Evolutionary transformation of rod photoreceptors in the all-cone retina of a diurnal garter snake

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Vertebrate retinas are generally composed of rod (dim-light) and cone (bright-light) photoreceptors with distinct morphologies that evolved as adaptations to nocturnal/crepuscular and diurnal light environments. Over 70 years ago, the "transmutation" theory was proposed to explain some of the rare exceptions in which a photoreceptor type is missing, suggesting that photoreceptors could evolutionarily transition between cell types. Although studies have shown support for this theory in nocturnal geckos, the origins of all-cone retinas, such as those found in diurnal colubrid snakes, remain a mystery. Here we investigate the evolutionary fate of the rods in a diurnal garter snake and test two competing hypotheses: (*i*) that the rods, and their corresponding molecular machinery, were lost or (*ii*) that the rods were evolutionarily modified to resemble, and function, as cones. Using multiple approaches, we find evidence for a functional and unusually blue-shifted rhodopsin that is expressed in small single "cones." Moreover, these cones express rod transducin and have rod ultrastructural features, providing strong support for the hypothesis that they are not true cones, as previously thought, but rather are modified rods. Several intriguing features of garter snake rhodopsin are suggestive of a more cone-like function. We propose that these cone-like rods may have evolved to regain spectral sensitivity and chromatic discrimination as a result of ancestral losses of middle-wavelength cone opsins in early snake evolution. This study illustrates how sensory evolution can be shaped not only by environmental constraints but also by historical contingency in forming new cell types with convergent functionality.

rhodopsin evolution | visual evolution | reptile vision | snake photoreceptors | visual pigment

How complex structures can arise has long fascinated evolutionary biologists, and the evolution of the eye, as noted by Charles Darwin (1), is perhaps the most famous example. Within the vertebrate eye, the light-sensing photoreceptors are complex, highly specialized cellular structures that can be divided into two general types based on their distinct morphologies and functions: cones, which are active during the day and contain cone opsin pigments, and rods, which mediate dim-light vision and contain rhodopsin (RH1) (2–4). The visual pigments contained in cone photoreceptors are classified into four different subtypes that mediate vision across the visible spectrum from the UV to the red (2). Although most vertebrate retinas are duplex, containing both cones and rods, squamate reptiles (lizards and snakes) are unusual, not only in having highly variable photoreceptor morphologies, but also for several instances of the absence of an entire class of photoreceptors, resulting in simplex retinas composed of only cones or rods (4).

In a seminal book published in 1942, Walls (4) hypothesized that, during evolution, vertebrate photoreceptors could transform from one type to another, a process that he termed photoreceptor "transmutation." As key examples of his theory, Walls (4) highlighted anatomical changes in the photoreceptors of snakes and geckos, two groups within which there have been significant shifts in diurnal and nocturnal activity patterns. Although several subsequent studies have investigated this hypothesis in geckos (5–9), whether the evolutionary transmutation of photoreceptors can happen in snakes remains an open question (10). Walls also noted a number of peculiar morphological adaptations in snake eyes, which he proposed were due to a subterranean phase early in snake evolution that led to degeneration of the ophidian visual system, resulting in loss of features common to other terrestrial vertebrates (4).

Colubrid snakes are an ideal group to study Walls's hypothesis of transmutation, due to their highly variable photoreceptor morphologies that range from all-cone in, at least some, diurnal species, such as *Thamnophis* (garter snakes), to all-rod in some nocturnal species, as well as species with the presumed ancestral condition of duplex retinas (4, 11). Previous studies in the diurnal colubrid *Thamnophis* have demonstrated an all-cone retina (4, 11–14), consisting of double cones and large single cones that express a long-wavelength pigment [presumably long wavelength-sensitive opsin (LWS)], and two classes of small single cone, one with a short-wavelength pigment [presumably short wavelength-sensitive 1 opsin (SWS1)] and the other with a middle-wavelength pigment, the identity of which is unclear (14). However, the ancestral condition for colubrids is likely to have been a duplex retina containing both rods and cones, similar to snakes such as pythons and boas, which have rods that express RH1, large single cones that express LWS, and small single cones that express SWS1 (Fig. 1) (4, 10, 11, 15, 16). The SWS2 and RH2

Significance

This study provides compelling evidence that the previously reported all-cone retina of a diurnal garter snake in fact contains a population of rod photoreceptors with the appearance, and presumably function, of cones. Our results suggest that the evolution of all-cone retinas occurred not through loss of rods but rather via the evolutionary transmutation of ancestral rods into more "cone-like" photoreceptors, to regain functionality that was lost during the early, possibly fossorial, origin of snakes. This study provides a better understanding of the process by which complex molecular/cellular structures and tissue types can evolve, and how, particularly for sensory systems, physiological constraints can be shaped by selective forces to produce evolutionary novelty.

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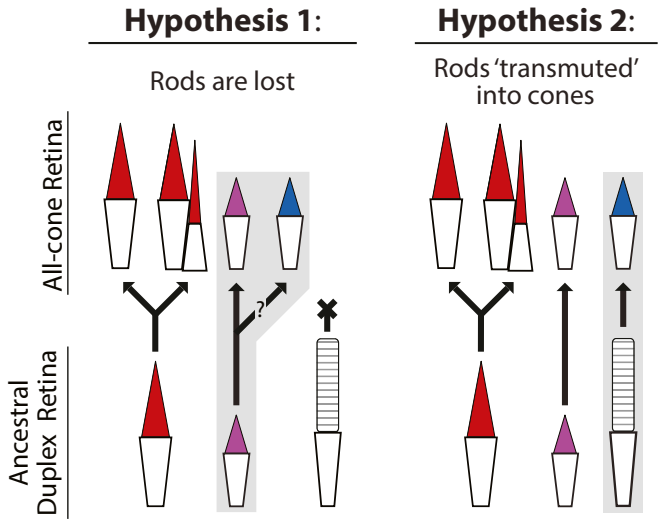

Fig. 1. Illustration of evolutionary pathways for two alternative hypotheses for the evolution of an all-cone retina from a duplex ancestor in diurnal colubrids. In hypothesis 1 the rod photoreceptors, along with RH1, are lost, and an additional cone type is derived from duplication of an existing cone or retained from an ancestral condition that was lost in other snakes. In hypothesis 2 the rod photoreceptor is evolutionarily modified into a cone photoreceptor, maintaining expression of RH1 and other rod-specific phototransduction machinery.

opsins, present ancestrally in vertebrates, appear to have been lost early in the evolution of snakes, perhaps as a result of their proposed fossorial origins (10, 17, 18).

Based on these findings, we can formulate two main hypotheses for the evolution of the all-cone retina of diurnal colubrids from the duplex ancestral condition (Fig. 1). The first is that the rods were lost, and RH1 and other components of the visual transduction cascade unique to rod photoreceptors were either lost or targeted to cones. The second hypothesis is that the rods were evolutionarily modified to resemble the appearance, and presumably the function, of cones. If the rods were modified to resemble cones, we might expect a subset of cones to possess molecular components, such as RH1, and morphological features consistent with a rod ancestry. To test these hypotheses, we examined the photoreceptors and visual pigments of a diurnal garter snake (*Thamnophis proximus*) by combining multiple methodologies including sequencing and molecular evolutionary analyses of opsin genes, microspectrophotometry (MSP) of intact photoreceptor cells, in vitro expression of visual pigments, and scanning and transmission electron microscopy (SEM and TEM) and immunohistochemistry of *T. proximus* retinas. The combined results of these experiments provide strong evidence that RH1 and other components of the rod visual transduction machinery are expressed in a subset of cone-like photoreceptors with rod ultrastructural features, and that the RH1-expressing "cones" are not true cones, as previously thought, but rather are modified (i.e., "transmuted"), cone-like rods. Our results shed new light on the evolutionary origins of the all-cone retinas of diurnal colubrid snakes, demonstrating how ancestral losses can be compensated by evolutionary modification of existing cellular structures.

Results

***T. proximus* Has an "All-Cone" Retina.** Scanning electron microscopy of *T. proximus* retina revealed only cells that could be identified as cones based on their gross morphology, including small, tapering outer segments and bulbous inner segments (Fig. 2 and *SI Appendix*, Fig. S1). We found no evidence of rods, such as those in, for example, python and boa retinas, which are quite distinct with long, slender inner and outer segments (15, 16). This finding is consistent with earlier studies of a closely related species, *Thamnophis sirtalis* (12–14), and with the condition described by Walls (4) for diurnal colubrids in general. Four cone types were identified in *T. proximus*: double cones, large single cones, and two seemingly distinct sizes of small single cones (Fig. 2*C*). These four cone types appear to be the same as those reported for *T. sirtalis* (14) and similar to those described for other caenophidian snakes with all-cone retinas (19). Sillman et al. (14) described two subtypes of small single cone in *T. sirtalis*, and we also found evidence for this in *T. proximus*, where some small single cones were substantially smaller than the others (see very small single cone, Fig. 2*C*), but this distinction was more subtle than that between the large single cones and small single cones and may be confounded by size variation of individual cells. As far as is known, pythons and boas have only large and small single cones, with no double cones (15, 16).

In *T. proximus*, large single cones and double cones account for ∼45% and 44% of the cones, respectively. The small single cones were rarer, accounting for the remaining 11% (∼9% small single and ∼2% very small single). Although four individuals were used for SEM, only a single complete retinal preparation was available to determine proportions. As a result, the level of individual variation in *T. proximus* photoreceptor proportions is unknown. Despite this, the proportions we found for *T. proximus* are similar to those found previously for *T. sirtalis* (14). Samples from different areas of the retina had similar proportions of the three photoreceptor cells, and there did not appear to be any strong distributional pattern or mosaic to the photoreceptors, such as that found in some other vertebrates (20–22), consistent with *T. sirtalis* (14).

***T. proximus* Possesses Three Visual Pigments.** Microspectrophotometry of intact photoreceptors from dissociated retina was used to determine the absorption spectra of the four morphological types of photoreceptor cells (*SI Appendix*, Table S1). The double cones and large single cones were found to possess a long-wavelength pigment with a peak absorbance (λ_{max}) of 542 nm (*SI Appendix*, Fig. S2), whereas the small single cones could be divided into two categories based on absorption characteristics: Some contained a medium-wavelength pigment with a λ_{max} of 482 nm (Fig. 3), and others possessed a short-wavelength pigment with a λ_{max} of 366 nm (*SI Appendix*, Fig. S2). The absorbance spectra of all three pigments fit the A1 chromophore profile. These results are similar to those found previously for *T. sirtalis* (14), except that the long-wavelength pigment is blue-shifted by ∼12 nm and the short-wavelength pigment is red-shifted by ∼6 nm, but differ from previous MSP in other snakes (*SI Appendix, Results*). The long- and short-wavelength pigments for both species are likely to be LWS and SWS1, respectively, based on their λ_{max} values and presence in other snakes, but the identity of the 482-nm pigment is unclear.

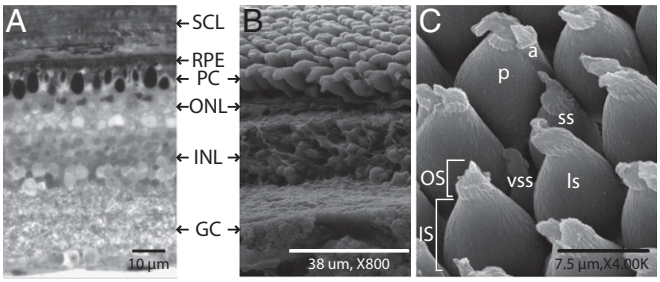

Fig. 2. Light and scanning electron microscopy of *T. proximus* retina. (*A* and *B*) Retinal cross-sections imaged using light (*A*) and electron (*B*) microscopy illustrating the layers of the retina. (*C*) SEM image of the retina illustrating the all-cone photoreceptor population with four different photoreceptor cell types. a, accessory member of double cone; GC, ganglion cell layer; INL, inner nuclear layer; IS, inner segment; ls, large single cone; ONL, outer nuclear layer; OS, outer segment; p, principal member of double cone; PC, photoreceptor cell layer; RPE, retinal pigment epithelium; SCL, scelera; ss, small single cone; vss, very small single cone.

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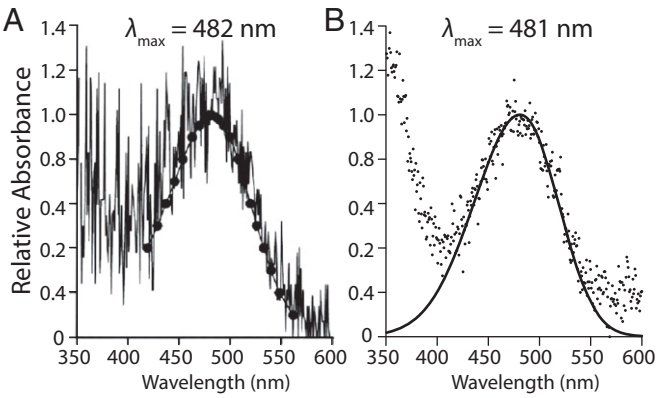

Fig. 3. Normalized absorbance spectra of (*A*) middle-wavelength visual pigment from intact photoreceptor cells measured by MSP and (*B*) in vitro expressed rhodopsin (RH1) from *T. proximus*. The filled circles and smooth curves of *A* are for the best-fit visual pigments calculated from A1-based template data. The λ_{max} values are the averages of measurements from multiple cells as shown in *SI Appendix*, Table S1. The λ_{max} of *B* was estimated by Govardovskii curve fitting.

RH1, LWS, and SWS1 Expressed in *T. proximus* Eye RNA and RH1 Maintained Under Normal Selective Pressures. Three full-length visual pigment genes were isolated from eye RNA using a combination of degenerate and RACE primers. These were identified using BLAST searches followed by phylogenetic analysis with other reptilian and vertebrate opsin sequences. These analyses identified the three opsin genes in *T. proximus* to be LWS, SWS1, and RH1 (GenBank accession nos. KU306727, KU306728, and KU306726, respectively; *SI Appendix*, Figs. S3–S5). *T. proximus* RH1 grouped with other snake RH1 sequences and was most closely related to the king cobra sequence, as expected based on the inferred species relationships (*SI Appendix*, Fig. S3) (23). The identification of an RH1 gene in the all-cone retina of *T. proximus* was surprising. Despite terrestrial vertebrates typically having RH1 that absorbs maximally around 500 nm (2), this raised the possibility that the 482-nm pigment identified by MSP may in fact be a highly blue-shifted rhodopsin. *T. proximus* RH1 has several distinctive residues, including S185 and S292. A292S is known to cause a substantial blue shift of λ_{max} in other vertebrate rhodopsins (24), whereas C185S has been shown to reduce transducin activation in vitro when mutated in bovine RH1 (25).

To determine whether expression in an all-cone retina altered evolutionary constraints on RH1, we analyzed selection patterns with PAML random-site, branch, branch-site, and clade models (*SI Appendix*, Fig. S3 and Table S2). The M0 model found an average ω (ratio of nonsynonymous to synonymous substitutions; d_N/d_S) of 0.07 and significant rate variation across sites (M3 vs. M0; *SI Appendix*, Table S2), as expected for a protein-coding gene under strong selective constraint. No evidence was found for positive selection on RH1 (ω > 1) either alignment-wide (M2a vs. M1a, M8 vs. M7; *SI Appendix*, Table S2) or in snakes, caenophidians, or *T. proximus* specifically, with the branch-site test. We found no evidence for loss of function in *T. proximus* RH1, which would be expected to result in an increased ω along this lineage; instead, the ω values for *T. proximus* did not differ significantly from background with either model (*SI Appendix*, Table S2), which is consistent with conserved function. This indicates that the RH1 gene in *T. proximus* is under strong selective constraint, similar to other vertebrates, despite it being expressed in an apparently all-cone retina.

***T. proximus* Rhodopsin Is Functional with a Highly Blue-Shifted λ_{max}.** To determine whether the *T. proximus* RH1 gene isolated from retinal mRNA encodes a functional visual pigment, the gene was ligated into the p1D4-hGFP II expression vector (26) and heterologously expressed in HEK293T cells. *T. proximus* RH1 properly bound and regenerated with 11-*cis*-retinal, producing a dark absorbance spectrum with a λ_{max} of 481 nm (Fig. 3 and *SI Appendix*, Fig. S6). This value is consistent with the MSP estimate of 482 nm for a subset of small single-cone photoreceptors (Fig. 3), strongly implying that RH1 is expressed in these cells. When bleached with light, the λ_{max} of *T. proximus* RH1 shifted to ∼380 nm, representing the biologically active metarhodopsin II intermediate and indicative of proper visual pigment function (27).

Rhodopsin and Rod Transducin Are Expressed in "Cone" Photoreceptor Cells. To further explore the possibility that components of the rod phototransduction cascade may be expressed in cone photoreceptors, we performed immunohistochemistry on retinal cryosections using two different antibodies: a rhodopsin antibody (4D2) and a rod-specific transducin antibody (K20).

As a positive control, we labeled mouse retina with both anti-rhodopsin (4D2) and anti-rod transducin (K20) antibodies (Fig. 4 *A*–*D*). We found RH1 localized to the rod outer segments and rod transducin localized to the inner segments, which was expected based on previous immunohistochemical characterizations of mouse retina using these antibodies (28). Because mouse retinas are highly rod-dominated, both RH1 and rod transducin were continuously distributed across the photoreceptor layer (Fig. 4*D*).

In *T. proximus*, staining for RH1 (4D2) was found in a small proportion of the cone photoreceptor cells. Staining was localized to the outer segment (Fig. 4*F*). This is consistent with previously unexplained staining of *T. sirtalis* retinas (*SI Appendix, Results*) (14). Rod transducin (K20) was also found in a subset of the cone photoreceptor cells, where staining was localized primarily to the inner segment and cell body of the photoreceptor (Fig. 4*G*). The presence of rod transducin in the inner segment is expected from retina exposed to light, unlike cone transducin, which does not translocate to the inner segment (29). This further supports the specificity of K20 for rod transducin to the exclusion of cone transducin. Double staining and analysis of the confocal z stack revealed that RH1 and rod transducin are present in the same cells and that there is some overlap of their localizations (Fig. 4 *H*–*J*). Combined with our MSP, sequencing, and in vitro expression results, the immunohistochemical results support the hypothesis that *T. proximus* RH1 is expressed in a "cone" photoreceptor cell.

A Subset of Small Single "Cones" Have Rod Ultrastructure. To further test the hypothesis that the rhodopsin-bearing cones are actually derived from rods, we examined the ultrastructure of the photoreceptors using TEM. Four different cone types were identified: double cones (*SI Appendix*, Fig. S7*A*), large single cones (*SI Appendix*, Fig. S7 *B* and *C*), and two types of small single cone (*SI Appendix*, Fig. S7 *B* and *C*). The double cones, large single cones, and first type of small single cone had the expected morphology, namely small tapering outer segments and bulbous inner segments with large ellipsoids (*SI Appendix*, Fig. S7 *A*–*C*) (12, 14). These cones also had the expected lamellar structure, where the outer-segment discs were open to the plasma membrane on one side (*SI Appendix*, Fig. S7 *D*–*F*, arrows). The other type of small single cone was noticeably distinct. These cells tended to have less-tapered outer segments and inner segments that were less bulbous and closer in width to the outer segments (Fig. 5 and *SI Appendix*, Fig. S7*C*). Additionally, the outer-segment discs of these cells were completely enclosed by plasma membrane (Fig. 5, arrows), which is a feature that is otherwise exclusive to, and characteristic of, rods (6, 14). Collectively, these results suggest that these cells are actually transmuted cone-like rods rather than true cones.

Discussion

In this study, we present several lines of evidence, both experimental and computational, to support the evolutionary transmutation of rods into "cone-like" photoreceptors in colubrid snakes. We found that despite a lack of apparent rod photoreceptors in its all-cone retina, which we confirmed by SEM, *T. proximus* possesses a rhodopsin gene (RH1), in addition to two cone opsins (SWS1,

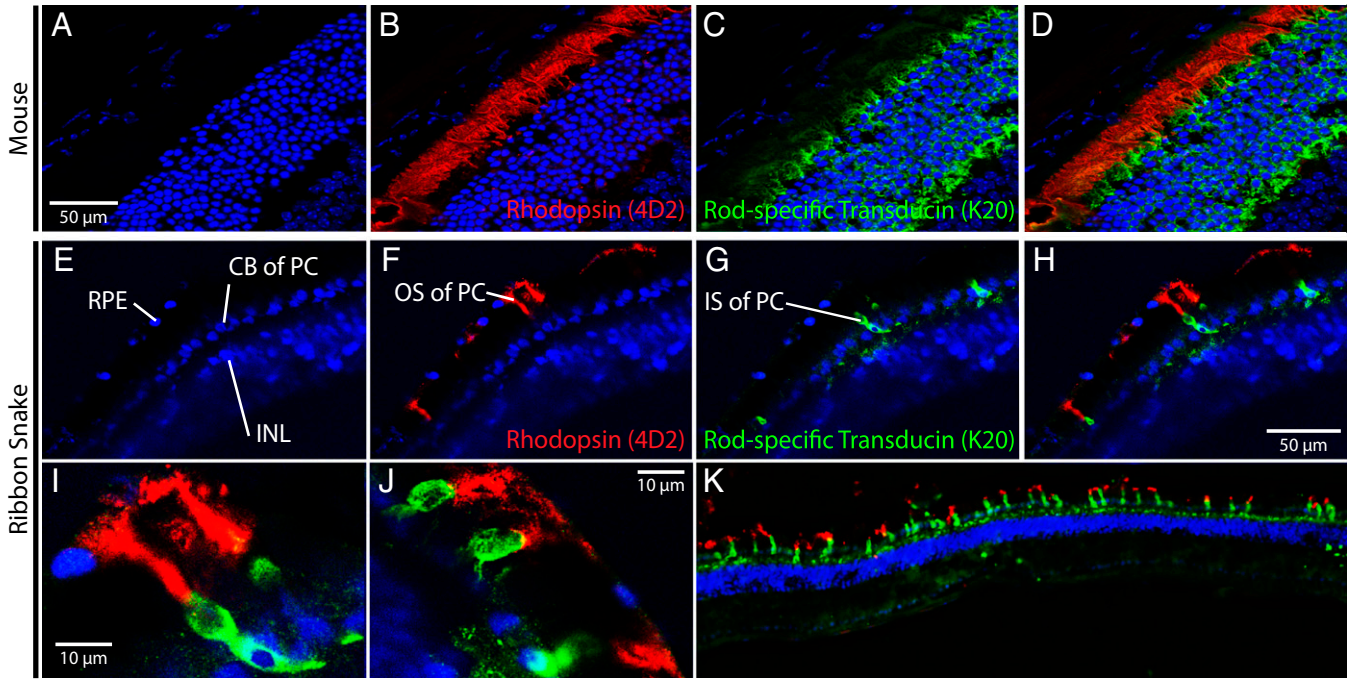

Fig. 4. Immunohistochemical staining of control (mouse; *A–D*) and *T. proximus* (*E–K*) transverse retinal cryosections with rhodopsin (4D2) and rod-specific transducin (K20) antibodies. Rhodopsin is found in a subset of cone cells localized to the outer segment (*F*). Rod-specific transducin is also found in a subset of these cells localized primarily to the inner segment (*G*). Double staining indicates that both rhodopsin and rod-specific transducin are found within the same cells (*H*), and this is confirmed in individual slices from the z stack (*I* and *J*). The section in *K* shows the broad distribution of rhodopsin and rod transducin-containing cells. Nuclear staining is shown in blue, rhodopsin (4D2) staining is shown in red, and rod-specific transducin (K20) staining is shown in green. CB, cell body.

LWS). Immunofluorescence staining demonstrated that RH1 is present in the outer segments of a subset of cone photoreceptor cells in *T. proximus* retina. Another rod-specific component of the phototransduction cascade, rod transducin, was found to colocalize in the same subset of photoreceptors. Despite its unusual expression in an all-cone retina, comparative sequence analyses showed *T. proximus* RH1 to be under strong selective constraint indicative of a functionally conserved protein-coding gene. When heterologously expressed in vitro, *T. proximus* RH1 was found to encode a photoactive visual pigment that is substantially blue-shifted in its absorption maxima, matching our spectral MSP measurements of intact photoreceptors. Finally, although the general morphology of the photoreceptors was indicative of an all-cone retina, close examination of the ultrastructure of individual cells using TEM revealed that a subset of "cones" in fact had rod features, including outer-segment discs that were completely enclosed by plasma membrane.

The finding that RH1 is expressed in a previously reported all-cone retina of the diurnal colubrid *T. proximus* raises several possible alternative hypotheses to those proposed in Fig. 1. The simplest is that RH1 is a nonfunctional pseudogene. Our molecular evolutionary analyses, however, indicate that RH1 has been maintained under strong selective constraint, and we found no evidence for a relaxation of selection. This implies that *T. proximus* RH1 is functional. To confirm this, we heterologously expressed *T. proximus* RH1 and found that it can bind retinal and activate in response to light. Another alternative is that, along with the loss of rods in diurnal colubrids, RH1 was relegated to a solely nonvisual role (e.g., maintenance of circadian rhythm) (30). Immunohistochemical staining of *T. proximus* retina revealed the presence of RH1 within cone photoreceptors, which strongly suggests that this is not the case. Last, RH1 may have been co-opted for expression in cones, possibly even coexpressed with a cone opsin. The coexpression of multiple types of cone opsin within individual cone cells has been found in rodents (31), salamanders (32), and cichlid fishes (33), but coexpression of a cone opsin and RH1 has not been reported. The presence of rod transducin along with rhodopsin implies that other components of the rod transduction machinery would have had to be co-opted as well. However, the finding of rod-specific ultrastructure argues against a simple shift in expression of rod-specific transduction machinery into a different cell type, although this idea could be addressed in future cell developmental studies. Currently, the most parsimonious explanation of our results is that the rhodopsin-containing cones of *T. proximus* are homologous to the rods of pythons and boas; that is, they are actually cone-like rods.

Although this study is the first molecular evidence, to our knowledge, of an evolutionary shift from rod to cone morphology, a transition in the opposite direction has been shown in nocturnal geckos. Geckos are hypothesized to have evolved from a lizard ancestor with an all-cone retina and to have evolved an all-rod retina during adaptation to a nocturnal lifestyle (4). A series of papers have shown that gecko "all-rod" retinas contain only cone opsins and cone phototransduction machinery (7–9), and that the "rods" have cone ultrastructural features (6) and function at a level intermediate between true rods and cones (9).

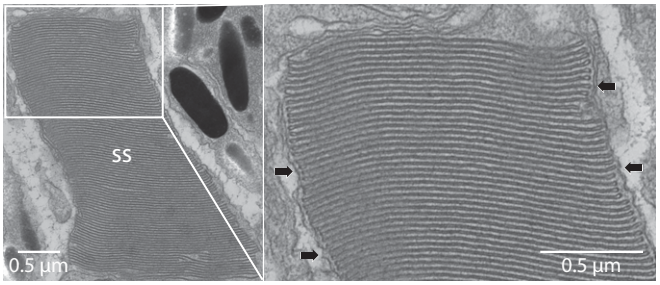

Fig. 5. TEM image of the outer segment of a *T. proximus* photoreceptor cell with rod ultrastructure. The arrows indicate the complete enclosure of the discs by the plasma membrane, which is a feature exclusive to rods.

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These findings support Walls's (4) contention that gecko rod photoreceptors are transmuted cones.

The evolutionary alterations in gecko rods are similar in nature to those found in our study in a subset of rhodopsin-staining cones, but in the opposite direction. *T. proximus* rhodopsin-staining cones have outer segments that resemble cones, but with rod ultrastructural features, and contain rod phototransduction machinery. Several intriguing and atypical features of the rod machinery within these photoreceptors are also consistent with a more cone-like function. The highly blue-shifted absorption spectrum of *T. proximus* RH1, unique among terrestrial vertebrates, is a shift toward wavelengths generally occupied by the cone opsin RH2, which is suggestive of a more cone-like physiology. *T. proximus* RH1 also has the mutation C185S, which has been shown to reduce transducin activation in bovine RH1 (25), which is more typical of cone opsins. Furthermore, the only electrophysiological study of *Thamnophis* (performed in *T. sirtalis*) (13) found no evidence for a separate rod (scotopic) visual response. Although these data all point to more cone-like characteristics, despite the rod machinery and ultrastructure, it is clear that further study is needed to explore the functional consequences of this evolutionary transition in *Thamnophis* and other diurnal colubrids.

A common property of photoreceptor transmutation appears to be substantial morphological changes to the outer segment. The correlation of rod-like cellular morphology with nocturnal species and cone-like morphology with diurnal species (4) suggests a functional relevance to outer-segment shape. Enlarged, rod-like outer segments are known to increase sensitivity by increasing cell volume and, as a result, the number of visual pigment molecules available to catch photons (3, 34). Recent theoretical work has proposed that the small tapering outer segments of cones may help to reduce self-screening of the visual pigments, increase signal-to-noise ratios, and allow light to more efficiently be focused on the outer segment by the ellipsoid (35). Interestingly, recent work has also suggested that reduction of RH1 expression alone can result in a more cone-like morphology, decreasing the photosensitivity of the cell and increasing the kinetics of the phototransduction cascade (34, 36, 37). A second striking difference in rod and cone morphology is the accessibility of the outer-segment discs to the plasma membrane. In cones the discs are open, which contributes to rapid response kinetics, whereas in rods the complete enclosure of the discs results in increased sensitivity to light (3). In the rod-like cones of nocturnal geckos the discs are partially enclosed, and this may contribute to their intermediate physiological properties. In *T. proximus*, the discs of the cone-like rods remain enclosed by the plasma membrane, but the extent to which this slows responses, and how it may have been overcome, would be an interesting area for future research.

The question remains as to why diurnal colubrids and nocturnal geckos have modified their rods and cones when many other groups that have transitioned between diurnality and nocturnality have not. Goldsmith (38) proposed that opsin gene loss might be a prerequisite for photoreceptor transmutation. At the time it was known that geckos had lost the RH1 and SWS2 opsins, but in this context it is interesting to note that snakes have also experienced opsin loss (RH2 and SWS2), likely as a result of their proposed burrowing origins (4, 10, 17, 18). Because the diurnal ancestors of geckos had already lost RH1, the advantage of transmuting cones into rods when adapting to a nocturnal lifestyle is clear. Nearly all highly diurnal animals, however, maintain a population of rods (2), presumably because even highly diurnal animals may encounter, or be active in, dim-light environments. In fact, only diurnal squamates are thought to have lost rods and thus have all-cone retinas (with the possible exception of the stellate sturgeon), and only geckos are known to have lost RH1 (2). Thus, the change to cone-like rods in diurnal snakes, and the corresponding reduction in dim-light visual capabilities, is unusual.

The extraordinary evolutionary shift from a duplex to an all-cone retina might be explained by the ancestral loss of the SWS2 and RH2 cone opsins in snakes, which results in low sensitivity to a large portion of the visual spectrum due to the lack of appreciable overlap between the LWS and SWS1 cone opsins (Fig. 6*A*). Not only would this largely preclude color vision, it would also severely limit the amount of visible light to which snakes would be sensitive. In primarily nocturnal snakes this may not be an issue, but in highly diurnal snakes, such as *Thamnophis*, there may be a significant advantage to increasing the range of spectral sensitivity. Inclusion of RH1 in the daylight (photopic) absorption spectrum would greatly enhance the range of spectral sensitivity and provide the basis for trichromatic color vision (Fig. 6*B*). This would also help to explain the unusual blue-shifted absorption spectra of *T. proximus* RH1. It is the most blue-shifted RH1 found so far in any terrestrial vertebrate, and it is also highly blue-shifted relative to other snake groups that tend to have burrowing and nocturnal habits, such as the sunbeam snake (10). The substantial blue shift could be important for chromatic discrimination and color vision, resulting in more even spacing in spectral tuning with LWS and SWS1 opsins. This effect on chromatic discrimination could be further enhanced by the slight red and blue shifting of SWS1 and LWS, respectively, relative to other snakes, such as the python (Fig. 6). It is not known whether diurnal colubrids possess color vision or whether the rod neural pathways in snakes, or more generally reptiles, can contribute to color vision. However, there is evidence that suggests that rods can contribute to color vision (39, 40). For example, human cone monochromats (individuals with only SWS1 cones and RH1 rods) are able to perceive color under mesopic conditions, where both the rods and cones are active (41). If rods are similarly able to contribute to color vision in snakes, the transition to cone-like rods may have provided an additional adaptive advantage, but testing this hypothesis will require studies both of retinal pathways in snakes and behavioral tests for color vision.

The unexpected results presented in this study that reveal a hidden class of photoreceptors in a previously characterized all-cone retina provide tantalizing clues to the diverse evolutionary pathways through which sensory adaptations may be achieved. Here we have shown that the all-cone retina of a diurnal colubrid evolved through modification of the rod photoreceptors, which may have allowed recovery of visual function that was lost during the presumed fossorial origins of snakes. Sensory systems in general may be particularly vulnerable to the need to compensate for ancestral loss of function in response to shifts in ecology. For example, a recent study showed that although sweet taste receptors were lost in the avian ancestor, hummingbirds have reacquired the ability to taste sweet compounds through modification of their savory taste receptor (42). The peculiar adaptive transitions necessitated by ancestral loss demonstrate how fascinating evolutionary novelty may arise even out of the limitations imposed by accidents of history.

Materials and Methods

See also *SI Appendix, Materials and Methods* for detailed descriptions.

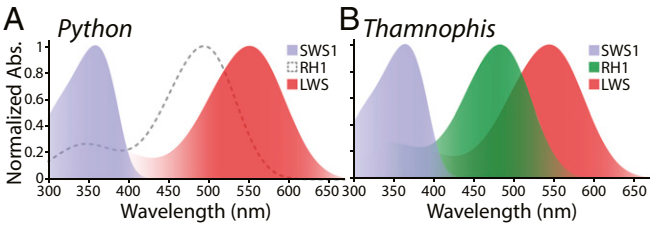

Fig. 6. Absorption spectra of *Python* (*A*) and *T. proximus* (*B*) based on Govardovskii curves illustrating the large gap in appreciable bright-light spectral sensitivity in *Python* between ~380 and 480 nm (*A*) that is filled by the presence of a blue-shifted rhodopsin expressed in a cone-like photoreceptor in *T. proximus* (*B*). This gap, and a corresponding increase in spectral overlap between pigments, is further decreased by slight red shifting of the SWS1 and slight blue shifting of the LWS pigments relative to *Python*. *Python* λ_{max} values are from ref. 15.

Animals. Adult *T. proximus* were obtained from a licensed retailer and euthanized under the approval of the University of Toronto Animal Care Committee. Eyes were extracted and prepared either for MSP, RNA extraction, or electron microscopy. Blood was collected for genomic (g)DNA extraction.

Microspectrophotometry. The methodology used for MSP measurements and analyses has been described previously (8, 14).

Phylogenetic and Molecular Evolutionary Analyses. Full-length RH1-, LWS-, and SWS1-coding sequences were sequenced from total RNA extracted from *T. proximus* eyes or from gDNA, using standard PCR, RACE, and GenomeWalker (Clontech) procedures. A representative set of vertebrate RH1, LWS, and SWS1 sequences were aligned with the *T. proximus* sequences, and gene trees were estimated with MrBayes 3 (43). The RH1 gene tree and alignment were analyzed with the codeml package of PAML 4 (44) using the random-site, branch, and branch-site models (45), as well as the clade model C (CmC) (46). Model pairs were compared using a likelihood ratio test (LRT) with a χ^2 distribution.

Rhodopsin Expression and Spectroscopic Assay. Rhodopsin was expressed and spectroscopically assayed as previously described (26, 47).

Immunohistochemistry. Retinae from *T. proximus* were processed for immunohistochemistry following sucrose infiltration. Stained cryosections were visualized via a Leica TCS SP8 confocal laser microscope. Primary antibodies used were the K20 antibody (Santa Cruz Biotechnology) and 4D2 anti-rhodopsin antibody. Alexa Fluor 488 goat anti-rabbit (Life Technologies) and the Cy-3 anti-mouse (Jackson ImmunoResearch) were used as secondary antibodies.

Electron Microscopy. Hemisections of *T. proximus* retinae were prepared for SEM and TEM following standard procedures. The detailed protocol is available in *SI Appendix, Materials and Methods*. SEM samples were examined with a Hitachi S2500 and images were acquired using a Quartz PCI. TEM sections were examined with a Hitachi H7000 and images were acquired using a digital camera (Advanced Microscopy Techniques).

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