Functional Shifts in Bat Dim-Light Visual Pigment Are Associated with Differing Echolocation Abilities and Reveal Molecular Adaptation to Photic-Limited Environments

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Abstract

Bats are excellent models for studying the molecular basis of sensory adaptation. In Chiroptera, a sensory trade-off has been proposed between the visual and auditory systems, though the extent of this association has yet to be fully examined. To investigate whether variation in visual performance is associated with echolocation, we experimentally assayed the dim-light visual pigment rhodopsin from bat species with differing echolocation abilities. While spectral tuning properties were similar among bats, we found that the rate of decay of their light-activated state was significantly slower in a nonecholocating bat relative to species that use distinct echolocation strategies, consistent with a sensory trade-off hypothesis. We also found that these rates of decay were remarkably slower compared with those of other mammals, likely indicating an adaptation to dim light. To examine whether functional changes in rhodopsin are associated with shifts in selection intensity upon bat *Rh1* sequences, we implemented selection analyses using codon-based likelihood clade models. While no shifts in selection were identified in response to diverse echolocation abilities of bats, we detected a significant increase in the intensity of evolutionary constraint accompanying the diversification of Chiroptera. Taken together, this suggests that substitutions that modulate the stability of the light-activated rhodopsin state were likely maintained through intensified constraint after bats diversified, being finely tuned in response to novel sensory specializations. Our study demonstrates the power of combining experimental and computational approaches for investigating functional mechanisms underlying the evolution of complex sensory adaptations.

Key words: **evolution of bat vision, meta II stability, evolution of protein function, likelihood-based codon models, clade models of molecular evolution, visual ecology.**

Article

Introduction

Comprising about 20% of all living mammals, bats are one of the largest and most striking mammalian radiations to have diversified in the nocturnal environment (Simmons 2005; Teeling et al. 2005). This is largely attributed to their self-powered flight and sophisticated ability to acoustically navigate in the dark using highly specialized laryngeal echolocation (Jones and Teeling 2006). Although echolocation is a compelling adaptation to explore nocturnal environments, this sensory innovation is not ubiquitous among bats. Old World fruit bats (Pteropodidae) are incapable of generating laryngeal echolocation calls, though tongue and wing-click-based echolocation have been reported in some pteropodid species (Yovel et al. 2011; Boonman et al. 2014). In contrast, all remaining bat families exhibit laryngeal echolocation (fig. 1A), which may be classified into two differing calling strategies. Low-duty cycle (LDC) echolocation represents the calling strategy adopted by the majority of bat species (Kalko and Schnitzler 1989; Brinkløv et al. 2009, reviewed in Jones and Teeling 2006), whereas high-duty cycle (HDC) calls are restricted to representatives of families Rhinolophidae and Hipposideridae (Schuller 1980; Vogler and Neuweiler 1983), and the mormoopid *Pteronotus parnellii* species complex (Dávalos 2006; Pavan and Marroig 2016). HDC arguably represents a more sophisticated type of echolocation ability as it allows for pulses to be separated from echoes based on frequency through Doppler shift compensation (Trappe and Schnitzler 1982; Hiryu et al. 2005), rather than based on time as occurs in LDC species (Kalko and Schnitzler 1989; Holderied and von Helversen 2003, reviewed in Fenton et al. 2012).

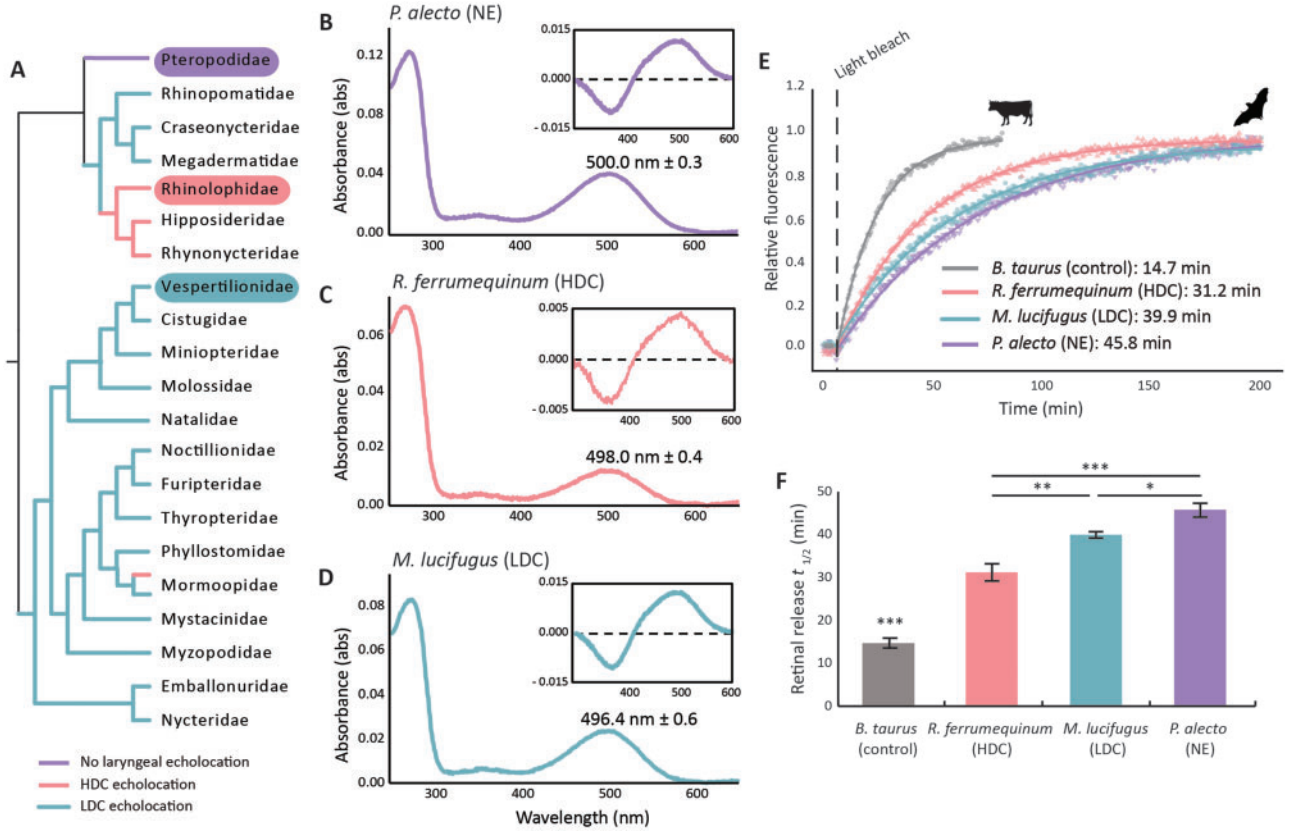

Fig. 1. Functional properties of bat rhodopsin in species with differing echolocation abilities. (*A*) Interfamily relationships of Chiroptera depicting diversity of echolocation abilities. Representative species of highlighted families were selected for in vitro protein expression. (*B–D*) Spectral absorbance curves of dark state rhodopsin and dark-light difference spectra (inset) of *Pteropus alecto* (*B*), *Rhinolophus ferrumequinum* (*C*), and *Myotis lucifugus* (*D*). Spectral absorbance peaks (λ_{Max}) are indicated and were estimated according to curve-fitting methods by (Govardovskii et al. 2000). (*E*) Fluorescence time courses of retinal release following rhodopsin light bleach in *P. alecto*, *R. ferrumequinum*, *M. lucifugus*, and bovine Rh1 control. Indicated half-lives ($t_{1/2}$) were estimated by fitting time courses to first-order exponential curves. (*F*) Statistical comparison of retinal release half-life averages where significant differences are observed between all three species of bats: *P. alecto* is the slowest, whereas *R. ferrumequinum* is the fastest, and *M. lucifugus* is intermediate. Retinal release half-lives of bats are all significantly slower than bovine. Error bars indicate SE. Significant differences are indicated by * ($P < 0.05$), ** ($P < 0.01$), and *** ($P < 0.001$). NE, no laryngeal echolocation; HDC, high-duty cycle echolocation; LDC, low-duty cycle echolocation.

The diversification of bats in nocturnal environments, accompanied by the evolution of echolocation, likely imposed varying constraints on the evolution of other sensory modalities, particularly the visual system. Although bats have eyes adapted to dim-light conditions (Neuweiler 2000) and rely on vision at night to some extent, particularly for long-range navigation (Boonman et al. 2013), anatomical, physiological, and molecular variation in the visual system among different lineages is often associated with differing echolocating abilities and provide support for a potential sensory trade-off. Old World fruit bats, which lack the ability of laryngeal echolocation, usually have larger eyes, specialized connections between retina and visual nuclei, and enlarged vision-associated areas in the brain (Baron and Jolicoeur 1980; Pettigrew 1986; Liu et al. 2015; Thiagavel et al. 2018) that likely support visually guided behaviors at night (Nelson 1965). Most laryngeal echolocating bats, by contrast, have smaller eyes, generally simpler visual systems and smaller visual centres in the brain (Pettigrew et al. 1988; Baron et al. 1996), which occur in parallel with a number of neuroanatomical specializations of the auditory system (Covey 2005). However, even among echolocating bats, a range of visual abilities exist. Vision appears to play a more important role in the biology of bats with less sophisticated echolocation abilities (LDC), which have been demonstrated to rely on visual cues for orientation, location of roosts, and even detection of prey (Bradbury and Nottebohm 1969; Bell 1985; Eklöf and Jones 2003; Ruczyński et al. 2011). On the other hand, the sophisticated echolocation abilities of HDC lineages are thought to have been accompanied by additional auditory specializations, such as the development of an acoustic fovea (Vater et al. 1985). This may have occurred at the expense of vision as observed by lower visual threshold (Liu et al. 2015), and is also supported by the loss of function of several vision-associated genes in HDC lineages (Zhao, Rossiter, et al. 2009; Shen et al. 2013; Dong et al. 2017).

Under typical nocturnal conditions, vision is initiated by the dim-light visual pigment rhodopsin (Rh1). This photosensitive transmembrane protein is a G protein-coupled receptor (GPCR) expressed in the outer segment of rod photoreceptor cells in the retina and comprises a protein moiety covalently bound to a 11-*cis* retinal chromophore (Palczewski et al. 2000).

Absorption of a photon triggers isomerization of chromophore to all-*trans* retinal, inducing a series of conformational changes in rhodopsin through several intermediates, including the metarhodopsin II (meta II) state (Choe et al. 2011). Meta II is the biologically active state that binds the G protein transducin and initiates the visual transduction cascade, ultimately resulting in rod hyperpolarization and propagation of a neural signal through the retina (Ebrey and Koutalos 2001). Release of the all-*trans* retinal through hydrolysis of the covalent bond followed by uptake of a new 11-*cis* molecule allow for rhodopsin to regenerate and regain photosensitivity (i.e., dark adaptation, Lamb and Pugh 2004).

Functional properties of rhodopsin are thought to play a major role in visual system specialization to photic-limited environments. Among those properties, the wavelength of maximum absorbance (λ_{Max}) has been the best investigated, along with the molecular underpinnings underlying this function (Hunt et al. 1996, 2001; Yokoyama et al. 2008; Dungan et al. 2016). Shifts in visual pigment λ_{Max} in response to spectral composition of photic environment are usually considered an adaptation to maximize photon capture and allow photoreceptor activation particularly when ambient light is limited (Bowmaker 2008). Although not as well investigated, nonspectral functional properties of rhodopsin may also be the target of evolutionary innovation, ultimately contributing to organismal fitness (Castiglione et al. 2017; Dungan and Chang 2017; Hauser et al. 2017). In particular, the light-activated rhodopsin (meta II) state plays a fundamental role in modulating the biochemical visual cascade (Kojima et al. 2014). Shifts in kinetic rates of meta II formation or decay are also thought to influence rod photosensitivity and visual performance in varying light environments, but have only recently become more appreciated through comparative in vitro approaches (Sugawara et al. 2010; Bickelmann et al. 2015; Dungan and Chang 2017; Hauser et al. 2017).

Although functional shifts in dim-light visual pigments have usually been associated with adaptive changes to photic environment in a number of vertebrate groups, rhodopsin function has been remarkably underexplored in bats. Microspectrophotometry measurements (Feller et al. 2009) and in vitro characterization of rhodopsin (Sugawara et al. 2010) suggest little variation in λ_{Max} between different bat species, which have peak sensitivity typical of other vertebrate Rh1 pigments. Conversely, kinetic differences in the rate of meta II formation have been observed in bats, indicating differences in photosensitivity and dim-light visual ability among species that may prioritize distinct sensory strategies while foraging (Sugawara et al. 2010). Surprisingly, differences in rhodopsin function have not been investigated yet in the context of other sensory adaptations of bats, such as the diverse echolocation abilities observed in Chiroptera, which may induce trade-offs with vision.

In contrast to experimental studies, much effort has been dedicated to computationally characterizing the selective pressures underlying bat *Rh1* evolution (Zhao, Ru, et al. 2009; Shen et al. 2010). Because of the ability of bats to perform visually guided behaviors in dim-light, most studies have focused on identifying signatures of adaptive molecular evolution in the protein-coding sequence, but failed to find evidence for positive selection acting on *Rh1*, either among Chiroptera or in specific bat lineages, identifying instead pervasive purifying selection acting upon bat *Rh1* sequences (Zhao, Ru, et al. 2009; Shen et al. 2010). By contrast, shifts in the intensity of selection in bat *Rh1* have been proposed to occur in response to differing echolocation abilities, which could provide support for potential differences in visual pigment function between species with distinct sensory specializations (Zhao, Ru, et al. 2009). However, these shifts in selection have been shown to emerge from comparisons of an inadequate null model that is prone to false positives (Weadick and Chang 2012).

To provide a better understanding of rhodopsin adaptations to dim light and evolution in response to auditory sensory specializations, we experimentally investigated rhodopsins of bat species representing each main type of echolocation and functionally characterized the purified visual pigments in vitro to determine both the peak wavelength of maximum absorbance as well as the kinetic rates of metarhodopsin II decay. We hypothesized that rhodopsin would exhibit functional variation in species with distinct echolocation abilities and predicted that functional differences would be associated with varying visual performances in dim light. While λ_{Max} showed minimal variation, we found that kinetic rates varied significantly among bats with differing echolocation abilities and were consistent with an expected reliance on dim-light vision. Interestingly, we found that rates of light-activated rhodopsin (meta II) decay in bats are among the slowest of any mammal studied to date, suggesting a unique and possibly bat-specific adaptation for vision in photic-limited conditions. To determine whether functional differences in bat rhodopsins were mediated by changes in evolutionary rates, we combined our experimental in vitro assays with computational analyses of selective constraint in bat *Rh1* sequences. We used codon-based likelihood clade models of sequence evolution to test whether distinct sensory specializations or life history of bats mediate shifts in selection constraint intensity in *Rh1* coding sequences. These models provide a useful statistical framework to test for long-term shifts in selection pressures associated with changes in ecology and life histories, and have been applied to the study of a variety of organisms and systems (Schott et al. 2014, 2018; Torres-Dowdall et al. 2015; Van Nynatten et al. 2015; Baker et al. 2016; Dungan et al. 2016; Castiglione et al. 2017, 2018; Hauser et al. 2017; Gutierrez et al. 2018). While no variation in constraint was observed in response to differing echolocation abilities, we detected a significant increase in selection accompanying the diversification of Chiroptera. Combined, our in vitro and in silico analyses suggest that adaptive changes in rhodopsin function occur in response to differing echolocation abilities and that large shifts in visual pigment kinetics that facilitate vision in dim light may have been acquired in ancestral lineages leading to bats, being maintained in extant species through strong evolutionary constraint.

Results

Bats with Distinct Echolocation Abilities Possess Rhodopsins with Similar Spectral Tuning Peaks

We used a heterologous expression system to express and functionally characterize in vitro rhodopsin pigments of bat species with differing echolocation, and potentially dim-light visual abilities: the nonlaryngeal echolocating (NE) *Pteropus alecto*, the high-duty cycle (HDC) echolocating *Rhinolophus ferrumequinum*, and the low-duty cycle (LDC) echolocating *Myotis lucifugus*. The expressed, purified wild-type bat rhodopsin pigments exhibited similar peaks of maximal absorbance (λ_{Max}) between the different species (fig. 1*B–D* and supplementary table S1, Supplementary Material online). The HDC *R. ferrumequinum* rhodopsin produced a λ_{Max} of 498.0 nm ± 0.4 (fig. 1*C*), similar to a different *Rhinolophus* sp. rhodopsin previously reported (Sugawara et al. 2010). In contrast, the LDC *M. lucifugus* rhodopsin exhibited a λ_{Max} of 496.4 nm ± 0.6 (fig. 1*D*), slightly blue-shifted compared with *R. ferrumequinum*. The NE *P. alecto* rhodopsin, on the other hand, yielded a slightly red-shifted λ_{Max} of 500.0 nm ± 0.3 (fig. 1*B*). All bat Rh1 pigments expressed heterologously in vitro produced functional pigments and activated in the presence of light, exhibiting a λ_{Max} of ≈ 380 nm following a 30 s light bleach (fig. 1*B–D*, inset), which is characteristic of the active metarhodopsin II (meta II) photointermediate state (Farrens and Khorana 1995; Sugawara et al. 2010).

Shifts in Rhodopsin Kinetics Occur in Bat Species with Differing Echolocation Abilities

We next used a fluorescence assay to infer the stability of the meta II state by monitoring the rate of *all*-trans retinal release from the rhodopsin binding pocket following light activation (Farrens and Khorana 1995; Schafer et al. 2016). In contrast to minimal variation in λ_{Max}, significant differences in retinal release half-lives ($t_{1/2}$) were observed among all three bat species (fig. 1*E* and *F*). The NE *P. alecto* rhodopsin produced the slowest rates of retinal release among all bats, with an estimated $t_{1/2}$ of 45.8 min ± 1.7, indicating significantly slower rates of meta II decay in this species relative to the other bat species examined (*t*-test $P < 0.05$, fig. 1*E* and *F* and supplementary table S2, Supplementary Material online). In contrast, the HDC *R. ferrumequinum* Rh1 exhibited a retinal release $t_{1/2}$ that was significantly shorter (31.2 min ± 2.0) compared with the other two bat species (*t*-test $P < 0.01$, fig. 1*E* and *F* and supplementary table S2, Supplementary Material online), suggesting faster rates of decay of the light-activated meta II conformation in the HDC species. Lastly, the retinal release $t_{1/2}$ of the LDC bat *M. lucifugus* was intermediate (39.9 min ± 0.7) and also significantly different from *P. alecto* and *R. ferrumequinum* (*t*-test $P < 0.05$, fig. 1*E* and *F* and supplementary table S2, Supplementary Material online). The significant variation in rates of retinal release among bats indicates that there are significant differences in meta II stability in species that utilize and/or prioritize different sensory modalities to navigate and forage. Because meta II corresponds to the signaling rhodopsin conformation, its stability may play a crucial role in signal transduction and amplification (Kojima et al. 2014), and therefore may have important implications for visual performance in photic-limited environments. This suggests that dim-light visual ability may differ in bats with differing echolocation capabilities.

Along with significant variation within bats, we also observed that the rates of retinal release in all bats were significantly different than a bovine rhodopsin control. Our half-life estimates for bovine rhodopsin ($t_{1/2}$ = 14.7 min ± 0.4, fig. 1*E* and *F*) were in agreement with prior literature (Farrens and Khorana 1995; Morrow and Chang 2015; Castiglione et al. 2017), but considerably shorter compared with all bat rhodopsins assayed in this study (*t*-test $P < 0.001$, supplementary table S2, Supplementary Material online). This suggests that meta II is significantly more stable in bats than in a model terrestrial rhodopsin. Although bovine rhodopsin is the best studied visual pigment, and has even served as a model for other GPCRs (Hofmann et al. 2009), recent studies have started to reveal greater variation in mammalian rhodopsin meta II stability than previously assumed (Bickelmann et al. 2012; Dungan and Chang 2017; Morrow et al. 2017). However, the striking differences in bat meta II stability relative to all other mammals studied to date are likely to represent functional differences associated with the unique life history and visual ecology of bats.

Long-Term Shifts in Selective Constraint in Bat Rh1 Do Not Occur in Response to Varying Echolocation Abilities

Because we identified significant functional differences in rhodopsin kinetics among bats, we next investigated whether variation in selection constraint in bat *Rh1* coding sequences occurred in response to differing sensory ecologies. In order to investigate these hypotheses, we first improved sampling in Neotropical bats by obtaining new rhodopsin sequences from the eye transcriptomes of six Neotropical species. These sequences, combined with recently published bat draft genomes (Parker et al. 2013), and others available in the public sequence databases, resulted in a data set that included 38 sequences, spanning about half of extant chiropteran families (supplementary table S11, Supplementary Material online). We analyzed this data set using random sites codon models (Yang 2007) to estimate overall form and strength of selection acting upon the Chiroptera *Rh1* sequences. Our random sites analyses were consistent between a maximum likelihood gene tree as well as a constrained species topology, and revealed that overall substitution rate ratios (d_N/d_S or ω) are very low in chiropteran *Rh1* sequences ($\omega_0 = 0.026$, supplementary tables S3 and S4, Supplementary Material online), which are comparable with previous literature (Zhao, Ru, et al. 2009; Shen et al. 2010). Significant among-site rate variation in ω, characteristic of functional protein-coding sequences, was also detected (M3 vs. M0, $P < 0.0001$), and no evidence for positive selection was observed (M8 vs. M8a, $P > 0.05$, supplementary tables S3 and S4, Supplementary Material online) as previously reported (Zhao, Ru, et al. 2009; Shen et al. 2010).

To examine whether long-term shifts in the intensity of selection in *Rh1* (hereafter also referred to as divergent selection, Schott et al. 2014, 2018; Baker et al. 2016) occur in response to the diverse echolocation abilities of bats, we analyzed the Chiroptera *Rh1* data set using codon-based clade models (CmC, Bielawski and Yang 2004), which allow a proportion of codon sites to evolve with a different ω along specified lineages in the phylogeny. We first isolated the nonlaryngeal echolocating species (NE) in the foreground partition and compared it to the echolocating lineages (fig. 2*A* and *B* and supplementary fig. S1 and table S5, Supplementary Material online), but found no support for different rates of ω in echolocating versus nonecholocating lineages (all comparisons are relative to the null model M2a_rel, which does not allow for divergent rates, Weadick and Chang 2012). Similarly, no evidence for significant differences in ω was observed when high-duty cycle (HDC) echolocation lineages were isolated in the foreground, and compared against nonecholocating (NE) and low-duty cycle (LDC) lineages. We also found no support for divergent selection underlying the evolution of *Rh1* when either the Old World or the New World HDC clades were individually tested as foreground partitions. Isolating NE and HDC partitions as separate foreground partitions, and comparing these against the remainder of the tree was not a significantly better fit either in comparison to the null M2a_rel model (supplementary table S5, Supplementary Material online). Finally, we also tested whether other ecological factors, such as diet, roosting behavior and foraging habitat, which may be associated with the use of visual information or exposure to varying light environment (Baron et al. 1996; Zhao, Rossiter, et al. 2009; Veilleux et al. 2013), produce shifts in selective constraint intensity in *Rh1*. While some of these ecological factors have been demonstrated to mediate divergent selection in other bat visual opsins (Gutierrez et al. 2018), we found that no partition model tested resulted in significant improvements over the null model in the bat *Rh1* data set (supplementary fig. S1 and table S7, Supplementary Material online).

Rhodopsin Experienced Increased Evolutionary Constraint following Bat Diversification

To investigate whether the functional differences in bat relative to other mammalian rhodopsins were associated with differing selective constraints underlying bat *Rh1* evolution, we carried out selection analyses using a larger data set that comprised 80 mammalian *Rh1* sequences, including representatives of crown mammalian groups along with bats (supplementary table S11, Supplementary Material online). We first analyzed this data set using random sites models and observed that mammal *Rh1* sequences have higher overall substitution rate ratios (M0, $\omega_0 = 0.036$) and a smaller proportion of sites (75%) under strong purifying selection (M3, $\omega_0 = 0.003$) compared with bats (89%, supplementary tables S3 and S4, Supplementary Material online). Next, we used clade models (CmC) to test for significant shifts in selective constraint (ω) in the branch leading to bats (i.e., episodic selection), or the entire Chiroptera clade (i.e., pervasive shift in ω following diversification) (fig. 2*A*). While no significant variation in evolutionary constraint was detected on the branch leading to Chiroptera, allowing a separate ω to be estimated for the entire bat clade resulted in a significantly better fit compared with the null M2a_rel model (fig. 2*C*). Our analysis supported a significant decrease in ω accompanying the diversification of bats, indicating a proportion of $\approx$ 20% of sites in bat *Rh1* are under significantly stronger purifying selection relative to other mammalian groups (supplementary tables S8 and S9, Supplementary Material online), as previously suggested (Zhao, Ru, et al. 2009). Intensified purifying selection in the clade containing the ancestral node of bats and all descending lineages, but not when the single branch leading to Chiroptera, was also supported by our analyses in PAML using Branch model (allows variation in ω along different lineages) and Clade model D (CmD, allows variation along lineages and codon sites, reviewed in Baker et al. 2016; Schott et al. 2018) (supplementary table S10, Supplementary Material online).

Discussion

In this study, we used a combination of in vitro protein assays and computational analyses to test whether the molecular evolution of the dim-light visual pigment rhodopsin is constrained by the differing echolocation capabilities and the distinct evolutionary history of bats. We expressed the rhodopsin visual pigment of the nonlaryngeal echolocating species *Pteropus alecto*, along with two echolocating species, the high-duty cycle *Rhinolophus ferrumequinum* and low-duty cycle *Myotis lucifugus*, and functionally characterized the pigments in the laboratory using absorbance and fluorescence-based spectroscopic assays. While little variation in bat rhodopsin spectral tuning was observed, our fluorescence assay revealed significant differences in the retinal release half-life ($t_{1/2}$) and in the stability of the light-activated rhodopsin (meta II state) between all three species, suggesting that differences in visual performance in dim light are likely associated with differing echolocation abilities in bats. Interestingly, retinal release $t_{1/2}$ of all bats was significantly slower compared with a bovine rhodopsin control, indicating marked functional differences in rhodopsin between bats and other mammals. We then tested whether functional differences in bat rhodopsin were associated with shifts in selective constraint (ω) in *Rh1* coding-sequences. While no divergent selection was identified in response to differing echolocation abilities, we found evidence for a significant increase in the intensity of evolutionary constraint accompanying the diversification of Chiroptera. Here, we discuss our results in the context of the ecology, evolutionary history and sensory biology of bats, and highlight the power of combining computational and experimental approaches to study the evolution of visual pigments.

Minimal Spectral Tuning Shifts in Bat Rhodopsin

We expressed the rhodopsin pigment of three species of bats with differing echolocation abilities and found that while each visual pigment produced a distinct peak of maximum absorbance, only small variation was detected in species with distinct sensory specializations. Relative to the HDC species

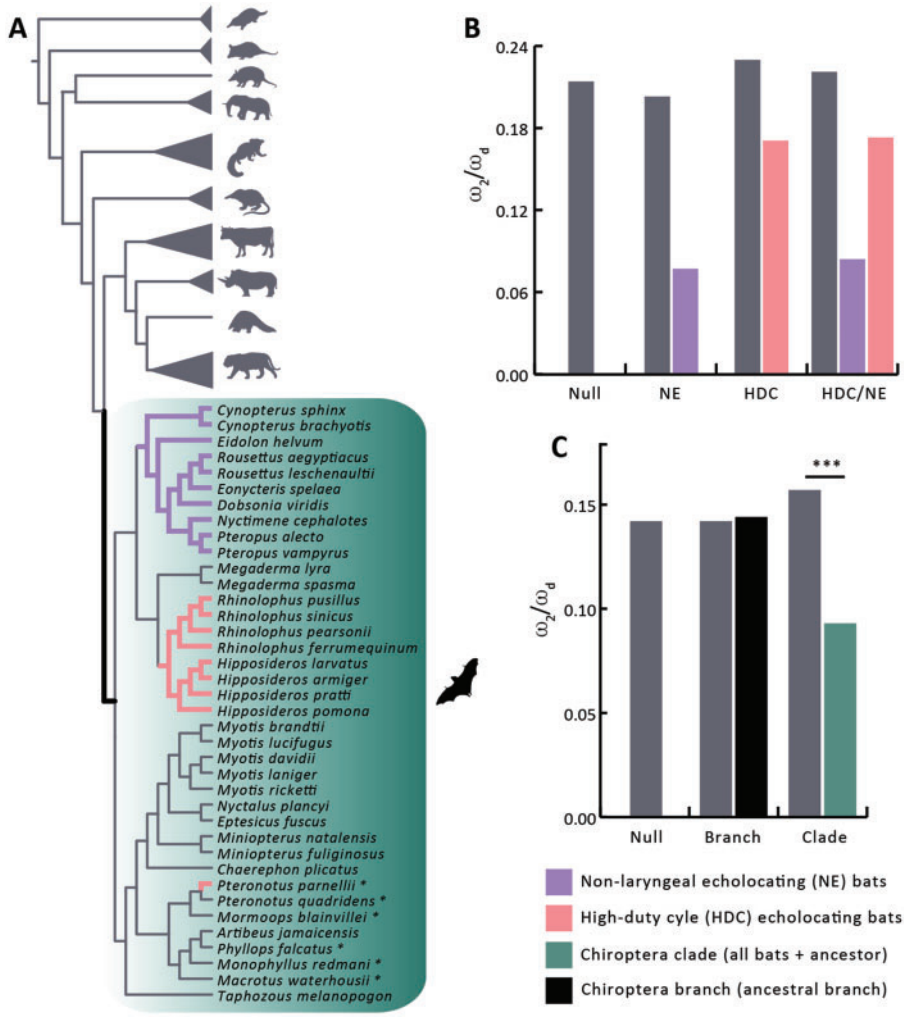

Fig. 2. PAML analyses of long-term shifts in the intensity of selective constraint acting upon bat *Rh1* sequences. (*A*) Schematic species topology (according to Teeling et al. 2005; Foley et al. 2016; Tarver et al. 2016) depicting *Rh1* sampling across mammals and bats. * indicates new sequences obtained in this study from eye transcriptomes. No laryngeal echolocating (NE) and high-duty cycle echolocating (HDC) lineages were highlighted and tested as foreground partitions for variation in rates of evolution using the bat *Rh1* subset. The branch leading to as well as the entire Chiroptera clade are also highlighted and were tested as foreground partitions for shifts in selection constraint intensity using the full mammal *Rh1* data set. (*B*) Comparison of divergent omega classes ω_d in CmC PAML analysis along with respective ω_2 from the null M2a_rel model using the bat *Rh1* subset. No significant differences in rates of evolution relative to the null model were identified in response to differing echolocation abilities. (*C*) Comparison of divergent omega classes ω_d in CmC PAML analysis and with corresponding ω_2 from the null model M2a_rel using the full mammal *Rh1* data set. Significant increase in selection constraint intensity was identified in the entire clade of bats but not in the single branch leading to Chiroptera. *** denotes $P < 0.001$.

R. ferrumequinum ($\lambda_{Max} \approx 498$ nm), the nonecholocating *P. alecto* rhodopsin was slightly red-shifted ($\lambda_{Max} \approx 500$ nm), whereas the LDC *M. lucifugus* produced a pigment with a minor blue shift ($\lambda_{Max} \approx 496$ nm), which is consistent with previous microspectrophotometry measurements of intact photoreceptors (Feller et al. 2009), and also similar to rhodopsin pigment of another LDC bat species previously expressed in vitro (Sugawara et al. 2010). Small shifts in rhodopsin λ_{Max} have been shown to occur in response to amino acid variation at sites 83 and 183 (Yokoyama et al. 2008; Dungan et al. 2016; van Hazel et al. 2016; Hauser et al. 2017) which are variable in *M. lucifugus* (N83) and *R. ferrumequinum* (L183). Mutation N83D has been shown to produce a 2 nm red-shift in a bat rhodopsin background (Sugawara et al. 2010), which could explain the minor blue-shift in *M. lucifugus* Rh1 relative to *R. ferrumequinum* (D83). In contrast, mutation L183M results in a 2 nm red-shift (Yokoyama et al. 2008), which may account for λ_{Max} differences between *R. ferrumequinum* and *P. alecto*.

These observed differences in rhodopsin λ_{Max} among bats are minimal and therefore unlikely to represent an adaptation to their spectral environment. Instead, the maximum absorbance peak of the three species we expressed in this study are within the range ($\approx$ 495–500 nm) of typical terrestrial mammalian rhodopsin pigments (fig. 3*A*, Yokoyama et al. 2008; Bickelmann et al. 2012; Morrow et al. 2017). Usually, adaptive shifts in visual pigment spectral tuning are observed in association with the composition of ambient light spectrum of an

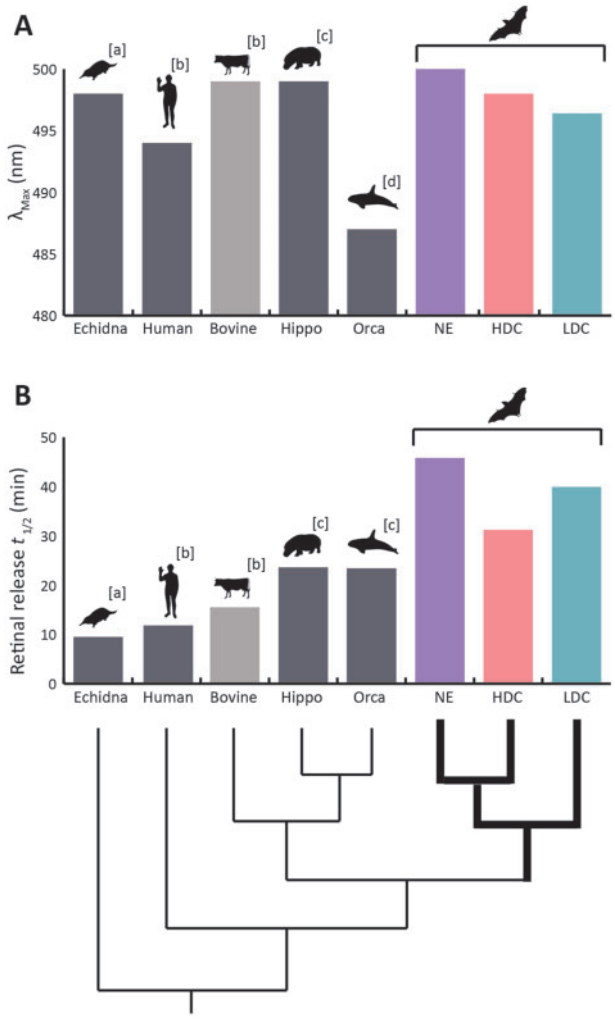

Fig. 3. Schematic comparison of rhodopsin (*A*) spectral absorbance peak (λ_{Max}) and (*B*) retinal release half-life ($t_{1/2}$) averages across bats and selected mammalian lineages according to [*a*] (Bickelmann et al. 2012), [*b*] (Morrow et al. 2017), [*c*] (Dungan and Chang 2017), and [*d*] (Dungan et al. 2016). NE, no laryngeal echolocation; HDC, high-duty cycle echolocation; LDC, low-duty cycle echolocation.

animal's habitat (Bowmaker 2008). In rhodopsin, blue-shifts are usually observed in aquatic lineages that dwell in underwater environments where light is predominantly blue-shifted, presumably maximizing photon capture and improving photosensitivity (Hunt et al. 1996, 2001; Dungan et al. 2016). In contrast, spectral composition of light in terrestrial environments is not as variable. Shifts in the ambient light spectrum are most noticeable at twilight and under starlight (Johnsen et al. 2006), and the spectral composition of ambient light is also influenced by vegetation coverage (Veilleux and Cummings 2012). However, spectral composition of environmental light in terrestrial environments is very similar from daytime to nighttime, only several orders of magnitude dimmer at night (reviewed in Warrant and Johnsen 2013). This absence of pronounced shifts in ambient light spectrum in terrestrial relative to aquatic environments may explain why rhodopsin λ_{Max} is fairly conserved across terrestrial mammals sampled to date (fig. 3A).

The Role of Light-Activated Rhodopsin Kinetics in Bat Visual Ecology

Unlike spectral tuning, pronounced kinetic differences in rhodopsin were detected among the three species of bats. The rate at which all-*trans*-retinal is released from the signaling rhodopsin conformation, which also corresponds to the rate of light-activated rhodopsin (meta II) decay (Schafer et al. 2016), differed significantly in bats with distinct echolocation capabilities. In the nonecholocating *P. alecto*, rhodopsin produced the slowest rates of retinal release, suggesting that meta II decays at a slower rate and is thus more stable in this species. In contrast, the fastest rates of retinal release were observed in *R. ferrumequinum*, indicating relatively lower stability and faster decay of the signaling conformation in a species that uses sophisticated HDC echolocation, whereas meta II exhibited intermediate stability in the LDC bat *M. lucifugus* relative to the other two species. Meta II underlies signaling of rod photoreceptor cells by initiating and likely modulating the biochemical visual cascade (Kojima et al. 2014; Schafer et al. 2016; Van Eps et al. 2017), and kinetics of the light-activated state has been described as an evolutionary innovation to photic-limited environments (Sugawara et al. 2010; Bickelmann et al. 2015; Dungan and Chang 2017; Hauser et al. 2017). Meta II stability is also associated with dark adaptation and regeneration of rhodopsin, which is determined by the rate of *all*-trans retinal release and replacement by a new 11-*cis* molecule (Ala-Laurila et al. 2006), which may be particularly relevant for organisms that must rapidly respond to variation in ambient light (Dungan and Chang 2017; Hauser et al. 2017). Because this process is physiologically limited, as it requires recycling of released *all*-trans and synthesis of 11-*cis* retinal through the retinoid cycle (Lamb and Pugh 2004; Ala-Laurila et al. 2006), meta II may also have a photoprotective role by preventing accumulation of *all*-trans by-products in the retina, which may become toxic at high concentrations (Maeda et al. 2008). This may have important implications for nocturnal mammals, which have larger proportion and density of rod cells (Peichl 2005), exhibit slower dark adaptation and may therefore be more susceptible to light bleaches than diurnal organisms (reviewed by Różanowska and Sarna 2005; Organisciak and Vaughan 2010).

Because meta II stability is associated with efficiency of visual cascade activation (Kojima et al. 2014), shifts in this functional property of rhodopsin may indirectly influence photoreceptor sensitivity and ultimately contribute to differing visual performances in dim light (Imai et al. 2007; Yue et al. 2017). Here, the significant variation in meta II stability observed in bats suggests differences in dim-light visual ability in species with distinct sensory specializations that appear to be associated to their sensory ecology. Nonlaryngeal echolocating species, such as *P. alecto*, rely on visual information to navigate, forage, and locate roosts and thus require a specialized visual system to operate under low light conditions. Along with anatomical and physiological specializations (Pettigrew 1986; Liu et al. 2015), enhanced dim-light abilities putatively mediated by a more stable meta II state may

facilitate signal amplification and visual performance in dim light. In contrast, HDC species rely heavily on acoustical information generated by sophisticated calling ability that allows these bats, such as *R. ferrumequinum*, not only to navigate but also to locate and continuously track prey using solely echolocation (Koselj et al. 2011). The sophisticated echolocation and corresponding auditory structures along with reduced eyes in HDC species supports decreased dependence on vision (Liu et al. 2015; Thiagavel et al. 2018), which may also be linked to decreased meta II stability. Many echolocating bats, however, may rely on visual cues to perform a number of behaviors under nocturnal light conditions, such as *M. lucifugus* and other LDC species (Williams et al. 1966; Bradbury and Nottebohm 1969; Bell 1985; Eklöf and Jones 2003; Ruczyński et al. 2011; Gutierrez et al. 2014). Therefore, increased meta II stability in LDC species relative to HDC bats may represent an evolutionary advantage to support various visual-based behaviors in dim light.

Alternatively, recent studies have suggested that meta II stability may be a mechanism to prevent phototoxicity and photodamage in the retina resulting from abrupt increases in chromophore release, which may be further improved in association with other components of the visual transduction cascade, such as arrestin (Sommer et al. 2014). This may contribute to enhanced vision in the varying light environments that bats with differing roosting ecologies encounter during the day. For example, the nonecholocating *P. alecto* roosts in exposed trees (Vardon et al. 2001) and may therefore be expected to encounter higher light levels resulting in light-bleaching of the retina as compared with the laryngeal echolocating *M. lucifugus* and *R. ferrumequinum*, which roost in more secluded environments (Fenton and Barclay 1980; Hooper and Hooper 2009). Therefore, the greater meta II stability observed in the rhodopsin of *P. alecto* might be associated with a photoprotective role linked with aspects of its ecology, which may have important implications for maintaining vision in limited light conditions.

Evolution of Dim-Light Vision and Sensory Specialization in Bats

Complex aspects of organismal ecology and evolutionary history have been demonstrated to mediate shifts in selection constraint intensity in coding sequences, ultimately contributing to adaptive changes in protein function (Storz et al. 2007; Li et al. 2008; Liu et al. 2014; Dungan et al. 2016; Castiglione et al. 2017). Using a comparative experimental approach, we observed significant differences in rhodopsin kinetics associated with possible evolutionary innovation for dim-light vision in species with differing echolocation abilities. Because the evolution of distinct types of echolocation is hypothesized to have imposed varying constraints on the visual system, we tested whether changes in rhodopsin function were associated with shifts in selection pressure in *Rh1* coding sequences in response to diverse echolocation capabilities of bats. While differences in selective constraint in bat *Rh1* have been reported to occur in response to echolocation (Zhao, Ru, et al. 2009), reanalyses of the same data set found no support for evolutionary divergence in bat *Rh1* when compared with a more adequate null model (Weadick and Chang 2012). Similarly, we found no differences in selection pressure in *Rh1* associated with echolocation ability in HDC and nonlaryngeal echolocating lineages when partitions were compared with a null model that assumed similar evolutionary rates for all bats (Weadick and Chang 2012). Because rhodopsin mediates dim-light vision, it is possible that *Rh1* experienced similar selective constraint in different bat species in response to a long evolutionary history in nocturnal environments (Teeling et al. 2005). Alternatively, the different models indicated variation in evolutionary rates in HDC and nonlaryngeal echolocating lineages, which might suggest variation in selective constraint in bat *Rh1* that may not be discernable due to the lack of statistical power. Rhodopsin sequences are extremely conserved among bats, with 90% of codon sites under high constraint, which would pose a challenge to analyses attempting to detect variation in evolutionary rates (Anisimova et al. 2001). Our data set represents the most comprehensive to date, including new sequences from Neotropical bat eye transcriptomes as well as from draft bat genomes (Parker et al. 2013). Although our bat *Rh1* data set represented the largest used for molecular evolutionary analyses to date, we were still unable to discern any variation in constraint in bat *Rh1*. It is possible that greater efforts in improving sampling across bat lineages could reveal interesting patterns of selection associated with echolocation or other ecological factors, as observed in other visual opsins (Wertheim et al. 2015; Gutierrez et al. 2018).

In contrast, our analyses of an expanded mammalian data set that included *Rh1* sequences of bats along with most representative mammalian lineages did reveal significant shifts in selection underlying the evolution of bat *Rh1*. We detected a significant increase in selection constraint intensity accompanying the diversification of Chiroptera, suggesting that a proportion of ~20% of sites in the *Rh1* is more conserved in bats than in other mammals. Because sites that contribute to function tend to be maintained under lower evolutionary rates (Fay and Wu 2003), intensified constraint in bat *Rh1* likely represents an evolutionary mechanism to retain function that may have great implications for organisms' fitness. Along with shifts in selection, our comparative experimental approach revealed that all bat rhodopsin pigments assayed in this study exhibited significantly greater stability of the light-activated meta II conformation compared with a model bovine rhodopsin. Until recently, kinetic properties of rhodopsin had not been explored from a comparative perspective, and shifts in meta II stability had rarely been observed (Dungan and Chang 2017; Hauser et al. 2017). Relative to bovine Rh1, faster rates of meta II decay have been observed in human (Morrow et al. 2017) and in the monotreme echidna rhodopsin (Bickelmann et al. 2012), whereas slower meta II decay had only been reported in hippopotamus and the killer whale Rh1 (Dungan and Chang 2017). Our study demonstrates that bats appear to have a substantially more stable meta II state compared with all other mammalian lineages in which this property has been examined so far (fig. 3*B*), and highlights functional differences in the dim-light visual pigment potentially associated with the distinct lifestyle

of bats. Given the discernable shift in intensity of selective constraint in bat *Rh1* sequences, and functional differences in bat rhodopsin kinetics relative to other mammals, it is possible that increases in evolutionary constraint underlie the remarkable shift of rhodopsin kinetics in bats and other mammalian groups.

Shifts in functional properties of rhodopsin are proposed to have occurred in response to limited photic environments during the nocturnal bottleneck period that accompanied the evolution and diversification of most mammalian lineages (Bickelmann et al. 2015; Fernández-Sampedro et al. 2016; Maor et al. 2017; Wu et al. 2017). These functional changes in rhodopsin are thought to have been mediated by accelerated evolution in the branch leading to the ancestral Theria (Bickelmann et al. 2015; Fernández-Sampedro et al. 2016). Among those functional shifts, progressive increases in meta II stability were observed leading to mammalian ancestors and have been hypothesized to represent evolutionary innovations to nocturnality, potentially promoting photosensitivity and visual performance in dim light (Bickelmann et al. 2015). Recent studies suggest that the most recent common ancestor of Chiroptera was able to echolocate, but likely retained a visual system specialized for performance in dim light (Wang et al. 2017; Thiagavel et al. 2018). Therefore, the specialized dim-light visual abilities found in bats (Neuweiler 2000) along with other sensory adaptations, such as echolocation, likely facilitated chiropteran diversification during the nocturnal bottleneck of mammalian evolution (Maor et al. 2017; Wu et al. 2017) as well as maintenance of a primarily nocturnal lifestyle in extant species.

Conclusions

Studies of rhodopsin evolution in bats offer a remarkable opportunity to understand not only how the visual system adapted to operate in photic-limited environments but also how it evolved in response to other dim-light sensory specializations, such as echolocation. While previous computational approaches provided initial insight into the selective forces mediating the evolution of bat dim-light vision, comparative experimental approaches of rhodopsin function have been extremely limited and as yet unexplored from the perspective of bat sensory ecology. Using in vitro expression and characterization of rhodopsin, we observed nonspectral functional differences in the dim-light visual pigment of bat species with differing echolocation abilities. This is the first time that rhodopsin pigments have been expressed and functionally tested in the context of other sensory adaptations of bats. The rhodopsin kinetic shifts we observed are likely associated with differences in dim-light visual performance among the three species, which appear to be consistent with a proposed hypothesis of sensory trade-off between vision and echolocation in Chiroptera and could be further investigated by functionally characterizing rhodopsin in additional bat species with differing sensory ecologies.

We also found impressive differences in rhodopsin kinetics that may distinguish bats from other mammalian groups. These differences are likely associated with enhanced dim-light abilities that may have evolved in response to the primarily nocturnal lifestyle of Chiroptera and may reflect functional changes that occurred during the nocturnal bottleneck period in which most bat lineages diversified. This functional innovation may have been acquired in ancestral lineages leading to Chiroptera, being maintained under strong evolutionary constraint after bat diversification. Studies aiming to reconstruct and functionally characterize rhodopsin of the ancestral Chiroptera and ancestral nodes leading to bats may help to clarify not only when in the evolutionary history of bats functional shifts may have occurred but may also provide some insight on the visual ecology and sensory specialization of early bats.

Materials and Methods

Rhodopsin Expression

The wild-type *Rh1* coding sequences of three bat species (*Pteropus alecto*, *Rhinolophus ferrumequinum*, and *Myotis lucifugus*) were synthesized by GeneArt (Invitrogen) with 5′ and 3′ restriction enzyme sites for insertion into a p1D4-hrGFP II expression vector (Morrow and Chang 2010). These species were selected for this experiment as they are representatives of the three major types of echolocation in bats, and all have genomic data available from which full-length Rh1 sequences were obtained. Expression vectors containing the *Rh1* sequence of each species were transiently transfected into HEK cells using Lipofectamine 2000 (Invitrogen) and harvested after 48 h, along with a bovine control. Expressed rhodopsin proteins were regenerated with 5 μM 11-*cis*-retinal, solubilized in 1% *N*-dodecyl-D-maltoside, and immunoaffinity purified using the 1D4 monoclonal antibody in the dark, as previously described (Morrow and Chang 2015; Castiglione et al. 2017, 2018).

Absorbance and Fluorescence Spectroscopy

The UV-visible absorption spectra of purified bat and bovine rhodopsin control samples was measured using a Cary 4000 double-beam spectrophotometer (Varian) at 25°C in the dark, and again after 30 s following light bleach with a fiber optic lamp (Dolan-Jenner, Boxborough, Massachusetts) to confirm pigment activation. Difference spectra were obtained by subtracting light spectra from dark spectra. Spectral absorbance peak (λ_{Max}) values were obtained by fitting a standardized template to the dark absorbance spectra (Govardovskii et al. 2000). To assess all-*trans* retinal release rates from light-activated rhodopsins, we measured intrinsic signals in tryptophan fluorescence that increase as residues become unquenched when the chromophore migrates from the binding pocket (Farrens and Khorana 1995). Fluorescence signals were measured with a Cary Eclipse spectrophotometer (Varian) at 20°C following a 30 s light bleach, as previously described (Morrow and Chang 2015; Castiglione et al. 2017, 2018). Retinal release half-life ($t_{1/2}$) values were estimated by fitting the fluorescence data to first-order exponential curves ($y = y_0 + a(1 - e^{-kt})$, where $t_{1/2} = \ln(2)/k$). All curve fittings resulted in adjusted r^2 values > 0.97. Retinal release half-life values were calculated using a minimum of three parallel

measurements of purified rhodopsin samples for each species assayed ($n \geq 3$ replicates, detailed in supplementary table S1, Supplementary Material online) and statistically compared through a two-tailed *t*-test with unequal variance.

Data Set Preparation, Sequence Alignment, and Phylogenetic Analysis

New Neotropical bat *Rh1* sequences were obtained from whole eye transcriptomes of six species (the mormoopid *Mormoops blainvillei*, *Pteronotus parnellii*, and *P. quadridens*, and the phyllostomid *Macrotus waterhousii*, *Monophyllus redmani*, and *Phyllops falcatus*) as previously described (Gutierrez et al. 2018). *Rh1* sequences were extracted from assembled transcriptomes using custom BLAST searches (discontiguous megablast, evalue cutoff of 1e-10) with full-length rhodopsin sequences obtained from available bat genomes as reference. Additional rhodopsin sequences were obtained from GenBank as well as identified from published bat draft genomes (Parker et al. 2013) through standalone BLAST. Along with new Neotropical rhodopsin sequences, our data set comprised sequences from 38 bat species, spanning 10 chiropteran families. Another 42 sequences of representative mammalian orders were also obtained from GenBank and included in the data set for tree estimation and molecular evolutionary analysis. Species list and accession numbers for sequences used in the study are provided in supplementary table S11, Supplementary Material online. Mammalian and chiropteran rhodopsin sequences (80 species total) were aligned by codon in MEGA 6.0 (Tamura et al. 2013) and used to estimate a tree through maximum likelihood in PhyML 3.0 (Guindon et al. 2010). The estimated gene tree recovered monophyletic relationships of most mammalian orders, including Chiroptera, but differed significantly from accepted species relationships at both deep and recent nodes both within Chiroptera as well as among other mammalian orders, as previously observed in other mammalian *Rh1* data sets (Zhao, Ru, et al. 2009; Dungan et al. 2016). Because of these differences, we constrained the gene tree to best recover inter- and intraordinal mammalian and chiropteran relationships according to recent literature (Teeling et al. 2005; Foley et al. 2016; Tarver et al. 2016).

Molecular Evolutionary Analyses

To estimate the form and strength of selection acting upon *Rh1* sequences, alignment and trees were analyzed with the codeml program in PAML 4.9a (Yang 2007). Random sites analyses (Yang et al. 2000) were carried out to estimate variation in the ratio of nonsynonymous to synonymous substitution rate ratio (d_N/d_S or ω) among sites in two different data sets of *Rh1* sequences: Chiroptera *Rh1* data set and an expanded data set that included bat and representative mammalian *Rh1* sequences. Random sites models were compared through likelihood ratio tests (LRTs) with χ^2 distribution to test for among-site variation in ω (M3 vs. M0) and positive selection (M2a vs. M1a, and M8 vs. M8a).

PAML's clade model C (CmC, Bielawski and Yang 2004) was used to test for long-term shifts in selection constraint in bat *Rh1* in response to differing echolocation abilities. We partitioned the chiropteran *Rh1* data set to test whether divergent evolutionary rates (ω_d) occur in nonlaryngeal echolocating (NE) and high-duty cycle (HDC) lineages in comparison to the remainder of the tree. The independent Old World and New World (*Pteronotus parnellii*) HDC clades were also examined by testing each individually as a foreground partition. Additionally, we analyzed whether other ecological variables that may influence either reliance on visual information (e.g., diet) or exposure to ambient light (e.g., roosting behavior and foraging habitat) by systematically partitioning the chiropteran *Rh1* data set as previously described (Gutierrez et al. 2018). A complete description of the various clade model partitions performed can be found in supplementary fig. S1 and table S6, Supplementary Material online. All CmC partition models were compared with the null model M2a_rel (Weadick and Chang 2012), which does not allow variation of ω in the divergent class of sites, using an LRT to test for significant shifts in selection constraint.

Second, we used the expanded mammal *Rh1* data set to test whether the dim-light visual pigment in bats experienced shifts in selection constraint intensity relative to other mammals. We separated either the branch leading to Chiroptera (i.e., test for episodic shift in evolutionary constraint) and also the entire chiropteran clade (i.e., test for shifts in selection following bat diversification) as foreground partition and analyzed in PAML using the branch model (Br), and the clade models CmC and CmD (reviewed in Baker et al. 2016; Schott et al. 2018). Significant variation and divergent selection were tested through an LRT to compare Br, CmC, and CmD against its null model M0, M2a_rel, and M3, respectively. Codons experiencing divergent selection in significant CmC partitions were identified by BEB analysis (Weadick and Chang 2012).

Supplementary Material

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

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