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

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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).

62 The family Colubridae is the most speciose family of snakes and encompasses a diverse
63 range of lifestyles and ecologies. Colubrid snakes have recently emerged as a compelling
64 group in which to study visual system evolution and adaptation (Schott et al., 2016;
65 Simões et al., 2015; Simões et al., 2016).
66 In the vertebrate retina, photoreceptor cells can be divided into two types based on
67 their overall structure and function: cones, which are active in bright light and contain
68 cone visual pigments (SWS1, SWS2, RH2, LWS) in a tapered outer segment, and rods,
69 which function in dim light and contain rhodopsin (RH1) in a longer, more cylindrical
70 outer segment (Bowmaker, 2008; Lamb, 2013; Walls, 1942). Reptilian retinas are unique
71 in having multiple retinal configurations among closely related species including all-rod
72 (Kojima et al., 1992), rod and cone (Sillman et al., 2001), and all-cone (Sillman et al.,
73 1997). In 1942, physiologist Gordon Walls outlined his theory of transmutation to explain
74 the evolutionary transformation of photoreceptors from one type to another (Walls,
75 1942). This phenomenon has since been investigated in nocturnal geckos, where cone
76 opsins are expressed in an all-rod retina in order to compensate for the evolutionary loss
77 of RH1 in a hypothesized diurnal, all-cone ancestor (Kojima et al., 1992; Taniguchi et al.,
78 1999). While the nocturnal henophedian snakes, such as boas and pythons, are known to
79 have duplex retinas expressing RH1, LWS and SWS1 in canonical photoreceptors
80 (Davies et al., 2009), the more derived diurnal colubrid snakes have been primarily
81 shown to posses simplex retinas comprising of all cone photoreceptors, with the fate of
82 the rod photoreceptor unknown (Caprette, 2005; Walls, 1942). Early studies of the
83 colubrid visual system found a green-sensitive visual pigment in addition to a red and a
84 blue pigment (Sillman et al., 1997) in the simplex retina, but were unable to distinguish
85 between a spectrally shifted rhodopsin in a transmuted rod or a potentially resurrected
86 RH2 cone opsin (Cortesi et al., 2015). More recently, a study from our group identified a
87 functional blue-shifted RH1 pigment in the all-cone retina of the ribbon snake
88 (*Thamnophis proximus*), and proposed that this resulted from a rod to cone evolutionary
89 transmutation in colubrid snakes that may have allowed for enhanced spectral
90 discrimination and even trichromatic colour vision (Schott et al., 2016). A recent study
91 that sequenced the opsins of several other colubrid snake species discovered the
92 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*

123 A Northern pine snake (*Pituophis melanoleucus melanoleucus,* adult) specimen and mice
124 (*Mus musculus,* adult, CD1*)* were obtained from a licensed source as commissioned by
125 the University Animal Care Committee (UACC). The specimen was sacrificed using an
126 approved euthanasia protocol. The eyes were enucleated and preserved in RNAlater or
127 4% paraformaldehyde.
128
129 *Total RNA extraction and cDNA synthesis*
130 The dissected whole eye was homogenized with TRIzol, and total RNA was isolated
131 using a phenol/chloroform extraction and ethanol precipitation. The first strand of
132 complementary DNA (cDNA) was synthesized using SuperScript III Reverse
133 Transcriptase (Invitrogen, Waltham, MA, USA) from RNA samples primed with a 3'
134 oligo-dT and a 5' SMART primer, following the protocol outlined by the SMART cDNA
135 Library Construction Kit (BD Biosciences, Franklin Lakes, NJ, USA). The second strand
136 complement was synthesized by long-distance PCR following the same protocol.
137 Visual pigment genes were isolated using a degenerate PCR strategy. Degenerate
138 primers based on an alignment of reptilian visual pigment sequences were used in
139 attempts to amplify partial sequences of the LWS, SWS1 and RH1 opsin genes with a
140 heminested strategy. GenomeWalker (Clontech, Mountain View, CA, USA) was
141 additionally used to obtain full-length sequences (Supplementary Table S1). Extracted
142 PCR products were ligated into the pJET1.2 blunt plasmid vector.
143
144 *Phylogenetic analysis*
145 A representative set of vertebrate visual opsin sequences was obtained from Genbank.
146 These sequences were combined with the three opsins genes sequenced from the pine
147 snake and aligned using MUSCLE (Edgar, 2004). The poorly aligned 5' and 3' ends of the
148 sequence were manually trimmed. Species list and accession numbers for all sequences
149 used in the study are provided in Supplementary Table S2. In order to confirm the
150 identities of the opsin genes from the pine snake, a gene tree was estimated using the
151 resulting alignment in MrBayes 3 (Ronquist and Huelsenbeck, 2003) using reversible
152 jump MCMC with a gamma rate parameter (nst=mixed, rates=gamma), which explores
153 the parameter space for the nucleotide model and the phylogenetic tree simultaneously.

154 The analyses were run for five million generations with a 25% burn-in. Convergence was
155 confirmed by checking that the standard deviations of split frequencies approached zero
156 and that there was no obvious trend in the log likelihood plot.
157
158 *Protein expression*
159 Full-length opsins sequences (RH1, SWS1, and LWS) were amplified from pJET1.2
160 vector using primers that added the *Bam*HI and *Eco*RI restriction sites to its 5' and 3'
161 ends, respectively, and inserted into the p1D4-hrGFP II expression vector (Morrow and
162 Chang, 2010). Expression vectors containing *P. melanoleucus* cone opsins and rhodopsin
163 genes were transiently transfected into cultured HEK293T cells (ATCC CRL-11268)
164 using Lipofectamine 2000 (Invitrogen, Waltham, MA, USA; 8 µg of DNA per 10-cm
165 plate) and harvested after 48 h. Visual pigments were regenerated with 11-*cis* retinal,
166 generously provided by Dr. Rosalie Crouch (Medical University of South Carolina),
167 solubilized in 1% dodecylmaltoside, and purified with the 1D4 monoclonal antibody
168 (University of British Columbia #95-062, Lot #1017; Molday and MacKenzie, 1983)
169 as previously described (Morrow and Chang, 2015; Morrow and Chang, 2010; Morrow
170 et al., 2011). RH1 and SWS1 pigments were purified in sodium phosphate buffers and
171 LWS was purified in HEPES buffers containing glycerol (as described in van Hazel et al.
172 (2013)). The ultraviolet-visible absorption spectra of purified visual pigments were
173 recorded using a Cary 4000 double beam spectrophotometer (Agilent, Santa Clara, CA,
174 USA). Dark-light difference spectra were calculated by subtracting light-bleached
175 absorbance spectra from respective dark spectra. Pigments were photoexcited with light
176 from a fiber optic lamp (Dolan-Jenner, Boxborough, MA, USA) for 60 s at 25°C.
177 Absorbance spectra for acid bleach and hydroxylamine assays were measured following
178 incubation in hydrochloric acid (100mM) and hydroxylamine (NH_2OH, 50mM),
179 respectively. To estimate λ_{max}, the dark absorbance spectra were baseline corrected and
180 fit to Govardovskii templates for A1 visual pigments (Govardovskii et al., 2000).
181
182 *Immunohistochemistry*
183 Fixation of pine snake eyes was conducted as previously described (Schott et al., 2016).
184 Briefly, after enucleating *P. melanoleucus* eyes in the light, they were rinsed in PBS

185 (0.8% NaCl, 0.02% KCl, 0.144% NaHPO4, and 0.024% KH2PO4, pH 7.4), fixed
186 overnight at 4ºC in 4% paraformaldehyde, infiltrated with increasing concentrations of
187 sucrose (5%, 13%, 18%, 22%, 30%) in PBS, and embedded in a 2:1 solution of 30%
188 sucrose and O.C.T compound (Tissue-Tek, Burlington, NC, USA) at -20º. The eyes were
189 cryosectioned transversely at -25ºC in 20 µm sections using a Leica CM3050 (Wetzlar,
190 Germany) cryostat, placed onto positively charged microscope slides, and stored at -80ºC
191 until use.
192 Slides were first rehydrated in PBS and then air-dried to ensure adhesion. Sections
193 were rinsed three times in PBS with 0.1% Tween-20 (PBT) and then incubated in 4%
194 paraformaldehyde PBS for 20 minutes. After rinsing in PBT and PDT (PBT with 0.1%
195 DMSO), the slides were incubated in a humidity chamber with blocking solution (1%
196 BSA in PDT with 2% normal goat serum) for one hour, incubated with primary antibody
197 diluted in blocking solution overnight at 4º in a humidity chamber. Antibodies used were
198 the K20 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA, sc-389, lot#:C1909,
199 dilution: 1:500) and RET-P1 anti-rhodopsin antibody (Sigma-Aldrich, St. Louis, MO,
200 USA, O-4886, lot#: 19H4839, dilution: 1:200).
201 After extensive rinsing and soaking in PDT (3 times for 15 minutes), secondary
202 antibody was added to the samples and incubated at 37º for one hour in a humidity
203 chamber. Secondary antibodies used for the fluorescent staining were the AlexaFluor-488
204 anti-rabbit antibody (Life Technologies, Waltham, MA, USA, A11034, lot#: 1298480,
205 dilution: 1:1000) and the Cy-3 anti-mouse antibody (Jackson ImmunoResearch, West
206 Grove, PA, USA, 115-165-003, dilution: 1:800). After rinsing with PBS, followed by
207 PDT, sections were stained with 10 µg/mL Hoescht for 10 minutes at room temperature.
208 The sections were then rinsed in PBS and PDT and mounted with ProLong® Gold
209 antifade reagent (Life technologies, Waltham, MA, USA) and coverslipped. Sections
210 were visualized via a Leica SP-8 confocal laser microscope (Wetzlar, Germany).
211
212 **Results**
213 *Full-length RH1, SWS1 and LWS opsin sequences found in Pituophis melanoleucus*
214 *cDNA*

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

221 All three opsin genes sequence contained the critical amino acid residues required
222 for proper structure and function of a prototypical opsin including K296, the site of the
223 Schiff base linkage with 11-*cis* retinal (Palczewski et al., 2000; Sakmar et al., 2002), and
224 E113, the counter-ion to the Schiff base in the dark state (Sakmar et al., 1989), as well as
225 C110 and C187, which form a critical disulfide bond in the protein (Karnik and Khorana,
226 1990). Both cone opsin genes also have the conserved P189 residue which is critical for
227 faster cone opsin pigment regeneration (Kuwayama et al., 2002).

228 Interestingly, *P. melanoleucus* RH1 has serine at site 185 instead of the highly
229 conserved cysteine, similar to several other snakes (Schott et al., 2016; Simões et al.,
230 2016). Mutations at site 185 have been shown to reduce both visual pigment stability
231 (McKibbin et al., 2007) and transducin activation *in vitro* (Karnik et al., 1988). Also, the
232 *P. melanoleucus* RH1 has N83 and S292, which are often found in rhodopsins with blue-
233 shifted λ_{max} values, and can also affect all-*trans* retinal release kinetics following
234 photoactivation (Bickelmann et al., 2012; van Hazel et al., 2016).

235 Based on known spectral tuning sites in LWS, *P. melanoleucus* has A285,
236 compared to T285 in *Thamnophis* snakes. T285A is known to blue-shift the LWS
237 pigment by 16-20 nm (Asenjo et al., 1994; Yokoyama, 2000). This suggests that the *P.*
238 *melanoleucus* LWS may be considerably blue-shifted relative to the LWS pigment in
239 *Thamnophis* snakes. Within *P. melanoleucus* SWS1, the phenylalanine at site 86 suggests
240 that the pigment will be absorbing in the UV, as is typical of reptilian SWS1 pigments
241 (Hauser et al., 2014). *Pituophis melanoleucus* SWS1, as well as other colubrids SWS1
242 (Simões et al., 2016), have hydrophobic residues at two spectral tuning sites, A90 and
243 V93. These sites are usually have polar or charged amino acid side chains (Carvalho et
244 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

276 al., 2011). Bovine wildtype rhodopsin was used as a control (Fig 2A). Pine snake
277 rhodopsin has a λ_{max} of 481nm (Fig 2B) which is similar to the measured λ_{max} of
278 rhodopsins from *T. proximus*, *T. sirtalis,* and *Arizona elegans* snakes (Schott et al., 2016;
279 Sillman et al., 1997; Simões et al., 2016). The drastic blue shift is expected given the
280 presence of the blue-shifting N83 and S292 amino acid identities (Bickelmann et al.,
281 2012; Dungan et al., 2016; van Hazel et al., 2016). *P. melanoleucus* rhodopsin expressed
282 similar to that of *T. proximus,* with a large ratio between total purified protein
283 (absorbance at 280nm) and active protein (absorbance at λ_{max}) that indicates that only a
284 small proportion of the translated opsin protein is able to bind chromophore and become
285 functionally active. One possible explanation for this effect is the S185 residue in *P.*
286 *melanoleucus* rhodopsin, as mutations at this site have been shown to affect the retinal
287 binding efficiency of rhodopsin pigments expressed *in vitro* (McKibbin et al., 2007).
288 Expression of pine snake SWS1 showed a much more favorable 280nm to λ_{max}
289 ratio (Fig 2C). We found that *P. melanoleucus* SWS1 pigment absorbs in the UV range
290 with a λ_{max} of 370nm, similar to the SWS1 λ_{max} of *Lampropeltis getula*, *Rhinocheilus*
291 *lecontei*, and *Hypsiglena torquata* (Simões et al., 2016) all of which have the most red-
292 shifted UV SWS measured among colubrid snakes.
293 Similar to the SWS1 expression, LWS also expressed quite well (Fig 2D). Fit to
294 A1 templates gave a λ_{max} of 534nm, which is blue-shifted relative to *Thamnophis* (Schott
295 et al., 2016; Sillman et al., 1997), but identical with LWS MSP measurements of *H.*
296 *torquata* (Simões et al., 2016) and very close to those of *L. getula*, *A. elegans*, and *R.*
297 *lecontei* (Simões et al., 2016).
298
299 *Opsin protein functional characterization*
300 In order to confirm the covalent attachment of the chromophore in *P.*
301 *melanoleucus* SWS1 pigments, the purified opsin was acid bleached (Fig 2C). We found
302 a shift of the λ_{max} from 370nm to 440nm, which indicates the presence of 11-*cis* retinal
303 covalently bound by a protonated Schiff base to a denatured opsin protein (Kito et al.,
304 1968), suggesting that the UV sensitivity of the pigment may be established by only the
305 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

368 cone-specific Müller cell retinoid cycle (Das et al., 1992) providing a dedicated pool of
369 11-*cis* retinal. These faster kinetic properties are hypothesized to be facilitated in cone
370 opsins via the relative "openness" of the chromophore binding pocket, which allows
371 water molecules, and therefore other small molecules like hydroxylamine, to access the
372 chromophore where they can participate Schiff base hydrolysis (Chen et al., 2012;
373 Piechnick et al., 2012; Wald et al., 1955). Rhodopsins, on the other hand, are optimized
374 for sensitivity and signal amplification; therefore, E122/I189 and a tighter overall
375 structure contribute to a slower active state decay allowing for the activation of multiple
376 G proteins (Chen et al., 2012; Starace and Knox, 1997), increased thermal stability
377 relative to cone opsins (Barlow, 1964), and a resistance to hydroxylamine (Dartnall,
378 1968). *Pituophis melanoleucus* rhodopsin shows adaptations to decrease the number of
379 G-proteins activated, and hydroxylamine reactivity which suggests that an open
380 chromophore binding pocket would enable water access to facilitate active state decay,
381 Schiff base linkage hydrolysis, and retinal regeneration (Chen et al., 2012) rates similar to
382 cone opsins. Spectroscopic assays measuring G protein activation and retinal release rates
383 have never been performed on colubrid rhodopsins, but would be an interesting direction
384 for future research characterizing this cone opsin-like rhodopsin.
385 Retinal immunohistochemistry localized *P. melanoleucus* rhodopsin protein in the
386 outer segment of a photoreceptor, as well as the presence of rod transducin in the inner
387 segment. Rod and cone transducin are thought to originate via duplication from on
388 ancestral gene (Larhammar et al., 2009) and both have been shown to function with all
389 opsins (Sakurai et al., 2007), therefor the presence and preservation of rod transducin in
390 the photoreceptor supports the theory that this is indeed a transmuted rod and not a cone
391 photoreceptor co-opting rhodopsin expression. Because the retinas were not dark adapted
392 prior to sacrifice, we can presume that under normal photopic light conditions, *P.*
393 *melanoleucus* rod transducin is cycled out of the outer segment of the cone-like rod, a
394 distinct rod property (Chen et al., 2007; Rosenzweig et al., 2007). In the light, rods cycle
395 transducin and recoverin out of the outer segment, and arrestin into it (Calvert et al.,
396 2006). This allows the rod to effectively shut down phototransduction under bleaching
397 conditions to prevent damage to the photoreceptor. Cones generally do not cycle
398 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

430 a variety of light levels (Jacobs et al., 1992). However, this study did not record any
431 scotopic (rod) response, nor did it record any photopic response from the SWS1-type
432 photoreceptors, which suggests that the results of the study may be incomplete or that the
433 scotopic pathways in the colubrid eye have degraded. Indeed, in high scotopic and
434 mesopic light levels, mammalian rod photoreceptors can and do use cone pathways (Kolb
435 et al.). The presence of rod bipolar cells and AII amacrine cells, both of which are
436 required in the rod-specific photoresponse pathway (Lamb, 2013), has never been
437 established in the colubrid retina..
438 Transmutation may be an attempt to compensate for the loss of the RH2 cone
439 opsin and the lack of spectral overlap between the LWS and SWS1 pigment, such that the
440 rod photoreceptor may have evolved cone-like functionality such that it could participate
441 in colour vision. In addition to the molecular modifications to *P. melanoleucus* rhodopsin
442 and the physiological modifications to the rod cell, the extreme blue-shift of the RH1
443 λ_{max}, which is quite rare for terrestrial rhodopsins, may itself be an adaptation for colour
444 vision, as a λ_{max} of ~480 nm is in the range of typical RH2 pigments (Lamb, 2013).
445 *Pituophis melanoleucus*, in comparison to the *Thamnophis* genera (Schott et al., 2016;
446 Sillman et al., 1997), has a narrower overall range of spectral sensitivities. There could be
447 two possible reasons for this narrowing. It could be that this narrowing of the spectral
448 ranges is to facilitate spectral overlap as an adaptation in *P. melanoleucus*. Or the
449 narrowing of the spectral range may simply be due to phylogenetic history, as *P.*
450 *melanoleucus* LWS and SWS1 absorb at similar wavelengths to its closest relatives
451 (Simões et al., 2016), which in turn could be an adaptation, but not one due to the specific
452 visual environment of *P. melanoleucus*. Trichromatic vision would be greatly
453 advantageous for a diurnal species (Ankel-Simons and Rasmussen, 2008; Heesy and
454 Ross, 2001), and perhaps sacrificing scotopic vision in order to achieve better mesopic
455 and photopic vision is possible, since other snake sensory systems adaptations, such as
456 chemoreception, could be sufficient in dim light environments (Drummond, 1985).
457 However, currently there is a lack of behavioral studies investigating trichromatic colour
458 discrimination in colubrid snakes under mesopic light conditions.
459 We hypothesize that rod-to-cone transmutation may be allowing colubrid snakes
460 to have a third cone-like photoreceptor, allowing for spectral sensitivity between SWS1

461 and LWS, possibly also trichromatic colour perception in mesopic light conditions. The
462 loss of RH1 in nocturnal geckos and the resulting transmutation of cone into rod
463 demonstrate that the visual system of squamates is capable of adapting to compensate for
464 previous functionality loss in different photoreceptor types. In colubrid snakes, and
465 possibly squamates in general, the rod/cone photoreceptor binary is not as distinct as it is
466 in other vertebrates and caution should be taken in classifying rod or cone photoreceptors
467 based on limited characterization.
468 In summary, we find that *P. melanoleucus,* like *T. proximus,* has an all-cone retina
469 derived through evolutionary transmutation of the rod photoreceptors. Furthermore, *P.*
470 *melanoleucus* rhodopsin is the first vertebrate rhodopsin to show hydroxylamine
471 reactivity similar to cone opsins. This study is also the first to demonstrate the functional
472 effects of transmutation in the retina of colubrid snakes. We suggest that transmutation in
473 colubrid snakes is an adaptation to diurnality and is compensating for the loss of RH2 by
474 establishing spectral sensitivity in a range where the existing SWS1 and LWS are not
475 sensitive, and possibly establishing trichromatic colour vision. Perhaps transmutation in
476 colubrid snakes may have contributed to the widespread success of the snake family
477 across such a vast range of ecologies and lifestyle. Ultimately, future work investigating
478 the functional effects of transmutation, from behavioral to molecular, will reveal the
479 significance of rod-to-cone transmutation in colubrid snakes.
480
481 **Competing interests**
482 The authors declare no competing or financial interests
483
484 **Author contributions**
485 N.B and B.S.W.C contributed to the conceptual design. V.T. provided mice and
486 experimental advice. N.B. and B.D. performed the research, and N.B., R.K.S, and
487 B.S.W.C analyzed data. N.B., R.K.S and B.S.W.C wrote the manuscript.
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493

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705 **Figure captions:**

706 **Figure 1:** Immunohistochemical staining of control (mouse, **A-C**) and pine snake (**D-F**)
707 transverse retinal cryosections with rhodopsin (RET-P1) and rod-specific-transducin
708 (K20) antibodies. Rhodopsin is found in a subset of cone-like photoreceptors localized to
709 the outer segment (**D**). Rod-specific transducin is also found in the same photoreceptor,
710 primarily to the inner segment (**E**). Double staining indicates that both rhodopsin and rod-
711 specific transducin are found within the same cell (**F**). Nuclei are shown in blue,
712 rhodopsin in red, and rod-specific transducin in green. Scale bars = 20 μm.
713

714 **Figure 2:** UV-visible dark absorption spectra of pine snake opsins. (**A**) Bovine wildtype
715 rhodopsin was used as a control for expressions. Dark spectra for pine snake (**B**)
716 rhodopsin (**C**) SWS1 and (**D**) LWS. Inset in (**A**), (**B**), and (**D**) is the dark-light spectra.
717 Inset in (**C**) is the dark-acid bleach spectrum. λ_{max} estimations are shown for each
718 pigment.
719

720 **Figure 3:** Hydroxylamine reactivity of pine snake (**A**) LWS and (**B**) RH1 pigments and
721 (**C**) bovine rhodopsin. Absorption values of the dark λ_{max} peak decrease over time (open
722 circles), while absorption of the retinal oxime at 360nm increase over time (solid circles).
723 The half-lives of the reactive opsins were determined via curve fitting exponential rise
724 and decay equations to data.
725

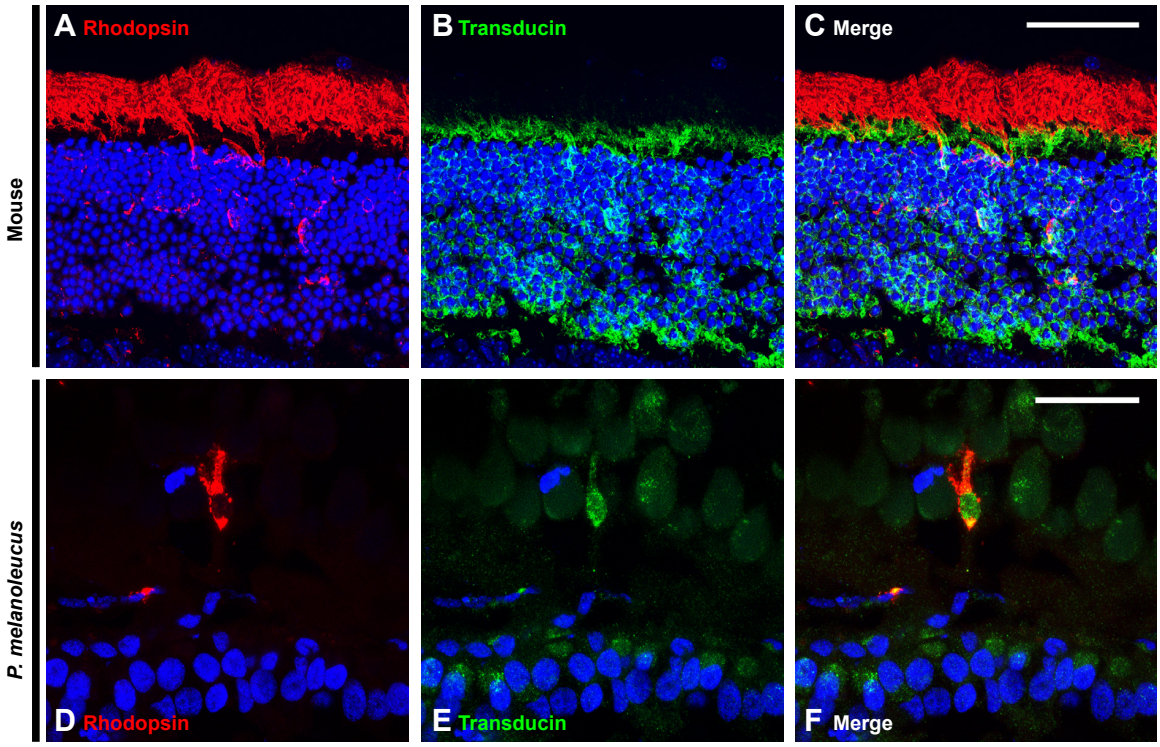
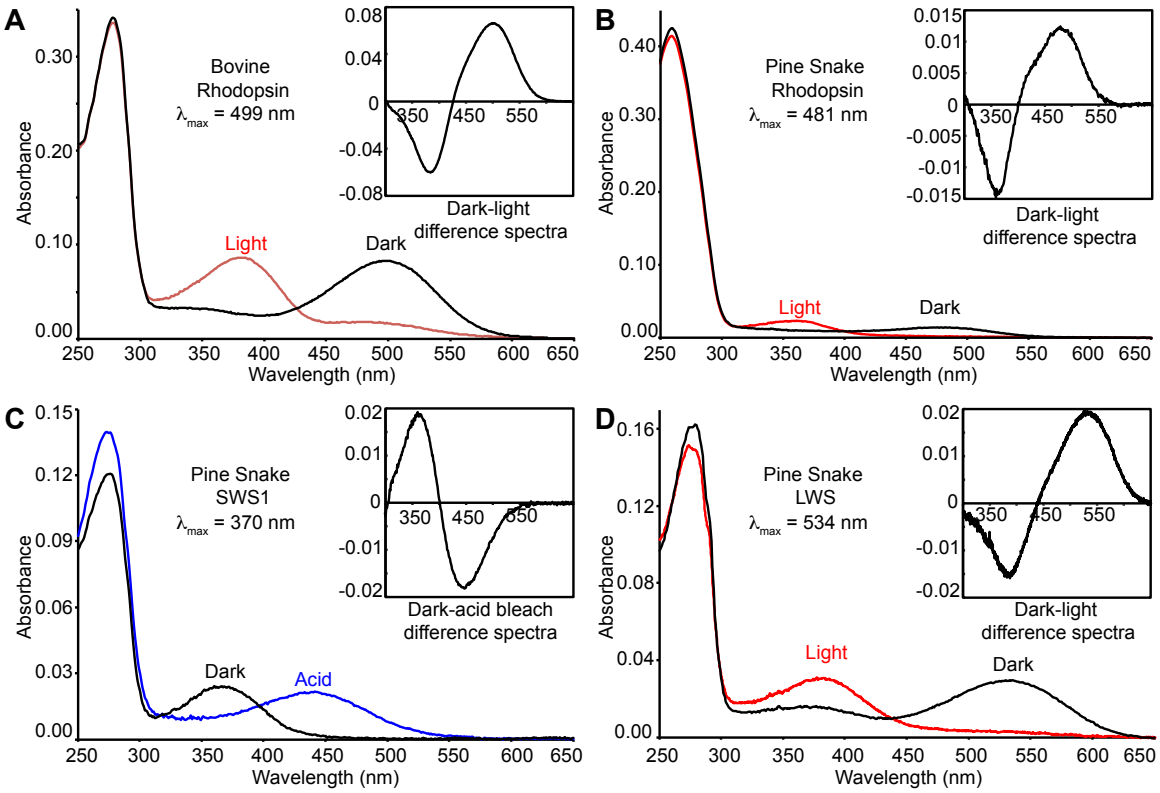

A Rhodopsin
B Transducin
C Merge
Mouse
P. melanoleucus
D Rhodopsin
E Transducin
F Merge

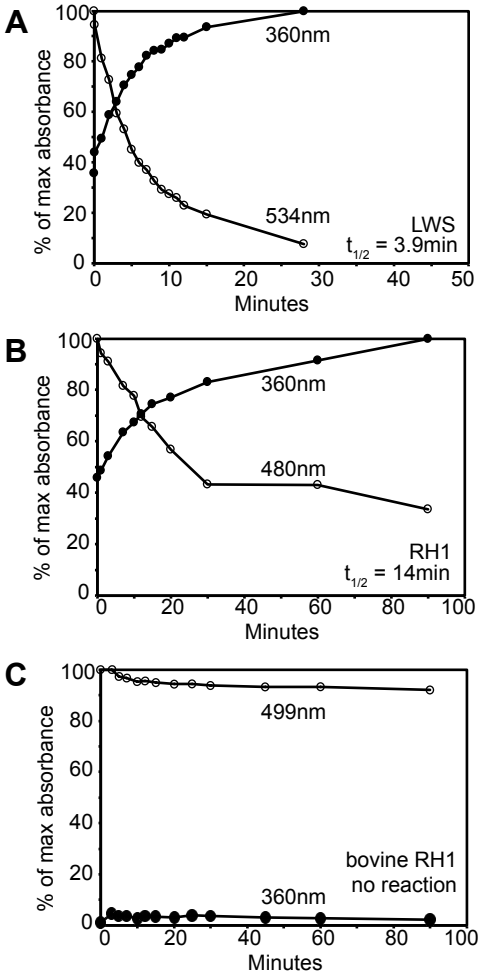
Figure 1



729

730 Figure 2

731



732

733 Figure 3

734