 2012; Dungan et al. 2016). Moreover, recent mutagenesis studies of orca rhodopsin demonstrate this site can also affect the decay of the active state of rhodopsin, which may have been adaptive for changes in light intensity in the terrestrial-aquatic transition (Dungan and Chang 2017). These findings suggest that this aspect of rhodopsin function may have been favoured in clear water (brighter) habitats inhabited by South American species (e.g., members of *Crenicichla* and *Teleocichla*) (supplementary fig. S8, Supplementary Material online).

In Central American cichlids, we identified two residues known to mediate shifts in rhodopsin function that undergo parallel substitutions in a number of lineages, and are also under positive selection. Recent mutagenesis work has identified that the M123I slightly extends the half-life of the active meta II rhodopsin in zebrafish, which may be favoured in dim conditions (Morrow and Chang 2015). Rhodopsin site 83 is of particular interest in vertebrates for its potential role in dim light adaptation. At site 83, aspartic acid (D) is nearly ubiquitous across vertebrate rhodopsin pigments, and other GPCRs (Breikers et al. 2001). An unusual substitution

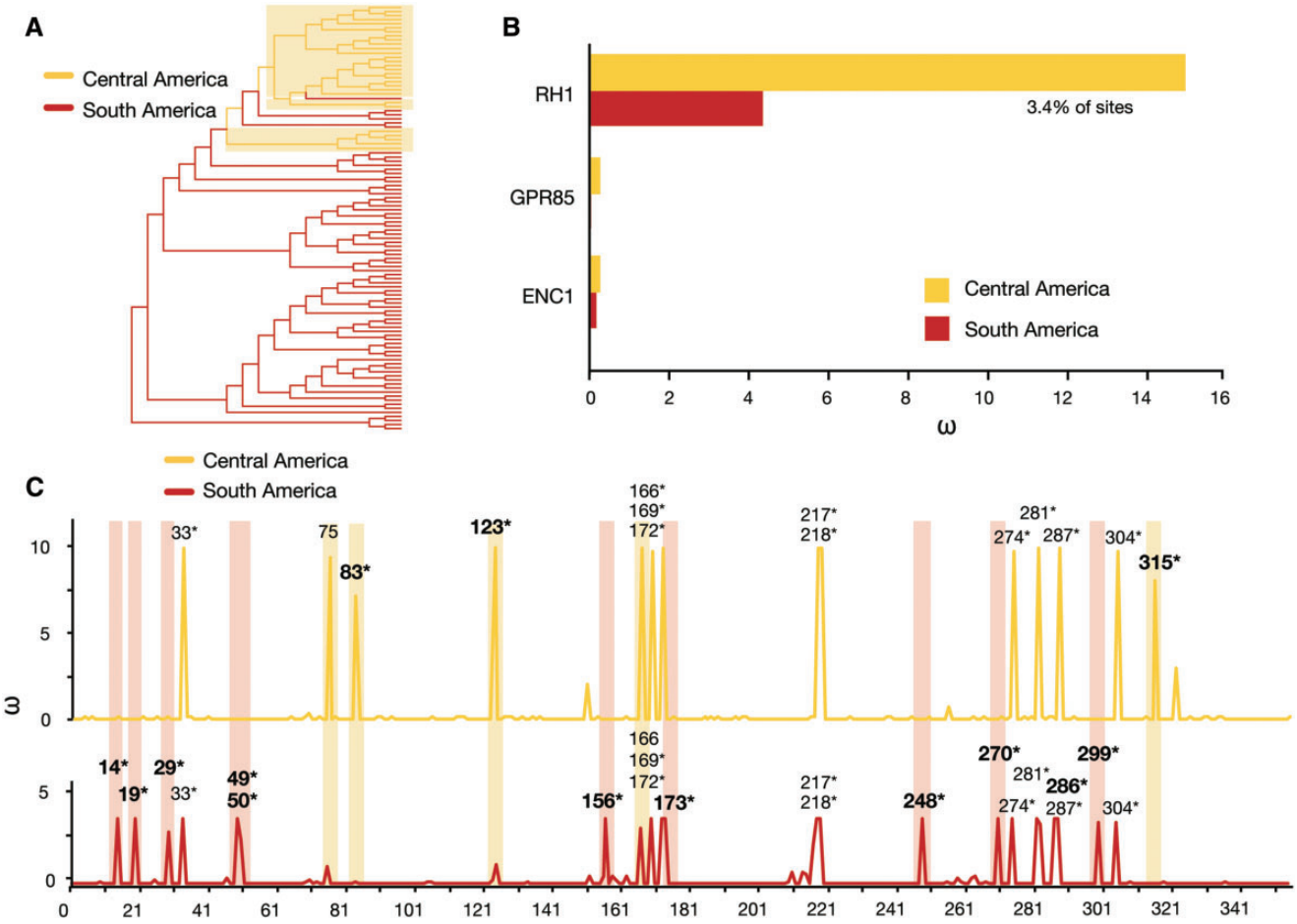

Fig. 2. Results of clade model and random sites analyses on South and Central American cichlid rhodopsin. (*A*) Species tree illustrating the best-fitting clade model partition (Central American species in foreground; South American species in background) (*B*) Bar graph depicting the differing levels of selection in the divergently selected site class as estimated by the CMC Central America partition on RH1, and two nonvisual control genes (GPR85, ENC1). (*C*) Random sites analyses conducted using PAML M8 on Central and South American cichlid RH1 data sets. Labelled sites were identified as positively selected via PAML BEB; those with an asterisk were also confirmed as under positive selection with FUBAR. Bolded sites are unique to either Central or South American cichlids (supported with both PAML BEB and FUBAR).

(D83N) is found in several vertebrate lineages adapted to light-limited environments, including bats, whales, and deep-water sculpin fishes (Hunt et al. 2001; Sugawara et al. 2010; Dungan et al. 2016). The D83N substitution has been hypothesized to be advantageous in dim environments due to its role in increasing the stability of active meta II rhodopsin, thereby favouring formation of the active state (Sugawara et al. 2010; van Hazel et al. 2016). Across cichlids, rhodopsin site 83 exhibits an unusual distribution (table 3). While African cichlids are dominated by D83, the D83N substitution occurs in three species of African lake cichlids inhabiting deep waters (and does not occur in close shallow water-dwelling relatives) and is therefore thought to be advantageous in dim light habitats (Sugawara et al. 2010). We found that Neotropical cichlids are unusual in that the predominant amino acid residue at site 83 is asparagine (N), rather than aspartic acid (D). A reverse substitution to the more common residue (N83D) occurs in three Central American species, and may perhaps induce a shift in function in Neotropical cichlid rhodopsin consistent with adaptation to habitats with more available ambient light. We expressed wild-type cichlid RH1 in vitro to investigate whether it exhibits kinetic properties consistent with those found in the RH1 pigments of other dim-light adapted vertebrates (e.g., Sugawara et al. 2010; Dungan and Chang 2017). We selected site 83 for further investigation via site-directed mutagenesis, due to the occurrence of the N83D mutation in several Central American cichlid species.

Site 83 Mediates a Functional Shift in Neotropical Cichlid Rhodopsin Kinetics

To gain further insight into the functional properties of Neotropical cichlid rhodopsin, we expressed pike cichlid (*Crenicichla frenata*) rhodopsin in vitro via heterologous protein expression. Wild-type Neotropical cichlid rhodopsin (N83) has a slightly blue-shifted spectral sensitivity of 496.5 nm relative to the model bovine rhodopsin ($\lambda_{max} = 500$ nm), a value consistent with what was found with microspectrophotometry (MSP) measurements on rods from the same species (Weadick et al. 2012). Wild-type *C. frenata* rhodopsin exhibited a significantly slower rate of retinal release relative to the bovine rhodopsin control, likely reflecting enhanced stability of the

Table 3. Variation at Important Functional Rhodopsin Site 83 across African and Neotropical Cichlids.

Major Clade	Common Residue	Unique Residue	Known Species with Unique Residue	Species Characteristics	Reference
African cichlids	D83	N83 (3 known species)	*Baileychromis centropomoides*	Lake Tanganyika; benthic habitats	Sugawara et al. (2010)
			Diplotaxodon macrops	Lake Malawi; deep water	
			Pallidochromis tokolosh	Lake Malawi; deep water	
Neotropical cichlids	N83	D83 (5 known species)	*Retroculus xinguensis*	Basal Neotropical; South America; Xingu river basin (clear water)	This study
			Retroculus sp.	Basal Neotropical; South America; Tocantins river basin (clear water)	
			Nosferatu bartoni	Heroini tribe; Mexico (clear lakes and rivers)	
			Herichthys cyanoguttatus	Heroini tribe; Mexico and United States (clear lakes and rivers)	
			Nandopsis haitiensis	Heroini tribe; Haiti and Dominican Republic (clear lagoons and rivers)	

Table 4. Spectral Absorbance Measurements and Retinal Release Half Lives of Neotropical Cichlid (*Crenicichla* frenata) Rhodopsin Measured In Vitro.

Species	Mutant	λ_{max} (nm)[a]	Retinal Release $t_{1/2}$ (min)[a]
Bos Taurus (control)	Wild-type (D83)	500.1 ± 0.11 (3)	14.23 ± 0.3 (5)
Crenicichla frenata	Wild-type (N83)	496.5 ± 0.35 (3)	40.10 ± 4.1 (5)
Crenicichla frenata	N83D	498.5 ± 0.32 (3)	27.89 ± 3.1 (3)

[a]Measurements are ± standard error with sample size in brackets.

active meta II state (table 4 and fig. 3*D* and *E*). To evaluate the effect that the reversal to D83, which occurred in several Central American cichlids, has on cichlid rhodopsin function, we mutated the site and expressed and assayed the mutant pigment. The N83D substitution produces a modest red shift in rhodopsin λ_{max} to 498.5 nm (table 4 and fig. 3*B*); however, it significantly accelerated the rate of retinal release, shortening the meta II half life by ~13 min (table 4 and fig. 3*D* and *E*). Both the wild-type and mutant pigment responded normally to light activation (fig. 3*C*).

Discussion

We used cross-species exon capture to target and sequence the rhodopsin gene from 101 Neotropical cichlid species. Using clade model analyses, we tested the hypothesis that invasion and subsequent diversification of cichlids within Central America facilitated a rapid divergence and shift in molecular evolutionary rates in rhodopsin, consistent with recent morphological and ecological evolutionary findings in this group (e.g., Arbour and López-Fernández 2014, 2016). We found a significant acceleration in rhodopsin evolutionary rates during cichlid diversification in Central America, and recovered unique sites under positive selection between South and Central America that may mediate adaptation to different photic environments. We also experimentally investigated a Neotropical cichlid rhodopsin pigment in vitro to provide both the first functional characterization of a Neotropical visual pigment, and to test the effect of variation at rhodopsin site 83. This site is thought to mediate increased sensitivity in dim light conditions in cichlids and other vertebrates, and the amino acid residue considered a dim light adaptation (N83) is nearly ubiquitous across Neotropical cichlids. On the other hand, our results also indicate that the N83D substitution present in some Central American cichlids may be more suitable for vision in brighter (i.e., clear water) environments.

Accelerated and Divergent Rhodopsin Evolution in Central American Cichlids

The clade model analyses implemented in this study identified substantial divergent positive selection in rhodopsin in Central American cichlid lineages relative to their South American counterparts. Family-wide analyses across both African and Neotropical cichlid rhodopsin have identified primarily ecological factors driving selection, revealing that while geography and phylogenetic history mediate divergent selection on rhodopsin, clade-based models accounting for lake versus riverine ecologies were the best fitting models overall (Schott et al. 2014; Torres-Dowdall et al. 2015). This previous work identified increased levels of positive selection in lake-dwelling Neotropical cichlids, comparable to findings in African lake cichlids (Torres-Dowdall et al. 2015). Our results suggest that this may be due to higher rates of rhodopsin molecular evolution in Central American cichlids overall, rather than lake versus riverine ecologies, since these lake sequences were not analyzed alongside related riverine Central American lineages, and our Central American lacustrine versus riverine partition was not supported to be under divergent selection. Most of the Central American lake diversity sampled to date encompasses recently diverged species flocks within the genus *Amphilophus* (e.g., Barluenga et al. 2006; Elmer et al. 2010; Elmer and Meyer 2011), whereas other lake-dwelling cichlids are also commonly found in riverine environments (e.g., *Parachromis*, *Archocentrus*). These lineages may not yet have accumulated sufficient variation in their rhodopsin gene to be detectable using interspecific

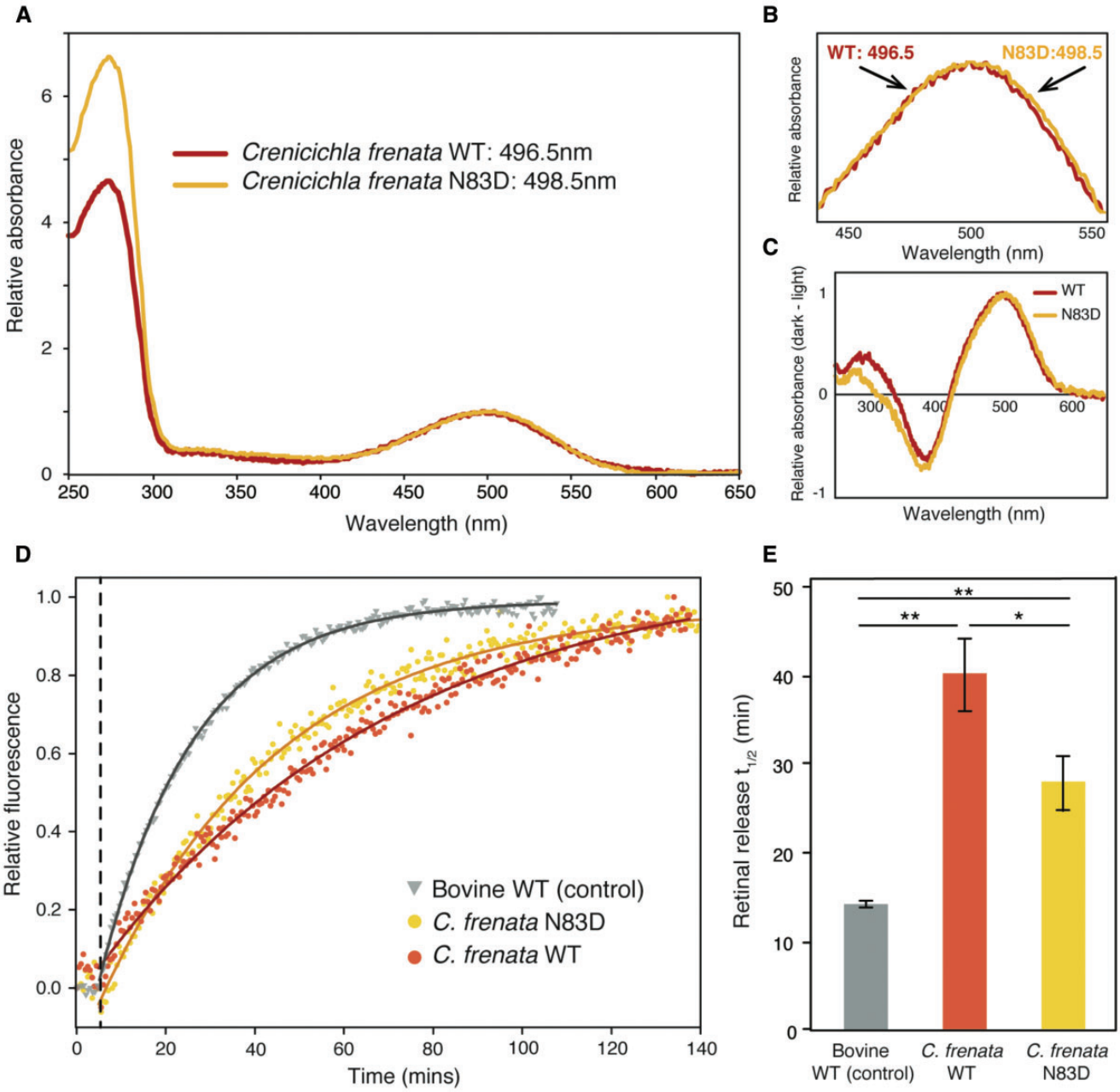

Fig. 3. Functional characteristics of wild-type Neotropical cichlid and N83D cichlid mutant rhodopsin. (*A*) Spectral absorbance curves of dark state rhodopsin (left) and dark-light difference spectra (right). Indicated spectral peaks (λ_{max}) were estimated according to Govardovskii et al. 2000. Absorbance peaks at 280 nm represent total protein. (*B*) Isolated λ_{max} peaks from panel A illustrating the 2nm red shift in the N83D mutant. (*C*) Dark-light spectra of rhodopsin illustrating response to light. (*D*) Fluorescence assays of retinal release rates following light activation of rhodopsin. (*E*) Average meta II half-lives estimated by fitting time courses with first-order exponential curves (panel D) where the N83D mutant cichlid rhodopsin has a significantly shorter half-life than wild-type cichlid. Error bars represent standard error.

comparative analyses. Indeed, it is likely that opsin expression differences, rather than sequence differences, modulate visual sensitivity with variation in ambient light in these recently radiated lake lineages (Torres-Dowdall et al. 2017).

The high levels of positive selection in Central American riverine cichlid rhodopsin may be mediated by a number of factors. First, there may be a greater diversity in aquatic habitats in Central America compared with South America (e.g., while both clear and turbid water are present in both regions, Central America has more lacustrine habitats relative to South America). Second, the release from competition with other South American fish lineages likely promoted a substantial increase in both phenotypic and lineage diversity in cichlids that colonized Central America (Arbour and López-Fernández 2016). The associated increase in ecomorphological specialization (e.g., specialized feeding behaviours such as substrate sifting, detritivory, molluscivory, algae-scraping, etc.) may have in turn expanded the visual niches available to Central American cichlids, driving increased levels of positive selection in RH1. However, further examination of variation in

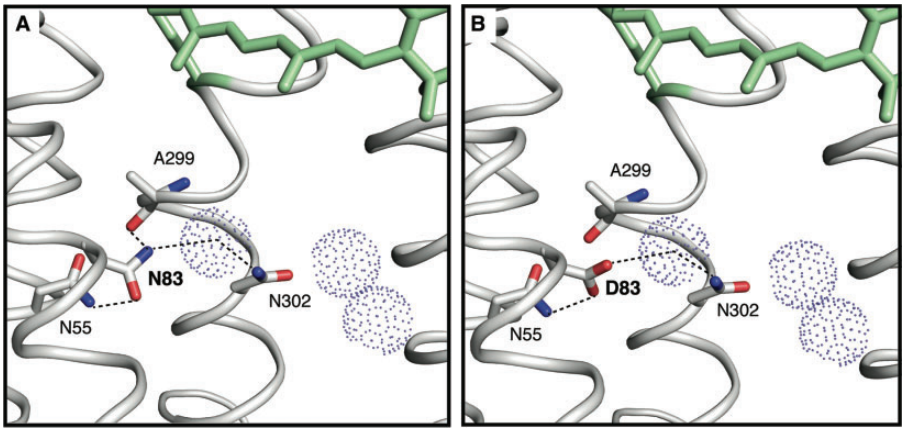

Fig. 4. (*A*) Crystal structure of active meta II rhodopsin (bovine; PDB = 3PQR) with *Crenicichla frenata* wild-type residue N83 and (*B*) Wild-type bovine rhodopsin (D83). Dotted lines indicate hypothesized H-bond interactions (Choe et al. 2011). The retinal chromophore is shown in green, and water molecules are represented by blue dots.

cone opsin genes in Central American cichlids will allow for a more complete picture of how visual system variation may have been influenced by the ecological opportunity encountered in Central America. Moreover, additional data on ambient lighting environments inhabited by South and Central American cichlids (e.g., whether certain species dwell primarily in black, turbid, and clear water, or a combination), will shed additional light on processes influencing visual pigment evolution on a macroevolutionary scale, since it is evident that, at the level of opsin gene expression, turbid versus clear water environments can induce rapid changes in Midas cichlid visual systems (Torres-Dowdall et al. 2017).

Divergence coupled with increases in molecular evolutionary rates has been detected in the dim-light visual pigments of fishes and other vertebrates undergoing macroevolutionary and ecological transitions. For instance, a study of rhodopsin in marine anchovies invading the red-shifted freshwater riverine environments of South America found that a clade model partition isolating (nonmonophyletic) freshwater-invading lineages was the most strongly supported (Van Nynatten et al. 2015). Rhodopsin evolution in cetaceans was also found to be influenced by foraging depth rather than by evolutionary history alone (Dungan et al. 2016). By extensively sampling rhodopsin for the Neotropical members of Cichlidae, we found that the invasion and subsequent diversification of Central American cichlids has substantially influenced rhodopsin evolution. The considerable increase in divergent diversifying selection in rhodopsin is concomitant with increased rates of morphological evolution and ecological niche expansion in the Central American cichlids (López-Fernández et al. 2013; Arbour and López-Fernández 2016). While South American cichlids underwent a decline in rates of morphological evolution over time, upon Central American invasion these evolutionary rates accelerated and approached those found at the beginning of the South American radiation, as cichlids encountered novel ecological opportunities (Arbour and López-Fernández 2016). Rhodopsin molecular evolutionary rates investigated in this study are consistent with these phenotypic findings, as we find evidence for positive diversifying selection in rhodopsin in South American cichlids, but much higher levels of selection in Central American lineages, often at unique amino acid sites.

Unique Rhodopsin Sites under Positive Selection in South and Central American Cichlids and Their Role in Adaptation to Photic Environment

Several positively selected rhodopsin sites overlap between South and Central American cichlids, as expected for lineages with shared evolutionary history. South American cichlids have a greater number of positively selected sites overall, which could be due to their distribution across seven separate tribes (Geophagini, Cichlasomatini, Heroini, Chaetobranchini, Astronotini, Cichlini, and Retroculini), or to the fact that, collectively, they inhabit a much larger area with more diverse ecological conditions (Sioli 1984; Costa et al. 2012). Site 299, which is under positive selection only in South American cichlids, may tune the pigment to accommodate this variation in water colour or transparency (Fasick and Robinson 2000; Dungan et al. 2016).

In Central American cichlids, we found that the levels of selection at positively selected sites were much higher overall. We recovered several positively selected sites in Central American cichlids that are known to mediate rhodopsin function either spectrally (166; Malinsky et al. 2015), kinetically (123; Morrow and Chang 2015), or both (83; Sugawara et al. 2010). Interestingly, in Central American cichlids, the M123I substitution favouring meta II rhodopsin stability found in several species is not consistent with their habitats (which are primarily clear water); however, the kinetic effects of this site may not be consistent across vertebrate groups—the wild-type rate of meta II decay in zebrafish is nearly seven times faster than in Neotropical cichlid rhodopsin (6.6 mins; Morrow and Chang 2015). Moreover, the effect of amino acid substitutions on rates of retinal release is likely highly dependent on protein context, and may differ among rhodopsins from different species (Dungan and Chang 2017). We focussed attention on site 83 as the N83 residue found throughout South (and many Central) American Neotropical cichlids,

which has frequently been identified as mediating adaptation to dim light in a variety of vertebrate rhodopsin pigments (Hunt et al. 1996; Sugawara et al. 2010; Dungan et al. 2016; van Hazel et al. 2016).

The Influence of Site 83 on Cichlid Rhodopsin Function in Variable Spectral Environments

The prevalence of N83 in most Neotropical cichlids is intriguing given that D83 is highly conserved among rhodopsin-like GPCRs, and is typically found only in select deep-water dwelling organisms due to its blue-shifting properties (Breikers et al. 2001; Hunt et al. 2001, Dungan et al. 2016). Recent work has begun to elucidate whether site 83 influences non-spectral (i.e., kinetic) properties of rhodopsin, specifically its interaction with other residues participating in the hydrogen bonding network of the protein (fig. 4). In African cichlids, D83 is the most prevalent residue, but N83 was identified in three deepwater-dwelling lineages (Sugawara et al. 2005, 2010). N83 accelerated formation of the active meta II state of rhodopsin, an effect likely favoured in dim light conditions since increased production of active rhodopsin could enhance signal amplification and pigment sensitivity (Sugawara et al. 2010). Here, we measured the rate of release of all-*trans*-retinal from the active pigment, which corresponds to the rate of decay of the active meta II state. The wild-type *C. frenata* pigment (N83) has a significantly extended retinal release half-life compared with bovine rhodopsin (D83), and zebrafish (D83); however, its λ_{max} is typical of most fish rhodopsins (Morrow and Chang 2015). The dim conditions found in some South and Central American rivers would likely favour the retention of an amino acid residue enhancing rhodopsin sensitivity such as N83. The N83D substitution produces a minimal shift in the λ_{max} of the pigment, which is consistent with findings in cichlids and other vertebrates (e.g., Sugawara et al. 2010, van Hazel et al. 2016). This is an expected result given its distance from the rhodopsin retinal chromophore (fig. 4), and the studies highlighted above have emphasized the role of site 83 as primarily mediating kinetic, rather than spectral, differences among rhodopsin pigments. Accordingly, the rate of retinal release (corresponding to rate of decay of the active state) accelerates markedly upon introduction of the N83D mutation, consistent with what been found in other rhodopsin mutagenesis experiments (Sugawara et al. 2010; Dungan and Chang 2017).

The transition to D83 occurs in three species of Neotropical cichlid in our data set: closely related Mexican species Nosferatu bartoni (also called Herichthys bartoni) and Herichthys cyanoguttatus likely evolved in lotic (i.e., fast-moving) clear streams and clear water lakes in Mexico, and continue to inhabit these environments today (Říčan et al. 2016, Qvist and Evjeberg 2009), and the Antillean *Nandopsis haitiensis*, which also inhabits clear lagoons and rivers (Stawikowski and Werner 1998) (supplementary fig. S8, Supplementary Material online). It is possible that N83, which provided enhanced dim light sensitivity, is no longer essential in clear water environments, where it is likely the ancestors of *Herichthys*, *Nosferatu*, and *Nandopsis* evolved (Říčan et al. 2016). Dark adaptation (recovery of rods from bleaching) is limited by decay of the meta II state; consequently, faster rates of retinal release may be advantageous in clearer environments where partial light bleaches may occur more frequently (Ala-Laurila 2006). Together, these results open several additional lines of inquiry concerning the effect of rhodopsin amino acid variation on Neotropical cichlid vision. First, Central American cichlid RH1 pigments that do not have the N83D mutation may be kinetically and/or spectrally tuned by other amino acid mutations, particularly in lineages inhabiting clear water environments. Second, additional mutagenesis work targeting sites that may evolve in concert with D83 in *Herichthys* and *Nosferatu* (e.g., 123 and 166; supplementary fig. S8, Supplementary Material online), as well as variable sites in South American cichlids (217, 299) will further elucidate functional differences among cichlid rhodopsin proteins and how such variation is tuned. Third, whether clear water-dwelling South American cichlids have variation at other rhodopsin sites that may accomplish a similar protein phenotype to the N83D mutation remains an open question. For instance, the S299A mutation occurs in several South American cichlid lineages (e.g., *Crenicichla*, *Teleocichla*) common in clear South American drainages such as the Xingu river basin (Albert and Reis 2011). Similarly to N83D, S299A also shortens the retinal release half-life of the active meta II conformation in other vertebrates (Dungan and Chang 2017).

It is important to note that while in this study we find strong evidence for positive selection across Neotropical cichlids, and substantial functional effects mediated by an amino acid substitution, in addition to (and often in absence of) opsin structural variation, visual changes coincident with rapid habitat transitions, activity pattern, depth, etc. may also be accomplished through other mechanisms in the visual system. For instance, recent work in Midas cichlids (found in Central American crater lakes) found that modifications to their visual system in response to turbid versus clear environments is not achieved through amino acid substitutions in opsin genes. Instead, increases in lens transmission, differential expression of opsins, and usage of A1 chromophore (which blue-shifts visual pigment absorbance relative to the A2 chromophore frequently found in freshwater fishes), accompanies parallel evolution of species in clearwater lakes (Torres-Dowdall et al. 2017). Future work on Neotropical cichlid visual systems could perhaps target a genus-level investigation of lineages with rhodopsin site D83 (e.g., across *Herichthys* and *Nosferatu*) to provide additional insight into the extent of the variation found at this important amino acid site. Furthermore, investigation of this N83D mutation may be coupled with analyses of differential opsin expression and lens transmittance, and such properties could be contrasted with turbid water-dwelling relatives.

Conclusions

Studies of Neotropical cichlid opsin diversity offer an important opportunity to investigate visual system evolution in fishes across broad temporal and spatial scales, and across a suite of different aquatic environments. We used a broad cross-species exon capture approach to sequence rhodopsin

across the Neotropical cichlid clade, obtaining genus-level representation for the majority of Neotropical lineages. Clade model analyses tested the hypothesis that the renewed ecological opportunity encountered by Central America-invading cichlid lineages promoted divergent positive selection on the dim light visual pigment. Contrary to previous findings, we did not detect divergent selection on rhodopsin mediated by ecological differences between lake and riverine habitats; rather, further sampling of riverine cichlids reveals that rhodopsin is under stronger levels of positive selection in Central American species overall, which includes all currently sampled Neotropical lake-dwelling taxa. Further sampling of Neotropical lake cichlids, however, would be needed to properly test this hypothesis.

In addition to differences in the strength of positive selection, we identified a number of sites likely mediating shifts in rhodopsin function in response to different spectral environments. We experimentally characterize the first Neotropical cichlid visual pigment in vitro, and use site directed mutagenesis of the important rhodopsin site 83 to reveal that this site mediates kinetic differences with respect to the active state pike cichlid rhodopsin. Light-activated wild-type pike cichlid rhodopsin (N83) was shown to have markedly extended retinal release rates, suggesting enhanced stability of the active meta II state contributing to increased photosensitivity in dim habitats. The N83D substitution, found in three clear water-dwelling Central American cichlid lineages, significantly accelerates the release of retinal from the light-activated pigment. This kinetic change may promote more rapid recovery of rhodopsin function following activation, an advantageous property in environments with increased light availability. Further visual ecological differences between the Central and South American lineages, particularly pertaining to nuptial colouration and sexual selection, may be revealed upon comparative investigations of cone opsin genes in these groups.

Materials and Methods

DNA Extraction

Neotropical cichlid tissue samples were obtained from both aquarium and wild-caught cichlid specimens and are deposited the Royal Ontario Museum's ichthyology collection. Tissue vouchers are listed in supplementary table S1, Supplementary Material online. Genomic DNA was extracted from muscle tissue with a QIAGEN DNeasy kit (Qiagen Inc, Santa Clara CA, USA), with the addition of RNAse A (Qiagen) following the manufacturer's protocol with the exception that final elutions used 2 × 50 µl ddH20 for a total of ~100 µl per sample. Library preparation and sequencing was performed at the Donnelly Sequencing Centre (University of Toronto).

Rhodopsin Sequencing and Assembly

Full-length RH1 coding sequences (1047 bp) from 101 species of Neotropical cichlid were obtained through cross-species targeted exon capture (described in Ilves and López-Fernández 2014; Ilves et al. 2017). Briefly, rhodopsin probes were designed from the African riverine *Oreochromis niloticus* (Nile tilapia) rhodopsin, and used to enrich extracted Neotropical cichlid gDNA for the region of interest. Full-length rhodopsin sequences were assembled using a custom assembly pipeline with BWA (Li 2013) for guided assembly against the *Oreochromis niloticus* and *Crenicichla frenata* rhodopsin sequences and the mpileup-bcf-vcfutils (Samtools) pipeline for consensus generation (see Schott et al. 2017 for full details). Average completeness of assembled RH1 reads was 99.3% across all Neotropical cichlids, with at least 10× depth of coverage. Assembled sequences did not differ between the data sets assembled with the two different reference sequences. We combined these data with three additional Neotropical cichlid rhodopsin sequences from Genbank for a total of 104 sequences (supplementary table S1, Supplementary Material online) (Weadick et al. 2012; Torres-Dowdall et al. 2015). Sequence data from two non-visual genes, GPR85 and ENC1 (frequently used phylogenetic markers in fishes; Betancur-R et al. 2013), were obtained using the exon capture approach described above for use as controls. Where applicable, select captured RH1 sequences ($n = 23$) were compared against RH1 sequences from the same species obtained via Sanger sequencing (Schott et al. 2014; Torres-Dowdall et al. 2015) to ensure accuracy.

Alignment and Phylogenetic Analyses

The rhodopsin sequences (104 species total) were aligned using MUSCLE codon alignment implemented in MEGA (Kumar et al. 2016). Rhodopsin gene trees for all Neotropical cichlids, as well as South and Central American cichlids separately, were estimated with PhyML 3 (Guindon et al. 2010). ML analyses were run under the GTR + G + I model with a BioNJ starting tree, best of NNI and SPR tree improvement, and aLRT SH-like branch support.

Molecular Evolutionary Analyses

To ensure monophyly of the major Neotropical cichlid tribes, analyses were performed on a species tree with established relationships (López-Fernández et al. 2010; Říčan et al. 2016; Ilves et al. 2017), with additional analyses carried out on a tree with a different, conflicting placement of the genus *Nandopsis* (supplementary fig. S2, Supplementary Material online). The placement of this genus had no effect on the results (supplementary table S4, Supplementary Material online). Two additional Neotropical lake cichlid rhodopsin sequences obtained from Genbank were placed on this topologically constrained species tree with RAxML (10000 runs; GTR + gamma model) (Stamatakis 2014).

To estimate the strength and form of selection acting on rhodopsin, the alignment, along with the species phylogeny (López-Fernández et al. 2010; Ilves et al. 2017), was analyzed with the codeml package of PAML 4 using the random sites models (M0, M1a, M2a, M2a_rel, M3, M7, M8a, and M8) (Yang 2007; Weadick and Chang 2012). Since PAML does not incorporate rate variation at synonymous sites (d_S), we also analyzed all Neotropical, Central, and South American cichlid data sets using the HYPHY FUBAR model (Kosakovsky Pond and Frost 2005; Kosakovsky Pond et al. 2005; Murrell

et al. 2013) implemented on the Datamonkey webserver (Delport et al. 2010) which is similar to the PAML random sites models, but allow for independently estimated d_S. Several different subsets of the RH1 data set were analyzed with the random sites models of PAML and FUBAR in order to assess differences in selective pressure among the various partitions: the full RH1 data set (Neotropical cichlids), the South American cichlids, Central American cichlids, and the Heroini, Cichlasomatini, and Geophagini tribes. Random sites analyses were repeated on the All Neotropical, South, and Central American data sets using the maximum likelihood gene trees.

PAML Clade Model C analyses (Bielawski and Yang 2004) were carried out on the species tree, using the M2a_rel model as the null model, which does not permit divergence in the foreground clade but allows for an unconstrained ω (Weadick and Chang 2012). Lineages encompassing the various clade model partitions performed on the Neotropical cichlid tree are shown in figure 1 and listed in detail in supplementary table S1, Supplementary Material online. To test whether differences in phylogenetic history in the most species-rich tribes (Cichlasomatini, Heroini, Geophagini) have driven rhodopsin divergence, each tribe was isolated as a foreground clade relative to the remainder of the tree, and then isolated as three separate partitions against the background to test if all three were undergoing divergent selection relative to each other and the background. To test whether ecological variables, in this case lake versus riverine environments, have driven selection on rhodopsin, Neotropical lake lineages as identified in Torres-Dowdall et al. (2015) were isolated as a foreground partition. Finally, we tested whether geographic differences, i.e., invasion of Central America, drove divergence in rhodopsin by isolating all Central American cichlid lineages in a foreground partition (Central vs. South America partition). An alternative partition was also tested that retained any embedded South American lineages placed in the foreground ("Central America clade") (supplementary fig. S3, Supplementary Material online). Nonnested CmC partitions were compared with AIC (Schott et al. 2014). The best-fitting CmC model was compared with a null model where the divergent site class of the foreground clade was constrained to equal one, creating a nested model with one fewer parameter to explicitly test for $\omega > 1$ (Chang et al. 2012; Schott et al. 2014). If the LRT between the unconstrained ($\omega > 1$) versus constrained model ($\omega = 1$) is significant, there is evidence for positive selection in this foreground partition.

To test whether differences between lake and riverine environments in Central American cichlids has influenced divergence in rhodopsin, Clade Model analyses isolating lacustrine species as a separate partition were also performed on the Central American cichlid subset (29 species total).

Finally, to ensure any significant divergence found in the full Neotropical cichlid RH1 data set was not due to genome-wide increases in molecular evolutionary rates or an artifact of small population size, we tested for evidence of significant divergent selection in 102 partial coding sequences of non-visual control genes GPR85 (738 bp) and ENC1 (1167 bp) obtained via the sequence capture approach. These sequences were tested for divergent selection under the best-fitting clade model partition for RH1.

Protein Expression and Functional Characterization

Wild-type rhodopsin coding sequences for the pike cichlid *Crenicichla frenata* (GenbankID: JN990733) were synthesized using GeneArt (Invitrogen) with 5′ and 3′ restriction sites for insertion into the p1D4-hrGFP II expression vector (Morrow and Chang 2010). The pike cichlid was chosen for this experiment as it had one of the best characterized visual systems among Neotropical cichlids, and spectral absorbance of its rods had been measured and therefore could be directly compared against measurements of expressed rhodopsin pigment (Weadick et al. 2012). The N83D mutation in pike cichlid wild-type RH1 was generated via site-directed mutagenesis (QuickChange II, Agilent). The N83D mutant was verified using a 3730 DNA Analyzer (Applied Biosystems) at the Centre for Analysis of Genome Evolution and Function (CAGEF) at the University of Toronto. Expression vectors containing wild-type and mutant rhodopsin were transiently transfected into HEK293T cells (Lipofectamine 2000, Invitrogen) and harvested after 48 h together with a bovine rhodopsin control. Expressed proteins were regenerated with 11-*cis*-retinal, solubilized in 1% N-dodecyl-D-maltoside, and purified using the 1D4 monoclonal antibody in the dark. We measured the UV-visible absorption spectra of purified rhodopsin samples using a Cary 4000 double-beam spectrophotometer (Agilent) at 20 °C in the dark, and again following 60 s of bleaching with white light to confirm activation. Difference spectra were calculated by subtracting light spectra absorbance values from dark spectra absorbance values. Spectral sensitivity (λ_{max}) values were estimated by fitting a standardized template to the dark absorbance spectra (Govardovskii et al. 2000). To determine release rates of all-*trans*-retinal from light activated rhodopsin (meta II), we measured intrinsic increases in tryptophan fluorescence that occur as residues are unquenched during chromophore exit from the binding pocket (Farrens and Khorana 1995). Fluorescence signals were measured with a Cary Eclipse fluorescence spectrophotometer (Agilent) at 20 °C following a 30s light bleach (Morrow and Chang 2015; van Hazel et al. 2016). Retinal release half-life ($t_{1/2}$) values were estimated by fitting the fluorescence time courses to first-order exponential curves ($y = y_0 + a(1 - e^{-kx})$, where $t_{1/2} = \ln(2)/k$). All curve fitting resulted in r^2 values >0.95. Retinal release half-life values were compared with a two-tailed *t*-test (unequal variance).

Protein Crystal Structure Visualization

The bovine meta II crystal structure (PDB 3PQR; Choe et al. 2011) was visualized using MacPyMOL (Schrodinger, LLC). The mutagenesis wizard in PyMOL was used to substitute D83 for N83 in the structure.

Data Accessibility

All sequences are deposited in the Genbank database and accession numbers are listed in supplementary table S1, Supplementary Material online.

Supplementary Material

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

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