Investigations on the Reptilian Spectacle

by

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AUTHOR'S DECLARATION

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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Abstract

The eyes of snakes and most geckos, as well as a number of other disparate squamate taxa, are shielded beneath a layer of transparent integument referred to as the "reptilian spectacle." Derived from the embryonic fusion of palpebral tissues, the spectacle contains a number of specializations of the skin to benefit vision while still allowing it to function as the primary barrier to the environment. For example, in nearly all species that possess it, it is markedly thinned compared to the surrounding integument and its keratinized scale is optically transparent. While the spectacle may thus seem ideally adapted to vision in allowing the eyes to be always unoccluded, it does have a few drawbacks. One such drawback is its vascularity, the implications of which are still not fully understood, but are explored herein. As no recent synthesis exists of the body of knowledge on reptilian spectacles, the first chapter of this thesis consists of a review of spectacle anatomy, physiology, adaptive significance and evolution to help put into context the following chapters that present original research. The second chapter describes the dynamics of blood flow through the spectacle vasculature of colubrid snakes, demonstrating three main points: (1) that the spectacle vasculature exhibits cycles of regular dilation and constriction, (2) that the visual perception of a threat induces vasoconstriction of its vessels, and (3) that spectacle vessels remain dilated throughout the renewal phase. The implications of these points are discussed. The third chapter describes the spectral transmittance of the shed spectacle scale, the only keratinized structure in the animal kingdom to contribute to the dioptric apparatus of the eye, as well as its thickness. Spectacle scale transmittance and thickness was found to differ dramatically between snakes and geckos and found in snakes to vary between families. The adaptive significance of the observed variation is discussed. The fourth chapter describes biochemical analyses of the shed spectacle scales of snakes and geckos and compares their composition to other scales in the integument. Spectacle scales were found to differ significantly from other scales in their keratin composition, and gecko spectacle scales in particular were found to lack ß keratin, that hard corneous protein thought to be common to all reptile scales. The concluding chapter will discuss where this research has brought the state of our knowledge on the spectacle and offers thoughts on potentially useful avenues for further research.

Acknowledgements

But for the need for brevity, this section risked dwarfing all others, so I beg forgiveness from all those who deserve far more heaping praise than I can offer them on these pages. Research cannot flourish in a vacuum and many hands and minds beyond my own contributed to this work, directly and indirectly. Infinite thanks are due to supervisor/facilitator/cheerleader Prof. Jake Sivak, and I am indebted as well to my advisors Drs. Tom Singer, Matt Vijayan and Jeff Hovis for their helpful suggestions at all stages. Thanks to Profs. Ralph Chou & Trefford Simpson who provided invaluable insight and suggestions on carrying out spectrophotometric measurements and intraocular imaging respectively. A great many thanks to Miriam Heynen and Elizabeth Martell, both of whom are always willing to share their bountiful knowledge of biochemistry and their technical mastery, and thanks as well to Dr. Lyndon Jones for allowing the use of all the equipment in his lab. Thanks to Dr. Vivian Choh and Gah-Jone Won for valuable discussions, for sharing their knowhow on lab techniques and for the use of lab supplies. My gratitude extends as well to Brian Chow and Raymond Ho for their advice on running gels both 1-D and 2-D and to Dr. Brendan McConkey for the use of the IEF system. Many thanks to and due recognition of Drs. Simone Schneider and Jyotsna Maram for their expert assistance with capturing *in vivo* confocal microscopy images. Vielen dank Dr. Marc Schulze und Alex Müntz for translating some German articles and to Peter Stirling and Kathy MacDonald for help in tracking down those and other historical articles. I am grateful to Robin Jones for assisting with the design and building of custom equipment and to Nancy Gibson who helped with housing and caring for the snakes and geckos and for sharing her remarkable insight into animal behaviour. This work depended greatly on the kind donations of shed snake skins from several sources. I would like to extend my gratitude to Rob Caza for sending sheds from his personal collection, to Bry Loyst and the Indian River Reptile Zoo in Indian River, Ontario, for generous donations of a diverse array of shed snake skins, and to Little Ray's Reptile Zoo in Ottawa, Ontario, for collecting and sending many sheds from their collection. I am indebted to Drs. Roger Sawyer and Matthew Greenwold of the University of South Carolina for the generous gift of ß keratin antibodies without which a significant portion of this work would not have been possible. Reproductions of historical images are courtesy of the Biodiversity

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List of Abbreviations & Symbols

ANOVA	Analysis of variance
cm	Centimeters
cpd	Cycles per degree
DTT	Dithiothreitol
g	Grams
HCl	Hydrochloric acid
kDa	kilodalton
IEF	Isoelectric focussing
IgG	Immunoglobulin G
IPG	Immobilized pH gradient
λ	Wavelength
$\lambda_{50\%}$	50% cutoff wavelength
M	Mean or Molar
µg	Micrograms
µm	Micrometers
mm	Millimeters
mM	Millimolar
MWS	Middle wavelength sensitive (cones)
n	Refractive index or sample size (context dependent)
NaCl	Sodium chloride
NIR	Near infrared
nm	Nanometers
p	Probability
pI	Isoelectric point
PVDF	Polyvinylidene fluoride
s	Seconds
SWS	Short wavelength sensitive (cones)
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TBS	Tris-buffered saline
TTBS	Tween 20 + tris-buffered saline
Univ-ß	Universal ß keratin antibody
UV	Ultraviolet

UV-A	Ultraviolet A (315-400 nm)
UV-B	Ultraviolet B (280-315 nm)
UV-C	Ultraviolet C (100-280 nm)

Chapter 1, The Reptilian Spectacle: A Review

Many squamates possess a layer of transparent integument that overlays their eyes, shielding them from the external environment. These “reptilian spectacles” are ubiquitous among snakes but also found in most geckos and in several other squamate families, such as among skinks, xantusiid night lizards, some lacertid and teiid lizards, and in many legless, burrowing reptiles such as amphisbaenids. This review will discuss the anatomy and physiology of the spectacle as well as its diversity and functional significance. The anatomical variation of the spectacle across families will be described with emphasis on its unusual traits that have no analogue in unspectacled vertebrates. The optical implications of the spectacle to vision will be discussed and will touch upon the implications of its shape and the properties of its hard, keratinized scale, which is unique in being the only keratinized structure in the animal kingdom to contribute to the dioptric apparatus of the eye. The diversity of spectacle types, including windowed eyelids, will be discussed and theories on the adaptive significance and evolution of the spectacle will be presented. Throughout, the holes in our knowledge of this strange and fascinating structure will be emphasized and suggestions for fruitful avenues of research will be made.

“Il est de connaissance presque vulgaire que la cornée des serpents est protégée par une écaille transparente que, sur les dépouilles épidermiques abandonnées par ces reptiles au course de l’été, on retrouve enchâssée dans les téguments de la tête sous l’apparence d’un petit verre de montre.”

“It is rather common knowledge that the cornea of snakes is protected by a transparent scale that, within epidermal sheds abandoned by these reptiles during the summer, we may find it encased in the integument of the head with the appearance of a little watch glass.”

- André Rochon-Duvigneaud 1916

Shed skin from the head of a snake showing the “watch glass” appearance of the scales that cover the eyes. In his later account of the snake eye, Rochon-Duvigneaud (1943) instead likened the scales to rigid contact lenses.
(photo by K. van Doorn)

Vision has been credited with sparking the incredible phyletic diversification of animals during the Cambrian explosion 545 million years ago (Nilsson 1996; Land and Nilsson 2002). Given the remarkable value of light perception and image formation, it is perhaps no wonder that most species have evolved structures, behaviours and biochemical mechanisms to protect the integrity of their eyes during the courses of their life cycles. While many invertebrates have eyes supported by hard chitinous material (eg. arthropods) and others are able to regenerate damaged or excised eyes (eg. gastropods, Flores Scarsso and Pellegrino de Iraldi, 1973), vertebrates have comparatively fragile eyes in that the optically transmissive window to the outside world, the cornea, is rather delicate compared to their integument and has limited regenerative abilities beyond the renewal of the epithelium and scarring of the stroma. Vertebrates have thus evolved protective extra-ocular structures, with eyelids and nictitating membranes (i.e. "third" eyelids) being the most familiar examples among terrestrial species. Another protective structure that evolved among some vertebrates, terrestrial and aquatic alike, consists of a layer of transparent integument overlaying the eyes, which acts as a permanent, immovable shield against the external environment. These integumentary "spectacles" are found in some fishes (which typically lack eyelids altogether) and a few amphibians, but find their greatest terrestrial presence among reptiles, with some lizards having them, and snakes in particular being ubiquitously equipped with them.

Given that there is no analogue to the spectacle in mammals (or birds), and that it thus precludes spectacled animals as models for most human ocular conditions, it is perhaps no source of wonder that gaps exist in our knowledge of it in such diverse areas as its anatomy, physiology, optics, evolution, and its implications to ecology and ethology. This review aims to bring together the current state of knowledge of the reptilian spectacle -- its anatomy and physiology, its adaptive significance, and its evolution -- and in doing so to highlight areas of limited knowledge, some of which will be addressed by the experiments described in the 3 chapters that follow. This review will begin with a description of the anatomy of spectacles, from a somewhat historical perspective, to provide a context for further discussions of their other biological characteristics.

1.1 Anatomy of the spectacle

The simplest description of a spectacle is that of transparent integument that overlays the eye. Walls (1942) recognized three main types of spectacle, which he referred to as primary, secondary, and tertiary types, differentiated from one another by their developmental origin and anatomical relationship with the eye. Primary spectacles, found in lampreys, are perhaps the most primitive form, composed of skin overlaying the eye, under which the eye is apposed directly against the dermis but remains unattached so it may move freely. Secondary spectacles are found in some fishes and differ from the primary type in that the cornea is fused with the dermis, but with sufficient loose tissue around the eye to still allow for rotation. Tertiary spectacles, found in reptiles, amphibians and again in some fishes, appear to be the most evolved form in which eyelids develop a transparent component and are manifested as "windowed" eyelids or altogether fuse over the eye. While similar to the primary type, the tertiary form differs in that the cornea of the eye is not apposed directly against the spectacle's dermis, but rather the posterior of the spectacle is lined with conjunctival epithelium, continuous with that of the eye, which encloses a fluid filled pocket between the spectacle and cornea, allowing the eye to rotate freely, lubricated by a fluid analogous to the tear layer of lidded vertebrates. Because only the tertiary spectacle is found in reptiles, this discussion will focus entirely on its characteristics. Historically, the nomenclature of the spectacle has been as varied as the linguistic heritages of the anatomists who have studied it and their beliefs of its origin: paupière, brille, lunette, apparecchio palpebrale... Modern English accounts have settled on "spectacle" and its German translation "brille" ('brill-uh'), and though this review will primarily make use of "spectacle," historical precedent necessitates certain brillar references.

1.1.1 The Spectacles of Snakes

There was little agreement among early anatomists and naturalists regarding the nature of the spectacle's relationship with the eye. While it was certainly understood by anyone who came across a snake's shed skin that its eye was covered by a transparent scale, as attested by Rochon-Duvigneaud's

quote above, the relationship between that shed scale and the eye remained open to debate and speculation on whether the scale was affixed to the cornea, to an eyelid, or if it simply floated over the cornea (reviewed in Cloquet 1821).

Cloquet (1821) is credited with offering the first thorough and accurate account of the gross spectacle anatomy and its relationship with the eye, an account from which most later researchers drew inspiration. His illustration of the relationship of the snake spectacle with the eye is reproduced in Figure 1-1. Making use of fine dissections, he recognized three main layers: 1- a hard corneous layer (ie. the ocular or spectacle scale) at the exterior, 2- the dermis, and 3- the inner conjunctival layer. Whereas the first two layers are homologous with the corneum stratum and dermis of the skin, the inner conjunctival layer is homologous with the palpebral conjunctiva that lines the inner surface of eyelids and is thus continuous with the scleral conjunctiva. Between the spectacle conjunctiva and the cornea of the eye is a fluid-filled cavity called by various names such as subspectacle space or conjunctival sac.

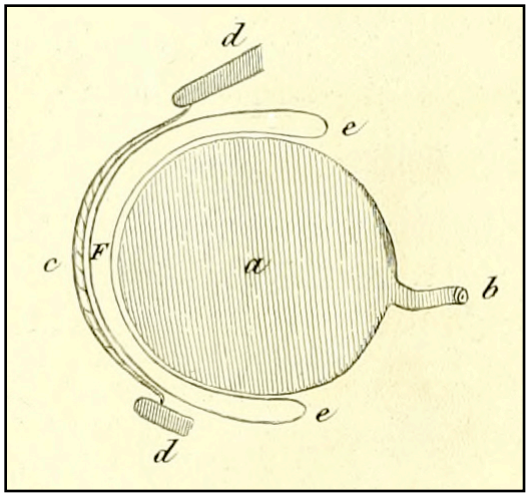


Fig. 1-1. The earliest accurate illustration of the spectacle's relationship with the eye. The spectacle (c) is separated from the eye (a) by the subspectacle space (F). Also labeled are the optic nerve (b), upper and lower periocular scales (d), and the fornix (e). Reproduced from Cloquet 1821.

Not until more than 50 years later, when Ficalbi (1888a, 1888b) published his monumental treatise of the reptile integument, was the histological structure of the spectacle well understood to have a far more complex layering nearly identical to that of the rest of the skin. Ficalbi recognized 5 main layers (Figure 1-2, next page), listed here from external to internal: 1- an external stratum

corneum, 2- an inner stratum corneum, 3- an epidermal "malpighian" layer, itself with with two layers: the stratum intermedium and stratum germinativum 4- a dermis, and 5- a thin inner conjunctival layer of partially overlapping squamous epithelia. In addition, his account and illustrations imply a clear zone between the inner and outer stratum corneum layers, which is possibly homologous to the mesos layer elsewhere in the reptile integument that is composed largely of lipoprotein lamellae (Maderson 1985).

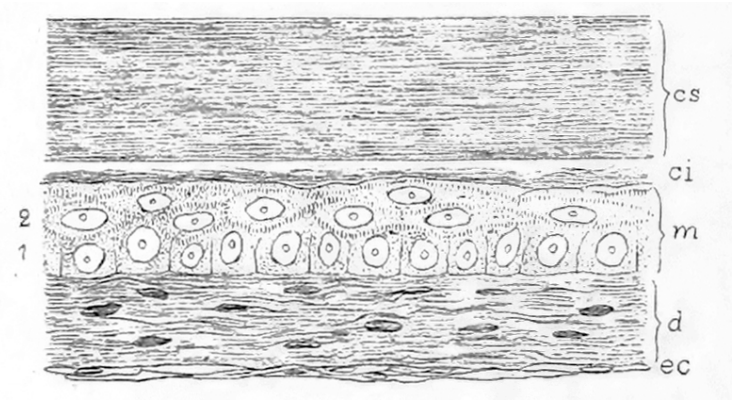


Figure 1-2. Early illustration of the layers of the spectacle. cs: outer corneum stratum, ci: inner corneum stratum, m: malpighian layer (1: stratum germinativum, 2: stratum intermedium), d: dermis, ec: conjunctival epithelium. Reproduced from Ficalbi 1888b.

The spectacle was thus shown to consist of all the same layers as the rest of the reptile integument, with the addition of a subdermal conjunctiva. It's important to note that Ficalbi's descriptions are specific to the resting phase of the snake integument, as the number of layers and their thicknesses increase during the renewal phase prior to moulting (Maderson 1998). Although excellent research has been done on the histology of the moulting integument of reptiles (Maderson 1985; Alibardi and Maderson 2003; Alibardi 2005), no studies have been published on the specifics of spectacle renewal, but it is unlikely to deviate significantly from that of the rest of the integument. *In vivo* histological images of the spectacle dermis and conjunctiva are shown in Figure 1-3 on the next page.

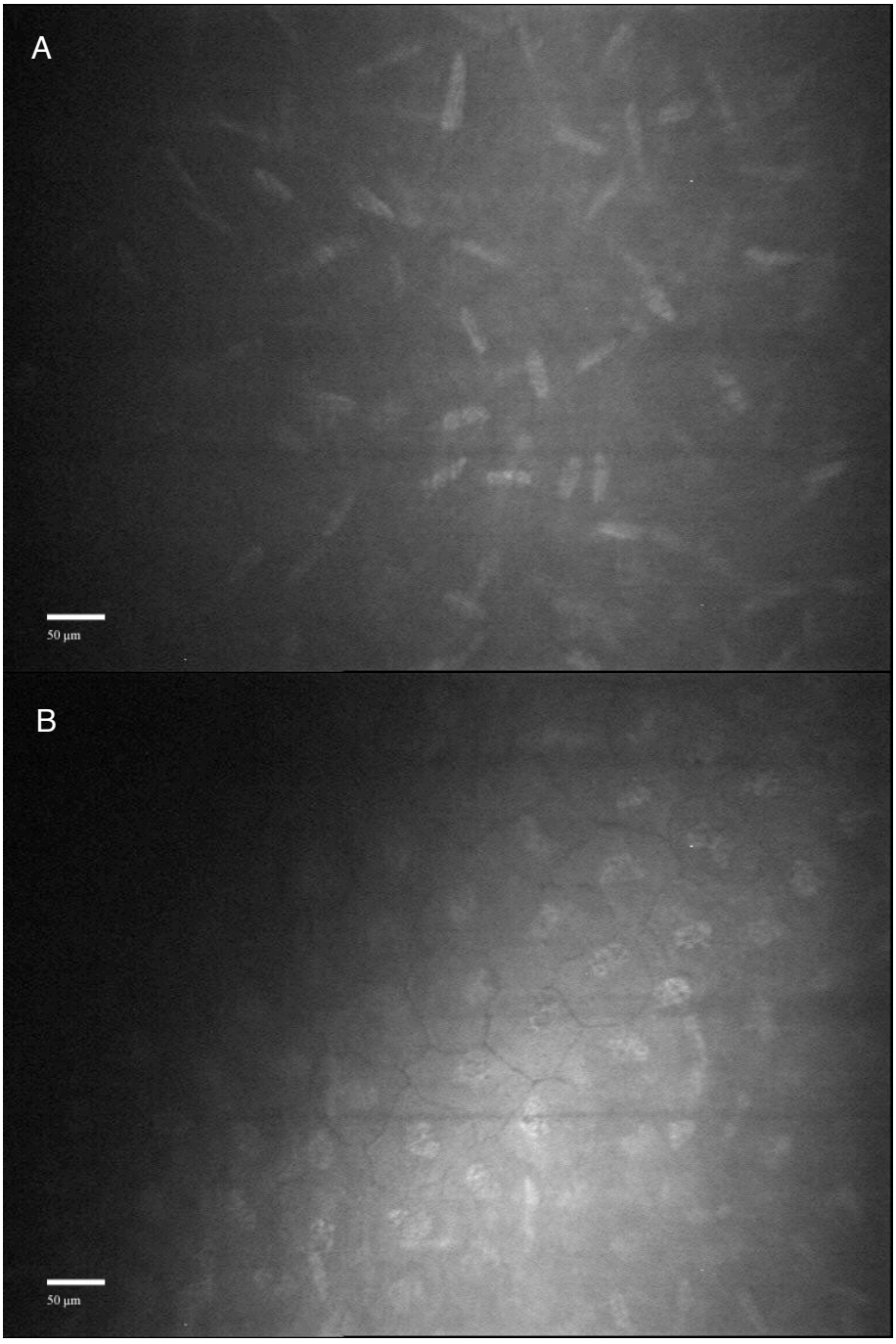


Figure 1-3. *In vivo* confocal microscope images of the spectacle dermis (A) and conjunctiva (B). Owing to its transparency, the spectacle is the only part of any terrestrial vertebrate integument to be amenable to *in vivo* microscopy, allowing for example the visualization of live fibrocytes in the spectacle dermis (A) and squamous cells of the spectacle conjunctiva (B) . This makes it of potential value in studying the physiology of the integument. The dark horizontal bands are artifactual. (unpublished photos by van Doorn, Maram, and Schneider)

The two layers he described in the snake spectacle are now known to consist of different types of keratin, just as are other scales in the snake integument (Maderson 1985). While the complex layering of the keratins have been studied in snakes (Alibardi and Toni 2005a), geckos (Alibardi and Toni 2005b) and other squamates (Alibardi and Toni 2006), no work has been published on the specifics of the spectacle scale. Chapter 4 will discuss the keratin composition of the squamate integument and spectacle scale in greater detail.

Although the spectacle shares the same overall anatomical structure as the skin, it is markedly more thin (Rochon-Duvigneaud 1943; Duke-Elder 1958). Due to tissue distortion that occurs during most histological preparations, the exact thickness of the whole spectacle had not been accurately ascertained until the development of modern ocular imaging techniques. Making use of ultrasonography, Hollingsworth *et al.* (2007) found spectacle thickness to vary between species. Its thickness is comparable between corn snakes (*Elaphe guttata*), California kingsnakes (*Lampropeltis getula californiae*) and ball pythons (*Python regius*), varying from 184 to 190 μm in these species, but is thicker in the gopher snake (*Pituophis melanoleucus*) at 220 μm. This variation may be largely due to the thicker spectacle scale of *P. melanoleucus* (see Chapter 3), indicating that the epidermis, dermis and conjunctiva together are of comparable thicknesses in all these species.

The surface of all scales of the snake integument bear microscopic ultrastructural features, such as micropits and interdigitating plates with varied stepping heights between the plates, somewhat similar in appearance to shingles on a roof (Hoge and Souza Santos 1953; Chiasson and Lowe 1989). The micropits have been suggested to serve as channels for sebaceous secretions (Chiasson *et al.* 1989). The morphology, patterning and density of these structures differ in different scales. Campbell *et al.* (1999) have shown that the spectacle scale of a python has larger plates with lower stepping heights than other scales, resulting in an overall smoother surface, which would improve transparency of the scale by reducing light scatter.

A curious feature of the spectacle that it shares with the rest of the integument is its vascularity. Other than the neural retinas of mammals and snakes and the corneas of Florida manatees (Harper *et al.* 2005), no other vertebrate is known to have blood vessels within the optically transmissive portions

of the eye, barring developmental anomalies or pathologies. The vascularity of the spectacle was first documented by Quekett (1852) who hazarded upon it by chance after injecting the vasculature of a rock python to study a neovascular anomaly of its lens capsule. His illustration is reproduced in Figure 1-4 and shows a complex and apparently irregular meshwork of anastomosing blood vessels with the degree of anastomosing being greater in the peripheral regions of the spectacle.

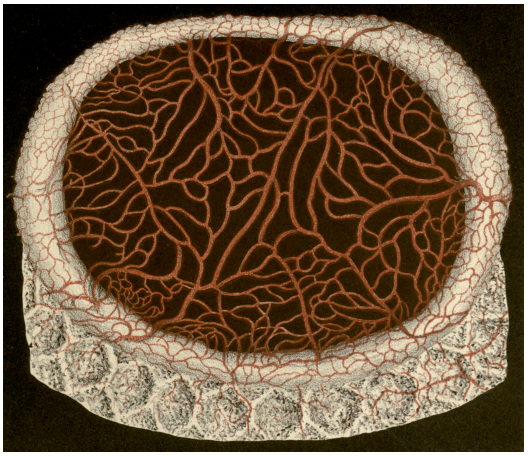


Figure 1-4. Earliest known illustration of the spectacle vasculature, drawn from the injected vasculature of a rock python (*Python molurus*). Reproduced from Quekett 1852.

Quekett's account seems to have been largely ignored or forgotten, as the next oldest account of the spectacle vasculature was offered by Ficalbi (1888b), whose neglect in citing Quekett is most likely due to his being unaware of the earlier author's work. Ficalbi's descriptions of the spectacle vasculature of the snake were also based on injections, by which he demonstrated that the spectacle dermis is permeated by blood vessels that lie mostly in the posterior region of the dermis and form, as he described it, an irregularly arranged anastomosing mesh across the whole of the spectacle. His illustration of the spectacle vasculature of a colubrid (Figure 1-5, next page) showed more precisely that the entry of the vessels into the spectacle occurs all around its circumference where they form complex anastomoses before adopting a largely dorso-ventral orientation at the center of the spectacle with a modest degree of anastomosing. Of interest is the noticeably different vascular layouts between Ficalbi's colubrid and Quekett's pythonid.

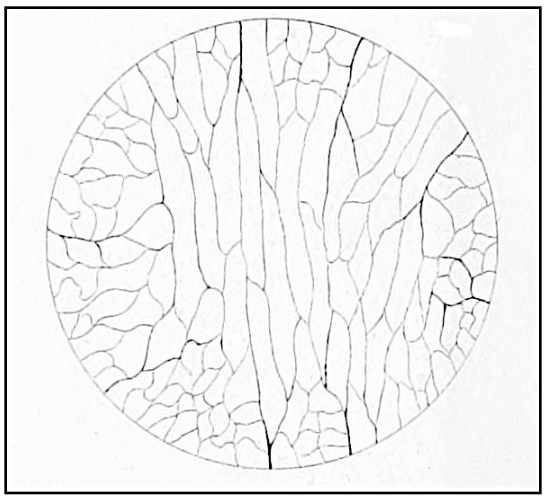


Figure 1-5. Illustration of the spectacle vasculature in *Hierophis viridiflavus*. Vessels enter from around the circumference of the spectacle, showing complex anastomoses in the periphery and largely dorso-ventral orientation at the center. Reproduced from Ficalbi 1888b.

The vascular anatomy of the spectacle was further explored by Manfred Lüdicke who mapped out the meshwork of blood vessels in several species of snake from several families (Lüdicke 1940, 1969, 1973, 1977; Lüdicke and Kaiser 1975). Lüdicke demonstrated that the arrangement of spectacle vessels varies between families. For example, those of colubrid snakes exhibit a predominantly dorso-ventral (i.e. ventral) arrangement with few anatomoses in the center (eg. Figures 1-5 and 1-6, next page), whereas those of boids, pythonids, acrochordids and aniilids are radially arranged with varying degrees of organization and anastomoses. The meshwork of *Gekko gecko* has a similar radial organization with entry of the vessels from around the circumference. Curiously and significantly, Lüdicke (1969) also found in the green vine snake (*Ahaetulla nasuta*, Colubridae), one of few snake species known to have foveas, that the distribution of vessels is such that the nasal region of the spectacle, which serves the temporally-located foveas and the binocular field, has a lower density of vessels than elsewhere in the spectacle. This suggests an adaptation specifically to minimize loss of visual clarity due to the vessels, a rather compelling theory given the highly visual nature of this species and one that will be further discussed later.

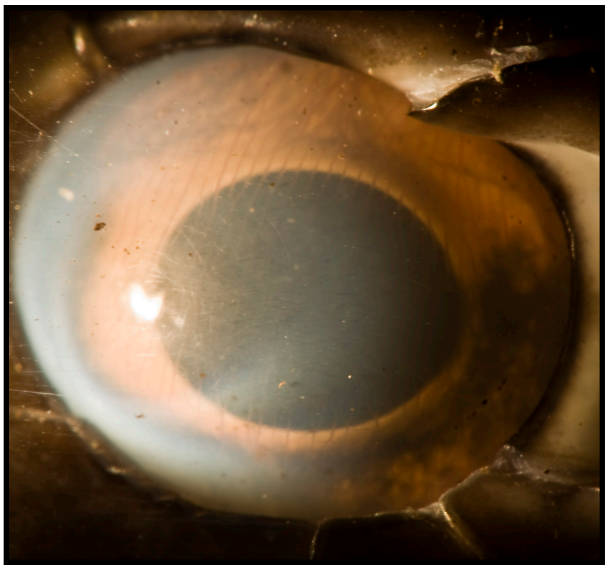


Figure 1-6. Photograph of the eye of a coachwhip snake, *Masticophis flagellum*, during the renewal phase. The vertically oriented blood vessels of the spectacle are visible around the pupil. The clouding of the eye is characteristic of the renewal phase of the integument. (photo by K. van Doorn)

Mead (1976) added to this work by showing the layout of spectacle blood vessels of crotaline vipers to have a radial arrangement as in boids and pythons and that elapid snakes (specifically a siamese cobra, *Naja naja kaouthia*) have a vertically oriented meshwork as in colubrids but with a reduction in vessel size at the centre of the spectacle and a much greater degree of complex anastomoses away from the optic axis. Although Mead made no mention of the adaptive significance of this latter point, it is again a compelling thought that this arrangement is such that visual disturbance due to the vessels might thus be minimized on the animals' optic axis, an anatomically distinct but functionally similar adaptation to that found in *A. nasuta*. Although Mead also reported examining the spectacle vasculature of a xenopeltid snake, but did not include a description or images of its organization. On the vascular flow through the spectacle vessels, Mead (1976) reported only that "the vessels [ ] fill without any obvious directional priority in the anesthetized animal."

Lüdicke's studies of spectacle vasculature also included the measurement of blood vessel diameters. In *Python reticulatus*, the diameters of the proximal afferent vessels are approximately 30 µm, large enough to pass several erythrocytes abreast. No values were given for the smaller branches, but it is evident from photographs of the injected vessels that their diameters decreased toward the center of the spectacle at the optic axis -- again hinting at an adaptation to minimize visual disturbance. In the case of *A. nasuta,* no values were given for the vessels' diameters, but a photograph of the

injected spectacles clearly shows that nasal vessels (in the forward visual field served by the foveas) have a considerably smaller diameter than median and temporal vessels. My own investigations of the spectacle vasculature have shown that the vessel diameters of corn snakes (*Elaphe guttata*, Colubridae) measure approximately 35-45 μm at full dilation, whereas in coachwhip snakes (*Masticophis flagellum*, Colubridae), the vessels measure 25-30 μm at full dilation (van Doorn, unpubl.). This difference between species may be due to the different emphases placed on visual acuity, with corn snakes being mostly nocturnal and coachwhip snakes being active and very rapid diurnal predators with large eyes whose ranges extend into open areas with comparatively few obstacles to vision (Greene 1997).

While the gross layout of the vascular meshwork will be constant throughout an individual's life, injuries to or pathologies involving the spectacle have been shown to cause neovascular proliferation within the spectacle (Maas *et al.* 2010). Such a response elsewhere in the integument may not significantly impact the organism, depending of course on its severity, but were it to occur in a visual snake like *A. nasuta*, the altered meshwork may have a deleterious effect on vision if the vessel density increases sufficiently to reduce retinal image contrast or if it were to present itself in high acuity areas of the visual field.

Not only are spectacles vascularized, but they also are innervated, a characteristic that again was first reported upon by Ficalbi (1888b) in his remarkably thorough account. Crevatin (1904), elaborating upon Ficalbi's preliminary research, described in detail the layout of spectacle nerves in two species of colubrid and one viperid. His findings showed that the nerves penetrate radially from the periphery into the spectacle dermis and form complex anastomoses (Figure 1-7). From the dermis, fine nerve endings extend into the epithelial layer at the base of the stratum corneum. Little research has been done on the spectacle innervation since Crevatin (1904). Jackson (1977) showed that colubrid snakes did not possess touch corpuscles on their spectacles, but that *Leptotyphlops dulcis* (Leptotyphlopidae), a fossorial blind thread snake, did possess them on regions covered by the oculolabial scale. It is not clear from Jackson's account if the corpuscles occurred on the region immediately overlaying the eye. Jackson and Shawary (1980) further demonstrated the absence of

specialized mechanoreceptor tubercles on colubrid spectacles, in contrast with scales elsewhere on their head. Of course this demonstrates nothing about what receptor types spectacles do possess, with the possible exception of the leptotyphlopid. It is likely that the spectacle innervation is at least partly sensory in function, given that nerve endings extend to the epidermis, but autonomic innervation to the spectacle vasculature may also be present as it is with all cutaneous vasculature (Baker *et al.* 1972; Rowell 1977), particularly in light of the spectacle vascular dynamics presented in Chapter 2.

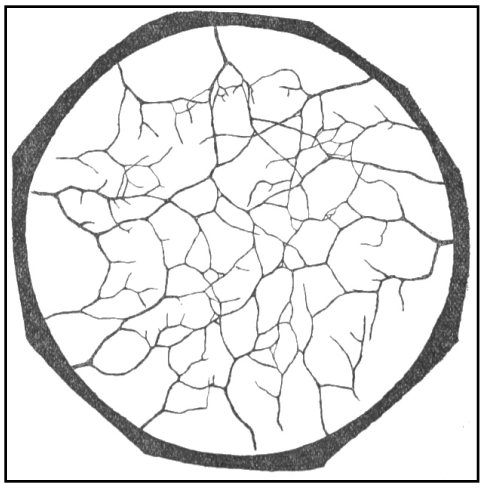


Figure1-7. Early illustration of the spectacle nerves of the colubrid snake *Natrix tessellata*. The nerves are seen penetrating radially into the spectacle from all around the circumference with complex anastomoses occurring in the branches. From Crevatin 1904.

An interesting aside about the spectacle nerves is that, as Crevatin (1904) observed and remarked upon with enthusiasm, their layout is similar to that of corneal nerves in other species. Human corneal nerves, for example, penetrate into the corneal stroma from around the corneolimbal circumference with only the exception of the dorsalmost and ventralmost areas. Within the stroma, they extend fine nerve endings toward the epithelium (Müller *et al.* 1997). Morphologically, the individual neurons of the spectacle are similar as well to those of human corneal nerves as observed with *in vivo* confocal microscopy (Figure 1-8, next page) where mitochondrial aggregations in the form of beads can be observed along the axons.

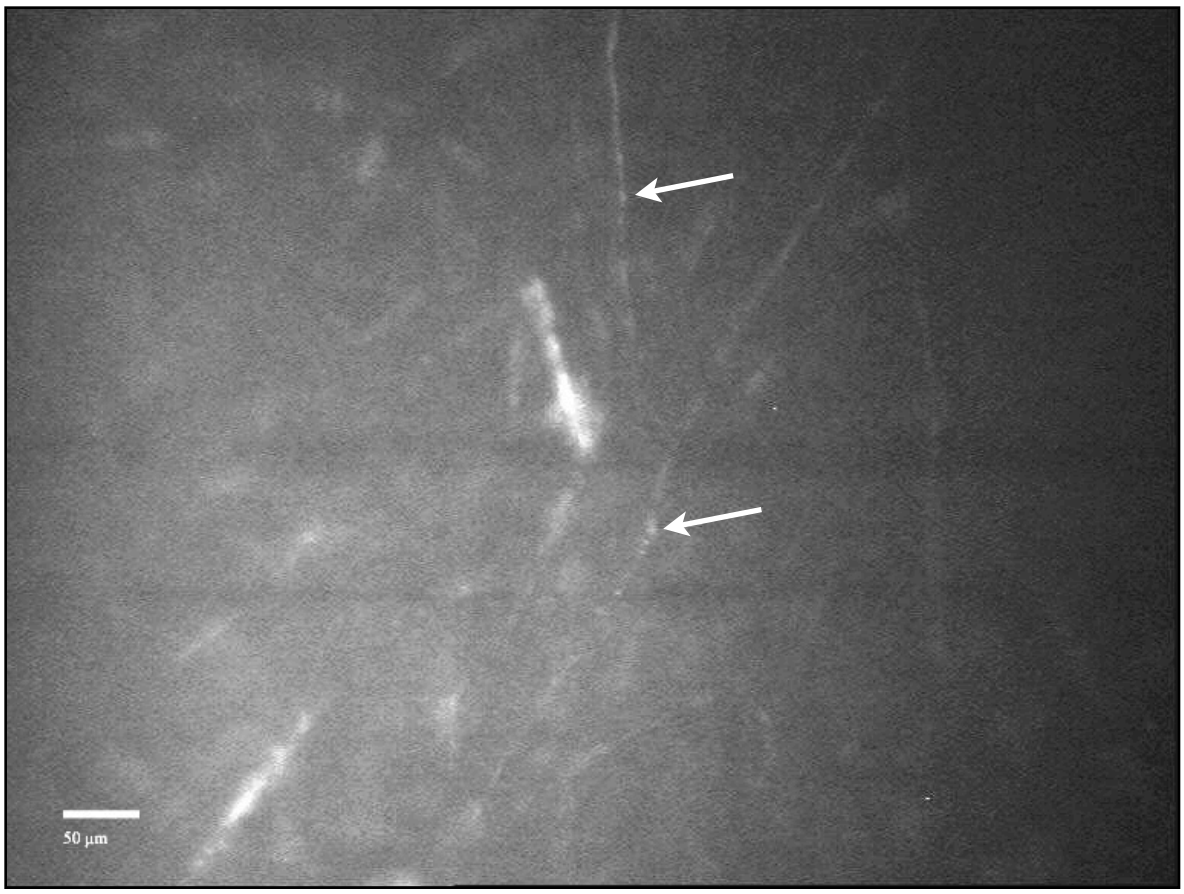


Figure 1-8. *In vivo* confocal microscopy image of a spectacle nerve of a coachwhip snake (*Masticophis flagellum*). The thin lines that extend more or less vertically are neurons. Aggregations of mitochondria can be seen as subtle "beads" along their length (indicated by white arrows). The faint elliptical spots correspond to fibrocytes in the dermis, while the brighter and longer spots have yet to be identified, but are observed primarily in the outer dermis (van Doorn, *unpubl. obs.*). The two dark bands running horizontally through the image are artifactual. The scale represents 50 μm. (unpublished photo by van Doorn, Maram, and Schneider)

1.1.2 The Spectacles of Scolecophidian Snakes

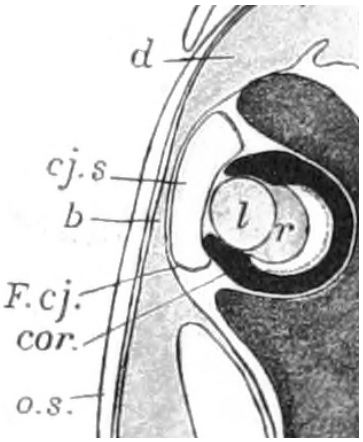


Figure 1-9. Early diagram of the typhlopid eye and its relationship with the spectacle. The spectacle dermis (d) is noticeably thinned compared to surrounding areas. cj. s. conjunctival sac; b. brille (spectacle); F. cj. fornix conjunctiva; cor. choroid/iris; o.s. outer stratum corneum; l. lens; r. retina. Reproduced from Eigenmann 1909.

All the descriptions thus far have been on alethinophidian snakes, that largest superfamily which includes all but the most basal snakes, the Scolecophidia or blind and thread snakes. These share similarities of the spectacle anatomy with alethinophidians (Eigenmann 1909; Foureaux *et al.* 2009), including the thinning of the tissues immediately overlaying the eye, but differ in the size of the scale covering the eye. In alethinophidians, the spectacle scale is sufficiently broad to cover the cornea of the eye and little beyond. In scolecophidians, however, the scale overlaying the eye extends well beyond its margins, in some cases covering a significant portion of the head. In these species, the scale is more properly referred to as the ocular scale, and in those where it extends to the mouth, it may be called the oculolabial scale. In all species, usage of the term "spectacle" should be restricted to the specialized integument immediately overlaying the eye.

A recently discovered species of leptotyphlopid, *Leptotyphlops macrops* ("larged eyed thread snake"), is distinguished in having much larger eyes than all other scolecophidians (Broadley and Wallach 1996). To accommodate the size of the eyes, a dome is formed in the ocular scale. The eyes of this unique snake may represent a transitional form in the evolution of snake eyes from the reduced scolecophidian form to the more sophisticated alethinophidian eye.

1.1.3 The Spectacles of Geckos

Most, but not all geckos, are spectacled. Only species of the family Eublepharidae bear true eyelids and lack any form of spectacle (*Eublepharis* = "proper eyelid bearer"). All other families contain only spectacled species (Underwood 1954).

Gecko spectacles have not been subject to as much research as those of snakes. Given that geckos will typically eat their shed skin to reclaim nutrients (Bustard and Maderson 1965), there would have been fewer conspicuous indications to begin with that their eye even had a scale. Furthermore, the ridge that surrounds their eye (the so-called "extra-brillar fringe") has the appearance of eyelids (Figure 1-10). This may explain the relative paucity of early gekkonid spectacle histology compared with snakes.

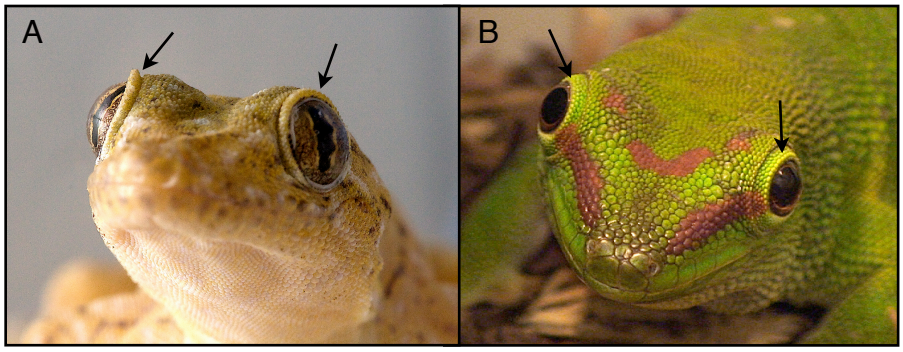


Figure 1-10. Portraits of a marbled gecko, *Gekko grossmanni* (A), and a giant day gecko, *Phelsuma madagascariensis grandis* (B), showing the extra-brillar fringes that have the appearance of opened eyelids, but are separate entities and remain fixed in most species. (photos by K. van Doorn)

The earliest modern account of the anatomy of the gecko spectacle was given by Müller (1830) who confirmed that Cloquet's findings of the basic layering of the spectacle applied as well to spectacled geckos as it does for snakes. Ficalbi's (1888b) histological study of the spectacle extended as well to geckos, but while his account of the snake spectacle is remarkable in its detail, that of the gecko spectacle is rather vague in mentioning that the gecko spectacle is "similar to snakes except in

having perhaps a more highly developed dermis and a thinner stratum corneum". It is not clear what constitutes a "more highly developed dermis," nor is it clear if he found a multi-layered stratum corneum as in snakes, but given the results presented in Chapter 4, it is possible that he did not. Likewise, he made no explicit claim about the spectacle being vascularized, though later researchers confirmed that it was (Lüdicke 1971; Mead 1976). Lüdicke, in his investigation of the ocular blood supply of *Gekko gecko*, showed the meshwork to be somewhat radial and irregular in arrangement and to exhibit a high degree of anastomosing. Notably, he also found the blood vessels to be smaller than those of snakes, measuring 4-17 μm in diameter. It is unclear, however, how a 4 μm vessel can pass the large nucleated erythrocytes of geckos, which are greater than 9 μm on the shortest dimension (Saint Girons and Saint Girons 1969; Starostová *et al.* 2005). Given the vagueness of Ficalbi's observations on the gecko spectacle, its innervation has yet to be truly established.

Unlike snakes, the scales of most geckos are small, in some cases resembling tubercules, and don't overlap (Pianka and Vitt 2003). In these species, the size of the spectacle scale thus makes it by far the largest of their integument, which, as in alethinophidian snakes, is just large enough to cover the cornea of the eye but no larger, being bordered by the extra-brillar fringe. In one gekkonid genus, *Ptenopus*, the extra-brillar fringes are hypertrophied, contain muscle fibers and are mobile, allowing the fringes to incompletely cover and shield the spectacle (Smith 1939; Bellairs 1948). Interestingly, this species burrows in dry sandy habitats (Haacke 1975), which has led Bellairs (1948) to suggest that the extra-brillar "eyelids" (spectaclids? brillids?) protect the spectacle from abrasive sand and dust, a curious arrangement given that the spectacle itself is typically considered to be protective against the very same (see the section on Adaptive Significance below).

1.1.4 The Spectacles of Amphisbaenids

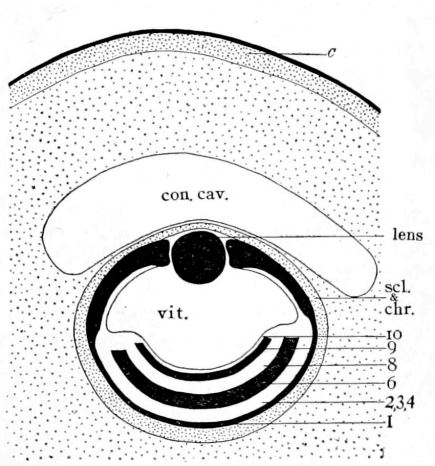


Figure 1-11. Early generalized diagram of the amphisbaenid eye and its relationship with the spectacle. c. outer covering of the eye; con. cav. conjunctival cavity; vit. vitreous humour; scl and chr. sclera and choroid; 1-10. layers of the retina. The dermis (unlabeled) remains thick in this species. Reproduced from Eigenmann 1909.

Like scolecophidian snakes, amphisbaenids are small, burrowing squamates with reduced eyes. And rather than having a spectacle scale, theirs is an ocular scale that extends beyond the margins of the eye, streamlining the head for burrowing, although in some the spectacle immediately overlaying the eye may protrude convexly outward (Gans 1978). Their spectacles are composed of all the same integumentary layers as other spectacled squamates: a stratum corneum, 2-layered epidermis, dermis and conjunctiva (Foureaux *et al.* 2009). Unlike other spectacled reptiles, the amphisbaenid spectacle is not always thinner than the surrounding integument and it may also be pigmented. The thickness and degree of pigmentation of the spectacle seems to vary between species, being thinner in some than the surrounding integument (eg. *Trogonophis weigmanni*, *Amphisbaena alba*, *Amphisbaena mertensi*, and *Leposternon infraorbitale*), of the same thickness and degree of pigmentation as the integument in others (eg. *Amphisbaena strauchi*, and *Amphisbaena darwinii*), or massively thickened and pigmented as in *Amphisbaena fuliginosa* (Fischer 1899; Bellairs and Boyd 1947; Gans 1978; Foureaux *et al.* 2009). These reports seem to indicate that thickness of the spectacle and its degree of pigmentation are positively correlated, suggesting that the emphasis placed on vision varies significantly among these fossorial squamates.

In those amphisbaenids with thinned spectacles, the thinning was shown by Foureaux *et al.* (2009) to be achieved by thinning each integumentary layer individually, as in other spectacled

squamates. Foureaux *et al.* also confirmed the presence of blood vessels in their spectacle. The blood vessels are quite large at ~50 μm and lie in the outer dermis, next to the stratum germinativum of the epidermis. This is in contrast with snakes in which the meshwork generally (though not exclusively) lies deeper, next to the conjunctiva.

It appears that no study has been done on the innervation of the amphisbaenid spectacle. Given its vascularity, it likely receives autonomic input, and given the burrowing lifestyle and overall reduced eyes of amphisbaenids, it would not be surprising to find mechanoreceptors on its surface.

1.1.5 The Spectacles And Windowed Eyelids of Other Squamates

While the spectacle may have been championed by snakes and geckos, it actually finds its greatest diversity among the many lacertilian families and genera in which it is manifested at any stage of sophistication from a moderately translucent lower eyelid to a fully sealed and immovable spectacle.

The simplest eyelid modification involves a thinning of its several layers, rendering it translucent. This form is seen for example in the lacertid *Eremias vermiculata* (Angel and Rochon-Duvigneaud 1941). An enlargement of the scales making up the window improves transparency by reducing the number of "seams" between scales. This form is found in *Eremias guttulata* and in the iguanid *Anolis argenteolus* and *Anolis lucius*, in which the eyelid windows are additionally pigmented (Williams and Hecht 1955). The most highly developed form of windowed eyelid involves replacing the multiple transparent scales with a single scale large enough to cover the cornea. This is seen for example in *Mabuya vittata* and *Leiolopisma fuscum* (Schwartz-Karsten 1933) and also some aquatic turtles (eg. *Lissemys punctata* and *Chelodina longicollis*, Johnson 1927), the only non-squamate reptiles to bear such eyelid modifications. Fully sealed spectacles are seen in a number of disparate families and genera, including Xantusiidae (night lizards), Pygopodidae (legless lizards evolved from geckos), in several families of burrowing legless lizards with reduced eyes (Dibamidae, Anelytropidae, Euchirotidae), in several genera of scincid lizards such as *Ablepharus* (snake-eyed skinks), *Morethia* and *Proablepharus*, in the lacertid genus *Ophisops* (snake-eyed wall lizards), and the teiid genera

Gymnophthalmus (spectacled tegus) and *Micrablepharus* (Walls 1942; Greer 1980; Greer 1983). This list is by no means exhaustive but hopefully conveys the diversity of eyelid modifications in reptiles.

The anatomy of windowed eyelids and spectacles in these species has been little studied beyond their superficial morphology. Mead (1976) reported finding blood vessels in the spectacle of a xantusiid night lizard but did not describe the layout of the meshwork. It is thus still not known if and how the eyelid windows and spectacles in these species are vascularized. Cross-sections of eyelid windows have been presented as diagrams (Angel and Rochon-Duvigneaud 1941; Bellairs and Boyd 1947), but no high-resolution studies of their histology have been published. The pressing question remains of whether eyelid windows are vascularized and whether the eyelid muscles and glands are arrayed in such a way to minimize their presence in the transparent portion. Curiously, *Ablepharus* and *Ophisops*, both fully spectacled, retain the depressor palpebralis inferioris muscle which inserts into the spectacle's inferior border (Underwood 1970).

1.2 Spectacle Development

The development of the spectacle has been described for snakes and geckos (Schwartz-Karsten 1933; Neher 1935; Bellairs and Boyd 1947; Bellairs 1948; Boughner *et al.* 2007). It generally consists of the proliferation over the developing eye of mesenchymal tissues until they meet and fuse, becoming transparent and void of all glands and typically of muscles otherwise found in eyelids (Underwood 1970). The tissues may take the form of eyelids, with the margin of the lower "lid" gradually progressing upward until it meets the upper lid and fuses, while in others, the extraocular tissues migrate inward from all around the circumference of the eye, gradually shrinking the aperture over the eye until it vanishes. While the former description of fusing eyelids may have the appearance of a truncated version of mammalian eyelid development, in which developing integumentary tissues fuse over the eye and then separate again as distinct eyelids (Addison and How 1921; Pearson 1980; Findlater *et al.* 1993), it should be emphasized that eyelid development in birds and reptiles does not appear to involve fusion at any stage (Hamburger and Hamilton 1951; Hays and Lecroy 1971; Billy

1988; Vieira *et al.* 2011). Thus the development of spectacles logically resembles a progression from that of lidded reptiles rather than a regression of the process observed in mammals. No published study of spectacle development in other reptiles, such as scincid or xantusiid lizards, has been found, so it is unknown if they too follow a similar principle, though it seems likely that they would given the precedent and the nature of tertiary spectacles.

1.3 Optics of the Spectacle

As the window to the outside world, the spectacle plays a crucial role in the quality of vision. This was briefly touched upon in the description of the spectacle blood vessel layouts of the previous section with the consideration that the blood vessels themselves might constitute an impediment to clear vision. While such visual consequences are speculative, the fact that no vertebrate (again, other than the Florida manatee) has non-retinal blood vessels in its visual field implies that visual clarity may be impacted by any degree of vascularization in the optical transmissive regions of the eye. As well, the asymmetric meshwork in the spectacle of *A. nasuta* that minimizes the density of vasculature in the most acute field of vision suggests an adaptation to minimize a loss of clarity due to the vessels.

Species in which pupils constrict to near pinhole dimensions would be most likely to suffer visually due to the increased depth of field resulting from such small apertures (Green *et al.* 1980) which, in tandem with the short focal lengths of snake eyes (Sivak 1977; Howland *et al.* 2004), might resolve the spectacle vessels in the retinal image. Even the horizontal slit pupil of the keen-eyed *A. nasuta* is able to constrict to sub millimeter widths, but it being fortunately horizontal, vision is thankfully saved as the increased depth of field and resolving capacity of its thin aperture would affect only horizontal lines in the visual field, not the vertical lines of the spectacle vessels.

Spectacle blood vessels are not alone responsible for potentially limiting visual clarity. The scale itself may acquire abrasions or collect debris during the course of the animal's activities (Figure 1-12, next page) that could reduce retinal image contrast or result in scotomas (i.e. blind spots). As Walls (1942) quipped: "[The] renewal of the [spectacle scale] often comes none too soon - as one

appreciates on observing the sadly scratched and dull appearance of the spectacle of a garter snake inhabiting such an abrasive place as a stone wall."

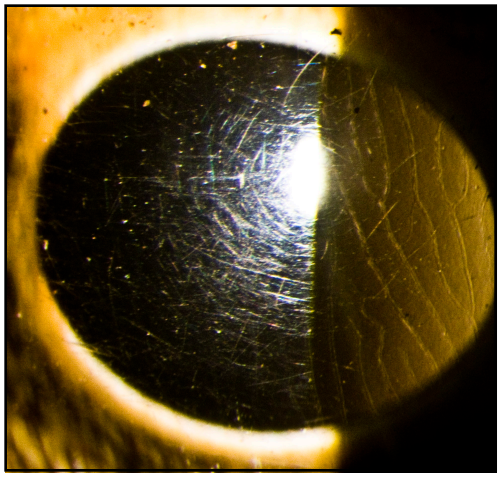


Figure 1-12: Photograph of the eye of a coachwhip snake, *Masticophis flagellum*, showing scratches on the spectacle scale and debris which accumulate during the regular activities of the animal. Coupled with the spectacle blood vessels visible on the right, these factors may have significant implications for the visual clarity of snakes. (photo by K. van Doorn)

During the renewal phase of the snake integument, when they generate a new stratum corneum to replace the old, the spectacle clouds over, effectively reducing vision to a low-contrast perception of low-spatial frequency forms and shapes. Curiously, this phenomenon does not occur in geckos, in which the renewal of the integument is a more gradual process with no external indication at any stage (Maderson 1964; Maderson 1966). The cause of the snake spectacle's opacification is unknown, although it is not exclusive to the spectacle as it occurs across the integument and is there manifested as a dulling of the animal's colouration. Possible causes might be edema (which for example can cause opacification of the cornea), the proliferation of keratinocytes and gradual keratogenesis that disturbs tissue organization, or the presence of eosinophils that invade the integument of snakes during the renewal phase (Maderson 1965).

The overall shape of the snake spectacle has a significant impact on the dioptric properties of the ophidian eye. While the corneas of most terrestrial vertebrates have a smaller radius of curvature than the rest of the globe, the spectacles of snakes typically have a greater radius of curvature (Walls 1940; Sivak 1977). Put another way, the surface of the snake eye is relatively flatter than that of any

other terrestrial vertebrate. The spectacle scale, being composed of keratin, has a higher refractive index ($n \geq 1.5$) than that of underlying tissues ($n = 1.36$-1.375 if similar to the cornea) (Valentin 1879a, 1879b), making it a thin lens. Sivak (1977) and Caprette (2005) calculated the power of the whole spectacle in several colubrids based on measurements of curvature and average refractive index of the whole spectacle and found the dioptric power of the spectacle to be relatively similar to the lens, in some cases slightly favouring the lens, in others the spectacle. A lens with such a high relative power results in a shorter focal length for the optical system, which in turn results in a lower f-number (i.e. greater retinal illumination) and lower image magnification, all other parameters being equal. This optical design is frequently seen in nocturnal (Roth *et al*. 2009) and aquatic or amphibious vertebrates (Sivak 1976; Northmore and Granda 1991; Brudenall *et al.* 2008; Walls 1942; Duke-Elder 1958). In comparison, the ratio of cornea:lens dioptric power in a diurnal iguana is approximately 3:1 (Sivak 1977, calculated based on data from Citron and Pinto 1973), and that of a (mostly) diurnal primate, *Homo sapiens*, is 2:1. It would therefore appear that the relative flatness of the spectacle constrains the snake eye, even that of diurnal species, to a predominantly nocturnal or amphibious optical design. Gecko eyes do not share this unusual morphology, nor do those of other spectacled lacertilians with well developed eyes. Rather they recall the eyes of lidded squamates in possessing highly curved corneas with consequent longer focal lengths.

The question of what anatomical characteristics allow the spectacle dermis to remain as transparent as possible with maximum transmittance of the visual wavelengths of light remains unanswered. It is conceivable (and likely) that the composition of the spectacle dermis is similar to that of the cornea, in which transparency is achieved by the orthogonal arrangement of collagen lamellae and by maintaining its hydration state within a narrow range (Maurice 1957; Cox *et al.* 1970; Freegard 1997) through passive and active means (Candia 2004). And as with retinal blood vessels, the spectacle blood vessel walls are transparent (Mead 1976) such that when constricted they are nearly invisible and difficult to discern even with slit lamp microscopy (van Doorn, *unpubl. obs.*).

The transparency of the spectacle scale on the other hand presents a novel problem, since most keratinous structures are translucent at best. The transparency of spectacle scales varies little with

hydration state (van Doorn, *unpubl. obs.*), so they are not dependent on the precise balancing of water flux as is the cornea. Campbell *et al.*'s (1999) work on the surface ultrastructure of a python's scales, described earlier, did show that the surface of the spectacle scale differs from others in having features which are less likely to cause optical scatter. The specific complement of keratins and their arrangement may also play a role in transparency. In chapter 4 of this thesis, results of investigations on the biochemical composition of spectacle scales will be presented, which is hoped can provide a foundation for further research to determine the relationship between scale composition and spectral transmittance.

The even and parallel boundaries between adjacent layers of the spectacle is unquestionably essential to achieving transparency. Because of the large difference between refractive indices of keratin and dermal tissues ($n_{keratin} - n_{dermis} \geq 0.13$), it is essential that the boundaries between the dermis and epidermis/stratum corneum remain as even and as parallel as possible to minimize random scatter and reflection of incident light. This can be understood by considering Figure 1-13.

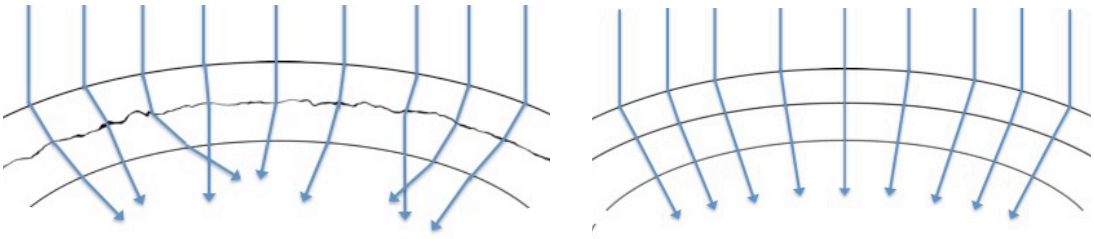


Figure 1-13. Diagram of the effects of uneven surface in adjoining layers with different refractive indices. In contrast with an optical system with smooth surfaces (B), the system with an uneven boundary exhibits scatter of the incident illumination (A). (diagram by K. van Doorn)

1.4 Physiology of the Spectacle

The barrier properties of the reptile integument to cutaneous fluid flux and respiration have been the subject of some research (Lillywhite and Maderson 1982; Feder and Burggren 1985). Unfortunately no work has been published to my knowledge on the particular characteristics of the spectacle.

As described above, the hydration state of the spectacle dermis is likely crucial to maintaining transparency. Unlike the cornea, the water flux of the spectacle has not been studied, so it is unclear how hydration of the dermis is controlled. As an analogue of the corneal endothelium, the spectacle conjunctiva would likely play a role in this, either actively or passively.

The capacity of the spectacle to obtain sufficient oxygen from the atmosphere and transmit it to the cornea may be of significance in explaining the continued presence of blood vessels in even highly visual geckos and snakes. While lidded vertebrates make use of atmospheric oxygen diffusion through the tear layer of open eyes to supply the cornea, the corneas of spectacled reptiles are not directly exposed to the atmosphere. In closed eyes, the palpebral vasculature supplies the tear layer with oxygen (Efron and Carney 1979) that in turn diffuses into the cornea. With limited oxygen diffusion through the spectacle scale, the oxygen necessary for cellular respiration in the spectacle and cornea thus is likely to require a vehicle in the form of a vascular supply to the region. This would explain why the corneas of even those species with reduced eyes and little capacity for acute vision continue to be spared from neovascularization.

Similarly, this may help in explaining why the spectacle vascular meshwork is seen more frequently in the posterior dermis of alethinophidians, next to the subspectacle space, while those of amphisbaenids occur more superficially. The small eye of amphisbaenids may allow sufficient oxygen to diffuse from iridial and limbo-scleral vasculature to the cornea due to the short distances involved. In contrast, the large eyes of alethinophidians would require a vascular plexus more proximal to the cornea. It appears that the spectacle vasculature of scolecophidians has not been described, but if this theory holds true, their spectacle vasculature would not be constrained to the deepest layer of the dermis.

1.5 Adaptive Significance of the Spectacle and its Evolution

"[The spectacle] is quite insensitive to touch. The cobra, python, and other snakes all allowed me to touch it, and even polish it with a rag, so as to get a clear view of the fundus, without any attempt at resistance or even sign of discomfort" - George Lindsay Johnson, 1927

While the validity of this claim of insensitivity remains untested, particularly given that the spectacle is innervated, this quote nevertheless sums up nicely the spectacle's protective character. The spectacle of extant snakes unquestionably serves a protective role as attested by the severely abraded snake spectacle in Figure 1-12. Of course its current adaptive significance, as with any trait, makes no implication of the selective pressures on its early evolution. Thus, a number of theories have been put forth to explain the evolution of the spectacle.

1.5.1 Mechanical Protection

Perhaps the most frequently recognized theory is that of mechanical protection from blowing sand and against obstacles during close-crawling, subterranean, and nocturnal locomotion (Rochon-Duvigneaud 1916; Walls 1934, 1940, 1942). In legless or short-legged organisms, the eyes will be exposed to any number of large obstacles in their path as well as to small rocks, twigs and other sharp, pointed, or abrasive objects that would pose quite a threat to an exposed cornea, particularly under restricted visual conditions such as at night. Arid environments carry the risk of blowing sand and dust, foreign particles that can cause not only discomfort, but serious harm to eyes, nictitans and eyelid conjunctiva. Snakes and geckos likely inherited their spectacles from their respective common ancestors (discussed further below), so they all possess them regardless of habitat, size and diel activity, but among other spectacled squamates, spectacles and windowed eyelids are indeed found most frequently in smaller species, in those that burrow, in nocturnal species, and in species that inhabit dry and semi-dry microclimates (Storr 1971; Greer 1983; Walls 1934; Walls 1942). Walls asserted that most extant

snakes have no need of a spectacle as they do not all fit the theory's requirement of nocturnal activity patterns and deserticolous habitat.

In discussing the protective nature of the spectacle, one should obviously consider its mechanical properties. Unfortunately these properties have eluded rigourous and systematic inquiry but have nevertheless elicited anecdotes such as Walls' and Johnson's quotes above and the following anecdote of my own: While studying a marbled gecko (*Gekko grossmanni*), a nocturnal arboreal species, I lightly rubbed my hand quite by accident against its spectacle. Remarkably (and regretfully), this resulted in a deformation on the spectacle surface, resembling a tear, which was clearly observable with a slit lamp. Fortunately, the gecko's damaged spectacle was improved after the following moult, and completely renewed after a second moult, the new stratum corneum showing nothing of the earlier blemish. As this indicates, the delicate gecko spectacle does not provide the same degree of robustness against ocular trauma as a snake's. No such deformation ever occurred by rubbing or abrading their spectacles, which attests to their durability. This fragility of the gecko spectacle may draw suspicion to the belief that the spectacle evolved for the purpose of mechanical protection, but again it must be borne in mind that current incarnations imply nothing of the original function. And while an arboreal gecko's habitat and lifestyle, nocturnal or not, may make it less likely to suffer insults to the eye than a snake that uses its head to push through anything in its path, and while it is equally endowed with the ability to regularly replace damaged spectacle scales, it nevertheless emphasizes the need to consider alternative theories of the function of spectacles.

1.5.2 Minimization of Evaporative Water Loss

Arnold (1973) proposed another such theory by suggesting that spectacles and windowed eyelids may minimize evaporative water loss from the cornea. According to Reichling (1957, cited in Arnold 1973), water loss from the tear layer could account for up to 20% of the water loss in *Lacerta agilis*, quite a high proportion considering this lizard has small eyes proportional to its body size. Evaporation from the surface of the eye would be of greater significance to smaller species with large eyes that are

diurnally active in hot and dry habitats. That smaller animals are more affected can be deduced from the greater surface area to volume ratio of their bodies (Mautz 1982) and the general inverse relationship between eye size and body size (Hughes 1977; Kiltie 2000). Put simply: small animals, already at greater risk from evaporative water loss due to their small size, have correspondingly larger eyes from which proportionally greater water loss can occur. Greer (1983) provided some evidence in support of this theory of spectacle evolution by correlating the presence of spectacles and windowed eyelids in scincid, teiid, and lacertid lizards with body size, habitat and diel activity patterns. Indeed, he found a higher proportion of small-bodied, diurnal species inhabiting drier habitat to have some form of eyelid modification. As a counter argument, smaller animals tend to be physically closer to their substrate and thus have their eyes closer to it as well, which might make them again more vulnerable to abrasions. Also blowing sand and dust, both entailing risk to the eye, occur most often in drier habitats. These points of course bring us back again to the theory that spectacles have a primary function of mechanical protection.

1.5.3 Protection Against Solar Radiation

Yet a third theory, originally proposed by Plate (1934), is that of protection against solar radiation. Williams and Hecht (1955) observed that, when exposed to bright sunlight, two species of anoline lizard would cover their eyes with their pigmented, windowed eyelids. As they emphasized: "[] eyelids in tetrapods always have *two* functions: to guard the eye against foreign objects and against excess light." While few extant species have such highly pigmented eyelid windows or spectacles, at least not in wavelengths that we can perceive, some snake spectacle scales do exhibit yellow or slight brown pigmentation (see Chapter 3), which may provide a degree of solar protection, particularly to the ultraviolet spectrum.

1.5.4 Evolution of the Spectacle

It is of course possible that all theories are valid and that spectacles have evolved for a number of different reasons, particularly given that they evolved independently several times.

Snakes and geckos are the only two taxa in which the spectacle occurs throughout (again excepting Eublepharidae) regardless of habitat and ecology. In both case, it is likely that they owe its presence to their respective common ancestors. The majority of geckos are predominantly nocturnal, suggesting nocturnality to be ancestral, which is in line with assertions that nocturnal species are more likely to have spectacles to protect the eyes from injury in low-light conditions. The absence of spectacles in Eublepharidae has thus been suggested as an ancestral trait of the taxon (Kluge 1967; Kluge 1987). Jonniaux and Kumaza (2008) suggest, based on molecular evidence, that spectacles either evolved independently in non-eublepharid geckos and sister taxon Pygopodidae or that eyelids evolved independently in Eublepharidae from a spectacled ancestor, as a reversal to a more primitive form. The significance of this latter scenario could not be overstated considering the need to re-evolve analogous musculature and innervation for eyelid opening and closure as well as the necessary secretory glands responsible for maintaining a tear layer. A thorough comparative study of the anatomy and innervation of the eyelids of these geckos, as well as the source(s) of the components of their tear layer, to ascertain similarity to or divergence from typical lacertilian eyelids, would be helpful in verifying the stage of spectacle evolution at which this reversal might have occurred.

The oldest fossil snake found dates to ~100 mya and several studies of genomic divergence have placed the earliest snake at 109-160 mya (reviewed in Vidal *et al*. 2009), so we may never know or fully understand what prompted their common ancestor to evolve a spectacle. Walls (1940, 1942) put forward a strong argument for the original snake having fossorial habits, based not only on the most primitive extant snakes, the Scolecophidia, being fossorial with extremely reduced eyes, but also on the unusual ocular anatomy of all snakes, which suggests a reduction in the need for acute vision occurred at some juncture in their evolution. This would have been followed by the redevelopment of functionally-analogous intraocular structures after abandoning the subterranean existence. While part

of his argument hinges on the presence of the spectacle, a fossorial existence may not have been the original inducement for its evolution. Instead, the common ancestor may have evolved a spectacle for completely different reasons and simply have been thus pre-adapted for burrowing (Bellairs and Underwood 1951).

Curiously, Walls (1942) also hinted at the optical similarity of the snake eye to the fish eye, noting the sphericity of the lens (and correspondingly high dioptric power) and the relative flatness of the ocular surface, both features which are also adaptations of aquatic and amphibious mammals and birds (Walls 1942; Sivak 1978; Howland and Sivak 1984), but he suggested nothing about a possible aquatic ancestry, in spite of contemporary hypotheses of such an ancestry (Cope 1869; Rochon-Duvigneaud 1916; Nopsca 1923) and despite discussing the two aquatic turtles with windowed eyelids, *Lissemys punctata* and *Chelodina longicollis*. The recent discovery of Cretaceous marine snakes that are suggested by some to be transitional forms between lizards and snakes (Caldwell and Lee, 1997; Lee *et al*. 1999; Rage and Escuillie 2000; Scanlon and Lee 2000) has prompted renewed interest in the aquatic theory (Caprette *et al*., 2004), although it has also been suggested that the fossils instead represent an advanced snake that had re-evolved legs (Tchernov *et al*. 2000; Zaher and Rieppel 2000). But whatever the habitat of the ophidian ancestor, underground, aquatic or otherwise, the snake eye, as with most fish eyes, has a flatter surface than other terrestrial vertebrates, as described above. This is consistent with both an aquatic existence, recalling the optical morphology of the fish eye, or a fossorial lifestyle in streamlining the head to minimize trauma while burrowing.

It is clear that only circumstantial evidence is available to answer the questions of what selective pressures have led to the evolution of spectacles. Unfortunately, spectacles do not readily fossilize. Until the discovery of a preserved spectacle (or windowed eyelid) in a putative ancestor of any currently spectacled species, our speculations on spectacle evolution must rely on the current evidence of snakes' unusual ocular anatomy, gekkotan molecular phylogenetics, and the variation among lizards of varied habitats and their eyelids, windowed eyelids and spectacles.

1.6 Conclusion

As the reader can ascertain from this review, it is clear that although the reptilian spectacle has intrigued researchers for over two centuries, many questions about it remain captivatingly unanswered. What are the visual implications of its vasculature and of abrasions on its surface? How does the spectacle achieve transparency and what compositional characteristics allow for a transparent spectacle scale? What makes the snake spectacle scale more resilient than that of a gecko? What sensory information is obtained from the spectacle innervation? What selective pressures are responsible for the evolution of a spectacle or ensure the continued presence of spectacles in any species? What factors in developmental genetics strongly decrease the likelihood of reversion to a lidded form as is thought to have occurred in Eublepharidae?

The research presented in the next three chapters will provide some answers to questions of the physiology of the spectacle vasculature, the spectacle scale's spectral transmittance, and the spectacle scale's biochemical composition. Of course, the answers will in turn raise many more questions to be addressed by future research.

1.7 Organization of the following chapters

The next three chapters present original research that is organized chronologically in the order in which the experiments were conducted. The investigations cover three main characteristics of the spectacle and its corneous scale: the physiology of its vasculature, the optical characteristics of the scale, and the biochemical composition of the scale as follows:

- Chapter 2 describes investigations on the blood flow patterns in reptilian spectacles, effectively adding the 4th dimension to the already well-described 3-dimensional anatomical structure of the spectacle blood vessels.

- In Chapter 3, findings on the varied spectral transmittance and thicknesses of spectacle scales will be presented, with comparisons within and between families and subfamilies.

- Chapter 4, the third and final experimental chapter, describes biochemical analyses of the spectacle scales, inspired in part by the findings of Chapter 3. As a result, and despite the chronological ordering of the chapters, these two chapters will cross-reference each other. An attempt was nevertheless made to organize the text in such a fashion that the thesis can be read through without flipping between sections.

- The fifth and final chapter will be a discussion of where this work has brought the state of knowledge on reptilian spectacles and offers suggestions of further avenues of research that are likely to provide valuable results.

Chapter 2, Blood Flow in the Reptilian Spectacle

This chapter describes investigations on the dynamics of blood flow in the reptilian spectacle both when animals are at rest and undisturbed and when they are presented with visual stimuli which are perceived to be threatening. This work was presented as posters at two international meetings: The Association for Research in Vision and Ophthalmology Annual Meeting 2008 (van Doorn & Sivak 2008a) and The Joint Meeting of Ichthyologists and Herpetologists (van Doorn & Sivak 2008b).

2.1 Introduction

This introduction will summarize the relevant background information of spectacle anatomy and physiology described in Chapter 1 so as to provide a context for the research presented here such that this chapter can be read as a standalone account.

The spectacle of snakes and geckos is a layer of transparent integument that overlays the eye, isolating it from the external environment. Arising from the fusion of embryonic tissues that would otherwise form eyelids (Schwartz-Karsten 1933; Neher 1935; Bellairs & Boyd 1947), the spectacle's anatomy is homologous with that of the skin in having a stratum corneum (the spectacle scale), a complex epidermis and a dermis, but differs in possessing a subdermal conjunctival layer similar to that of eyelids (Ficalbi 1888b; Walls 1942).

Unlike either the skin or most eyelids however, the spectacle is optically transparent, ideally suited to vision but for one characteristic that it shares with the rest of the integument: its vascularity (Figure 2-1).

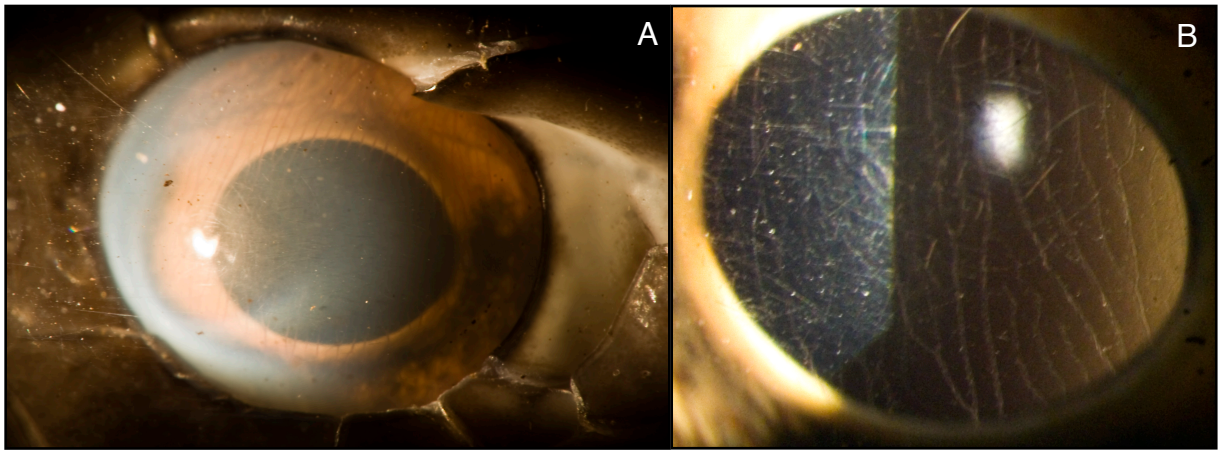


Figure 2-1. Photographs of the eye of a coachwhip snake, *Masticophis flagellum*, during the renewal phase of the integument (**A**) and with retro- and cross-illumination (**B**), showing the dorso-ventrally arrayed spectacle blood vessels. (photos by K. van Doorn)

The presence of blood vessels in the spectacle dermis was first demonstrated by Quekett (1852). The vascular supply to and anatomical layout of the snakes' spectacle blood vessels was later

described in great detail by Lüdicke (1940; 1969; 1973; 1977; Lüdicke & Kaiser 1975) who showed the arrangement of blood vessels to vary between families. The spectacle vessels of colubrids differ from boids, pythonids, aniilids, and acrochordids in having a dorso-ventral orientation to the blood vessels rather than the latter's radial organization of the spectacle vasculature. Mead (1976) further showed the spectacle vessels of elapids to have a dorso-ventral organization similar to colubrids and that of vipers to have a radial organization.

That an optically transmissive region of the visual apparatus is vascularized is quite remarkable, as no other terrestrial vertebrate has blood vessels in the optically transmissive non-retinal regions of the eye (Walls 1942; Duke-Elder 1958). This suggests that any degree of vasculature in the visual field can have a negative impact on visual clarity, and the vision of snakes might thus be constrained by the presence of the spectacle blood vessels. Supporting this assertion, the green vine snake, *Ahaetulla nasuta* (Colubridae), one of few snakes known to have foveas, exhibits a naso-temporal asymmetry in the density of spectacle vessels, with the nasal region having a lower vascular density than elsewhere in the spectacle (Lüdicke 1969). The spectacles of other colubrids described by Lüdicke, none of which are foveated, show no perceivable asymmetry, which suggests the unusual arrangement in *A. nasuta* to be an adaptation of the spectacle vessel organization to maximize visual clarity.

Though the spatial layout of spectacle blood vessels has been well described in several species, little commentary was made on the blood flow dynamics within those vessels but for Mead (1976) who, noting the transparency of the blood vessel walls, observed erythrocytes flowing through them but stated only that "the vessels [ ] fill without any obvious directional priority in the anesthetized animal." One may consider that alternatively or in addition to spatial adaptations in the layout of the vascular meshwork, temporal adaptations in blood flow dynamics could benefit vision by means of constricting and emptying the spectacle blood vessels in times of visual need, such as when tracking predators, effectively removing the vessels from the visual field altogether. The work described here provides experimental evidence of such an adaptation.

2.2 Methods & Materials

The experiment described here involved the high-magnification imaging of spectacle blood flow in colubrid snakes and attempt to characterize the spectacle vascular dynamics under varying conditions, including at rest, when moulting, and when a visual threat is presented.

2.2.1 Animals

The experimental subjects were 3 coachwhip snakes (*Masticophis flagellum*, Colubridae) obtained from a local pet store and private keepers. They ranged in age from 2 to 5 years, with the following sizes: 130 cm (snout to vent) & 445 g; 120 cm & 320 g; and 97 cm & 240 g. The snakes were housed in separate terraria equipped with burrows and water dishes. Ambient temperature was kept at approximately 25˚C with daytime basking spots of ~31˚C, and lighting was on a 12:12 h light:dark cycle. They were fed to satiety once per week with frozen/thawed adult mice. A fourth specimen was excluded from the experiments due to high apparent stress levels.

2.2.2 Experimental Equipment and Setup

All observations were made using a Topcon SL-5D slit lamp to which was mounted a video camcorder (Sony HC-7) via a beam splitter. The subjects' eyes were illuminated with a near infrared(NIR)-filtered light source using a combination of cross- and retro-illumination. NIR was used to minimize disturbance to the subjects, as it is not perceptible by coachwhips who lack the infrared-sensing pit organs found in crotaline vipers and in some boas and pythons. This required modifying the slit lamp by placing near-infrared (NIR) filters in the light path between the lamp and condenser. The filters consisted of either exposed negative photographic film (Kodak Portra 160VC on a polyester base) or unexposed positive film (Kodak Ektrachrome Duplicating Film on a polyester base) processed to full density (see Figure 2-2, next page, for transmission spectra).

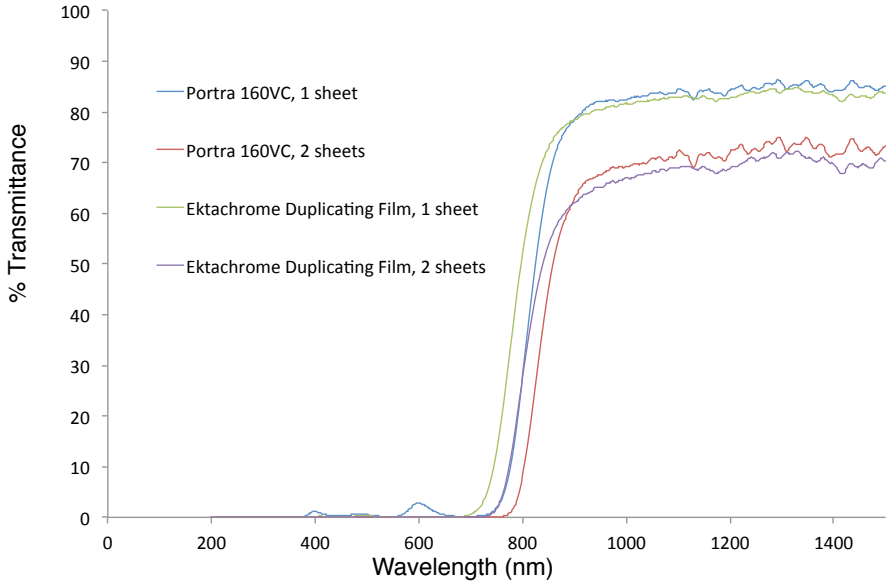


Figure 2-2. Transmission spectra of photographic films used as NIR filters. Single sheets provide insufficient blockage of the visible spectrum: Ektachrome duplicating film because it passes far red wavelengths, and Portra 160VC because it has several windows of transmission in the visible range. Double layering either film provides sufficient blockage of the visible spectrum, though this also attenuates some NIR, necessitating an increase in the gain of the imaging system.

Using photographic film as a NIR filter has the advantage of cost and adaptability of the material - it is thin, deformable and easy to cut to shape. The drawback is that in addition to blocking the visible spectrum, it also attenuates some NIR, effectively acting as a neutral density filter that limits the brightness of the image obtained.

Because non-visible illumination was used, the camcorder was modified by replacing its NIR-blocking filter with a clear glass window of equal thickness. Using a custom-fashioned filter adapter, the camera was mounted by its filter thread to a beam splitter on the slit-lamp which permitted monitoring of the image on its LCD screen. With the camera's zoom lens set to the longest focal length and using the 40x objective of the slit lamp, the system was able to resolve a lower limit of approximately 12 µm which is just sufficient to resolve individual erythrocytes that measure ~12.6 µm on the short axis in *M. flagellum* (Hartman & Lessler 1964).

To allow for extended high-magnification observations, it was necessary for the animals to remain still. To restrict a subject animal's mobility, it was placed within a custom-built acrylic box transparent to both visible and NIR wavelengths (see Figure 2-3 for transmission spectrum) and with internal dimensions of 3.5 cm x 5 cm x 95 cm (H x D x W).

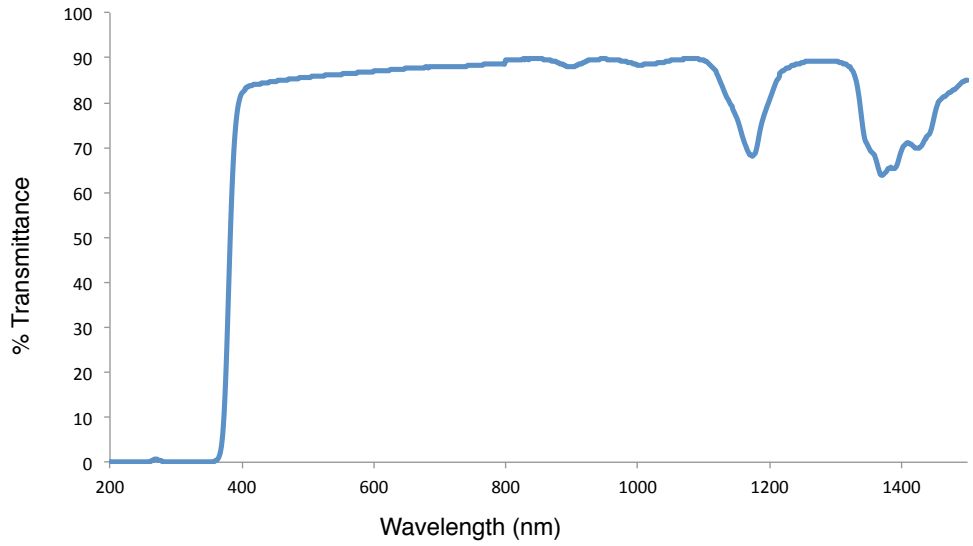


Figure 2-3. Spectral transmission of the acrylic material used to fashion the holding box. It exhibits high spectral transmission throughout the visible and NIR ranges.

In practice, this box was large enough that the animal was unrestrained, but was nevertheless constrained to a small space. After an initial period of agitation upon placement in the box, coachwhip snakes were found to settle down and remain mostly stationary, making them an ideal model animal for this type of observation (quite unlike corn snakes that absolutely refuse to remain still under the same conditions).

The box was mounted on a tripod, allowing for easy placement of one of the animal's eyes to within the focal range of the slit-lamp, and allowing for quick minor adjustments throughout the experiment to compensate for slight shifts in the animal's position. Figure 2-4 (next page) illustrates the experimental setup.

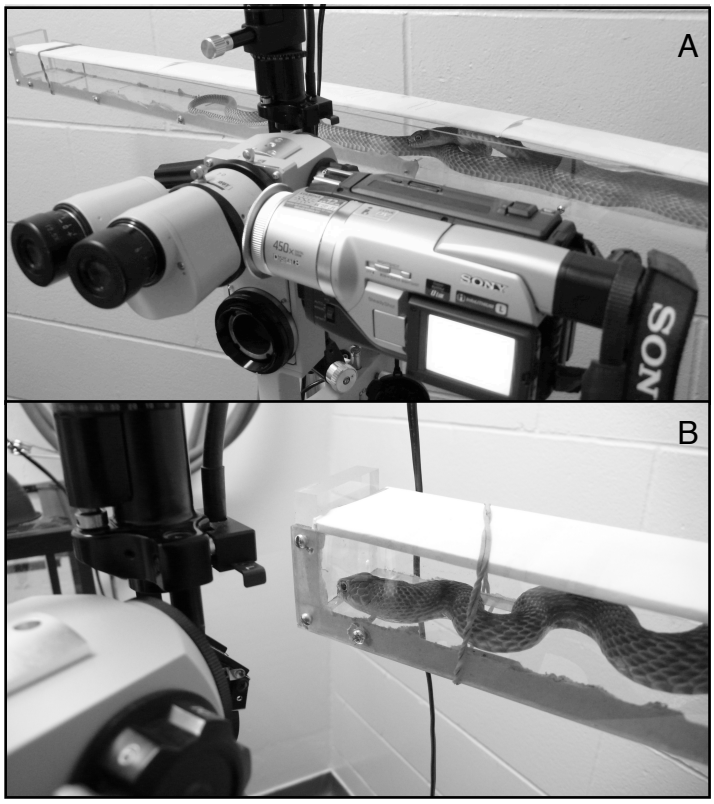


Figure 2-4. Experimental setup. A: the whole imaging system with the snake in a holding box (the curtains that obscure the experimenter are not shown). B: a coachwhip snake with its eye next to the pane of the holding box.

To prevent the snake from observing the experimenter, curtains were mounted on the slit-lamp table, which allowed only the slit-lamp objectives and illumination arm to be visible to the snake. Thus hidden, the experimenter was free to manipulate the controls of the slit-lamp and enter data without visually alerting the animal.

2.2.3 Experimental Protocol

Animals were left to acclimate in the transparent box for 30 minutes prior to data collection, during which time the box and slit-lamp were adjusted to bring one of the animal's eyes into view. After this setup and acclimation time, the experimenter began recording to the nearest second the times when blood flow in the spectacle began and stopped. Owing to the transparency of the spectacle blood vessel walls, it proved difficult to reliably observe their constriction and dilation, so the presence and absence of erythrocytes was used instead as the measure. Data were recorded for 70 consecutive minutes.

At 30 minutes into the experiment, a potential threat to the snake was simulated by having the white-coated experimenter step out from behind the curtain for 8 minutes, and perform routine laboratory activities within 1.5 meters of the boxed snake. It should be noted that in the case of these captive coachwhips, a white-coated human was deemed an effective threatening stimulus as evidenced by their exhibition of vigilance behaviour and occasional attempt to hide or flee when their terraria were approached.

After the 8 minutes, the experimenter returned behind the curtain. The simulated threat was repeated 16 minutes later, when the experimenter again stepped out from behind the curtain for 8 minutes. The experiment was concluded 8 minutes after cessation of the second simulated threat. The timeline of a trial is illustrated in Figure 2-5. The decision to present the threat for 8 minutes and do comparisons on blocks of 8 minutes was based on preliminary observations that the spectacle blood flow could cease for up to approximately this amount of time under the pilot experimental conditions.

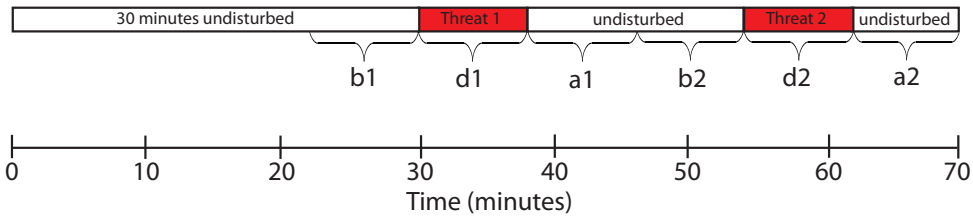


Figure 2-5. Timeline of an experimental trial. Each trial lasted for 70 minutes, with the subject remaining undisturbed for the first 30 minutes. This was followed by 8 minutes during which a visual threat was presented (Threat 1), after which it was removed to leave the subject undisturbed for 16 minutes before the threat was presented a second time for 8 minutes (Threat 2). The trial ended after the subject was left undisturbed for another 8 minutes. b1, d1, a1, b2, d2, a2 refer to the 8 minute blocks before, during and after the first and second threats from which statistical comparisons were made.

Each of the three snakes were tested 7 times on as many days, ensuring that they were only tested once per day. Although observations were made during the snakes' moulting phase, experiments were not conducted during this time due to clouding of the eye, changes in behaviour, and changes in

spectacle blood flow dynamics which became apparent in preliminary studies and which will be discussed. All experimental observations were done at an ambient temperature of 27˚C under fluorescent illumination of approximately 290 lux.

All experimental procedures were in accordance with the animal utilization guidelines of the University of Waterloo, the Canadian Council of Animal Care, and the Ontario Animals for Research Act.

2.2.4 Data Analysis

From the datasets were extracted the amount of time of each period with flow and each period without flow for each trial of each subject. To determine if there was a significant difference in the initial 30 minute undisturbed phase between the durations of each period, between experimental subjects, or if habituation was taking place between trials, comparisons were made between the durations using univariate multifactorial ANOVA with the following three factors: 1- day of the experiment (of which there were 7), experimental subject (of which there were 3), and presence or absence of flow (binary factor). Probability values of Type 1 errors equal to or less than 0.05 were considered statistically significant.

To determine if there was any change in the proportion of spectacle blood flow during periods of perceived threat, the total durations of periods without flow were converted to sine-transformed proportions between 0 and 1 and compared with the 8 minutes of observed blood flow both before and after each threat presentation. Three factors were again taken into consideration in these analyses: the day of the experiment, the threat event within each experiment (of which there were 2), and the block of time either before, during, or after the presented threat. The data were analyzed with univariate multifactorial repeated-measures ANOVA, and calculations were corrected with the Greenhouse-Geisser epsilon to account for non-sphericity in the data. Statistical significance was again set a threshold of 0.05.

All statistical analyses were performed using the Systat 13 and Mystat 12 statistical software packages.

2.2.5 Additional Observations

In addition to the main experiment, observations were made on spectacle blood flow in physically restrained snakes (hand-held), in moulting snakes, and in geckos.

The first attempts to document spectacle blood flow were done whereby the snake's head was handheld while making observations with a slit-lamp or stereomicroscope. Although at the time, this was merely an attempt to stabilize the animal's head, in retrospect it's understood that this will have elicited a strong sympathetic response, so observations done under these conditions were reviewed in this light.

Spectacle blood flow was observed and video-recorded in snakes that were were in the renewal phase of their integument as judged by the opacification of the spectacle. Observations were made on coachwhip snakes (*M. flagellum*) and corn snakes (*Elaphe guttata*). Unlike the situation in the main experiment, moulting corn snakes tended to remain still within the experimental box long enough to make observations. The experimental setup for observing the corn snakes was identical to that for the coachwhip snakes including the transparent acrylic holding box, the NIR-modified slit-lamp and camera, and an ambient temperature of 27ºC with approximately 290 lux of illumination. The only difference in the case of a yearling individual was the size of the holding box being reduced to 2 cm x 2 cm x 15 cm (H x D x W). Based on the assumption that visual clarity would be poor due the clouded spectacle, the effect on blood flow of a perceived threat was elicited either by physically touching the snakes or by jostling their holding box.

Attempts were made to observe spectacle blood flow in coachwhips while food (frozen/thawed mice) was presented. A juvenile mouse was held by 10 cm forceps and inserted into the holding box. In no case did a snake attempt to feed, so this avenue was discontinued.

Observations were also attempted on 2 gekkonid geckos: an adult marbled gecko (*Gekko grossmanni*) and an adult giant day gecko (*Phelsuma madagascariensis grandis*). The experimental setup in this case was similar to that involving the snakes, except for the dimensions of the transparent acrylic holding box which were 3.5 cm x 10 cm x 25 cm (H x D x W). In the case of the day gecko, its behaviour proved unsuitable due to its unwillingness to remain stationary, so observations could only be realistically made on the marbled gecko.

2.3 Results

2.3.1 Spectacle blood flow in undisturbed snakes

During the initial 30 minutes of the trials, when snakes were at rest and undisturbed, significant differences were found between the durations of flow (M = 57 s, SD = 49 s) and empty periods (M = 115 s, SD = 80 s) when data from all snakes were pooled ($p < 0.000$). Generally, durations of flow were shorter than empty periods (see Table 2-1). Differences between individual snakes were seen in the durations of flow periods ($p = 0.006$), but not in empty periods ($p = 0.640$). In the cases of both flow period and empty period durations, there were differences between trials ($p_{(\text{flow duration})} = 0.000$; $p_{(\text{empty duration})} = 0.017$), suggesting significant variation from day to day. The means and standard deviations of flow and empty periods for all subjects are tabulated in Table 2-1. Figure 2-6 (next page) is a graphical representation of spectacle blood flow and empty periods over the initial 30 undisturbed minutes during two trials in separate snakes. A video recording of the onset and cessation of spectacle blood flow in a coachwhip snake is available in Appendix A.

	Flow (s)				Empty (s)			
	Mean	SD	Min	Max	Mean	SD	Min	Max
Snake 1	47	17	12	114	130	71	44	490
Snake 2	66	42	13	214	116	91	7	399
Snake 3	59	68	7	458	101	76	12	360

Table 2-1: Durations of spectacle blood flow and empty periods while undisturbed. The means, standard deviations, minima and maxima are shown for each experimental subject taken from all 7 trials.

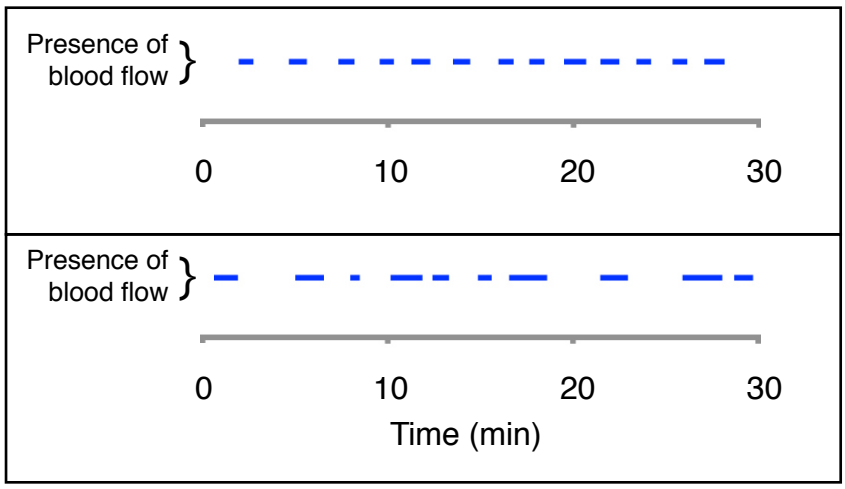


Figure 2-6. Two example trials showing blood flow patterns in undisturbed snakes. Blue dashes indicate when spectacle blood flow was apparent in the initial 30 minute undisturbed phase of two separate snakes.

2.3.2 Effect of threat perception on spectacle flow

Figure 2-7 (next page) illustrates the effects of threat perception on spectacle blood flow in two representative trials. During the 8 minutes of perceived threat, the mean duration of individual flow events was reduced to 33.5 s (standard deviation: 17.6 s), down from 57 s, when data from all subjects were pooled. As well, the total proportion of time during which flow occurred was reduced when compared with the 8 minutes prior to and after the presented threat ($p = 0.011$). No difference was found between trials ($p = 0.633$) nor between the first and second threat events within a trial ($p = 0.150$). Interaction terms between any or all of the 3 factors were also found to not be significant. Figure 2-8 (next page) shows a box plot of the proportion of time during which blood occurred before, during, and after each threat event.

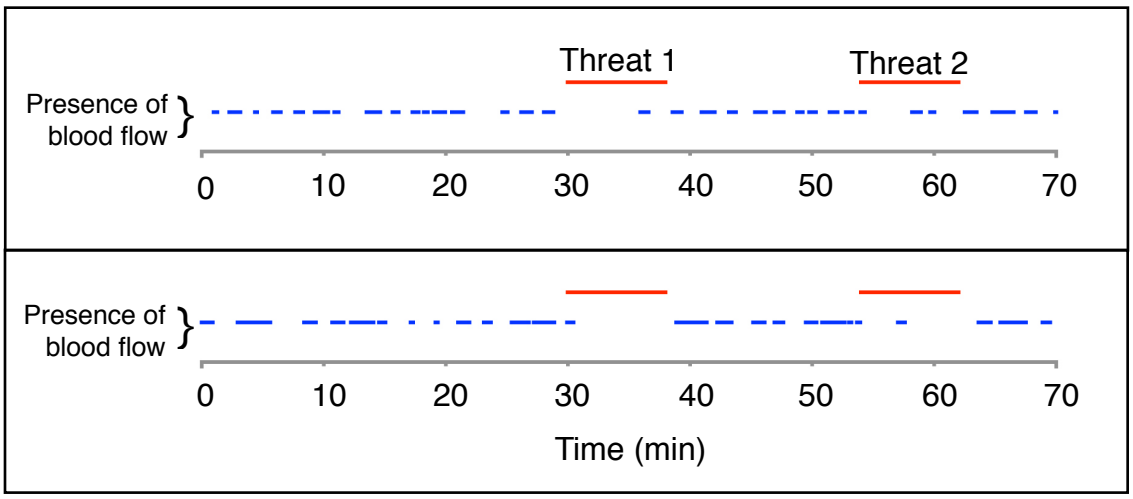


Figure 2-7. Graph of two representative trials. The blue lines indicate when spectacle blood flow occurred. The periods during which the threats were presented are indicated by the red lines labeled "Threat 1" and "Threat 2". A visual appraisal is sufficient to determine that the total duration of blood flow during the threat presentations is less than when the subject was undisturbed.

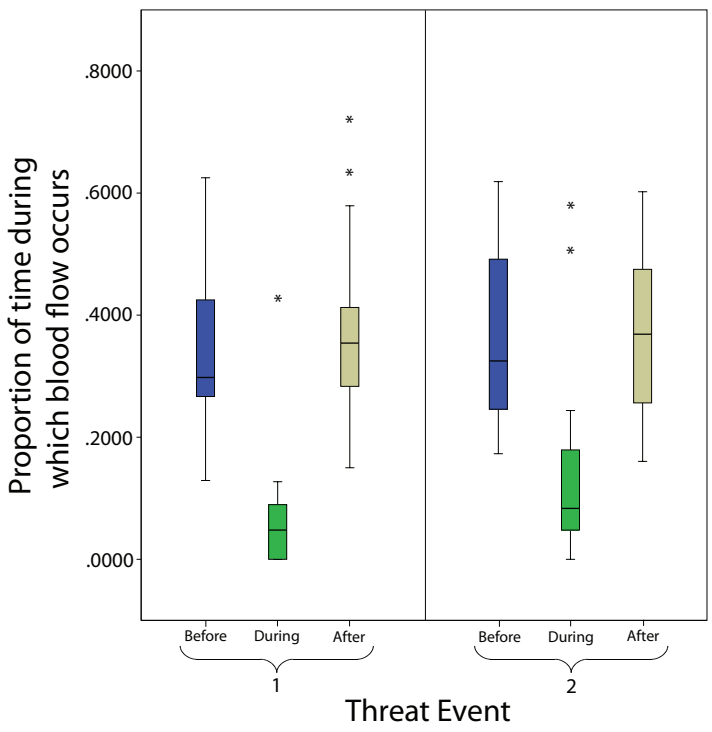


Figure 2-8. Plot of the proportion of time during which spectacle blood flow occurred before, during and after each threat event. The plots show the mean (horizontal bar), the 25%-75% intervals and the standard deviation, as well as outliers. It can be seen that the proportion of flow during the threat events is less than the 8 minute blocks before and after each threat was presented. As well the 25-75% intervals are very similar in the 8 minutes before and after each threat, indicating a rapid return to baseline after the threat is removed.

2.3.3 Additional Observations

Spectacle blood flow was found to altogether stop when a subject was physically restrained, presumably due to a strong sympathetic response. Due to what was perceived as unnecessary stress that could negatively impact subsequent experiments, these observations were not carried out systematically to determine how long the vessels would remain empty during physical restraint.

During the renewal phase of the integument, spectacle blood flow was found to remain constant when the snakes were undisturbed - i.e. there was no constriction or emptying of the blood vessels. When handled or otherwise disturbed, blood flow would slow or stop for brief periods on the order of 1-10 s, but the spectacle blood vessels remained fully dilated and did not empty. Erythrocytes merely remained motionless within the vessels. A video recording of spectacle blood flow in a moulting juvenile corn snake is available in Appendix B.

2.3.4 Spectacle blood flow in geckos

Attempts at observing spectacle flow in the marbled gecko were largely unsuccessful, except for one brief moment when a single erythrocyte was noticed in the upper quadrant of the spectacle and quickly progressed upward out of view. The significance of this apparent lack of spectacle blood flow during experimental conditions will be discussed.

2.4 Discussion

The purpose of this study was foremost to document and characterize blood flow dynamics in the snake spectacle under various conditions according to factors both endogenous (when at rest and during moulting) and environmental (when a threat is visually perceived) and, in doing so, determine if these dynamics could support a mechanism for mitigating visual clarity loss due to the blood vessels.

Three characteristics of spectacle blood flow were apparent from these experiments: (1) regardless of whether the animal is at rest or disturbed, blood flow is discontinuous, except during the moulting phase; (2) the visual perception of a potentially threatening organism induces a reduction in the proportion of time during which blood flow occurs in the spectacle; (3) spectacle blood flow during the integument renewal phase remains strong and, though flow can stop briefly if the animal is disturbed, the vessels do not constrict or empty. While any visual consequences owing to the presence of the spectacle blood vessels are speculative, the discussions here will assume that they are, based on the facts that (1) no terrestrial vertebrate has non-retinal blood vessels in the transmissive regions of the eye and (2) Lüdicke's findings that the spatial distribution of spectacle blood vessels in *Ahaetulla nasuta*, a foveated vine snake, minimized the density of the vascular meshwork in the foveal and binocular fields. Only one study has measured the acuity of a snake (Baker *et al.* 2007), which was found in the midland banded water snake (*Nerodia sipedon pleuralis*) to be approximately 4.9 cycles/degree (cpd) using evoked telencephalic potentials. As a reference, the acuity of cats and dogs measured with similar techniques are respectively 3.2-6.5 cpd and 12.6 cpd (Berkley & Watkins 1973; Odom *et al.* 1983), and that of rats is ~0.44-1.2 cpd depending on specific technique used (Boyes & Dyer 1983). The water snake's acuity is thus quite good for a small eye and Baker *et al.* (2007) commented that larger-eyed snakes like coachwhips may well achieve higher acuity results. Although the acuity of coachwhips has not been measured, their large eyes, their ecology and their behaviour all imply a high reliance on vision (Greene 1997), suggesting that their visual clarity would be negatively impacted by blood flow through the spectacle vasculature. One anatomical advantage the coachwhip may have in minimizing the resolution of the spectacle vasculature in the retinal image is its

comparatively large pupil size even at full constriction (*pers. obs.*), because a larger aperture minimizes depth of field (Green *et al.* 1980). In tandem with the short focal lengths of snake eyes (Sivak 1977; Howland *et al.* 2004), species with small pupillary apertures at full constriction, such as some pythons and boas (Greene 1997), are more likely to perceive the spectacle vessels.

The cyclical nature of flow through the spectacle vessels may act to reduce their negative impact on vision, particularly in light of the transparency of the blood vessel walls (Mead 1976). When the vessels are absent of flow, they are hardly visible even with a slit lamp. The animals' perception and observation of potential threats therefore depends partly on the likelihood that the spectacle vessels were empty at the time the threats presented themselves. While these blood vessels are a permanent, immobile fixture of the spectacle, their location within the visual field will shift when the eyes are turned. As a result, visual adaptation to the blood vessels (eg. from Troxler's effect in which stationary targets appear to fade or disappear, Troxler 1804) would only occur when the eyes remain still for extended periods (Lettvin *et al.* 1968). It was found that at rest the coachwhip's eyes remain perfectly steady (*pers. obs.*), far more stable than a human subject's whose eyes exhibit constant minute shifts in gaze direction. This remarkable ability of snakes to maintain a steady gaze may thus eliminate their perception of the spectacle vessels as well as benefit their perception of motion through Troxler's effect.

Although likely to be subject to sympathetic innervation, the factors responsible for timing the resting cycles of dilation and constriction of the spectacle vessels remain unknown. However, as cutaneous vasculature, these vessels may be involved in thermoregulation (Bartholomew 1982). In pilot experiments, the proportion of spectacle flow appeared to be related to some degree on ambient temperature, with lower temperatures resulting in longer periods without flow (*pers. obs.*). This is consistent with the animals being moved from a warm terrarium (25-31ºC) to a lower ambient temperature, which would result in cutaneous vasoconstriction to minimize heat loss and maintain core temperature post-transfer (Morgareidge & White 1969; Rice & Bradshaw 1980; Bartholomew 1982).

The rapid recovery in the proportion of blood flow after removal of the threat suggests a neural mechanism is involved in control of spectacle blood flow. The question remains whether the observed

vascular changes were occurring only in the spectacle or if they occurred throughout the whole integument. After all, and in spite of its transparency and unique attributes, the spectacle is part of the integument, and at least in mammals, general sympathetic responses may be accompanied by localized cutaneous vasoconstriction (Nalivaiko & Blessing 1999; Blessing 2003) concurrently with localized cutaneous vasodilation (Vianna & Carrive 2005). An application of the same experimental methodology to observe cutaneous vasculature in other regions of the integument was unsuccessful, as the surface capillaries could not be discerned even at high magnification, possibly due to the size difference between these and the comparatively large spectacle capillaries, or because the translucency of the scales optically blurs structures beneath them. It also remains unknown whether the reported observations were due to a generalized sympathetic response or to a blood flow control mechanism specific to aiding vision. The end result is the same however: when a potential threat must be tracked or targeted, emptying and constriction of the spectacle blood vessels occurs. This would be of visual benefit in preparation of an attack or an escape that requires improved acuity to effectively carry out.

With regard to the constant spectacle blood flow during the moulting phase, this is presumably necessary to support the cellular proliferation involved in the generation of a new stratum corneum (Maderson 1985, 1998) as well as to bring to the region eosinophils which are associated with the sloughing process (Maderson 1965). This constant blood flow is therefore likely to occur across the animals' integument, bringing with it possible thermoregulatory implications during the renewal phase of the integument.

The difficulty in imaging blood flow in the gecko spectacle may be accounted for by its small blood vessels which were measured by Lüdicke (1971) to be 4-17 µm in width. It is not clear how a 4 µm blood vessel can pass a large gekkonid erythrocyte that measures at least 9 µm on the short axis (Saint Girons & Saint Girons, 1969; Starostová *et al.* 2005), but it is possible that the erythrocytes were just small enough to mostly go unresolved by the imaging system. It is also possible that *Gekko grossmanni* simply exhibits little blood flow to the spectacle under stressful conditions, either by having a greater resistance to anoxia or by capitalizing on the iridial blood flow to supply the cornea

and spectacle via diffusion through the aqueous or by the limbal blood supply via diffusion through the subspectacle fluid.

Though spectacle vessels are present in all snakes and likely in all spectacled squamate (see Chapter 1), it is comparatively unusual, but not unknown, for blood vessels to be present in the optical path of the anterior segment of other vertebrates' eyes. It can occur, for example, among mammals and birds in the form of corneal neovascularization associated with an underlying pathology (Lee *et al.* 1998; Chang *et al.* 2001; Williams & Whitaker 1994; Maggs *et al.* 2008). The Florida manatee, *Trichechus manatus latirostris*, exhibits non-pathological corneal vascularity (Harper *et al.* 2005), and although the anatomical distribution of the manatee's corneal vessel network has been described in great detail, the author is unaware of any work describing its corneal blood flow dynamics. Given the comparatively low visual acuity of these animals (Bauer *et al.* 2003), the evolution of compensatory blood flow mechanisms seems unlikely.

Further research will be necessary to determine if the results described here hold true for other species of snakes and squamates with spectacles or windowed eyelids, as well as other vertebrates that possess other forms of spectacle (eg. fish) and transparent eyelids (eg. frogs and turtles).

As integument, the reptilian spectacle, being adapted to the ocular need of tissue transparency, offers an unprecedented value as a means to study cutaneous vascular physiology. In combination with their large erythrocytes that are relatively easier to image than those of mammals, snakes may be an excellent model animal for studying peripheral vascular dynamics. As discussed above, a comparatively low proportion of spectacle blood flow was noticed in early trials when a snake was moved from a warm terrarium to a lower ambient temperature. The imaging techniques described here could be of significant utility in studying cutaneous blood flow dynamics during thermoregulation, or for any other purpose where the quantification of cutaneous vascular flow is called for.

Chapter 3, Spectral Transmission of Snake and Gecko Spectacle Scales

This chapter presents investigations of the spectral transmission and thicknesses of shed snake and gecko spectacle scales. A portion of this work was presented as a poster at the XXth biennial meeting of the International Society for Eye Research (ISER) in 2010.

3.1 Introduction

The spectral properties of the ocular media, from the cornea to the photoreceptors of the retina, play a significant role in tuning transmitted light as it transverses the optical tissues of the eye. Tissues may filter out harmful ultraviolet (UV) radiation or increase the contrast of the retinal image, as with the yellow lenses of squirrels (Walls 1931; Chou & Cullen 1984), some reptiles including snakes and geckos (Walls 1942; Röll *et al.* 1996; Röll 2000) and some fishes (Walls & Judd 1933; Kennedy & Milkman 1956; Muntz 1973), or the oil droplets in bird and reptile photoreceptors (Walls 1942; Rodieck 1973). The spectral transmittance and absorption of the various ocular tissues and fluids have been studied in all vertebrate taxa with some having received more attention than others. While they have been well described for fish (review in Muntz 1972; Muntz 1973; Hawryshyn *et al.* 1985; Chou & Hawryshyn 1987; Douglas & McGuigan 1989; Siebeck & Marshall 2001; Litherland *et al.* 2009) and mammals (Pitts 1959; Boettner & Wolter 1962; Norren & Vos 1974; Chou & Cullen 1984), comparatively few investigations have been done on individual media in birds (Emmerton *et al.* 1980; Håstad *et al.* 2009) and very little on reptiles (sea snakes: Hart *et al.* 2012) and amphibia in which only lens (Kennedy & Milkman 1956) and whole eye (Govardovskiĭ & Zueva 1974) measurements have been published.

Snakes and geckos uniquely offer an extra layer to the visual apparatus in the form of the spectacle, that corneous layer of integument that overlays their eyes. This spectacle may further tune the spectrum of light by absorbing or reflecting wavelengths that are unnecessary for or deleterious to an animal's vision (due to chromatic aberration or scatter, Sivak 1982; Sivak & Mandelman 1982) or that are potentially damaging to ocular tissues. But despite the potential of the spectacle in tuning transmittance spectra, it has not been subject to much research in this area. The spectral transmittance of the whole spectacle, including the scale, epidermis and dermis, has been published only for hydrophiid sea snakes (Hart *et al.* 2012).

Reptilian spectacles, as described in Chapter 1, are composed of soft tissues (dermal stroma and epithelia) and hard keratin. The dermis of the spectacle, presumably composed largely of collagen

like the cornea, may exhibit similar spectral properties. In no other species however does keratin contribute to the optical structures of the eye, as the corneal epithelium of non-spectacled species is non-keratinized. Keratin, therefore, is a unique material in the layered structure of the eye and thus may exhibit unique spectral characteristics and provide an extra "degree of freedom" in the evolution of ocular filtering.

The modest translucency of keratin in most biological structures has resulted in few studies of its spectral transmittance in the visible and ultraviolet wavelengths (for the sake of convenience, the term "visible spectrum" will be used to refer to that range visible to humans, that is from approximately 400 to 750 nm). It has been described for horse hair (Bendit & Ross 1961), human stratum corneum (Bruls *et al.* 1984) and for keratin films made from human hair extracts (Reichl *et al.* 2011), all of which show high transmittance through the visible range but whose spectral profiles differ slightly in the far UV-A range. Reichl *et al.* did not report on transmittance of human hair extracts below 300 nm, but both horse hair and human stratum corneum showed a characteristic peak in the UV-C at ~254 nm. The only account on the transmissive properties of the spectacle scale alone appears to have been by Safer *et al.* (2007) who reported on the transmittance only of the infrared spectrum.

The aim of the research presented here was primarily to document the spectral transmittance of shed spectacle scales of snakes and geckos to gauge similarity with known keratin spectra and to determine whether any observed variation might be accounted for by evolutionary relationships. Because of the role played by the spectacle in mechanical protection of the eye (Walls 1942), the thickness of the scales was also considered to determine if and how trade-offs might occur in balancing thickness and spectral transmittance.

3.2 Methods & Materials

This experiment involved the spectral transmittance and thickness measurements of spectacle scales from shed snake and geckos skins. Comparisons were made within and between families and correlations between spectral transmittance and thickness were calculated.

3.2.1 Shed skins

Shed skins from 43 species of snake (6 boids, 7 pythonids, 10 viperids, 3 elapids, 17 colubrids) were collected from personally owned snakes and from generous donations by private pet owners and zoos. The species investigated and the number of sheds from each species are summarized in Table 3-2, which due to its length is placed at the end of the chapter on page 74. As well, sheds from 2 species of gekkonid gecko, a giant day gecko (*Phelsuma madagascariensis grandis*) and a marbled gecko (*Gekko grossmanni*) were locally collected.

Because moulting snakes will often soak themselves in water to assist in softening the skin, sheds were air dried upon local collection or receipt from off-site sources. They were then stored in paper envelopes until ready to be analyzed. This was done to prevent spoiling of the sheds which was found to occur with hydrated sheds kept in plastic bags.

3.2.2 Spectrophotometry

Spectacle scales were cut from the shed skins and mounted with adhesive tape onto a sample holder with either an 8 mm aperture for larger scales or a 1 mm aperture for smaller scales. The sample holder was secured into a Varian Cary 500 UV-VIS-IR dual-beam spectrophotometer such that the scanning beams were passed through the scale from front to back (i.e. from surface to interior). Measurements were made in the range of 200-750 nm in 2 nm increments. Scans were performed on dry scales, as it was found that hydrated scales would gradually dry out during the scan, resulting in slight vertical shifts within the transmittance curves.

The scales of both the right and left eyes were measured for each specimen and the results averaged. A few specimens had only one useful spectacle scale in the shed, so measurements in these cases include only the one.

3.2.3 Spectacle thickness measurements

The thickness of spectacle scales were determined using a thickness gauge designed for measuring the thickness of hard contact lenses. Some samples, including the 3 elapids, were omitted due to having undergone biochemical analysis (presented in Chapter 4) prior to thickness measurements.

3.2.4 Analytical methods

Spectral transmittance curves were plotted for visual inspection of the spectra. The 50% cutoff wavelengths ($\lambda_{50\%}$) were determined from the raw data of each sample. To test for species and family differences in $\lambda_{50\%}$, Kruskal-Wallis analysis of variance on ranks was used due to potential non normality of the data (the number of Elapid samples was too low to test for normality). Differences between families were assessed with Dunn's multiple comparisons. Spectacle scale thicknesses were compared between families using the same methods. Correlations between thickness and $\lambda_{50\%}$ values was assessed with Spearman's R correlation coefficient on ranks. For all analyses, the threshold for statistical significance was set to a Type I error probability of 0.05. Analyses were done using the Statistica 10 software package.

3.3 Results

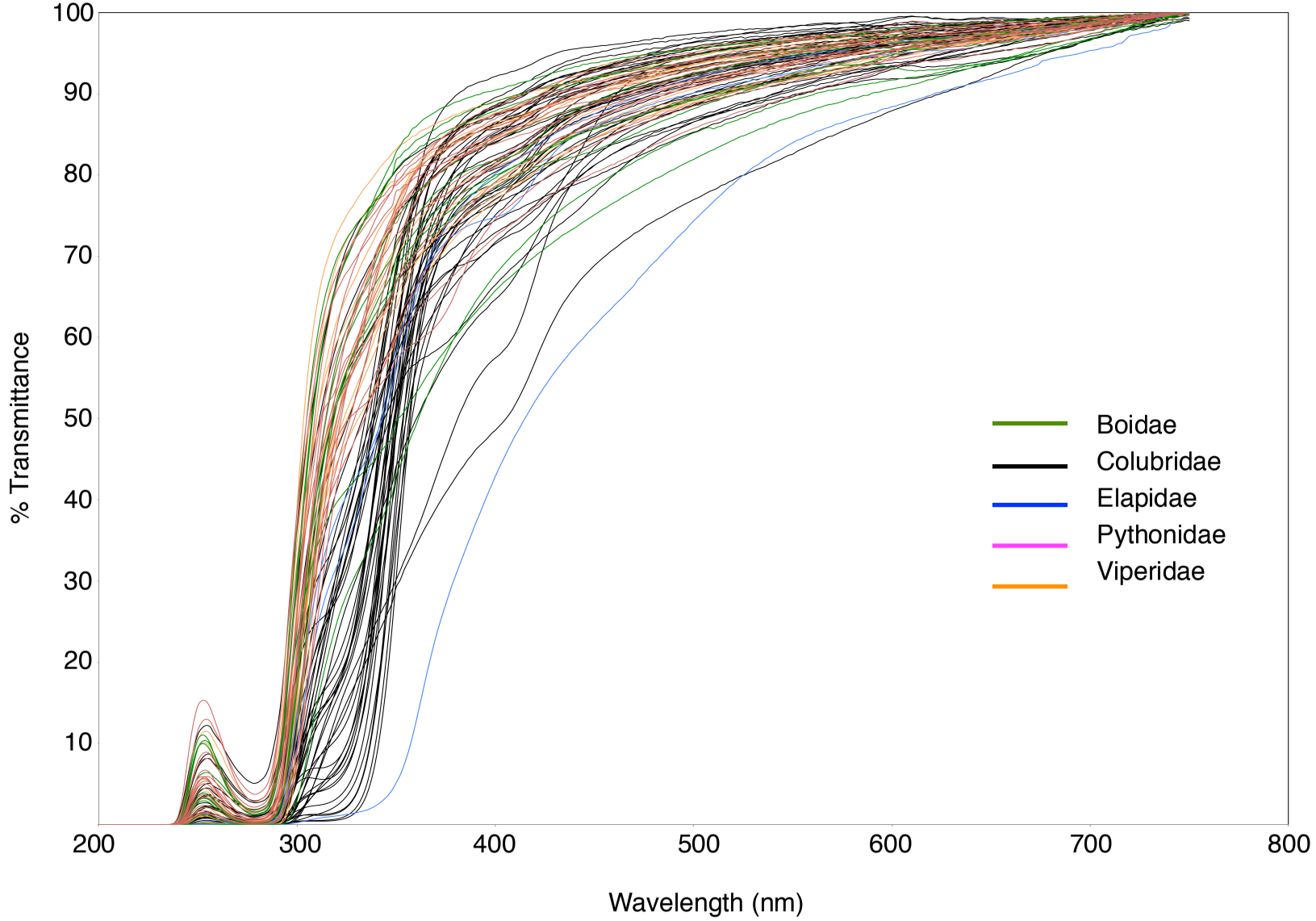
3.3.1 Snake Spectacle Scale Transmittance

The spectral transmittance curves of every snake sample measured in this study are plotted in Figure 3-1, which, although cluttered, clearly shows the variation encountered. With most species, the spectacle scales exhibit relatively high transmittance throughout the so-called visual spectrum. A gradual tapering from 750 nm is evident and could possibly be accounted for by an increase in scatter with shorter wavelengths due to scratches and irregularities on either surface of the scale. As well, though great care was taken to properly center the scales in the scanning beam's path, slight decentration would reduce the measured transmittance due to refraction. Within the ultraviolet (UV) range (i.e. < 400 nm), significant variation is apparent in the profiles, with the degree of tapering and the cutoff wavelengths varying noticeably between species and families. Means, minima and maxima for each family and subfamily shown in Table 3-2. The $\lambda_{50\%}$ of individual sheds can be found in Table 3-2 on page 74.

Family	Subfamily	Mean $\lambda_{50\%}$ (nm)	Min $\lambda_{50\%}$	Max $\lambda_{50\%}$
Boidae		324	305	361
	Boinae	319	305	361
	Erycinae (*Charina bottae*)	351	n/a	n/a
Colubridae		347	312	406
	Colubrinae	344	312	358
	Xenodontinae (*Heterodon platirhinos*)	382	360	406
Elapidae		367	342	415
Pythonidae		318	305	334
Viperidae		317	303	331
	Crotalinae	316	303	325
	Viperinae (*Bitis gabonica*)	331	n/a	n/a

Table 3-1. Means, minima, and maxima of $\lambda_{50\%}$ of each family and subfamily. Subfamilies Erycinae, Xenodontinae, and Viperinae are represented by only one species. Those values marked as "n/a" indicate only one specimen was available for that subfamily.

Figure 3-1. Spectral transmittance curves of all the snake spectacle scales in the study, organized by family.



The spectral transmittance curves of individual families are plotted in Figure 3-2 (next page). The spectacle scales of boas (Fig 3-2A) of the subfamily Boinae generally have high transmittance throughout the visible and UV-A spectra, with mean $\lambda_{50\%}$ of 319 nm. An exception is the green anaconda with a significant reduction in transmittance beginning around 400 nm resulting in a $\lambda_{50\%}$ of 361 nm. The one sample of an erycine boa, the rubber boa, exhibits a spectacle transmittance curve with a gradual tapering beginning at greater wavelengths and a $\lambda_{50\%}$ of 351 nm. A peak at 254 nm in the UV-C is apparent.

Spectacle transmittance of the Pythonidae (Fig 3-2B) is similar to that of Boinae in that transmittance remains high through the visible and UV-A spectra until cutting off abruptly in the far UV-A (mean $\lambda_{50\%}$ = 318 nm) and exhibit the same peak in the UV-C. The burmese python spectacle does exhibit a gradual decrease in transmittance from 400 nm, but not to the same degree as in the green anaconda.

The spectacle scale transmittance of Viperidae (Fig 3-2C) is similar in most respects to Pythonidae and Boinae.

Elapids (Fig 3-2D), in contrast, exhibit cutoffs at higher wavelengths (mean $\lambda_{50\%}$ = 367 nm). Among the elapids, the snouted cobra has a particularly unusual spectacle transmittance profile with a gradual reduction in transmittance apparent even above 500 nm. Its spectacle scale is in fact yellowish in appearance. No appreciable transmittance occurs in the UV-C range.

The greatest intrafamilial diversity (perhaps on account of the greater number of samples) is found within the colubrids (Fig 3-2E), in which $\lambda_{50\%}$ varies from 312 to 406 nm, some species with a transmittance peak in the UV-C, others without. While the spectacle scales of most species transmit highly through the visual and UV-A spectra with relatively sharp cutoffs, those of *Heterodon platirhinos*, the only sample of subfamily Xenodontinae (all others being of Colubrinae), attenuates UV and even the shorter wavelengths of the visible spectrum. Among the Colubrinae, the spectacles of most species present similar profiles but with shifted cutoff wavelengths. For example, those of *Lampropeltis* cutoff further in the UV-A near 310 nm, whereas *Masticophis flagellum*, the various species of *Pituophis* and several other genera have $\lambda_{50\%}$ closer to 350 nm.

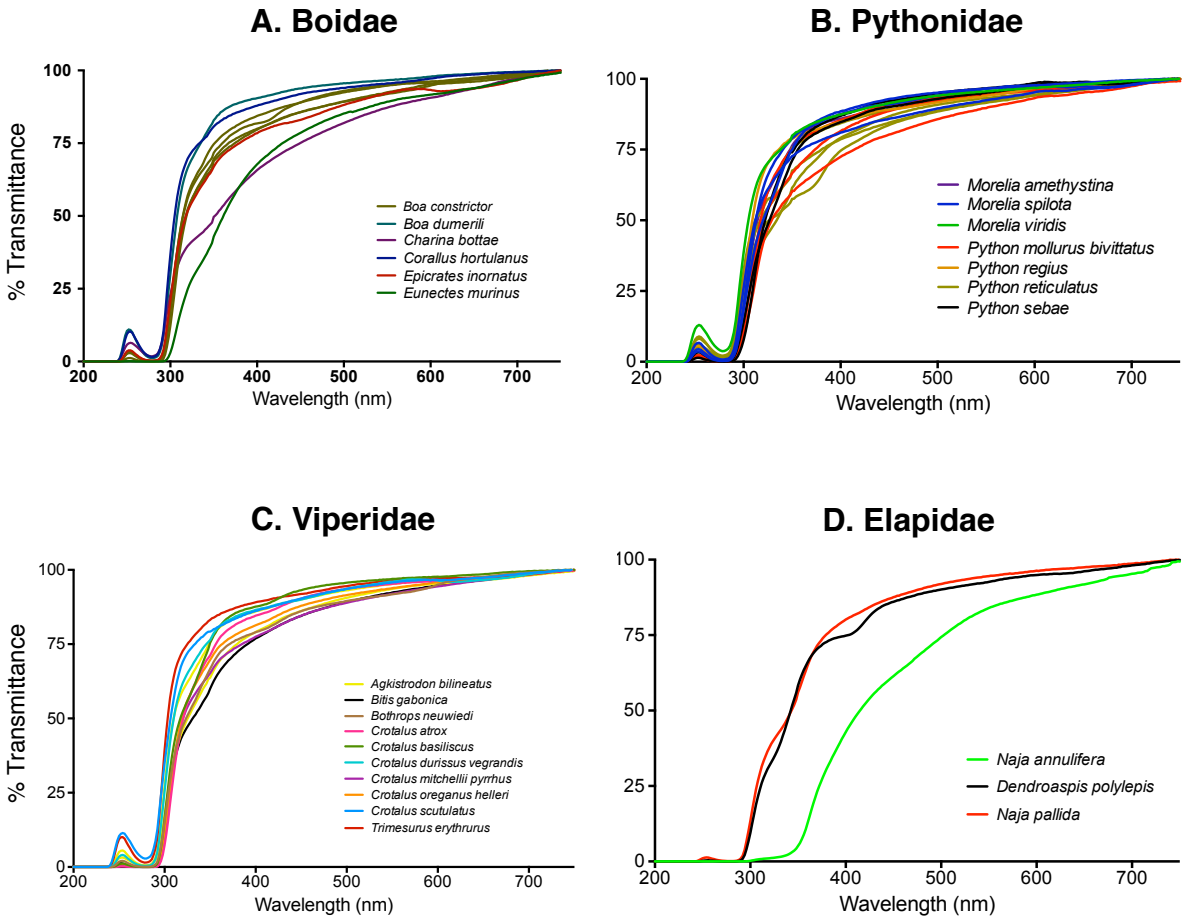


Figure 3-2. Spectacle scale transmittance spectra of individual families.

Transmittance profiles are generally similar in appearance with main differences being the lateral shift in the cutoff and the presence or absence of a peak at 254 nm. Unusual specimens in the Boidae (**A**) are *Eunectes murinus* (green anaconda) and *Charina bottae* (rubber boa) that show greater attenuation of short wavelengths compared with other boids. Pythonids (**B**) and viperids (**C**) all are generally similar. Among elapids (**D**), *Naja annulifera* (snouted cobra) stands out in having the lowest UV transmittance of any species with significant attenuation of even visible wavelengths. Of the colubrids (**E**), the genus *Lampropeltis* has the lowest spectacle scale $\lambda_{50\%}$ and the xenodontine *Heterodon platirhinos* the highest with greater UV blockage.

The spectacle scale transmittance spectra of hatchling and juvenile reticulated pythons (*Python reticulatus*) are plotted in Figure 3-3. Although not very dissimilar in profile from the older specimens, the hatchling shed does exhibit a slightly higher transmittance in the UV-A and an unusual "hump" at approximately 320 nm. While not likely of visual relevance to the animal, it may be indicative of the differences in its keratin composition (Chapter 4).

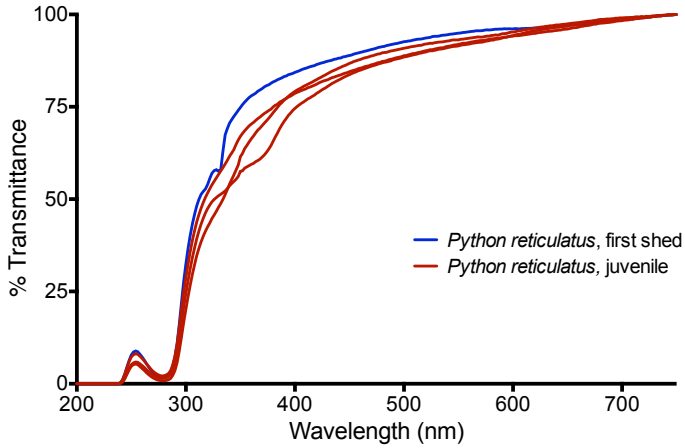


Figure 3-3. Spectacle scale transmittance spectra of *Python reticulatus*. Variations in transmittance spectra may show age-dependent Intraspecific variability. The first shed post-hatch shows an unusual "hump" in the transmittance curve that may be indicative of its different composition (discussed further in Chapter 4).

Spectacle scale transmittance spectra of individual colubrid genera and species are plotted in Fig. 3-4 (next page). All species of *Pituophis* (Fig 3-4A) show similar transmittance profiles. Greater variation is apparent in the genus *Elaphe* (Fig 3-4B) with differences occurring within even the *Elaphe obsoleta* complex. *Masticophis flagellum* (Fig 3-4C), the coachwhip snake, typically exhibits a very sharp cutoff at around 350 nm, although in one specimen suffering from chronic anorexia the transmittance spectrum extends further into the UV-A, possibly suggesting that overall health can influence transmittance spectra. Variation is seen as well in the genus *Lampropeltis* (Fig 3-4D), in which *Lampropeltis alterna*, the grey-banded kingsnake, exhibited the lowest $\lambda_{50\%}$ among the colubrids (min $\lambda_{50\%}$ = 312 nm).

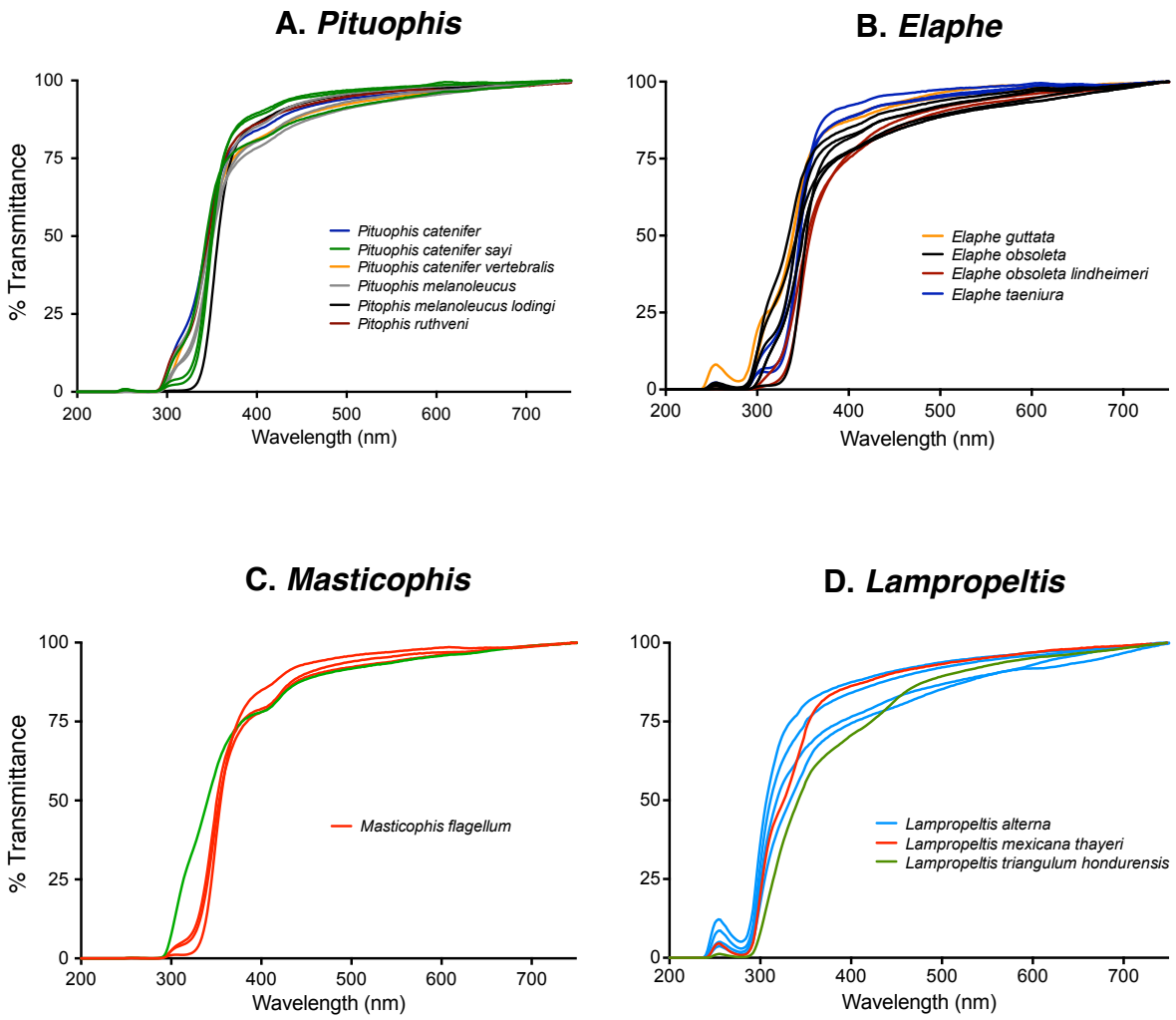


Figure 3-4. Spectacle scale transmittance spectra of individual colubrid genera. Transmittance spectra have generally similar profiles varying mainly in lateral shifts in the cutoff. The UV-C window at 254 nm is insignificant in all but *Elaphe guttata* (**B)** and *Lampropeltis* (**D**). Samples of *Masticophis flagellum* (**C**) have consistently high transmittance and sharp cutoffs near 350 nm, but for one individual that suffered from chronic anorexia (green trace).

3.3.2 Gecko Spectacle Scale Transmittance

The spectacle scale transmittance spectra of geckos, in sharp contrast with that of snakes, shows near 100% transmittance throughout the visible and UV-A ranges with a drop in transmittance occurring in the UV-B, but rising again in the UV-C with a characteristic peak at 254 nm before cutting off completely at ~240 nm (Fig. 3-5).

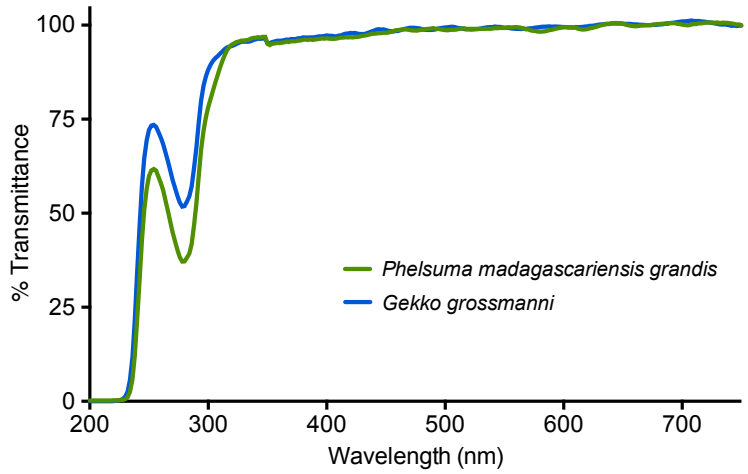


Figure 3-5. Spectacle scale transmittance spectra in gekkonid geckos. Gecko spectacles exhibit exceptionally high transmittance through the visible and UV spectra, close to or at 100% until dropping somewhat in the UV-B, before peaking again at 254 nm in the UV-C.

3.3.3 Statistical Analyses of $\lambda_{50\%}$

The $\lambda_{50\%}$ grouped by family are plotted in Figure 3-6 (next page). K-W test on ranks indicated a significant difference between families ($p < 0.0001$). Individual comparisons between families indicate that Colubridae exhibits significant differences in $\lambda_{50\%}$ compared with Pythonidae and Viperidae. No other significant difference was detected. These results are summarized in Table 3-3 (next page). However, as can be observed in Fig. 3-6, not only are the mean $\lambda_{50\%}$ of Elapidae considerably higher than Pythonidae and Viperidae, but all $\lambda_{50\%}$ are higher. A greater sample of elapids would be necessary to statistically establish a conclusive difference.

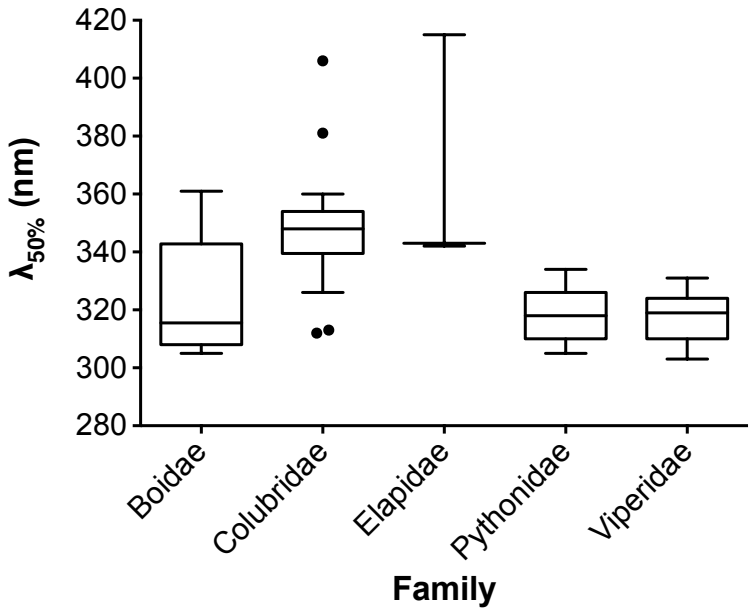


Figure 3-6. 50% cutoff wavelengths of spectacle scales grouped by family. Colubridae differs significantly from Pythonidae and Viperidae. The boxes show means and 25th and 75th percentiles, while whiskers are drawn according to Tukey's method (1.5 times the interquartile distance, truncated at the lowest and highest values). Statistical outliers in Colubridae are *Heterodon platirhinos* (higher $\lambda_{50\%}$) and *Lampropeltis alterna* (lower $\lambda_{50\%}$).

	Boidae	Colubridae	Elapidae	Pythonidae	Viperidae
Boidae (n = 6)		0.2812	0.8936	1.000	1.000
Colubridae (n = 17)	0.2812		1.000	0.0049	0.0023
Elapidae (n = 3)	0.8936	1.000		0.1242	0.1353
Pythonidae (n = 7)	1.000	0.0049	0.1242		1.000
Viperidae (n = 10)	1.000	0.0023	0.1353	1.000	

Table 3-3. Adjusted P values of multiple comparisons of $\lambda_{50\%}$ between families. Using Dunn's multiple comparisons on ranks, colubrid spectacle scales were found to differ significantly in transmittance spectra from those of pythonids and viperids. The small sample of elapids preluded significance despite a large apparent difference from boids, pythonids, and viperids.

3.3.4 Spectacle Scale Thickness

Mean spectacle scale thickness of each family is tabulated in table 3-4. The thicknesses of the spectacle scales for each specimen are tabulated in table 3-2 on page 74. The spectacle scales of geckos are quite thin at 3-4 μm, thinner than even the thinnest among snakes (mojave rattlesnake, *Crotalus scutulatus*, at 5 μm, followed by the green tree python, *Morelia viridis*, at 10 μm). The thickness of snake spectacle scales varies even within species, though the cause of this variation is unknown.

The thickness of spectacle scales differs significantly between families (K-W $p < 0.0001$). Thicknesses grouped by family are plotted in Fig. 3-7 (next page). Colubrids have significantly thicker spectacle scales than all other families measured, with no differences found between the other families (Table 3-5, next page).

Family	Mean Thickness (μm)
Boidae	16
Colubridae	30
Pythonidae	16
Viperidae	14

Table 3-4. Mean thicknesses of spectacle scales grouped by family. Colubrids have significantly thicker spectacle scales than boids, pythonids, and viperids, which differ little among themselves.

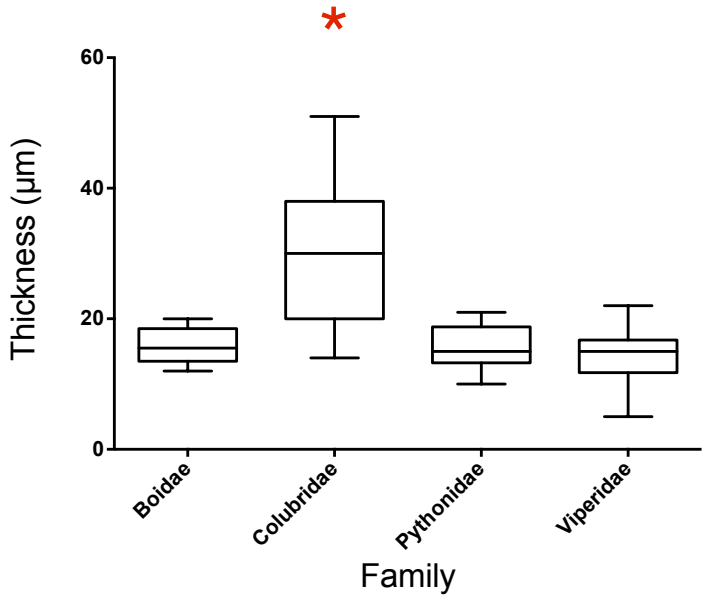


Figure 3-7. Plot of spectacle scale thicknesses grouped by family. Colubridae is seen to differ significantly from the other families (red asterisk) in having a greater mean spectacle scale thickness. In no other family does the highest value match or exceed the Colubridae mean. The boxes show the mean and 25th and 75th percentiles and the whiskers are drawn according to Tukey's method (1.5 times the interquartile distance, truncated at the lowest and largest values).

	Boidae	Colubridae	Pythonidae	Viperidae
Boidae (n = 4)		0.0303	1.000	1.000
Colubridae (n = 15)	0.0303		0.0084	0.0155
Pythonidae (n = 6)	1.000	0.0084		1.000
Viperidae (n = 5)	1.000	0.0155	1.000	

Table 3-5. Adjusted P values of multiple comparisons of spectacle scale thickness between families. Using Dunn's multiple comparison test on ranks, colubrid spectacle scale thickness is found to differ statistically from that of all other families.

3.3.5 Correlation of spectacle scale thickness and $\lambda_{50\%}$

Figure 3-8 shows a plot of thickness versus $\lambda_{50\%}$ of all snake families combined. Correlation analysis was significant ($p < 0.0001$) and fairly strong (Spearman's rho = 0.7713). Correlation analyses within individual families are summarized in Table 3-6 (next page). The only statistically significant comparisons were for colubrids as a whole ($p = 0.0007$, Spearman's rho = 0.6320) and within its subfamily Colubrinae ($p < 0.0001$, Spearman's rho = 0.7338). No other correlation was found in other families and subfamilies.

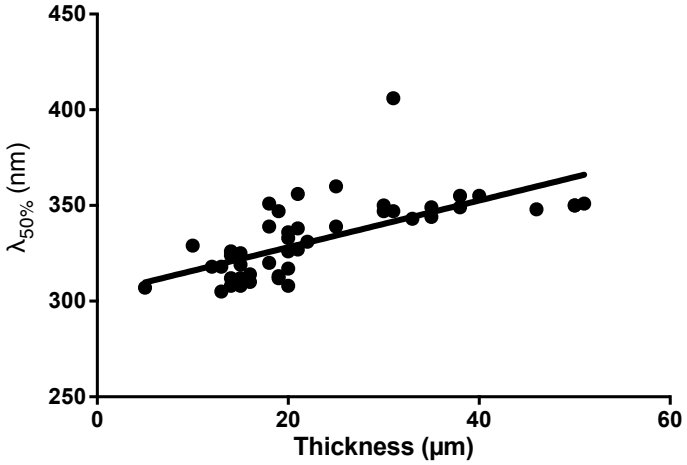


Figure 3-8. Correlation and regression plot of $\lambda_{50\%}$ versus scale thickness. Data from all families are pooled and show a positive and significant correlation (Spearman's rho = 0.7713, $p < 0.0001$). A linear regression line is drawn to assist in visualizing the trend.

Family	Spearman's rho	p
Colubridae	0.6320	0.0007
Colubridae (Colubrinae only)	0.7338	< 0.0001
Boidae	0.2899	0.5778
Boidae (Boinae only)	-0.0513	0.9000
Pythonidae	-0.0989	0.7246
Viperidae	0.7084	0.1167
Viperidae (Crotalinae only)	0.4588	0.5000

Table 3-6. Intrafamilial correlation analyses of spectacle scale $\lambda_{50\%}$ and thickness. A significant correlation is seen only in Colubridae.

3.4 Discussion

The purpose of this study was to determine if variation exists in the transmittance spectra of spectacle scales and, if so, whether evolutionary relationships or ecological factors could account for any differences. Significant differences were indeed found between families and unique spectra were observed in a few species, attesting to the diversity of which the spectacle is capable and its significance in tuning the spectrum of incident light.

3.4.1 Differences Between Families in Spectacle Scale Transmittance Spectra

Most spectra exhibit high transmittance in the visible and near UV-A ranges but begin to differ in the middle UV-A as evidenced by the variation in cutoff wavelengths. The Colubridae differ markedly in spectacle scale transmittance from Pythonidae and Viperidae, as well as most Boidae other than the green anaconda (*Eunectes murinus*) and rubber boa (*Charina bottae*). The high $\lambda_{50\%}$ of Elapidae seems to parallel that of colubrids.

A point should be made that most of the samples within any given family were not only of a specific subfamily, but also from a restricted number of genera, somewhat skewing the representation. Single species were available from other subfamilies, and in some cases, the spectacle scales of these presented rather different transmittance spectra. The xenodontine hognose snake, *Heterodon platirhinos*, showed the highest $\lambda_{50\%}$ (mean $\lambda_{50\%}$ = 382 nm, Max = 406 nm) among all colubrids, blocking much of the UV-A spectrum. Among the Boidae, the erycine rubber boa (*Charina bottae*) has a spectacle scale with a high $\lambda_{50\%}$ (351 nm), second only to that of the green anaconda ($\lambda_{50\%}$ = 361 nm) within the Boidae. The spectacle scale transmittance of the viperine Gaboon viper, *Bitis gabonica* ($\lambda_{50\%}$ = 331 nm), also is higher than that of crotaline vipers (mean $\lambda_{50\%}$ = 316 nm). That such differences were found in these samples warrants further investigation to determine if they are representative of their respective subfamilies.

The $\lambda_{50\%}$ of spectacle scales is correlated with their thickness, particularly in colubrids, although it's unclear if thickness is the cause of and $\lambda_{50\%}$ the effect. The association may be indirect by

virtue of both being characteristic of colubrids. The question then remains of why colubrid spectacle scales are thicker and have higher $\lambda_{50\%}$ than other families, Elapidae and specific boids excluded. To speculate on this, a consideration of the functional effects of $\lambda_{50\%}$ and thickness is called for.

3.4.2 Functional Differences in Spectacle Scale Transmittance Spectra & Thickness

Functional differences between transmittance spectra would relate to both the perceptual capabilities of these animals and to the protection against harmful radiation afforded by the $\lambda_{50\%}$. Some species of snake and gecko have been shown to possess UV-A-sensitive cones (Loew 1994; Loew *et al.* 1996; Sillman *et al.* 1997; Sillman *et al.* 1999; Sillman *et al.* 2001; Davies *et al.* 2009; Yang 2010; Hart *et al.* 2012), suggesting that the visual perception of UV-A wavelengths is a common trait throughout these taxa. While the UV-A spectrum spans a broad region from 315-400 nm, it should be borne in mind that vision in this region will be restricted to the specific spectral sensitivity of an animal's short-wavelength sensitive (SWS) cones (or medium wavelength cones (MWS) with absorbance spectra extending into the UV-A), which varies between species according to their expressed opsins. The retinal absorbance spectra of three snakes included in this study have been characterized (*Thamnophis sirtalis* (Sillman *et al.* 1997), *Python regius* (Sillman *et al.* 1999), and *Boa constrictor* (Sillman *et al.* 2001)) with each being found to possess a UV-sensitive visual pigment with an absorbance peak around 360 nm, well above the cutoff frequency of the spectacle scale (*T. sirtalis*: 338 nm; *P. regius*: 309 nm; *B. constrictor*: 314-317 nm). Although UV perception is widespread and used in navigation (Coemans & Vos 1992; Hawryshyn 2010), foraging (Viitala *et al.* 1995; Siitari *et al.* 1999), and communication with and recognition of conspecifics (Fleishman *et al.* 1993; for a review, see Honkavaara *et al.* 2002), short wavelength radiation has also been implicated in ocular pathologies such as cataractogenesis and photochemical retinal damage (Sliney 1986; Taylor 1989; Wu *et al.* 2006), although most of these data are from mammals with little data available for reptiles.

The conspicuously yellow spectacle scale of the snouted cobra stands out in recalling the yellow lenses and corneas of some diurnal terrestrial vertebrates (Walls 1931; Walls & Judd 1933;

Walls 1942; Chou & Cullen 1984) and fishes (Walls & Judd 1933; Walls 1942; Kennedy & Milkman 1956; Muntz 1973), which are thought to function as barriers to UV and/or to increase contrast. Whatever the functional significance of its yellow spectacle, it certainly restricts vision in the UV-A and attenuates a significant portion of the blue region. The somewhat brown colouration of the spectacles of the hognose snake spectacle as well may function as a modest UV filter. The nature of the colouration in either of these species is not known, but may be contributed by pigments deposited in the scale during keratogenesis. Alternatively, it may result from staining by the animals' substrate, such as by tannins or quinone pigments.

The function of a thicker spectacle scale may relate to the spectacle's protective role against injury. Snakes and geckos probably owe their spectacles to a single common ancestor (see Chapter 1), but in other squamates such as scincids, the presence of a spectacle seems to correlate roughly with body size (Walls 1942; Greer 1983). This suggests that the value of a spectacle is less for larger species. While some species of python and boid achieve much greater sizes than colubrids, many are comparably sized as are crotaline vipers, indicating that body size within a family is unlikely to have influenced spectacle scale thickness. There is significant overlap in the habitats occupied by the colubrids and vipers in this study, so this as well is unlikely to influence scale thickness. Where the colubrids overall differ from vipers, boids and pythons is in their speed, which makes them more susceptible to ocular insults during locomotion (*Masticophis flagellum* is especially fast), and in their lack of an efficient envenomation mechanism or larger size, making them more vulnerable to predators and uncooperative prey. This latter point has been observed in a population of island tiger snake (*Notechis scutatus*) with a disproportionately high incidence of blindness due to injury by adult gulls' attempts at protecting their nests (Bonnet *et al.* 1999).

3.4.3 Activity Patterns & Ocular Morphology

An attempt to deduce relationships between diel activity patterns and $\lambda_{50\%}$ would be somewhat confounded by several of the sampled species not being conveniently classifiable as strictly diurnal or

nocturnal (Brattstrom 1952; Klauber 1997). The nominally nocturnal *Boa constrictor,* for example, is occasionally active diurnally (Martínez-Morales & Cuarón 1999; Chiaraviglio *et al.* 2003; Romero-Nájera *et al.* 2007), and some species of colubrids (eg. black rat snakes, *Elaphe obsoleta*) and crotaline vipers (eg, *Crotalus atrox*, *Crotalus viridis*) have been shown to shift their activity patterns according to latitude and seasonal temperatures (Gauthier 1967; Landreth 1973; Golan *et al.* 1982; Sperry *et al.* 2010), effectively flip-flopping between being diurnal and nocturnal or crepuscular.

We can nevertheless consider individual species with clear habits. The colubrid coachwhip snake (*Masticophis flagellum*) is an active diurnal predator inhabiting prairie, open pine forest and semi-arid habitats (Greene 1997). With a large minimum pupil size restricted by the protrusion of the lens through the pupil (van Doorn, *unpubl. obs*.) and no eyelids to shield them from incident radiation, the eyes of a coachwhip would be exposed to very high doses of UV. One might then speculate that its spectacle scale has such a sharp cutoff around 350 nm to permit high sensitivity over most of the visually relevant UV-A spectrum while shielding the eye from the most damaging shorter wavelengths. However, the coachwhip spectacle scale's high $\lambda_{50\%}$ may equally be a side-effect of the necessity for a thicker scale (up to 50 μm) to shield it from abrasive substrates, from ocular injury during strikes, or from a prey item disagreeing with its fate.

The rather low $\lambda_{50\%}$ of crotaline vipers, regardless of habitat and diel pattern, suggests the spectacle scale has not evolved a protective function against UV in this subfamily. Unlike coachwhips and many other colubrids (including many in this study such as *Pituophis, Elaphe,* and *Lampropeltis*), crotaline vipers have pupils that can constrict to far smaller apertures than a coachwhip as the lens does not protrude through the pupil (Beer 1898; Michel 1932). These small apertures are in themselves protective of harmful radiation to the eye. Many arboreal boids and pythonids, also with small minimum pupillary apertures, would receive a far lower dose of potentially harmful UV wavelengths than deserticolous crotaline vipers, yet they differ little in their spectacles' $\lambda_{50\%}$ with only the erycine rubber boa and the semi-aquatic, mostly nocturnal anaconda having spectacle scales with measurably higher UV cutoffs. Many elapids (but by no means all) also have minimum pupillary diameters constrained by the protrusion of the lens (Duke-Elder 1958; Greene 1997). This restriction could

conceivably influence the evolution of $\lambda_{50\%}$ (or vice versa). An analysis of the relationship between $\lambda_{50\%}$, pupil size and lens/iris relationship may thus be beneficial.

3.4.4 On Gecko Spectacle Scales

Compared with snake spectacle scales, those of geckos have extraordinarily high transmittance. While thinner than snakes at 3-4 μm, they are not much thinner than a mojave rattlesnake's (5 μm), yet the latter still exhibits the same transmittance profile as other viperids, including the strong attenuation of the UV-B and a much smaller peak at 254 nm than the gecko. Although the arboreal *Gekko grossmanni* is largely nocturnal and may need little protection from UV radiation (nor would its minuscule pupils allow much through anyway), *Phelsuma madagascariensis grandis* is active diurnally and exposed to as much UV as an arboreal snake, yet its spectacle scale lets pass a tremendous dose of UV comparable with *G. grossmanni*. Clearly, the gecko spectacle scale is simply engineered for maximal transmittance of all wavelengths transmissible by keratin.

3.4.5 Physical reasons for variation in $\lambda_{50\%}$

As previously mentioned, it is unclear if thickness directly influences $\lambda_{50\%}$, but if so, it would not be the only factor. The coefficients of correlation calculated in the study indicate that thickness accounts only for some of the variability, and even then only in Colubridae. Other factors influencing the $\lambda_{50\%}$ should be considered.

The surface ultrastructure of the spectacle scale may be one such factor. Using scanning electron microscopy, Campbell *et al.* (1999) found the spectacle scale of a python to be generally more smooth with fewer surface ridges than in other scales of the integument. As well, they reported observations of the spectacle exhibiting less optical scatter than pit organ scales. I am unaware of any reports on the surface ultrastructure of spectacle scales in other species of snake nor any species of gecko. The ultrastructure of spectacle scales and its potential relationship with their spectral properties deserves further investigation.

Another potential factor that may govern spectral transmittance of the spectacle scale is the biochemical composition of the keratins that make up the scales. While much research has been conducted on the keratin composition of snake and gecko skin (Alibardi & Toni, 2005a, 2005b; Toni & Alibardi 2007a), no previous work has been done on the specifics of the spectacle keratins. This gap in our knowledge inspired the work described in Chapter 4, which will present the first investigation of the keratin composition specifically of reptilian spectacles.

Table 3-2. List of individual spectacle scale samples used in the study, including 50% cutoff wavelengths and thicknesses. The list is organized by family, subfamily, and binomial nomenclature.

Family	Subfamily	Species	Common name	$\lambda_{50\%}$ (nm)	Thickness (μm)
Gekkonidae	Gekkoninae	*Gekko grossmanni*	Marbled gecko	243	4
Gekkonidae	Gekkoninae	*Phelsuma madagascariensis grandis*	Giant day gecko	246/266	3
Boidae	Boinae	*Boa constrictor*	Boa Constrictor	317	20
Boidae	Boinae	*Boa constrictor*	Boa Constrictor	314	16
Boidae	Boinae	*Boa dumerili*	Dumeril's Boa	308	15
Boidae	Boinae	*Boa dumerili*	Dumeril's Boa (juvenile)	308	14
Boidae	Boinae	*Corallus hortulanus*	Garden Tree Boa	305	
Boidae	Boinae	*Epicrates inornatus*	Puerto Rican Boa	318	12
Boidae	Boinae	*Eunectes murinus*	Green Anaconda	361	
Boidae	Erycinae	*Charina bottae*	Rubber Boa	351	18
Colubridae	Colubrinae	*Bogertophis subocularis*	Transpecos Ratsnake	336	20
Colubridae	Colubrinae	*Drymarchon couperi*	Indigo Snake	350	50
Colubridae	Colubrinae	*Elaphe guttata*	Corn snake	339	25
Colubridae	Colubrinae	*Elaphe guttata*	Corn snake (hatchling)	339	18
Colubridae	Colubrinae	*Elaphe obsoleta*	Black Ratsnake	347	19
Colubridae	Colubrinae	*Elaphe obsoleta*	Black Ratsnake	354	
Colubridae	Colubrinae	*Elaphe obsoleta*	Black Ratsnake	334	
Colubridae	Colubrinae	*Elaphe obsoleta*	Black Ratsnake	344	
Colubridae	Colubrinae	*Elaphe obsoleta lindheimeri*	Texas rat snake (leucistic)	355	38
Colubridae	Colubrinae	*Elaphe obsoleta lindheimeri*	Texas Rat snake (leucistic)	358	

Colubridae	Colubrinae	*Elaphe taeniura*	Beauty snake	343	33
Colubridae	Colubrinae	*Elaphe taeniura*	Beauty snake	347	30
Colubridae	Colubrinae	*Elaphe taeniura*	Beauty Snake	347	31
Colubridae	Colubrinae	*Lampropeltis alterna*	Grey-banded Kingsnake	313	19
Colubridae	Colubrinae	*Lampropeltis alterna*	Grey-banded Kingsnake	333	20
Colubridae	Colubrinae	*Lampropeltis alterna*	Grey-banded Kingsnake	312	14
Colubridae	Colubrinae	*Lampropeltis mexicana thayeri*	Thayer's Kingsnake	326	20
Colubridae	Colubrinae	*Lampropeltis triangulum hondurensis*	Honduran MIlksnake	342	
Colubridae	Colubrinae	*Masticophis flagellum*	Eastern Coachwhip	355	40
Colubridae	Colubrinae	*Masticophis flagellum*	Eastern Coachwhip	340	
Colubridae	Colubrinae	*Masticophis flagellum*	Eastern Coachwhip	350	50
Colubridae	Colubrinae	*Masticophis flagellum*	Western Coachwhip	354	
Colubridae	Colubrinae	*Pituophis catenifer*	Gopher Snake	348	46
Colubridae	Colubrinae	*Pituophis catenifer*	Gopher Snake	344	30
Colubridae	Colubrinae	*Pituophis melanoleucus*	Bullsnake	349	38
Colubridae	Colubrinae	*Pituophis melanoleucus*	Bullsnake	344	35
Colubridae	Colubrinae	*Pituophis melanoleucus*	Bullsnake	350	30
Colubridae	Colubrinae	*Pituophis melanoleucus*	Northern Pine Snake	350	
Colubridae	Colubrinae	*Pituophis melanoleucus*	Northern Pine Snake	351	50
Colubridae	Colubrinae	*Pituophis melanoleucus*	Southern Pine Snake	349	35
Colubridae	Colubrinae	*Pituophis melanoleucus lodingi*	Black Pine Snake	357	

Colubridae	Colubrinae	*Pituophis ruthveni*	Louisiana Pine Snake	348	
Colubridae	Colubrinae	*Spilotes pullatus*	Tiger Rat Snake	356	
Colubridae	Colubrinae	*Thamnophis sirtalis parietalis*	Red-sided garter snake	338	21
Colubridae	Xenodontidae	*Heterodon platirhinos*	Hognose Snake	381	
Colubridae	Xenodontidae	*Heterodon platirhinos*	Hognose Snake (hatchling)	406	31
Colubridae	Xenodontidae	*Heterodon platirhinos*	Hognose Snake (hatchling)	360	25
Elapidae		*Dendroaspis polylepis*	Black Mamba	342	
Elapidae		*Naja annulifera*	Snouted Cobra	415	
Elapidae		*Naja pallida*	Red Spitting Cobra	343	
Pythonidae		*Morelia amethystina*	Amethystine Python	310	16
Pythonidae		*Morelia spilota*	Carpet Python	313	
Pythonidae		*Morelia spilota*	Carpet Python	312	19
Pythonidae		*Morelia spilota*	Carpet Python	320	18
Pythonidae		*Morelia viridis*	Green Tree Python	305	13
Pythonidae		*Python mollurus bivittatus*	Burmese Python	308	20
Pythonidae		*Python mollurus bivittatus*	Burmese Python	329	10
Pythonidae		*Python mollurus bivittatus*	Burmese Python (juvenile)	319	15
Pythonidae		*Python regius*	Ball Python	309	15
Pythonidae		*Python reticulatus*	Reticulated Python (first shed)	312	15
Pythonidae		*Python reticulatus*	Reticulated Python (juvenile)	326	14
Pythonidae		*Python reticulatus*	Reticulated Python (juvenile)	318	13
Pythonidae		*Python reticulatus*	Reticulated Python (juvenile)	334	

Pythonidae		*Python sebae*	Rock Python	322	
Pythonidae		*Python sebae*	Rock Python	327	21
Viperidae	Crotalinae	*Agkistrodon bilineatus*	Mexican Mocassin	310	15
Viperidae	Crotalinae	*Agkistrodon bilineatus*	Mexican Mocassin	325	15
Viperidae	Crotalinae	*Bothrops neuwiedi*	Jararaca Pintada	324	14
Viperidae	Crotalinae	*Crotalus basiliscus*	Mexican West Coast Rattlesnake	317	
Viperidae	Crotalinae	*Crotalus durissus vegrandis*	Uracoan Rattlesnake	310	15
Viperidae	Crotalinae	*Crotalus mitchellii pyrrhus*	Southwestern Speckled Rattlesnake	320	
Viperidae	Crotalinae	*Crotalus oreganus helleri*	Southern Pacific Rattlesnake	319	
Viperidae	Crotalinae	*Crotalus scutulatus*	Mojave Rattlesnake	307	5
Viperidae	Crotalinae	*Crotalus atrox*	Western Diamondback	320	
Viperidae	Crotalinae	*Trimesurus erythrurus*	Redtail Viper	303	
Viperidae	Viperinae	*Bitis gabonica*	Gaboon Viper	331	22

Chapter 4, Biochemical Analysis of the Spectacle Scale with an Emphasis on Beta(ß) Keratins

This chapter describes investigations on the biochemical composition of spectacle scales of snakes and geckos.

4.1 Introduction

The reptilian spectacle is a layer of specialized integument that overlays the eyes of snakes, most geckos, and a varied array of other squamates (Walls 1942). A hard, optically transparent scale forms its outer surface. This scale is of a highly specialized nature, as few other reptilian scales achieves the same degree of optical transparency (see Chapter 3).

Ficalbi (1888b) established that the snake spectacle scale consists of two main layers, an internal and an outer stratum corneum, separated by a clear layer (see Figure 1.2 in Chapter 1). Given that the spectacle scale likely has the same anatomical layering as the rest of the reptile integument, the two main layers should correspond with the alpha (inner) and beta (outer) layers, distinguished by the predominant type of keratin of which they are composed, while the clear layer is likely to be an artifactual separation of the mesos layer that frequently occurs during histological procedures. Little research has been done on the fine anatomy of the spectacle stratum corneum specifically, but if it is assumed to be layered similarly to the integument, an intact spectacle would contain 4 layers (Maderson 1964; Landmann 1986; see Figure 4-1, next page): 1) an oberhautchen layer on the surface, which consists of surface ultrastructural features; 2) a hard beta layer beneath this, often thick in snakes, called as such because it is primarily composed of beta(ß) keratins; 3) the mesos layer, consisting of thin repeating layers of keratin and lipid; and (4) an alpha layer, referred to as such because it is primarily (but not exclusively) composed of α keratin (Maderson 1965; Maderson 1966; Alexander & Parakkal 1969; Maderson 1998; Alibardi 2005). Two additional α keratin layers form during the renewal phase: (5) the lacunar layer and (6) the clear layer. These are thought to be involved in shedding the old, outer stratum corneum and remain attached to the shed skin. Ficalbi briefly mentioned that the gecko spectacle is similar to a snake's other than perhaps a thinner stratum corneum. No other research has been published on the fine histology of the gecko spectacle, so the spectacle scale's layered structure is not known. Between scales is the hinge region which contains less or no ß keratin and is therefore more elastic, allowing for an unrestrained range of motion and

permitting growth between moults and expansion of the body wall in snakes during ingestion and digestion (Hinkley *et al.* 2002).

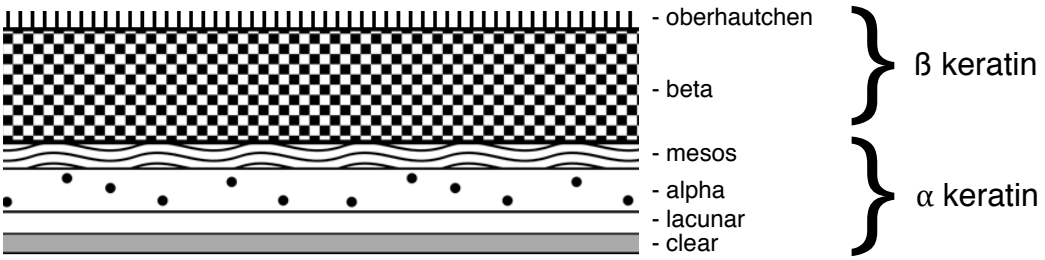


Figure 4-1. Diagram of the various layers of a shed reptilian scale. The layers can be grouped according to the predominant keratin of which they're composed, whether ß keratin or α keratin. The outer ß keratin layers consist of the oberhautchen ("upper skin/cuticle") that contribute to ultrastructural features on the scale surface and the beta layer that provides the primary mechanical barrier to the environment. The inner α keratin layers consist of the mesos layer that, with its interleaved lipid deposits, constitutes part of the permeability barrier, the alpha layer that contributes elasticity to the scale. The thin clear and lacunar layers develop only during the renewal phase.

Reptilian scales thus contain α keratin just as the stratum corneum of all tetrapods (some amphibians excepted), that resilient protein from which fur, nails and dander are made. But they additionally contain beta(ß) keratin, a harder and more rigid protein (Maderson 1964; Klein *et al.* 2010) that is expressed only in reptiles and birds where it forms or contributes to hard structures such as scales, feathers, claws, turtle scutes, adhesive gecko toe pads and bird bills (Alibardi 2003; Toni *et al.* 2007). These two distinct types of keratin can be distinguished by their size, molecular organization and material properties. ß keratins are ~8-26 kDa in size, have a core that forms classical beta-pleats (Fraser & Parry 1996; Toni *et al.* 2007) and as previously mentioned form structures that are mechanically more rigid. α keratins are ~40-70 kDa intermediate filaments that assemble into alpha helices and form integumentary structures that are generally more flexible.

The thicknesses of the various corneous layers of the integument appears to vary between species, although no systematic study has been done. Spectacle scales vary in thickness as was shown in Chapter 3, just as do other scales (van Doorn, *unpubl.*). Proportions of the various keratins could

thus be optimized to meet not only the specific needs of the organism but also allows the properties of individual regions to be tailored (Klein *et al.* 2010). Different ß keratins have been associated with specializations of the reptilian integument such as the adhesive toe pads of geckos (Alibardi & Toni 2005b).

A highly conspicuous example of scale specialization again is seen in reptilian spectacles in which the scale is optically transparent, in sharp contrast with most or all other scales which at best are only translucent (Figure 4-2).

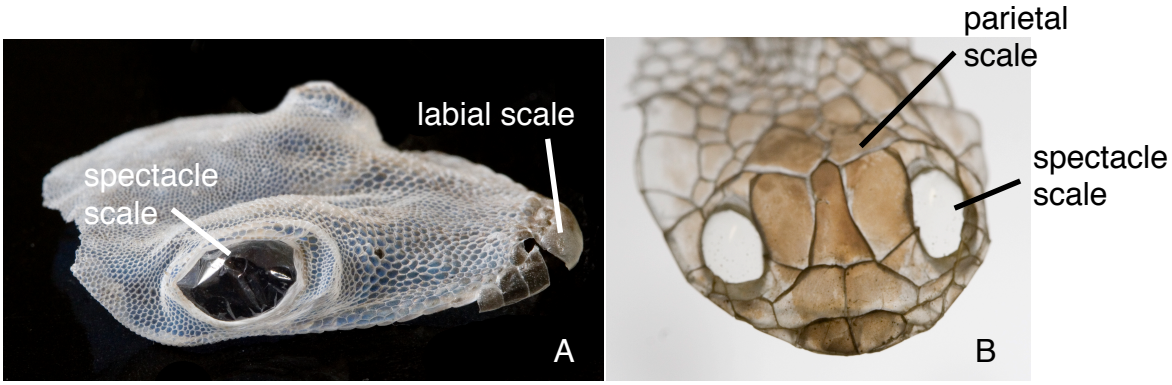


Figure 4-2. Moults from the head of a marbled gecko, *Gekko grossmanni* (A), and a coachwhip snake, *Masticophis flagellum* (B). In both species, the transparency of the spectacle scale is immediately apparent, contrasting with all other scales which are translucent at best and may be pigmented. The creases in the gecko spectacle scale attest to its pliability compared with that of the snake, which though not apparent from the photo's perspective, is far less pliable and almost recalls a hard contact lens. The labial scales of the gecko also show a degree of specialization by being larger and more rigid than the rest of the moult. Photos by K. van Doorn.

Chapter 3 established that spectacle scales vary in their spectral properties, with those of geckos exhibiting extremely high transmission throughout the visual and ultraviolet spectrum, while those of snakes maintained high transmission only through the visual and the higher UV-A spectra, showing significant differences between families in their cutoff frequencies. Compared with a snake's spectacle scale, the gecko's is also far more delicate, being easily damaged beyond the superficial scratches and abrasions often seen on snakes (*pers. obs.*, see Chapter 1).

In spite of considerable research on the biochemical nature of the reptilian integument, including recent efforts at sequencing the keratins expressed within it (Maderson 1964; Maderson 1998; Sawyer *et al.* 2000; Alibardi & Sawyer 2002; Alibardi 2003; Alibardi *et al.* 2007a, 2007b; Dalla Valle *et al.* 2009), the biochemical composition of the spectacle scale has thus far avoided scrutiny. An analysis of the spectacle scale's composition in snakes and geckos may therefore help to explain the differences in spectral transmission and rigidity. The research here will present electrophoretic analyses showing that the spectacle keratin complement differs from other scales in the integument, that it differs between other species, including congeneric ones, and it differs between snakes and geckos.

4.2 Methods & Materials

4.2.1 Sample Collection and Solubilization

Shed skins were collected from snakes (*Masticophis flagellum*, *Elaphe guttata*) and geckos (*Phelsuma madagascariensis grandis*, *Gekko marmoratus*) kept on site as well as from zoos and private pet owners (all other species). The spectacle scales from most sheds were used in the study of spectral transmission described in Chapter 3. Scales of interest were cut out, taking care to remove all surrounding material to ensure only the hard scales were used, with the exception of gecko dorsal scales which are too small to individually excise. Scales were briefly washed in turn in distilled water and 100% ethanol, ensuring that all debris was removed, and allowed to air dry. They were then placed in a solubilization buffer consisting of 7 M urea, 2 M thiourea, 25 mM tris-HCl (pH 8.5), and 5% ß-mercaptoethanol. Protease inhibitors were omitted due to thiourea's significant antiproteolytic property at concentrations of 2 M (Castellanos-Serra & Paz-Lago, 2002). Samples were left to digest for 12 hours at room temperature, then pulse sonicated on ice until visibly broken into smaller pieces, and again left at room temperature for 36 hours before being centrifuged and the supernatant collected and frozen at -80ºC. This method combines characteristics of two other established methods for keratin extraction. The recipe (the 2 M thiourea in particular) and 48 hour extraction time were inspired by the Shindai Method (Nakamura *et al.*, 2002) for extracting alpha keratins from human hair, while the higher urea content, mechanical pulverization, and room temperature digestion were adopted from methods used in the extraction of α and ß keratins from snake integument (Toni & Alibardi 2007a, who cite Sybert *et al.*, 1985, as the source of their method).

Protein concentrations were measured using a Bradford colorimetric assay (Bio-Rad Protein Assay, Bio-Rad) on samples diluted between 10-100 times with double-distilled water, which not only brought the protein concentrations to within the linear range of the assay, but also reduced the urea concentrations to levels that would not interfere with its accuracy.

4.2.2 SDS-PAGE

Prior to electrophoresis, samples were diluted with solubilization buffer to equalize the concentrations of protein between samples and again diluted 1:1 with 2x SDS reducing buffer (2% SDS, 25% glycerol, 5% ß-mercaptoethanol, 0.0625 M Tris-HCl pH 6.8, 1% bromophenol blue). As proteins would already have been denatured by the highly chaotropic solubilization buffer, the samples were not boiled, but instead sat at room temperature for 15 minutes prior to loading.

4.2.3 Electrophoresis

Samples (0.1 µg) were separated on 12% or 15% homogenous polyacrylamide gels using a discontinuous buffer system at 180V for 45 minutes. Gels were then either stained immediately with colloidal coomassie (Bio-Safe Coomassie Stain, Bio-Rad, or EZBlue, Sigma), or equilibrated in transfer buffer (25 mM tris, 192 mM glycine, pH 8.3) for 20-30 minutes prior to membrane transfer.

4.2.4 Western Blotting

Gels and 0.2 µm pore size Immun-Blot PVDF membranes (Bio-Rad) were both equilibrated in transfer buffer for at least 20 minutes prior to being transferred at a constant 350 mA for 1 hour using a Bio-Rad Mini Trans-Blot. The membranes were then twice rinsed for 15 minutes in tris-buffered saline (TBS:20 mM Tris-HCl pH 7.5, 500 mM NaCl) and blocked for 1 hour with Tween-TBS (TTBS: TBS + 0.05% Tween 20) with 1% skim milk. The universal ß keratin (univ-ß) antibodies which were then used as primary antibody probes were a generous gift from Dr. Roger Sawyer of the University of South Carolina who produced them to alligator ß keratins. These were diluted 1:1500 in TTBS with 1% skim milk and incubated with the membrane for 1 hour at room temperature. Membranes were then twice rinsed in TTBS for 15 minutes and incubated with secondary antibodies conjugated with horseradish-peroxidase (goat anti-rabbit IgG, Bio-Rad) diluted 1:3000. They were then rinsed three times in TTBS for 15 minutes to remove all protein not bound to the membrane and then twice in TBS

for 15 minutes to remove all residual Tween 20. Following this, either a chemiluminescent reagent (Amersham ECL Prime) was applied for 3 minutes and the resultant luminescence either photographed or scanned using a chemifluorescence imager (Storm 840, Molecular Dynamics), or the membrane was incubated with a colorimetric reagent (Opti-4CN, Bio-Rad) for 1-10 minutes, rinsed for 15 minutes in deionized water, and documented with a desktop scanner.

4.2.5 2-Dimensional Electrophoresis

2-Dimensional electrophoresis was performed on coachwhip spectacle and parietal scales to expose isoforms with varying pI's.

4.2.6 Isoelectric Focusing (IEF)

Samples were diluted with rehydration buffer (7 M urea, 2 M thiourea, 5% glycerol, 20mM DTT, 0.5% immobilized pH gradient (IPG) buffer ampholytes (Bio-Lyte 3/10, Bio-Rad) 0.005% bromophenol blue) and applied to 7 cm nonlinear pH 3-10 IPG strips (Immobiline DryStrip, GE Healthcare) which were then left to rehydrate for 12 hours. IEF was performed using an Ettan IPGPhor II (GE Healthcare) according to the following protocol: 500 V for 30 min, gradient to 1000 V for 30 min, gradient to 5000 V for 90 min, 5000 V for 35 min, 500 V up to 12 hours until the strips were removed. On completion, the strips were stored at -20ºC until the second dimension could be run. Due to the high density of ß keratins aggregating at similar pH, two separations with different amounts of loaded protein (3.75 µg and 12.5 µg) were performed for each sample to adequately resolve all proteins.

4.2.7 2nd Dimension (SDS-PAGE)

The frozen IPG strips were thawed and immersed for 15 minutes in equilibration buffer (6 M urea, 50 mM Tris-HCl, 30% glycerol, 2% SDS, 0.005% bromophenol blue, 2% w/v DTT), followed by another 15 minutes in the same buffer with 4% w/v iodoacetamide replacing the DTT. The strips were then

placed atop a 13% polyacrylamide gel, overlaid with agarose, and separated at a constant 0.2 mA for 1 hour. The gels were stained with colloidal coomassie and documented with a desktop scanner. Image enhancement was performed to extract maximum information from the gels. Estimates of pH were made based on the manufacturer's published pH gradient profile for the specific IPG strips.

4.3 Results

4.3.1 Keratins of Coachwhip and Corn Snake Scales

The proteins of spectacle, parietal (see Figure 4-2), and ventral scales of 3 coachwhip snakes were compared to determine differences between the scales and to ensure consistency of the methodology between samples. The gel is shown in Figure 4-3.

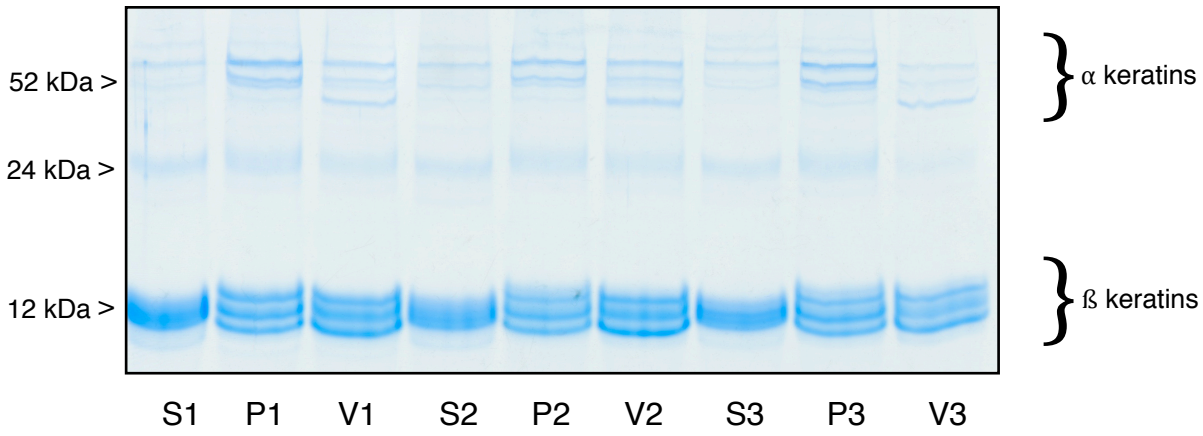


Figure 4-3. SDS-PAGE of spectacle (S), parietal (P) and ventral (V) scale proteins of coachwhip snakes. The numbers in the lane labels refer to three different snakes. The spectacle scales differ markedly in their ß keratin complement compared with parietal and ventral scales and differ also in the proportion of keratins. A faint, diffuse 24 kDa band appears in all samples.

The results are comparable between the 3 individuals and show protein bands in the regions expected of α and ß keratins. The spectacle scales (S1, S2, S3) contain an abundance of a 12 kDa protein that corresponds with the ß keratin region and a lower proportion of a ~52 kDa protein that corresponds to α keratins and associated proteins. The latter are known to be present in snake skin based on cross-reactivity with anti-mammalian antibodies to loricrin, sciellin, filaggrin and transglutaminase (Alibardi & Toni 2005a). This greater proportion of ß to α keratin in the coachwhip spectacle scale may be due to its being thicker than other scales (van Doorn, *unpubl. obs.*) - if a thickened beta layer is responsible for the overall increase in thickness, then the equalized amount of protein in the loaded samples will be manifested as a variation in the α:ß ratio. The parietal and ventral

scales differ noticeably in consisting of 3 closely spaced proteins centered around 12 kDa in the ß keratin region and a greater expression of proteins corresponding with the α keratin region. In all scales a faint and diffuse band appears at 24 kDa. An immunoblot of the same samples with the univ-ß antibody (Figure 4-4) confirmed that the 12 kDa bands in parietal and ventral scales correspond with ß keratins as do the faint 24 kDa bands. The antibody reacted only very weakly with the spectacle scale samples, which is assumed to be due to low affinity rather than the absence of ß keratins.

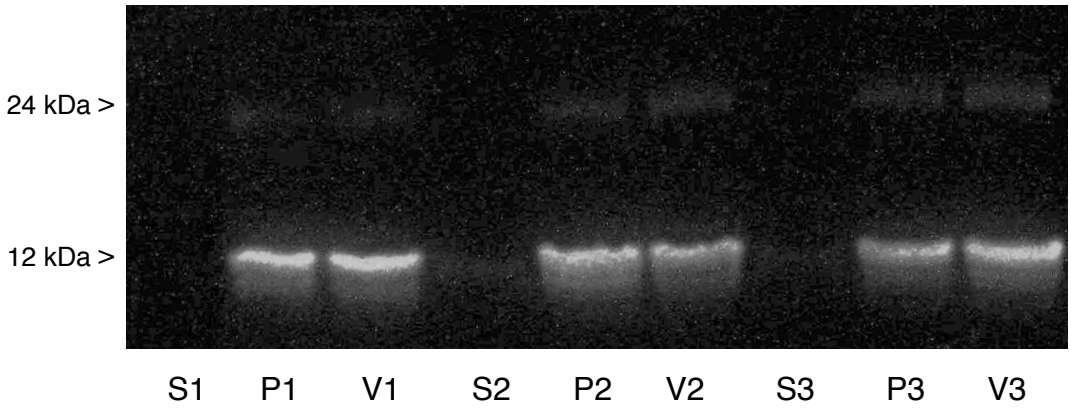


Figure 4-4. ß keratin immunoblot of spectacle (S), parietal (P) and ventral (V) scale proteins of coachwhip snakes. The numbers in the lane labels refer to three different snakes. The univ-ß antibody has a high affinity for the ß keratins of the parietal and ventral scales and a very low affinity for those of the spectacle scale.

The antibody was further verified with corn snake spectacle, parietal and ventral scale samples. A coomassie stained gel is shown in Figure 4-5 (next page) with a corresponding immunoblot in Figure 4-6 (next page), demonstrating again the low affinity of the antibody for spectacle ß keratins. Of note in Figure 4-5 is the variation between spectacle scales of the two individuals (S1 vs S2). While both have a highly expressed ß keratin just larger than 12 kDa, the first individual, which was amelanistic (i.e. albino) exhibits a second ß keratin just under 17 kDa. This second ß keratin was also observed in a different and unrelated amelanistic individual, while the result from a different non-amelanistic corn snake was similar to S2 (data not shown).

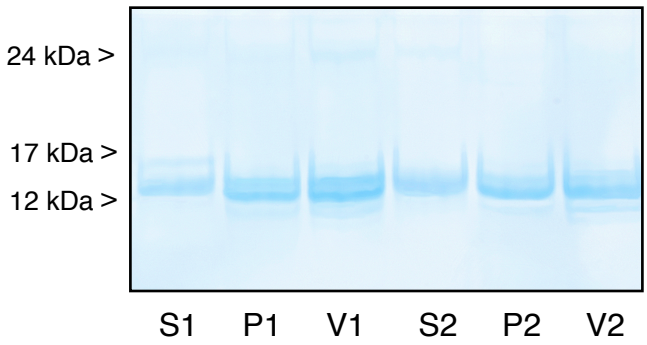


Figure 4-5. SDS-PAGE of spectacle (S), parietal (P) and ventral (V) scale proteins of corn snakes. The numbers in the lane labels refer to two different snakes.

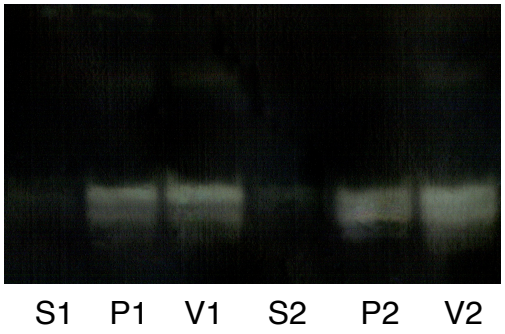


Figure 4-6. ß keratin immunoblot of spectacle (S), parietal (P) and ventral (V) scale proteins of corn snakes. The numbers in the lane labels refer to two different snakes. The low affinity of the univ-ß antibody for corn snake spectacle ß keratins is evident.

Because of the low reactivity of the univ-ß antibody for spectacle keratins, it was further evaluated with spectacle scales from several more species of different families, including the xenodontine hognose snake (Figure 4-7, next page)). This ensured that spectacle scales do indeed contain significant ß keratin as expected, but that spectacle keratins vary significantly in their reactivity with the antibody, and that colubrine (but not xenodontid) colubrid spectacle scales generally seem to lack the epitope to which the antibody is most specific.

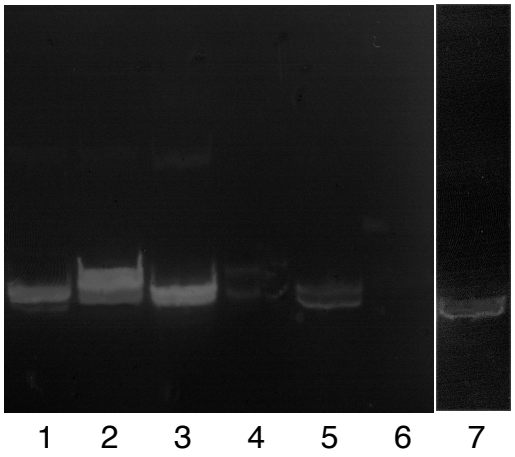


Figure 4-7. ß keratin immunoblot of snake spectacle scale proteins. 1: Southern Pacific rattlesnake (*Crotalus oreganus helleri*, Viperidae); 2: boa constrictor (*Boa constrictor*, Boidae); 3: green anaconda (*Eunectes murinus*, Boidae); 4: Mexican west coast rattlesnake (*Crotalus basiliscus*, Viperidae); 5: western diamondback rattlesnake (*Crotalus atrox*, Viperidae); 6: Louisiana pine snake (*Pituophis ruthveni*, Colubridae); 7: hognose snake (*Heterodon platirhinos*, Colubridae, subfamily Colubrinae).

4.3.2 Keratins of Gecko Spectacle, Labial and Head scales.

Comparisons of the proteins of gecko scales are shown in Figure 4-8 (*Gekko grossmanni*, next page) and Figure 4-9 (*Phelsuma madagascariensis grandis*, next page). Gecko spectacle scales contain no ß keratin. *G. grossmanni* expresses two forms of α keratin in the range of 54-65 kDa and *P. m. grandis* expresses a single form of 55-60 kDa. The labial and head scales however are largely composed of ß keratin with a single tight α keratin band in *P. m. grandis* and no detectable α keratin in *G. grossmanni*. In both species, the diversity of ß keratins in these scales is greater than in the snake scales and their sizes are larger on average, clustering around 17-18 kDa and ranging from ~12-22 kDa. The univ-ß antibody reacted with most of the putative ß keratins, but also exhibited non-specific binding to the α keratin of *P. m. grandis*, a problem noted by previous authors with other anti-ß keratin antibodies (Alibardi & Toni 2005b). To determine if other proteins were present in quantities too small to detect with the standard methodology, a sample of *P. m. grandis* labial scale was overloaded in one gel and the image heavily processed (Figure 4-9C). This demonstrated the presence of several other proteins present in minute quantities, including two proteins slightly larger than 24 kDa, a diffuse band of 38 kDa, and two proteins between this and the single α keratin. The larger proteins of 38 to ~50 kDa may correspond with loricrin, filaggrin-like protein, sciellin and/or transglutaminase, all accessory cornification proteins shown by Alibardi & Toni (2005b) to occur in the gecko integument.

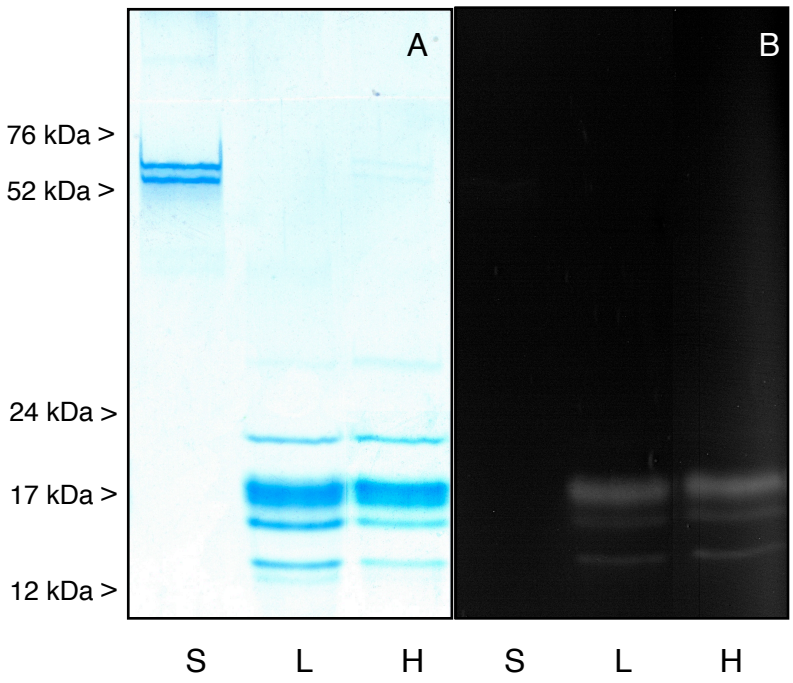


Figure 4-8. Spectacle (S), labial (L) and head (H) scale proteins of *Gekko grossmanni*. A: SDS-PAGE stained with coomassie; B: univ-ß immunoblot.

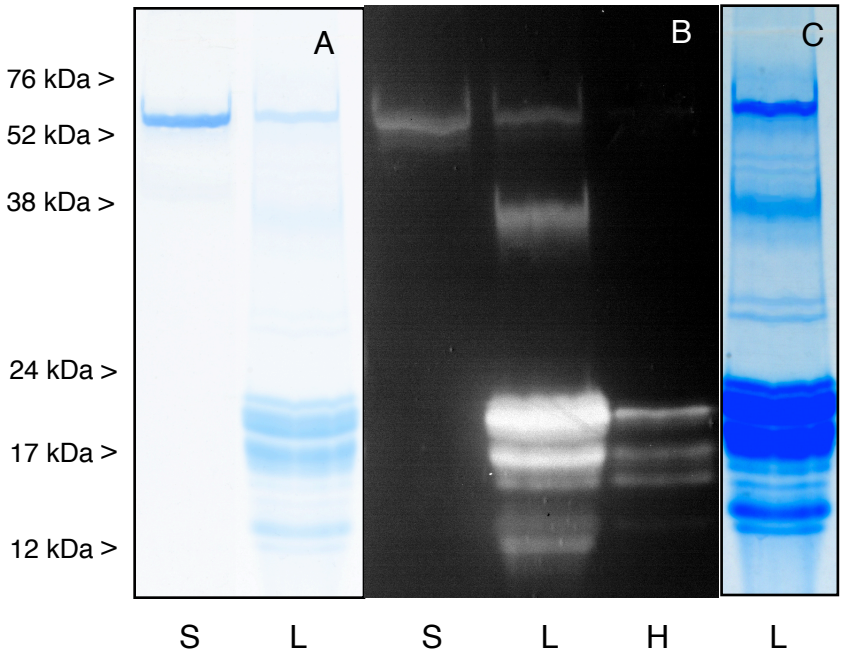


Figure 4-9. Spectacle (S), labial (L) and head (H) scale proteins of *Phelsuma madagascariensis grandis*. A: SDS-PAGE stained with coomassie. B: univ-ß immunoblot. C: overloaded and image processed labial scale sample showing proteins expressed in low quantities.

Having successfully identified as ß keratins the lower molecular weight proteins of snake and gecko scales, all subsequent electrophoretic separations were stained only with coomassie.

4.3.3 Comparative Assessment of Snake Spectacle ß Keratins

SDS-PAGE was done on boid, pythonid, elapid, viperid and additional colubrid spectacle scale samples to determine the differences in ß keratin complement. Images of the stained gels are on the following two pages (Figures 4-10 to 4-14).

Boid spectacle scales (Figure 4-10) contain one or two highly expressed forms of 12-13 kDa, except for the erycine rubber boa, *Charina bottae*, with three distinct forms and a smaller 8.5 kDa protein, which may still correspond to a ß keratin.

Pythonid spectacle samples resolved poorly (Figure 4-11), but show several ß keratins between 12 and 17 kDa. Of interest is the first shed post-hatch of the reticulated python (lane 2), which differs from the adult (lane 3) in expressing an extra, larger 17 kDa ß keratin but also clearly contains less α keratin with only a faint band detectable at 52 kDa.

Four ß keratin from 12-17 kDa are apparent in elapid spectacle scales (Figure 4-12). Likewise, crotaline viper spectacle scales (Figure 4-13) contain up to four 12-17 kDa ß keratins.

Colubrid samples are shown in Figure 4-13. In the genera *Pituophis* (lanes 1, 2) and *Elaphe* (lanes 3, 4, 5), the spectacle scales contain one highly expressed ß keratin band of ~ 14 kDa and one lower expressed of 12 kDa, similar to the coachwhip and corn snakes in showing a single highly expressed band with or without an associated low density band. The transpecos rat snake (*Bogertophis subocularis*) spectacle scale (lane 6) has two closely spaced ß keratins at ~ 14 kDa and a diffuse band at 12 kDa. The ß keratins of the hognose snake spectacle scale (lane 7), the only xenodontid investigated, exhibit greater size diversity than the colubrine colubrids in showing 4 clear bands between 12 and ~15 kDa.

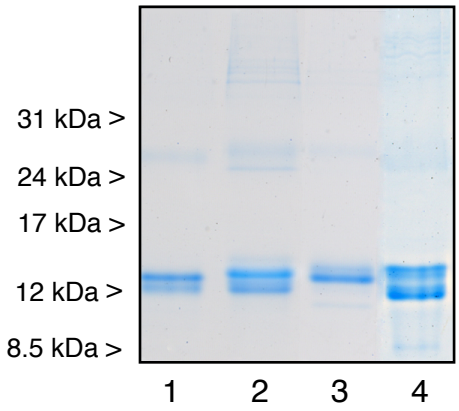


Figure 4-10. Spectacle scale proteins of boids. 1: green anaconda (*Eunectes murinus*); 2: boa constrictor (*Boa constrictor*); 3: garden tree boa (*Corallus hortulanus*); 4: rubber boa (*Charina bottae*, Erycinae). Lane 4 required significant image processing due to the very small amount of rubber boa sample available.

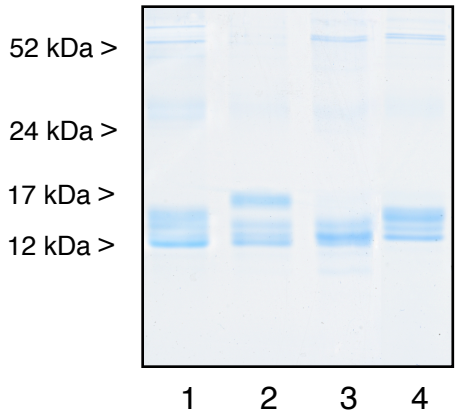


Figure 4-11. Spectacle scale proteins of pythonids. 1: carpet python (*Morelia spilota*); 2: reticulated python first shed (*Python reticulatus*); 3: reticulated python (*Python reticulatus*); 4: rock python (*Python sebae*). The embryonic reticulated python (lane 2) show an extra ß keratin band not seen in the adult. As well its band at 52 kDa, likely an α keratin, is much weaker.

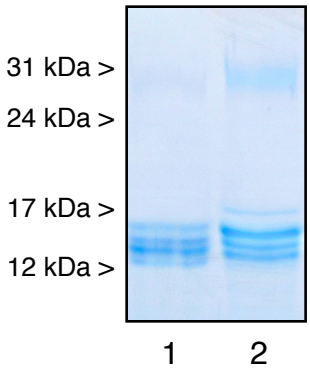


Figure 4-12. Spectacle scale proteins of elapids. 1: red spitting cobra (*Naja pallida*); 2: snouted cobra (*Naja annulifera*). Elapids show several distinct ß keratin bands between 12 and 17 kDa.

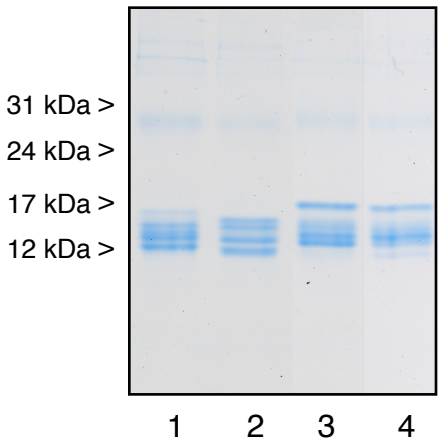


Figure 4-13. Spectacle scale proteins of crotaline vipers. 1: Western diamondback rattlesnake (*Crotalus atrox*) 2: Mexican west coast rattlesnake (*Crotalus basiliscus*); 3: Southern Pacific rattlesnake (*Crotalus oreganus helleri*); 4: Southwestern speckled rattlesnake (*Crotalus mitchellii pyrrhus*). Like the elapids, viperids show several distinct ß keratin bands between 12 and 17 kDa.

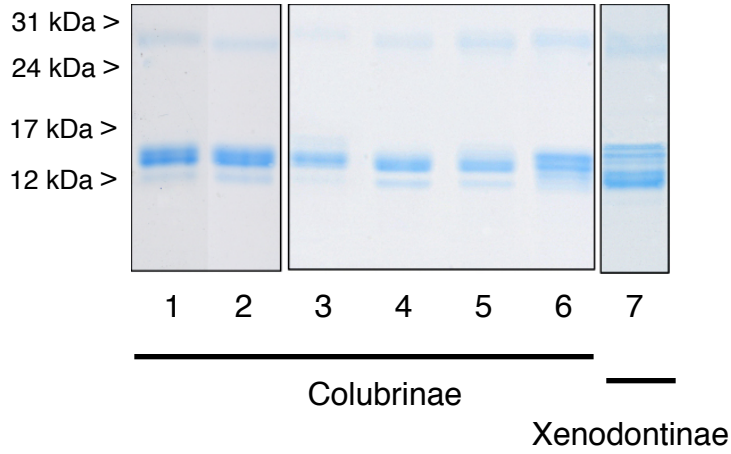


Figure 4-14. Spectacle scale proteins of colubrids. 1: Louisiana pine snake (*Pituophis ruthveni*); 2: northern pine snake (*Pituophis melanoleucus*); 3: black rat snake (*Elaphe obsoleta*); 4: Texas rat snake (*Elaphe obsoleta lindheimeri*); 5: tiger rat snake (*Spilotes pullatus*); 6: transpecos rat snake (*Bogertophis subocularis*); 7: hognose snake (*Heterodon platirhinos*, Xenodontinae).

4.3.4 2D Electrophoretic Comparison of Coachwhip Snake Spectacle and Parietal Scale Proteins

To determine the extent to which keratin isoforms of differing pI's contribute to the composition of the spectacle scale versus other scales, 2-dimensional electrophoresis was performed on coachwhip spectacle and parietal scale samples.

The ß keratins of the spectacle scale (Figure 4-15, next page) are most dense at 12 kDa in the range of pH 4-5, but several isoforms are present up to pH 7.5. In contrast, the ß keratins of the parietal scale (Figure 4-16, page 97) are more heavily aggregated around 12 kDa and pH 4-5, although a few very low concentration ß keratins are visible at pH ~5.3 and 6.5. The diffuse 24 kDa ß keratin band visible in the 1-D SDS-PAGE and immunoblots appears here as an aggregation between 24-31 kDa at pH 4.5.

Paralleling the 1-D SDS-PAGE results, the spectacle scale shows fewer α keratins though the sizes and pH ranges are comparable with those of the parietal scale. They range from 40-64 kDa in the acidic to neutral region. An 8.5 kDa protein of neutral pH is present in both scales.

In all gels, one or more <8.5 kDa spots of pH 4 were visible but surrounded by significant quantity of breakdown products, as evidenced by the heavy smearing around them. The exact stage at which the breakdown occurred is not clear, but given the extent of the horizontal smearing it had to be before or during the IEF step. Keratins are relatively stable at temperatures up to at least 50ºC (Nakamura *et al.* 2002), so temperature fluctuations during the overnight IEF are unlikely the culprits. The rehydration buffer prior to IEF differs little from the sample buffer other than the addition of glycerol and ampholytes, but the equilibration buffer does contain iodoacetamide, a strong alkylating agent, which is lacking in all other buffers. An attempt to separate samples with 1-dimensional SDS-PAGE after adding excess iodoacetamide did not resolve any previously unnoticed peptides, so the densest spots are most likely artifacts of the protein breakdown.

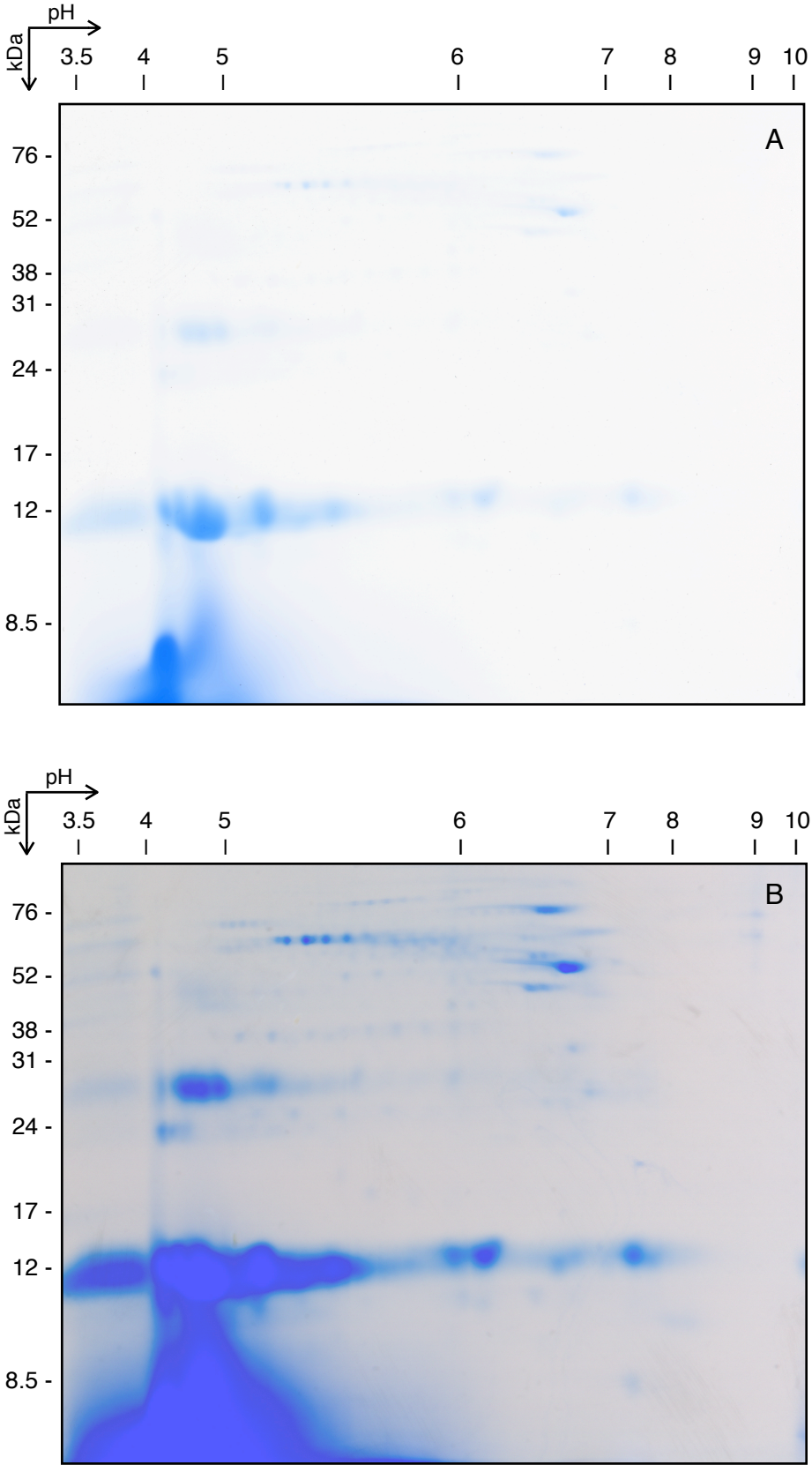


Figure 4-15. 2D electrophoresis of coachwhip spectacle scales. A: 3.75 μg of loaded protein to adequately resolve the main ß keratins. B: 12.5 μg of loaded protein and image enhancement to resolve low concentration proteins and isoforms. The majority of ß keratins are grouped between pH 4-5 and at 12 kDa. Several 12 kDa ß keratin isoforms are present at higher pH and may correspond to different post-translational modifications.

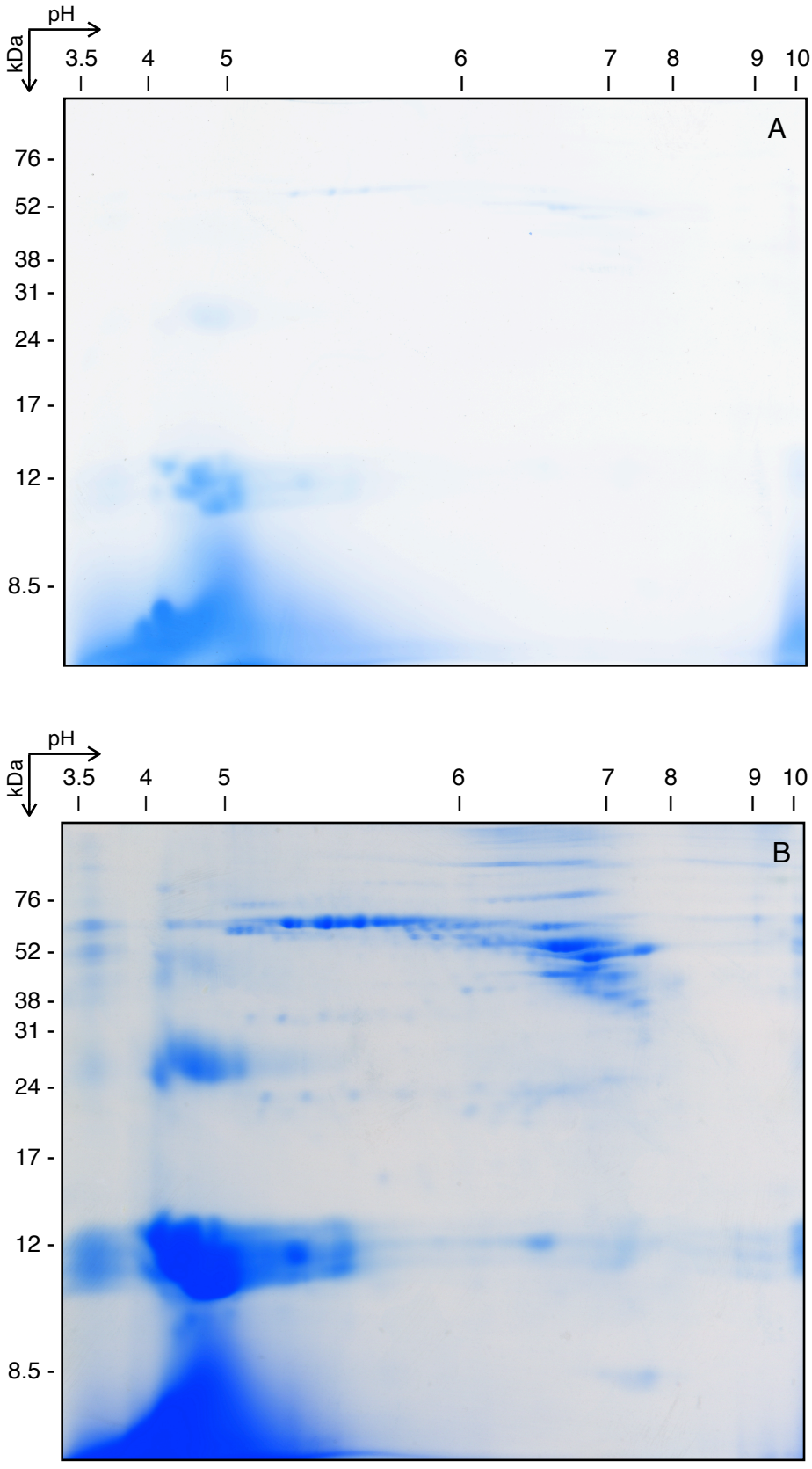


Figure 4-16. 2D electrophoresis of coachwhip parietal scales. A: 3.75 μg of loaded protein to adequately resolve the main ß keratins. B: 12.5 μg of loaded protein and image enhancement to resolve low concentration proteins and isoforms. As with the spectacle scale, most ß keratins are grouped between pH 4-5 and weight in the range of 12 kDa. Comparatively fewer ß keratin isoforms are present at higher pH, suggesting less (or different) post-translational modifications taking place.

4.4 Discussion

The purpose of this study was to determine if and how the biochemical composition of snake and gecko spectacle scales differs from that of non-spectacle scales in the hope of offering insight into whether a scale's optical properties may be related to its composition. Differences in keratin composition were indeed found between spectacle scales and other scales of the integument in both snakes and geckos. Differences were also found between the spectacle scales of various families and species of snake. Each of these findings will be discussed in turn.

4.4.1 Keratins of the Snake Spectacle Scale Versus Other Scales

Compositionally, coachwhip snake spectacle scales differ from other scales in the integument by containing not only a greater proportion of ß keratin relative to other cornification proteins, but also a greater number of ß keratins of differing pI. The significance of a higher ß:α ratio suggests either a thicker beta layer and/or thinner alpha or mesos layer(s). Based on observations of the shed coachwhip snake spectacle scale being more rigid and thicker than other scales (van Doorn, *unpubl. obs.*), it is more likely to have a thicker beta layer, possibly to provide greater mechanical protection to the eye, to prevent deformation of the eye or spectacle during accommodation or rotation of the globe, or to tune its transmission spectrum.

The dense zone at ~12 kDa of acidic pH 4-5 ß keratin mirrors that previously reported in the overall integument of the corn snake, but contrasts with the carpet python, *Morelia spilota*, and western diamondback rattlesnake, *Crotalus atrox,* that have mostly neutral to slightly basic ß keratins in their integument (Toni *et al.* 2007; Toni & Alibardi 2007a). Toni *et al.* (2007) suggested that ß keratins are primarily basic proteins, but that they may become acidic through post-translational modifications. If so, then ß keratins of the colubrid subfamily Colubrinae may receive similar post-translational modifications as in both known cases (the corn snake (Toni *et al.* 2007) and the coachwhip snake (this study)), the ß keratins migrate to the acidic region during isoelectric focussing. The significance of these post-translational modifications is not clear, although Toni & Alibardi

(2007a) have theorized that basic ß keratins may associate with the primarily acidic α keratins of snakes during differentiation of keratinocytes. Lacking basic ß keratins, at least in the shed skin, this theory may not extend to colubrine snakes, unless different ß keratins than observed in this study are expressed in the intact integument prior to complete cornification of the keratinocytes.

Regarding the diffuse immunoreactive bands at 24 kDa in the 1D SDS-PAGE and the dense collection of spots between 24 and 31 kDa in the 2D gel, these are likely attributed to protein aggregation which is known to occur with extracted ß keratins (Shames *et al.* 1991; Sawyer *et al.* 2003; Alibardi & Toni 2005b).

4.4.2 Comparative Investigation of Spectacle ß Keratins

The ß keratins that make up the spectacle scale vary from species to species, indicating that transparency is not restricted to a single specific ß keratin or isoform. In fact, the link between transparency and keratin composition alluded to above should still be considered circumstantial. Factors such as keratin fiber layout and surface ultrastructure may also influence optical quality and spectral transmission. If keratin fibrils are highly organized and evenly spaced, an analogy can be drawn with the cornea of the eye that achieves transparency by the orthogonal arrangement of and precise spacing between collagen fibers (Maurice 1957; Cox *et al.* 1970). Campbell *et al.*'s (1999) work on the surface ultrastructure of the python spectacle showed that it exhibits fewer irregularities than other scales, suggesting that this may be significant in ensuring transparency of reptilian scales.

ß keratin expression in snake spectacle scales varies between families. The ß keratins expressed by boine and colubrine species differ little in size, whereas the erycine boid, xenodontine colubrid, pythonids, elapids and viperids express a range of ß keratins varying in size. The ß keratins in colubrids differ little in size but do differ greatly in pI, at least in the coachwhip snake. Further investigations should be done to determine if spectacle scales of other species, colubrids and otherwise, also contain ß keratins of diverse pI and whether they are basic as in the integument of the

carpet python and western diamondback rattlesnake or predominantly acidic as in the spectacles of coachwhip snakes.

The spectacle scale of hatchling reticulated pythons, representing the embryonic stratum corneum, contains a larger ß keratin not found in the adult and also have a much lower proportion of α keratin. It has been shown that the stratum corneum of hatchling snakes have a particularly thin mesos layer which nearly doubles in thickness after the first shed (Tu *et al.* 2002). This is thought to increase the efficacy of the water permeability barrier, which is not altogether necessary in the fluidic environment of the egg, but is absolutely essential upon hatching, especially in arid and semi-arid environments inhabited by the subject snakes of Tu *et al.*'s study (the California kingsnake, *Lampropeltis getula*). This increase in the the ratio of α:ß keratin would explain the low α keratin signal in the hatchling reticulated python. The difference in ß keratin complement is unclear. While the hatchling spectacle scale's spectral transmission varies slightly after the first shed (see Chapter 3), it is not likely enough to affect vision. The biochemical difference may not be exclusive to the spectacle scale and may be again for permeability or for necessary mechanical properties of scales *in ovo*.

4.4.3 Keratins of Gecko Scales

Gecko spectacle scales appear to be composed exclusively of α keratin. Being generally softer than ß keratin, this explains the relative ease with which the spectacles are damaged. And although this study does not conclusively relate biochemical composition with transparency, the absence of any ß keratin may conceivably be cause for the gecko spectacle's exceptionally high spectral transmission.

This absence also speaks to the modest need for mechanical protection of the eye in these arboreal animals. Unlike snakes that may use their heads to persistently penetrate substrates or that need to withstand potential retribution from large prey items, the eyes of arboreal geckos rarely encounter anything more harmful than a speck of dust or the occasional collision during their rapid and seemingly reckless locomotion. Nevertheless, any damage to the delicate surface risks damaging the underlying mesos layer. This layer is normally well shielded by the hard beta layer, but in the gecko

spectacle, this layer may be particularly vulnerable to damage that could impair its function as a permeability barrier (Landmann 1981). This of course presupposes that the gecko spectacle has a mesos layer, since no high resolution microscopic studies of the gecko spectacle have yet been published.

Not all geckos are arboreal however. The burrowing gecko *Ptenopus* inhabits arid and sandy environments and is possibly the only spectacled species to have evolved mobile lids to cover the spectacle (Bellairs 1948). This condition has been difficult to explain as the spectacle has generally been considered the epitome of ocular shielding. If *Ptenopus* were to lack ß keratin in the spectacle like the geckos in this study, this may explain the need to evolve protection for the soft spectacle, particularly given its burrowing habits and the preponderance of abrasive sand in its environment. The integrity of the mesos layer will be especially important in this genus to minimize cutaneous water loss in the arid climate. An analysis of the keratin composition of *Ptenopus*' spectacle would be especially valuable to better understand why it evolved novel eyelid analogues.

The absence of ß keratin also implies the absence of oberhautchen or surface micro-ornamentation. These ultrastructural features are thought to be found on all reptilian scales (Hoge & Souza Santos 1953; Ruibal 1968; Chiasson & Lowe 1989; Chiasson *et al.* 1989), but given the absence of ß keratin in the gecko spectacle, the spectacle scale may have a completely different surface ultrastructure from any known scale. For example, mutant scaleless snakes that do not express ß keratin lack the more elaborate micro-ornamentation of scaled snakes, instead having an undulating surface of α keratin less than 200 nm thick, directly beneath which lies the mesos layer (Toni & Alibardi 2007b). Because of the potential relationship between scale surface ultrastructure and transparency (Campbell *et al.* 1999), an electron microscopic study of the gecko spectacle surface may be helpful to determine if the gecko spectacle's high transmission can be accounted for by its lack of ß keratin oberhautchen.

4.4.4 Conclusion

This research was inspired by the variation observed in the spectral transmission results reported in Chapter 3. While no direct link between keratin content and spectral transmission could be made, the results do indicate that the composition of the spectacle scale is different from other scales, both in snakes and in geckos. Furthermore, the variation in the ß keratin content of spectacle scales of different species and families indicates that transparency is not restricted to a single ß keratin or isoform, but rather that ß keratins in general, as well as α keratins, may have the potential of achieving transparency.

Chapter 5, Summary and Concluding Remarks

Though minuscule in area compared with the whole of the integument, the reptilian spectacle presents numerous specializations of the skin to permit acute vision while still maintaining the integument's protective role. The research presented in this thesis covered two such specializations: (1) the vascular dynamics that act to minimize the effect of the spectacle vasculature in the visual field, and (2) the transparent scale that has a different composition than scales elsewhere on the integument and in some cases exhibits potentially adaptive transmittance spectra.

The spectacle vasculature presents a unique visual problem in that no other known vertebrate (manatees excepted; Harper *et al.* 2005) has blood vessels in the optically transmissive portions of the eye other than the retina. What's more, the eyes of spectacled reptiles rotate freely beneath their spectacles, causing shifts in the location of the vessels within the visual field, which would interfere with adaptation (Troxler 1804; Lettvin *et al.* 1968). Lüdicke's (1969) finding of the asymmetry of *Ahaetulla nasuta*'s spectacle vasculature and Mead's (1976) finding of the vessel walls being transparent, just as are retinal blood vessel walls (Martin 2009), further reinforce the supposition that the spectacle vessels can be detrimental to vision (from an unspectacled species' perspective) and hint at the evolutionary tweaking that's taken place to maximize spectacled animals' visual clarity. Cutaneous vasculature is especially apt at regulating flow via vasomotor mechanisms (Fredericq 1882; Hertzman 1959; Fox and Edholm 1963; Kellogg Jr. 2006), leading one to wonder if the spectacle blood vessels may react to endogenous or exogenous stimuli and if such stimuli were to induce constriction of the vessels, all visual problems might be solved (for a time). The results presented in Chapter 2 demonstrated that a neural mechanism does exist to enable spectacle vessels to constrict when a sympathetic response is incurred. The potential for future research is certainly not lacking, as the results raise several interesting questions on the mechanism involved and its prevalence among spectacled species. Is the mechanism observed in snakes comparable across species, whether highly visual (eg. *A. nasuta*) or not (eg. blind snakes)? Is the mechanism present in species with windowed eyelids? Is the mechanism engaged only when faced by a perceived threat to facilitate defensive and

escape behaviours or also during predation? Is it specific to the spectacle or a manifestation of a generalized sympathetic response of the whole integument? How to reconcile this mechanism with thermoregulatory mechanisms? As with all scientific findings, the questions raised in the aftermath are numerous.

Turning then to the transparency of the scale, another trait unique to the reptilian spectacle, it was asked how this optical keratinized structure might tune the spectrum of incident light to suit the animal's visual needs and possibly its need for protection again damaging radiation. After all, the spectacle scale is the outermost layer and the first encountered by incoming radiation. Only in the snouted cobra, *Naja annulifera*, does the yellow spectacle scale block sufficient short-wavelength radiation to convincingly be considered a UV filter, but the yellow filter may have evolved instead (or in addition) to increase contrast by blocking shorter wavelengths most prone to scattering or dispersion (Sivak 1982; Sivak & Mandelman 1982). In other species, the role of the spectacle scale in UV protection or contrast enhancement remains ambiguous (eg. the coachwhip's sharp cutoff at 350 nm) or is completely absent (eg. pythonids, viperids, *Lampropeltis*). The diversity of transmission profiles and cutoff wavelengths and the correlation of these with taxonomic family suggest that cutoff wavelengths may not be adaptive for many species, but may instead reflect some other characteristic of the spectacle which may be adaptive. Scanning electron microscopy of the surface of the spectacle scale to compare ultrastructural features between species with differing transmission profiles may be helpful in clarifying the contribution of oberhautchen to spectral transmittance.

One such characteristic is the thickness of the spectacle scale. That thickness and $\lambda_{50\%}$ were correlated only in Colubridae and that thickness varied between families points to the potentially multiple roles played by the spectacle and especially to the wide degree of mechanical protection that it affords. The question remains of why colubrid spectacle scales are so much thicker on average than those of pythonids, boids and viperids. As discussed in Chapter 3, this may reflect the vulnerability of the colubrid eye during rapid locomotion or to predators and retaliating prey. The material properties of snake scales have recently come under scrutiny (Klein *et al.* 2010; Klein and Gorb 2012) with

evaluations of the elastic modulus and hardness of the inner and outer stratum corneum. Similar studies on spectacle scales would be helpful to determine their value in mechanical protection.

The variation in spectral transmittance patterns that were observed in Chapter 3 inspired the biochemical analysis of Chapter 4 to determine if and how the biochemical composition of the spectacle scales differ between species and between different scales of the same species. The results of the analysis showed that spectacle scales do indeed have a different composition from other scales and that variations are seen between species. Differences between families could be seen as well considering the ß keratins of colubrine colubrids exhibit the least variation in molecular weight, while several other families like the elapids and viperids consistently displayed several ß keratin bands of differing molecular weights. The diversity of the keratins that make up a single scale thus showed that there isn't a single compositional factor that is responsible for optical transparency, although the molecular properties responsible that allow for transparent keratin structures remain unknown. The diversity of α and ß keratins in terms of their pI, molecular weight and presumptive post-translational modifications says nothing about their respective optical characteristics, particularly given the complex composition of each scale. So although the results indicate clearly that spectacle scale composition is unique and variable, the door remains open to more in-depth research on the material and biophysical properties of the keratins.

The gecko spectacle scale has surprised in several ways. Not only does it have outstanding transmissive properties but it appears to lack the hard, corneous ß keratins presumed to be present in all squamate scales (Maderson 1985; Landmann 1986). These two characteristics may well be related. Perhaps ß keratins broadly attenuate UV or perhaps the absence of ß keratin oberhautchen (i.e. scale surface ultrastructural features) minimizes scatter of the shorter wavelengths. In either case, the gecko spectacle deserves further investigation. Scanning electron microscopy of the spectacle scale surface would clarify the presence or absence of surface features. High-magnification immunohistochemistry or transmission electron microscopy may be useful in determining whether a beta layer is present or if the layering of the gecko spectacle appears similar to the scales of scaleless snakes that lack ß keratin (Toni & Alibardi 2007b).

Although a step to better understanding the biology of the spectacle, the findings of these experiments may also have more immediate applicability. Keratin is a potentially useful biomaterial (Rouse and Van Dyke 2010; Sierpinski *et al.* 2008; Vincent 1990) and spectacle keratins in particular may be useful for applications that require optical transparency. Reichl *et al*. (2011) describe the synthesis of keratin films from human hair as a potential support and protective barrier in ocular surface reconstruction. Injured corneas and conjunctivas benefit from having a transparent protective barrier and structural support to allow a degree of vision while healing. Amniotic membranes for example have been used in ocular surface reconstruction (Fernandes *et al.* 2005), but as Reichl *et al.* (2011) point out, the spectral transmittance of amniotic membrane is poor, showing obvious scatter. Reichl *et al.*'s keratin films based on human hair extracts have improved transmission, although they still fall short of the transmittance of reptilian spectacles. Providing that biocompatibility of reptilian keratins can be assured, spectacle keratins may be a suitable and preferable alternative to human hair keratin for this usage.

As with all research, the experiments described in this thesis answered some questions, left others unanswered, and provided serendipitous answers to questions unasked. Nearly 200 years since Cloquet's (1821) seminal account of the "paupière des serpens," the reptilian spectacle continues to intrigue, withholding secrets to be uncovered by future research.

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Appendix A

One cycle of spectacle blood flow in the resting coachwhip

This appendix is a video file of one cycle of blood flow in a coachwhip snake's spectacle vasculature. The file name of this video file is "Appendix_A_-_spectacle_flow_at_rest.mov". The false colour cast is due to the near-infrared capture. The video begins with spectacle vessels constricted and therefore invisible. The dark lines and spots across the image are scratches and abrasions in the spectacle scale. At 11 seconds into the clip, blood flow begins in a ventral to dorsal direction. It ceases again just before the end of the clip.

Appendix B

Spectacle blood flow in a juvenile corn snake during the renewal phase

This appendix is a video file of spectacle blood flow during the renewal phase of a juvenile corn snake. The file name of this video file is “Appendix_B_- _spectacle_flow_during_renewal_phase.mov”. The many dark lines are scratches in the spectacle scale. The false colour cast is due to the near-infrared capture.