The evolution of color vision in nocturnal mammals

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Nonfunctional visual genes are usually associated with species that inhabit poor light environments (aquatic/subterranean/nocturnal), and these genes are believed to have lost function through relaxed selection acting on the visual system. Indeed, the visual system is so adaptive that the reconstruction of intact ancestral opsin genes has been used to reject nocturnality in ancestral primates. To test these assertions, we examined the functionality of the short and medium- to long-wavelength opsin genes in a group of mammals that are supremely adapted to a nocturnal niche: the bats. We sequenced the visual cone opsin genes in 33 species of bat with diverse sensory ecologies and reconstructed their evolutionary history spanning 65 million years. We found that, whereas the long-wave opsin gene was conserved in all species, the short-wave opsin gene has undergone dramatic divergence among lineages. The occurrence of gene defects in the short-wave opsin gene leading to loss of function was found to directly coincide with the origin of high-duty-cycle echolocation and changes in roosting ecology in some lineages. Our findings indicate that both opsin genes have been under purifying selection in the majority bats despite a long history of nocturnality. However, when spectacular losses do occur, these result from an evolutionary sensory modality tradeoff, most likely driven by subtle shifts in ecological specialization rather than a nocturnal lifestyle. Our results suggest that UV color vision plays a considerably more important role in nocturnal mammalian sensory ecology than previously appreciated and highlight the caveat of inferring light environments from visual opsins and vice versa.

bats opsin gene sensory tradeoff echolocation selection

Vision plays one of the most important roles in the survival of an individual, underpinning numerous key behaviors such as foraging, predator avoidance, and mate recognition. Color vision is conferred by the cone photopigments, each comprising an opsin transmembrane protein and a 11-cis-retinal chromophore (1, 2). Diversity in the properties and arrangement of photoreceptors in vertebrates reflects the evolutionary malleability of this system in response to specific visual challenges (3). Opsin proteins can be classified into medium/long wavelength sensitive (M/LWS) and short-wavelength-sensitive (SWS) based on the wavelength of their peak light sensitivity. Comparisons of visual pigments across taxa indicate that spectral tuning and, therefore, the wavelength of peak light sensitivity (λ_{max}) are modulated by 5 key critical amino acid sites in M/LWS opsins (4) and at least 11-aa sites in SWS opsins (5).

Most mammals possess both classes of opsin, with the M/LWS sensitive to green-red and the SWS1 sensitive to blue-violet (6), and a greater proportion of cones containing the former (85–95%) than the latter (only 5–15%) (3). Reported exceptions to this visual state include a number of monochromats, such as the blind mole rat (7), cetaceans (8), and the flying squirrel (9), all of which have acquired loss-of-function mutations in their SWS1 opsin. Such losses have been typically explained by relaxed selection from inhabiting poor photopic environments (3). Recently, the presence of a functional SWS1 opsin in several lineages of prosimians has been used to refute the longstanding hypothesis of nocturnality in ancestral primates (10). To understand further how opsin genes have adapted in mammals and to investigate whether functionality can indeed be used to infer activity patterns, we undertook an extensive survey of visual genes in bats, which are considered the sensory specialists (11) and arguably show the greatest adaptation for nocturnality of all of the vertebrates.

The unique ability of most bats to orient using laryngeal echolocation without the need for vision, coupled with their characteristically small eyes, has led to speculation that laryngeal echolocation and nocturnality coevolved, and that an evolutionary "tradeoff" occurred between vision and hearing in bats (12). Using this logic, it is hypothesized that bats have occupied a nocturnal niche for >52 million years, because this is the age of the oldest bat fossil that shows evidence of echolocation capabilities (13). To infer the impact of nocturnality on the evolution of vision in mammals, we sequenced the *SWS1* opsin gene (2.2 kb) in 32 species of bat and the *M/LWS* opsin gene (3.2 kb) in 14 species of bats. We included bats from both major lineages (Yangochiroptera and Yinpterochiroptera) and species that varied in their acoustic and roosting ecology [see supporting information (SI) Table S1 and Fig. 1]. We analyzed our sequences alongside the published opsin sequences of 35 additional and phylogenetically diverse mammal species, representing the largest single analyses of mammalian opsins to date (Table S1). We predicted that bats should show evidence for loss of function throughout their phylogenetic tree because of their long history of nocturnality and evolution of echolocation.

Results

Sequence Alignment and Analyses Based on the ORF. ***M/LWS opsin.*** For the *M/LWS* opsin sequences, phylogenetic reconstruction based on both Maximum Likelihood and Bayesian methods supported the published consensus species tree for mammals (see Fig. 2). *M/LWS* sequences were highly conserved across all species examined, including bats, and showed an intact ORF and consensus mammalian intronic splice sites, suggesting the gene is functional. Maximum likelihood estimates of the ratio of nonsynonymous to synonymous substitution rates (d_N/d_S or ω) on each branch of the tree were uniformly low among bat lineages (0.00–0.48), indicating the *M/LWS* opsin gene has been subject to purifying selection during the radiation of bats (Table S2). Estimated omega values were also below 1 under other models of variation in selection across branches that we examined, confirming a lack of positive selection (Table S3). Examination of 5 critical sites revealed 2 substitutions that are known

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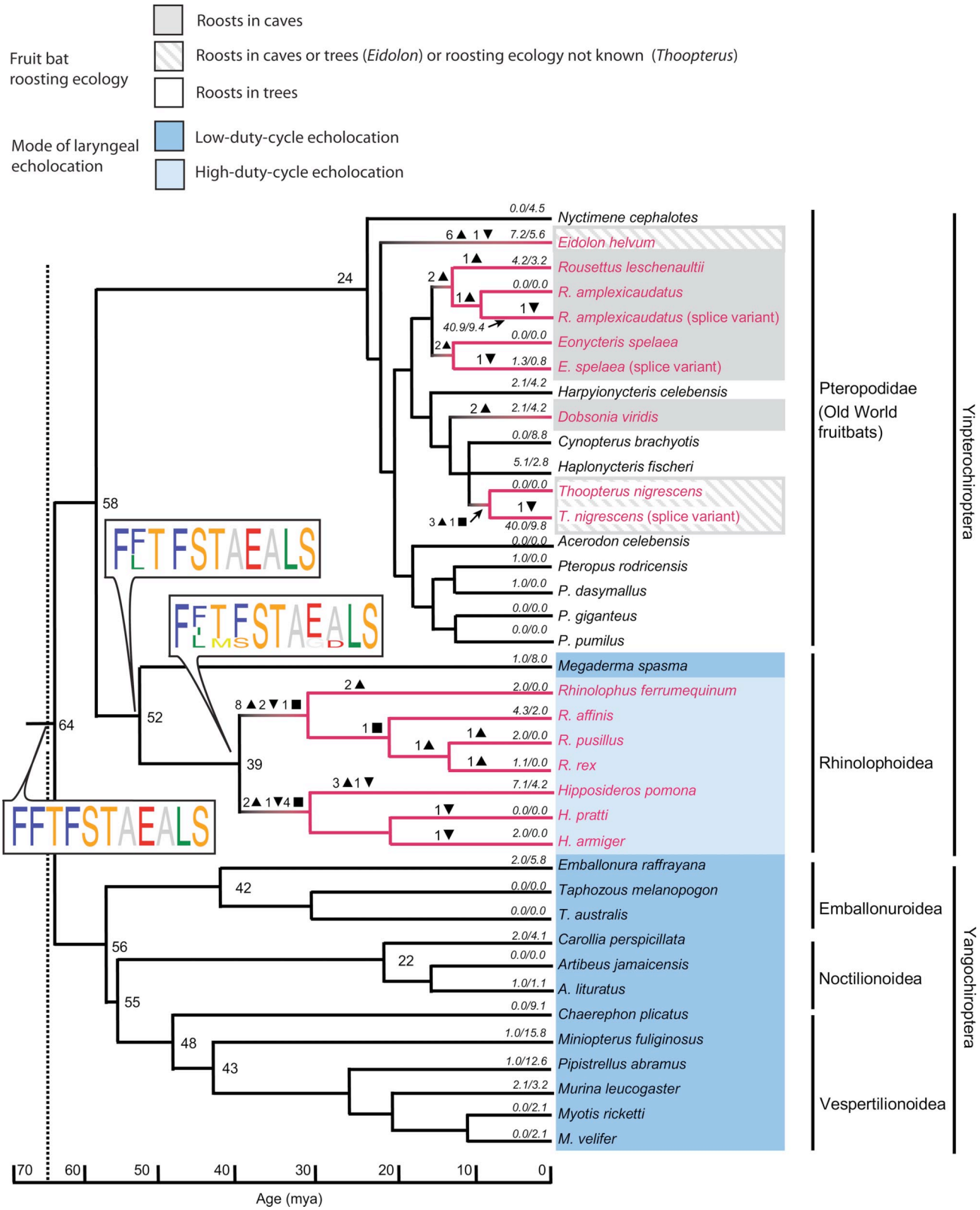

Fig. 1. *SWS1* species tree showing substitution rates, indels and stop codons based on the ORF. The tree topology and divergence dates follow consensus published phylogenies (*SI Text*). Numbers of insertions and deletions in the ORF are shown on the branches by downward (▼) and upward oriented (▲) triangles, respectively. Ancestral stop codons were inferred by probabilistic reconstruction and are denoted by squares (■), and critical sites implicated in spectral tuning were obtained using the same method for all functional ancestral nodes predating stops. Stops found in the tip sequences are shown in Fig. 3. Key changes in the critical spectral tuning amino acid sites are shown by sequence logos in which the height of amino acids at a given position are proportional to their posterior probability. Species with sequences containing stop codons are shown in red font, whereas the loss of functionality inferred from indels or stops is depicted by red branches. Branch length represents millions of years and numbers at the nodes represent divergence time in millions of years (*SI Text*). Numbers along the terminal branches are the d_N/d_S ratios after removing indels and stops, calculated by PAML (40) (see also *SI Text*).

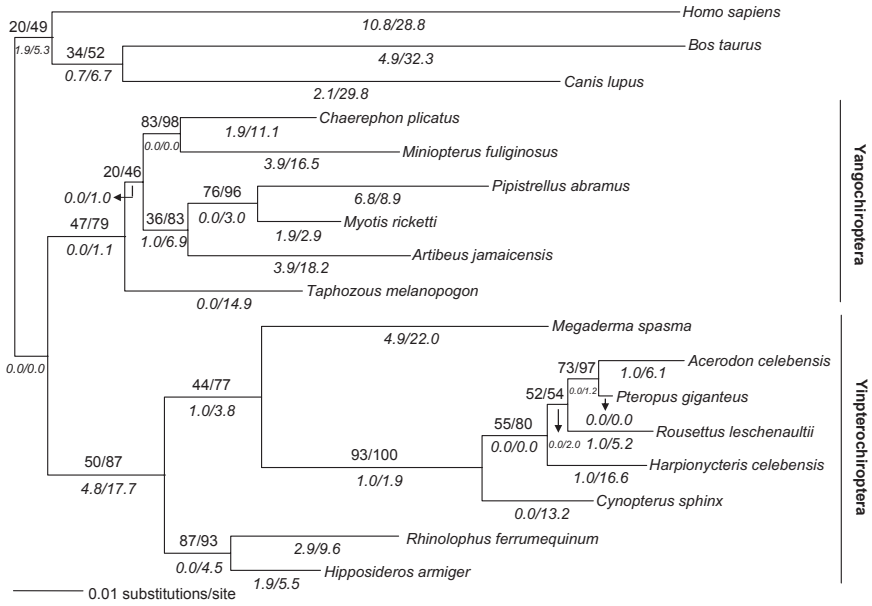

Fig. 2. The TBR maximum likelihood tree (-ln likelihood = 2,103.5) for the *M/LWS* dataset under the GTR+Γ+I model of sequence evolution. Numbers above the branches are the ML bootstrap values/Bayesian posterior probabilities as percentages, 100* = clades that received 100% ML bootstrap support and had posterior probabilities of 1; numbers below the branches are the d_N/d_S values estimated by PAML (40) (see also *SI Text*).

to influence spectral tuning (T285A and S180A) (1). The former occurred in most bats, was independent of phylogenetic relationships, and has been shown to reduce peak light absorption from 560 to 553 nm. The latter was recorded in one species only (*Myotis ricketti*) and has been shown to reduce absorption to 545 nm (1).

SWS1 opsin. Surprisingly, the *SWS1* opsin gene showed a sharply different evolutionary trajectory to the *M/LWS* opsin gene in bats. Phylogenetic analyses of the *SWS1* opsin gene differed from the recently published consensus tree (11) and instead showed an alternate topology in which bats with laryngeal echolocation formed a single monophyletic clade resembling the traditional grouping of Microchiroptera (Figs. S1 and S2). However, this could not be considered as a robust result, because statistical support for this clade was very weak, with a maximum-likelihood bootstrap value of 0.27 and a Bayesian posterior probability of 0.63 in Fig. S1 and similar values in Fig. S2. Moreover, a Shimodaira–Hasegawa test revealed no significant difference between the published species tree and our gene tree (Table S4). Branch lengths varied greatly among lineages, consistent with different selection pressures (Figs. S1 and S2, Table S2).

Among the echolocating bats studied, all 12 species examined from the major suborder of echolocating species (Yangochiroptera) were found to possess a functional *SWS1* opsin characterized by an intact ORF (Fig. 1) and conserved mammalian intronic splice sites (Fig. S3). In each case, the 11 critical amino acid sites implicated in spectral tuning matched those of the ancestral and consensus mammalian sequence, which is known to encode an UV sensitive pigment (Fig. 1). All of these bats use ''low-duty-cycle'' echolocation, in which the emitted calls are relatively short in duration and do not overlap in time with the returning echoes (11). Similarly, of the echolocating species studied that belong to the other major suborder (Yinpterochiroptera), the only representative to use low-duty-cycle echolocation (*Megaderma spasma*) (11) was also found to be the only 1 to possess an intact UV sensitive shortwave opsin (Fig. 1).

In all of the other surveyed members of the Yinpterochiroptera that use laryngeal echolocation (rhinolophids and hipposiderids), we found evidence that the *SWS1* opsin gene had accrued multiple insertions and deletions (indels) and premature stop mutations that disrupted the ORF and was thus nonfunctional (Figs. 1 and 3, Fig. S4). These bats have evolved a specialized form of echolocation in which calls are characterized by a constant frequency component, are relatively long in duration and are separated by shorter intervals (''high-duty-cycle echolocation'').

To ascertain when the functionality of the *SWS1* opsin gene was lost in these species we developed a probabilistic model of codon evolution to allow us to incorporate stop codons in our analyses and reconstruct the ancestral amino acid sequences based on the ORF alignment (Fig. 1, *SI Text*). Amino acid reconstructions suggest that stop codons arose independently at the ancestral nodes of both the families Hipposideridae and Rhinolophidae, and that both stops and indels appear at different positions in the 2 lineages.

We also found evidence of several cases of loss-of-function of *SWS1* in the sequences of 6 of the nonecholocating Old World fruit bats, although 9 closely related fruit bat genera possessed ORFs (Fig. 1, Fig. S4). Reconstructions of amino acid sequences revealed no ancestral stop codons among these taxa, suggesting independent losses (Fig. 1). Of those genera with nonfunctional SWS1 opsins, as inferred by stop or frameshift mutations, 3 (*Rousettus*, *Dobsonia*, and *Eonycteris*) roost in caves in poor photopic conditions, and one roosts in caves (*Eidolon*) occasionally (Fig. 1).

The strong contrast in the selective constraints acting on the *SWS1* opsin gene among different lineages of bats was also demonstrated by the results of branch model tests of selection. Of those *SWS1* genes considered to be functional (i.e., without stops or indels), we found no evidence that the dN/dS ratio (ω) calculated for ancestral branches of the fruit bats, Yinpterochiroptera or Yangochiroptera was significantly greater than that estimated using a model in which the ratio was fixed across the tree (Tables S2 and S3). Probabilistic reconstructions of the 11 critical sites implicated in spectral tuning (5) revealed that these sites are unlikely to have undergone significant evolutionary change in any branch of bats since their radiation, and the opsin has thus remained UV sensitive (Fig. 1). Thus, the independent losses of SWS1 in some bat lineages are probably not a consequence of changes in peak spectral sensitivity.

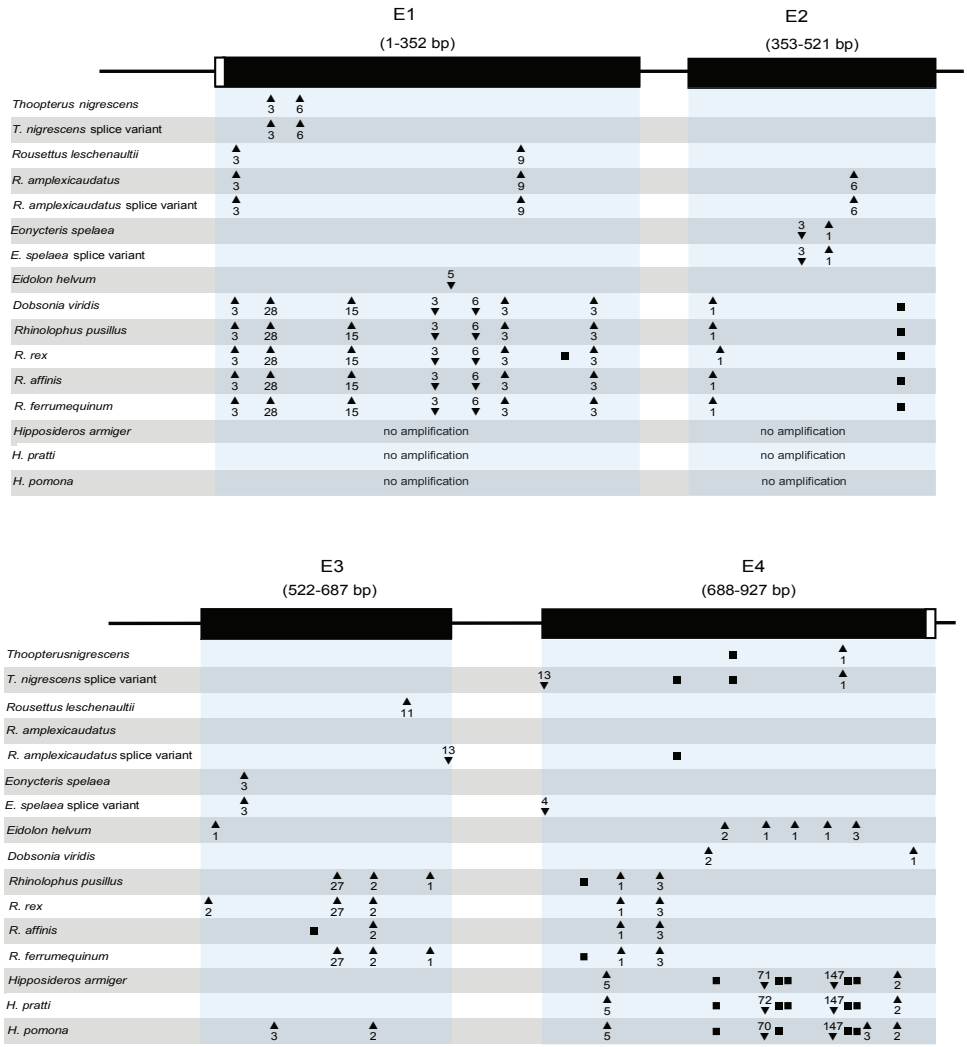

Fig. 3. Schematic to show *SWS1* bat sequences with insertions (▼), deletions (▲) and stops (■) in the ORF. Sizes of indels are given in base pairs.

Own Reading-Frame Analyses. To assess the extent of frameshift mutations resulting from indels, we removed gaps, translated each sequence in its own frame, and aligned the amino acids (Fig. S5, *SI Text*). Sequences with stop codons typically showed lower similarity to the consensus alignment than sequences with no stops (Fig. S5). Exceptions were the potential splice variant of *Eonycteris spelaea*, which had no stop yet low similarity score, and the sequence of *Thoopterus nigrescens*, which had a stop yet high similarity score (*SI Text*). The former case suggests that this potential splice form does not exist in nature, whereas the latter case supports our assertion that the loss of SWS1 in fruit bats has occurred recently, a conclusion that is also suggested by sliding window analyses (Fig. S6). Indeed, apparent elevated substitution rates were recorded mainly at or downstream of stop codons, indicating that both of these features in fact reflect shifts in frame because of indels rather than relaxed selection in fruit bats (Fig. S6). Sliding windows also revealed the impact of indels on genetic divergence, even where no stop codons are present (*Dobsonia* and *Rousettus*, Fig. S6).

Discussion

The ability of bats to orient and hunt using sound in complete darkness (echolocation) (14) represents one of the most striking examples of nocturnal sensory innovation in vertebrates. However, the extent to which bats use vision has long been debated. Early reports of all-rod-retinae suggested that bats lack color vision (3), whereas more recent visual studies of the visual capabilities in bats have yielded conflicting and inconsistent findings. The amplification of intact genes encoding the visual cone opsins (long/medium and short wavelength) in 3 species of bats suggested that bats possess dichromatic color vision (15). However, immunocytochemical examinations of cone type and density in 6 Old World fruit bats (Pteropodidae) indicated that all 6 species had long-wavelength cones, but that only 2 had short-wavelength cones (16), suggesting that some fruit bats are monochromats and so incapable of color vision. A recent behavioral study showed that one echolocating bat species can see UV visual stimuli; however, this was attributed to an adaptive change in the sensitivity of rods rather than true cone dichromatic color vision (17). Thus, the extent to which bats use vision to interpret their environment and therefore the evolutionary consequence of being a nocturnal specialist is unknown and, as these recent results (15–17) show, the answer is likely to be much more complicated than previously believed.

The study of functional genes within a phylogenetic framework can provide powerful insights into the relationship between genes, ecology, and behavior (18–21). Our phylogenetic and molecular evolutionary analyses of 2 classes of visual opsin gene reveal dramatic contrasting evolutionary histories in bats. All 14 species examined were found to possess a functional *M/LWS*

opsin gene tuned to red light, despite their long history of nocturnality and, in most cases, use of laryngeal echolocation. Indeed, a functional M/LWS opsin appears to have been retained for >80 million years, since bats diverged from other mammals (22). This supports other studies that have reported strong conservation of the M/LWS opsin in mammals (10, 15, 23). One possible explanation for such conservation in the face of nocturnality is that the M/LWS opsin might have additional roles to vision, such as in controlling the circadian rhythms of physiological processes (see ref. 23).

By comparison, the shortwave opsin (*SWS1*) shows much greater evolutionary divergence among mammals, and especially within bats. Differential conservation or losses via indels or stops appear to correspond closely to differences in species' sensory ecology. The retention of an intact UV sensitive shortwave opsin in taxa with 'low-duty-cycle' echolocation, both in all members of the Yangochiroptera, and independently in the Yinpterochiroptera taxon *Megaderma spasma* (11) suggests that these species are dependent on short wave vision for orientation and/or hunting, despite being nocturnal. At the same time, evidence of independent losses of shortwave opsin functionality early in the evolution of the Hipposideridae and Rhinolophidae lineages (extant members of which possess high-duty-echolocation) indicates that these losses are broadly coincident with the evolution a novel form of echolocation (Fig. 1). Here, bats exploit Döppler shifts to produce calls of lower frequencies than their returning echoes, allowing them to separate their calls from the returning echoes in terms of frequency rather than time and so receive a more continuous flow of acoustic information (24–26). This is considered perhaps the most advanced nocturnal sensory adaptation within mammals (27).

Such contrasting trajectories in the visual ecology of 2 main groups of echolocating bat within a nocturnal niche indicates that the neural ''picture'' obtained by the 2 divergent forms of echolocation is likely to differ markedly. We postulate that low-duty-cycle echolocators augment their acoustic ''image'' with shortwave vision, whereas the evolutionary innovation of high-duty-cycle echolocation has rendered dichromatic color vision redundant. This apparent tradeoff between vision and hearing is further supported by the recent discovery that a key cochlear protein implicated in high frequency audition has undergone a burst of adaptive selection in high-duty-cycle echolocators (21) that coincides with the loss of SWS1 function reported here. This highly specialized form of echolocation allows these bats to detect acoustic glints created by flapping insects and is considered especially well adapted for hunting in dense vegetation (narrow-space specialists) (11). Although tradeoffs between species' evolutionary adaptations have long been assumed to occur through ecological specialization, evidence for postulated evolutionary sensory tradeoffs is poor. Indeed, although most mammal species are assumed to be sensory specialists with 1 sensory modality enhanced above the others, most species also show multiple sensory modalities throughout their evolution and, where sensory losses occur, they are typically associated with a cessation of sensory input rather than via a tradeoff per se. The origin of a novel sensory modality (high-duty-cycle echolocation) within the evolutionary timeframe considered in this study means that we are able to directly relate the gain of one sense with the loss of another.

We also found evidence of the independent loss of a functional shortwave opsin in a number of fruit bat lineages, supporting the findings of emerging but taxonomically limited immunocytochemical studies (16). The retention of a functional *SWS1* gene in obligate tree roosters, yet loss in those species that roost in caves, indicates that cave roosting is associated with a relaxation in selective constraint. The close correspondence between the lack of *SWS1* functionality and roosting ecology, coupled with the lack of ancestral stops (Fig. 1), strongly suggests that cave roosting in fruit bats represents a recent behavioral innovation that has evolved independently several times (28). We also cannot rule out the possibility that the loss of SWS1 opsin in cave roosting fruit bats might also correspond to an undetected sensory tradeoff, perhaps because of an increase in olfactory capabilities, as is hypothesized for ancestral placental mammals (29), although this scenario cannot account for such close ties with roosting environment.

Reconstruction of ancestral amino acid sequences (30) and synthesis of their expressed pigments (31) suggest that the ancestral vertebrate short-wave opsin was UV (UV) sensitive (λ_{max} ≈360 nm). Derived critical site replacements in some lineages of amphibian, bird and mammal have usually been explained as adaptations to different light environments (5, 32), whereas cases of loss-of-function appear related to the cessation of sensory input (9). Conversely, the reconstruction of the ancestral intact shortwave opsin in primates has been used to infer diurnality in extinct species (10). Our finding, that the SWS1 opsin has been under purifying selection in the majority of echolocating bats and a large number of fruit bats despite a long history of nocturnality, shows that such logic is at best an over-simplification and strongly indicates that UV color vision is likely to play a considerably more important role in bat sensory ecology than previously appreciated. UV vision in mammals was until recently considered to be restricted to rodents and marsupials, with other orders showing either a loss or a switch to violet (3). The addition of most bats to this group advances our understanding of the extent to which vertebrates are able to perceive and use UV light (33, 34) and leads us to question the validity of earlier claims of color blindness in a nectarivorous bat (17) and diurnality in ancestral primates (10). More generally, the detected tradeoff reported here between vision and echolocation in bats supports the longstanding but weakly supported assumption that tradeoffs are indeed associated with ecological specializations and highlights the need to explore evolutionary hypotheses in phenotypically and phylogenetically divergent taxa.

Materials and Methods

Taxon Coverage. We amplified and sequenced ≈2.2 kb of the short wavelength opsin gene (*SWS1*) from exon 1 to exon 4 in 32 species of bats that vary in their acoustic and roosting ecology. Sequencing was based on genomic DNA and, where possible, from retinal mRNA (*SI Text*, Table S1). These data were supplemented with sequences from an additional 35 mammal species obtained from GenBank, comprising 4 marsupials, 1 proboscidean, 4 rodents, 19 primates, 1 canid, 1 suid, 1 bovid, 1 equid, and 3 other bats. In total, the *SWS1* dataset included 67 mammals. We also amplified and sequenced the *M/LWS* opsin gene in a subset of 14 bat species (Table S1) and added the sequences of 3 outgroups (1 primate, 1 bovid, and 1 canid) in our analyses. GenBank accession numbers and species names are given in Table S1.

Nucleotide Alignment, Phylogenetic and Molecular Evolution Analysis. Nucleotide sequences were aligned in the ORF using CLUSTAL X (35) and modified by eye with SE-AL (36). Intron-exon boundaries were identified with reference to published sequences, and, in the case of *SWS1*, c-DNA sequences obtained in this study. The best-fit model of nucleotide evolution was determined by Modeltest (37), and Maximum Likelihood and Bayesian phylogenetic reconstruction methods were performed with PAUP 4.0b10 (38) and MrBayes 3.1.1 (39), respectively (see also *SI Text*). We also applied a Maximum Likelihood approach to test for differences in selection pressure, using the CODEML program of PAML version 4 (40) (see also *SI Text*).

Ancestral Sequence Reconstruction. Ancestral sequences were reconstructed under an explicit phylogenetic model of coding sequence evolution in a Maximum Likelihood framework. Our model of coding sequence evolution is similar to the models of Muse and Gaut (41), extended to include substitutions to and from stop codons and so that substitution rates depend on the nucleotide composition. We modeled separate rates for changes between synonymous and nonsynonymous codons for each branch of the tree, and an additional parameter describing the rate of sense codons changing to stop

codons. Rates of change between codons also depended on the nucleotide composition at each codon position (see also *SI Text*).

Protein Sequence Alignment and Sliding Window Analysis. The indels of *SWS1* protein coding sequences among bats were removed and the sequences translated in their own reading frames using SE-AL (36) and realigned using CLUSTAL X (35) and T-Coffee (42). The sequences were then examined for stop codons, indicative of loss-of-function. We derived similarity scores of each sequence relative to the multiple sequence alignment using T-Coffee (see also *SI Text*). We also measured deviation from the functional state by estimating average rates of nonsynonymous (d_N) and synonymous (d_S) substitutions per site, and the d_N/d_S (ω) ratio, for a sliding window of 90 nt with a step size of 9 nt (Fig. S6) in the software SWAAP 1.0.2 (43).

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