Cone-like rhodopsin expressed in the all cone retina of the colubrid pine snake as a potential adaptation to diurnality

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Running title (30/40 characters): Snake rods adapt to diurnality

Keywords (5/3-6): rod and cone photoreceptors, photoreceptor transmutation, rhodopsin, visual pigments, visual evolution, reptile vision

Number of pages:
Number of tables:
Number of figures:

Summary Statement

The all cone retina of the colubrid snake, *Pituophis melanoleucus* contains a blue-shifted rhodopsin with cone opsin-like properties, which may have been adaptive in diurnal snakes.

Abstract

Colubridae is the largest and most diverse family of snakes, with visual systems that reflect this diversity, encompassing a variety of retinal photoreceptor organizations. The transmutation theory proposed by Walls postulates that photoreceptors could evolutionarily transition between cell types in squamates, but few studies have tested this theory. Recently, evidence for transmutation and rod-like machinery in an all cone retina has been identified in a diurnal garter snake (*Thamnophis*), and it appears that the rhodopsin gene at least may be widespread among colubrid snakes. However, functional evidence supporting transmutation beyond the existence of the rhodopsin gene remains rare. We examined the all cone retina of another diurnal colubrid, *Pituophis melanoleucus*, distantly related to *Thamnophis*. We found that *P. melanoleucus* expresses two cone opsins (SWS1, LWS) and rhodopsin (RH1) within the eye. Immunohistochemistry localized rhodopsin to the outer segment of photoreceptors in the all-cone retina of the snake and all opsin genes produced functional visual pigments when expressed *in vitro*. Consistent with other studies, we found that *P. melanoleucus* rhodopsin is extremely blue-shifted. Surprisingly, *P. melanoleucus* rhodopsin reacted with hydroxylamine, a typical cone opsin characteristic. These results support the idea that the rhodopsin-containing photoreceptors of *P. melanoleucus* are the products of evolutionary transmutation from rod ancestors, and suggests that this phenomenon may be widespread in colubrid snakes. We hypothesize that transmutation may be an adaptation for diurnal, brighter-light vision, which could result in increased spectral sensitivity and chromatic discrimination with the potential for colour vision.

Introduction

Reptiles are known for their impressive array of visual adaptations and retinal organizations, which reflect distinct ecologies and evolutionary histories (Walls, 1942).

The family Colubridae is the most speciose family of snakes and encompasses a diverse range of lifestyles and ecologies. Colubrid snakes have recently emerged as a compelling group in which to study visual system evolution and adaptation (Schott et al., 2016; Simões et al., 2015; Simões et al., 2016).

In the vertebrate retina, photoreceptor cells can be divided into two types based on their overall structure and function: cones, which are active in bright light and contain cone visual pigments (SWS1, SWS2, RH2, LWS) in a tapered outer segment, and rods, which function in dim light and contain rhodopsin (RH1) in a longer, more cylindrical outer segment (Bowmaker, 2008; Lamb, 2013; Walls, 1942). Reptilian retinas are unique in having multiple retinal configurations among closely related species including all-rod (Kojima et al., 1992), rod and cone (Sillman et al., 2001), and all-cone (Sillman et al., 1997). In 1942, physiologist Gordon Walls outlined his theory of transmutation to explain the evolutionary transformation of photoreceptors from one type to another (Walls, 1942). This phenomenon has since been investigated in nocturnal geckos, where cone opsins are expressed in an all-rod retina in order to compensate for the evolutionary loss of RH1 in a hypothesized diurnal, all-cone ancestor (Kojima et al., 1992; Taniguchi et al., 1999). While the nocturnal henophedian snakes, such as boas and pythons, are known to have duplex retinas expressing RH1, LWS and SWS1 in canonical photoreceptors (Davies et al., 2009), the more derived diurnal colubrid snakes have been primarily shown to posses simplex retinas comprising of all cone photoreceptors, with the fate of the rod photoreceptor unknown (Caprette, 2005; Walls, 1942). Early studies of the colubrid visual system found a green-sensitive visual pigment in addition to a red and a blue pigment (Sillman et al., 1997) in the simplex retina, but were unable to distinguish between a spectrally shifted rhodopsin in a transmuted rod or a potentially resurrected RH2 cone opsin (Cortesi et al., 2015). More recently, a study from our group identified a functional blue-shifted RH1 pigment in the all-cone retina of the ribbon snake (*Thamnophis proximus*), and proposed that this resulted from a rod to cone evolutionary transmutation in colubrid snakes that may have allowed for enhanced spectral discrimination and even trichromatic colour vision (Schott et al., 2016). A recent study that sequenced the opsins of several other colubrid snake species discovered the widespread presence of full-length rhodopsin genes in species with supposed simplex

93 retinas that were previously presumed to have lost rod/rhodopsins (Simões et al., 2016).
94 However, detailed characterizations of colubrid snake opsins and photoreceptors in the
95 context of the theory of evolutionary transmutation still remain rare.
96 To further test the hypothesis of widespread transmutation in colubrid snakes, and
97 its potential functional consequences, we examined the visual system of the Northern
98 Pine Snake (*Pituophis melanoleucus*), a diurnal colubrid snake distantly related to *T.*
99 *proximus*. *Pituophis melanoleucus* inhabits the eastern half of the United States and
100 Canada (Stull, 1940) and spends relatively short intervals on the surface during the day to
101 forage for prey such as small mammals and birds, and to create new burrows (Diller and
102 Wallace, 1996; Himes, 2001). While *P. melanoleucus* has been found to possess an all-
103 cone retina (Caprette, 2005), similar to previous diurnal colubrids snakes studied (Schott
104 et al., 2016; Sillman et al., 1997), unlike other strongly diurnal colubrids such as the
105 garter snake, *P. melanoleucus* is more secretive and is thought to spend a considerable
106 amount of time burrowing (Gerald et al., 2006).
107 In this study, we investigate whether there is evidence of photoreceptor
108 transmutation from rods into cones in the all-cone retina of *P. melanoleucus* via
109 functional characterization, cellular localization, and molecular evolutionary analyses of
110 its visual pigment (opsin) genes. We isolated three opsins genes from *P. melanoleucus*:
111 SWS1, LWS and RH1. Immunohistochemistry of the retina localized rhodopsin (RH1)
112 protein and rod transducin to the inner and outer segments of a small subset of
113 photoreceptors, suggesting that *P. melanoleucus* supports the theory of rod-to-cone
114 transmutation in diurnal colubrids. All three opsins were successfully expressed *in vitro*
115 and displayed properties characteristic of fully functional visual pigments. Additionally,
116 spectroscopic assays revealed that *P. melanoleucus* rhodopsin is sensitive to
117 hydroxylamine, which is more typical of cone opsins and is suggestive of more cone-like
118 functional properties. This study provides further evidence for a fascinating evolutionary
119 transformation in the retinas of colubrid snakes, with implications for reptiles in general.
120
121 **Materials and Methods**
122 *Animals*

A Northern pine snake (*Pituophis melanoleucus melanoleucus,* adult) specimen and mice (*Mus musculus,* adult, CD1*)* were obtained from a licensed source as commissioned by the University Animal Care Committee (UACC). The specimen was sacrificed using an approved euthanasia protocol. The eyes were enucleated and preserved in RNAlater or 4% paraformaldehyde.

Total RNA extraction and cDNA synthesis

The dissected whole eye was homogenized with TRIzol, and total RNA was isolated using a phenol/chloroform extraction and ethanol precipitation. The first strand of complementary DNA (cDNA) was synthesized using SuperScript III Reverse Transcriptase (Invitrogen, Waltham, MA, USA) from RNA samples primed with a 3' oligo-dT and a 5' SMART primer, following the protocol outlined by the SMART cDNA Library Construction Kit (BD Biosciences, Franklin Lakes, NJ, USA). The second strand complement was synthesized by long-distance PCR following the same protocol.

Visual pigment genes were isolated using a degenerate PCR strategy. Degenerate primers based on an alignment of reptilian visual pigment sequences were used in attempts to amplify partial sequences of the LWS, SWS1 and RH1 opsin genes with a heminested strategy. GenomeWalker (Clontech, Mountain View, CA, USA) was additionally used to obtain full-length sequences (Supplementary Table S1). Extracted PCR products were ligated into the pJET1.2 blunt plasmid vector.

Phylogenetic analysis

A representative set of vertebrate visual opsin sequences was obtained from Genbank. These sequences were combined with the three opsins genes sequenced from the pine snake and aligned using MUSCLE (Edgar, 2004). The poorly aligned 5' and 3' ends of the sequence were manually trimmed. Species list and accession numbers for all sequences used in the study are provided in Supplementary Table S2. In order to confirm the identities of the opsin genes from the pine snake, a gene tree was estimated using the resulting alignment in MrBayes 3 (Ronquist and Huelsenbeck, 2003) using reversible jump MCMC with a gamma rate parameter (nst=mixed, rates=gamma), which explores the parameter space for the nucleotide model and the phylogenetic tree simultaneously.

The analyses were run for five million generations with a 25% burn-in. Convergence was confirmed by checking that the standard deviations of split frequencies approached zero and that there was no obvious trend in the log likelihood plot.

Protein expression

Full-length opsins sequences (RH1, SWS1, and LWS) were amplified from pJET1.2 vector using primers that added the *Bam*HI and *Eco*RI restriction sites to its 5' and 3' ends, respectively, and inserted into the p1D4-hrGFP II expression vector (Morrow and Chang, 2010). Expression vectors containing *P. melanoleucus* cone opsins and rhodopsin genes were transiently transfected into cultured HEK293T cells (ATCC CRL-11268) using Lipofectamine 2000 (Invitrogen, Waltham, MA, USA; 8 µg of DNA per 10-cm plate) and harvested after 48 h. Visual pigments were regenerated with 11-*cis* retinal, generously provided by Dr. Rosalie Crouch (Medical University of South Carolina), solubilized in 1% dodecylmaltoside, and purified with the 1D4 monoclonal antibody (University of British Columbia #95-062, Lot #1017; Molday and MacKenzie, 1983) as previously described (Morrow and Chang, 2015; Morrow and Chang, 2010; Morrow et al., 2011). RH1 and SWS1 pigments were purified in sodium phosphate buffers and LWS was purified in HEPES buffers containing glycerol (as described in van Hazel et al. (2013)). The ultraviolet-visible absorption spectra of purified visual pigments were recorded using a Cary 4000 double beam spectrophotometer (Agilent, Santa Clara, CA, USA). Dark-light difference spectra were calculated by subtracting light-bleached absorbance spectra from respective dark spectra. Pigments were photoexcited with light from a fiber optic lamp (Dolan-Jenner, Boxborough, MA, USA) for 60 s at 25°C. Absorbance spectra for acid bleach and hydroxylamine assays were measured following incubation in hydrochloric acid (100mM) and hydroxylamine (NH_2OH, 50mM), respectively. To estimate λ_{max}, the dark absorbance spectra were baseline corrected and fit to Govardovskii templates for A1 visual pigments (Govardovskii et al., 2000).

Immunohistochemistry

Fixation of pine snake eyes was conducted as previously described (Schott et al., 2016). Briefly, after enucleating *P. melanoleucus* eyes in the light, they were rinsed in PBS

(0.8% NaCl, 0.02% KCl, 0.144% NaHPO4, and 0.024% KH2PO4, pH 7.4), fixed overnight at 4ºC in 4% paraformaldehyde, infiltrated with increasing concentrations of sucrose (5%, 13%, 18%, 22%, 30%) in PBS, and embedded in a 2:1 solution of 30% sucrose and O.C.T compound (Tissue-Tek, Burlington, NC, USA) at -20º. The eyes were cryosectioned transversely at -25ºC in 20 µm sections using a Leica CM3050 (Wetzlar, Germany) cryostat, placed onto positively charged microscope slides, and stored at -80ºC until use.

Slides were first rehydrated in PBS and then air-dried to ensure adhesion. Sections were rinsed three times in PBS with 0.1% Tween-20 (PBT) and then incubated in 4% paraformaldehyde PBS for 20 minutes. After rinsing in PBT and PDT (PBT with 0.1% DMSO), the slides were incubated in a humidity chamber with blocking solution (1% BSA in PDT with 2% normal goat serum) for one hour, incubated with primary antibody diluted in blocking solution overnight at 4º in a humidity chamber. Antibodies used were the K20 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA, sc-389, lot#:C1909, dilution: 1:500) and RET-P1 anti-rhodopsin antibody (Sigma-Aldrich, St. Louis, MO, USA, O-4886, lot#: 19H4839, dilution: 1:200).

After extensive rinsing and soaking in PDT (3 times for 15 minutes), secondary antibody was added to the samples and incubated at 37º for one hour in a humidity chamber. Secondary antibodies used for the fluorescent staining were the AlexaFluor-488 anti-rabbit antibody (Life Technologies, Waltham, MA, USA, A11034, lot#: 1298480, dilution: 1:1000) and the Cy-3 anti-mouse antibody (Jackson ImmunoResearch, West Grove, PA, USA, 115-165-003, dilution: 1:800). After rinsing with PBS, followed by PDT, sections were stained with 10 µg/mL Hoescht for 10 minutes at room temperature. The sections were then rinsed in PBS and PDT and mounted with ProLong® Gold antifade reagent (Life technologies, Waltham, MA, USA) and coverslipped. Sections were visualized via a Leica SP-8 confocal laser microscope (Wetzlar, Germany).

Results

Full-length RH1, SWS1 and LWS opsin sequences found in Pituophis melanoleucus cDNA

To determine the identities of the visual pigments in *P. melanoleucus,* eye cDNA and gDNA was screened for opsin genes. Three full-length opsins were amplified, sequenced, and analyzed phylogenetically with a set of representative vertebrate visual opsins (Table S1) using Bayesian inference (MrBayes 3.0) (Ronquist and Huelsenbeck, 2003). This analysis confirmed the identity of the three opsin genes as RH1, LWS, and SWS1 (Fig. S1-S3).

All three opsin genes sequence contained the critical amino acid residues required for proper structure and function of a prototypical opsin including K296, the site of the Schiff base linkage with 11-*cis* retinal (Palczewski et al., 2000; Sakmar et al., 2002), and E113, the counter-ion to the Schiff base in the dark state (Sakmar et al., 1989), as well as C110 and C187, which form a critical disulfide bond in the protein (Karnik and Khorana, 1990). Both cone opsin genes also have the conserved P189 residue which is critical for faster cone opsin pigment regeneration (Kuwayama et al., 2002).

Interestingly, *P. melanoleucus* RH1 has serine at site 185 instead of the highly conserved cysteine, similar to several other snakes (Schott et al., 2016; Simões et al., 2016). Mutations at site 185 have been shown to reduce both visual pigment stability (McKibbin et al., 2007) and transducin activation *in vitro* (Karnik et al., 1988). Also, the *P. melanoleucus* RH1 has N83 and S292, which are often found in rhodopsins with blue-shifted λ_{max} values, and can also affect all-*trans* retinal release kinetics following photoactivation (Bickelmann et al., 2012; van Hazel et al., 2016).

Based on known spectral tuning sites in LWS, *P. melanoleucus* has A285, compared to T285 in *Thamnophis* snakes. T285A is known to blue-shift the LWS pigment by 16-20 nm (Asenjo et al., 1994; Yokoyama, 2000). This suggests that the *P. melanoleucus* LWS may be considerably blue-shifted relative to the LWS pigment in *Thamnophis* snakes. Within *P. melanoleucus* SWS1, the phenylalanine at site 86 suggests that the pigment will be absorbing in the UV, as is typical of reptilian SWS1 pigments (Hauser et al., 2014). *Pituophis melanoleucus* SWS1, as well as other colubrids SWS1 (Simões et al., 2016), have hydrophobic residues at two spectral tuning sites, A90 and V93. These sites are usually have polar or charged amino acid side chains (Carvalho et al., 2011; Hauser et al., 2014). These functional significance of these hydrophobic

245 residues have yet to be characterized, and suggests that caution should be taken in
246 applying spectral tuning predictions on squamates SWS1 pigments.
247
248 *Immunohistochemistry*
249 Because *P. melanoleucus* has an all-cone retina, we used immunohistochemistry
250 to determine if both rhodopsin and the rod G protein transducin are expressed in cone
251 photoreceptors. We performed fluorescent immunohistochemistry on the transverse
252 cryosections of the retina of *P. melanoleucus* with the rhodopsin antibody (RET-P1) and
253 a rod-specific transducin antibody (K20). Both antibodies have been previously shown to
254 be selective across a range of vertebrates (Fekete and Barnstable, 1983; Hicks and
255 Barnstable, 1987; Osborne et al., 1999; Schott et al., 2016). We also used these antibodies
256 on a CD1 mouse retina, following similar preparation, as a positive control.
257 Our results showed rhodopsin localized to the outer segments of select
258 photoreceptors of the *P. melanoleucus* retina (red, Fig 1D), whereas the rod transducin
259 localized to the inner segment (green, Fig 1E). The small amount of colocalization
260 between rhodopsin and transducin in the inner segment (yellow, Fig 1F) is expected as
261 the animal wasn't dark-adapted prior to sacrifice, as rod transducin translocates to the
262 inner segment when exposed to bright light(Calvert et al., 2006; Elias et al., 2004). This
263 pattern is consistent with rhodopsin and transducin staining in the *T. proximus* retina
264 (Schott et al., 2016) and the previously unexplained results of rhodopsin detected in the
265 retina of *T. sirtalis* (Sillman et al., 1997).
266 As expected, CD1 mouse retina had strong rhodopsin fluorescence (red, Fig 1A)
267 in the outer segment and strong rod transducin staining (green, Fig 1B) in the inner
268 segment, consistent with the rod dominant mouse retina. The lack of colocalization is
269 consistent with a light-adapted retina (Calvert et al., 2006; Elias et al., 2004) (Fig 1C).
270
271 *In vitro expression*
272 Complete coding sequences of the *P. melanoleucus* RH1, LWS, and SWS1 opsins
273 were cloned into the p1D4-hrGFP II expression vector (Morrow and Chang, 2010).
274 Expression vectors were then transfected into HEK293T cells and the expressed protein
275 was purified with the 1D4 monoclonal antibody (Morrow and Chang, 2015; Morrow et

al., 2011). Bovine wildtype rhodopsin was used as a control (Fig 2A). Pine snake rhodopsin has a λ_{max} of 481nm (Fig 2B) which is similar to the measured λ_{max} of rhodopsins from *T. proximus*, *T. sirtalis,* and *Arizona elegans* snakes (Schott et al., 2016; Sillman et al., 1997; Simões et al., 2016). The drastic blue shift is expected given the presence of the blue-shifting N83 and S292 amino acid identities (Bickelmann et al., 2012; Dungan et al., 2016; van Hazel et al., 2016). *P. melanoleucus* rhodopsin expressed similar to that of *T. proximus,* with a large ratio between total purified protein (absorbance at 280nm) and active protein (absorbance at λ_{max}) that indicates that only a small proportion of the translated opsin protein is able to bind chromophore and become functionally active. One possible explanation for this effect is the S185 residue in *P. melanoleucus* rhodopsin, as mutations at this site have been shown to affect the retinal binding efficiency of rhodopsin pigments expressed *in vitro* (McKibbin et al., 2007).

Expression of pine snake SWS1 showed a much more favorable 280nm to λ_{max} ratio (Fig 2C). We found that *P. melanoleucus* SWS1 pigment absorbs in the UV range with a λ_{max} of 370nm, similar to the SWS1 λ_{max} of *Lampropeltis getula*, *Rhinocheilus lecontei*, and *Hypsiglena torquata* (Simões et al., 2016) all of which have the most red-shifted UV SWS measured among colubrid snakes.

Similar to the SWS1 expression, LWS also expressed quite well (Fig 2D). Fit to A1 templates gave a λ_{max} of 534nm, which is blue-shifted relative to *Thamnophis* (Schott et al., 2016; Sillman et al., 1997), but identical with LWS MSP measurements of *H. torquata* (Simões et al., 2016) and very close to those of *L. getula*, *A. elegans*, and *R. lecontei* (Simões et al., 2016).

Opsin protein functional characterization

In order to confirm the covalent attachment of the chromophore in *P. melanoleucus* SWS1 pigments, the purified opsin was acid bleached (Fig 2C). We found a shift of the λ_{max} from 370nm to 440nm, which indicates the presence of 11-*cis* retinal covalently bound by a protonated Schiff base to a denatured opsin protein (Kito et al., 1968), suggesting that the UV sensitivity of the pigment may be established by only the presence of F86.

306 *P. melanoleucus* LWS (Fig 3A) and RH1 (Fig 3B) were tested for hydroxylamine
307 reactivity, which assesses the accessibility of the chromophore-binding pocket to attack
308 by small molecules. If hydroxylamine can enter the binding pocket, it will hydrolyze the
309 Schiff base linkage, resulting in an absorbance decrease of the dark peak and the increase
310 of a retinal oxime peak at 363nm. Rhodopsins are thought to be largely non-reactive in
311 the presence of hydroxylamine (Dartnall, 1968) (Fig 3C) due to their highly structured
312 and tightly packed chromophore binding pockets relative to cone opsins, which often
313 react when incubated in hydroxylamine (van Hazel et al., 2013). *P. melanoleucus* LWS
314 reacted to hydroxylamine, as expected, with a $t_{1/2}$ of ~3.9 min (Fig 3A), a time within the
315 range of cone opsins (Das et al., 2004; Ma et al., 2001). As the λ_{max} of *P. melanoleucus*
316 SWS1 is 370nm, it was not tested as we would not be able to distinguish the retinal
317 oxime peak from the λ_{max} peak. Interestingly, *P. melanoleucus* rhodopsin also reacted to
318 hydroxylamine with a $t_{1/2}$ of ~14 min (Fig 3B), unlike the bovine rhodopsin control that
319 did not react (Fig 3C). This implies that the chromophore binding pocket of *P.*
320 *melanoleucus* rhodopsin has a more open configuration relative to other rhodopsin
321 proteins, a property more typical of cone opsins.
322
323 **Discussion**
324 Recently, there has been mounting evidence supporting the theory of
325 transmutation in photoreceptor evolution, proposed by Walls in 1942, which outlines the
326 evolutionary transformation of one photoreceptor type into another in reptilian retinas.
327 Evidence of cone to rod transmutation in nocturnal geckos has been extensively
328 demonstrated using both cellular and molecular techniques (Crescitelli, 1956; Dodt and
329 Walther, 1958; Kojima et al., 1992; McDevitt et al., 1993; Röll, 2001; Sakami et al.,
330 2014; Tansley, 1959; Tansley, 1961; Tansley, 1964; Zhang et al., 2006), while evidence
331 of rod-to-cone transmutation in colubrid snakes remains somewhat sparse (Schott et al.,
332 2016). In order to demonstrate rod-to-cone transmutation in the retina, there needs to be
333 evidence of a functional rod machinery in a photoreceptor with some rod-like features in
334 a retina with appears, superficially, to consist of only cones. Certainly, the presence of
335 RH1 genes and MSP data suggests transmutation has occurred in several colubrid species
336 (Hart et al., 2012; Sillman et al., 1997; Simões et al., 2015; Simões et al., 2016), but

337 further investigation is required in order to firmly state transmutation is present in the
338 retinas of these colubrid snakes as there are multiple alternate explanations possible (RH1
339 in the genome but not expressed, rhodopsin expressed but not functional, a cone cell co-
340 opting rhodopsin etc). There is only one colubrid snake species for which cellular and
341 molecular evidence for transmutation has been reported, *Thamnophis proximus* (Schott et
342 al., 2016).

343 This study provides strong evidence that supports the hypothesis that
344 photoreceptor transmutation has occurred in the retina of *P. melanoleucus*. As *P.*
345 *melanoleucus* is not closely related to snakes in the genus *Thamnophis*, this suggests that
346 transmutation may be widespread in colubrid snakes. However, the functional
347 significance of transmutation in colubrid snakes still has not been established. In geckos,
348 the advantage of cone-to-rod transmutation is more straightforward as these nocturnal
349 animals are most likely compensating for the loss of RH1 in their diurnal ancestor. We
350 propose that transmutation in colubrids may have occurred as an adaptation to diurnality
351 that provided *P. melanoleucus* with a cone-like rod photoreceptor that operates at brighter
352 light levels, perhaps as a compensation for the loss of the RH2 cone opsins. Our finding
353 of a highly blue-shifted rhodopsin with more cone-like functional properties, as indicated
354 by hydroxylamine reactivity, support this hypothesis.

355 *Pituophis melanoleucus* rhodopsin shows hydroxylamine reactivity, a canonical
356 cone opsin property (Wald et al., 1955). With a reaction half-life of ~14min, the *P.*
357 *melanoleucus* rhodopsin reacts much quicker and closer to cone opsin speeds (Das et al.,
358 2004; Ma et al., 2001) than previous rhodopsins that have reacted when incubated in
359 hydroxylamine, like the echidna (Bickelmann et al., 2012) and the anole (Kawamura and
360 Yokoyama, 1998) which react over hours. The RH1 sequence contains both E122 and
361 I189, which are known to mediate the slower decay and regeneration kinetics typical of
362 rhodopsin (Imai et al., 1997; Kuwayama et al., 2002). Conversely, the presence of serine
363 rather than cysteine at site 185, in rhodopsin has been shown to activate fewer G proteins
364 (Karnik et al., 1988) and mutation at site 185 has been shown to reduce the thermal
365 stability of the protein (McKibbin et al., 2007), both characteristics being more typical of
366 cone opsins. Cones have been optimized for fast regeneration, with cone opsin meta-
367 intermediate states being short lived compared to rhodopsin (Imai et al., 2005), and a

cone-specific Müller cell retinoid cycle (Das et al., 1992) providing a dedicated pool of 11-*cis* retinal. These faster kinetic properties are hypothesized to be facilitated in cone opsins via the relative "openness" of the chromophore binding pocket, which allows water molecules, and therefore other small molecules like hydroxylamine, to access the chromophore where they can participate Schiff base hydrolysis (Chen et al., 2012; Piechnick et al., 2012; Wald et al., 1955). Rhodopsins, on the other hand, are optimized for sensitivity and signal amplification; therefore, E122/I189 and a tighter overall structure contribute to a slower active state decay allowing for the activation of multiple G proteins (Chen et al., 2012; Starace and Knox, 1997), increased thermal stability relative to cone opsins (Barlow, 1964), and a resistance to hydroxylamine (Dartnall, 1968). *Pituophis melanoleucus* rhodopsin shows adaptations to decrease the number of G-proteins activated, and hydroxylamine reactivity which suggests that an open chromophore binding pocket would enable water access to facilitate active state decay, Schiff base linkage hydrolysis, and retinal regeneration (Chen et al., 2012) rates similar to cone opsins. Spectroscopic assays measuring G protein activation and retinal release rates have never been performed on colubrid rhodopsins, but would be an interesting direction for future research characterizing this cone opsin-like rhodopsin.

Retinal immunohistochemistry localized *P. melanoleucus* rhodopsin protein in the outer segment of a photoreceptor, as well as the presence of rod transducin in the inner segment. Rod and cone transducin are thought to originate via duplication from on ancestral gene (Larhammar et al., 2009) and both have been shown to function with all opsins (Sakurai et al., 2007), therefor the presence and preservation of rod transducin in the photoreceptor supports the theory that this is indeed a transmuted rod and not a cone photoreceptor co-opting rhodopsin expression. Because the retinas were not dark adapted prior to sacrifice, we can presume that under normal photopic light conditions, *P. melanoleucus* rod transducin is cycled out of the outer segment of the cone-like rod, a distinct rod property (Chen et al., 2007; Rosenzweig et al., 2007). In the light, rods cycle transducin and recoverin out of the outer segment, and arrestin into it (Calvert et al., 2006). This allows the rod to effectively shut down phototransduction under bleaching conditions to prevent damage to the photoreceptor. Cones generally do not cycle transducin out of the outer segment of the photoreceptor under normal light conditions

399 (Chen et al., 2007). This suggests that the rhodopsin-expressing photoreceptors in the
400 retina of *P. melanoleucus* would not be able to generate a photoresponse in normal
401 daylight, and thus if this cone-like rod is participating in colour vision with the canonical
402 cones in the retina, it would likely only be under mesopic light conditions where both
403 photoreceptor cell types can be active.
404 Our microscopy results of the *P. melanoleucus* retina additionally revealed a
405 cone-like rod which still looks distinct in comparison to the other cones. The cone-like
406 rod outer segment and inner segment had similar diameters with a relatively long outer
407 segment, while the surrounding cones had distinctly large ellipsoids in the inner segment,
408 and proportionally smaller outer segments. Rod photoreceptor morphology is also
409 generally specialized to maximize sensitivity with long cylindrical outer segments (Lamb,
410 2013). Cone morphological specializations, however, are thought to enable selective
411 colour vision, a faster phototransduction and visual pigment regeneration, while also
412 minimizing metabolic load by miniaturizing the overall structures with large ellipsoids
413 that tunnel light onto smaller tapered outer segments (Harosi and Novales Flamarique,
414 2012). Previous EM studies on the retina of *T. proximus* showed that the membrane discs
415 unique to rods in the outer segment are still present in the transmuted photoreceptor
416 (Schott et al., 2016). Interestingly, a reduction of RH1 expression levels has been shown
417 to reduce the size of the outer segment of rods, in addition to lowering the
418 photosensitivity and altering the kinetics of the cell to be more cone-like (Makino et al.,
419 2012; Rakshit and Park, 2015; Wen et al., 2009). Currently, the relative expression levels
420 of RH1 in the retinas of colubrid snakes have not been measured. There are additional
421 specializations in the synaptic structures that reflect the different priorities in rod and
422 cone function (Lamb, 2013), but the synaptic structure of the cone-like rod also remains
423 uninvestigated.
424 Results from this study suggest that transmutation is modifying the function of a
425 subset of photoreceptors in the retina of *P. melanoleucus*. These modifications may serve
426 to lower the sensitivity and signal amplification of the photoreceptor, supporting the
427 hypothesis of a more cone-like function. However, the type of signal these transmuted
428 rods send to the brain is still unknown. Rods and cones are known to have distinct ERG
429 responses, but *T. sirtalis* is the only colubrid snake with ERG measurements performed at

a variety of light levels (Jacobs et al., 1992). However, this study did not record any scotopic (rod) response, nor did it record any photopic response from the SWS1-type photoreceptors, which suggests that the results of the study may be incomplete or that the scotopic pathways in the colubrid eye have degraded. Indeed, in high scotopic and mesopic light levels, mammalian rod photoreceptors can and do use cone pathways (Kolb et al.). The presence of rod bipolar cells and AII amacrine cells, both of which are required in the rod-specific photoresponse pathway (Lamb, 2013), has never been established in the colubrid retina..

Transmutation may be an attempt to compensate for the loss of the RH2 cone opsin and the lack of spectral overlap between the LWS and SWS1 pigment, such that the rod photoreceptor may have evolved cone-like functionality such that it could participate in colour vision. In addition to the molecular modifications to *P. melanoleucus* rhodopsin and the physiological modifications to the rod cell, the extreme blue-shift of the RH1 λ_{max}, which is quite rare for terrestrial rhodopsins, may itself be an adaptation for colour vision, as a λ_{max} of ~480 nm is in the range of typical RH2 pigments (Lamb, 2013). *Pituophis melanoleucus*, in comparison to the *Thamnophis* genera (Schott et al., 2016; Sillman et al., 1997), has a narrower overall range of spectral sensitivities. There could be two possible reasons for this narrowing. It could be that this narrowing of the spectral ranges is to facilitate spectral overlap as an adaptation in *P. melanoleucus*. Or the narrowing of the spectral range may simply be due to phylogenetic history, as *P. melanoleucus* LWS and SWS1 absorb at similar wavelengths to its closest relatives (Simões et al., 2016), which in turn could be an adaptation, but not one due to the specific visual environment of *P. melanoleucus*. Trichromatic vision would be greatly advantageous for a diurnal species (Ankel-Simons and Rasmussen, 2008; Heesy and Ross, 2001), and perhaps sacrificing scotopic vision in order to achieve better mesopic and photopic vision is possible, since other snake sensory systems adaptations, such as chemoreception, could be sufficient in dim light environments (Drummond, 1985). However, currently there is a lack of behavioral studies investigating trichromatic colour discrimination in colubrid snakes under mesopic light conditions.

We hypothesize that rod-to-cone transmutation may be allowing colubrid snakes to have a third cone-like photoreceptor, allowing for spectral sensitivity between SWS1

and LWS, possibly also trichromatic colour perception in mesopic light conditions. The loss of RH1 in nocturnal geckos and the resulting transmutation of cone into rod demonstrate that the visual system of squamates is capable of adapting to compensate for previous functionality loss in different photoreceptor types. In colubrid snakes, and possibly squamates in general, the rod/cone photoreceptor binary is not as distinct as it is in other vertebrates and caution should be taken in classifying rod or cone photoreceptors based on limited characterization.

In summary, we find that *P. melanoleucus,* like *T. proximus,* has an all-cone retina derived through evolutionary transmutation of the rod photoreceptors. Furthermore, *P. melanoleucus* rhodopsin is the first vertebrate rhodopsin to show hydroxylamine reactivity similar to cone opsins. This study is also the first to demonstrate the functional effects of transmutation in the retina of colubrid snakes. We suggest that transmutation in colubrid snakes is an adaptation to diurnality and is compensating for the loss of RH2 by establishing spectral sensitivity in a range where the existing SWS1 and LWS are not sensitive, and possibly establishing trichromatic colour vision. Perhaps transmutation in colubrid snakes may have contributed to the widespread success of the snake family across such a vast range of ecologies and lifestyle. Ultimately, future work investigating the functional effects of transmutation, from behavioral to molecular, will reveal the significance of rod-to-cone transmutation in colubrid snakes.

Competing interests

The authors declare no competing or financial interests

Author contributions

N.B and B.S.W.C contributed to the conceptual design. V.T. provided mice and experimental advice. N.B. and B.D. performed the research, and N.B., R.K.S, and B.S.W.C analyzed data. N.B., R.K.S and B.S.W.C wrote the manuscript.

Funding

This work was supported by a National Sciences and Engineering Research Council (NSERC) Discovery Grant (To B.S.W.C), a Vision Science Research Program Scholarship (to N.B. and R.K.S), and an Ontario Graduate Scholarship (to R.K.S).

References

Ankel-Simons, F. and Rasmussen, D. T. (2008). Diurnality, nocturnality, and the evolution of primate visual systems. *Am. J. Phys. Anthropol.* **137**, 100–117.

Asenjo, A. B., Rim, J. and Oprian, D. D. (1994). Molecular determinants of human red/green color discrimination. *Neuron* **12**, 1131–1138.

Barlow, H. B. (1964). Dark-adaptation: a new hypothesis. *Vision Research* **4**, 47–58.

Bickelmann, C., Morrow, J. M., Müller, J. and Chang, B. S. W. (2012). Functional characterization of the rod visual pigment of the echidna (Tachyglossus aculeatus), a basal mammal. *Vis. Neurosci.* **29**, 1–7.

Bowmaker, J. K. (2008). Evolution of vertebrate visual pigments. *Vision Research* **48**, 2022–2041.

Calvert, P. D., Strissel, K. J., Schiesser, W. E., Pugh, E. N., Jr and Arshavsky, V. Y. (2006). Light-driven translocation of signaling proteins in vertebrate photoreceptors. *Trends in Cell Biology* **16**, 560–568.

Caprette, C. L. (2005). Conquering the Cold Shudder: The Origin and Evolution of Snake Eyes. 1–122.

Carvalho, L. S., Davies, W. L., Robinson, P. R. and Hunt, D. M. (2011). Spectral tuning and evolution of primate short-wavelength-sensitive visual pigments. *Proc. Biol. Sci.* **279**, rspb20110782–393.

Chen, J., Wu, M., Sezate, S. A. and McGinnis, J. F. (2007). Light Threshold–Controlled Cone α-Transducin Translocation. *Invest. Ophthalmol. Vis. Sci.* **48**, 3350–6.

516 **Chen, M.-H., Kuemmel, C., Birge, R. R. and Knox, B. E.** (2012). Rapid Release of
517 Retinal from a Cone Visual Pigment following Photoactivation. *Biochemistry* **51**,
518 4117–4125.

519 **Cortesi, F., Musilová, Z., Stieb, S. M., Hart, N. S., Siebeck, U. E., Malmstrøm, M.,**
520 **Tørresen, O. K., Jentoft, S., Cheney, K. L., Marshall, N. J., et al.** (2015).
521 Ancestral duplications and highly dynamic opsin gene evolution in percomorph
522 fishes. *Proc. Natl. Acad. Sci. U.S.A.* **112**, 1493–1498.

523 **Crescitelli, F.** (1956). The nature of the gecko visual pigment. *J. Gen. Physiol.* **40**, 217–
524 231.

525 **Dartnall, H.** (1968). The photosensitivities of visual pigments in the presence of
526 hydroxylamine. *Vision Research* **8**, 339–358.

527 **Das, J., Crouch, R. K., Ma, J.-X., Oprian, D. D. and Kono, M.** (2004). Role of the 9-
528 Methyl Group of Retinal in Cone Visual Pigments †. *Biochemistry* **43**, 5532–5538.

529 **Das, S. R., Bhardwaj, N., Kjeldbye, H. and Gouras, P.** (1992). Muller cells of chicken
530 retina synthesize 11-cis-retinol. *Biochem J* **285 (Pt 3)**, 907–913.

531 **Davies, W. L., Cowing, J. A., Bowmaker, J. K., Carvalho, L. S., Gower, D. J. and**
532 **Hunt, D. M.** (2009). Shedding Light on Serpent Sight: The Visual Pigments of
533 Henophidian Snakes. *Journal of Neuroscience* **29**, 7519–7525.

534 **Diller, L. V. and Wallace, R. L.** (1996). Comparative ecology of two snake species
535 (Crotalus viridis and Pituophis melanoleucus) in Southwestern Idaho. *Herpetologica*
536 **52**, 343–360.

537 **Dodt, E. and Walther, J. B.** (1958). *[Spectral sensitivity and the threshold of gecko*
538 *eyes; electroretinographical studies on Hemidactylus turcicus & Tarentola*
539 *mauritanica.]*. Pflugers Archiv: European journal of ….

540 **Drummond, H.** (1985). The role of vision in the predatory behaviour of natricine snakes.
541 *Animal Behaviour* **33**, 206–215.

542 **Dungan, S. Z., Kosyakov, A. and Chang, B. S. W.** (2016). Spectral Tuning of Killer
543 Whale (Orcinus orca) Rhodopsin: Evidence for Positive Selection and Functional
544 Adaptation in a Cetacean Visual Pigment. *Mol. Biol. Evol.* **33**, 323–336.

545 **Edgar, R. C.** (2004). MUSCLE: multiple sequence alignment with high accuracy and
546 high throughput. *Nucleic Acids Res* **32**, 1792–1797.

547 **Elias, R. V., Sezate, S. S., Cao, W. and McGinnis, J. F.** (2004). Temporal kinetics of
548 the light/dark translocation and compartmentation of arrestin and alpha-transducin in
549 mouse photoreceptor cells. *Mol. Vis.* **10**, 672–681.

550 **Fekete, D. M. and Barnstable, C. J.** (1983). The subcellular localization of rat
551 photoreceptor-specific antigens. *J. Neurocytol.* **12**, 785–803.

552 **Gerald, G. W., Bailey, M. A. and Holmes, J. N.** (2006). Movements and activity range
553 sizes of Northern Pinesnakes (Pituophis melanoleucus melanoleucus) in Middle
554 Tennessee. *Journal of herpetology* **40**, 503–510.

555 **Govardovskii, V. I., FYHRQUIST, N., REUTER, T., KUZMIN, D. G. and Donner,**
556 **K.** (2000). In search of the visual pigment template. *Vis. Neurosci.* **17**, 509–528.

557 **Harosi, F. I. and Novales Flamarique, I.** (2012). Functional significance of the taper of
558 vertebrate cone photoreceptors. *J. Gen. Physiol.* **139**, 159–187.

559 **Hart, N. S., Coimbra, J. P., COLLIN, S. P. and Westhoff, G.** (2012). Photoreceptor
560 types, visual pigments, and topographic specializations in the retinas of hydrophiid
561 sea snakes. *J. Comp. Neurol.* **520**, 1246–1261.

562 **Hauser, F. E., van Hazel, I. and Chang, B. S. W.** (2014). Spectral tuning in vertebrate
563 short wavelength-sensitive 1 (SWS1) visual pigments: Can wavelength sensitivity be
564 inferred from sequence data? *J. Exp. Zool. B Mol. Dev. Evol.* **322**, 529–539.

565 **Heesy, C. P. and Ross, C. F.** (2001). Evolution of activity patterns and chromatic vision
566 in primates: morphometrics, genetics and cladistics. *Journal of Human Evolution* **40**,
567 111–149.

568 **Hicks, D. and Barnstable, C. J.** (1987). Different rhodopsin monoclonal antibodies
569 reveal different binding patterns on developing and adult rat retina. *Journal of*
570 *Histochemistry & Cytochemistry* **35**, 1317–1328.

571 **Himes, J. G.** (2001). Burrowing ecology of the rare and elusive Louisiana pine snake,
572 Pituophis ruthveni (Serpentes : Colubridae). *Amphibia-Reptilia* **22**, 91–101.

573 **Imai, H., Kojima, D., Oura, T., Tachibanaki, S., Terakita, A. and Shichida, Y.**
574 (1997). Single amino acid residue as a functional determinant of rod and cone visual
575 pigments. *Proc. Natl. Acad. Sci. U.S.A.* **94**, 2322–2326.

576 **Imai, H., Kuwayama, S., Onishi, A., Morizumi, T., Chisaka, O. and Shichida, Y.**
577 (2005). Molecular properties of rod and cone visual pigments from purified chicken
578 cone pigments to mouse rhodopsin in situ. *Photochem. Photobiol. Sci.* **4**, 667–8.

579 **Jacobs, G. H., Fenwick, J. A., Crognale, M. A. and Deegan, J. F., II** (1992). The all-
580 cone retina of the garter snake: spectral mechanisms and photopigment. *J. Comp.*
581 *Physiol. A Neuroethol. Sens. Neural. Behav. Physiol.* **170**, 701–707.

582 **Karnik, S. S. and Khorana, H. G.** (1990). Assembly of functional rhodopsin requires a
583 disulfide bond between cysteine residues 110 and 187. *J Biol Chem* **265**, 17520–
584 17524.

585 **Karnik, S. S., Sakmar, T. P., Chen, H. B. and Khorana, H. G.** (1988). Cysteine
586 residues 110 and 187 are essential for the formation of correct structure in bovine
587 rhodopsin. *Proc. Natl. Acad. Sci. U.S.A.* **85**, 8459–8463.

588 **Kawamura, S. and Yokoyama, S.** (1998). Functional characterization of visual and
589 nonvisual pigments of American chameleon (Anolis carolinensis). *Vision Research*
590 **38**, 37–44.

591 **Kito, Y., Suzuki, T., Azuma, M. and Sekoguti, Y.** (1968). Absorption spectrum of
592 rhodopsin denatured with acid. *Nature* **218**, 955–957.

593 **Kojima, D., Okano, T., Fukada, Y., Shichida, Y., Yoshizawa, T. and Ebrey, T. G.**

(1992). Cone visual pigments are present in gecko rod cells. *Proc. Natl. Acad. Sci. U.S.A.* **89**, 6841–6845.

Kolb, H., Nelson, R., Fernandez, E. and Jones, B. eds. *Webvision*.

Kuwayama, S., Imai, H., Hirano, T., Terakita, A. and Shichida, Y. (2002). *Conserved Proline Residue at Position 189 in Cone Visual Pigments as a Determinant of Molecular Properties Different from Rhodopsins†*. American Chemical Society.

Lamb, T. D. (2013). Progress in Retinal and Eye Research. *Prog Retin Eye Res* **36**, 52–119.

Larhammar, D., Nordström, K. and Larsson, T. A. (2009). Evolution of vertebrate rod and cone phototransduction genes. *Philos. Trans. R. Soc. Lond., B, Biol. Sci.* **364**, 2867–2880.

Ma, J. X., Kono, M., Xu, L., Das, J., Ryan, J. C., Hazard, E. S., Oprian, D. D. and Crouch, R. K. (2001). Salamander UV cone pigment: sequence, expression, and spectral properties. *Vis. Neurosci.* **18**, 393–399.

Makino, C. L., Wen, X.-H., Michaud, N. A., Covington, H. I., DiBenedetto, E., Hamm, H. E., Lem, J. and Caruso, G. (2012). Rhodopsin Expression Level Affects Rod Outer Segment Morphology and Photoresponse Kinetics. *PLoS ONE* **7**, e37832–7.

McDevitt, D. S., Brahma, S. K., Jeanny, J.-C. and Hicks, D. (1993). Presence and foveal enrichment of rod opsin in the “all cone” retina of the American chameleon. *The Anatomical Record* **237**, 299–307.

McKibbin, C., Toye, A. M., Reeves, P. J., Khorana, H. G., Edwards, P. C., Villa, C. and Booth, P. J. (2007). Opsin stability and folding: The role of Cys185 and abnormal disulfide bond formation in the intradiscal domain. *Journal of Molecular Biology* **374**, 1309–1318.

Morrow, J. M. and Chang, B. S. W. (2015). Comparative Mutagenesis Studies of

620 Retinal Release in Light-Activated Zebrafish Rhodopsin Using Fluorescence
621 Spectroscopy. *Biochemistry* **54**, 4507–4518.

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

625 **Morrow, J. M., Lazic, S. and Chang, B. S. W.** (2011). A novel rhodopsin-like gene
626 expressed in zebrafish retina. *Vis. Neurosci.* **28**, 325–335.

627 **Osborne, N. N., Safa, R. and Nash, M. S.** (1999). Photoreceptors are preferentially
628 affected in the rat retina following permanent occlusion of the carotid arteries. *Vision*
629 *Research* **39**, 3995–4002.

630 **Palczewski, K., Kumasaka, T., Hori, T., Behnke, C. A., Motoshima, H., Fox, B. A.,**
631 **Le Trong, I., Teller, D. C., Okada, T., Stenkamp, R. E., et al.** (2000). Crystal
632 structure of rhodopsin: A G protein-coupled receptor. *Science* **289**, 739–745.

633 **Piechnick, R., Ritter, E., Hildebrand, P. W., Ernst, O. P., Scheerer, P., Hofmann, K.**
634 **P. and Heck, M.** (2012). Effect of channel mutations on the uptake and release of the
635 retinal ligand in opsin. *Proc. Natl. Acad. Sci. U.S.A.* **109**, 5247–5252.

636 **Rakshit, T. and Park, P. S. H.** (2015). Impact of Reduced Rhodopsin Expression on the
637 Structure of Rod Outer Segment Disc Membranes. *Biochemistry* **54**, 2885–2894.

638 **Ronquist, F. and Huelsenbeck, J. P.** (2003). MrBayes 3: Bayesian phylogenetic
639 inference under mixed models. *Bioinformatics* **19**, 1572–1574.

640 **Rosenzweig, D. H., Nair, K. S., Wei, J., Wang, Q., Garwin, G., Saari, J. C., Chen, C.**
641 **K., Smrcka, A. V., Swaroop, A., Lem, J., et al.** (2007). Subunit Dissociation and
642 Diffusion Determine the Subcellular Localization of Rod and Cone Transducins.
643 *Journal of Neuroscience* **27**, 5484–5494.

644 **Röll, B.** (2001). Gecko vision - retinal organization, foveae and implications for
645 binocular vision. *Vision Research* **41**, 2043–2056.

646 **Sakami, S., Kolesnikov, A. V., Kefalov, V. J. and Palczewski, K.** (2014). P23H opsin
647 knock-in mice reveal a novel step in retinal rod disc morphogenesis. *Hum. Mol.*
648 *Genet.* **23**, 1723–1741.

649 **Sakmar, T. P., Franke, R. R. and Khorana, H. G.** (1989). Glutamic acid-113 serves as
650 the retinylidene Schiff base counterion in bovine rhodopsin. *Proc. Natl. Acad. Sci.*
651 *U.S.A.* **86**, 8309–8313.

652 **Sakmar, T. P., Menon, S. T., Marin, E. P. and Awad, E. S.** (2002). Rhodopsin:
653 insights from recent structural studies. *Annu Rev Biophys Biomol Struct* **31**, 443–484.

654 **Sakurai, K., Onishi, A., Imai, H., Chisaka, O., Ueda, Y., Usukura, J., Nakatani, K.**
655 **and Shichida, Y.** (2007). Physiological properties of rod photoreceptor cells in
656 green-sensitive cone pigment knock-in mice. *J. Gen. Physiol.* **130**, 21–40.

657 **Schott, R. K., Müller, J., Yang, C. G. Y., Bhattacharyya, N., Chan, N., Xu, M.,**
658 **Morrow, J. M., Ghenu, A.-H., Loew, E. R., Tropepe, V., et al.** (2016).
659 Evolutionary transformation of rod photoreceptors in the all-cone retina of a diurnal
660 garter snake. *Proc Natl Acad Sci USA* **113**, 356–361.

661 **Sillman, A. J., Govardovskii, V. I., Röhlich, P., Southard, J. A. and Loew, E. R.**
662 (1997). The photoreceptors and visual pigments of the garter snake (Thamnophis
663 sirtalis): a microspectrophotometric, scanning electron microscopic and
664 immunocytochemical study. *J Comp Physiol A* **181**, 89–101.

665 **Sillman, A. J., Johnson, J. L. and Loew, E. R.** (2001). Retinal photoreceptors and
666 visual pigments in Boa constrictor imperator. *J. Exp. Zool.* **290**, 359–365.

667 **Simões, B. F., Sampaio, F. L., Jared, C., Antoniazzi, M. M., Loew, E. R., Bowmaker,**
668 **J. K., Rodriguez, A., Hart, N. S., Hunt, D. M., Partridge, J. C., et al.** (2015).
669 Visual system evolution and the nature of the ancestral snake. *J. Evol. Biol.* **28**,
670 1309–1320.

671 **Simões, B. F., Sampaio, F. L., Loew, E. R., Sanders, K. L., Fisher, R. N., Hart, N. S.,**
672 **Hunt, D. M., Partridge, J. C. and Gower, D. J.** (2016). Multiple rod–cone and

673 cone–rod photoreceptor transmutations in snakes: evidence from visual opsin gene
674 expression. *Proc. Biol. Sci.* **283**, 20152624–8.

675 **Starace, D. M. and Knox, B. E.** (1997). Activation of transducin by a Xenopus short
676 wavelength visual pigment. *J Biol Chem* **272**, 1095–1100.

677 **Stull, O. G.** (1940). *Variations and Relationship in the Snake of the Genus Pituophis,*
678 *United States.. National Museum. Washington, DC*. Smithsonian Institution. Bulletin.

679 **Taniguchi, Y., Hisatomi, O., Yoshida, M. and Tokunaga, F.** (1999). Evolution of
680 visual pigments in geckos. *FEBS Lett.* **445**, 36–40.

681 **Tansley, K.** (1959). The retina of two nocturnal geckos Hemidactylus turcicus and
682 Tarentola mauritanica. *Pfl*[illegible]*gers Archiv* **268**, 213–220.

683 **Tansley, K.** (1961). The retina of a diurnal gecko, Phelsuma madagascariensis
684 longinsulae. *Pflugers Arch Gesamte Physiol Menschen Tiere* **272**, 262–269.

685 **Tansley, K.** (1964). The gecko retina. *Vision Research* **4**, 33–IN14.

686 **van Hazel, I., Dungan, S. Z., Hauser, F. E., Morrow, J. M., Endler, J. A. and Chang,**
687 **B. S. W.** (2016). A comparative study of rhodopsin function in the great bowerbird
688 (Ptilonorhynchus nuchalis): Spectral tuning and light-activated kinetics. *Protein*
689 *Science* n/a–n/a.

690 **van Hazel, I., Sabouhanian, A., Day, L., Endler, J. A. and Chang, B. S. W.** (2013).
691 Functional characterization of spectral tuning mechanisms in the great bowerbird
692 short-wavelength sensitive visual pigment (SWS1), and the origins of UV/violet
693 vision in passerines and parrots. *BMC Evol. Biol.* **13**, 250.

694 **Wald, G., Brown, P. K. and Smith, P. H.** (1955). Iodopsin. *J. Gen. Physiol.*

695 **Walls, G. L.** (1942). *The vertebrate eye and its adaptive radiation [by] Gordon Lynn*
696 *Walls.* Bloomfield Hills, Mich.,: Cranbrook Institute of Science.

697 **Wen, X.-H., Shen, L., Brush, R. S., Michaud, N., Al-Ubaidi, M. R., Gurevich, V. V.,**

Hamm, H. E., Lem, J., DiBenedetto, E., Anderson, R. E., et al. (2009). Overexpression of Rhodopsin Alters the Structure and Photoresponse of Rod Photoreceptors. *Biophys. J.* **96**, 939–950.

Yokoyama, S. (2000). Molecular evolution of vertebrate visual pigments. *Prog Retin Eye Res* **19**, 385–419.

Zhang, X., Wensel, T. G. and Yuan, C. (2006). Tokay Gecko Photoreceptors Achieve Rod-Like Physiology with Cone-Like Proteins†. *Photochem. Photobiol.* **82**, 1452.

Figure captions:

Figure 1: Immunohistochemical staining of control (mouse, **A-C**) and pine snake (**D-F**) transverse retinal cryosections with rhodopsin (RET-P1) and rod-specific-transducin (K20) antibodies. Rhodopsin is found in a subset of cone-like photoreceptors localized to the outer segment (**D**). Rod-specific transducin is also found in the same photoreceptor, primarily to the inner segment (**E**). Double staining indicates that both rhodopsin and rod-specific transducin are found within the same cell (**F**). Nuclei are shown in blue, rhodopsin in red, and rod-specific transducin in green. Scale bars = 20 μm.

Figure 2: UV-visible dark absorption spectra of pine snake opsins. (**A**) Bovine wildtype rhodopsin was used as a control for expressions. Dark spectra for pine snake (**B**) rhodopsin (**C**) SWS1 and (**D**) LWS. Inset in (**A**), (**B**), and (**D**) is the dark-light spectra. Inset in (**C**) is the dark-acid bleach spectrum. λ_{max} estimations are shown for each pigment.

Figure 3: Hydroxylamine reactivity of pine snake (**A**) LWS and (**B**) RH1 pigments and (**C**) bovine rhodopsin. Absorption values of the dark λ_{max} peak decrease over time (open circles), while absorption of the retinal oxime at 360nm increase over time (solid circles). The half-lives of the reactive opsins were determined via curve fitting exponential rise and decay equations to data.

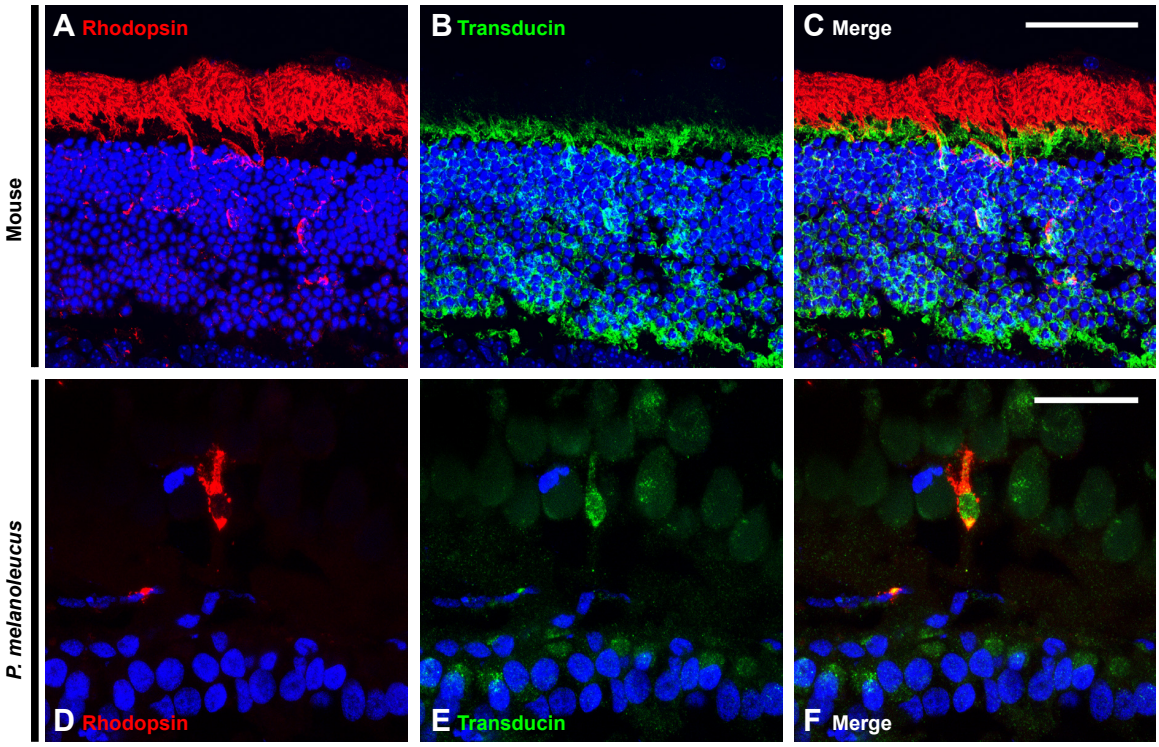
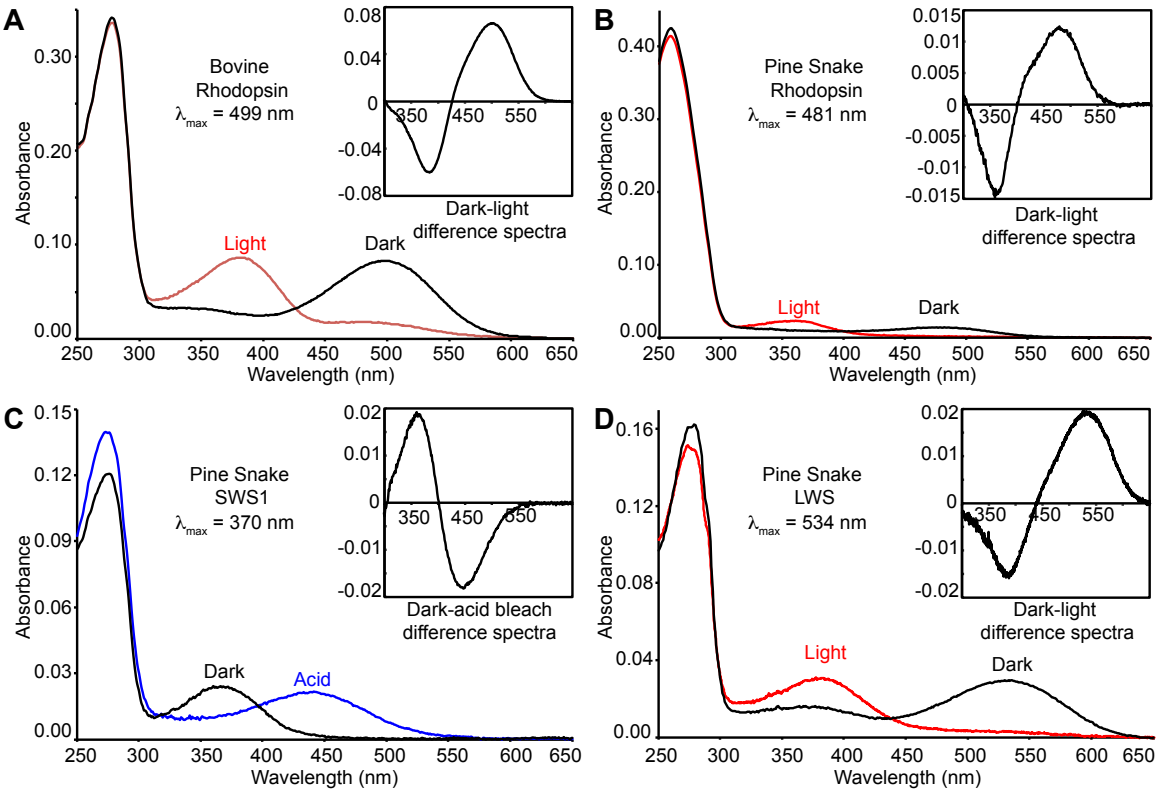


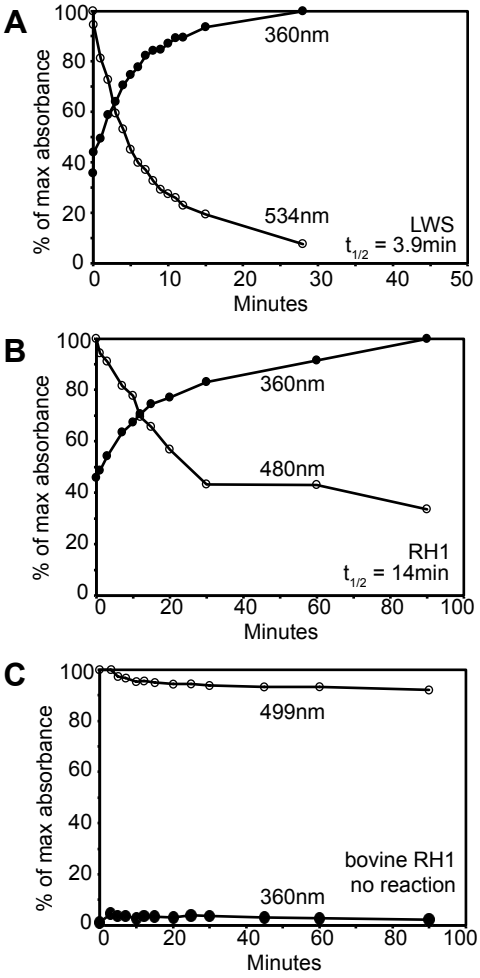
Figure 1



729

730 Figure 2

731



732

733 Figure 3

734