

RESEARCH ARTICLE

Blood flow dynamics in the snake spectacle

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SUMMARY

The eyes of snakes are shielded beneath a layer of transparent integument referred to as the ‘reptilian spectacle’. Well adapted to vision by virtue of its optical transparency, it nevertheless retains one characteristic of the integument that would otherwise prove detrimental to vision: its vascularity. Given the potential consequence of spectacle blood vessels on visual clarity, one might expect adaptations to have evolved that mitigate their negative impact. Earlier research demonstrated an adaptation to their spatial layout in only one species to reduce the vessels’ density in the region serving the foveal and binocular visual fields. Here, we present a study of spectacle blood flow dynamics and provide evidence of a mechanism to mitigate the spectacle blood vessels’ deleterious effect on vision by regulation of blood flow through them. It was found that when snakes are at rest and undisturbed, spectacle vessels undergo cycles of dilation and constriction, such that the majority of the time the vessels are fully constricted, effectively removing them from the visual field. When snakes are presented with a visual threat, spectacle vessels constrict and remain constricted for longer periods than occur during the resting cycles, thus guaranteeing the best possible visual capabilities in times of need. Finally, during the snakes’ renewal phase when they are generating a new stratum corneum, the resting cycle is abolished, spectacle vessels remain dilated and blood flow remains strong and continuous. The significance of these findings in terms of the visual capabilities and physiology of snakes is discussed.

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INTRODUCTION

The reptilian spectacle is a layer of transparent integument that overlays the eyes of some squamate reptiles, isolating them from the external environment. Ubiquitous among snakes and nearly so in geckos (family Eublepharidae being the exception), spectacles have also evolved in several other disparate squamate taxa, including for example xantusiid night lizards and some scincids, teiids and lacertids (Walls, 1942; Greer, 1983). Arising from the fusion of embryonic tissues that would otherwise form eyelids (Schwartz-Karsten, 1933; Neher, 1935; Bellairs and Boyd, 1947), the spectacle’s anatomy is homologous with that of the skin in having a stratum corneum (the spectacle scale), an epidermis and a dermis, but differs in that the posterior-most layer is conjunctival in origin, similar to eyelids (Ficalbi, 1888; Walls, 1942). The spectacle remains unattached to the eye, behaving essentially as a fixed, immobile window beneath which the eye rotates freely.

Unlike either the skin or most eyelids, the spectacle is optically transparent, ideally suited to vision but for one characteristic that it shares with the rest of the integument: its vascularity (Fig. 1). The presence of blood vessels in the snake spectacle dermis was first noted by Quekett (Quekett, 1852). The vascular supply to and anatomical layout of the spectacle blood vessels was later described in great detail by Lüdicke (Lüdicke, 1940; Lüdicke, 1969; Lüdicke, 1971; Lüdicke, 1973; Lüdicke, 1977; Lüdicke and Kaiser, 1975) and Mead (Mead, 1976), who showed the arrangement of blood vessels to vary taxonomically between families. The organization of spectacle vessels in colubrid and elapid snakes differs from that of boids, pythonids, aniliids,

crotaline vipers and acrochordids in having a vertically oriented layout rather than a radial arrangement.

That an optically transmissive region of the visual apparatus is vascularized is quite remarkable as few other tetrapods possess non-pathological, non-retinal vasculature in the light path. This suggests that blood vessels in the visual field can have a negative impact on visual clarity and that spectacle blood vessels therefore could conceivably constrain their owners’ visual capabilities because of optical scatter, absorption and possibly even perception of the vessels themselves as entoptic phenomena (i.e. the visualization of structures within one’s own eyes, such as our own perception of ‘floaters’ located in our vitreous body). Supporting this assertion, the green vine snake, *Ahaetulla nasuta* (Colubridae), one of few snake species known to possess a fovea (Duke-Elder, 1958), 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). This region supplies the species’ anteriorly oriented foveal and binocular visual fields. The spectacles of other colubrids described by Lüdicke, none of which are foveated, show little or no perceivable asymmetry, which suggests the unusual arrangement in *A. nasuta* to be an adaptation to the spectacle vessel organization to minimize visual disturbance in the region of highest acuity of this highly visual species.

Though the spatial layout of spectacle blood vessels has been well described in several species, little commentary has been made on the blood flow dynamics within those vessels but for Mead (Mead, 1976) who, noting the transparency of the blood vessel walls, observed erythrocytes flowing through them but stated only that,

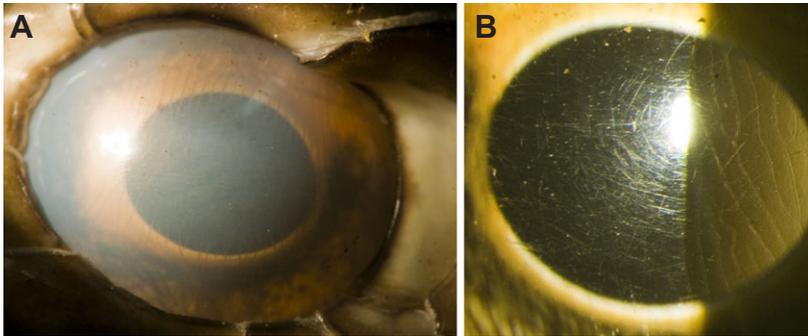


Fig. 1. Blood vessels in the spectacle of a coachwhip snake (*Masticophis flagellum*). (A) Image taken during the renewal phase of the integument when the spectacle becomes cloudy. The vessels are most apparent in the region that overlays the iris–pupil boundary because of their higher contrast with the background in this region. (B) The spectacle under retro-illumination, showing the vessels in the illuminated anterior portion of the pupil on the right side. The vessels are dorso-ventrally arranged as is typical for colubrid snakes. Debris and scratches are visible on the spectacle scale (particularly the left side), attesting to its protective role.

‘The vessels ... fill without any obvious directional priority in the anesthetized animal’. Mead made no comment on blood flow in non-anesthetized, unrestrained animals. One may consider that alternatively or in addition to spatial adaptations in the layout of the spectacle’s 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, effectively removing the vessels from the visual field altogether. Here, we describe studies that provide experimental evidence of such an adaptation by imaging spectacle blood in the coachwhip snake (*Masticophis flagellum*) when it is at rest and when a potential threat is visually perceived.

MATERIALS AND METHODS

Animals

The main experimental subjects were three coachwhip snakes, *M. flagellum* (Colubridae) (Shaw 1802) 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 length and 445 g; 120 cm and 320 g; and 97 cm and 240 g. The snakes were housed in separate terraria equipped with burrows and water dishes. Ambient temperature was kept at $\sim 27^{\circ}\text{C}$ with daytime basking spots of $\sim 31^{\circ}\text{C}$, and lighting was on a 12 h:12 h light:dark cycle. Snakes were fed once per week with frozen/thawed mice. Additional observations were made during the renewal phase of the integument on a juvenile corn snake, *Pantherophis guttatus* (formerly *Elaphe guttata*; Colubridae) (Linnaeus 1766), kept in similar conditions.

Experimental setup

Observations of spectacle blood flow were made using a modified slit-lamp that had a near-infrared (NIR) low-pass filter in the light path between the lamp and condenser so that it emitted only NIR wavelengths. The animals’ eyes were illuminated using combinations of retro-illumination and oblique illumination. Retro-illumination consists of reflecting light off the retina to backlight objects within or in front of the eye so that they appear dark against a brightly illuminated pupil, whereas oblique illumination involves illuminating structures from the side to give objects a more three-dimensional appearance. A NIR-sensitive camcorder (Sony HC7 with its NIR-blocking filter removed) was mounted to a beam splitter on the slit-lamp to allow blood flow to be monitored on the camcorder’s LCD screen. With the zoom lens set to the longest focal length and the slit-lamp set to a $40\times$ objective, the system was measured with a graticule to resolve a lower limit of $\sim 12\mu\text{m}$, which is just sufficient to observe individual erythrocytes that, in *M. flagellum*, measure about $12.6\mu\text{m}$ on the short axis (Hartman and Lessler, 1964).

To restrict a subject animal’s mobility, it was placed within a transparent acrylic box with internal dimensions of $3.5\times 5\times 95\text{ cm}$.

In practice, this box was large enough that the animal was not restrained but was nevertheless constrained to a small space. The box was mounted on a tripod, easing 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 small shifts in the animal’s position. It was found that after an initial 1–3 min of agitated movement within the box, coachwhip snakes settle down and tend to remain nearly motionless under the experimental conditions described here, making them excellent model animals for high-magnification observations over long periods.

To prevent the snake from visually observing the experimenter, curtains were mounted on the slit-lamp table, allowing only the slit-lamp objectives and mirror to be visible to the snake. Thus hidden, the experimenter was free to manipulate the controls of the slit-lamp and enter data without alarming the snake.

Experimental protocol

Animals were allowed to acclimate in the box for 10–15 min prior to data collection. After this time, the experimenter began recording to the nearest second when blood flow in the spectacle began and stopped. Data were recorded for 70 min. An explanation should be made on the nomenclature of blood flow used in this report. Because the vessel walls of the spectacle blood vessels are transparent, it proved difficult to image their dilation and constriction. As a result, the nomenclature used in this article will primarily be descriptive of the presence and absence of blood flow, rather than on the vessels’ degree of dilation and constriction.

The timeline of an experimental trial is shown in Fig. 2. No stimulus was presented during the initial 30 min of the trial to permit collection of baseline blood flow data. At 30 min into the trial, a potential threat to the snake was simulated by having the experimenter step out from behind the curtain for 8 min, and perform routine laboratory activities within 1.5 m of the boxed snake. It should be noted that in the case of these captive coachwhip snakes, a human was deemed an effective stimulus, as demonstrated by their vigilant behaviour and occasional attempts to hide or flee when their terraria were approached. After the 8 min, the experimenter returned behind the curtain. The simulated threat was repeated 16 min later, when the experimenter again stepped out from behind the curtain for 8 min. The experiment was concluded 8 min after cessation of the second simulated threat.

Each of the three snakes was tested seven times in as many days, thus ensuring that they were only tested once per day. Although independent observations were made during the snakes’ moulting phase, experiments were not conducted during this time because of clouding of the spectacle, general changes in behaviour, and changes in spectacle blood flow dynamics, which became apparent in preliminary studies and which will be discussed below. All

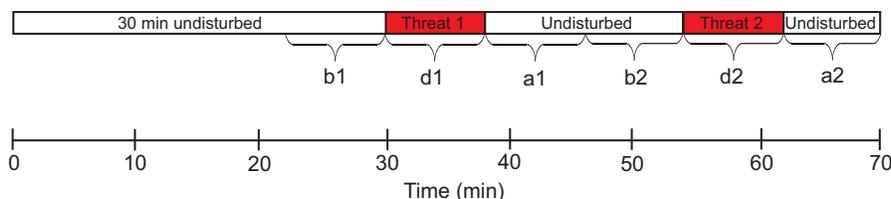


Fig. 2. Timeline of an experimental trial. Each trial lasted for 70 min, with the snake remaining undisturbed for the first 30 min. This was followed by an 8 min period during which a visual threat was presented (Threat 1), after which the threat was removed to leave the subject undisturbed for 16 min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

observations were made at an ambient temperature of 27°C and ambient fluorescent illumination of ~290 lx.

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.

Data analysis

Two sets of analyses were performed. The first was performed on the initial 30 min undisturbed phase to determine whether there was a significant difference between the durations of periods with and without flow, between experimental subjects, or whether habituation was taking place between trials. These analyses were done using univariate multifactorial ANOVA with the following three factors: (1) day of the trial (of which there were 7), (2) experimental subject (of which there were 3), and (3) the presence or absence of flow as a binary value.

The second set of analyses was performed to determine whether there was any change in the proportion of spectacle blood flow during periods of perceived threat. To this end, the total duration of periods without blood flow was converted to sine-transformed proportions between 0 and 1 and compared with the 8 min of observed blood flow both before and after each threat presentation. Three factors were taken into consideration in these analyses: (1) the day of the experiment (of which there were 7), (2) the threat event within each experiment (of which there were 2), and (3) whether the block of time was before, during or after the presented threat. These data were analysed using univariate multifactorial repeated-measures ANOVA, and calculations were corrected with the Greenhouse–Geisser epsilon to account for non-sphericity in the data. Probability values equal to or less than 0.05 were considered statistically significant.

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

RESULTS

Spectacle flow in undisturbed snakes

During the initial 30 min of the trials, when snakes were at rest and undisturbed, significant differences ($P=0.000$) were found between the durations of flow (mean=57 s, s.d.=49 s) and empty periods (mean=115 s, s.d.=80 s) when data from all snakes were pooled. In any given trial, durations of flow were generally shorter than the empty periods. Differences between individual snakes were seen in the durations of flow periods ($P=0.006$) but not empty periods ($P=0.640$). Differences were also observed between trials in the durations of both flow periods ($P=0.000$) and empty periods ($P=0.017$), indicating significant variation from day to day. Fig. 3 is a graphical representation of spectacle blood flow and empty periods over the initial 30 undisturbed minutes during two trials in different snakes.

Effect of threat perception on spectacle flow

The effect of threat perception on spectacle blood flow is illustrated in Fig. 4. During the 8 min of perceived threat, the mean duration of individual flow events was reduced to 33.5 s (s.d.=17.6 s), down from 57 s (s.d.=49 s), when data from all subjects were pooled. Additionally, the total proportion of time during which flow occurred was reduced when compared with the 8 min prior to and after the presented threat ($P=0.011$). No difference was found between trials ($P=0.633$) or between the first and second threat events within a trial ($P=0.150$). Interaction terms between any or all of the three factors were also found to be not significant. Fig. 5 shows a box plot of the proportion of time during which spectacle blood flow occurred before, during and after each threat event.

Additional observations

Spectacle blood flow was found to stop altogether when a subject was physically restrained, presumably due to a strong sympathetic response. Because of 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 the snakes were handled or otherwise disturbed, blood flow would slow or stop for brief periods of 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. See supplementary material Movie 1 for a video recording of spectacle blood flow in a moulting juvenile corn snake.

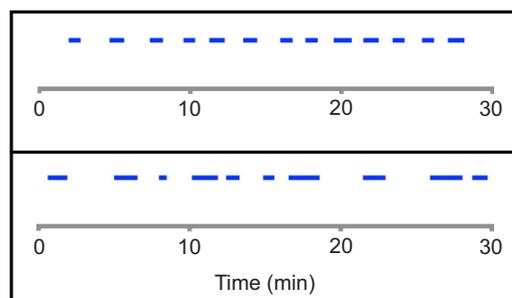


Fig. 3. Representative trials in two different snakes illustrate when spectacle blood flow occurred (blue lines) in the initial 30 min undisturbed phase. Although the pattern in the lower graph is less regular than that in the upper graph, cycles of blood flow and emptying of the spectacle vessels are nevertheless evident in both. In particular, the variability in duration of flow and empty periods is clearly apparent.

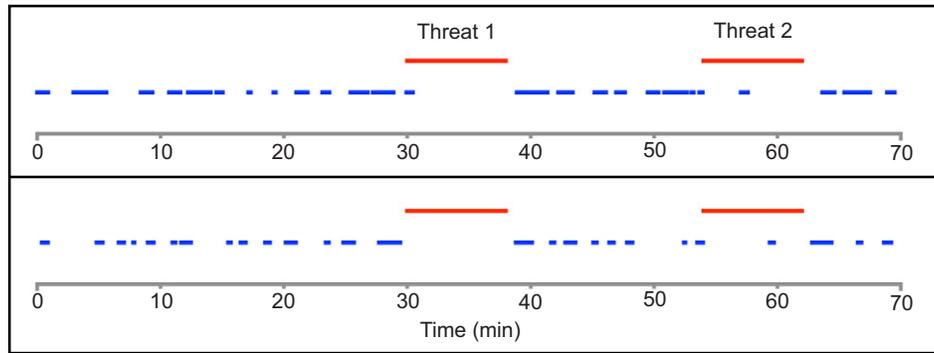


Fig. 4. Graph of two representative trials. The blue lines indicate when spectacle blood flow occurred. The periods during which the threats were presented are indicated by the red lines labelled 'Threat 1' and 'Threat 2'. A visual appraisal is sufficient to determine that the total duration of blood flow during the threat presentations is shorter than when the subject was undisturbed.

DISCUSSION

The primary purpose of this study was 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 potential threat is visually perceived) and, in so doing, to determine whether 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 spectacle blood flow occurs; and (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.

Prior to discussing these findings in turn, a brief discussion of visual acuity of snakes is in order. While any visual consequences due to the presence of blood vessels have not been verified experimentally, this discussion will assume that they limit visual capacity, based on the fact that few vertebrates have non-retinal blood vessels in the optically transmissive portions of the eye and that Lüdicke's (Lüdicke, 1969) findings of the spatial distribution of blood vessels in *A. nasuta*'s spectacle show a reduced density of vasculature in the foveal and binocular fields, which we can infer to be an adaptation to minimize visual disturbance in these fields. Baker and colleagues (Baker et al., 2007) provide one of the few published measures of visual acuity in snakes and found the midland banded water snake (*Nerodia sipedon pleuralis*) to have an acuity of ~ 4.9 cycles deg^{-1} by recording evoked telencephalic potentials. As a reference, the acuities of cats and dogs measured with similar techniques are respectively 3.2–6.5 and 12.6 cycles deg^{-1} (Berkley and Watkins, 1973; Odom et al., 1983); that of rats is ~ 0.44 – 1.2 cycles deg^{-1} , the measure varying according to the specific technique used (Boyes and Dyer, 1983). The water snake's acuity is thus quite respectable for a small eye, and Baker and colleagues (Baker et al., 2007) commented that larger-eyed snakes like coachwhips (as used in the present study) may well achieve higher acuity results. Although the acuity of coachwhips has not been measured, their large eyes and their ecology and 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, similar to *A. nasuta*. One anatomical

advantage the coachwhip may have in minimizing the perception of its own spectacle vasculature is the protrusion of its crystalline lens through its pupil, which sets a lower boundary on pupil constriction. A comparatively larger pupil minimizes the depth of field of an eye (Smith and Atchison, 1997), suggesting that coachwhips, as well as other species with larger pupils, are less likely to perceive the spectacle vessels as entoptic phenomena. Conversely, those species with pupils that constrict to thin slits, such as some boids, pythonids and viperids, would be more susceptible to casting perceptible images of the spectacle vessels on their retinas, especially given the short focal lengths of snake eyes (Sivak, 1977; Howland et al., 2004), which further increase the depth of field.

The cyclical pattern 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 flow is absent, the vessels are particularly challenging to observe even with a slit-lamp. The animals' initial perception of potential threats therefore depends partly on the likelihood that the spectacle vessels were empty at the time the threats presented themselves (assuming that discontinuous flow is also characteristic in the wild even at rest), particularly when the target is at the threshold of their acuity under the given conditions. While these blood vessels are necessarily permanent, immobile fixtures of the spectacle, their location within the visual field will dynamically vary with rotation of the eyes. As a result, visual adaptation to the blood vessels [e.g. from Troxler's effect in which stationary images on the retina 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 remarkably steady, exhibiting few to no saccades compared with a human subject, whose eyes exhibit constant minute shifts in gaze direction. This ability of snakes to maintain a steady gaze may thus eliminate their perception of the spectacle vessels, similarly to humans' (and presumably other mammals') psychophysical adaptation to their own retinal vasculature.

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, it was found that the proportion of spectacle flow appeared to be related to some degree to ambient temperature, with lower temperatures resulting in longer periods without flow. This is consistent with the animals being moved from a heated terrarium to a lower ambient

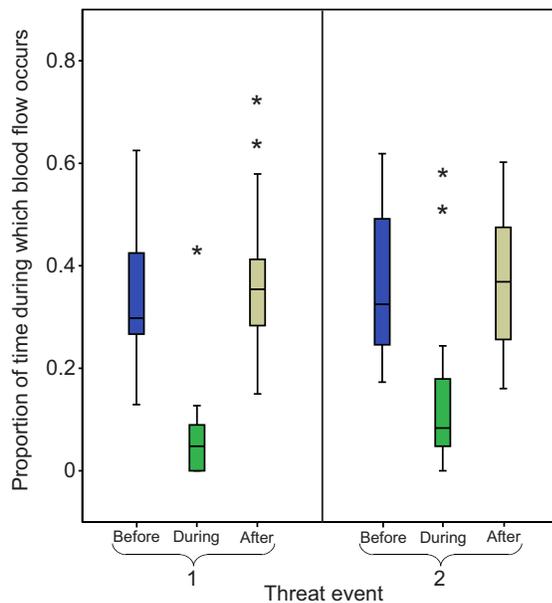


Fig. 5. Plot of the proportion of time during which spectacle blood flow occurred before, during and after each threat event. The plots, which are based on seven trials on each of three coachwhip snakes, show the mean (horizontal bar), the 25–75% intervals and the standard deviation, as well as outliers. It can be seen that the proportion of flow during the threat events is less than that during the 8 min blocks before and after each threat was presented. The 25–75% intervals are similar in the 8 min before and after each threat, indicating a rapid return to baseline after the threat is removed.

temperature, which would result in cutaneous vasoconstriction to minimize heat loss and maintain core temperature (Morgareidge and White, 1969; Rice and Bradshaw, 1980; Bartholomew, 1982). It is certainly possible that the transfer to the transparent box and/or merely being held within it was stressful enough to the experimental animals to affect the durations of the flow and empty periods. This could explain the variation observed between trials, but there is unfortunately no simple way to test this.

The rapid return to resting state blood flow dynamics after the removal of threatening stimuli suggests a neural mechanism is involved in the regulation of spectacle blood flow. The question remains whether the observed vascular changes were occurring only in the spectacle or whether they occurred across the whole integument. After all, and in spite of its transparency and unique attributes, the spectacle is part of the integument, and general sympathetic responses may be accompanied by localized cutaneous vasoconstriction (Nalivaiko and Blessing, 1999; Blessing, 2003) concurrently with localized cutaneous vasodilation (Vianna and 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, an emptying and constriction of the spectacle blood vessels occurs. This would be of visual benefit in

anticipation of a strike or an escape that requires improved acuity to be effectively carried out.

With regard to the constant spectacle blood flow during the renewal phase of the integument, this is presumably necessary to support the cellular proliferation involved in the generation of a new stratum corneum (Maderson, 1985; Maderson, 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.

Given that blood vessels are well known to occur in the spectacle dermis of snakes and have also been described in geckos, xantusiid night lizards (Mead, 1976) and amphisbaenids (Foureaux et al., 2010), they may be a common characteristic of spectacled squamates in general. In contrast, few non-squamate tetrapods have blood vessels in the optical path of their eyes, and those that do restrict them to the cornea. These are species that have reduced eyes [the giant salamander *Megalobatrachus* – see Duke-Elder, citing Tawara and Kurose (Duke-Elder, 1958; Tawara, 1933; Kurose, 1956)], or are known to exhibit modest visual acuity [the Florida manatee *Trichechus manatus latirostris* (Rochon-Duvigneaud, 1943; Bauer et al., 2003; Harper et al., 2005)], or possess keratinized corneas [the armadillo *Dasyurus* (Duke-Elder, 1958)], the armadillo paralleling spectacled squamates in maintaining a keratin barrier over the eye, which has been suggested to inhibit the diffusion of atmospheric oxygen into the cornea (Walls, 1942). Unlike the case with spectacled reptiles, the image (whether blurry or sharp) of corneal vessels cast upon these animals' retinas would remain stationary even with shifts in gaze, thus benefitting from Troxler's fading of stabilized retinal images. The only other species known to maintain blood vessels in the optical path are those with transparent nictitating membranes such as penguins (Sivak and Glover, 1986).

Further research will be necessary to determine whether the vascular dynamics described here hold true for other species of snakes and squamates with spectacles or windowed eyelids, as well as in species that possess transparent nictitating membranes. Attempts to image blood flow in the gecko spectacle using the same techniques as used with the snakes were largely unsuccessful as on only one occasion was a single blood cell observed in a specimen of marbled gecko, *Gekko grossmanni* (K.v.D. and J.G.S., unpublished observations). This might be accounted for by its smaller spectacle vessels, which measure 4–17 μm in width (Lüdicke, 1971), or by its small erythrocytes, which measure as little as 9 μm on the short axis (Saint Girons and Saint Girons, 1969; Starostová et al., 2005), taxing the resolving power of the imaging system. It is also possible that *G. grossmanni* simply exhibits little blood flow in the spectacle under stressful laboratory conditions, either by having a greater resistance to anoxia, by supplying the cornea and spectacle *via* diffusion from the iridial or limbal vasculature, and/or *via* the diffusion of atmospheric oxygen into and through the spectacle.

As integument, the reptilian spectacle, being adapted to the ocular need of tissue transparency, offers unprecedented value as a means to study cutaneous vascular physiology. In combination with their large erythrocytes, which are easier to image than those of mammals, snakes in particular may be an excellent model animal for studying peripheral vascular dynamics as demonstrated by these experiments. The imaging techniques described here could be of significant utility in studying cutaneous blood flow dynamics during thermoregulation, or for any other purpose that calls for the quantification of cutaneous vascular flow.

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AUTHOR CONTRIBUTIONS

K.v.D. designed and performed the research; K.v.D. and J.S. wrote the paper.

COMPETING INTERESTS

No competing interests declared.

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