The molecular origin and evolution of dim-light vision in mammals

ARTICLE *in* EVOLUTION · OCTOBER 2015

Impact Factor: 4.61 · DOI: 10.1111/evo.12794

8 AUTHORS, INCLUDING:

Constanze Bickelmann

Museum für Naturkunde - Leibniz Institute fo…

17 PUBLICATIONS **76** CITATIONS

SEE PROFILE

Ilke Van Hazel

University of Toronto

6 PUBLICATIONS **22** CITATIONS

SEE PROFILE

Johannes Müller

Museum für Naturkunde - Leibniz Institute fo…

84 PUBLICATIONS **1,278** CITATIONS

SEE PROFILE

Belinda S W Chang

University of Toronto

59 PUBLICATIONS **1,257** CITATIONS

SEE PROFILE

doi:10.1111/evo.12794

The molecular origin and evolution of dim-light vision in mammals

Constanze Bickelmann,[1,2] James M. Morrow,[3,4] Jing Du,[4] Ryan K. Schott,[4] Ilke van Hazel,[4] Steve Lim,[3] Johannes Müller,[1] and Belinda S. W. Chang[3,4,5,6]

[1]*Museum für Naturkunde, Leibniz-Institut für Evolutions- und Biodiversitätsforschung, 10115 Berlin, Germany*

[2]*E-mail: constanze.bickelmann@mfn-berlin.de*

[3]*Department of Cell and Systems Biology, University of Toronto, Toronto, ON M5S 3G5, Canada*

[4]*Department of Ecology and Evolutionary Biology, University of Toronto, Toronto, ON M5S 3B2, Canada*

[5]*Centre for the Analysis of Genome Evolution and Function, Toronto, ON M5S 3B2, Canada*

[6]*E-mail: belinda.chang@utoronto.ca*

Received June 1, 2015
Accepted September 27, 2015

The nocturnal origin of mammals is a longstanding hypothesis that is considered instrumental for the evolution of endothermy, a potential key innovation in this successful clade. This hypothesis is primarily based on indirect anatomical inference from fossils. Here, we reconstruct the evolutionary history of rhodopsin—the vertebrate visual pigment mediating the first step in phototransduction at low-light levels—via codon-based model tests for selection, combined with gene resurrection methods that allow for the study of ancient proteins. Rhodopsin coding sequences were reconstructed for three key nodes: Amniota, Mammalia, and Theria. When expressed in vitro, all sequences generated stable visual pigments with λ_{MAX} values similar to the well-studied bovine rhodopsin. Retinal release rates of mammalian and therian ancestral rhodopsins, measured via fluorescence spectroscopy, were significantly slower than those of the amniote ancestor, indicating altered molecular function possibly related to nocturnality. Positive selection along the therian branch suggests adaptive evolution in rhodopsin concurrent with therian ecological diversification events during the Mesozoic that allowed for an exploration of the environment at varying light levels.

KEY WORDS: **Ancestral sequence reconstruction, codon-based likelihood models, dN/dS, mammalian evolution, nocturnality, rhodopsin evolution.**

The early Mesozoic rise of mammals (sensu Kielan-Jaworowska et al. 2004) from premammaliaform cynodonts was a cornerstone in vertebrate evolution (Kemp 2005). With over 5000 extant species and at least 4000 fossil taxa, mammals are a highly diverse and successful clade, having evolved an enormous number of ecological specializations (for review see Luo 2007). The emergence of endothermy is considered a major event in mammalian evolution, enabling mammals to be independent of ambient temperatures, evolve higher metabolic rates, and have longer activity periods (Jerison 1971; Crompton et al. 1978). The evolutionary origin of mammalian endothermy has historically been viewed as a by-product of an adaptation to a nocturnal lifestyle, based on the observation that living insectivorous hedgehogs, tenrecs, and shrews are ecomorphologically similar to early mammalian fossil taxa (Jerison 1971; Crompton et al. 1978). In fact, a recent study of nonmammalian synapsids using linear morphometrics on scleral dimensions supports the view that mammals and their ancestors had adapted, possibly several times independently, to at least dim-light conditions during synapsid history (Angielczyk and Schmitz 2014). The results rectify the traditional view that nocturnality may have evolved in response to predation pressures from diurnal dinosaurs, whose activities may have been restricted to daytime due to their dependence on solar radiation (Clarke and Pörtner 2010).

Ecological specializations such as endothermy and nocturnality are reflected primarily in the physiology of an organism, or in its soft parts such as eye shape and size (Ross and Kirk 2007; Hall et al. 2012), but further evidence has recently been

EVOLUTION
INTERNATIONAL JOURNAL OF ORGANIC EVOLUTION

detected in the hard parts of a fossil phenotype (Angielczyk and Schmitz 2014). Even though the hypothesis of ancestral mammalian nocturnality has gained increasing support, inferences at the molecular level are scarce (Davies et al. 2012). However, if this hypothesis is correct, one would expect functional adaptations in systems involved in vision and light detection, the most important of which are the visual pigments that initiate the visual transduction cascade (Yau 1994; Blumer 2004).

Rhodopsin is the visual pigment responsible for mediating the critical first step of dim-light vision in vertebrates (Burns and Baylor 2001). The 11-*cis*-retinal chromophore, bound through a protonated Schiff base linkage at Lys-296 in the chromophore binding pocket of rhodopsin, is converted to all-*trans*-retinal upon activation by a photon (Ebrey and Koutalos 2001). This results in a series of conformational changes in rhodopsin, eventually leading to the biologically active metarhodopsin II, which exists in an equilibrium with other metarhodopsin intermediates (Ritter et al. 2004; Vogel et al. 2004). For rhodopsin to regenerate in vivo, all-*trans*-retinal must first be released, which involves hydrolysis of the existing Schiff base linkage, and dissociation of retinal from opsin (Hofmann et al. 1992; Pulvermüller et al. 1997). Rhodopsin is a highly specialized chromophore-protein complex, where small changes in the amino acid sequence can profoundly affect its structure and function, ultimately resulting in changes to the visual capacities of an organism. Therefore, we hypothesize that a nocturnal lifestyle in early mammals would have involved adaptive changes in their visual system, especially in rhodopsin. These adaptive changes are likely to be detected by computational tests for selection acting on the coding sequence of the gene, and through functional characterization of inferred ancestral proteins expressed in vitro (Thornton 2004; Yang 2007; Zhao et al. 2009a,b); in the present study, we employ both approaches to test this hypothesis.

Methods

COMPUTATIONAL ANALYSES

Protein-coding nucleotide sequences of 26 vertebrate rhodopsins downloaded from GenBank, and the *Thamnophis proximus* rhodopsin sequence (Yang 2010), were aligned using MEGA4 (Table S1) (Tamura et al. 2007). A gene tree was inferred by maximum likelihood (ML) using PhyML 3 under the GTR+G+I model with a BioNJ starting tree, the best of NNI and SPR tree improvement, and aLRT SH-like branch support (Anisimova and Gascuel 2006; Guindon et al. 2010). The topology of this tree disagreed with the expected species relationships (Fig. S1). This is common with rhodopsin gene trees, which are known to produce biased phylogenetic reconstructions (Chang and Campbell 2000). As a result, we instead used a species phylogeny based on previous studies (Bininda-Emonds et al. 2007; Meredith et al. 2007; Murphy et al. 2007; Wible et al. 2007; Asher and Helgen 2010) for the computational analyses (Fig. 1). Taxa were sampled from a broad range of tetrapods, with one or two representatives from each major group, in order to maximize phylogenetic coverage and divergence (Fig. 1; Fig. S1). Coelacanth and lungfish were included as outgroups (Fig. 1; Fig. S1).

Ancestral sequence reconstructions were carried out using codon-based marginal reconstruction implemented in the software package PAML 4.2b (Yang et al. 1995; Yang 2007). The random sites codon models were compared in order to find the best-fitting model (Table S2). To test the robustness of the ancestral reconstruction we generated and assayed three alternate ancestral mammal sequences. One was inferred by weighted random samplings derived from the posterior probability distribution following Gaucher et al. (2003). The other two were produced by replacement of a site reconstructed with low posterior with a different residue in the posterior distribution (V39A, V49L).

In addition to ancestral sequence reconstruction, we used codon models in PAML to test for positive selection on the branches leading to reconstructed nodes. Specifically, branch-site models were used to test for positive selection on the amniote, mammalian, and therian branches. The branch-site model has four site classes: (0) $0 < \omega_0 < 1$ for all branches; (1) $\omega_1 = 1$ for all branches; (2a) $\omega_{2a} = \omega_{2b} \geq 1$ in the foreground and $0 < \omega_{2a} = \omega_0 < 1$ in the background; and (2b) $\omega_{2b} = \omega_{2a} \geq 1$ in the foreground and $\omega_{2b} = \omega_1 = 1$ in the background. The branch-site model is compared to a null model where ω_{2a} and ω_{2b} are constrained to equal one in both the background and the foreground. Statistical significance was assessed by likelihood ratio tests (LRTs) between the different nested models (Yang 2007). Positively selected sites were identified by Bayes Empirical Bayes (BEB) for the branch-site model (Yang et al. 2005).

SYNTHETIC GENE CONSTRUCTION AND IN VITRO EXPRESSION

The protein-coding sequences of the reconstructed ancestral Amniota, Mammalia, and Theria rhodopsins, as well as the resampled Mammalia sequence, were optimized for expression in mammalian cells and artificially synthesized (Geneart AG, Regensburg, Germany). Two additional ancestral Mammalia sequences were created by site-directed mutagenesis (V39A, V49L). Sites were mutated in the reconstructed ancestral mammal rhodopsin background to another residue in the posterior distribution. Site-directed mutagenesis primers were designed to induce the specified amino acid substitutions at these sites (Table S3), with mutagenesis performed via PCR with *Pfu* polymerase (Fermentas) employing standard cycling conditions.

All constructs were cloned into the p1D4-hrGFP II expression vector (Morrow and Chang 2010). After transformation into

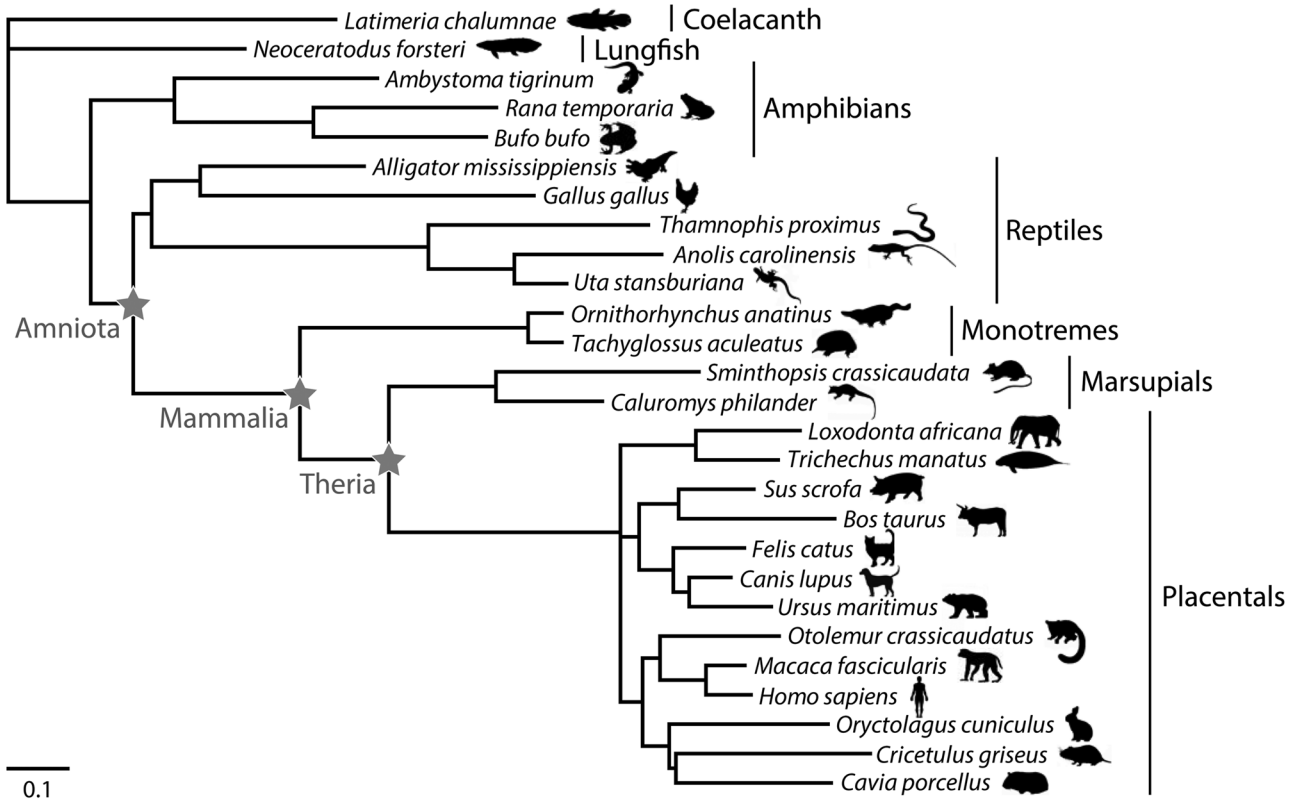

Figure 1. **Tetrapod phylogeny used in this study, with coelacanth and lungfish as outgroups. The topology is based on species relationships from recent studies (Bininda-Emonds et al. 2007; Meredith et al. 2007; Murphy et al. 2007; Wible et al. 2007; Asher and Helgen 2010). Branch lengths are proportional to the number of nucleotide substitutions per codon as estimated by the M3 model of PAML. Nodes with inferred ancestral rhodopsins are indicated by a gray star.**

α-Select Silver Competent Cells (Bioline), plasmids were purified with a Plasmid Maxi Kit (Qiagen). All vector sequences were confirmed using a 3730 DNA Analyzer (Applied Biosystems). Expression vectors were used to transfect HEK293T cells (8 μg/plate) using Lipofectamine 2000 (Invitrogen). Cells were harvested 48 hours post-transfection, washed three times with harvesting buffer (PBS, 10 μM aprotinin, 10 μM leupeptin) and incubated in 5 μM 11-*cis*-retinal to regenerate visual pigments. Samples were then solubilized in solubilization buffer (100 mM NaCl, 1 mM $CaCl_2$, 50 mM Tris pH 7, 1% dodecyl maltoside) and immunoaffinity purified with the 1D4 monoclonal antibody (Molday and MacKenzie 1983). Purified visual pigment samples were eluted in sodium phosphate buffer (50 mM NaPhos, 0.1% dodecyl maltoside, pH 7).

The ultraviolet-visible absorption spectra of purified bovine and ancestrally reconstructed rhodopsins were recorded in the dark at 25°C using a Cary 4000 double-beam spectrophotometer (Varian). Activation was measured by a blue shift in peak absorbance to ~380 nm following light bleaching, representing the active metarhodopsin II state. Dark-light difference spectra were generated by subtracting metarhodopsin II spectra from respective dark state spectra. All λ_{MAX} values were calculated after fitting absorbance spectra to a standard template for A1 visual pigments (Govardovskii et al. 2000). The protocol used to determine retinal release rates of visual pigments was modified from that of Farrens and Khorana (1995). Briefly, 0.08–0.15 μM visual pigment samples in submicrofluorometer cell cuvettes (Varian) were bleached for 30 seconds at 20°C using a Fiber-Lite MI-152 Illuminator external light source (Dolan-Jenner), using a filter to restrict wavelengths of light below 475 nm. Fluorescence measurements were integrated for 2 seconds at 30-second intervals using a Cary Eclipse fluorescence spectrophotometer (Varian). The excitation wavelength was 295 nm [1.5 nm slit width] and the emission wavelength 330 nm [10 nm slit width]; no noticeable pigment bleaching by the excitation beam was detected. Retinal release was demonstrated through a sharp initial rise in intrinsic tryptophan fluorescence, representing a decrease in fluorescent quenching of W265 by the retinal chromophore. Data from the initial rise was fit to a three variable, first order exponential equation [$y = y_o + a(1 - e^{-bx})$], with half-life values calculated based on the rate constant 'b' [$t_{1/2} = \ln 2/b$]. All curve fitting resulted in r^2 values of greater than 0.95. Differences in retinal release rates were assessed using a two-tailed *t*-test with unequal variance, where *P*-values of < 0.05 were deemed significant.

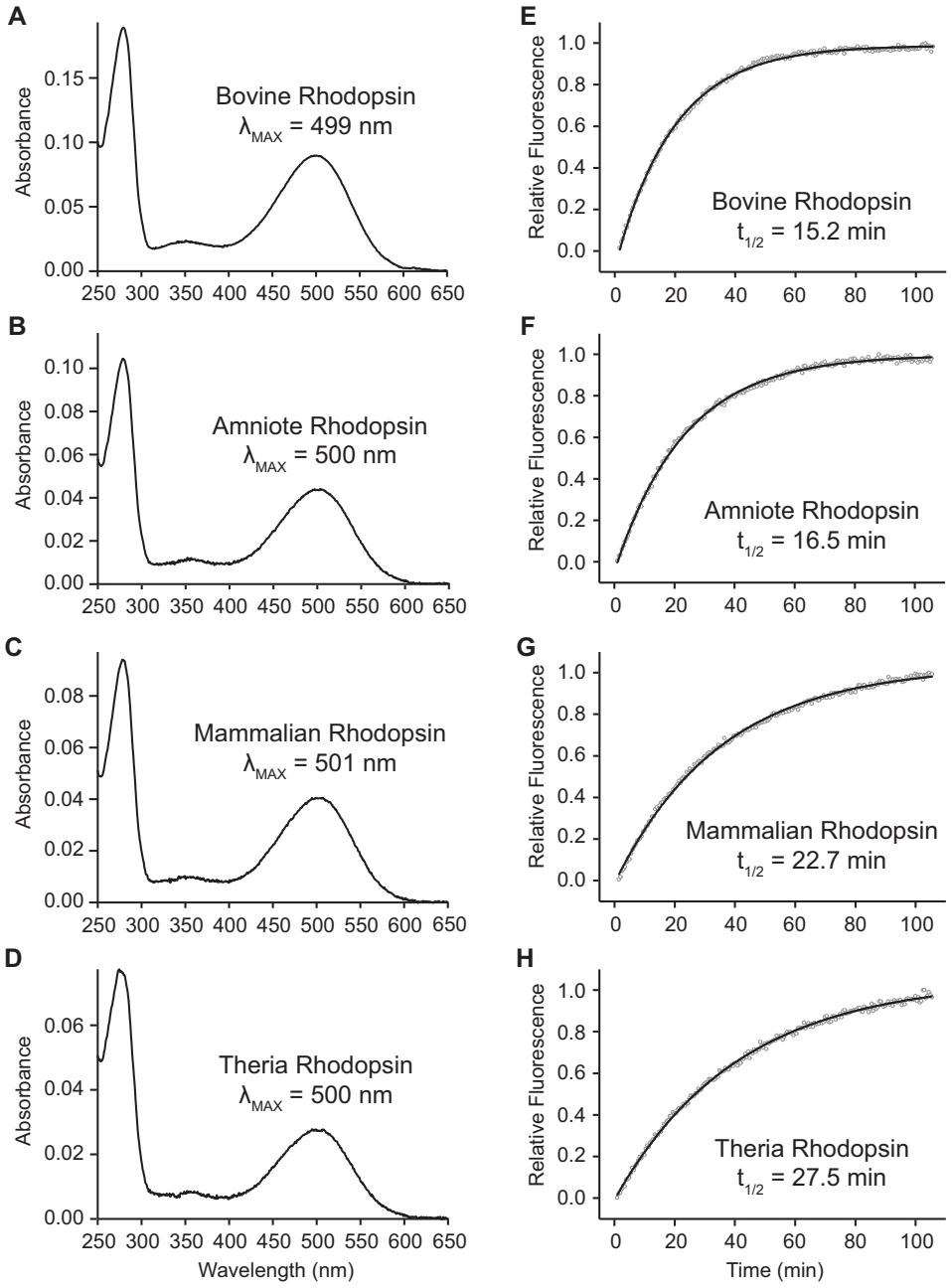

Figure 2. Absorption spectra (A–D) and retinal release fluorescence data (E–H) of rhodopsins inferred for Amniota (B, F), Mammlia (C, G), and Theria (D, H) nodes. Bovine rhodopsin is the control (A, E).

Results and Discussion

ANCESTRAL GENE RECONSTRUCTION

In order to test the hypothesis that the origin and successful rise of the mammalian clade took place in a nocturnal environment, ancestral rhodopsin coding sequences for the nodes Amniota (reptiles, mammals), Mammalia (monotremes, marsupials, placentals), and Theria (marsupials, placentals) were inferred by the M3/M7 codon models implemented in PAML 4.2b (Yang et al. 1995; Yang 2007). M3 and M7 were the best-fitting random sites models and resulted in identical ancestral sequences (Table S2). The amniote and mammal ancestral sequences had 95% similarity (18 variable amino acids), whereas the amniote and therian sequences had 92% similarity (28 variable amino acids). As expected, the mammalian and therian sequences were more similar with only 10 variable amino acids (97% similarity). The majority of sites were reconstructed with high posterior probability (Fig. S2) with only nine, eight, and five amino acids reconstructed with less than 80% posterior probability for the amniote,

mammalian, and therian ancestral sequences, respectively, and only one site reconstructed with less than 50% posterior probability (Table S4). Since the reconstructed ancestral sequences represent the most probable sequence out of a distribution, it is still useful to assess the robustness of the results to variation in the reconstruction (Gaucher et al. 2003; Pollock and Chang 2007; Bar-Rogovsky et al. 2015). Thus, we inferred three additional ancestral mammalian sequences, one by weighted random resampling from the posterior distribution and two by site-directed mutagenesis of residues that were reconstructed with low posterior probability to an alternate residue in the distribution. The resampled ancestral mammalian sequence differed from the most probable mammalian sequence by seven amino acids and thus provides a strong test for the effect of uncertainty in the reconstruction.

Inferred sequences were expressed in vitro and functionally assayed (Table 1; Fig. 2). As would be expected of properly folded and functional visual pigments, all expressed ancestral rhodopsins were stable in the dark, and converted to a biologically active metarhodopsin II intermediate when exposed to light (Fig. 2B–D), as indicated by a shift in the absorption maximum (λ_{MAX}) to approximately 380 nm, the characteristic λ_{MAX} of metarhodopsin II (Baumann and Bender 1973). The λ_{MAX} estimate for our control, bovine rhodopsin, was 499 nm, which matches previous well-established results (Fig. 2A) (Oprian et al. 1987). Reconstructed ancestral rhodopsins (Amniota, Mammalia, and Theria), as well as additional inferred sequences of Mammalia, had λ_{MAX} values similar to bovine rhodopsin, at around 500 nm (Table 1; Fig. 2B–D). The similarities in λ_{MAX} values among ancestral mammalian rhodopsins is consistent with the lack of variation in known visual pigment spectral tuning sites among these sequences (Lin and Sakmar 1996).

We also assessed the rate of retinal release from light-activated rhodopsin by monitoring the increase in fluorescence following release of all-*trans*-retinal from the binding pocket, an assay used to evaluate the stability of the activated metarhodopsin II state (Farrens and Khorana 1995). Retinal release rates of all ancestral visual pigments were measured alongside a bovine rhodopsin control, and are reported as relative half-life values (rel-$t_{1/2}$). Retinal release half-life measurements for bovine rhodopsin ($t_{1/2} = 15.2$ min; Table 1; Fig. 2E) were consistent with previously published results (Farrens and Khorana 1995; Yan et al. 2002). However, retinal release half-life values for the ancestral Amniota, Mammalia, and Theria rhodopsins were 1.2, 1.6, and 1.9 times greater than bovine, respectively, with both the ancestral Mammalia and Theria rhodopsins being significantly different (Table 1; Fig. 2F–H). The alternate ancestral Mammalia sequences did not have significantly different retinal release rates than the most probable sequence, suggesting that the assayed phenotype is robust to uncertainty in the reconstruction (Table 1). These findings suggest variation in the function of activated rhodopsin at the early stages of mammalian evolution, as well as at the split of crown monotremes and eutherians (placentals and marsupials).

Eighteen amino acid substitutions occurred along the branch leading from the ancestral Amniota node to the ancestral Mammalia node, which led to a significantly slower rate of retinal release in ancestral Mammalia rhodopsin. One substitution that likely contributed to this difference is V189I, which has previously been shown to slow retinal release (Morrow and Chang 2015). This is not surprising, considering site 189 is a known functional determinant of visual pigments (Kuwayama et al. 2002) and is part of the "retinal plug" that shields the chromophore from the extracellular environment (Sakai et al. 2010). Meanwhile, another pair of substitutions (L216M, S260A) are located near an opening between transmembrane helices 5 and 6 in the activated state (Choe et al. 2011) that is the hypothesized site of retinal release (Wang and Duan 2011). There are also three substitutions (A39V, L49V, V308M) at sites that have been shown not to influence retinal release, either in this study or previously (Piechnick et al. 2012; Morrow and Chang 2015). Finally, four of the substitutions are in the C-terminus (I318L, T333A, A336T, T340S), which are more likely to mediate rhodopsin phosphorylation than retinal release (Ohguro et al. 1995; Greene et al. 1997).

Our results show that light-activated ancestral Mammalia and Theria rhodopsins release retinal significantly slower than Amniota and bovine rhodopsin (Table 1; Fig. 2E–H). However, the association of retinal release with other biochemical properties of vision, and its influence on physiological aspects of vision, are still unclear. Our previous study suggests that the D83N substitution in the nocturnal echidna leads to slower retinal release (Bickelmann et al. 2012). This substitution has also been associated with an increase in the formation rate of metarhodopsin II in bats and deep-water cichlids, and has been postulated to be adaptive for dim-light vision in those animals (Sugawara et al. 2010). There is also evidence that retinal release may affect dark adaptation, the recovery of sensitivity of the visual system following exposure to light (Hecht et al. 1937; Ala-Laurila et al. 2006). For larger light bleaching events, the metabolism of new 11-*cis*-retinal in the retinal pigment epithelium is thought to be the rate limiting step of dark adaptation (Mahroo and Lamb 2004; Lamb and Pugh 2006; Lee et al. 2010). On the other hand, existing pools of 11-*cis*-retinal in the rod outer segments may be able to accommodate for light bleaches of smaller magnitude (Azuma et al. 1977; Cocozza and Ostroy 1987), suggesting that under these conditions, faster retinal release may be beneficial for dim-light vision, allowing for a shorter period of dark adaptation. Additionally, a recent study highlighted a possible link between active state lifetime and dark state thermal isomerization in rhodopsin (Yanagawa et al. 2015), which suggests a potential connection between light-activated stability and photosensitivity in the dark. In this context,

Table 1. Retinal release rates and λ_{MAX} values of bovine and ancestrally reconstructed rhodopsins.

Rhodopsin	Retinal release rate, relative to bovine rhodopsin (min)	λ_{MAX} (nm)
Bovine	1.0 ± 0.00 (16)	499.4 nm
Ancestral amniota	1.2 ± 0.14 (4)	499.8 nm
Ancestral mammalia	1.6 ± 0.22 (4)*	501.3 nm
Ancestral mammalia V39A	1.9 ± 0.15 (4)*	500.9 nm
Ancestral mammalia V49L	1.6 ± 0.14 (3)*	500.4 nm
Ancestral mammalia resampled	2.1 ± 0.41 (3)*	501.7 nm
Ancestral theria	1.9 ± 0.15 (4)*	500.2 nm

Includes three additional ancestral Mammalia sequences inferred to assess the robustness of the ancestral reconstruction. *Indicates *P*-value of < 0.05 when compared to bovine rhodopsin using a two-tailed *t*-test with unequal variance.

it is interesting to speculate that changes in retinal release rates may be associated with shifts in visual capabilities associated with life-history transitions in early mammalian ancestors. Therefore, while the precise mechanism by which variation in retinal release may influence adaptations to dim-light vision remains unclear, it continues to be an interesting question to pursue from both a physiological and evolutionary perspective.

TESTS FOR SELECTION

Along with in vitro expression of ancestral rhodopsins, we also applied tests of selection in order to detect positive selection in rhodopsin coding sequences that may lead to functional change (Table S5). Assuming a switch from diurnality to nocturnality at the origin of mammals, one would expect to find positive selection indicating adaptive changes along the branch leading from Amniota to Mammalia. However, in contrast to initial expectations, we did not find any evidence of positive selection along the mammalian branch (Table S5). Instead, our analyses revealed positive selection, using the branch-site model, along the branch leading to Theria (Table S5). The elevated dN/dS ratio is supported by a total of seven sites (13, 37, 225, 290, 345, 346, 348), identified by Bayes Empirical Bayes (BEB) analysis with a posterior probability greater than 90% (Table S6). Even though this finding may appear to contradict our initial assumption, as well as the results from the reconstruction methods employed in this study, the detected positive selection along the therian branch might be explained by the ecological diversification of Mesozoic mammals (Luo 2007). Indeed, there is evidence for five ecological diversification events during the early evolution of this clade, including the appearance of lifestyles typically seen in modern mammals (Luo 2007). For example, already by the early Jurassic, theriiform taxa such as *Docofossor*, *Agilodocodon*, *Fruitafossor*, *Repenomamus*, *Volaticotherium*, *Henkelotherium,* and *Vincelestes*, had evolved fossorial, scansorial, arboreal, and volant ecologies (Luo and Wible 2005; Meng et al. 2006; Luo 2007; Luo et al. 2015; Meng et al. 2015). Assuming that the earliest mammals had indeed been nocturnal, it seems plausible that rhodopsin may have undergone major changes in response to invading new habitats at different light levels; these adaptations are likely to be detected as positive selection by codon model analyses of sequence evolution.

Selection was nevertheless acting upon mammalian rhodopsin in another way: in a previous study, we detected a high number of synonymous substitutions along the mammalian branch, resulting in a preference for G/C-ending codons over A/T (Chang et al. 2012). A preferred use of codons with a fourfold degeneracy at the 3rd position has been found to increase mRNA stability and tRNA translation efficiency (Ikemura 1985; Shabalina et al. 2006; Drummond and Wilke 2008), resulting in an increased number of rhodopsin molecules within the retina, and facilitating vision at low-light levels (Chang et al. 2012). Here, we hypothesize that these changes in codon bias were evolutionarily followed by adaptive changes in rhodopsin coding sequences along the therian branch due to ecological specializations at varying light levels.

Our study found molecular support for the hypothesis that nocturnality was likely present in early mammals and their ancestors, which presumably was a selective advantage in the evolution of endothermy, a potential mammalian key innovation, followed by changes in rhodopsin as a consequence of adapting to new light habitats in the therian lineage. We employed two different molecular approaches to study rhodopsin: in vitro expression of ancestrally reconstructed sequences to assess functional differences, and likelihood-based tests for selection to detect potential selective pressures. Our results are congruent with previous studies that support the view of ancestral mammalian nocturnality from an ecomorphological perspective (Jerison 1971; Crompton et al. 1978; Kemp 2005; Angielczyk and Schmitz 2014). The findings contribute to an improved understanding of early mammalian evolution, highlighting the importance of the interplay between morphology, palaeontology, and molecular biology when addressing large-scale evolutionary questions.

ACKNOWLEDGMENTS

We are thankful to M. Meixner, R. Schreiber and A. Sonntag (Berlin, Germany), as well as members of the Chang Lab (Toronto, Canada) for valuable help in the lab. 11-*cis* retinal was generously provided by R. Crouch (Charleston, USA). We thank Z.-X. Luo (Chicago, USA) for enriching discussions on early mammalian ecology and diversity. Comments by L.A. Tsuji (Toronto, Canada) helped improve earlier versions of the manuscript. This work was supported by a a research grant from the Deutsche Forschungsgemeinschaft (Mu 1760/2-3; to C.B. and J.M.), an Ontario Graduate Scholarship and a Vision Science Research Program Scholarship (to R.K.S.), a National Sciences and Engineering Research Council Discovery grant (to B.S.W.C.), and a Human Frontier Science Program grant (to B.S.W.C.). C.B., B.S.W.C., and J.M. designed the study. C.B., J.D., and R.K.S. compiled the dataset and performed computational sequence analyses, including ancestral reconstructions. C.B., J.M.M., I.V.H., and S.L. performed in vitro experiments. C.B., J.M., J.M.M., and B.S.W.C. analyzed and interpreted the experimental data. C.B., B.S.W.C., J.M., J.M.M., and R.K.S. helped to draft the manuscript. All authors read and approved the final manuscript.

CONFLICT OF INTEREST

The authors declare they have no competing interests.

LITERATURE CITED

Ala-Laurila, P., A. V. Kolesnikov, R. K. Crouch, E. Tsina, S. A. Shukolyukov, V. I. Govardovskii, Y. Koutalos, B. Wiggert, M. E. Estevez, and M. C. Cornwall. 2006. Visual cycle: dependence of retinol production and removal on photoproduct decay and cell morphology. J. Gen. Physiol. 128:153–169.

Anisimova, M., and O. Gascuel. 2006. Approximate likelihood-ratio test for branches: a fast, accurate, and powerful alternative. Syst. Biol. 55:539–552.

Angielczyk, K. D., and L. Schmitz. 2014. Nocturnality in synapsids predates the origin of mammals by over 100 million years. Proc. R. Soc. B. 281:20141642.

Asher, R. J., and K. M. Helgen. 2010. Nomenclature and placental mammal phylogeny. BMC Evol. Biol. 10:102.

Azuma, K., M. Azuma, and W. Sickel. 1977. Regeneration of rhodopsin in frog rod outer segments. J. Physiol. 271:747–759.

Bar-Rogovsky, H., A. Stern, O. Penn, I. Kobl, T. Pupko, and D. S. Tawfik. 2015. Assessing the prediction fidelity of ancestral reconstruction by a library approach. Protein Eng. Des. Sel. Advance online publication. Available at http://peds.oxfordjournals.org/content/early/2015/08/13/protein.gzv038.long

Baumann, C. H., and S. Bender. 1973. Kinetics of rhodopsin bleaching in the isolated human retina. J. Physiol. 235:761–773.

Bickelmann, C., J. M. Morrow, J. Müller, and B. S. W. Chang. 2012. Functional characterization of the rod visual pigment of the echidna (*Tachyglossus aculeatus*), a basal mammal. Vis. Neurosci. 29:211–217.

Bininda-Emonds, O., M. Cardillo, K. E. Jones, R. D. E. MacPhee, R. M. D. Beck, R. Grenyer, S. A. Price, R. A. Vos, J. L. Gittleman, and A. Purvis. 2007. The delayed rise of present-day mammals. Nature 466:507–512.

Blumer, K. J. 2004. The need for speed. Nature 427:20–21.

Burns, M. E., and D. A. Baylor.2001. Activation, deactivation, and adaptation in vertebrate photoreceptor cells. Ann. Rev. Neurosci. 24:779–805.

Chang, B. S. W., and D. L. Campbell. 2000. Bias in phylogenetic reconstruction of vertebrate rhodopsin sequences. Mol. Biol. Evol. 17:1220–1231.

Chang, B. S. W., J. Du, C. Weadick, J. Müller, C. Bickelmann, D. Yu, and J. M. Morrow. 2012. The future of codon models in studies of molecular function: ancestral reconstruction and clade models of functional divergence. Pp. 145–163 *in* G. M., Cannarozzi, and A. Schneider, eds. Codon models in evolution. Oxford Univ. Press, Oxford.

Choe, H. W., Y. J. Kim, J. H. Park, T. Morizumi, E. F. Pai, N. Krauss, K. P. Hofmann, P. Scheerer, and O. P. Ernst. 2011. Crystal structure of metarhodopsin II. Nature 471:651–655.

Clarke, A., and H. O. Pörtner. 2010. Temperature, metabolic power and the evolution of endothermy. Biol. Rev. Camb. Philos. Soc. 85:703–727.

Cocozza, J. D., and S. E. Ostroy. 1987. Factors affecting the regeneration of rhodopsin in the isolated amphibian retina. Vis. Res. 27: 1085–1091.

Crompton, A. W., C. R. Taylor, and J. A. Jagger. 1978. Evolution of homeothermy in mammals. Nature 272:333–336.

Davies, W. I., S. P. Collin, and D. M. Hunt. 2012. Molecular ecology and adaptation of visual photopigments in craniates. Mol. Ecol. 21: 3121–3158.

Drummond, D. A., and C. O. Wilke. 2008. Mistranslation-induced protein misfolding as a dominant constraint on coding-sequence evolution. Cell 134:341-352.

Ebrey, T., and Y. Koutalos. 2001. Vertebrate photoreceptors. Prog. Retin. Eye Res. 20:49–91.

Farrens, D. L., and G. Khorana. 1995. Structure and function in rhodopsin. Measurement of the rate of metarhodopsin II decay by fluorescence spectroscopy. J. Biol. Chem. 270:5073–5076.

Gaucher, E. A., J. M. Thomson, M. F. Burgan, and S. A. Benner. 2003. Inferring the paleoenvironment of ancient bacteria on the basis of resurrected proteins. Nature 425:285–288.

Govardovskii, V. I., N. Fyhrquist, T. Reuter, D. G. Kuzmin, and K. Donner. 2000. In search of the visual pigment template. Vis. Neurosci. 17:509–528.

Greene, N. M., D. S. Williams, and A. C. Newton 1997. Identification of protein kinase C phosphorylation sites on bovine rhodopsin. J. Biol. Chem. 272:10341–10344.

Guindon, S., J. F. Dufayard, V. Lefort, M. Anisimova, W. Hordijk, and O. Gascuel. 2010. New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. Syst. Biol. 59:307–321.

Hall, M. I., J. M. Kamilar, and E. C. Kirk. 2012. Eye shape and the nocturnal bottleneck of mammals. Proc. R. Soc. B. 279:4962-4968.

Hecht, S., C. Haig, and A. M. Chase. 1937. The influence of light adaptation on the subsequent dark adaptation of the eye. J. Gen. Physiol. 20: 831–850.

Hofmann, K. P., A. Pulvermüller, J. Buczytko, P. Van Hooser, and K. Palczewski. 1992. The role of arrestin and retinoids in the regeneration pathway of rhodopsin. J. Biol. Chem. 267:15701–15706.

Ikemura, T. 1985. Codon usage and tRNA content in unicellular and multicellular organisms. Mol. Biol. Evol. 2:13–34.

Jerison, H. J. 1971. More on why birds and mammals have big brains. Am. Nat. 105:185–189.

Kemp, T. S., ed. 2005. The origin and evolution of mammals. Oxford Univ. Press, New York.

Kielan-Jaworowska, Z., R. L. Cifelli, and Z.-X. Luo, eds. 2004. Mammals from the age of dinosaurs: origins, evolution, and structure. Columbia Univ. Press, New York.

Kuwayama, S., H. Imai, T. Hirano, A. Terakita, and Y. Shichida. 2002. Conserved proline residue at position 189 in cone visual pigments as a determinant of molecular properties different from rhodopsins. Biochemistry 41:15245–15252.

Lamb, T., and E. Pugh. 2006. Phototransduction, dark adaption, and rhodopsin regeneration. Invest. Ophthal. Vis. Sci. 47:5138–5153.

Lee, K. A., M. Nawrot, G. G. Garwin, J. C. Saari, and J. B. Hurley. 2010. Relationships among visual cycle retinoids, rhodopsin phosphorylation,

and phototransduction in mouse eyes during light and dark adaptation. Biochemistry 49:2454–2463.

Lin, S. W., and T. P. Sakmar. 1996. Specific tryptophan UV-absorbance changes are probes of the transition of rhodopsin to its active state. Biochemistry 35:11149–11159.

Luo, Z.-X. 2007. Transformation and diversification in early mammal evolution. Nature 450:1011–1019.

Luo, Z.-X., and J. R. Wible. 2005. A late Jurassic digging mammal and early mammalian diversification. Science 308:103–107.

Luo, Z.-X., Q.-J. Meng, Q. Ji, D. Liu, Y.-G. Zhang, and A. I. Neander. 2015. Evolutionary development in basal mammaliaforms as revealed by a docodontan. Science 13:760–764.

Mahroo, O. A., and T. D. Lamb. 2004. Recovery of the human photopic electroretinogram after bleaching exposures: estimation of pigment regeneration kinetics. J. Physiol. 554:417–437.

Meng, J., Y. Hu, Y. Wang, X. Wang, and C. Li. 2006. A mesozoic gliding mammal from northeastern China. Nature 444:889–893.

Meng, Q.-J., Q. Ji, Y.-G. Zhang, D. Liu, D. M. Grossnickle, and Z.-X. Luo. 2015. An arboreal docodont from the Jurassic and mammaliaform ecological diversification. Science 13:764–768.

Meredith, R. W., M. Westerman, J. A. Case, and M. S. Springer. 2007. A phylogeny and timescale for marsupial evolution based on sequences for five nuclear genes. J. Mamm. Evol. 15:1–36.

Molday, R. S., and D. MacKenzie. 1983. Monoclonal antibodies to rhodopsin: characterization, cross-reactivity, and application as structural probes. Biochemistry 22:653–660.

Morrow, J. M., and B. S. W. Chang. 2010. The p1D4-hrGFP II expression vector: a tool for expressing and purifying visual pigments and other G protein-coupled receptors. Plasmid 64:162–169.

——. 2015. Comparative mutagenesis studies of retinal release in light-activated zebrafish rhodopsin using fluorescence spectroscopy. Biochemistry 54:4507–4518.

Murphy, W. J., T. H. Pringle, T. A. Crider, M. S. Springer, and W. Miller. 2007. Using genomic data to unravel the root of the placental mammal phylogeny. Genome Res. 17:413–421.

Ohguro, H., J. P. Van Hooser, A. H. Milam, and K. Palczewski. 1995. Rhodopsin phosphorylation and dephosphorylation in vivo. J. Biol. Chem. 270:14259–14262.

Oprian, D. D., R. S. Molday, R. J. Kaufman, and H. G. Khorana. 1987. Expression of a synthetic bovine rhodopsin gene in monkey kidney cells. Proc. Natl. Acad. Sci. USA 84:8874–8878.

Piechnick, R., E. Ritter, P. W. Hildebrand, O. P. Ernst, P. Scheerer, K. P. Hofmann, and M. Heck. 2012. Effect of channel mutations on the uptake and release oft he retinal ligand in opsin. Proc. Natl. Acad. Sci. USA 109:5247–5252.

Pollock, D. D., and B. S. W. Chang. 2007. Dealing with uncertainty in ancestral sequence reconstruction: sampling from the posterior distribution. In Ancestral Sequence Reconstruction, D. A. Liberles, ed. Oxford University Press; Oxford. 85–94.

Pulvermüller, A., D. Maretzki, M. Rudnicka-Nawrot, W. C. Smith, K. Palczewski, and K. P. Hofmann. 1997. Functional differences in the interaction of arrestin and its splice variant, p44, with rhodopsin. Biochemistry 36:9253–9260.

Ritter, E., K. Zimmermann, M. Heck, K. P. Hofmann, and F. J. Bartl. 2004. Transition of rhodopsin into the active metarhodopsin II state opens a new light-induced pathway linked to Schiff base isomerization. J. Biol. Chem. 279:48102–48111.

Ross, C. F., and E. C. Kirk. 2007. Evolution of eye size and shape in primates. J. Hum. Evol. 52:294–313.

Sakai, K., Y. Imamoto, T. Yamashita, and Y. Shichida. 2010. Functional analysis of the second extracellular loop of rhodopsin by characterizing split variants. Photochem. Photobiol. Sci. 9:1490–1497.

Shabalina, S. A., A. Y. Ogutsov, and N. A. Spiridonov. 2006. A periodic pattern of mRNA secondary structure created by the genetic code. Nucl. Acid. Res. 34:2428–2437.

Sugawara, T., H. Imai, M. Nikaido, Y. Imamoto, and N. Okada. 2010. Vertebrate rhodopsin adaptation to dim light via rapid meta-II intermediate formation. Mol. Biol. Evol. 27:506–519.

Tamura, K., J. Dudley, M. Nei, and S. Kumar. 2007. MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol. Biol. Evol. 24:1596–1599.

Thornton, J. W. 2004. Resurrecting ancient genes: Experimental analysis of extinct molecules. Nat. Rev. Genet. 5:366–375.

Vogel, R., J. Ruprecht, C. Villa, T. Mielke, G. F. Schertler, and F. Siebert. 2004. Rhodopsin photoproducts in 2D crystals. J. Mol. Biol. 338:597–609.

Wang, T., and Y. Duan. 2011. Retinal release from opsin in molecular dynamics simulations. J. Mol. Recognit. 24:350–358.

Wible, J. R., G. W. Rougier, M. J. Novacek, and R. J. Asher. 2007. Cretaceous eutherians and Laurasian origin for placental mammals near the K/T boundary. Nature 447:1003–1006.

Yan, E. C., M. A. Kazmi, S. De, B. S. Chang, C. Seibert, E. P. Marin, R. A. Mathies, and T. P. Sakmar. 2002. Function of extracellular loop 2 in rhodopsin: glutamic acid 181 modulates stability and absorption wavelength of metarhodopsin II. Biochemistry 41:3620–3627.

Yang, Z. 2007. PAML 4: phylogenetic analysis using maximum likelihood. Mol. Biol. Evol. 24:1586–1591.

Yang, Z., S. Kumar, and M. Nei. 1995. A new method of inference of ancestral nucleotide and amino acid sequences. Genetics 141:1641–1650.

Yau, K.-W. 1994. Phototransduction mechanism in retinal rods and cones. Invest. Ophthalmol. Vis. Sci. 35:9–32.

Yang, C. G. Y. 2010. Rod-like properties of small single cones: transmutated photoreceptors of garter snakes (*Thamnophis proximus*). Master Thesis, Department of Cell and System Biology, University of Toronto, Toronto.

Yang, Z., W. S. W. Wong, and R. Nielsen. 2005. Bayes empirical Bayes inference of amino acid sites under positive selection. Mol. Biol. Evol. 22:1107–1118.

Zhao, H., S. J. Rossiter, E. C. Teeling, C. Li, J. A. Cotton, and S. Zhang. 2009a. The evolution of colour vision in nocturnal mammals. Proc. Natl. Acad. Sci. USA 106:8980–8985.

Zhao, H., B. Ru, E. C. Teeling, C. G. Faulkes, S. Zhang, and S. J. Rossiter. 2009b. Rhodopsin molecular evolution in mammals inhabiting low light environments. PloS One 4:e8326.

Associate Editor: J. Storz
Handling Editor: J. Conner

Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Table S1. Rhodopsin sequences.
Table S2. Random sites model parameters.
Table S3. Primer sequences.
Table S4. Amino acid sites reconstructed with low posterior probability.
Table S5. Positive selection tests.
Table S6. List of condon sites under positive selection.
Figure S1. Rhodopsin maximum likelihood phylogeny.
Figure S2. Histogram of posterior probabilities of ancestrally reconstructed sites.