Morphology of the Elygium and Developing Umbraculum in the Eye of *Amietia vertebralis* Tadpoles

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ABSTRACT The elygium is a darkly pigmented projection over the pupil of the tadpoles of certain species that live mostly at high altitudes. It has been suggested that this structure shades the retina, protecting it from high UV levels. In post-metamorphic individuals, the elygium is replaced by a structure known as an umbraculum. Confusion arose in the past from the inconsistent use of terminology when referring to these two structures. While they may serve the same function, these structures differ fundamentally in structure and origin. Our investigation of the ultrastructure of the eye in *Amietia vertebralis* tadpoles, using electron and light microscopy, revealed that the elygium consists of an accumulation of melanophores situated within the inner cornea, whereas the umbraculum is a dorsal extension of the iris pigment epithelium, analogous to the ventral pupillary nodule, which is present in most frog species. In *A. vertebralis*, the umbraculum starts to develop on the iris of older tadpoles (Gosner [1960] stage 37 Herpetologica 16:183–190), medial to the overlying elygium. A smaller, ventral elygium is also present in this species and is similar in structure to the dorsal elygium. The development of the elygium over a six-month period is also described. J. Morphol. 274:551–556, 2013.

KEY WORDS: *Amietia vertebralis*; elygium; microscopy; ultrastructure; umbraculum

INTRODUCTION

The vertebrate eye has been the subject of extensive research, especially in the fields of embryology, evolution, regeneration, histology, and anatomy, as well as medical research (Chow and Lang, 2001; Imokawa et al., 2004; Sudha, 2007; Fernald, 2009; Wu et al., 2011). One of the least understood structures in the eye of some vertebrates is the umbraculum, a fleshy projection of the iris into or over the pupil, which is believed to shade the eye from excessive light (Ewer, 1952; Van Dijk, 1966) and UV radiation (Channing et al., 2012). The umbraculum of the rock hyrax (*Procavia capensis*), which enables them to spot raptors overhead without being hindered by the sun's glare (Millar, 1973), the operculum in several whale species, skates and teleost fish eyes (Walls, 1963; Mass, 2001), the corpora nigra of the dorcas gazelle and of the camel (Rahi et al., 1980), and the umbraculum (Fig. 1A) in adult frogs (Du Preez and Carruthers, 2009) all serve as umbrellas for diurnal eyes that are typically exposed to high-light intensities (Walls, 1963). A similar structure, the elygium (Fig. 1B), is found only in the eye of some amphibian tadpoles. It can either be a deeply pigmented zone arising from the middorsal margin of the iris distal to the pupil (ocular elygium) or a pigmented layer in the cornea located at the skin-cornea junction, in which case it is known as the epidermal elygium (Van Dijk, 1966).

Although elygia and umbracula are not common in any given amphibian assemblage, they have been documented in a number of anurans from around the world (Ewer, 1952; Walls, 1963; Van Dijk, 1966; Wassersug et al., 1981; McDiarmid and Altig, 1999; Channing, 2001; Dziminski and Anstis, 2004; Du Preez and Carruthers, 2009). Among the southern African anurans umbracula have been documented in adults of only four species, *Vandijkophrynus angusticeps*, *Hyperolius horstockii*, *Amietia umbraculata,* and *A. vertebralis* (see Mann, 1931; Du Preez and Carruthers, 2009), and elygia in the tadpoles of six species, *A. dracomontana*, *A. umbraculata*, *A. vertebralis*, *Strongylopus wageri*, *S. grayii,* and *Leptopelis natalensis* (Channing, 2001; Du Preez and Carruthers, 2009; Kruger, 2010).

A thorough literature review revealed that little is known about these ocular structures. Furthermore, the use of the terms elygium and umbraculum has not been consistent, giving rise to confusion. Not only do these structures occur in different places in the eye (Van Dijk, 1966) but also in different life stages. Ewer (1952) correlated the pupillary index for light and darkness adaptation and described the cellular structure of the umbraculum of *A. umbraculata*. Wassersug et al. (1981) described the presence of darkly pigmented skin covering the eye of the tadpole of *Philautes*

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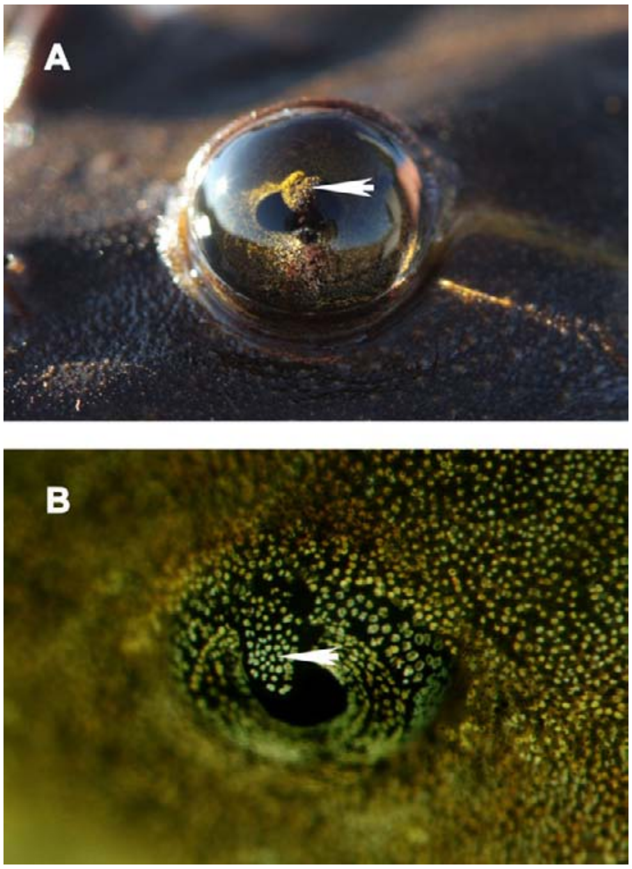

Fig. 1. A prominent umbraculum in the eye of an adult *Amietia umbraculata* (**A**) and an elygium in the eye of the tadpole of *A. vertebralis* (**B**). Both of these structures are flecked with iridophores. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com].

carinensis dorsomedially, which we assume represents an elygium. Candioti et al. (2005) noted the presence of an umbraculum in the tadpole of *Eupsophus calcaratus*. With the description of the dorsal and ventral elygia and the developing umbraculum in the eye of the tadpole of *A. vertebralis* in this article, we hope to resolve the confusion and emphasize the differences between these two ocular structures.

MATERIALS AND METHODS

To study tadpole development and in particular the elygium development of *Amietia vertebralis*, 20 tadpoles were collected in June 2009 near the headwaters of the Bilanjil River (28.760611S, 28.899417E, 2,966 m a.s.l.). Tadpoles were identified using a binomial key from Kruger et al. (2011). These tadpoles were placed into a shallow glass tank with sloping sides and that formed part of a simulated flow-through river model. The water was circulated from the pump passed through a MGW Lauda water cooling system (Type DLK-15, Western Germany) which maintained the water at 16°C in the autumn months (March–May, 2010) and 10°C in the winter months (June–August, 2010). At two-week intervals, 10 tadpoles were randomly selected, staged, and measured. Measurements were taken from March 7, 2010 until August 26, 2010 (Table 1).

Four *Amietia vertebralis* tadpoles (Gosner, 1960; stage 37) collected in September 2012 at the same locality in the Bilanjil River, were euthanized using 10 mg/l of MS222 (tricaine methane sulphonate), and fixed in TODD's solution (Todd, 1986). These tadpoles were used for light and transmission electron microscopy (TEM). The research satisfied the institutional requirements of animal ethics and adhered to the local legal requirements.

Biological Sample Preparation for Microscopy

Tadpoles were fixed overnight at 4°C with a glutaraldehyde–paraformaldehyde-(GA/PFA)-mixture modified after TODD's fixative (2.5% GA, 1.25% PFA, 0.03% $CaCl_2$, 0.03% Picric acid, pH 7.4) in preparation for TEM.

The eyes were excised from the tadpole, placed in fresh TODD's and placed on a TAAB rotator (4 r.p.m.) inside a fume cabinet to ensure proper perfusion of tissue with chemicals, before washed three times for 15 min in 0.05 mol l^{-1} cacodylate buffer. Eyes were post fixed in 1% OsO_4 for 1 h and then washed three times in distilled water for 15 min. Samples were stained with 2% uranyl acetate for 1 h and washed again three times in distilled water for 15 min. Samples were then dehydrated in an ethanol series (50%, 70%, 90%, and 100% × 2 for 15 min each). Care was taken to avoid exposure of the samples to dry air during this entire process. The 100% ethanol was replaced with 100% LR white resin for 10 min (Hard Grade Acrylic Resin, London Resin Company Ltd., England). The eyes were placed in fresh LR white resin twice for 1 h each and left overnight in the third resin substitute. The eyes were

TABLE 1. A summary of biweekly measurements of the elygium and the eye of the tadpole of A. vertebralis over a period of six months

Reading	Total length (mm)	Gosner	Elygium area (μm^2)	Elygium length (μm)	Elygium base (μm)	Lens diameter (μm)	Lens area (μm^2)	Ocular diameter (μm)
1	23.39	26	48,973	267	475	415	132,522	1,253
2	23.83	26	61,109	253	432	430	235,964	1,236
3	23.79	26	63,921	267	448	412	134,944	1,223
4	25.74	27	58,883	233	471	438	158,504	1,297
5	27.94	28	86,723	293	544	466	181,827	1,414
6	26.46	28	67,262	259	499	416	157,759	1,275
7	28.59	29	97,873	290	587	468	194,094	1,454
8	35.41	30	160,535	345	803	555	288,826	1,761
9	37.55	30	152,823	351	800	502	266,234	1,777
10	31.03	30	825,31	274	567	471	174,548	1,471
11	34.43	30	90,765	296	602	501	193,302	1,570
12	35.6	31	107,061	328	667	534	224,713	1,679

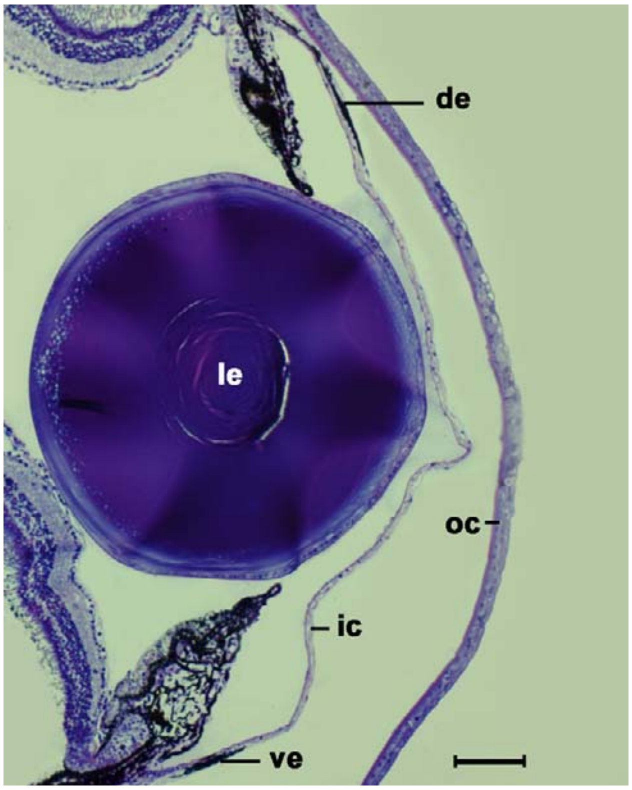

Fig. 2. A three micron sagittal section through the eye of *Amietia vertebralis*, showing the pigmented zones of the dorsal and ventral elygium in close association with the inner cornea. Section stained with toluidine blue and neufuchsin. de, dorsal elygium; ic, inner cornea; le, lens; oc, outer cornea; ve, ventral elygium. Scale bar = 100 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com].

orientated in a way that allowed sagittal sections (to the eye's perspective) to be made through the umbraculum. Capsules containing the tissue imbedded in resin were cured overnight in an oven at 65°C.

The resin capsule was trimmed and mounted in an automated microtome. Sections of 20 μm were cut until the area of interest was reached at which time the sections were reduced to 10 μm and then to 1.6 μm. Progress was monitored after every tenth section by evaluating a "monitor" section. Some sections from the center of the eye were stained with hematoxylin and eosin and others with toluidine blue for light microscopy.

The capsule was removed from the microtome and trimmed into a tight square surrounding the elygium. This was performed using a Nikon stereo microscope. A new microtome blade was then used to cut 0.4 μm sections to determine the elygium position, while monitoring every tenth section. Three 0.4 μm sections were transferred to a drop of dH_2O on a clean microscope slide. Xylene fumes were used to relax the thin film sections floating on the water. A small copper grid (75 μm mesh) was used to pick up each section. One grid was selected for TEM. The cellular structure of the elygium and underlying umbraculum were photographed with a Philips CM-10, 80 kV transmission electron microscope.

RESULTS

Elygium Position and Morphology

In the sagittal section through the eye of the of *A. vertebralis* tadpole in Gosner (1960, stage 37) (Fig. 2), the retinal layers, iris, inner and outer cornea, lens, dorsal elygium, and ventral elygium are easily distinguished. The developing umbraculum appears as a pigmented dilation of the pigment epithelium of the iris, extending above the dorsal aspect of the lens (Fig. 3). Dorsal to the developing umbraculum, the elygium can be observed as a layer of elongate melanocytes within the inner cornea. The elygium and developing umbraculum thus coexist in the same developmental stage (Gosner, 1960; 37). The relatively thick outer corneal epithelium consists of two to three layers of cubic epithelial cells. Tadpoles of *A. vertebralis* also possess a ventral elygium (Fig. 2), which coexists with the dorsal elygium.

Ultrastructure of the Elygium and Developing Umbraculum

The cornea consists two main layers, namely, the outer and inner cornea. The outer cornea consists of a corneal epithelium (cuboidal epithelial cell), stroma (collagen fibres arranged in layers), and a thin squamous endothelial layer. The inner cornea consists of a layer of densely compacted collagen fibrils in close association with mesenchymal cells (stroma) and lined with a thin inner squamous endothelial layer (Fig. 4). Bowman's layer appears to be absent. The elygium is formed by a layer of melanocytes that lie within the inner cornea between the endothelium and the stroma. The

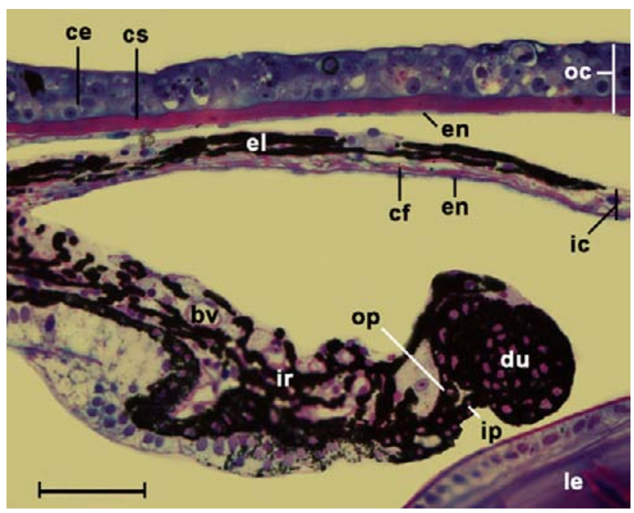

Fig. 3. Sagittal section of the iris of *Amietia vertebralis*, showing the outer cornea, inner cornea, dorsal elygium, developing umbraculum, and the iris. bv, blood vessel; ce, cornea epithelium; cf, collagen fibres; cs, cornea stroma; du, developing umbraculum with rounded melanocytes; el, dorsal elygium with its elongate melanocytes; en, endothelium (both at the inner and outer cornea); ic, inner cornea; ip, inner pigment epithelium of the iris; ir, pigmented cells of the iris; le, lens; oc, outer cornea; op, outer pigment epithelium of the iris. Scale bar = 50 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com].

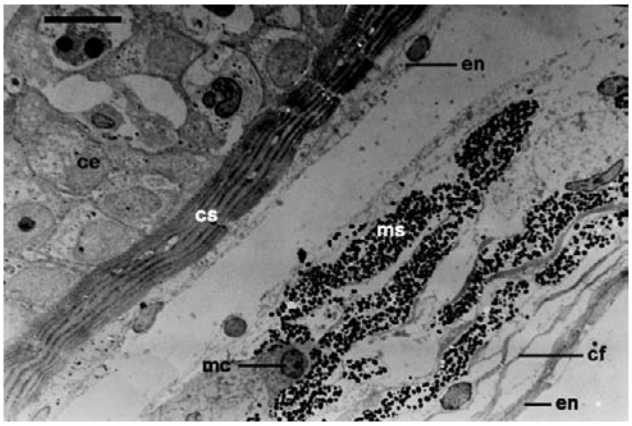

Fig. 4. Transmission electron micrograph of a cross-section of the cornea in the elygium-containing area, showing the cellular composition of the different layers of the outer and inner cornea and the melanosome-filled cells of the elygium. ce, layered cuboidal epithelium; cs, cornea stroma collagen fibres; en, endothelium (both at the inner and outer cornea); mc, mesenchymal cell; ms, melanosomes containing melanin. This melanocyte layer forms the elygium. Scale bar = 10 μm.

melanocytes are elongated and filled with melanosomes.

The developing umbraculum, a dilation of the anterior and posterior iris pigment epithelium, appears to consist of a mass of compact melanocytes (Fig. 5), which forms the umbraculum of the adult frog after metamorphosis. The developing umbraculum has the same cellular structure as the pigmented epithelium of the iris, consisting of densely pigmented melanocytes. Both the umbraculum and elygium are flecked with iridophores (Fig. 1A and B).

Elygium Development

During early development (Gosner, 1960; 26–27), the elygium width was broad and spanned nearly

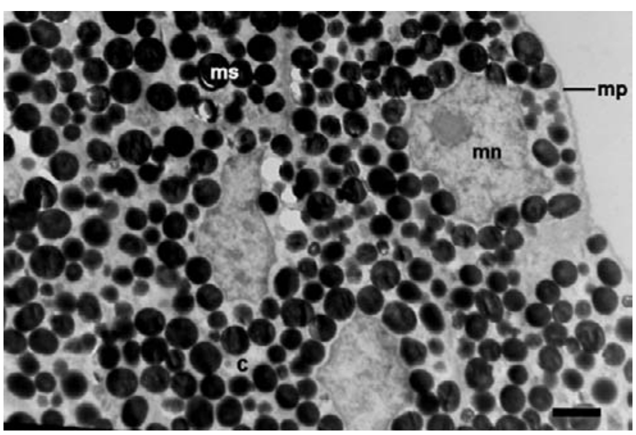

Fig. 5. Transmission electron micrograph of a sagittal section showing the cellular composition of the pigment cells within the developing umbraculum. c, cytoplasm; mn, melanocyte nucleus; mp, melanocyte plasmalemma; ms, melanosome containing melanin. Scale bar = 1 μm.

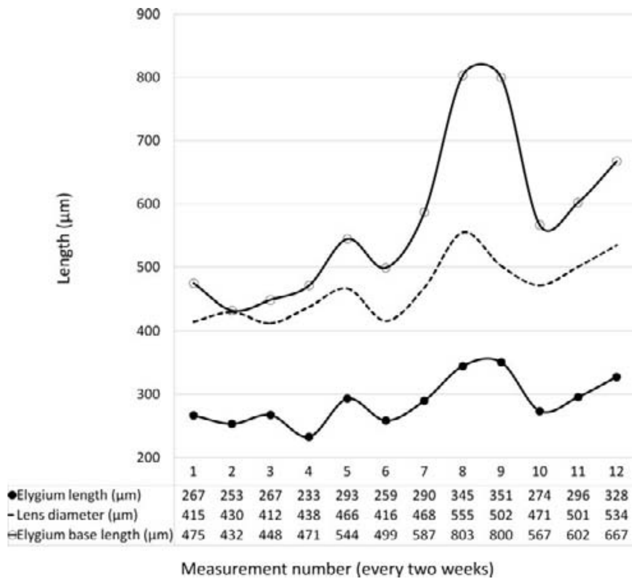

Fig. 6. A line chart showing the development of the elygium length, elygium base length, and the lens diameter over a period of six months.

the full diameter of the lens (Fig. 6). At later developmental stages (Gosner, 1960; 28–31), the width of the elygium relative to the diameter of the lens increases more rapidly than its length, which only increases slightly (Table 1; Fig. 7).

DISCUSSION

Although the presence of elygia and umbracula in anurans is well known, little research has been done on the morphology of these structures. The term umbraculum was first applied to amphibians by Mann (1931) with reference to the eyes of *Hyperolius horstockii* and *Bombina bombina*. Bush (1952) described the umbraculum of *Amietia umbraculata* as a large, brightly colored, circular extension of the dorsal margin of the iris, whereas Ewer (1952) described the histological structure of the umbraculum and ventral pupillary nodule of *A. umbraculata*. Van Dijk (1966, 1972) was the first to assign the term “elygium" to tadpoles and described the external appearance of this structure. Van Dijk (1966) distinguished between ocular and epidermal elygia, referring to the ocular elygium as a pigmented layer arising from the margin of the iris distal to the pupil, and the epidermal elygium is a pigmented layer in the skin above the eye. The exact morphological position of these two types of elygia has never been clearly described. Subsequently, the terms umbracula and elygia were not consistently applied by authors describing new species. Passmore and Carruthers (1979) and Lambiris (1988a) noted the presence of an umbraculum in adult *A. umbraculata*. Wassersug et al. (1981) described a darkly pigmented

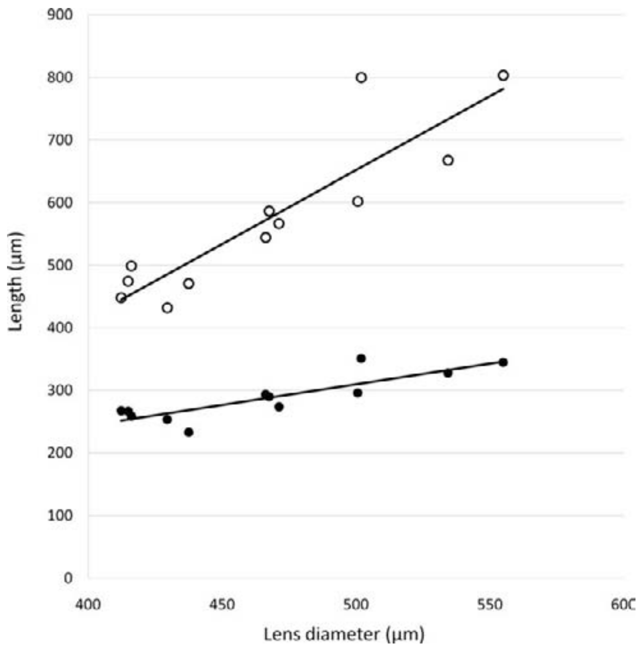

Fig. 7. A scatter plot showing the correlation of elygium length (solid circles) and elygium base length (open circles) to lens diameter.

area of skin occurring dorsomedially in the eye of the tadpole of *Philautes carinensis*, but did not name this structure. Lambiris (1988b) noted that the umbraculum and elygium have different anatomical origins, but did not provide further details. McDiarmid and Altig (1999) noted the presence of ocular elygia in certain *Bufo*, *Hyla*, *Mantidactylus*, *Pseudacris*, and *Rana* species. Channing (2001) noted that the tadpoles of *Amietia dracomontana* possess an umbraculum, whereas Du Preez and Carruthers (2009) refer to the same structure as an elygium. Channing (2001) notes the presence of an umbraculum in the tadpole of *Strongylopus hymenopus*, which is lost in adults. Du Preez and Carruthers (2009) referred to the presence of an umbraculum in adults of *Vandijkophrynus angusticeps*, *Amietia umbraculata*, and *A. vertebralis* and in the case of tadpoles, referred to the elygium in *A. dracomontana*, *A. umbraculata*, *A. vertebralis*, *Strongylopus wageri*, *S. grayii*, and *Leptopelis natalensis*. In addition, the term umbraculum has also been used to describe the pigmented cornea in the eyes of other nonAfrican tadpoles (Dziminski and Anstis, 2004; Candioti et al., 2005; Doughty et al., 2009; Nunez and Úbeda, 2009; Basso et al., 2011). No literature distinguishes between ocular and epidermal elygia.

This study shows that the tadpoles of *A. vertebralis* possess both dorsal and ventral elygia, which are located in the inner cornea and are equivalent to the epidermal elygium described by Van Dijk (1966). We did not observe any structure that could be regarded as equivalent to the ocular elygium, described by Van Dijk (1966), as "arising from the iris distal to the pupil." The developing umbraculum, which occurs as a dilation of the pupillary margin of the iris in the late-stage tadpoles of *A. vertebralis* does not appear to have been recognized previously.

Ewer (1952) and Channing (2001) suggested that all South African frogs and tadpoles from high altitude montane habitats are likely to possess elygia and umbracula, but thus far these structures are known from only six of a possible 25 montane species and are thus not ubiquitous in this ecological guild. As noted by Ewer (1952), the postulated function of the umbraculum, that of shading the eye, is speculative and the adaptive significance of this structure and of the elygium has still to be demonstrated. Additional data on the occurrence of umbracula and elygia, and on the habits of the species possessing them, are much needed. Because of the numerous melanophores and iridophores they possess, it appears that both the elygium and umbraculum have a light absorbing or shading function. The developing elygium expands first in width and then in length. It appears if the elygium width is of more functional importance to shade the pupil, implying a greater selection pressure in wider elygia. The elygium is positioned in such a way that it will protect the retina of tadpoles with dorsolaterally positioned eyes, when sunlight shines directly from above. The fact that the umbraculum starts to develop in conjunction with a functional elygium in the tadpole of *A. vertebralis*, ensures that the eye is never without the protection of a dorsal orbital appendage even during metamorphosis, when the elygium starts to disappear.

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