MICROORNAMENTATION ON SNAKE SCALES

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DECLARATION

I declare that this dissertation is my own, unaided work unless specifically acknowledged in the text. It has not been submitted before for any degree or examination in any other university, nor has it been prepared under the aegis or with the assistance of any other body or organisation or person outside the University of the Witwatersrand, Johannesburg.

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ABSTRACT

The morphology and functionality of surface microornamentation in southern African snakes is well-established in terms of resulting optical effects. Velvet-blackness, a type of optical effect is produced when light incident on a scale is scattered by microornamentation. I tested microornamentation from *Bitis arietans* dorsal exuvia for these optical effects. Scales were excised from shed skins of *B. arietans* and sputter coated with ±15 nm Gold-Palladium to control the effect of pigment. Spectral intensity (SI) of three scale regions of known micro-topography was recorded using spectrophotometry over the visible spectrum only as a measure of reflectivity of the scales. Given that surface roughness (the deviation of a membrane's surface topography from an ideal surface) is a product of the size of surface asperities and its degree of randomness, the three scale regions in order of decreasing surface roughness were dark (dorsal) scales, pale (dorsal) scales, and ventral scales. Measures of SI on dorsal and ventral scale regions revealed lowest SI on dark dorsal scales and highest on ventral scales. In general, the level of micro-structuring was inversely proportional to SI. To test if optical effects are angle-dependent, I measured differences in SI between normal (90°) and oblique (45°) angles of incidence. Differences in SI between 90° and 45° were significant for all scale regions which revealed that while microornamentation produces optical effects at both normal and oblique incidence, the effect is greater at 45°. Given that SI varies with surface roughness such that dark scales have a lower SI than pale scales, I conclude that scale colour in *B. arietans* is a product of optical effects created by microornamentation. The optical effect may improve the visual camouflage of *B. arietans* during ambush. While microornamentation is best known for its optical effects, it may have other functions. I examined the microornamentation in *Bitis schneideri* (Namaqua Dwarf Adder) using electron microscopy and found small, tooth-like protuberances distributed uniformly across the scales and across all body regions. I measured the distance between adjacent denticles which I compared to the mean dimensions of sand grains from two study sites: Noup, within the distribution of *B. schneideri*, and Tswalu, outside of it but with significantly smaller grains. The space between denticles is smaller than sand grains from both sites. Due to its physical characteristics, microornamentation in *B. schneideri* has the potential to restrict sand grains from direct contact with the scale surface. These results suggest that microornamentation can function to shield the integument from sand abrasion in *B. schneideri*. This study demonstrates specific functions of microornamentation in the ecology of two species of *Bitis*.

Keywords

Functional anatomy, microscopy, animal microanatomy, microornamentation, *Bitis*, scanning electron microscopy, optical effects, integumentary shielding

STRUCTURE OF THE REPORT

The background and scope of this study on the function of microornamentation in southern African snakes is presented in chapter one. In chapter two, I present an extensive review of progress in the field of microstructure-mediated optical effects. Studies on microstructure-mediated optical effects in butterflies have contributed significantly to our understanding of various types of optical effects, the morphology of the microstructures that produce them, and the synergist effect of pigments in producing optical effects. I report on my findings regarding the function of microornamentation in *Bitis arietans* in chapter three, and in *B. schneideri* in chapter four. I conclude the report with the outcomes of this study and future directions in chapter five.

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2. A REVIEW OF PROGRESS IN MICROSTRUCTURE-MEDIATED OPTICAL EFFECTS

3. DOES MICROORNAMENTATION PRODUCE OPTICAL EFFECTS IN THE PUFF ADDER, *BITIS ARIETANS*?

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CHAPTER 1

INTRODUCTION

The terms 'microornamentation' (Ruibal 1968), or 'microdermatoglyphics' (Dowling *et al.* 1972), refer to the overall structure of three-dimensional microscopic structural features of the outer epidermal layer of an organism, the oberhautchen surface (Maderson 1985, Irish *et al.* 1988). Microornamentation has a variety of functions. For example, microornamentation has an anatomic function in movement (Spinner *et al.* 2013a). It may aid physiological function; due to its manipulation of the flow of electromagnetic radiation, microornamentation has been suggested to play a role in thermoregulation (Nijhout 1991). Microscopic and nanostructures are known to alter the physical properties of scales, changing the reflectance properties and wettability. Hierarchically complex microornamentation on the dark scales of *Bitis rhinoceros* (West African Gaboon Adder) creates a black-velvet structural effect which intensifies the appearance of dark scales (Spinner *et al.* 2013b). Further, the microornamentation alters the surface tension of the scale reducing dirt and water adhesion (Spinner *et al.* 2014). Both effects of microornamentation, namely structural blackness and self-cleaning are indicated in improving the West African Gaboon Adder's camouflage in leaf litter of the forest floor. Ambush foragers depend on camouflage to remain undetected by both predators and prey (Miller *et al.* 2015) and the strong selective pressure on camouflage may have driven the evolution of the complex microornamentation on its scales.

While snake microornamentation remains poorly studied, the genus *Bitis* has received the most attention on the topic including reports on the microornamentation of *B. caudalis* (Price 1982), *B. rhinoceros* (Spinner *et al.* 2013a, Spinner *et al.* 2014) and *B. arietans* (Singh 2014, Singh and Alexander 2017). The present report developed from these earlier studies and presents the findings of two studies on microornamentation in *B. arietans* and *B. schneideri*. These two species represent opposites in the range of diversity found in *Bitis* in body size, degree of specialisation, and extent of distribution. On one hand *B. arietans* is a large-bodied viper with an extensive distribution across sub-Saharan Africa and is found in a variety of environments (Alexander and Marais 2007). On the other, *B. schneideri* is a small-bodied viper restricted to only the loose sands of the west coast of southern Africa with a relatively small distribution (Turner and Maritz 2014).

This study contributes to the growing body of evidence of microornamentation in *Bitis* which will make it possible to compare the effects of environment, body size, and phylogenetic relatedness on microornamentation. Due to their ambush foraging ecology, both *B. arietans* and *B. schneideri* rely on camouflage to remain undetected by both predators and prey (Miller *et al*. 2015). To increase its foraging success, *B. arietans* is under strong ecological selection for improved camouflage and *B. schneideri* is under strong ecological selection for reducing the effects of abrasion from burrowing in sand.

The morphology and functionality of microornamentation in southern African snakes is under-studied. As a result the function of scale microstructures in the biology of southern African snakes is poorly understood. Previous studies detail the morphology of microornaments, but with only speculative suggestions as to its possible function. For example, Singh (2014) documents the morphology of the scales in *B. arietans*, which show significant differences between the dark and pale scales, similar to that reported by Spinner *et al*. (2013b) in *B. rhinoceros*. However, Singh (2014) provided no experimental or conclusive evidence that demonstrate any optical effects of microornamentation, or indeed any other function. Microstructures are known to change the physical properties of scales, and are well studied in insects where key roles have been identified. Based on the similarity of the microanatomy of certain snake scales to butterfly scales, further studies on microornamentation in snakes has the potential to contribute significantly to the study of microstructure-mediated changes to an animal's integument. Whereas Price (1982) argues that microornamentation is a phylogenetic character rather than an ecological adaptation, the overarching aim of this study is to find a measurable function of microornamentation in the biology of two southern African snakes. This study aims to add to a growing body of knowledge that supports the idea that microornamentation has specific functions, and is not just the product of phylogenetic relatedness. This aim is expanded into two sub-aims with objectives as follows:

Aim 1: To assess if microornamentation produces an optical effect in Bitis arietans

Rationale: The roughness of a surface determines its ability to reflect an incident light beam. Rough surfaces reflect diffusely, whereas smooth surfaces reflect specularly. Since microornamentation is a random arrangement on the scale surface of *B. arietans*, it is expected to promote diffuse reflectance.

- Key research question: Does microornamentation produce optical effects in *B. arietans*?
    - Objective 1.1: Measure the reflectance properties of the scales of *B. arietans*, and compare differences in reflectance between scales with microornamentation (treatment) as compared to that without (control)
        - Hypothesis: Scales with microornamentation have an overall lower reflectance than scales without
    - Objective 1.2: Test for differences in reflectance between angles of incidence for scales with microornamentation as compared to that without
        - Hypothesis: Scales with microornamentation show differences in reflectance at different angles of incidence

Aim 2: To assess if microornamentation reduces contact area between sand and scales in Bitis schneideri

Rationale: The Namaqua Dwarf Adder is endemic to the west coast of South Africa where it buries itself beneath a narrow upper layer of sand. The sand grains are angular due to its geological history. The integument of *B. schneideri* risks damage from abrasion when burrowing in sand and during windy conditions.

- Key research question: Can microornamentation reduce area of direct contact of sand grains and the integument in *B. schneideri*?
  - Objective 2.1: Compare the size of the spaces between ornaments on the scales of *B. schneideri* to the size of sand grains from its distribution
    - Hypothesis: The space between ornaments is less than the size of sand grains

CHAPTER 2

LITERATURE REVIEW: FUNCTIONAL MICROSTRUCTURES IN BIOLOGICAL SYSTEMS

Introduction

Progress in the field of microornamentation in animals has focused mainly on the histology of the epidermis especially during the sloughing cycle (Maderson 1964, Irish *et al.* 1988). Microornamentation in African snakes, however, remains poorly studied. Hierarchically complex microornamentation on the dark scales of *Bitis rhinoceros* (West African Gaboon Adder) creates a black-velvet structural effect which intensifies the appearance of dark scales (Spinner *et al.* 2013b). Further, microornamentation alters the surface tension of the scale, reducing dirt and water adhesion (Spinner *et al.* 2014). The two effects of microornamentation reported by Spinner *et al.* (2013b), namely structural blackness and self-cleaning, are indicated in improving *B. rhinoceros*'s camouflage against the leaf litter of the forest floor. Other than the work on *B. rhinoceros* by Spinner *et al.* (2013a, 2013b) and Spinner *et al.* (2014) almost no information is available and thus, conclusive evidence on the functionality of microornamentation in the biology of African snakes remains scarce. My study aims to add to the body of knowledge on the form and function of microornamentation in southern African snakes through conclusive evidence of specific functions in snake biology.

Studies on butterfly wing scale microstructures are most abundant, and thus butterflies have become model organisms for understanding a wide variety of optical effects caused by microornamentation. Studies on the microstructures of insect wings report its function in producing structural colours (Vukusic and Stavenga 2009), reducing wettability (Wang et *al.*

2007), and reducing the proliferation of microorganisms (Kelleher *et al.* 2015). While microstructures are indicated in various functions, the production of optical effects is the best studied. Butterflies, probably due their popularity in Europe, have received considerable interest from physicists and biologists alike. As a result, most reports on microstructure-mediated optical effects are from butterfly wing scales. The principles of the physics governing the optical effects on wing scales are equally applicable with those of snake scales, and form the theoretical foundation for the interpretation of the results from this study.

Microstructure-mediated Optical Effects: An ecological perspective

Almost all animals are exposed to the effects of light, either via sunlight or moonlight. Sunlight introduces colour, produced from electromagnetic radiation in the range of 300-700 nm through pigment and structural means (Fox 1976). The effect of colour creates variation in the natural world that can be to an animal's advantage or disadvantage. Other than the physiological functions of colour, such as absorption of light, temperature regulation and UV protection, for example, colours create an organism's appearance (Endler 1981). Selection with regards to the visual appearance of an animal has resulted in the evolution of fascinating methods of evading detection (crypsis); avoiding detection (mimicry and camouflage); signals for attracting mates (communication and behaviour), signalling danger (aposemy), territoriality and pollinator-host recognition (Endler 1981). The recognition of an animal's defining traits is important to members of the same species, as well as its natural predators and prey (Endler 1981) and is central to its various ecological relationships.

How structural colour is produced on scales

Structural colours are produced through the physical manipulation of electromagnetic radiation and not through the selective absorption of light by pigments (Vukusic 2006). Physical, or structural colours originate through the interaction of incident electromagnetic radiation (including visible, ultraviolet and near-infrared light in the wavelength range 300-

700 nm) with ordered microscopic ultrastructure that is on the same length scale as the wavelength of light (Vukusic and Stavenga 2009; Umbers 2011). In butterflies, microstructures on the scale surface of the organism act like a prism to refract the incident light beam, as a diffraction grating to diffract the light beam, or as a polarisation plane to scatter the light beam. The velocity of the light beam is retarded during refraction resulting in spectral colours. The wavelength of light that is reflected gives the scale surface the appearance of colour. For example, surfaces reflecting wavelengths of 450-490 nm appear blue (Umbers 2011). The majority of insect structural colouration is created by multi-layer interference where the cuticle is composed of alternating layers of high and low refractive index (Vukusic and Sambles 2003). Biological systems are complex and both chemical and physical colours can combine to produce striking effects.

Butterflies, a model organism for studying microstructure-mediated optical effects

The colourful appearance of butterflies results from the millions of microscopic scales covering their wings (Tada *et al*. 1998). The name Lepidoptera is derived from this feature and is Greek for ‘scaly winged’ (Dawson 2006). Butterflies have a more remarkable diversity of microscopic scale architectures than other insects, due partially to the processes of cuticle formation. Each scale is an evagination of the cuticle (Vukusic 2006)and has an upper and lower lamina. The upper lamina consists of ridges, micro-ribs and cross ribs forming a fine grid-like structure (Tada *et al.* 1998). From this basic structure of the scale, diverse architectures have arisen that produce optical effects. The fact that butterflies are under stronger selective pressures for colour-based intra-specific visual signalling for both mate-attraction and territoriality (Vukusic 2006) than other insects may also explain the diversity of structural colours, and why it is a model organism for structural colour research.

An insect’s appearance is often the combined effect of structural colours and chemical pigments, such as melanins, flavonoids and pterins (Vukusic 2006). Pigments also absorb stray wavelengths of light, thus yielding spectral purity (Vukusic and Sambles 2003). Special effects such as iridescence (or interference colours) occur as a result of physical structural colours in conjunction with underlying pigments under special optical conditions. Some of the best-studied examples of structural colour are from butterflies in the genera *Morpho* (Fig. 2.1) and *Papilio*. The genus *Morpho* is endemic to Northern and Central America (Kinoshita and Yoshioka 2005) and is known for its resplendent blue wings (Fig. 2.2 (a)) The underlying pigment is only visible under angles of incident light which do not produce the structural blue effect (Fig. 2.2 (b)). The size of the microstructures on the wing scales is in the dimension of the wavelength of blue light (Fig. 2.2 (c)). Therefore, light is diffracted by the microstructures to reflect short wavelengths of light, namely blue. The underlying pigments further absorb diffracted wavelengths other than blue so that the combined effect of structural colour and the underlying pigment produces high spectral purity of the blue wings of *Morpho* (Ingram and Parker 2008). Experiments on *Morpho rhetenor* where the underlying pigment was bleached, reveal a significant loss of spectral purity (Fig. 2.3, Ingram and Parker 2008). The effect of pigment in association with structural colour enhances spectral purity yielding a higher grade of colour, or special effects that change with light intensity or viewing angle.

Fig. 2.1 Butterflies from the genus *Morpho* are model organisms for studying structural colour. (Ingram and Parker 2008).

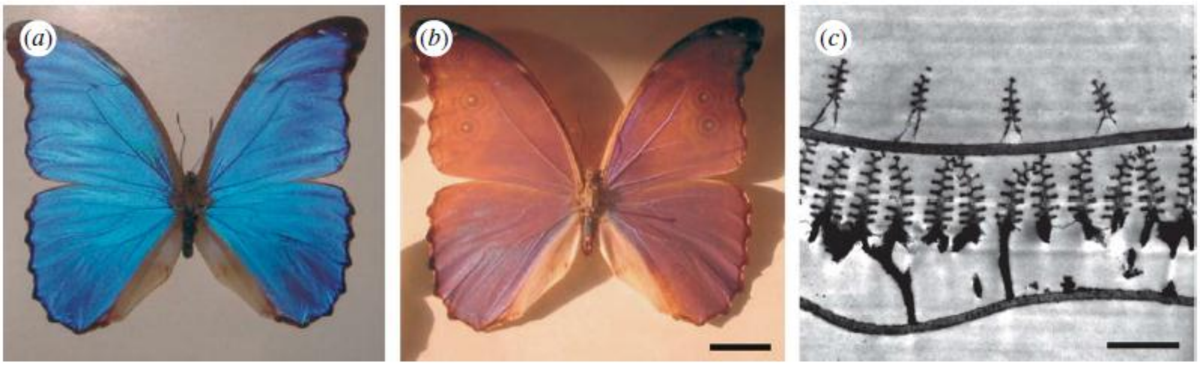

Fig. 2.2 The tropical butterfly *Morpho didius* is known for its resplendent blue appearance.When light is incident at a normal angle, the butterfly appears blue due to structural colours (a). When light is incident at an oblique angle, only the brown pigment of the scales is visible (b). The structural colour is produced by the refraction of light by microstructures on its wing scales (c). (Ingram and Parker 2008).

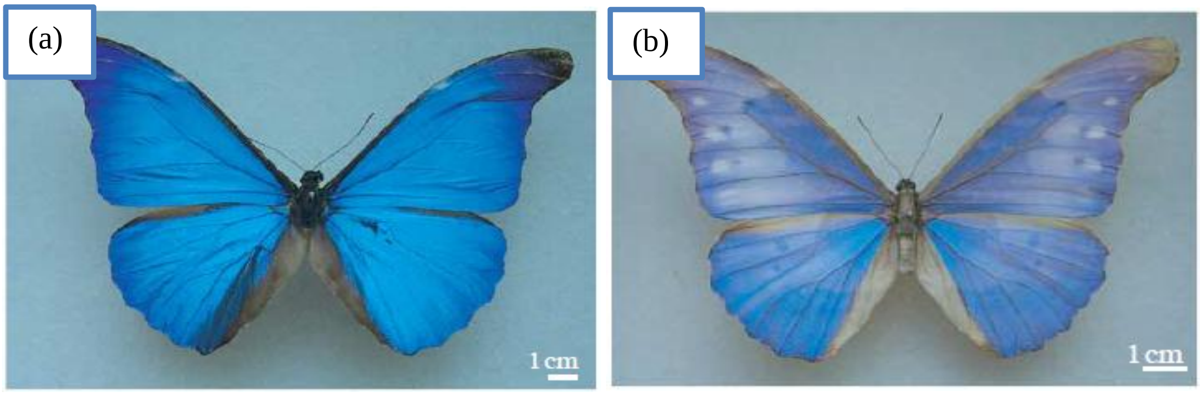

Fig. 2.3 *Morpho rhetenor* is a resplendent blue butterfly whose appearance is created by structural blue (a). When scales were bleached of the underlying pigment, a loss of spectral purity occurs (b) (Ingram and Parker 2008).

The appearance of an animal with angle-dependent structural effects varies with the angle of incident light. Thin-film interference is the mechanism underlying the production of structural colour in *Papilio blumei*, a tropical butterfly from Indonesia with a full wingspan of 10 cm (Tada *et al*. 1998). The basic anatomy of the wing scales is not very different to most butterflies, except for as many as ten layers of lamina separated by air gaps (Tada *et al.* 1998). These thin-film layers are curved to form a series of frames separated by ridges and cross ribs, shown in cross section in Tada *et al.* (1998). The thickness of each layer is matched to the order of wavelength of light so that thin-film curved microstructures result in specular reflection that is termed ‘non-planar’ because the microstructures change the angle of reflected light (Tada *et al*. 1998). Observed from different viewing angles, the reflected beam is a different colour, primarily a result of thin-film non-planar spectral reflection (Tada *et al*. 1998). Interesting colour effects result according to the angle of incidence of the light beam and the angle at which an observer views it: at a 45° angle, *P. blumei* appears green; at 0°, bluish-green; at -20°, blue; at -45°, purple and at -90°, white (Tada *et al*. 1998). Correspondingly, the butterfly appears differently at different times of day according to the angle of incidence of sunlight. Thus the non-planar optical effects produced by thin-film interference improve the camouflage of the butterfly due to the diversity of effects that vary with viewing angle.

Microstructure-mediated structural colour can help the organism thermoregulate better. *Papilio ulysses* has blue wings bordered by scales of rich black (Fig. 2.4, Vukusic *et al*. 2004). Studies on the black scales of *P. ulysses* revealed that the rich black appearance of these scales is due to structural colour (Vukusic *et al*. 2004). For equal amounts of absorbing pigment, scales with microstructures are more efficient absorbers of incident radiation. This creates a superior shade of black (Vukusic *et al.* 2004). The tapered ridges of the nanostructures serve as an anti-reflective mechanism which reduces the extent of back-scatter when incident light first encounters the scale (Vukusic *et al.* 2004). Light incident upon the microstructures in *P. ulysses* is more efficiently scattered toward the underlying pigment for re-absorption (Vukusic *et al*. 2003). Owing to the quality of the blackness and hence the higher absorption of incident light, the structurally assisted blackness has a role in the thermoregulation of the wings (Vukusic *et al*. 2004). In this case, an indistinct border created by the black scales has also been shown to render the coloured region less conspicuous as a defence mechanism (Silberglied 1984). Studies on *P. ulysses* resulted in a breakthrough in the study of structural colour because it shed light on the significant role of microscopic structures in creating the appearance of blackness (Vukusic *et al*. 2003).

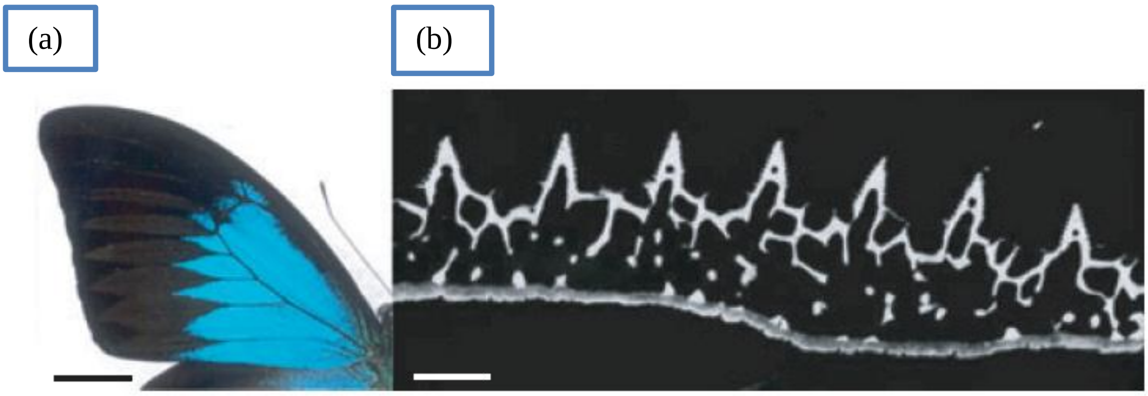

Fig. 2.4 The butterfly *Papilio ulysses* shows two types of black scales, glossy and matt (a. The structural colour is produced by microstructures (right) which vary in morphometrics. Scale bar: (a) 1cm, (b) 600 nm.

Structural colour as an ecological adaptation

Structural colours offer benefits that pigmentary colours do not. Pigmentary colour is often characterised by low intensity and a lack of directional effects (Fox 1976). The quality of structurally produced colour is superior to that of pigmentary colour and includes superior reflectance, brighter colour, and superior visibility than colours produced through pigments (Vukusic 2010). Structural colour is often characterised by directional effects, fluorescence, and colour reflectivity beyond the wavelength range of pigmentation (Vukusic *et al.* 2004). Furthermore, since the microscopic architectures of butterfly wing scales can modify and control the reflection and absorption of light off the wing surface, there are biological advantages relating to camouflaging, ultra-long signalling, orientation-dependent colour and thermoregulation (Vukusic 2006, Vukusic 2011, Tada *et al.* 1998). The butterfly *Papilio palinurus*, for example, has wing scales that emit a green structural colour (Vukusic 2006). This colour is due to the combination of yellow and blue wavelengths produced by different parts of each microstructure across the wing. The blue component is significantly linearly polarised such that only the visual system of a conspecific would recognise the blue signal. This offers the butterfly the advantage of camouflaged intra-specific signalling: appearing green to a predator yet blue to a conspecific (Vukusic 2006). In this example, the predation cost associated with a bright appearance is reduced. Since the blue is produced of strongly polarised light it requires a specialised visual system to be perceived.

Structural colours generated by continuous multi-layers can generate a strong reflection of one colour by polarisation. This is extremely advantageous in facilitating ultra-long distance intra-specific signalling (Vukusic 2006). In the butterfly *Morpho rhetonor*, for example, long-distance signalling of 400-800 m is achieved by the brilliant blue structural colour created by multi-layer interference (Vukusic and Sambles 2003; Vukusic, 2006). Structurally produced green iridescence in the chelicerae of the red-backed jumping spider, *Phidippus johnsoni* has also been associated with intra-specific male signalling (Ingram *et al*. 2011). In this example, the signal is also received by the visual system of a member of the same species (Ingram *et al.* 2011). The visual system of this animal shows peak sensitivity to 532 nm in the green range (Ingram *et al.* 2011) and thus the predation cost associated with a bright appearance is reduced, since the green produced requires a specialised visual system to be perceived.

Angle-dependent effects are a feature of structural colour that cannot be produced by regular pigmentary colour. According to the angle of incident light and viewing angle (Vukusic and Sambles 2003) a butterfly has the advantage of appearing differently under different circumstances of its orientation. At different times of the day, the incident solar radiation will be at different angles. According to the butterfly’s orientation its appearance will change. Thus, it may be able to signal to conspecifics and yet evade the risk of predators due to the orientation at which it is perceived by each. This may help the butterfly reduce predation risks when in a disadvantageous position due to a somewhat dynamic camouflage. In the morning hours the angle of incidence of light is still low, resulting in the absence of iridescent effects at this time (Tada *et al*. 2008). The absence of iridescent effects may promote camouflage and thus reduce its risk of predation (Tada *et al*. 2008).

Although physical structural colours are usually associated with brighter colour and higher reflectivity, certain nanostructures are also involved in reducing reflectivity with important biological functions in insect visual systems (Vukusic and Sambles 2003). Specific nanostructures match the optical impedance of neighbouring media and reduce broadband reflection. This enhances the photon collection efficiency of the compound eye (Vukusic and Sambles 2003) and has been strongly selected for in insect visual systems from early on in the evolution of the visual system, as fossilised Diptera from the Eocene period are found with such features (Land and Nillson 2001).

Electromagnetic radiation between the wavelengths 300 nm (ultraviolet) to 700 nm (red) are seen as colours (Umbers 2011). In the evolution of animal visual systems, the ability to perceive a specific range of wavelengths arose and was lost many times (Umbers 2011). Animals, in general, are able to perceive the range 400-700 nm. Birds are able to perceive between 300 nm (ultraviolet) to 700 nm (red); while insects, typically between 300 nm (ultraviolet) to 600 nm (green) (Umbers 2011). In a natural environment, the perception of colour depends on many factors. These include the background against which objects are seen, the clarity of the atmospheric conditions and the amount and colour of the ambient light available (Umbers 2011). Unfortunately, the current biological understanding of animal visual systems is restricted to the anatomical and physiological mechanisms of lenses and receptors. However, the type of information that travels to the brain and how it is processed and interpreted by the brain remains largely unclear (Schnitzer and Meister 2003). This also raises the question as to why butterflies predominantly reflect the range between UV to green, whereas they can see a wide range of colours (Ingram and Parker 2008).

Conclusion

Scientists from various fields have shown a keen interest in the study of colour production. Recent collaborations among biologists, chemists and physicists have elucidated more clearly the science underlying the phenomenon of structural colour. However, the drawback of the interest of researchers from various backgrounds has been the use of non-standardised terminology in published literature (Ingram and Parker 2008). Studies have indicated structural colour to have several biological functions, including long-distance signalling of up to 800 m (Vukusic and Sambles 2003). Colour signalling is responsible for intraspecific signalling amongst male butterflies, and therefore male butterflies are under stronger selection for resplendent colour as in *Morpho*. The principles of geometric optics reported in the examples of butterflies can inform the study of structural colour in snakes with microstructures.

CHAPTER 3

DOES MICROORNAMENTATION PRODUCE OPTICAL EFFECTS IN THE PUFF ADDER, *BITIS ARIETANS*?

Aspects of this chapter have been presented at the following conferences:

Microornamentation on Snake Scales
12th Conference of the Herpetological Association of Africa (Gobabeb, Namibia, November 2014)

A Micromorphometric Analysis of the Microornamentation of Snake Scales
7th Cross-Faculty Symposium (University of Witwatersrand, Johannesburg, March 2016)

Microstructure-mediated optical effects in southern African snakes
Nanophotonics and Micro/Nano Optics International Conference (Paris, France, December 2016)

Microstructure-mediated optical effects in *Bitis arietans*
13th Conference of the Herpetological Association of Africa (Bonamanzi, South Africa, January 2017)

Aspects of this chapter has been published in the following conference proceedings:

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ABSTRACT

To remain undetected by predators and prey, ambush foragers have a greater reliance on camouflage than do active foragers. It follows that ambush foragers are under intense selection for effective camouflage. *Bitis arietans* (Puff Adder) is an extreme ambush forager that has large, leaf-like micro-ornaments on its dark dorsal scales (Singh 2014, Singh and Alexander 2017). The level of micro-structuring on dark scales differs significantly to adjacent pale scales. This raises the question—how does microornamentation change the scale properties of dark scales? Because of evidence of optical effects in other species of *Bitis*, I investigated how microornamentation changes the reflective properties of dark scales. In this study I used spectrophotometry to measure how reflective dark and pale dorsal scales are. Ventral scales were measured to control for colour and structuring. Further, I measured the scale's reflectivity at normal (90°) and oblique (45°) angles of incidence over the visible spectrum on dark, pale, and ventral scale regions of *B. arietans*. Dark scales, when controlled for pigment, had a lower spectral intensity profile compared to other scale types at both normal and oblique angles of incidence. Further, the spectral intensity of dark scales was lower at oblique incidence than at normal incidence. Differences in reflective properties of scales in *B. arietans* correspond well with observed level of microstructuring. I suggest a microstructure-mediated optical effect is produced by microornamentation in *B. arietans*. The optical effect will tend to reduce the visibility of the snake against a similarly textured surface of low spectral intensity such as soil or leaf litter, and as a result may improve the snake's camouflage.

INTRODUCTION

Animals and plants use colour to define their physical appearance which becomes central to their ecological relationships. The nature of the colour organisms employ in camouflage, aposemy, and mimicry may be chemical (pigment-based), or physical (optical effects), or a combination of both (Fox 1976). While chemical-based colouration is produced by underlying pigments (Fox 1976), physical colour is produced through optical effects. Of the two mechanisms of colour production, optical effects produce more intense colours (Kinoshita and Yoshioka 2005) which can vary with angle of incidence (Tada *et al*. 1998). Other than producing colours, microstructures can also change scale properties, such as reducing the reflective properties of the scale (Spinner *et al.* 2013b) which can render the region less conspicuous as a defence mechanism (Silberglied 1984).

A light ray incident on a surface is reflected off in one of two ways, depending on the texture of the surface. Off a smooth surface, a light ray is completely reflected, and produces a real image (Hecht 2002). This type of reflection is known as 'specular reflection'. Off a rough surface, a light ray is scattered, and fails to produce a real image (Hecht 2002). This type of reflection is known as 'diffuse reflection'. Because each beam is scattered, the spectral intensity measured off a rough surface is always lower than the spectral intensity measured off a smooth surface (Hecht 2002). In snakes, microstructures (termed 'microornamentation') are found on the oberhautchen surface of a scale (Maderson 1985) which determine the type of reflection produced, either specular or diffuse (Vukusic and Stavenga 2009). The black hour-glass markings along *Bitis rhinoceros* (West African Gaboon Adder) are characterised by an optical effect termed 'velvet-blackness' produced by almost complete scattering of incident light by microornamentation (Spinner *et al*. 2013). The optical effect camouflages the animal against textured backgrounds of low reflectance, such as leaf litter. The microornamentation improves the camouflage in *B. rhinoceros* by reducing the reflectivity of dark scales (Spinner *et al*. 2014).

Singh (2014) found that microornamentation was present on dorsal scales of *B. arietans* (Puff Adder) but absent on ventral scales. Although microornamentation was present on dark dorsal scales (Fig. 3.1) and pale dorsal scales (Fig. 3.2), the height of microornamentation on dark scales was significantly greater than on pale scales. The findings of Singh (2014) required further investigation to measure the differences in spectral intensity of scale regions to understand if microornamentation produces optical effects on the scales of *B. arietans.* Given that *B. arietans* is an ambush forager which remains in ambush positions for extended periods of time, and its reliance on camouflage to avoid detection by predators and prey (Glaudas *et al.* 2017), I hypothesized that microornamentation on dark scales may function in improving the overall camouflage of *B. arietans*. In this study I used spectrophotometry to measure the spectral intensity of dark and pale dorsal scales as a measure of its reflective properties. Further, I measured the scale's reflectivity at normal (90°) and oblique (45°) angles of incidence over the visible spectrum on dark, pale, and ventral scale regions of *B. arietans*.

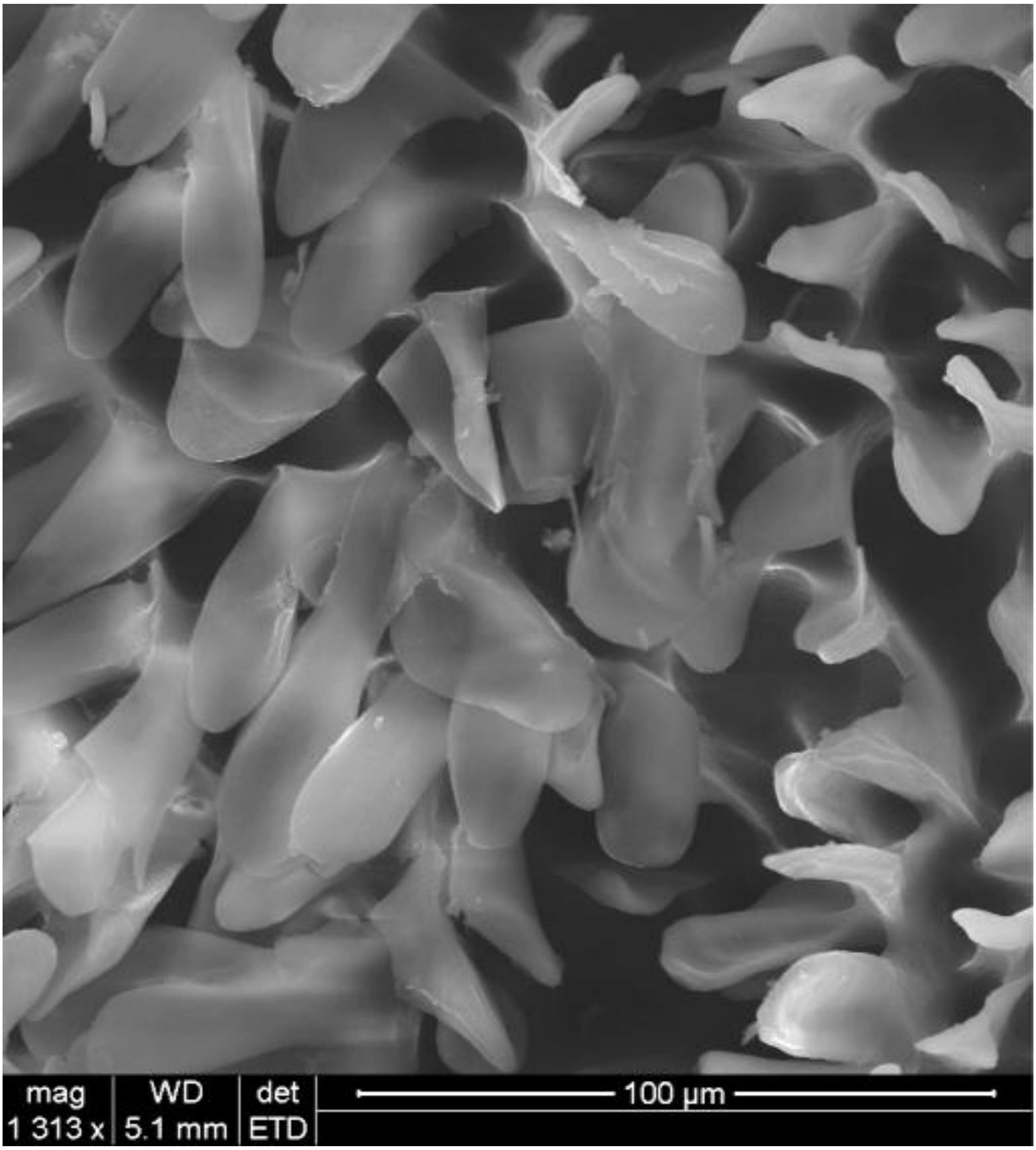

Fig. 3.1 A SEM micrograph showing the microornamentation on a dark scale region in *Bitis arietans* (Singh 2014). Scale bar 100 μm.

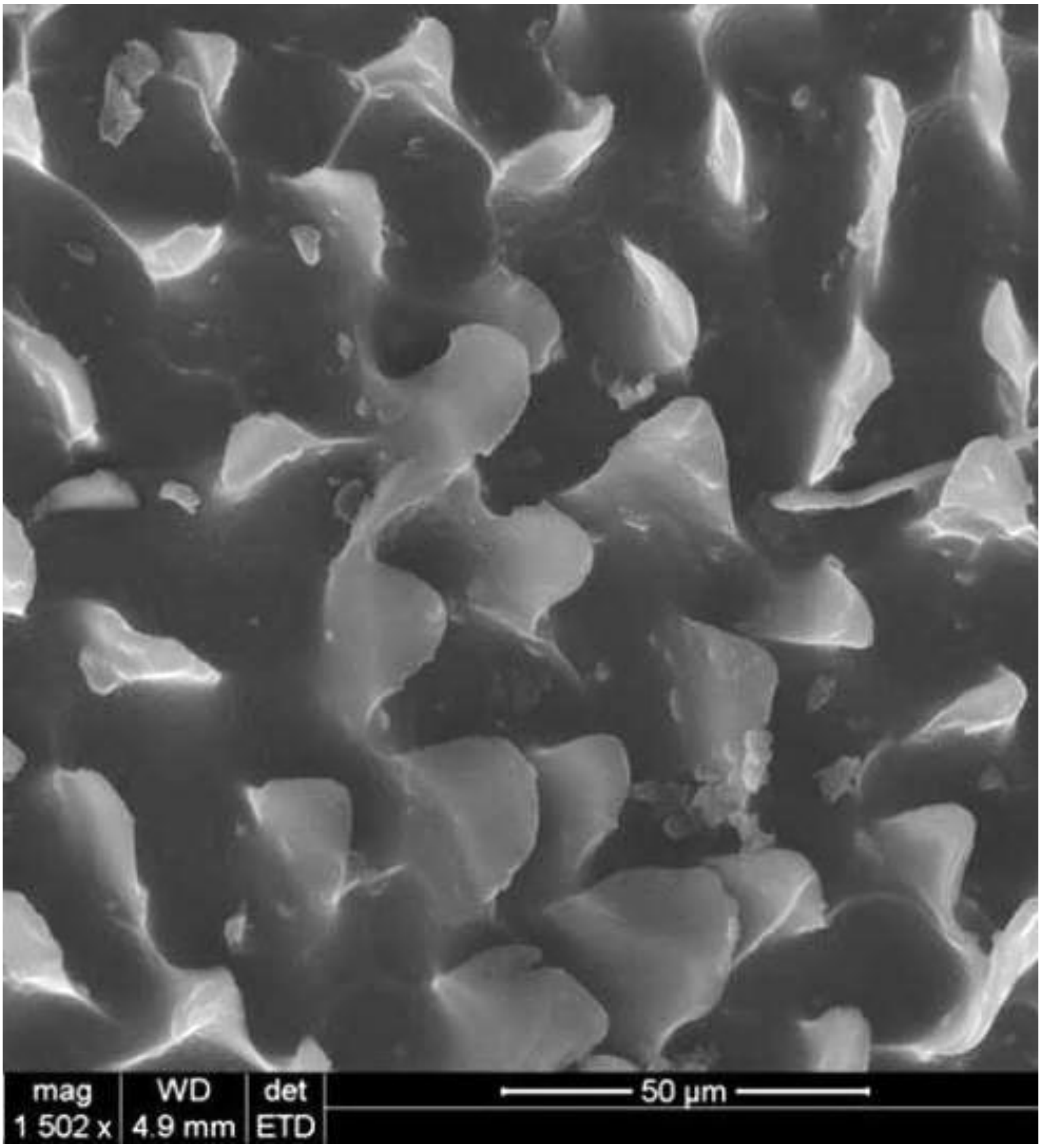

Fig. 3.2. A SEM micrograph showing the microornamentation on a pale scale region in *Bitis arietans* (Singh 2014). Scale bar 50 μm.

METHODS

Shed skins (exuvia) from three *B. arietans* were collected from the National Zoological Gardens, Pretoria (Reference: NZG/RES/P/001/F/02 P15/03). Skins were protected from environmental degradation by being sealed in re-sealable plastic bags, in a moisture and light-controlled storage facility. Samples were excised from two areas of the dorsal exuviam (dark and pale), and the ventral exuviam from three individual Puff Adders (*n* = 3) and mounted using onto discs of filter paper using double-sided carbon tape. Dorsal and ventral samples were sputter-coated with 15 nm of Gold-Palladium using an Emitek 950x sputter coater (Emitech Ltd., England), to remove pigmentary effects (based on Spinner *et al.* 2013b). Scales from ventral exuviam were used as a control for differences between dorsal scales and angles of incidence, as they completely lack microornamentation and pigment and are not expected to show differences. In this study, dark and pale dorsal scales (treatment) were tested for differences in reflectivity against ventral scales (control). Spectral intensity was used as a measure of reflectivity at two angles of incidence, oblique (45°) and normal (90°).

I took spectral readings in triplicate (based on Place 2012) for each region over the visible spectrum (approximately 200 to 850 nm) at normal (90°) and oblique (45°) angles using a USB2000 spectrometer (Ocean Optics Inc, Dunedin, Florida, USA.) with an optical fibre (diameter 200 mm) held 2 mm (Spinner *et al.* 2013a) above the scale surface and collected the reflected light through a monochromator (Ocean Optics Inc, Dunedin, Florida, USA) by an optical fibre. The light was generated from a balanced deuterium tungsten halogen light source (DH-2000-BAL, Ocean Optics Inc, Dunedin, Florida, USA) that produces light from ultraviolet to near infrared (wavelength range from 200 to 1100 nm). Data were recorded and processed with the software Spectral Suite (Ocean Optics, Inc, Dunedin, Florida, USA).

I tested the mean spectral intensity for normal (90°) and oblique (45°) angles of incidence for normality using a Kolmogorov-Smirnov normality test using GraphPad InStat 3.10 (GraphPad Software, San Diego California USA). I tested (by region: dark, pale, and ventral) for significant differences between 90° and 45° for each region using a Wilcoxon matched-pairs signed-ranks test in GraphPad InStat 3.10 (GraphPad Software, San Diego California USA). Spectral intensity at each angle of incidence (90° and 45°) was tested for significant differences for dark, pale and ventral scale regions using a Friedman test (repeated measures ANOVA for non-parametric data) followed by a Dunn's multiple comparison post-test among all scale regions in GraphPad InStat 3.10 (GraphPad Software, San Diego-California, USA).

RESULTS

Kolmogorov-Smirnov (KS) normality tests revealed that spectral intensity (SI) data at 90°incidence were non-normal for dark dorsal (KS = 0.16, $P < 0.0001$), pale dorsal (KS = 0.16, $P < 0.0001$), and ventral scales (KS = 0.18, $P < 0.0001$). Spectral data at 45°incidence were non-normal for dark dorsal (KS = 0.16, $P < 0.0001$), pale dorsal (KS = 0.16, $P < 0.0001$), and ventral scales (KS = 0.18, $P < 0.0001$).

The spectral intensity of dark regions was highly significantly different ($P < 0.0001$) between normal (90°) and oblique (45°) angles of incidence with the SI at 45° significantly lower than at 90° over the visible spectrum (Fig. 3.3). For pale regions, spectral intensity was highly significantly different ($P < 0.0001$) between normal and oblique angles of incidence with the SI at 45° significantly lower than at 90° over the visible spectrum (Fig. 3.4). The spectral intensity of ventral regions at normal and oblique angles of incidence were significantly different ($P < 0.0001$) with the SI at 45° significantly lower than at 90° over the visible spectrum (Fig. 3.5). Differences between spectral intensity at each angle of incidence was extremely significant ($P < 0.0001$) at normal (90°: $Fr = 3174.7$) and oblique (45°: $Fr = 949.8$) among dark and pale dorsal regions, and ventral region. The significance was further confirmed by Dunn's multiple comparison post-test at $P < 0.001$ level of significance. At normal incidence, the maximum SI measured for ventral scales is far greater than dorsal regions: ~20 times greater than dark regions, and ~12 times greater than pale regions (Fig. 3.6). At oblique incidence, the SI for dark scales and ventral scales are similar, peaking just above 150 counts in the middle of the visible spectrum, and the SI of pale scales is the highest of the three, peaking just below 300 counts, almost twice that of dark scales (Fig. 3.7).

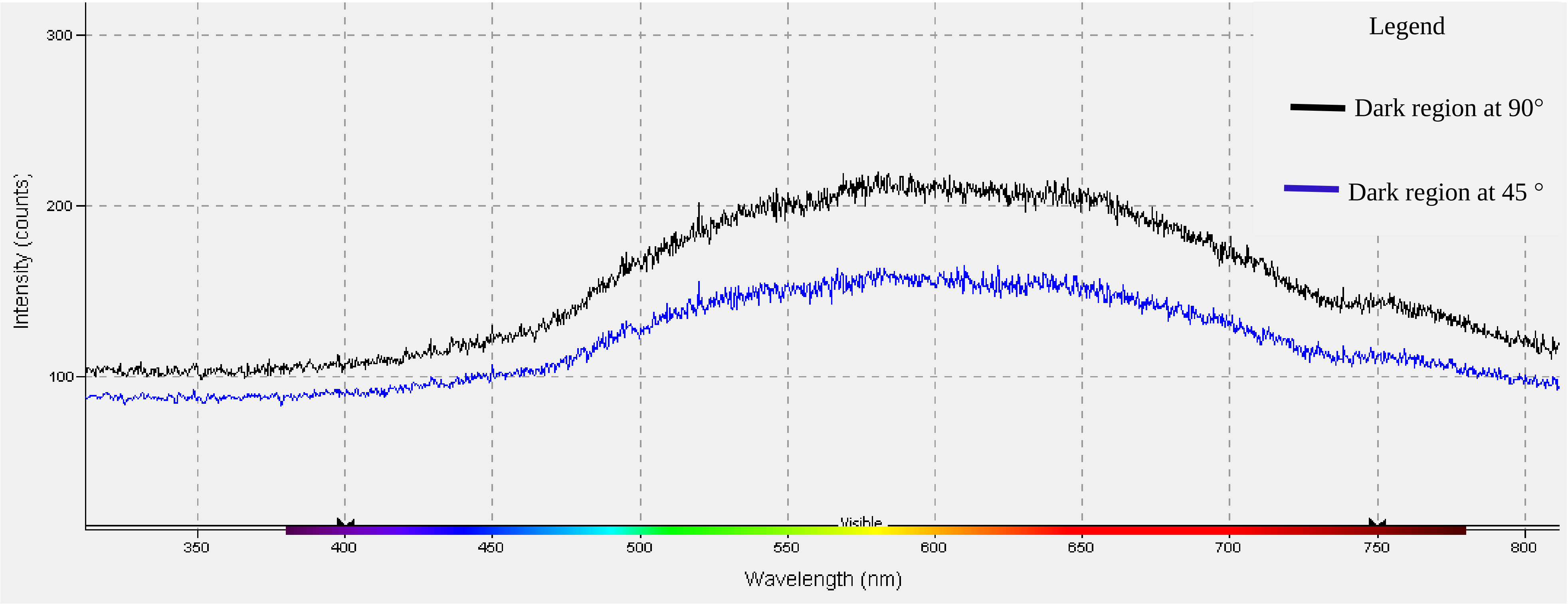

Fig. 3.3 Spectral Intensity (counts) across the wavelengths (nm) of the visible spectrum at oblique (45°; blue) and normal (90°; black) incidence for dark scale regions from *Bitis arietans*.

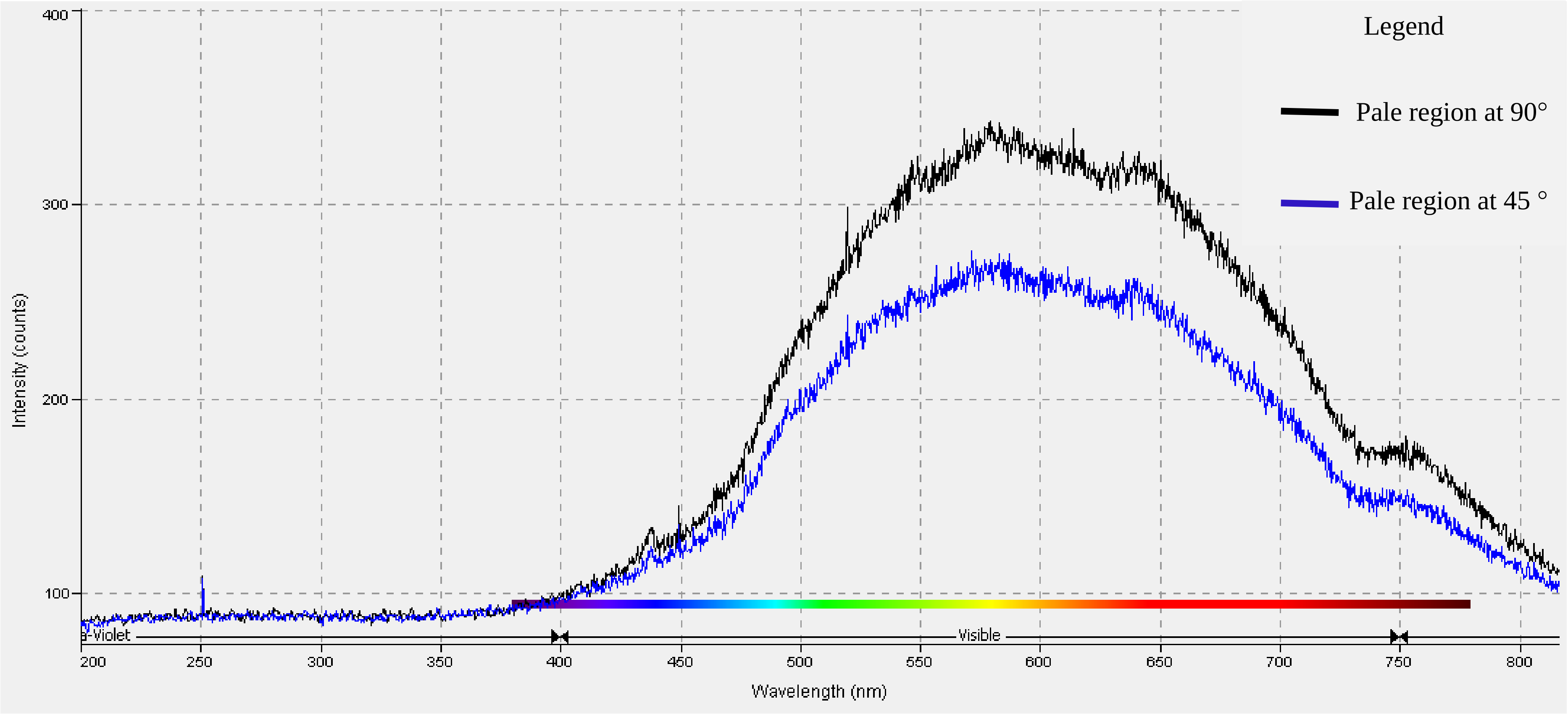

Fig. 3.4 Spectral Intensity (counts) across the wavelengths (nm) of the visible spectrum at oblique (45°; blue) and normal (90°; black) incidence on pale scale regions from *Bitis arietans*.

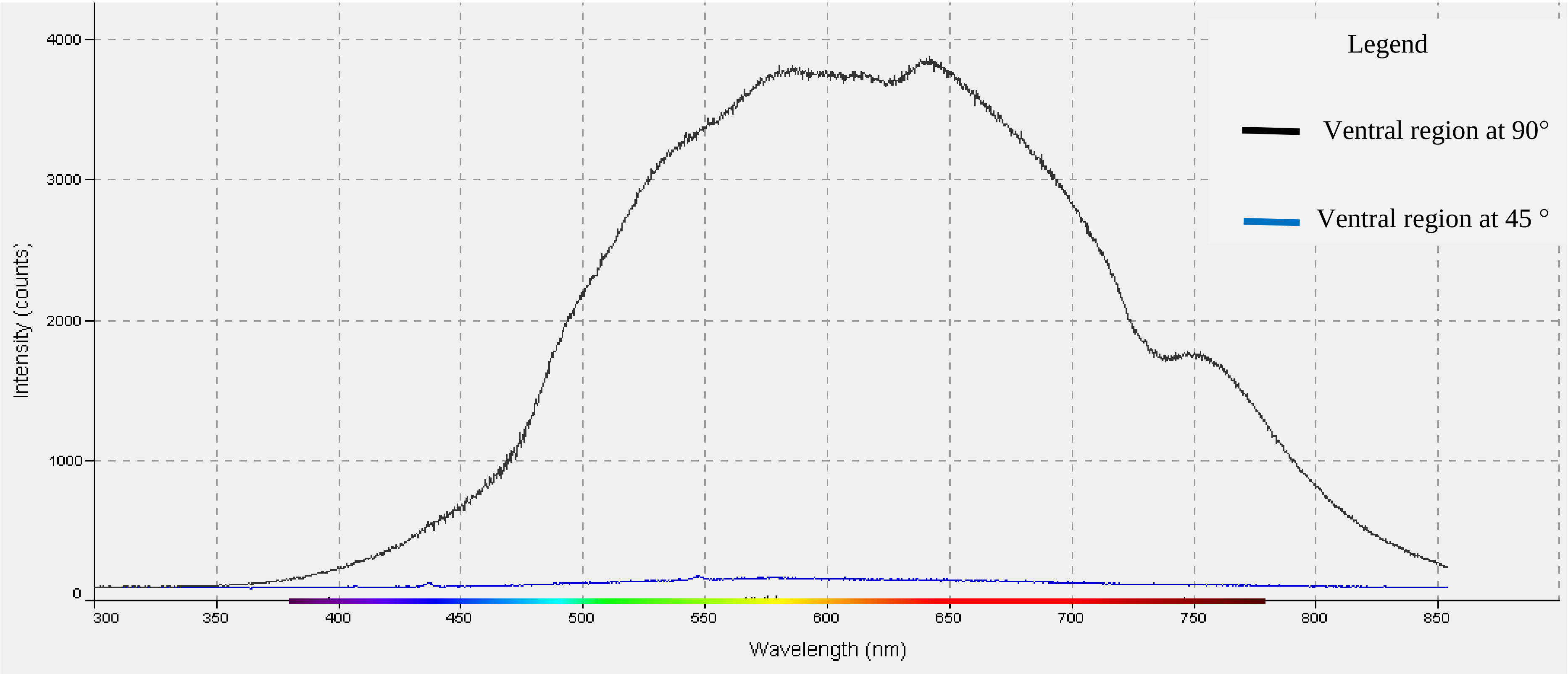

Fig. 3.5 Spectral Intensity (counts) across the wavelengths (nm) of the visible spectrum at oblique (45°; blue) and normal (90°; black) incidence on ventral scale regions from *Bitis arietans*.

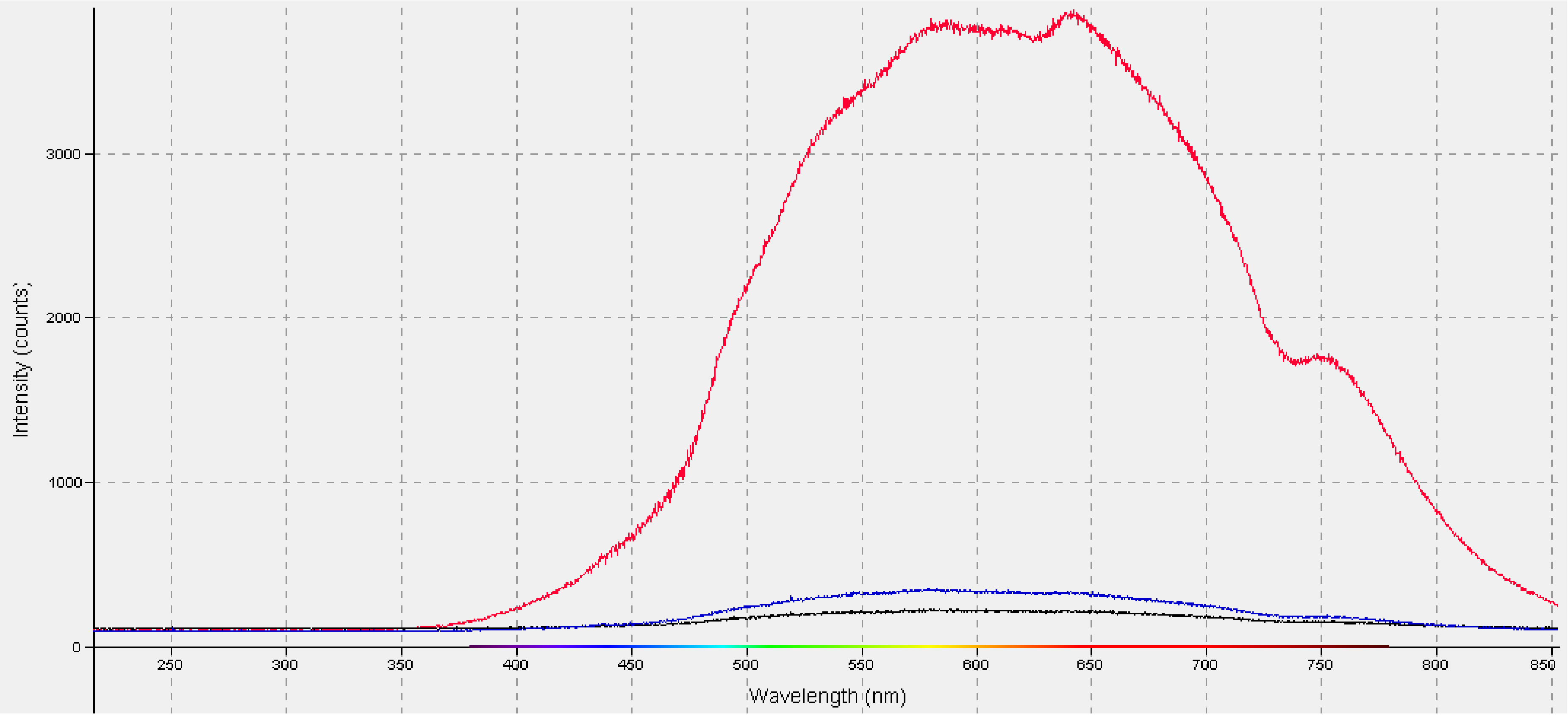

Fig. 3.6 Spectral Intensity (counts) across the wavelengths (nm) of the visible spectrum at normal (90°) incidence on ventral (red), pale (blue), and dark (black) scale regions from *Bitis arietans.*

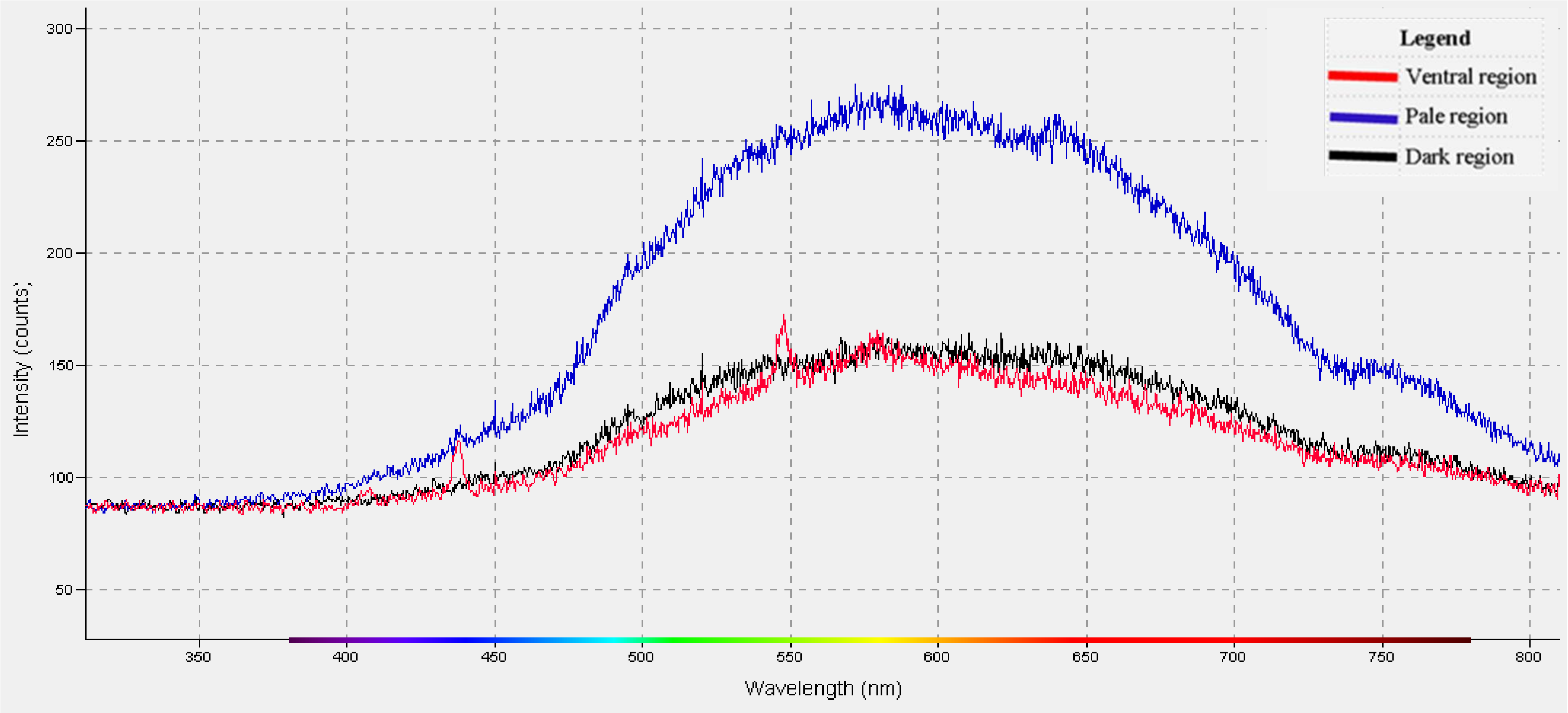

Fig. 3.7 Spectral Intensity (counts) across the wavelengths (nm) of the visible spectrum at oblique (45°) incidence for ventral (red), pale (blue), and dark (black) scale regions from *Bitis arietans*. At oblique incidence, the SI for dark scales and ventral scales are similar, the SI of pale scales is the highest of the three, almost twice that of dark scales. The similarity between dark and ventral scales' SI is owing to the fact that the incident beam is almost completely absorbed on dark scales, and almost completely reflected on ventral scales.

DISCUSSION

I measured spectral intensity of pale and dark dorsal scales and ventral scales of *Bitis arientans*. This revealed that spectral intensity of scales with microornamentation (dorsal scales) is lower than scales without (ventral scales), and the spectral intensity of dark regions is far lower than that of pale regions. The low spectral intensity of microstructured surfaces (pale and dark dorsal scales) can be explained as the scattering of incident light on the microornamentation which creates a rough texture. Within each scale region, spectral intensity differed between 90° and 45° incidence. The SI recorded for dark scales at 90° and 45° angles of incidence differed significantly. The SI was significantly lower at 45° than at 90°. The scattering effect is greater at non-normal angles of incidence, and may be due to the fact that the microstructures are irregularly arranged. For ventral scales, SI recorded at 90° and 45° differed significantly, and the recorded SI was lower at 45°. My results show that SI is indirectly proportional to variation of surface roughness. Differences in SI among dark, pale, and ventral regions were significant at both normal (90°) and oblique (45°) angles. This indicates that the level of microstructuring has a significant impact on spectral intensity. For each region, spectral intensity was lower at oblique (45°) incidence than normal (90°) incidence, indicating that the effect of angle of incidence has a significant impact on the spectral intensity for each region. However, SI is significantly different between normal and oblique angles of incidence for the control and varies by an order of magnitude in intensity counts. Ventral scales from exuviam have no microornamentation and no pigment and therefore make an effective control against dorsal scales.

Because of the absence of surface ornaments on the ventral scale it functions as an almost-perfect reflector and shows specular reflection. Due to the different types of reflection produced by scales, the differences measured have to be interpreted in the context of specular reflection in order not to prematurely discarded ventral scales as a control. Both the photo-source and photo-detector are housed within the probe of the spectrometer. So, a beam incident at 90°, is reflected along the same path back to the photo-receiver. The intensity of the beam which returns is compared to the intensity of the incident beam to calculate percentage reflection. When a beam is incident at 45° on a mirror-like surface, the beam is efficiently reflected away from the photo-detector at 45° (now 90° from incident beam), and is thus recorded as a low SI. The difference shown in these angles of incidence (Fig. 3.6) results from the beam reflecting perpendicularly to the photo-source, and thus is undetected by the photo-receiver. The SI differences on ventral scales are not due to microstructuring, and therefore ventral scales are a valid control to dorsal scales.

Microornamentation is found only on the dorsal scales in *B. arietans.* This fact eliminates any functions to locomotion. As the microornamentation varies in height with scale colour regions, indication is given that it functions with enhancing pigment and manipulating light to create structural effects. The wide ranging optical effect is perhaps more biologically relevant than if it were restricted to normal incidence only. When one considers the rounded body of the snake, its scales are facing different directions (both because the body is rounded, and often curled up). This means that reflectivity will vary because the scales face different orientations. The dorsal and lateral regions are exposed to light. In nature, optical effects that are restricted to angles of incidence are limited in their usefulness as they are only produced under specific light conditions and orientations of the snake's body (Kinoshita and Yoshioka 2005). The lateral and dorsal scales are roughly perpendicular to each other for a curled up snake. This means that when light is incident on the snake its either normal on dorsal and oblique on lateral, or *vice versa* which mean that for a snake curled in ambush its dorsal region is experiencing oblique incident light at dawn and dusk, and normal incidence during the middle of the day. Since the dorsal region is a greater area, we can deduce that the snake as a whole is far less reflective (and likely less detectible) at dusk and dawn than during the day. Optical effects in *B. arietans* have a wide range of applicability allowing the optical effect to play a significant role in the camouflage of *B. arietans*.

My findings of optical effects in *B. arietans* compare very closely with that reported by Spinner *et al.* (2013b) in *Bitis rhinoceros* in four main ways: (a) there is more complex microornamentation found on the dark scales than on pale scales, (b) there is no distinguishable or significant microstructures on ventralia, (c) spectral intensity is lower on scales with microornamentation (dorsal) than scales without (ventral), (d) spectral intensity is lower on dark than pale scales even when the effect of pigment is removed. Based on the differences in spectral intensity between pale and dark regions, Spinner *et al.* (2013b) suggests that the microstructures in *B. rhinoceros* produce an optical effect of velvet-blackness, which may improve camouflage, and may function to increase thermal absorbance by increasing absorbance capacity. Furthermore, according to Spinner *et al.* (2014) the microornamentation in *B. rhinoceros* alters the surface energy of the scales, making them hydrophobic and self-cleaning. This allows them to retain their rich black appearance and improves the camouflage of the snake. Due to the close similarity of microornamentation's form and function between *B. rhinoceros* and *B. arietans* on all accounts described above, it may well be that the microornamentation in *B. arietans* functions in a similar way to that of *B. rhinoceros* in mediating changes in surface energy, and can be measured in a future study.

Ventral scales of exuvia make the most suitable control against dark and pale scales dorsal scales of exuvia, being of the same biological material as dorsal scales yet completely lacking in microstructures or pigment. It is difficult to completely remove the effect of pigment using bleaching, and this may damage the microstructures. My method of coating with AuPd is effective and non-destructive, as the layer is thin enough to not obscure the optical effect of microstructures, and the coating has a known refractive index. It is also simple, and easy to apply. The experiments are easy to perform as they are carried out under laboratory-controlled conditions. Variation between samples is low for any scale type within an individual and amongst individuals, so few replicates are needed.

Further research may be undertaken to understand the function of microornamentation in terms of (a) whether other ambush foraging snakes, or snakes heavily dependent on camouflage have microornamentation on their dorsal surface, (b) whether microstructures have other biological effects in *B. arietans* such as thermal absorbance and hydrophobicity/self-cleaning, as with the microornamentation in *B. rhinoceros* (Spinner *et al.* 2013b, Spinner *et al.* 2014), (c) does microornamentation function in chemical crypsis?

Bitis arietans is one of the most abundant and widespread vipers in Africa. Its extreme ambush foraging biology makes it a model organism for research. Its success as an ambusher depends on effective camouflage to escape detection by both predators and prey (Alexander and Marais 2007) including chemical crypsis to avoid detection (Miller *et al.* 2015). In addition to chemical crypsis, microstructure-mediated optical effects are another adaptation that improves the success of *B. arietans* as an ambush forager. This study shows that microstructures create optical effects such as velvet-blackness on the scales of *B. arietans* and contributes to the body of knowledge regarding the mechanisms of camouflage in *B. arietans.* The results of this study contributes to our understanding of visual camouflage in *B. arietans* as a product of microstructure or structural colour and adds a new dimension to our understanding of the ecology of southern African snakes.

CONCLUSION

This study showed that dark dorsal scales of *B. arietans* are less reflective than pale dorsal scales, demonstrated by their lower spectral intensity. The reflectivity of scales is mediated by the degree of microstructuring, and is independent of scale colour. In *B. arietans* dark scales have better developed microornamentation than pale scales, and concomitantly show a lower reflectivity. In this study, the optical effect in *B. arietans* was recorded at normal and oblique angles of incidence, which indicates that the optical effect is angle-dependent. I suggest that the optical effect created by microornamentation in *B. arietans* contributes to its visual camouflage, and thus has an important function in the ecology of this species. Improving the camouflage of the snake improves its success in ambush-foraging episodes by reducing the probability of detection by predators and prey, and suggests an ecological basis (or selection) for microornamentation.

CHAPTER 4

COULD MICROORNAMENTATION PROTECT THE NAMAQUA DWARF ADDER, *BITIS SCHNEIDERI* AGAINST ABRASION?

ABSTRACT

Bitis schneideri is a sand dune specialist that occurs in the western extremes of the deserts of southern Africa. While in ambush, *B. schneideri* is exposed to wind-blown sand and its dorsal integument is thus exposed to damage from abrasion. This study aims to investigate if microstructures on *B. schneideri* scales reduce contact between sand particles and the dorsal integument, resulting in reduced mechanical injury by abrasion. Electron micrographs revealed denticulate microornamentation across the scale surface. Spaces between denticles were compared to the dimensions of sand grains from two locations: Namaqualand, where *B. schneideri* is found, and grains are angular, and Kalahari, where *B. schneideri* does not occur, but grains are significantly smaller. I found that the average-sized sand particle from each study site was significantly larger than the intra-denticular distance. These results indicate that scale microornamentation reduces the direct contact between sand particles and the integument since the denticles can keep sand grains from contact with the basal skin layer. Furthermore, because the sand grains from the Kalahari were also larger than the intra-denticular distance the scale microornamentation can protect it from abrasion from sand grains outside of its range, I suggest that the range of *B. schneideri* is not limited by abrasion.

INTRODUCTION

The integument of an organism has three important functions, namely, protection of underlying tissues, sensory detection, and homeostasis (Raven and Johnson 1990). Protection of the underlying tissues is especially important during locomotion, where the integument contacts a substrate, posing the risk of mechanical damage. The extent of mechanical damage to the integument can be calculated from the effect of abrasion between two bodies in stationary or sliding contact described by Archard (1956):

$$Q = K\,W\,L / H \qquad \ldots (1)$$

In equation 1, Q is the total volume of wear debris produced, K is a dimensionless constant, W is the total normal load, L is the sliding distance, and H is the hardness of the softest contacting surfaces. The relationship between the variables described by Archard (1956), above, help us understand the effect of abrasion to an animal's integument.

The volume of debris produced, Q, is the amount of damage to the animal's integument and is of concern to biologists. Abrasive wear between nominally flat surfaces in sliding contact is dependent on the real area of contact between the surfaces (Archard 1953). Alexander (1996) measured the surface area of ventral scales from exuvia of *Hemachatus haemachatus* (Rinkhals), and reported that the ventral scales made up 30% of the total body surface area. This means that up to 30% of a snake's integument is at risk of damage from the effect of abrasion during locomotion. This proportion of risk is much higher than most similarly sized animals moving over an equal distance.

The effect of abrasion, Q, is in direct proportion to the sliding distance, L, and inversely proportional to the hardness of the softer contacting surface, H, the amount of damage to snake integument could, conceivably, be very high since snakes slide their entire body over the substrate. The risk of wear is exacerbated by the fact that snakes do not have hard tissue reinforcement, such as nails, at the contacting surface. In order to enable locomotion, the integument must be soft and flexible yet structurally adapted to withstand mechanical injury. This is largely achieved through scales which are composed of alpha- and beta-keratin (Jackson and Reno 1975, Spearman 1977). This quaternary protein structure gives the integument its ability to shield itself from mechanical injury but allows flexibility for movement (Toni and Alibardi 2007).

Bitis schneideri (Boettger 1886), the Namaqua Dwarf Adder, is the world's smallest viperid (Maritz and Alexander 2012, Branch 1988). The species uses a peculiar shuffling movement to bury itself into loose sand by which it may camouflage itself from both predators and prey (Alexander and Marais 2007). When burrowing *B. schneideri*'s integument risks damage from the abrasive action of sand particles. Furthermore, while it lies in ambush for most of the daylight hours (Maritz 2012), its dorsal scales are exposed to wind-blown sediment.

Characteristics of wind-blown sands in desert environments

Sand is defined as 'any substance consisting of non-cohesive particles' usually of quartz (Bagnold 1941) within a range of 62.5-2000 µm in diameter (Greeley and Iversen 1985). Sand may be transported by two main processes: fluvial or aeolian transport. Aeolian transport is further divided into three categories, dependent on the energy of the wind and size of the particles: suspension, whereby dust is transported under turbulent flow (Laity 2008), saltation, a high energy sub-population of sand grains set into motion by high-speed winds that blow over the surface of a bed of sand (Anderson *et al*. 1991), and creep, whereby large particles move short distances along the sand bed set into motion by the impact of saltating particles (Bagnold 1941).

An ideal sand sample comprises grains of identical size and shape (Bagnold 1941) but is hardly a reflection of a real sample. Because of the long-lifespan of quartz, quartz-derived sand may be involved in several geological and transportation processes (Iversen and Greeley 1985). Therefore samples of desert sands are typically polymodal representative of more than one transport or depositional process being involved (Sun 2002). Wind-blown sand is usually a combination of fine sand and fine dust, each component represents a load transported through winds of different energy over different distances (Sun 2002). Apart from the fact that transport mode determines grain size, geological processes have a measurable impact on the shape and surface texture of sand grains (Bagnold 1941). The roundness of the grain is a reflection of the energy of the medium transporting it (Kasper-Zubillaga 2009). Fluvial and marine transported sand is generally sharper and more angular (Bagnold 1941) due to high energy, long-shore transport and collision between grains in sub-aqueous environments (Krinsley and Donahue 1968), while roundness is characteristic of aeolian transport (Laity 2008). *Bitis schneideri* lives in the sands of the desert dunes, and is subject to some level of disturbance from all three processes of sand transportation. Under turbulent conditions, *B. schneideri* risks significant abrasion from the angular, fluvial sediments.

Aim

The aim of this study was to test if the arrangement of microornamentation across an individual scale serves to minimise direct contact between sand and the scale surface. Based on field observations of the burrowing behaviour of *B. schneideri* (Alexander and Marais 2007) and the fact that grains of fluvial origin are reported to be angular (Kasper-Zubillaga 2009), I hypothesized that microornamentation on the scales of *B. schneideri* functions to reduce mechanical abrasion during ambush. I predicted that the arrangement of microornamentation on *B. schneideri* scales prevents sand grains from coming into direct contact with the basal layer of the scale.

METHODS

Study animal

Bitis schneideri (Boettger 1886), the Namaqua Dwarf Adder is endemic to southern Africa, found from the mouth of the Olifants River in the Western Cape, South Africa to Luderitz Bay in south-western Namibia (Branch 1998). Its distribution includes part of Namaqualand Sandveld, Richtersveld, Namaqualand Hardeveld, and Bushmansland where it inhabits the saline, semi-vegetated sandy areas close to the coast (Turner and Maritz 2014). Although the species occupies a fairly large sparsely inhabited area (extent of occurrence = 28 491 km^2) it is limited to loose sands near the coast (area of occupancy = 10 684 km^2) which are threatened by mining (Turner and Maritz 2014). This species was previously classified as Vulnerable by Branch (1988) but has now been re-classified as of Least Concern by the Red Data List (Turner and Maritz 2014). Individuals are relatively sedentary, with a mean daily displacement that varies from 0.8 m/day for females during winter to 47.3 m/day for males during spring (Maritz and Alexander 2012).

Study site A

The Namaqualand is a richly biodiverse region that comprises about a quarter of the area of the succulent Karoo (Desmet 2007). The coastal plain consists mainly of red, yellow, and grey sands (Francis *et al.* 2007). Along the coast, grey sands predominate, and are of most recent aeolian origin (Francis *et al.* 2007). This sand type is regic and calcareous and is apedal (i.e., shows little evidence of structure; Cowling *et al.* 1999). Sediment in Namaqualand is a product of fluvial transport from the Orange River followed by aeolian transport from the mouth of the Orange River to the dunes of the Succulent Karoo (Thomas and Shaw 1991). Sand was sampled from Noup, Northern Cape Province, South Africa. This site forms part of the natural distribution of *B. schneideri.*

Study site B

The Kalahari is a geological unit of about 2.5 million km^2, which makes up a substantial part of the southern African interior (Thomas 2002, Thomas and Shaw 1991). Its soils are characteristically made up of fine aeolian-deposited sediment (Thomas and Shaw 1991). The relative components of Kalahari soil indicate that Kalahari soil consists mainly of sand, 96.44%, with small fractions of silt, 2.18 %, and clay, 1.38 % (Dougill and Thomas 2004). Within the Kalahari, sand was sampled from Tswalu, Northern Cape province, South Africa (27.2961° S, 22.3943° E). Despite the fact that this site does not form part of the range of *B. schneideri*, I sampled grains from this site as an example of grains that are smaller in size and different in shape (due to different transportation processes) to the grains from the natural distribution of *B. schneideri* to test the hypothesis that *B. schneideri* is restricted to its range by the size of sand grains relative to its scale microornamentation.

Sand grain size measurement

I examined sand particles from Noup (*n* = 125) and Tswalu (*n* = 125) to calculate mean grain size. For each study site, approximately 0.1 g of sample was mounted on an aluminium stub using double-sided carbon tape. These were evaporated with ±10 nm of carbon using an Emitek 950x turbo evaporator (Emitech Ltd., England), and sputter coated with ±10 nm of chromium using a Quorum Q150 TES sputter coater (Quorum Technologies, Kent, UK). I made observations at an accelerating voltage of 30.00 kV using a FEI Nova 600 Nanolab FIB/ SEM (FEI, Munich, Germany) and examined SEM micrographs using the software *CellSens Dimension* version 1.12 (Olympus Corporation, Germany) to calculate the mean dimensions of a sand grain from each site. I randomly selected grains (*n* = 125) from micrographs by using a random path selection method and measured grains in terms of the grain's 'length' (the largest diameter) and 'breadth' (shortest diameter orthogonal to the largest diameter).

Intra-denticular distance

Specimens of *Bitis schneideri* were donated to this study by Bryan Maritz (University of Western Cape) from his research work on the ecology of the species (see Maritz 2011). These specimens were collected from a population of free-ranging individuals from the coastal dune fields in the Northern Cape Province of South Africa (Noup: 30°08'S, 17°12'E). I removed dorsal scales from laboratory specimens preserved in 70% ethanol (*n* = 5) which I dehydrated (Anderson and Bancroft 2005) and mounted onto aluminium stubs using double-sided carbon tape. These were evaporated with ±10 nm of carbon using an Emitek 950x turbo evaporator (Emitech Ltd., England), and sputter coated with ±5 nm of chromium using a Quorum Q150 TES sputter coater (Quorum Technologies, Kent, UK). I made observations at an accelerating voltage of 30.00 kV using a FEI Nova 600 Nanolab FIB/ SEM (FEI, Munich, Germany) and measures of scale microornamentation from SEM micrographs of *B. schneideri* using the software *CellSens Dimension* version 1.12 (Olympus Corporation, Germany). These measurements included (a) the intra-denticular distance, defined as the distance between two adjacent denticles on the scale surface (*n* = 150), and (b) the length of denticles (*n* = 14) from mid-base to tip.

Data analysis

Intra-denticular distances, sand grain length and breadth data were tested for normality using a Kolmogorov-Smirnov normality test. The mean length and breadth of grains were compared between sites using a one-way ANOVA and a Tukey-Kramer multiple comparison post-hoc test among all groups in GraphPad InStat 3.10 (GraphPad Software, San Diego California USA).

RESULTS

Dorsal scales of *B. schneideri* have a prominent, but incomplete keel. The keel extends from the apex disappearing just before the rounded base at the anterior edge of the scale. The posterior apical end of the scale is sharply tapered. Microstructures are clearly visible on the scale surface under the scanning electron microscope (Fig. 4.1). Microstructures are denticulate (tooth-shaped), with a mean height of 31.6 ± 5 μm (mean ± SD) measured from base to tip, and the intra-denticular distance (*n* = 150) was 24.8 ± 4 μm (mean ± SD).

Kolmogorov-Smirnov (KS) normality test revealed that measurements of intra-denticular distance were normal (KS = 0.05, $P > 0.10$). Measurements of sand grains from Site A were normal for length (KS = 0.12, $P > 0$) and breadth (KS = 0.9, $P > 0.10$). Measurements of sand grains from Site B were normal for length (KS = 0.12, $P > 0$) and breadth (KS = 0.1, $P > 0.10$). The dimensions of sand grains (length ± SD x breadth ± SD) sampled from Site A (372 ± 90 μm x 324 ± 137) was significantly different to Site B (146 ± 22 μm x 111 ± 22 μm both in length (Q = 15.940, $P < 0.001$) and breadth (Q = 23.330, $P < 0.001$). The grains from the Site A were angular: their mean length was significantly different to mean breadth (Q = 5.135, $P < 0.01$), whereas the grains from Site B were rounded: the mean length is not significantly different to mean breadth (Q = 2.713, $P > 0.05$). I found that sand grains from Site A was significantly larger than the intra-denticular distance, both in length (Q = 39.0, $P < 0.001$) and breadth (Q = 45.3, $P < 0.001$). Despite the fact that the grains from Site B were significantly smaller than that from Site A, they were still significantly larger than the mean intra-denticular distance, both in length (Q = 12.3, $P < 0.001$) and breadth (Q = 8.8, $P < 0.001$).

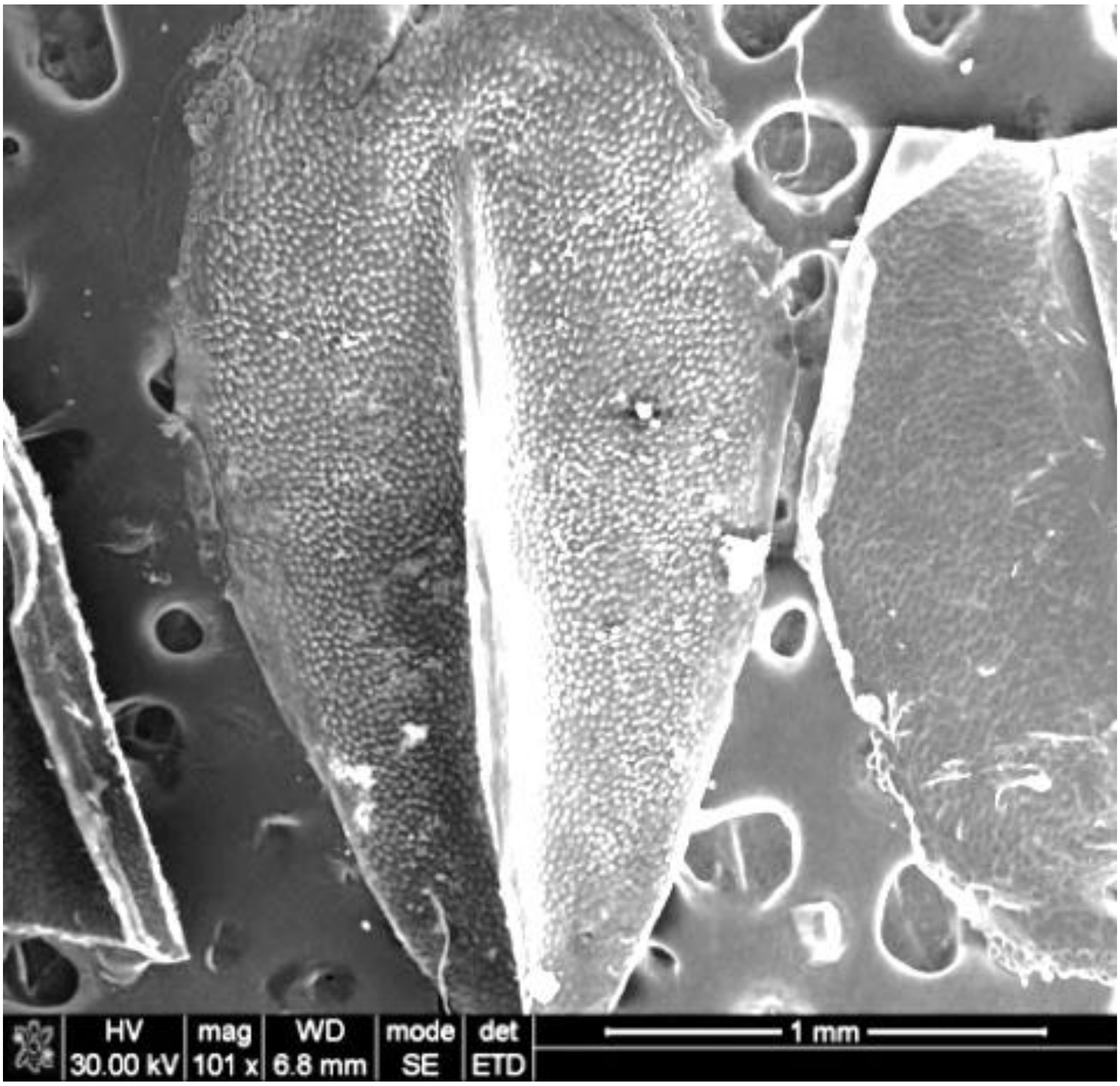

Fig. 4.1. (a) Micrograph showing a dorsal body scale of *Bitis schneideri*. The scale is keeled and covered in denticular microornamentation. Scale bar: 1 mm.

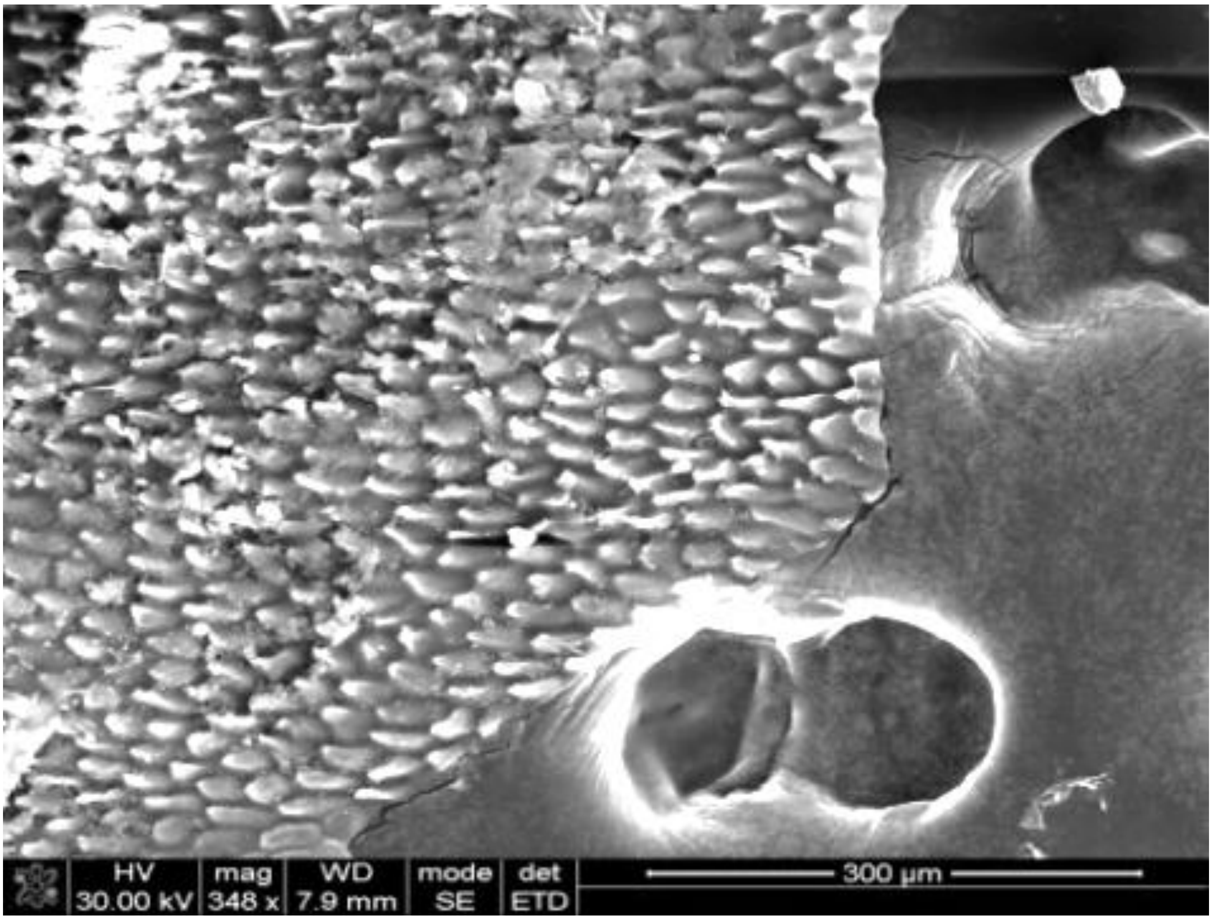

Fig. 4.1 (b) Micrograph showing a portion of a dorsal scale of *Bitis schneideri* showing denticle microornamentation in detail. Scale bar: 300 μm.

DISCUSSION

The microornamentation present on the scales of *B. schneideri* are distributed in an arrangement whereby the distance between adjacent ornaments is significantly smaller than the dimensions of sand particles from its range. To extend the relevance of the comparison, I compared the intra-denticular distance to the dimensions of sand grains from Site B, which is outside the range of *B. schneideri*. I found that the sand grains from Site B were significantly smaller than that of Site A, but still significantly larger than the intra-denticular spaces. The sand grains from Site A have a different geological history to that of Site B that accounts for the difference in its size and shape (Bagnold 1941). From Site B, grains are of aeolian origin

(Thomas and Shaw 1991) and their rounded appearance is consistent with wind-blown sediments (Bagnold 1941, Laity 2008, Kasper-Zubillaga 2009). It is expected that at Site B grains are of small size classes because the smaller grains are more easily carried by the wind (Sun 2002). Sand grains from Site A are polymodal (Sun 2002) or the product of more than one transport process: they are a product of fluvial transport from the Orange River followed by aeolian transport from the mouth of the Orange River to the dunes of the Succulent Karoo (Thomas and Shaw 1991). It follows that their appearance is angular, consistent with particles that have undergone fluvial transport in which high impact collisions result in sharp grains (Bagnold 1941).

Microornamentation has been reported to have a variety of functions, but no previous studies suggest that microornamentation functions to reduce abrasion. The function of microstructures such as hairs, cilia, spines, and spicules are well known for their function in reducing physical contact between harmful particles and delicate tissues in a variety of biological systems (Raven and Johnson 1990). Similarly, the function of keratinised structures is well known for its function in shielding tissue from damage. For example, all primates have keratin-based nails as accessory organs of the integumentary system (Lewin 2009) which offer anatomical shielding to sensitive receptors concentrated at the finger-tips (Raven and Johnson 1990). Microornamentation- the keratin outgrowths of the oberhautchen of snakes (Jackson and Reno 1975) can reduce the abrasion to the integument of psammophillous snakes such as *B. schneideri* if it has the correct physical arrangement.

The physical characteristics of microornamentation in *B. schneideri* make it suitable to shield the integument from abrasion. The denticulate ornaments which cover the dorsal scales of *B. schneideri* are outgrowths of the oberhautchen surface, the outermost layer of the epidermis (Maderson 1964). Thus, it is composed of the same material of the oberhautchen surface, namely keratin (Maderson 1964, Jackson and Reno 1975, Spearman 1977). The structural properties of keratin increase the hardness of the contacting surface. Because the effect of abrasion, *Q*, is indirectly proportional to hardness, *H* (equation (1), Archard 1953, Archard 1956), it follows that keratinised surfaces, such as the oberhautchen, are structurally adapted to reduce the effects of abrasion such as fracture and wear (Aghababaei *et al.* 2016).

The arrangement of microornamentation in *B. schneideri* can reduce the contact area of sand grains with the scale surface thereby shielding the integument from damage. Reducing sand contact with the integument has obvious benefits to *B. schneideri* because of its psammophillous lifestyle. Microornamentation can reduce the risk of mechanical damage posed by sand that is driven at high speeds by wind and from burrowing in sand. Even though the size of particles from each study site differed significantly in size, both were larger than the intra-denticular distance. The microornamentation on *B. schneideri* can reduce the contact abrasion from sand grains from Site A and B sampled in this study. The sand particles at Noup, which forms the natural distribution of *B. schneideri*, are primarily derived from fluvial transport down the Orange River and thereafter driven by wind to from the Orange River delta south to Noup. Despite the process of saltation the grains maintain a high-level of angularity. The potential damage from the angular grains is exacerbated by the fact that it is blown at high speeds and can inflict a significant abrasive damage to *B. schneideri*'s dorsal integument.

Sand grains from within and beyond the range of *B. schneideri* are significantly larger than the spaces between micro-ornaments. Sand grains are defined as non-cohesive particles of quartz (Bagnold 1941) within a range of 62.5-2000 µm in diameter (Greeley and Iversen 1985). Therefore, in general, sand grains will be larger than the intra-denticular space of *B. schneideri*. From the findings of this study, the restricted distribution of the species (Turner and Maritz 2014) cannot be explained by the hypothesis that areas outside the range have sand that would be more abrasive due to smaller grains. In fact, the Kalahari should be less abrasive because the grains are rounded (Thomas and Shaw 1991). The range limitation of *B. schneideri* must be due to other limiting factors other than particle size and angularity. Maritz (2011) suggests that an organism's distribution is due to the product of its biology, and the small home range of *B. schneideri* can be explained by the species' sedentary lifestyle with low levels of dispersal (Maritz and Alexander 2012). Microornamentation in *B. schneideri* probably alleviates the abrasion to its integument from the angular grains of Namaqualand rather than limits it to the area.

Body size in *Bitis* shows a reduction in desert specialists such as *B. schneideri* (Maritz and Alexander 2012). While this has been traditionally explained as an adaptation to resource availability, Maritz and Alexander (2012) explain the shift as a behavioural adaptation to exploit the upper layer of loose sand, in camouflage and thermal uptake. From this, it follows that this behaviour may have driven a strong selection to reduce scale abrasion. Consistent with the explanation of Maritz and Alexander (2012) I suggest that these behavioural adaptations also drove the reduction in the size and shape *of B. schneideri* microornamentation. In comparison, the large leaf-like structures of *B. arietans* (Singh 2014, Singh and Alexander 2017) are floppy and easily sheared off the scale by sand contact during burrowing, offering minimal protection to the integument. The microornamentation in *B. arietans* is not under this selection because the species is not known to burrow into sand as *B. schneideri* does. A trend toward reduced, erect denticles in *B. schneideri* likely offers a more rigorous defence to the abrasive angular sand grains of Namaqualand.

CONCLUSION

The scale surface of *B. schneideri* is characterised by denticulate microstructures. The mean intra-denticular distance is significantly smaller than the average sand particle from the surroundings. This suggests that microornamentation reduces direct contact between sand particles and the integument. I suggest that microornamentation in *B. schneideri* shields the integument from mechanical abrasion due to contact with sand particles during burrowing and wind-driven sand over the dorsal integument. This study contributes to our knowledge of the diversity of microornamentation in the genus *Bitis.* The findings of this study contribute to our understanding of the diversity of animals' adaptations to the desert environment and contribute to a growing body of knowledge of specific functions associated with microornamentation in southern African snakes.

CHAPTER 5

CONCLUSION

The primary aim of this study was to investigate specific functions of microornamentation in two species of *Bitis*. Using electron microscopy, I present the scale microornamentation of *Bitis arietans* and *B. schneideri* and experimental evidence to support the function of microornamentation. This is the first study to document the microornamentation in *B. schneideri*. My micrographs of microornamentation in *B. arietans* and *B. schneideri* can be used in studies on taxonomic relationships, and studies on the adaptations of desert organisms to their environment. This study contributes to an emerging body of knowledge on the microornamentation of southern African snakes, particularly as an adaptation of the snake to its environment.

The morphometric characteristics of the microornamentation of *B. schneideri* reveal that microornamentation in *B. schneideri* can reduce the direct contact of the integument with sand grains from its natural distribution. Keratin, the primary component of microornamentation, has structural properties which offer the integument shielding from contact abrasion. I suggest that the denticulate microornamentation on the scales of *B. schneideri* is as an adaptation to reduce abrasion from contact of the integument with sand grains during burrowing, and from exposure to sand blown at high wind speeds over the dorsal integument during foraging. To the best of my knowledge, this is the first study to show microornamentation in an integumentary shielding function. These results add to the growing body of knowledge about the functional role of microornamentation in southern African snakes. Further, my data may help scientists understand the distribution of *B. schneideri* because this study shows that sand grain size does not limit the species to its distribution. The microornamentation of *B. schneideri* can reduce the severity of abrasion from the angular grains of its range.

In this study, I observed that the microornamentation of each study species differed vastly in overall size and shape. The denticulate ornamentation on the scales of *B. schneideri* is far reduced in size compared to ornamentation on the dark scales in *B. arietans.* I observed in many instances under SEM that the large ornaments in *B. arietans* had collapsed under their weight and several were broken. It seems reasonable that the ornaments in *B. schneideri* are reduced in size and shape, as a result of the psammophillous lifestyle of the species. Thus, the denticulate microornamentation in *B. schneideri* seems well suited to its desert environment and adds to our knowledge of how desert animals have specialised to their environmental extremes. The reduction in size of microornamentation from *B. arietans* to *B. schneideri* is consistent with reduction in overall body size in *Bitis* arid extremes. However, this study suggests that the denticulate microornamentation in *B. schneideri* allows the species to exploit the upper layer of sand.

The microornamentation on the dark scales regions of *B. arietans* is significantly different in height to that of the pale regions. I tested differences in spectral intensity while controlling for pigment and structuring by using ventral scales. I demonstrated an effective protocol to cancel the effect of pigment residual in scales excised from *B. arietans* exuviam by coating scales with ± 15 nm of AuPd. This method was adapted from Spinner *et al*. (2013b). It proved a better way to control for pigment than bleaching, which was reported in previous studies to damage microstructure (e.g., Ingram and Parker 2008). My study demonstrates an effective protocol that yields spectral intensity with statistically significant results.

The larger ornaments of dark regions scatter incident light better than pale regions. Measurements of spectral intensity at 90° incidence show that pale regions are significantly more reflective than dark regions. The large ornaments on dark regions create a rough surface profile, which scatters light more effectively than the ornaments on pale regions. Because light is efficiently scattered by microornamentation dark scales are diffuse reflectors. The ventral regions, devoid of microornamentation, are highly reflective compared to dark and pale regions. These results confirm that microornamentation in *B. arietans* create a rough surface profile which promotes diffuse reflection causing a significant reduction in reflective index of the scale. These results add to the emerging body of knowledge about microstructure-mediated changes to the reflective index of biological surfaces.

The overall effect of microornamentation on dorsal scales of *B. arietans* is that microornamentation scatters incident light at both normal and oblique angles of incidence. The scattering effect lowers the spectral intensity of dark dorsal scales of *B. arietans* making the snake less reflective, so that it can more easily match a background with the same reflective index as its scales, such as leaf litter or soil. Dorsal scales have a mix of high and low reflectance that reduces the chances of the snake being detected by predators and prey when against a background of a similar low spectral intensity such as leaf litter or soil. The optical effect produced by microornamentation improves the Puff Adder’s camouflage during ambush. The outcomes of this study demonstrate direct functionality of microornamentation in the biology of *B. arietans,* with several opportunities for further research. Apart from visual crypsis, the findings of this study can direct answering questions about the mechanism of chemical crypsis (Miller *et al.* 2015) in *B. arietans.*

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