Rod-like Properties of Small Single Cones: Transmutated Photoreceptors of Garter Snakes (*Thamnophis proximus*)

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science
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Abstract

While nocturnal basal snakes have rod-dominant retinae, diurnal garter snakes have all-cone retinae. Previous work from the Chang lab identified three visual pigments expressed in the photoreceptors of *Thamnophis proximus*: SWS1, LWS and RH1. I further characterized *T. proximus* photoreceptors using electron microscopy, immunohistochemistry, and *in vitro* protein expression. *T. proximus* have four types of morphological cones: double cones, large single cones, small single cones, and very small single cones. Some small single cones have rod-like features, such as rod-like outer-segment membranes and a lack of micro-droplets. Immunohistochemistry showed that rod-specific transducin is expressed in some *T. proximus* photoreceptors. *In vitro* expression of *T. proximus* RH1 produced a functional rhodopsin with $\lambda_{\text{max}}$ at 485nm, which corresponds to microspectrophotometry measurement from some small single cones. Current results suggest that small single cones of *T. proximus* may have evolved from ancestral rods, and secondarily acquired a cone-like morphology as adaptation to diurnality.
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<tr>
<td>Gαi</td>
<td>G-protein α-subunit</td>
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<tr>
<td>GPCR</td>
<td>G-protein coupled receptors</td>
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<tr>
<td>LWS</td>
<td>Long-wavelength sensitive</td>
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<td>λmax</td>
<td>Spectral absorbance peak</td>
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<td>MWS</td>
<td>Middle-wavelength sensitive</td>
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<td>MSP</td>
<td>Microspectrophotometry</td>
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<td>RH1</td>
<td>Rhodopsin</td>
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<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
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<tr>
<td>SWS</td>
<td>Short-wavelength sensitive</td>
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<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
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<td>UV</td>
<td>Ultra-violet</td>
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1 Introduction

1.1 Retina and Photoreceptors

The vertebrate retina is a light sensitive tissue lining the inner surface of the eye. The vertebrate retina contains these main classes of cells: photoreceptors, horizontal cells, bipolar cells, amacrine cells, and ganglion cells (Masland, 2001; Wassle, 2004). Light travels through the ganglion cell layer, inner nuclear layers, outer nuclear layers, and finally reaches the photoreceptors. Each photoreceptor has an outer segment, an inner segment, and synaptic terminals (Figure 1). The outer segment is the membranous site specialized for photon detection, and the inner segment contains the nucleus and organelles such as mitochondria and Golgi apparatus for cellular metabolism (Kawamura and Tachibanaki, 2008). The inner segment of a typical vertebrate photoreceptor can be further divided into three regions: ellipsoid, paraboloid and myoid (Fein and Szuts, 1982). The ellipsoid is packed with mitochondria to supply the cell with metabolic energy such as ATP; the paraboloid contains intracellular vacuoles and glycogen granules; the myoid region houses organelles such as Golgi apparatus for protein synthesis (Fein and Szuts, 1982).

Two basic classes of photoreceptors can be found in most vertebrate retinae: rods and cones. Rods are more light-sensitive receptors for the dim-light (scotopic) visual pathway, while cones are optimized for day-light (photopic) visual pathway (Schultze, 1866). The different sensitivities of rods and cones originate in their distinct morphological, biochemical properties, and neuronal synapses. In vertebrate retina, rods and cones are often distinguishable based on morphology: rods are tall, slender cells with elongated outer segments; cones are shorter and stouter cells with tapering outer segments. The large outer segments of rods have increased surface area for photon capture, boosting visual sensitivity in low light environment. Inside the outer segments of rods are individualized membranous discs unattached to the plasma membrane. In contrast, outer segment membranes of cones are formed by invaginations of the plasma membrane. This distinguishing feature of the outer segment membranes has been extensively applied when characterizing rod versus cone photoreceptors. However, there are substantial differences between rods and cones beyond their morphology. For example, rods have a high convergence onto rod bipolar cells, which improves the signal-to-noise ratio of the rod-pathway (Kefalov, 2003). Although this thesis focuses on the difference between rods and cones based on
morphology and biochemistry, it is important to remember that post-receptor neurons play an important role in the visual pathways as well.

Figure 1. Schematic diagram of a rod and a cone photoreceptor. Note the outer segment membrane disks of rods, and the presence of oil-droplets in some cones.
Vertebrates live in environments that vary in intensity and spectral range of light. Duplex retinas with rods and cones enable animals to see during day and night. Vertebrate visual systems have an extensive array of adaptations to different lifestyle and visual environment. One such adaptation is through varying the proportion of rods and cones. In mammals, for example, rod-cone ratios roughly correlate with the daily activity pattern (Peichl, 2005). Nocturnal species have between 0.5% and 3% cones among their photoreceptors, crepuscular and arrhythmic species have between 2% and 10% cones, and diurnal mammals show a larger range of cone proportions from 8% to 95% cones (Ahnelt and Kolb, 2000; Peichl et al., 2000). The current understanding is that all mammals have a duplex retina containing rods and cones (Peichl, 2005). Although in some cases, vertebrates may appear to have all-rod or all-cone retinae as extreme adaptations to a strongly nocturnal or diurnal lifestyle, respectively (Kolmer, 1936; Walls, 1942; Rochon-Duvigneaud, 1943). For example, diurnal mammals such as tree shrews (*Tupaia belangeri*) appear to have “pure-cone” retina (Samorajski et al., 1966; Tigges et al., 1967). Based on EM, rod-like cells were later found to make up about 4% of the photoreceptors (Foelix et al., 1987). These photoreceptors do not have typical rod-like morphology, since they are relatively short and narrow cells. They are distinguished from cones based on staining pattern with toluidine blue (Foelix et al., 1987). The cone-dominant retina of *T. belangeri* is attributed to its great reliance on photopic vision, and its visually guided behavior (Samorajski, 1966).

Cone photoreceptors can be further classified into subtypes according to cellular features or protein expression. *Homo sapiens*, for example, have rods and three spectral classes of single cones. These different cone types are responsible for our color vision. However, human retina is rather simple when compared to that of some birds. Bird retina contains rods, four spectral classes of single cones, and a class of double cones (Hart, 2007). The bird cones also possess an array of color filters in the form of oil droplets. Found in the inner segment of cones, an oil droplet is a large spherical organelle with high lipid content (Johnston and Hudson, 1976). The oil droplets can be clear and non-pigmented, like those in chondrostean fishes (Walls and Judd, 1933), anuran amphibians (Hailman, 1976), geckos (Ellignson et al., 1995), monotremes (Walls, 1942) and marsupials (O’day, 1935; Arrese et al., 2002; Arrese et al., 2005). In birds, turtles, and lizards, however, the oil droplets can have a pale green, greenish yellow, golden yellow or ruby red coloration (Walls and Judd, 1933; Robinson, 1994; Bailes et al., 2006). The colored oil
droplets can enhance color discrimination by filtering out specific wavelength of light (Vorobyev, 2003).

Oil droplets are found in many reptiles but not in snakes, since ancestral snakes lost oil-droplets as an adaptation to fossorial lifestyle (Walls, 1942). Instead, the photoreceptors of common garter snakes (*Thamnophis sirtalis*) are packed with microdroplets of high refractive index (Wong, 1989). The microdroplets of a snake photoreceptor may function collectively to replace a single oil droplet as light filters (Wong, 1989). Since the oil droplets are cone-specific organelles, it will be interesting to examine whether snake cones differentially express microdroplets.

Different subtypes of photoreceptors can form regular retinal mosaic patterns, commonly found in fish species. In the velvet cichlids (*Astronotus ocellatus*), for example, the cone photoreceptors are arranged in a repeating square mosaic pattern with one single cone surrounded by four double cones (Braekevelt, 1992). These regularly spaced arrangements of cells have predictable pattern relative to neighboring cell types. There are two primary hypotheses for photoreceptor mosaic patterning in vertebrates: one postulates that cell fate is determined by a series of inductive and sequential events, based on preexisting mosaic position (Raymond et al., 1995; Stenkamp and Cameron, 2002; Raymond and Barthel, 2004); and another postulates that differential adhesion between cell subtypes establishes mosaic position (Galli-Resta, 2001; Mochizuki, 2002; Reese and Galli-Resta, 2002; Tohya et al., 2003). Migratory fish such as rainbow trout (*Oncorhynchus mykiss*) depend on well-defined square cone mosaic pattern for polarized light detection (Hawryshyn, 2000). Salmonid and trout species can alter their retinal mosaic during different lifestages, through ontogenetic loss of UV sensitivity (Allison et al., 2003). Rapid retinal development of most model systems makes photoreceptor developmental events difficult to study (Hoke et al., 2006). Mosaic pattern of photoreceptors have not been reported from any snake species.

Microscopy has been employed extensively in comparative studies of surface morphology, internal organelles, and mosaic pattern of photoreceptors. The present study used electron microscopy to characterize the cellular features of *Thamnophis proximus* photoreceptors. The reasons for studying this particular species of snake are discussed in later sections. When combined with molecular data of phototransduction proteins, the photoreceptors can be better characterized to provide a more accurate reflection on their evolution and functional capability.
1.2 Visual Pigments and Photo-transduction

In vertebrate photoreceptors, visual pigments capture light energy and activate the phototransduction cascade. A visual pigment is made up of an opsin protein bound to an 11-cis retinal. Opsin proteins belong to the super family of G protein-coupled receptors (GPCR), characterized by the seven-transmembrane domain, and the ability to activate a G-protein. The best studied opsin protein is the rhodopsin (RH1) of bovine, readily available in large quantity from the rod photoreceptors of cows. When regenerated with 11-cis retinal, bovine RH1 pigments in their dark state absorb at around 500nm (Figure 2). After photons strike the outer segment membrane, light energy induces a series of conformational changes to the retinal, eventually breaking its bond with the opsin. This is also known as light bleaching the visual pigment. When the visual pigment is in its biologically active conformation, known as the meta-II stage, it binds and activates the G-protein transducin. Transducin then activates cGMP phosphodiesterase (PDE) which hydrolyzes cGMP. The drop in cGMP concentration causes closure of cation channels in photoreceptor plasma membrane, leading to hyperpolarization of the plasma membrane and neuronal signaling (Hargrave et al., 1993; Ebrey and Koutalos, 2001). The shut-off mechanism of opsins are regulated by phosphorylation and arrestin binding (Kefalov, 2003). The only action of light in vision is to isomerize the retinal from 11-cis to all-trans configuration (Hubbard and Kropf, 1958), all the subsequent changes in chemistry and physiology are “dark” consequences. Therefore the dark state absorbance is usually referred to as the absorbance of a visual pigment, even though each intermediate stage has its own characteristic absorbance.

The absorbance range of a photoreceptor is dependant on what opsin is expressed in their outer segment membranes. Specialized in dim-light detection, RH1 pigments of most vertebrates absorb at around 500nm (Figure 3). For their high-sensitivity to photon, RH1 pigments are often expressed in rod photoreceptors for dim-light detection. In cone photoreceptors, four spectrally distinct classes of cone opsins can be found. Light from 355-440nm is absorbed by short-wave sensitive class SWS1, 410-490nm is absorbed by another short-wave sensitive class SWS2, 480-535nm is absorbed by middle-wave sensitive class RH2, 490-570nm is absorbed by middle- to long-wave sensitive class LWS (Yokoyama, 2000; Bowmaker, 2008). So far, three classes of opsins have been sequenced from sunbeam snakes (Xenopeltis unicolor) and python (Python regius): RH1, SWS1, and LWS (Davies et al., 2009).
Visual pigments play an important role in the functional difference between rod and cone photoreceptors. Rhodopsin pigments are typically 100 times more photosensitive than cone pigments, but rod response kinetics are several times slower (Baylor, 1987). Different kinetics between rod and cone pigments translate into high-sensitivity of rod photoreceptor, but a slower recovery from light bleaching compared to cones. Complete recovery of mammalian photoreceptors takes around 20 min for a rod (Thomas & Lamb 1999), but only 20 ms for a cone (Kenkre et al. 2005). A part of this difference is due to a 10-fold longer lifetime of meta-II state of rhodopsin, and its lowered rate of spontaneous isomerization. By one account, red cone pigment isomerizes spontaneously 10000 times more frequently than rod pigments (Kefalov, 2003). Cone pigments are more prone to spontaneous isomerization than rod pigments, contributing to the dark-noise of cone photoreceptors.
Vertebrates evolved visual pigments about 500 million years ago, before the appearance of jaws (Bowmaker, 2008). Four classes of cone opsin evolved from gene duplication, followed by the rod opsin class that arose from the duplication of RH2 opsin class. The primary selective pressure driving the opsin evolution is the spectral range and intensity of light of the visual environment (Bowmaker, 2008). Vertebrate opsins are tuned to specific spectral regions based on a number of spectral tuning sites. A spectral tuning site is an amino acid substitution which results in a shifted λmax of opsins. A single nucleotide substitution can lead to a different amino acid at the tuning site, resulting in altered interaction between the opsin and chromophore, thus a spectral shift. This shift, even based on one amino acid change, can range from a few nanometers to greater than 60nm (Wilkie et al., 2000). Rhodopsin can be spectrally tuned by several amino acid residues, such as site 83 and 292. With D83 and A292, wild-type bovine RH1 pigments absorb at 500nm, but after substituting with N83 and S292, the λmax was shifted to 485nm (Fasick and Robinson, 1998). Amino acid N83 is found in rhodopsin of bottle-nosed dolphin and deep-water teleost fish as an adaptation to monochromatic deep-water environment (Fasick and Robinson, 1998; Suguwara et. al., 2009). However, these amino acid substitutions can not be explained exclusively by deep-water adaptation, since it is also found in terrestrial vertebrates like elephants (Yokoyama et. al., 2005). In later sections, spectral tuning sites of snake opsins will be further discussed.

![Figure 3. Phylogenetic relationship between opsin families, and the respective range of λmax (spectral absorbance peak) according to recent reviews (Yokoyama, 2000; Bowmaker, 2008).](image-url)
Another way of visual adaptation based on the four ancestral cone classes is the loss of one or more cone classes, or the gain of new opsin genes by further gene duplication. There are many examples of visual pigment loss during species evolution. Mammals lost SWS2 and RH2 pigments, while retaining three functional opsin genes: SWS1, M/LWS, and RH1 (Yokoyama and Yokoyama 1996; Ebrey and Koutalos, 2001). The SWS2 and RH2 pigments were lost some time before the divergence of modern mammals (Levenson and Dizon, 2003). In addition, modern cetaceans and several amphibious mammals lack functional SWS visual pigments (Fasick et al. 1998; Peichl & Moutairou 1998; Peichl et al. 2001). In these marine mammals the loss of SWS cones is adaptive for vision under water in photopic conditions (Peichl et al., 2001).

While some vertebrates have lost certain visual pigments, others have gained novel visual pigments through gene duplication. It has been suggested that genome wide duplication occurred at the base of teleost radiation (Amores et al., 1998; Meyer and Malaga-Trillo, 1999; Postlethwait et al., 2000). Further genome duplications have occurred in salmonids, goldfish, and carp (Larhammar and Risinger, 1994). Zebrafish (*Danio rerio*), for example, have two red (LWS-1 and LWS-2), four green (RH2-1, RH2-2, RH2-3, and RH2-4), and single blue (SWS2) and ultraviolet (SWS1) opsin genes in the genome (Chinen et al., 2003). Mutations in the duplicated copy can lead to divergent evolution resulting in two or more spectrally distinct pigments within single opsin classes, exemplified by the two short-wave SWS1 and SWS2 opsin classes (Bowmakers, 2008). In snakes, three types of opsins are sequenced from two basal snakes, SWS1, LWS, and Rh1 (Davies et al., 2009). It is unclear whether other snakes, especially derived species, have gained or lost any opsin beyond the three.

Visual pigments have varying absorbance range and kinetics, but with respect to downstream signaling of phototransduction cascade, opsins are biochemically equivalent (Kefalov, 2003). Besides opsins, rods and cones express different sets of proteins for the subsequent phototransduction cascade, such as rod-specific and cone-specific transducin (Strathmann, et al., 1990). Identifying rod- and cone-specific downstream proteins has been used previously to characterize photoreceptors of Tokay geckos (Zhang et al., 2006). Even though the gecko photoreceptors are rods based on morphology and light response, only cone-specific versions of photo-transduction proteins were found, including transducin (Zhang et al., 2006). Transducin is a heterotrimeric G protein with α, β and γ subunits. Most interest in G proteins has been focused on the α-subunit
(Gαt), since it binds and hydrolyzes GTP into GDP. Antibodies that label rod- and cone-specific Gtα are commercially available.

K-20 antibody, for example, is an affinity-purified rabbit polyclonal antibody raised against rod α-transducin. K-20 antibody has been used as a rod marker in mice (Kerov et al., 2007), hamsters (Boughter et. al., 1997), and pigeons (Wada et. al., 2000). In two species of diurnal rodents (Arvicanthis ansorgei and Lemniscomys barbarus), only the cell body of rods but not cones is labeled with K-20 (Bobu, 2008). K-20 antibody also distinguished the rod-like photoreceptor from the cone-like cells in the primitive retina of lamprey (Muradov et al., 2008). In addition, K-20 antibody is used as rod-specific transducin marker for western blots of retinal extract on model organisms such as chicken and mice (Cai et al., 2001; Bell et al., 2000; Kasahara et al., 2001; Sokolov et al., 2004). K-20 antibody has not been used in snake species before this study, and there is no molecular data on phototransduction cascade of T. proximus.
1.3 Garter Snakes

Garter snakes (*Thamnophis*) are a genus of snakes from the family Colubridae. They are perhaps the most common and widespread snakes in North America (Rossman et al., 1966). Garter snakes are found in every state in the mainland United States, in Canada from the Maritime Provinces west to British Columbia, and through much of Mexico and Central America. They are often chosen as study subjects by biologists and ecologists, because they are abundant, easy to capture, and usually harmless to human (Rossman et al., 1966).

Garter snakes display a wide range of scale variations, pattern polymorphism, and sexual dimorphism. Ruthven (1908) thought that attempts to classify *Thamnophis* “has long stood in the minds of herpetologists as a synonym for chaos.” At least 30 species of *Thamnophis* have been recognized and the number is most likely to increase in future taxonomic studies. The common garter snakes, *Thamnophis sirtalis*, were once considered to be the base stock from which the other taxa arose (Ruthven, 1908), but recent phylogenetic studies based on combined DNA sequences and allozyme data put *T. sirtalis* as sister species with western ribbon snakes, *T. proximus* (de Queiroz and Lawson, 1994).

*Thamnophis proximus* is a diurnal garter snake species known as the western ribbon snakes (Rossman et al., 1966). Western ribbon snakes mainly feed on amphibians and their larvae, but they are also hunters for small fish and reptiles (Tinkle, 1957; Clark, 1974). Foraging behavior has been described for *T. proximus*, for example, using rapid and repeated striking motion to probe for frogs hiding in vegetation (Wendelken, 1978). Feeding behavior of garter snakes have been described qualitatively (Ford 1995) and quantitatively (King and Turmo, 1997).

Vision is an important sense for garter snakes. Prey movement was shown to be important in the feeding behaviour of colubrid snakes (Herzog and Burghardt, 1974). A similar preference for visually detected movement was demonstrated in adult *T. sirtatis* (Burghardt and Denny, 1983). Experienced water snakes orient to and capture fish that contrast with their background, instead of those that are color-matched (Czaplicki & Porter, 1974). Several *Thamnophis* species were observed making air-to-water attacks on aquatic prey when the distance or angle of the prey precluded any tongue contact, and the snakes repeatedly made underwater attacks on fish well outside tongue-flicking range (Drummond, 1983). While chemical cues can facilitate the attack
in experienced or ingestively naive snakes, garter snakes rely on vision for both initial detection of and orientation to prey (Drummond, 1985; Teather, 1991).

Besides hunting, garter snakes also use vision for mate recognition and chase sequence during reproduction (Perry-Richardson et al., 1990). Female red-sided garter snakes (*Thamnophis sirtalis parietalis*) exhibit a complex array of behavioral patterns for reproduction (Whittier and Crew, 1986). Females may mate once, more than once, or not at all from spring to late summer before returning to winter hibernation. Large year-to-year variation in reproductive rates of female garter snakes suggest that other factors may play an important role in mating frequencies, such as nutrition and stored energy reserves (Whittier and Crew, 1986). The reproductive pressure for male *Thamnophis* species can be intense. In Manitoba, male *T. sirtalis* emerge from hibernation ready to engage in courtship with females as they emerge a few days later (Rossman et al., 1966). The severely biased operational sex ratio leads to the formation of mating balls, due to clusters of males courting a single female. Under intense sexual selection pressure, visual cues are used by garter snakes for recognizing and chasing mates (Perry-Richardson et al., 1990).
1.4 Evolution of Snake Visual System

While there are many behavioral studies on garter snakes, snake visual systems deserve special attention (Walls, 1942). As a comparative biologist, Walls (1942) reported that the eyes of snakes are drastically different from other reptilian species. Instead of mobile eye-lids, snakes have a modified layer of scale covering the cornea, known as spectacle. Corneas of lizards are bared and are connected to ciliary muscle and scleral ossicles, both of which are lacking in snakes. Snakes also have achieved an entirely different mechanism for focusing light on the retina, by moving the lens in- or outwards (Caprette et al., 2004). Photoreceptors of snakes also lacked paraboloid and myoid region found in other vertebrate species (Walls, 1942).

After examining the eyes of an extensive collection of snake species, Walls (1942) proposed that snake ancestors must have undergone a period of visual degeneration, followed by regeneration of the visual system. After primitive snakes regained functional duplex retinæ, their photoreceptors further “transmuted” to produce the enormous diversity of the ophidian retina (Walls, 1942). In higher snakes, the duplex retina with both rods and cones is also primitive while all subsequent modifications are based on a “viperine” pattern with four types of photoreceptors: large single cones, double cones, small single cones, and rods (Underwood, 1951; 1966). While duplicity is the ancestral state of snake visual system, it has been lost several times and never regained in some species (Underwood, 1966).

Transmutation of snake photoreceptors can be better understood by comparing the visual system of basal snakes (henophidians) to derived snakes (caeonophidians). Three species of henophidian snakes have been characterized for their visual system: ball python (Python regius), boa (Boa constrictor), and sunbeam snakes (Xenopeltis unicolor). A member of primitive Boidae family, ball python is partly nocturnal and found primarily in West and Central African grasslands (De Vosjoli, 1990). Another Boidae snake, boas are found in Central and South America, also known to have nocturnal lifestyle (McGinnis and Moore, 1969). Common in south Asian countries, sunbeam snakes are nocturnal species with semi-fossorial lifestyle, distinguished by the iridescent sheen of their scales (O’Shea, 2007).

Henophidian snakes studied so far have duplex retinæ with a large number of rods expressing RH1, and a small number of cones expressing LWS and SWS1 (Table 1). The retina of ball
python has been characterized using scanning electron-microscopy (SEM) and microspectrophotometry (MSP). *P. regius* have a duplex retina dominated by rods (90%) with long, narrow outer segments, and two types of single cones (Sillman et al., 1999). The rods of *P. regius* express a rhodopsin with $\lambda_{\text{max}}$ of 494nm. Their large single cones express LWS pigments with $\lambda_{\text{max}}$ of 551nm, and their rare small single cones express SWS1 pigments with $\lambda_{\text{max}}$ of 360nm. The retina of *Boa constrictor imperator*, another henophidian snake, was also examined using SEM and MSP (Sillman et al., 2001). The retina of boa is highly dominated by rods (89%) over cones (11%). The rods express RH1 with $\lambda_{\text{max}}$ of 495 ± 2 nm. Boas have two types of single cones distinguishable by size. The more common large single cones expressed LWS with $\lambda_{\text{max}}$ of 549 ± 1 nm, and the rare small single cones express SWS1 with $\lambda_{\text{max}}$ of 357 ± 2 nm (Sillman et al., 2001). Rods also dominate the retina of sunbeam snakes (*X. unicolor*), expressing RH1 pigments with $\lambda_{\text{max}}$ around 499nm, while a smaller number of cones expressing LWS with $\lambda_{\text{max}}$ between 558 and 562nm (Davies, et al., 2009). The SWS1 gene is expressed in the ocular cDNA of *X. unicolor*, but this cone type was so rare that MSP was unable to pick up the short-wavelength signal (Davies, et al., 2009).

Although some henophidian snakes have rod-dominant retinas, caenophidian snakes such as diurnal garter snakes have been found to have an all-cone retina. For example, the common garter snakes (*Thamnophis sirtalis*) have four major morphological types of cones: double cones, large single cones, small single cones, and very small single cones (Wong, 1989; Sillman et al., 1997). Using SEM, the photoreceptor population was determined to be 45% double cones, 40% large single cones, and 15% small single cones (Sillman et al., 1997). The small single cones were further divided into two subtypes. Using MSP, it was found that the large single cones and double cones have $\lambda_{\text{max}}$ of 554nm, and the small single cones have $\lambda_{\text{max}}$ of either 482nm or 360nm (Sillman et al., 1997). In the same study, Sillman and colleagues (1997) found that some small single cones are labeled with at least two rhodopsin-specific antibodies (AO and B6). Despite this evidence, it was concluded that *T. sirtalis* retinas have only cones (Wong, 1989; Sillman et al., 1997). This conclusion is confirmed by Electroretinogram (ERG) on garter snakes, as the retinal gross potential peaked from 550nm to 570nm under varying experimental settings (Jacobs et al, 1992).
Henophidian snakes have rod-dominant retina because nocturnal vision is important for their survival. Diurnal caenophidian snakes such as common garter snakes have only cones, because they are mostly active during the day. Visual systems of snakes are adapted to their particular visual ecology and life-style. Vertebrates in general adapt their visual systems to their environments, one of many ways they can achieve this is through photoreceptor transmutation. Photoreceptor transmutation in snakes can be better understood by drawing a comparison between the visual systems of basal snakes and derived snakes. Photoreceptor morphology and visual pigments have been characterized in basal snakes (Sillman et al., 1999; Davies et al., 2009), yet no opsin data from derived species such as garter snakes has been published. Only one garter snake species, *T. sirtalis*, has been studied for photoreceptor physiology and morphology so far (Jacob et al., 1992; Wong, 1989; Sillman et al., 1997). To understand the evolution of snake visual systems, the retina of garter snakes and other caenophidian snakes should be further characterized.

Table 1. Summary of published data from recent papers on visual system of snakes.

<table>
<thead>
<tr>
<th>Henophidian Snakes</th>
<th></th>
<th>Photoreceptors</th>
<th>Opsins</th>
<th>λ max</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Python regius</em></td>
<td>ball python</td>
<td>rods (90%)</td>
<td>Rh1</td>
<td>494nm</td>
<td>Sillman et al., 1999</td>
</tr>
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<td></td>
<td></td>
<td>large single cones</td>
<td>LWS</td>
<td>551nm</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>small single cones</td>
<td>SWS1</td>
<td>360nm</td>
<td></td>
</tr>
<tr>
<td><em>Boa constrictor imperator</em></td>
<td>boa</td>
<td>rods (89%)</td>
<td>?</td>
<td>495nm</td>
<td>Sillman et al., 2001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>large single cones</td>
<td>?</td>
<td>549nm</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>small single cones</td>
<td>?</td>
<td>357nm</td>
<td></td>
</tr>
<tr>
<td><em>Xenopeltis sunicolor</em></td>
<td>sunbeam snake</td>
<td>rods (predominant)</td>
<td>Rh1</td>
<td>497nm</td>
<td>Davies et al., 2009</td>
</tr>
<tr>
<td></td>
<td></td>
<td>cones</td>
<td>LWS</td>
<td>550nm</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>very rare cones?</td>
<td>SWS1</td>
<td>361nm</td>
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<table>
<thead>
<tr>
<th>Caenophidian Snakes</th>
<th></th>
<th>Photoreceptors</th>
<th>Opsins</th>
<th>λ max</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Thamnophis sirtalis</em></td>
<td>common garter snake</td>
<td>double cones (45%)</td>
<td>?</td>
<td>554nm</td>
<td>Wong, 1989; Sillman et al., 1997</td>
</tr>
<tr>
<td></td>
<td></td>
<td>large single cones (40%)</td>
<td>?</td>
<td>554nm</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>small single cones (15%)</td>
<td>?</td>
<td>482nm</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>very small single cones</td>
<td>?</td>
<td>360nm</td>
<td></td>
</tr>
</tbody>
</table>
1.5 Visual System of *Thamnophis proximus*

The Chang lab and its collaborators have been studying the visual system of a species of garter snakes, *Thamnophis proximus*, commonly known as the western ribbon snake. From the retinal cDNA library of *T. proximus*, postdoctoral fellow Johannes Muller sequenced the full-length SWS1 opsin gene, undergraduate student Mengshu Xu sequenced the full-length LWS opsin gene, and undergraduate student Natalie Chan sequenced the full-length RH1 opsin gene. Phylogenetic analysis was carried out by fellow graduate student Jingjing Du based on reptile sequence alignments generated by me. Microspectrophotometry (MSP) was carried out on the *T. proximus* by Dr. Ellis Loew from Cornell University.

Microspectrophotometry (MSP) is a technique for measuring the visual pigment absorbance from individual photoreceptors. A MSP machine works by generating a precise beam of light of known wavelength, passing it through the outer segments of individual photoreceptors mounted on a microscope slide, and measuring the absorbance due to visual pigments using a detector on the output end. Since each visual pigment family has its own characteristic absorbance range, repeatedly measuring the same type of photoreceptors gave us a good idea about what visual pigments are expressed in a particular type of photoreceptors.

Previous MSP results published on *T. sirtalis* were experimentally carried out by Dr. Ellis Loew at Cornell University (Sillman et al., 1997). Six *T. proximus* were sent him, and Dr. Ellis Loew performed MSP on their photoreceptors. At least three types of visual pigments were identified by MSP from *T. proximus* photoreceptors (Figure 4). Based on MSP, *T. proximus* have the same three types of visual pigments as *T. sirtalis* (Table 1). Based on measurements from two large single cones, five principal double cones, and eight accessory double cones of *T. proximus*, one type of visual pigments have $\lambda_{max}$ around 544 ± 1.2 nm, within range of a LWS type pigment. Small single cones can be distinguished into two subtypes based on MSP. Out of twelve small single cones, four have $\lambda_{max}$ around 365 ± 1.0 nm, most likely due to a SWS1 type pigment. The other eight small single cones absorb around 483 ± 1.5 nm, which is within the range of RH2 pigments or blue-shifted RH1 pigments. Finally, the very small single cones do not produce any absorption according to MSP.
Figure 4. Summary of MSP measurements of *T. proximus* photoreceptors, showing $\lambda_{\text{max}}$ (nm) that met the selection criteria in terms of noise and bandwidth, and sample absorbance from each type of pigments (Ellis Loew, email communication). Median, standard deviation (S.D.) and sample size are also shown. Experimental methods of MSP have been described previously (Loew, 1994).
Based on opsin sequences obtained by Johannes Muller, Mengshu Xu, and Natalie Chan from the retinal cDNA library of *T. proximus*, a phylogenetic analysis was carried out by lab member Jingjing Du (unpublished), to determine the phylogenetic relationship between *T. proximus* opsins to other reptilian opsins (Figure 5). Opsin sequences from sunbeam snake, python, iguana, gecko, true lizard, anole, and alligator species were included in the phylogenetic analysis. Beside garter snake opsin genes sequenced by the Chang lab, other sequences used for generating the tree are from NCBI database: (1) RH1 opsins of sunbeam snake (*Xenopeltis unicolor*), FJ497233; python (*Python regius*) FJ497236; iguana, (*Uta stansburiana*), DQ100323; alligator (*Alligator mississippiensis*), U23802; (2) RH2 opsins of lacerta (*Podarcis sicula*), AY941829; iguana (*Uta stansburiana*), DQ100324; gecko (*Gekko gecko*), M92035; (3) SWS2 opsins of: iguana (*Uta stansburiana*), DQ100326; anole (*Anolis carolinensis*); (4) SWS1 opsins of sunbeam snake (*Xenopeltis unicolor*), FJ497234; python (*Python regius*), FJ497237; iguana (*Uta stansburiana*), DQ100325; gecko (*Phelsuma madagascariensis*), AF074045; (5) LWS opsins of python (*Python regius*), FJ497238; sunbeam snake (*Xenopeltis unicolor*), FJ497235; and gecko (*Gekko gecko*), M92036.

Vertebrate visual opsins can be classified into five families. The three opsin genes from *T. proximus* clustered within SWS1, LWS, and RH1 families. For SWS1 and LWS opsin families, the garter snake genes always cluster with opsins of python and sunbeam snake. While the general phylogenetic relationship between snakes and reptiles are well established, the phylogenetic relationship within snake species is debatable, depending on whether molecular (Figure 6A) or osteological data (Figure 6B) is used to resolve phylogeny (Lee and Scanlon, 2002; Vidal and Hedges, 2004).

For the LWS and SWS1 families, the opsin phylogeny agrees with molecular tree (Figure 6A) from Vidal and Hedges (2004). For the RH1 opsin family, the garter snake forms a monophyletic group with iguana (*Uta stansbriana*), instead of grouping with python and sunbeam snakes. This phylogenetic position is not supported by the molecular tree or the osteological tree.
Figure 5. Tree topologies reconstructed by Chang lab graduate student, Jingjing Du. Amino acid sequences of opsin genes were analyzed with Bayesian method in MrBayes 3.12. MrBayes is a free software program which performs Bayesian inference of phylogeny. Blosum model was chosen as the substitution model from a distribution of mixed amino acid models in MrBayes 3.12. The tree is drawn proportional to the branch length. Numbers associated with major nodes indicate Bayesian posterior probabilities. *T. proximus* sequences are boxed.
Figure 6. Phylogenetic relationship of reptilian species used in our analysis. Tree A is supported by molecular data (Vidal and Hedges, 2004), while Tree B is supported by osteology and soft anatomy (Lee and Scanlon, 2002).
Since *T. proximus* photoreceptors are expected to have cone-like morphology like those of *T. sirtalis*, the expression of RH1 in their retina is unusual. To find out whether *T. proximus* RH1 have interesting amino acid substitutions, its amino acid sequence was aligned with published RH1 sequences from sunbeam snake, python, iguana, and bovine (Figure 7).

Several key amino acid residues are conserved in *T. proximus* rhodopsin. The site for chromophore attachment and its counterion, K296 and E113, are conserved in *T. proximus* RH1. Palmitoylation sites conserved in rhodopsins but not in cone opsins, C322 and C323, are found in *T. proximus* RH1. The disulfide bond between C110 and C187 is likely conserved in *T. proximus* RH1. Another characteristic residue, E122, is conserved in *T. proximus* RH1, which is typical of RH1 and RH2 classes, but not found in other cone opsins (Imai et al., 1997).

The RH1 of *T. proximus* also have amino acid substitutions that are typically associated with cone opsins. RH1 class opsins exhibit a kink around the double glycine residues, G89 and G90, which brings G90 closer to E113, and gives rhodopsin a tighter retinal binding pocket (Nickle and Robinson, 2007). In RH2 and SWS2 classes, only G90 is conserved, while in SWS1 and M/LWS classes, neither glycine residue is conserved. These two residues are V89 and G90 in *T. proximus* RH1, suggesting a looser conformation of the retinal-binding pocket compared to bovine rhodopsin, perhaps more comparable to RH2 and SWS2 opsins.

S185 is a unique amino acid substitution for *T. proximus* RH1. With its close proximity to the disulfide bond, C185 is conserved in most vertebrate species whose RH1 sequence is available. Cysteine is a hydrophobic amino acid with a nonpolar thiol side chain, while serine is classified as a polar amino acid due to its hydroxyl group. C185S substitution is rarely found in vertebrate RH1, and its functional implications are unknown.

At least two amino acid residues from *T. proximus* RH1 have known spectral tunings properties: N83 and S292. Besides RH1 of *T. proximus*, N83 is found in python, sunbeam snakes, side blotched lizards, while D83 is found in bovine. N83 is also found in diving mammals such as bottle-nosed dolphin (Fasick and Robinson, 1998), deep-sea fish (Hunt, 2001), and deep-water lake cichlids (Sugawara et. al., 2009), which lead to the suggestion that it is an adaptation to monochromatic deep-water environment. However, N83 can not be explained exclusively by
deep-water adaptation, since it is also found in terrestrial vertebrates like elephants (Yokoyama et. al., 2005) and now in garter snakes.

Residue 292 is another known spectral tuning site for rhodopsin. It is conserved as A292 in python, sunbeam snake, lizards and bovine rhodopsin genes. However, S292 is found in T. proximus, dolphins (Fasick and Robinson, 1998), and deep-water lake cichlids (Sugawara et. al., 2005). A292S evolved several times independently in deep-water cichlid species, suggesting parallel evolution at this particular site (Sugawara et. al., 2005). The particular combination of N83 and S292 has been found to blue-shift RH1 by at least 10nm in dolphin, making them important substitutions for adaptation to deep-water marine environment (Fasick and Robinson, 2000). The T. proximus RH1 could be blue-shifted, according to MSP peak at 483 ± 1.5 nm, due to the combined effect of N83 and S292.

Figure 7. Amino acid sequence alignment of RH1 expressed in T. proximus, sunbeam (X. unicolor), python (P. regius), iguana (U. stansbriana), and cow (B. tauras), translated from nucleotide sequences. The transmembrane domains are denoted by the black boxes below the alignment. The three highlighted amino acid residues are N83, S185, and S292. A dot represents the same amino acid as T. proximus RH1, while a line represent a gap.
1.6 Objectives of Present Study

Based on published data on snake visual system and previous research in the Chang lab prior to my arrival, *T. proximus* may express RH1 pigments in cone-like photoreceptors. To better characterize the *T. proximus* photoreceptors and look for evidence of transmutation, three hypotheses were tested in the present study.

**Hypothesis 1: Based on photoreceptor morphology, *T. proximus* have four types of cones and no rods.**

*T. proximus* is a sister species to the common garter snakes *T. sirtalis*, therefore I expect them to have comparable retinal morphology. Since *T. sirtalis* is known to have all-cone retina (Sillman et al., 1997), I expect to find the same types of photoreceptors in *T. proximus*. To establish photoreceptor morphology, *T. proximus* retinae can be examined with electron-microscopy. Scanning electron microscopy (SEM) can be used to determine surface morphology, while transmission electron microscopy (TEM) can be carried out to examine internal anatomy of the photoreceptors.

**Hypothesis 2: Rod-specific transducin can be found in some *T. proximus* photoreceptors.**

Research conducted by previous members of the Chang lab isolated both cone opsin genes (SWS1 and LWS), as well as rod opsin gene (RH1) from *T. proximus* retinal cDNA library. I expect to find more rod-specific downstream proteins, such as the transducin (Gαt). To determine whether rod-specific Gαt maybe expressed in the retina, K-20 antibody was used in the present study to probe the cryosections of *T. proximus* photoreceptors for rod-specific Gαt.

**Hypothesis 3: RH1 pigments are expressed in some small single cones.**

According to MSP results from Dr. Ellis Loew, some small single cones have λmax at 483 ± 1.5 nm. To determine if RH1 might be expressed in these small single cones, the λmax of rhodopsin pigments can be measured *in vitro* by protein expression using mammalian cell culture. *T. proximus* rhodopsin gene was cloned into an over-expression vector, expressed *in vitro*, regenerated with 11-cis retinal, and measured for spectral absorbance using a spectrophotometer.
2 Materials and Methods

2.1 Animal Subjects

This study was approved by the U of T animal Care Committee and conforms to the Guide to the Care and Use of Experimental Animals Vol. 2, as determined by the Canadian Council of Animal Care regarding relevant guidelines for the care of experimental animals. Adult *Thamnophis proximus* were bought from licensed reptile dealers in Ontario, namely Boreal Scientific and Ward’s Natural Science. The animals were ordered through the departmental animal facility and handled according to animal care protocol. The snakes were sacrificed by decapitation before enucleating the eyes. *T. proximus* can be distinguished based on a pair of yellow dots on their heads, which are absent from *T. sirtalis*, the common garter snakes.

2.2 Electron Microscopy

Electron microscopy was carried out in the Cell and Systems Biology Imaging Facility. In total, ten retinas from five *T. proximus* were processed for EM: four retinas for SEM, two retinas for TEM, and four retinas for SEM again in a repeated trial. After hemisection of the *T. proximus* eye, the eyecup was placed in 3% glutaraldehyde (SPI Supplies) 0.1 M phosphate buffer, pH 7.8, where the retina was separated from its pigmented epithelium. After separation, the retina was kept in fixative at room temperature overnight. The tissue was then washed in 0.1 M phosphate buffer to remove primary fixative. The retina was incubated in secondary fixative, 1.0% osmium tetroxide in 0.1 M phosphate buffer, for one hour at room temperature. The tissue was dehydrated by immersion in increasing concentrations of ethanol (two 5 min immersion in each of 30, 50, 70, 80, 90% ethanol). From this point on the preparations for SEM and TEM become different.

Tissues for SEM were soaked four times in 100% ethanol for 3 minutes each. They were then infiltrated with Hexamethyldisilizane (HMDS) series for 30 minutes at 3:1, then 1:1, then 1:3 ethanol to HMDS ratio respectively. To displace as much ethanol in tissue sample with HMDS, the final infiltration was three 30 min immersion in 100% HMDS. The HMDS in tissue was allowed to volatilize overnight. The “dried” retina was taped onto a specimen holder, with photoreceptors facing outwards, then Sputter coated (50sec) with gold-palladium using the Bal-
Tec SCD050. The sample was examined with the Hitachi S-2500 at 20 kV and acquired images using Quartz PCI.

Tissues for TEM were soaked four times in 100% ethanol for 10 minutes each. The sample is then infiltrated with modified Spurr’s epoxy resin (16.4g ERL4221, 23.6g NSA, 5.72g DER736, and 0.4g DMAE) for 30 min. The sample was then embedded in flat molds, and all blocks polymerized overnight in 65°C oven. Semithin sections (0.5-1 micron) were cut from the blocks and stained with Toluidine blue (Fisher BioReagents, BP107-10) and methylene blue (British Drug House Ltd., England, #201383) mixture for 1 minute. Ultrathin sections (60 – 90 nm) were also cut from the blocks and picked up using high transmission grids. Grids were stained with 3% uranyl acetate in 50% methanol for 45 min and post-stained with Reynold’s lead citrate for 15 min. These ultra-thin sections were examined with the Hitachi H-7000 at 75 kV and images were acquired using AMT 11 megapixel digital camera.

Overall, more than one hundred SEM images were taken from six pieces of retinal tissues. These tissues are well preserved, whereas other pieces of *T. proximus* retinal tissues were not used because they became damaged during SEM preparation. One piece of retinal tissue with particularly well-preserved cellular integrity was used for calculating photoreceptor ratios across nine zones. In total, more than one hundred and fifty TEM images were taken from over twenty ultra-thin sections, made from two retinæ from two different *T. proximus* individuals.

### 2.3 Immunohistochemistry

Four retinæ from two *T. proximus* were processed for immunohistochemistry. After enucleation of eyes from *T. proximus*, the eyecups were placed in 1% glutaraldehyde prepared in 0.1 mol·l\(^{-1}\) phosphate buffer, where they remained for 1h. The eyes were then washed thoroughly, first in 0.1 mol·l\(^{-1}\) phosphate buffer and then in 0.1 mol·l\(^{-1}\) TRIS-HCl buffer. Two of the eyes were processed for retinal whole-mount, by surgically separating the retinæ from eye cups and removing the pigment epithelium. Each retinal tissue as a whole was then placed on a glass microscope slide, under a cover slide that was slightly elevated to avoid crushing the tissue. For the cryosections, two *T. proximus* eyes were dehydrated with ethanol and embedded in resin. A Leica CM3050 cryostat was used to cut serial semithin (0.2µm) tangential sections from the central part of each retina. The sections were treated with K-20 antibody (Santa-Cruz) diluted 1:1000 for 30 min. K-20 is an affinity purified rabbit polyclonal antibody raised against a peptide
mapping within a highly divergent domain of \( G_{\alpha 1} \). K-20 was used on retinal sections from pigeons (Wada et. al., 2000) and rodents (Boughter et. al., 1997) to selectively label rod photoreceptors. The secondary antibody used for both whole-mount and cryosection was Alexa Fluor 488 diluted 1:1000, a goat anti-rabbit IgG antibody. The sections were placed in 1% solution of bovine serum albumin in phosphate-buffered saline to block non-specific binding site. The sections were exposed to the primary antibody K-20 for 2 h, then Alexa Fluor 488 for 1 h. The sections were mounted with Prolong Gold and photographed in a Zeiss Axioscope. The whole mount was mounted with Prolong Gold as well. Due to the scarcity of \( T. \) proximus retinal tissue samples and the technical challenges of removing the RPE from smaller snakes, no negative control was available for the present immunohistochemistry study.

2.4 Cloning into p1D4

Working with \( T. \) proximus SWS1, LWS, or RH1 opsin genes in cloning vector pJET, primers were designed to add EcoRI restriction site to the 5’ and BamHI site to the 3’ end of the opsin genes. The amplified bands were ligated into the pJet blunt end cloning vector. The genes were isolated from the pJet vector with EcoRI and BamHI restriction endonucleases, and ligated into the p1D4 vector treated with the same restriction enzymes. The p1D4 vector consists of the monoclonal antibody 1D4 epitope, which were the last 9 amino acids from bovine rhodopsin, fused to the pIRES vector at the multiple cloning site. The expression construct containing the desired opsin inserts were sequenced to rule out mutations due to polymerase errors.

Mutants of \( T. \) proximus RH1 were generated by using QuickChange site-directed mutagenesis kit (Stratagene). To make a point mutation on a gene, a pair of mutagenesis primers was designed to anneal to the desired region of the gene, with one mismatch between the primer sequences and the gene sequences, hence the mutation. All three point-mutants were generated by altering one nucleotide in the annealing regions through PCR. The PCR product was treated with Dpn I endonuclease, which specifically digest the methylated and hemimethylated DNA, therefore destroying the parental DNA template and selecting for mutation-containing synthesized DNA. All DNA products that were subjected to mutagenesis were sequenced to rule out spurious mutation.
2.5 *in vitro* Expression and Purification

The expression constructs containing *T. proximus* RH1 genes in p1D4 vector were transfected into mammalian cells. This experiment was repeated ten times in total. The expressions were carried out according to previously described methods (Han et al. 1996), essentially by transient transfection into HEK293T cells using Lipofectamine 2000 (Life Technologies), harvested after 47 h, regenerated in 5 mM 11-cis retinal, solubilized in 1% n-dodecyl-β-D-maltoside detergent, and immunoaffinity purified using batch methods with the 1D4 monoclonal antibody. Bovine RH1 opsin was expressed using the same methods each time as a positive control. For each *in vitro* protein sample, absorbance spectroscopy was performed at 25°C using a Varian Cary4000 spectrophotometer, using quartz cuvettes with a 1-cm pathlength. The solubilization buffer was first measured as the blank. For each regenerated opsin sample, dark spectrum was recorded first, and then light spectrum was recorded after bleaching the sample with full-spectrum white light for 30 seconds. The difference spectrum can be produced by subtracting the light spectrum from the dark spectrum. To determine λ_max, the difference spectrum was then fitted to a standard Govardovskii rhodopsin A1 template (Govardovskii, 2000).
3 Results

3.1 Scanning Electron Microscopy

All the data presented in the results section, including electron microscopy, immunohistochemistry, \textit{in vitro} protein expression were generated by me.

To characterize the photoreceptors based on morphology, retina of \textit{T. proximus} was examined with scanning electron microscopy (SEM). The gross morphology of the \textit{T. proximus} retina shows both similarities to basal snakes and adaptations to diurnal lifestyle. The basic stratification pattern of retinal cells (Figure 8A) appears to be the same as Boidae snakes (Walls, 1942). Underneath the pigment epithelium layer are the outer segments of photoreceptors. Below the outer segments are the ellipsoid regions of photoreceptors, which are darkly stained with toluidine blue due to high mitochondrial content (Figure 8B). The nucleus and synaptic terminals of the photoreceptors form the outer nuclear layer. In a duplex retina, the nucleus of rods and cones tend to occupy different zones of the outer nuclear layer, such as found in Boidae snakes (Walls, 1942). The outer nuclear layer of \textit{T. proximus} retina is made up of a relatively thin layer of cone nucleus, compared to the inner nuclear layer. A nocturnal animal will tend to have a retina with a thicker outer nuclear layer to accommodate a high number of rod nuclei (Wong, 1989).

Based on cellular morphology, vertebrate photoreceptors can be cone-like, which are shorter and stouter cells with tapering outer segments, or rod-like, which are taller and slender cells with enlarged outer segments. Under SEM, all photoreceptors found in \textit{T. proximus} retina are cone-like (Figure 9), resembling those from its sister species, \textit{T. sirtalis} (Wong, 1989; Sillman et al., 1997). None of the photoreceptor of \textit{T. proximus} resembles the rods found in henophidian snakes (Sillman et al., 1999; 2001). Some photoreceptors of \textit{T. proximus} appear to be arranged in a pattern of concentric rings (Figure 9).

One type of double cone and three types of single cones can be distinguished under SEM (Figure 10). A \textit{T. proximus} double cone is composed of a large principal member and a small accessory member. The two members are joined together by their outer segments, but each member has a separate stack of outer segment membranes. The \textit{T. proximus} single cones can be classified,
based on their relative size, as large single cones, small single cones, or very small single cones. The large single cones of *T. proximus* are comparable in size to the principal members of its double cones.

It becomes apparent from SEM, that the retina of *T. proximus* has an abundance of double cones and large single cones. More scarce are the small single cones, and the very small single cones are rarely found. To assess the relative abundance of each type of photoreceptors, nine zones across the surface of a *T. proximus* retina were surveyed under SEM (Figure 11). Large single cones and double cones were very common, making up about 45% and 44% of the total photoreceptor population respectively. The small single cones and very small single cones make up about 9% and 2% of total photoreceptor population. The overall relative abundance of each type of photoreceptors is comparable between *T. proximus* and *T. sirtalis* (Sillman et al., 1997; Table 1).
Figure 8. A cross-sectional view of *T. proximus* retina, several layers of retinal tissue can be distinguished under SEM (A) and light microscopy (B). From the posterior end of the eye, the layers are R.P.E. - retinal pigment epithelium; P.C. – photoreceptor cells; O.N.L - outer nuclear layer; I.N.L - inner nuclear layer; G.C - ganglion cells. Scale bar = 5um. The outer nuclear layer of *T. proximus* retina is relatively thin compared to the inner nuclear layer.
Figure 9. Scanning electron micrographs of the retina of *T. proximus*. All photoreceptors are cone-like based on morphology. The white dotted line marks the concentric-ring pattern of *T. proximus* photoreceptors. Calibration bar: 5 µm.
Figure 10. Scanning electron micrographs of the retina of *T. proximus*. There are four types of cones based on morphology: *p*- principal double cone; *a*- accessory double cone; *ls*- large single cone; *ss*- small single cone; *v*- very small single cone. Calibration bar: 5 µm.
Figure 11. Nine zones were surveyed from a *T. proximus* retina under SEM (A). The four types of photoreceptors were visually scored from SEM from each zone, which were then converted into percentages (B). An average percentage for each type of photoreceptor was calculated by taking the means from all nice zones (C). The overall ratio of different photoreceptors types were calculated to be 44% double cones, 45% large single cones, 9% small single cones, and 2% very small single cones.
3.2 Transmission Electron Microscopy

To look for distinguishable cellular features of rods and cones, ultra-thin sections of *T. proximus* retina were examined with transmission electron microscopy (TEM). The ellipsoids of snake photoreceptors are usually darkly stained due to high mitochondrial content (Walls, 1942; Wong, 1989). Two types of ellipsoids can be found in *T. proximus* cones. TEM shows that the principal members of double cones (Figure 12B) and large single cones (Figure 13C) have similar ellipsoids, but small single cones (Figure 13B) and accessory member of double cones (Figure 12B) have another type of ellipsoids.

This difference is partly due to clusters of brightly stained micro-droplets in the ellipsoids, Micro-droplets were found in the mitochondrial cristae of *T. sirtalis* photoreceptors (Wong, 1989). Upon closer examination, micro-droplets are differentially expressed in *T. proximus* photoreceptors. Micro-droplets are found in the principal member of double cones (Figure 12B) and in large single cones (Figure 13C). The accessory member of double cones (Figure 12B) and some small single cones (Figure 13B) do not have micro-droplets in their outer segments. Instead, electron-dense pigments are found in the small single cones and accessory double cones. These dark pigments are even smaller in size than micro-droplets, and they can be occasionally found scattered amongst micro-droplets (Figure 13C).

The micro-droplets have only been found in snake species, and they are thought to function collectively to replace oil-droplets, which were lost in snakes but found in lizard and bird cones (Walls, 1942; Wong, 1989). When cone lattice were directly observed through the pupil of live garter snakes, dark holes in the cone lattice were also observed (Land and Snyder, 1985; Wong, 1989). These holes could contain structures slightly smaller than the visible cones themselves (Land and Snyder, 1985). It is possible that these dark holes are the small single cones. They may appear dark due to the accumulation of dark pigments in their inner segment. Since TEM can only be photographed in black-and-white, the color of the droplets remains unknown.

The outer segment of a photoreceptor is the membranous site specialized for photon detection (Kawamura and Tachibanaki, 2008). Rods and cones are known to differ in their outer segment membranes (Cohen 1972). Rods have outer segment membranes that are discontinuous from plasma membranes, while cones have outer segment membranes that are open to extracellular media. Under TEM, some small single cones of *T. proximus* were found to have rod-like outer-
segment membranes, which are disk-like and discontinuous from the plasma membrane (Figure 14B).

The very small single cones were found to lack any outer-segment membrane (Figure 14A). For a functional visual pigment to participate in phototransduction cascade, it must be properly packed in the outer segment membranes. This is perhaps why MSP can not detect any spectral absorbance from these very small single cones.
Figure 12. Transmission electron microscopy of a tangential section of *T. proximus* retina, showing (A) the outer segment of principal (pc) and accessory (ac) member of a double cone; (B) close-up of the boxed area in (A), micro-droplets are found in principal member but not accessory member. Scale bars = 0.5 µm. Dark arrows: electron-dense pigments; white arrows: micro-droplets.
Figure 13. Transmission electron microscopy of a tangential section of *T. proximus* retina, showing (A) the outer segment of a small single (ss) cone and a large single (ls) cone; (B) electron-dense pigments in the small single cone; (C) micro-droplets and electron-dense pigments in the large single cone. Scale bars = 1 µm. Dark arrows: electron-dense pigments; white arrows: micro-droplets.
Figure 14. Transmission electron microscopy of a tangential section of *T. proximus* retina, showing (A) the outer segments of a very small single (vs) cone and a small single (ss) cone; (B) outer segment membranes of the small single cone from (A). Note the absence of micro-droplets from both cones, and the lack of outer segment membranes from the very small single cone. The outer segment membranes of this small single cone are mostly discontinuous from the plasma membrane. Scale bar = 1 µm.
3.3 Immunohistochemistry

Besides visual pigments, rods and cones also differ in expression of downstream phototransduction proteins, such as transducin. Since RH1 may be expressed in the retina of *T. proximus*, it is interesting to find out whether rod-specific transducin is also expressed. K-20 targets rod-specific transducin alpha subunit, and selectively stained only rods in rodents (Boughter et. al., 1997), pigeons (Wada et. al., 2000), and lampreys (*Petromyzon marinus*) (Muradov et. al., 2008). In two species of diurnal rodents (*Arvicanthis ansorgei* and *Lemniscomys barbarus*), only the cell body of rods but not cones were labeled with K-20 (Bobu, 2008).

The retina of *T. proximus* was isolated and processed for retinal whole-mount and cryosection, before incubating with K-20 antibody. The retinal whole-mount shows the photoreceptor layer from a top-down view (like the SEM), while the cryosection shows the cross-section of the retina (like the TEM). In both methods of preparation, a subpopulation of photoreceptors was stained by the antibody K-20 (Figure 15). Positive staining with K-20 antibody suggests that some *T. proximus* cones are expressing rod-specific transducin, an important upstream component of the photo-transduction cascade. It is unclear at this point whether these positively stained cones are small single cones. A negative control was not carried out due to sample limitations. This preliminary result warrants further investigation into the downstream photo-transduction cascades in *T. proximus*. 
Figure 15. Fluorescence micrographs of retinal wholemount (A) and cryosection (B) of *T. proximus*. Photoreceptors appear green in retinal wholemount to indicate rod-specific transducin antibody (K-20) reactivity. The cryosection is shown with a light-microscopy image as reference for retinal cell layers, and the arrows indicate positively stained photoreceptors. Calibration bar: 30 µm.
3.4 RH1 in vitro Expression

Based on MSP data and opsin gene sequences from *T. proximus*, the RH1 may be responsible for the 483 ± 1.5 nm MSP peak. To test whether *T. proximus* RH1 absorbs at 483 ± 1.5 nm, wild-type *T. proximus* RH1 gene was expressed in vitro using previously described methods (Chang et al., 2002). The full length opsin cDNA sequence was cloned into over-expression vector p1D4, which is a vector constructed by fellow graduate student James Morrow. In vitro expressed *T. proximus* RH1 appears to be a functional pigment with blue-shifted λmax compared to bovine RH1 control.

Bovine rhodopsin has been established as a model system for opsin. Bovine RH1 in p1D4 was used to transfect HEK293T cells as a positive control, because the transfection protocol was established and optimized for bovine RH1. After harvesting and regeneration with 11-cis retinal, the in vitro expressed bovine rhodopsin pigments were measured for spectral absorbance in the dark, and then after bleaching with light. Dark-state of bovine RH1 pigments absorb at 500nm, as measured by the dark spectrum (Figure 16A). After bleaching the sample with light, the light spectrum shows a decrease of the 500nm peak, and an increase of the 380nm peak (Figure 16A). The 380nm peak was also noticeable from the dark spectrum. This is most likely due to light leak during sample preparation, which prematurely activates the in vitro RH1 pigments. Between the dark and light spectra, the shift in absorbance is mostly due to the activation of dark-state RH1 by light, and the accumulation of Meta-II intermediates.

To reduce artifacts and noise in absorbance spectra, a difference spectrum of bovine RH1 can be produced by mathematically subtracting the light spectrum from the dark spectrum (Figure 16B). The resulting 500nm peak corresponds to the λmax of bovine RH1, and the dip in absorbance at 380nm is due to an accumulation of Meta-II intermediates after bleaching with light. The formation of Meta-II intermediates is an important feature of RH1 pigments, since these intermediates activate the G-protein, transducin. A peak around 500nm and a dip at 380 nm from the difference spectrum indicates that the in vitro bovine RH1 pigments absorb at 500nm and can form Meta-II intermediates once light activated.

Compared to bovine RH1, *T. proximus* RH1 was more challenging to express in vitro. Small scale transfections of *T. proximus* RH1 produced dark and light spectra without noticeable
absorbance peaks (Figure 17A). The difference spectrum of *T. proximus* RH1 produced a noisy peak that seems to be blue-shifted from 500nm, while the dip in absorbance at 380nm was also not as pronounced as bovine RH1 (Figure 17B).

On a western blot, the *in vitro* expressed opsins are detected with 1D4 monoclonal antibody (Figure 18). Both the bovine and *T. proximus* RH1 pigments are 34kDa according to their amino acid sequences. While bovine RH1 was visualized on western blot, *T. proximus* was only detectable after the *in vitro* sample was concentrated four times.
Figure 16. Dark and light absorbance spectra (top), and the difference spectra (bottom) of \textit{in vitro} expressed RH1 visual pigments of bovine, transfected with one plate of cells.
Figure 17. Dark and light absorbance spectrum (A), and the difference spectrum (B) of *in vitro* expressed RH1 visual pigments of *T. proximus*, transfected with six plates of cells.
Figure 18. Western blot of *in vitro* expressed RH1 pigments of bovine and *T. proximus* using antibody against 1D4 epitope. On the right lane, the *T. proximus* RH1 sample was four times as concentrated as the sample in the middle lane. Based on amino acid composition, RH1 proteins have molecular weight of approximately 34kDa.
4 Discussion

4.1 Morphology of Photoreceptors

All photoreceptors of *T. proximus* are short and stout cells with tapering outer-segments. Based on cellular morphology, four types of cones can be identified in *T. proximus* retinae: double cones, large single cones, small single cones, and very small single cones. Based on SEM from *T. proximus*, their photoreceptors resemble the cones identified in the sister species *T. sirtalis* (Wong, 1989; Sillman et al., 1997). TEM images show that some *T. proximus* small single cones have morphological features that distinguish them from other cone types.

*T. proximus* photoreceptors are similar in size compared to their counter-parts in *T. sirtalis*. In *T. proximus*, the inner segments of double cones and large single cones are about 9-11µm in length, and the inner segments of small single cones are about 4-6 µm in length. The very small single cones are difficult to identify and measure; due to the small size and rareness. In *T. sirtalis*, the inner segments are 7-10um for double and large single cones, 3-5 µm in length for small single cones (Wong, 1989). In both *T. sirtalis* and *T. proximus*, inner segments of small single cones are about half the size of large single cones. The inner segment lengths of *T. proximus* photoreceptors were estimated from both SEM and TEM images, since there are limitations to both techniques. SEM images are two-dimensional representations of three-dimensional surface structures, so structures in greater depth of view appear smaller in size. SEM images are also usually taken from an angle, which makes accurate measurement of photoreceptors even more difficult. TEM sections can show the vertical cross-section of individual photoreceptors, but only a few of the photoreceptors in section are through the central vertical plane of the photoreceptors. To get a more accurate measurement of photoreceptor size, more ultra-thin sections for TEM would be needed.

Based on SEM, the cone photoreceptors of *T. proximus* are morphologically distinct from the rod photoreceptors of henophidian snakes. Rods of boas are tightly packed, with elongated outer segments that can exceed 20 µm in length (Sillman et al., 2001). The tapering outer segments of *T. proximus* photoreceptors are 3-5 µm in length. Due to the conical shape of inner segments, the *T. proximus* photoreceptors appear to be more loosely packed than rods from boas. After hundreds of SEM images were taken from eight pieces of *T. proximus* retinae, not a single
photoreceptor was found to resemble rods of henophidian snakes. Therefore based on cellular surface morphology, *T. proximus* do not appear to have typical rod shaped photoreceptors.

Based on photoreceptors scored from nine zones of the retinal surface from a *T. proximus* retina (Figure 11), large single cones and double cones were very common, making up about 45% and 44% of the total photoreceptor population respectively. The small single cones and very small single cones make up about 9% and 2% of total photoreceptor population. In *T. sirtalis*, similar photoreceptor ratios were found (Sillman et al., 1997). Any minor differences in photoreceptor ratios between the two species could be due to individual variation, but it could also be due to ecological differences. The differences in photoreceptor ratios are more striking when the all-cone retina of *T. proximus* is compared with the rod-dominant retinæ of boas and pythons, where more than 90% of photoreceptors are rods (Sillman et al., 1999; 2001). The rod dominant retina of henophidian snakes could be an adaptation to nocturnal environment, while the all-cone retina of *T. proximus* may be an adaptation to diurnal environment.

Previous studies on garter snake photoreceptors reported irregular mosaic pattern (Wong, 1989; Sillman et al., 1997), as opposed to the regular square mosaic found in fish (Braekevelt, 1992). Although photoreceptors of *T. proximus* also appear be arranged in irregular mosaic pattern, some cones appear to form concentric-ring pattern under SEM (Figure 9). This type of photoreceptor mosaic has not been reported by previous studies on snakes. Although some photoreceptors of *T. proximus* appear to be in a concentric ring pattern, this observation needs to be repeated in other snakes, and the functional implications of this cone mosaic pattern require further investigations.

Even though all the *T. proximus* photoreceptors have cone-like surface morphology, some small single cones were found to have rod-like outer segment membranes under TEM (Figure 14B). Disk-like outer segment membranes are one of the hallmarks of rod photoreceptors, and they may be crucial to the rod’s function under dim light (Lamb, 2009). While some small single cones have rod-like outer segment membranes, the current TEM result can not conclude whether all small single cones have rod-like disks, or maybe some small single cones have cone-like invaginations. This is due to technical limitations of TEM. Ultra-thin sections of TEM are often not cut through the central plane of the photoreceptors; therefore a large single cone could appear to look like a small single cone in size. The principal member of a double cone can also look like
a large single cone under TEM, if the section did not capture the accessory member. However, when rod-like disks of outer segments are found using TEM, they are always associated with small single cones.

Besides their rod-like disks, these small single cones can also be distinguished from other cone types by their ellipsoids. Under TEM, two types of ellipsoids can be found in the photoreceptors of *T. proximus*. Principal double cones and large single cones have ellipsoids packed with microdroplets (Figure 12B; Figure 13C). Some small single cones (Figure 13B and 14A) and accessory double cones (Figure 12B) do not have microdroplets in their ellipsoids. Microdroplets have been previously found in *T. sirtalis* (Wong, 1989), although it was not clear whether they were ubiquitously expressed in all cones, or differentially expressed in certain types of cones. In the one hundred and fifty TEM images taken from over twenty ultra-thin sections of *T. proximus* retinae, the microdroplets are only found in large single cones and principal double cones.

There have been speculations on the function of microdroplets, one of which proposes that these microdroplets may function collectively as an oil droplet (Wong, 1989). Found in the ellipsoids of cones, single oil droplets are continuous spherical structures with diameters of 2-3 µm (Hart et al., 2000). Smaller in size, microdroplets of *T. proximus* photoreceptors are around 0.1-0.15 µm in diameter, and found scattered throughout the ellipsoids. Like microdroplets of *T. sirtalis* (Wong, 1989), microdroplets of *T. proximus* are spheroids because in every plane of section they remained circular in shape.

Instead of microdroplets, some small single cones have a large amount of electron-dense pigments in their ellipsoids (Figure 13B and 14A). These electron-dense pigments have not been previously reported in snakes, since they are much smaller in size compared to microdroplets. These electron-dense pigments appear to be tiny spheroids, and their small size makes accurate measurement of diameter a difficult task. These electron dense pigments are also found occasionally in other *T. proximus* cones (Figure 13C), but they are found most abundant in the small single cones, which have rod-like disks and lack micro-droplets. These electron dense pigments are unlikely to be TEM artefacts, since they are not found outside ellipsoids, and they appear to be most abundant in the ellipsoids of small single cones, and to a lesser degree in accessory double cones. The accumulation of these dark pigments could make a photoreceptor
appear darker in the snake retinal mosaic *in vivo*. Photoreceptors with darker ellipsoids and smaller in size have been noted by previous studies on garter snake retinæ (Land and Snyder, 1985; Wong, 1989). Large single cones and principal double cones appear to have brighter ellipsoids in the retinal mosaic (Land and Snyder, 1985; Wong, 1989), possibly due to funnelling of light by the microdroplets (Wong, 1989).

Even though snake microdroplets are not single oil droplets, there appears to be a conserved pattern between localization of microdroplets of snakes and single oil-droplets of birds. Oil droplets are not found in rods or the accessory member of double cones of birds (Hart et al., 2000) and turtles (Kolb and Jones, 1982). Since oil droplets may act as light filters, the lack of oil droplets in rods could be an adaptation for enhanced photo-sensitivity. TEM on *T. proximus* photoreceptors shows that microdroplets are not expressed in some small single cones (Figure 13B; Figure 14A), or the accessory member of double cones (Figure 12B). Since rods don’t have oil droplets, a lack of microdroplets in some small single cones could be a rod-like feature. These small single cones also have rod-like outer segment disks (Figure 14B). The rod-like cellular features suggest that some small single cones could be the candidates for expressing RH1 pigments. The functional roles of the small single cones can be better understood by considering data from RH1 *in vitro* expression.
4.2  *In vitro* Expression of *T. proximus* RH1

In total, ten rounds of *in vitro* expression of *T. proximus* RH1 pigments were carried out, and for each round at least one plate of HEK293T cells were transfected with bovine RH1 as positive control. Bovine RH1 pigments can be reliably expressed as positive control, whereas *T. proximus* RH1 pigments were challenging to express and measure *in vitro*.

Bovine RH1 is a model system for studying GPCR protein (Pierce et al., 2002) and its crystal structure was the first GPCR to be elucidated (Palczewski, 2000). Bovine RH1 is therefore commonly expressed as a positive control of *in vitro* studies. My sample data of bovine RH1 pigments show *in vitro* $\lambda_{\text{max}}$ of 500nm for dark-state RH1, and 380nm peak from accumulation of Meta-II intermediates after bleaching (Figure 16). This corresponds to the established $\lambda_{\text{max}}$ of bovine RH1 *in vitro* (Yokoyama 2000). This result is typical of bovine RH1 pigments from every *in vitro* expression experiment carried out by me. Bovine RH1 pigments expressed *in vitro* can also be visualized on Western blot using 1D4 antibody (Figure 18).

In contrast, *in vitro* expressed *T. proximus* RH1 pigments as solubilized sample do not show any noticeable absorbance peak before or after bleaching (Figure 17A). The sample data shows spectral absorbance of *T. proximus* RH1 pigments harvested from six plates of HEK293T cells. There appears to be a trend of increasing absorbance in shorter wavelength, but it is most likely an artifact due to the Tris-based buffer used for solubilization, which tends to absorb in the UV. The resulting difference spectrum of *T. proximus* RH1 does eliminate the trend (Figure 17B), but the absorbance peak is still too low and too noisy for calculating $\lambda_{\text{max}}$. Western blot results also indicate that *T. proximus* RH1 pigments are expressed in lower quantities compared to bovine RH1 pigments (Figure 18). *T. proximus* RH1 pigments only become visible on Western blots after the sample is further concentrated. *T. proximus* RH1 pigments appear to be similar in size as bovine RH1, as expected from their molecular weights of about 34 kDa. The *T. proximus* RH1 pigments may be difficult to express and measure *in vitro* for several reasons.

First of all, the transfection protocol was optimized for bovine RH1, but it may not be ideal for expressing RH1 pigments of cold-blooded reptiles such as snakes. Vertebrate RH1 pigments are sensitive to temperatures, and there are considerable variations between species (Hubbard, 1958). The HEK293T cells transfected with over-expression construct were incubated at 37°C for
Garter snakes live in environments that can fluctuate between below freezing to around 50°C (Lysenko and Gillis, 1980; Peterson, 1987). Garter snakes prefer to maintain their body temperatures between 25-30°C through behavioral changes, such as basking or under cover (Peterson, 1987). When environmental temperatures reach more than 30°C, garter snakes maintained their preferred body temperature over 90% of the time (Peterson, 1987). It’s unlikely for a garter snake to have body temperature of 37°C for any extended period of time. The stability of *T. proximus* RH1 pigments may be hampered by the incubation steps of *in vitro* expression protocol. RH1 pigments of *X. unicolor* were expressed *in vitro* at 37°C from twelve plates of cells, and show λmax of 497nm only according to difference spectrum (Davies et al., 2009). At 37°C, snake RH1 pigments may be more difficult to express *in vitro* than bovine RH1 pigments.

Secondly, *T. proximus* RH1 have multiple amino acid differences compared to bovine RH1, and these amino acid differences could destabilize the protein structure of *T. proximus* RH1, or lead to a lowered amplitude of spectral absorbance. There are a number of amino acid substitutions found in *T. proximus* RH1, some of which are highly conserved in other vertebrate RH1 genes. The lowered amplitude in spectral absorbance could be due to the unique amino acid changes in *T. proximus* RH1, such as S185, I11, I130, Q150, L159, and V169. Some of these *T. proximus* residues, such as Q150 are not found in rod-dominant species such as bovine, as well as sunbeam snakes and pythons. RH1 pigments of sunbeam snakes and pythons have been expressed *in vitro* by previous study (Davies et al., 2009). These unique substitutions could potentially alter the protein structure of *T. proximus* RH1, leading to the lowered absorbance level (or sensitivity) compared to RH1 pigments of other vertebrates, such as the bovine and henophidian snake. To confirm this, reverse mutants of *T. proximus* RH1 with single amino acid substitution can be expressed in the future for comparison of absorption level.

In addition, *in vitro* expressed *T. proximus* RH1 pigments were tagged with 1D4 epitope, which is useful for affinity purification or antibody detection. Many *in vitro* studies have used 1D4 epitope for expressing vertebrate pigments (e.g. Pointer et al., 2007; Cowing et al., 2008; Yokoyama, 2000). In our lab, zebrafish opsins have been expressed successfully with 1D4 epitope using the same protocol (James Morrow, unpublished). However, this epitope could also alter the protein structure of *T. proximus* RH1. Other studies have found increased stability of GPCRs by eliminating the C-terminal tail (Rosenbaum et al., 2007). Extending the tail by fusing
the RH1 opsin with a 1D4 epitope could further decrease the stability of *T. proximus* RH1. To maintain opsin stability while tagging it with 1D4 epitope, *T. proximus* RH1 could have the C-terminal tail truncated before tagging with the epitope.

The low absorption level of *T. proximus* RH1 *in vitro* could also suggest that these pigments do not have a functional role in vision. Only 9% of *T. proximus* photoreceptors are small single cones, and only some of them absorb around 483nm according to MSP (Figure 4). ERG of garter snakes found that the retinal gross potential peaked from 550nm to 570nm under varying experimental settings (Jacobs et al., 1992). Therefore the absorbance from LWS pigments are likely the dominant contributor to visual signals sent to the brain. However, MSP result from some small single cones indicates that their outer segments do absorb at 483 ± 1.5 nm (Figure 4). If *T. proximus* RH1 pigments are not responsible for this absorption peak, may be there is another unidentified RH2 type pigment. Previous members of the lab have looked for RH2 sequence from *T. proximus* cDNA library but could not find any (Mengshu Xu, Natalie Chan, personal communication). RH2 pigments have been found in other reptile species. In Italian wall lizards (*Podarcis sicula*), RH2 opsin have been isolated and shown to participate in photic entrainment of behavioural rhythms (Pasqualetti et al., 2003). In side-blotched lizard (*Uta stansburiana*), RH2 opsin was isolated from the parietal-eye, which mediate global detection of dawn and dusk instead of image-forming vision (Su et al., 2006). Besides SWS1, LWS, and RH1, other opsins have not been reported in snake species so far. Previous study on henophidian snakes tried to isolated SWS2 and RH2 from retinal cDNA library but failed to find them (Davies et al., 2009). It is possible that the other opsins, such as SWS2 and RH2, are expressed in the retina, escaping both degenerate PCR and MSP detection. Since SWS2 and RH2 opsins have not been sequenced from any snakes, degenerate primers could be designed from lizard species to look for snake SWS2 and RH2 opsins. The genomic sequences of *T. proximus* could also be useful for identifying other opsins such as SWS2 and RH2. Nucleic acid probes can be designed from genomic sequences for Southern-blot to look for other opsins.

To confirm that the poor absorbance of *T. proximus* RH1 is not due to my experimental errors, a large scale expression using 36 plates of HEK293T cells was carried out by fellow graduate student James Morrow. The RH1 gene was re-cloned into expression vector before transfection. Based on the large scale expression result, the absorption level from purified *T. proximus* RH1 pigments is about 100 times lower than bovine RH1 (Figure 19). The resulting difference
spectrum (Figure 20) suggests that the *T. proximus* RH1 pigments can form Meta-II intermediates, indicated by the dip in absorbance around 380nm. There also appears to be an absorbance peak that may match the MSP signal from some small single cones. To determine the λmax of *T. proximus* RH1 *in vitro*, template-fitting was carried out on the difference spectrum.

Absorbance spectra of visual pigments can be represented by a common template (Dartnall, 1953; Lamb 1995; Govardovskii, 2000). While the λmax of visual pigments can be tuned to different wavelengths, the shape of the absorbance curve remains the same. Although this assumption is built upon empirical data, many studies have searched for the universal template of visual pigments and the underlying physical factors (Ebrey and Honig, 1977; MacNichol, 1986; Partridge and De Grip, 1991; Lamb 1995; Gorvadovski, 2000). Although the most recent template by Gorvadovski (2000) is based on MSP data, this template has been used extensively to fit difference spectra of *in vitro* expressed visual pigments of rodents (Parry et al., 2004), birds (Carvalho, et al., 2007; Pointer et al., 2007), sharks (Davies et al., 2009), lampreys (Davies et al., 2007), marsupials (Cowing et al., 2008; Hunt et al., 2009), and snakes (Davies et al., 2009).

According to template fitting of the large-scale difference spectrum, *T. proximus* RH1 pigments have *in vitro* λmax of 485nm (Figure 20B). This value may correspond to the MSP λmax of 483 ± 1.5 nm from some *T. proximus* small single cones. MSP λmax can be slightly different from *in vitro* λmax, because other proteins or structures of the outer-segments could skew the absorption. In *X. unicolor*, rod outer segments have MSP λmax of 499nm, while the RH1 pigments have *in vitro* λmax of 497nm (Davies et al., 2009). Compared to *X. unicolor*, *T. proximus* RH1 pigments have a blue-shifted λmax of 485nm, which could be due to residues N83 and S292 (Figure 7). Double-mutants of bovine RH1 with N83/S292 substitutions have λmax blue-shifted to 485nm (Fasick and Robinson, 1998). Based on amino acid sequences, RH1 of sunbeams and pythons both have residues N83/A292, instead of N83/S292 in *T. proximus*. In RH1 pigments of lake cichlids, those with N83/S292 have the most blue-shifted λmax, while those with one of N83 or S292 residues are still blue-shifted but to a lesser degree (Sugawara et. al., 2005). These substitutions could explain why rhodopsin pigments of sunbeams (*X. unicolor*) and pythons (*P. regius*) have less blue-shifted λmax compared to *T. proximus*. Further expression of N83D and S292A mutants of *T. proximus* are required to determine whether these spectral tuning sites are responsible for the blue-shifted λmax of snakes RH1 pigments.
Figure 19. Dark absorbance of \textit{in vitro} expressed rhodopsins, from 36 plates of cells transfected with \textit{T. proximus} RH1, and 4 plates of cells transfected with bovine RH1 as control. These are the same samples expressed and purified by James Morrow as shown in Figure 20. Note the difference in absorbance between bovine and \textit{T. proximus} RH1 pigments at 500nm.
Figure 20. Dark and light absorbance spectrum (A) of large-scale in vitro expressed RH1 visual pigments of *T. proximus*, transfected and purified by James Morrow using thirty-six plates of cells. Curve-fitting using the difference spectrum (B) of *T. proximus* RH1 from large scale expression, using previously described templates (Govardovskii, 2000): the dots represent data points from difference spectrum, and the line represents the absorbance predicted by curve-fitting. The $\lambda_{\text{max}}$ predicted by curve-fitting is 485nm.
4.3 Transmutation of Small Single Cones

Based on the current EM, *in vitro* expression, and MSP data from *T. proximus*, RH1 pigments may be expressed in some small single cones, with a blue-shifted \( \lambda_{\text{max}} \) of 485 nm. The expression of RH1 pigments in the all-cone retina of *T. proximus* is an unusual combination, since rhodopsin pigments are typically found in morphological rods of most vertebrates, including henophidian snakes such as *X. unicolor* and *P. regius* (Davies et al., 2009). Based on cellular and molecular evidence, these small single cones may have evolved from rods of ancestral snakes.

The evolutionary transition between rods and cones was coined “transmutation” by Walls (1942). According to Walls’ transmutation theory, the first rods were transmutated from cones, and this transition has occurred in the reverse direction in some snakes (Walls, 1942). There is growing evidence in support of photoreceptor transmutation. Rod photoreceptors are specialized dim-light detectors evolved from cones: the elongated outer segments of rods are a derived feature after diverging from cones, and rhodopsin pigments evolved high sensitivity after diverging from cone pigments (Bowmaker, 2008). In mammals, the entire rod pathway is built upon existing cone pathways (Strettoi et al., 1992). Molecular data and phylogenetic studies found that LWS pigments are the most basal family while the RH1 pigments are the most derived (Okano et al., 1992). In most vertebrates the cones and rods have become so specialized (i.e. typical rod-like morphology matched with expression of RH1), that any evidence of past transmutation has been obscured. On the other hand, evidence of photoreceptor transmutation has been found in a few vertebrate species, such as in nocturnal geckos.

The nocturnal Tokay gecko (*Gekko gecko*) is well studied for photoreceptor transmutation. Their photoreceptors are rod-like in terms of morphology and electrophysiology (Pedler and Tilly, 1964; Tansley, 1964; Rispoli et al., 1993; Kleinschmidt and Dowling, 1975). There are three types of photoreceptor cells in nocturnal geckos – type A single rods, type B double rods, and type C double rods (Underwood, 1951). Gecko photoreceptor cells have three cone-type visual pigments. The first visual pigment, P521, is similar to chicken red-sensitive cone visual pigment (iodopsin) belonging to M/LWS subfamily (Kojima et al., 1992). The second visual pigment P467 has the highest sequence similarity to chicken green-sensitive cone visual pigment and is also highly related to a subfamily of rhodopsin, RH2, which are found in both rods and cones.
The third visual pigment P364 is a short-wavelength-sensitive cone-like pigment (Yokoyama and Blow, 2001). Besides cone-type pigments, only cone-specific sequences were obtained for phototransduction proteins, including transducin α-subunit, phosphodiesterase catalytic and inhibitory subunits, cyclic nucleotide-gated channel and arrestin (Zhang et al., 2006). Phylogenetically, nocturnal rod-only gecko and modern diurnal cone-only geckos both evolved from a common ancestor with only cones (Northcut and Butler, 1974; Roll, 2000). Rod-shaped photoreceptors of geckos were derived from ancestral cone-like photoreceptors, since cone-specific phototransduction proteins were still expressed (Walls, 1934; Zhang et al., 2006). The retina of Tokay gecko is a good example of how the morphology of photoreceptor cells can evolve independently of visual pigments (Kojima et al., 1992).

Results from the present study suggest that morphology and visual pigments may have also evolved independently in *T. proximus*. While all *T. proximus* photoreceptors have cone-like ultra-structure, some small single cones have rod-like cellular features. These small single cones have rod-like outer segment membrane disks, which are usually only found in rods. RH1 pigments may be expressed in these rod-like disks, since MSP peak from some small single cones may correspond to *in vitro* $\lambda_{\text{max}}$ of *T. proximus* RH1 pigments. Unlike large single cones and double cones, some small single cones were found to lack microdroplets. Instead they have an accumulation of tiny electron-dense pigments. Considering that *T. proximus* is a diurnal snake, these dark pigments may function as neutral light filters, since *T. proximus* RH1 pigments may be used for day-light vision.

There is good reason to speculate that RH1 should participate in day-light vision of *T. proximus*. One possible function is to provide signal contrast with the other cones. Cone photoreceptors and cone pigments are usually responsible for providing signal contrast and color vision. For *T. proximus*, the $\lambda_{\text{max}}$ for its cone pigments, SWS1 and LWS, are perhaps too far apart (360nm and 550nm) for signal contrast. There is no record of any vertebrate using only SWS1 and LWS for color vision, but studies now suggest that rods can participate in color vision in human. Blue-cone monochromats have rods and only one type of cones, but these patients have color vision within a limited spectral and intensity range (Reitner et al., 1991). At this point, we can only speculate on the possibility that *T. proximus* can use rhodopsin for wavelength discrimination with short-wavelength and long-wavelength signals. This hypothesis can be tested through
behavioral studies. Food rewards have been used to study visual capability of vertebrates such as goldfish, but it may be more complicated to elicit a response from snakes, as they have high chemosensitivity as well. Instead, behavioral experiments may be carried out to take advantage of the optomotor response, well studied in invertebrates (e.g. McCann and MacGinitie, 1965) and vertebrates. Behavioral studies measuring optomotor response are carried out in a variety of vertebrate species, such as newts (Manteuffel and Himstedt, 1978), cichlids (Kroger et al., 2003), salamanders (Joseph et al., 1973), and the tuatara (Ireland and Gans, 1977). Optomotor (or optokinetic) responses have adaptive significance for orientation, visual acuity, and distinguishing moving objects (Walls, 1962). The experimental setup will involve putting a snake inside an optomotor-drum, a revolving chamber lined with contrasting vertical stripes on the inside. The drum is spun, and if snakes can distinguish the two colors represented on the vertical stripes, then behaviors typically associated with the optomotor response can be scored.

Our current evidence suggests that while one population of small single cones may be expressing RH1 pigments, another population may be expressing SWS1 pigments according to MSP. It is unknown what proportion of small single cones expresses each pigment, and it is unclear whether cones expressing SWS1 pigments have different morphological features from cones expressing RH1. Without knowing these answers, we can not rule out another possibility, that instead of expressing one type of visual pigments, small single cones could be co-expressing both SWS1 and RH1 pigments. Co-expression of LWS and SWS pigments has been discovered in mammalian photoreceptors (Szél et al., 2000; Lukáts et al., 2005). The co-expressed pigments are both linked to the phototransduction cascade (Lyubarsky et al., 1999; Ekesten et al., 2002), which turns the cone into a spectral broadband detector (Peichl, 2005). Snakes like *T. proximus* could be co-expressing SWS1 and RH1 pigments in their small single cones, perhaps using them as broadband detectors. MSP data can not rule out this possibility due to limitation of techniques, since only absorbance data that fit the standard absorbance curve was selected. Co-expression of RH1 and SWS1 pigments could broaden the absorbance curve detected by MSP, making these photoreceptors appear to be anomalies. Co-expression of visual pigments has not been reported in reptilian species, so perhaps the small single cones of *T. proximus* could be candidates for future studies on co-expression of visual pigments in reptiles.

In diurnal colubrids such as *T. proximus*, the LWS pigments are likely the major contributor to visual signals. Large single cones and double cones could be expressing LWS pigments
according to MSP on *T. proximus*. Combined, these two types of cones make up about 85-90% of the total photoreceptor population. ERG result from *T. sirtalis* also indicates that LWS pigments are the major contributor to retinal gross potential (Jacobs et al., 1992). The other two pigments, SWS1 and RH1, are likely to be expressed in small single cones of *T. proximus*. In basal snakes such as boas and pythons, LWS pigments are expressed in large single cones, SWS1 pigments in small single cones, but RH1 pigments are expressed in rods. One possible evolutionary scenario is that these rods secondarily evolved cone-like morphology, as some caenophidian snakes took on more diurnal lifestyle. It’s also possible that the rods degenerated, but the rod-pathways were maintained in a small number of cones. To know the answer, the visual systems of more nocturnal colubrids should be characterized in the future. For example, a nocturnal colubrid species called Texas Night Snake (*Hypsiglena torquata*) is known to have all-rod retina (Walls, 1942; Stovall, 1976). Studying nocturnal colubrids will provide contrast to the current results from diurnal *T. proximus*.

The retinala of *T. proximus* lack typical rods, but rod pigments could be expressed in small single cones. The RH1 pigments may still be functional and could provide signal contrast with LWS and SWS1 pigments. Cellular features such as rod-like outer segment membranes of some small single cones may be crucial for the proper function of RH1 pigments. Other features such as microdroplets may be used for correcting Stiles-Crawford effect, while electron-dense pigments perhaps functional as neutral filters for RH1 pigments. Evidence of transmutation has not been found in henophidian snakes (Davies, 2009), but our evidences suggest that some *T. proximus* small single cones could be derived from ancestral rods. Besides geckos, evidence of photoreceptor transmutation has not been published in other vertebrate species. There are many other questions left unanswered by the current data, therefore future experiments are outlined in the last section of the discussion.
4.4 Future Directions

The most immediate goal in the near future should be the expression of *T. proximus* RH1 mutants. Several sites have been identified in the RH1 sequence, some of which have been known to have spectral tuning properties (e.g. 83 and 292), others are unique to the western ribbon snake (e.g. 185). Three mutants, N83D, S185C, and S292A, have been generated and cloned into p1D4 vector (Figure 21). By *in vitro* expression of these RH1 mutants for site 83 and 292, it is expected that $\lambda_{\text{max}}$ will be shifted closer to 500nm. While mutants for site 185 may or may not have spectral tuning properties, it is important to measure the change in meta-II decay rate and protein kinetics, as varying this site may have functional consequence for the visual pigment beyond spectral tuning.

Figure 21. Amino acid sequence alignment of wild-type *T. proximus* RH1 (CY527mx), mutants (CY378mp2, CY528mx, CY534mx) and expected RH1 sequence in p1D4 expression vector (Tp RH1v). CY378mp2 is miniprep DNA that has N83D mutation; CY528mx is maxiprep DNA that has S185C mutation; and CY534mx is maxiprep DNA that has S292A mutation. These sequences are derived from contigs using sequencing results. The RH1 gene is denoted by a black line with boxed area representing the transmembrane domains. The 1D4 epitope is denoted by a double-line. The restriction sites used for cloning are represented by the black and grey triangles. The stop codon is denoted by an asterisk. Vector sequences that are not part of the *in vitro* expressed protein are not denoted.
Further immunohistochemistry study should be carried out on the *T. proximus* retina, using antibodies targeting rod-specific transducin (K-20), cone-specific transducin (I-20), as well as N-terminus of rhodopsin. Even though the preliminary staining result shows that the rod-specific transducin antibody K-20 labeled some photoreceptors in the *T. proximus* retina. In future staining, an effort can be put into distinguishing the cell-type of labeled photoreceptors, perhaps by measuring the size of outer segments.

The ophidian double cones were thought to be evolved within snakes, although double-unit photoreceptors have been found extensively in teleosts, amphibians, reptiles, and birds (Underwood, 1951; Walls, 1967; Crescitelli, 1972). There have been speculations on the function of double cones, such as increasing packing density (Pedler and Tilly, 1964), analyzing polarized light (Underwood, 1970), sampling a limited visual field with two instruments (Cohen, 1963), or detecting intensity differences (Stovall, 1976). In *T. proximus*, the principal member of double cones have the same ellipsoid region as large single cones, while the accessory members lack microdroplets, like small single cones. The two members of the double cones may combine their absorption signals, one for the intensity of light while the other for the wavelength information. There is still much to learn about these double cones.

TEM have also captured some very small single cones as they appear as budding photoreceptors (Figure 22). The very small single cones are another mystery, as they lack outer segment membranes and do not produce a MSP signal. One possibility is that these very small single cones are the precursors to the accessory members of double cones. Since large single cones (Figure 13) and principal double cones (Figure 12) appear to have similar ellipsoid regions, a double cone is perhaps formed by one large single cone and one very small single cone. To test this hypothesis would require a better understanding of the developmental stages of these ophidian photoreceptors.
Figure 22. Transmission electron microscopy of a tangential section of *T. proximus* retina, Arrows indicate the very small single cone. Note the absence of outer segment membranes. Scale bar = 5µm.
Developmental studies of photoreceptors have been carried out in visual system of migratory fish, such as rainbow trout. Rainbow trout (*O. mykiss*) is known to degenerate their SWS1 cones and lose UV sensitivity before migration from rivers to ocean (Hawryshyn, 2000). This transformation is called Smoltification, and it is triggered by elevated thyroid levels. Smoltification and the loss of UVS cones can also be induced by treating juveniles with thyroid hormone (Browman and Hawryshyn, 1994). Thyroid hormone levels control the shedding behavior in snakes (Chiu and Lynn, 1971). Garter snakes are known to get blue-eyed 4 days before skin shedding (King and Turmo, 1997). Clear-eyed garter snakes have different visual capabilities when compared to blue-eyed individuals, such as shorter latency to move, but blue-eyed snakes have significantly greater response distance (King and Turmo, 1997). It is possible that cycling thyroid hormone levels play an important role in the visual system of snakes, which may be measured by the relative expression level of visual pigments with respect to developmental events. Developmental study of the photoreceptors may be difficult to carry out, but it will provide useful information about the cell-fate of *T. proximus* photoreceptors.

One more observation on *T. proximus* visual system should be noted: the shape of the *T. proximus* lens. During dissection of the retina, the lens appears to be a spherical structure, which is confirmed under SEM (Figure 23). Cracking of the lens is likely an artifact of SEM preparation, but it reveals the layered structure of the *T. proximus* lens. Spherical lenses are not common in vertebrates, except in fish. Spherical lenses have different refractive properties from the thinner, more elastic ones, like those found in mammals. Attached to ciliary muscles, the thin lens can be stretched to accommodate focusing on different depths of view. Garter snakes like *T. proximus* do not have ciliary muscles; instead they have a spherical lens which can be moved inward or outward of the focal axis (Walls, 1942). The spherical lens of fish, such as those found in carps, have been recently shown to have multifocal properties (Malkki and Kröger, 2005). The spherical lens of garter snakes is unique amongst reptiles (Walls, 1942). Like many other unique features of the snake visual system, they have evolved as snakes have to adapt to new habits and different lifestyles. Studying their visual system will shed light on the evolutionary history of snakes, and will contribute evidence to solving the phylogenetic relationships between snake species.
Figure 23. Scanning electron micrographs of the lens of *T. proximus*. It has a spherical shape, with cracking induced from SEM preparation. Calibration bar: 0.1mm.
Appendix 1. Amino acid sequence alignment of SWS1 expressed in *T. proximus*, *X. unicolor*, *P. regius*, *U. stabriana*, and *G. gecko*. The transmembrane domains are denoted by the black boxes below the alignment, which are predicted for *T. proximus* SWS1 gene online using TMHMM Server Version 2.0 (http://www.cbs.dtu.dk/services/TMHMM/). In the alignment sequences, a dot represents the same amino acid as *T. proximus* SWS1, while a line represent a gap. Residue F86 of TM2 is boxed with solid line.
Appendix 2. Amino acid sequence alignment of LWS expressed in *T. proximus*, *X. unicolor*, *P. regius*, *O.anatinus*, and *T. guttata*. The transmembrane domains are denoted by the black boxes below the alignment, which are predicted for *T. proximus* LWS gene online using TMHMM Server Version 2.0 (http://www.cbs.dtu.dk/services/TMHMM/). In the alignment sequences, a dot represents the same amino acid as *T. proximus* LWS, while a line represents a gap.
Appendix 3. Amino acid sequence alignment of *T. proximus* SWS1 clone (CY541mx) and expected SWS1 sequence in p1D4 expression vector (Tpx_SWS1v). CY541mx is maxiprep DNA, with one putative polymorphism I205V. The sequence for CY541mx is derived from contig using sequencing results. The SWS1 gene is denoted by a black line with boxed area representing the transmembrane domains, which are predicted online for *T. proximus* SWS1 gene using TMHMM Server Version 2.0 (http://www.cbs.dtu.dk/services/TMHMM/). The 1D4 epitope is denoted by a double-line. The restriction sites used for cloning are represented by the black and grey triangles. The stop codon is denoted by an asterisk. Vector sequences that are not part of the *in vitro* expressed protein are not denoted.
Appendix 4. Amino acid sequence alignment of *T. proximus* LWS clone (CY344mp1) and expected LWS cDNA sequence (Tpx_LWS). CY344mp1 is miniprep DNA in pJET cloning vector, with three putative mutations: D18E, I146T, and S244T. The sequence for CY344mp1 is derived from contig using sequencing results. The LWS gene is denoted by a black line with boxed area representing the transmembrane domains, which are predicted online using TMHMM Server Version 2.0 (http://www.cbs.dtu.dk/services/TMHMM/). There are three amino acid differences between the two sequences, none of the three residues from CY344mp1 were found in other snake species (Appendix 2). Therefore these should be mutated back to wild-type residues before protein expression.
References


