The Evolution of Cutaneous Senses in Marine Snakes (Hydrophiinae)

A dissertation by

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“It’s not easy when you’re not catching snakes”

-Arne R. Rasmussen

Summary

Front-fanged elapid snakes (subfamily: Hydrophiinae) have invaded marine habitats twice: the oviparous sea kraits that diverged approximately 18 million years ago and the viviparous sea snakes that diverged approximately six million years ago. Due to these recent marine transitions, marine hydrophiine snakes are embedded within closely-related and extant terrestrial lineages. Within this phylogenetic context, I investigated two questions concerning two cutaneous senses in marine snakes: 1) How has the sense of touch evolved in the transition from land to sea? and 2) How has a novel phototactic trait arisen in sea snakes?

Marine snakes possess small scale organs ('sensilla') that are presumptive mechanoreceptors widely thought to be co-opted for detecting water motion (*i.e.* hydrodynamic reception in homoplasy with the lateral line of fish). To test this hypothesis and infer ancestral and derived functions for scale sensilla, I used morphological techniques (quadrate sampling, scanning electron microscopy) to quantify sensilla traits (number, density, area, coverage) among 19 species of terrestrial and marine elapids. After accounting for effects of allometry (head size) and phylogeny (shared descent), I used Bayesian analyses to reconstruct ancestral sensilla traits in sea kraits and sea snakes. I also characterised ultrastructure (histology, immunohistochemistry, transmission electron microscopy) of scale sensilla on the head and tail of two species of sea snakes, *Aipysurus laevis* and *Hydrophis stokesii*, which indicate interspecific variation but overall structural similarities with mechanosensory sensilla in terrestrial snakes. These results provide the first evidence for a mechanosensory function for scale sensilla among sea snakes, and a basis for further studies to test for physiological and behavioural responses to water motion among marine snakes.

In addition to scale mechanoreceptors, many lineages of sea snakes have conspicuous scale protuberances (*e.g.* spines, rugosities) with various purportedly sensory and non-sensory adaptive functions. I examined the morphology (scanning electron microscopy, histology) of sexually-dimorphic scale protuberances in turtle-headed sea snakes, *Emydocephalus annulatus*. Taken together with behavioural data, these morphological results suggest complex mechanosensory roles related to courtship and mating behaviours in this species.

Finally, I investigated the evolution and molecular basis of a novel phototactic trait in sea snakes. The movement of tail in response to light detection via the skin ('tail phototaxis') is a sensory trait shared by aquatic vertebrates with secretive habits, elongate bodies and paddle-shaped tails, *i.e.* hagfish, lamprey, aquatic amphibians and sea snakes. I conducted behavioural tests in eight species of sea snakes, developing a preliminary hypothesis for the evolutionary origin of this trait within a small clade of *Aipysurus* sea snakes. I also quantified tail damage in

museum specimens to test whether the probability of sustaining tail injuries is influenced by tail phototactic ability in snakes. I then profiled skin transcriptomes of phototactic snakes to identify candidate phototaxis genes, which can be used to understand the parallel evolution of this trait among vertebrates.

This thesis provides the basis for future research on the sensory ecology and evolution of marine snakes. The integrative methods employed speaks to power of these approaches in resolving fundamental questions in evolutionary biology, particularly how novel traits can arise from existing variation.

Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint award of this degree.

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Contributions

Chapter 2: The evolution of scale sensilla in the transition from land to sea in elapid snakes

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Chapter 4: Up close and personal: The role of enlarged scale organs in tactile foreplay of turtle-headed sea snakes (Emydocephalus annulatus, Hydrophiinae)

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Chapter 1

Introduction

Chapter 1 Introduction

Sensory transitions that accompany major adaptive shifts

The perception of our surrounding environment is created by our sensory systems, which are underpinned by complex neural pathways that receive and integrate stimuli (*e.g.* visual systems, Figure 1.1). Sensory systems evolve to be attuned to specific qualities of prominent signals within the environment. For example, seawater is a relatively dense medium that propagates acoustic signals very well and thus influences sound communication systems in marine taxa (Montgomery and Radford, 2017) and dim-light conditions of subterranean habitats can alter or impoverish visual systems to the point of loss of vision (Cronin *et al.*, 2014; O'Carroll and Warrant, 2017). Because sensory systems are the primary interface an organism has with the environment, studying the ways in which sensory systems have undergone change can serve as an indicator of how organisms respond to major ecological shifts during evolution.

Repeated invasions of new environments or substantial changes in ecological niches within clades (*i.e.* replicate adaptive shifts) can provide a phylogenetic comparative framework in which to study how organisms shift their sensory systems during adaptive transitions (Pagel, 1999; Rezende and Diniz-Filho, 2012). The transition from sea to land was a major adaptive shift in the evolution of vertebrates, and subsequent re-invasion of aquatic habitats has evolved independently in multiple lineages of tetrapods (reviewed in Thewissen and Nummela, 2008). While these 'secondarily-aquatic' lineages can be constrained by their past adaptations to terrestrial habits, they may also benefit from the shift in sensory 'landscape'. The greater density of water compared to air, for example, dramatically increases sound propagation underwater, which has afforded an opportunity for echolocation and long-range communication in cetaceans (Thewissen and Nummela, 2008). Similarly, pinnipeds have co-opted tactile mechanoreceptors based on hairs (vibrissae) to detect lingering vibrations or 'hydrodynamic trails' generated by the movement of prey (Dehnhardt and Mauck, 2008a). In this thesis, I explore the evolution of overlooked cutaneous senses in the largest radiation of secondarily-aquatic reptiles, marine snakes.

Aquatic transitions in snakes

Almost all major clades of snakes have taxa that rely on aquatic habitats for some aspect of their life history (reviewed in Thewissen and Nummela, 2008). Many species hunt in freshwater systems with notable examples being green anacondas (*Eunectes*) of the family Boidae, water pythons (*Liasis*) of the family Pythonidae, North American water snakes (*Nerodia, Thamnophis*) of the family Colubridae, and piscivorous cottonmouths (*Agkistrodon*) of the family Viperidae.

Several snakes are highly adapted to marine habitats including at least 30 species of Asian mud snakes (Homolopsidae) that inhabit coastal habitats (*e.g.* mangroves, salt marshes) and three species of fully-aquatic file snakes (Acrochordidae) found in freshwater, brackish and marine water habitats (Figure 1.2A) (Murphy, 2012; Rasmussen *et al.*, 2011). The snakes that have most successfully colonised marine habitats are the two independently aquatic lineages of the subfamily Hydrophiinae (Elapidae): eight species of sea kraits and at least 60 species of sea snakes (Figure 1.2A). Although many of the taxa outlined above are called 'marine snakes' (Rasmussen *et al.*, 2011; Udyawer *et al.*, 2018), in this thesis I use the term strictly to refer to marine hydrophiine snakes (*i.e.* sea kraits and sea snakes *sensu* Heatwole, 1999). Studies of trait evolution in marine snakes has been enhanced with the availability of a partially-resolved, dated phylogeny for the group (Lee *et al.,* 2016; Sanders *et al.,* 2013).

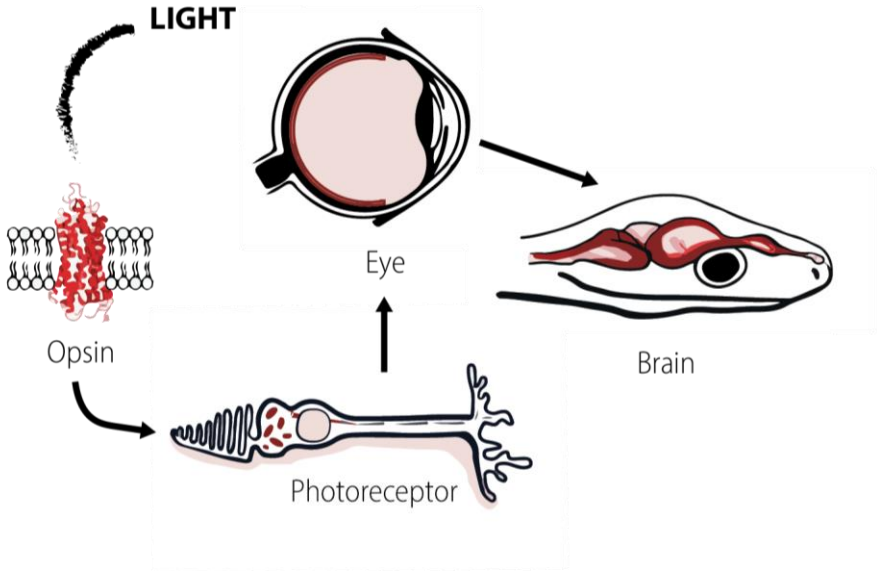

FIGURE 1.1 The generalised visual system of a vertebrate. Light is received by visual pigments, G-protein coupled receptor-class 'opsins', that are bound with light-absorbing chromophores derived from vitamin A. These visual pigments are found in high concentrations, embedded in the folded and stacked membranes of photoreceptors, which are the main cells responsible for light detection in the retinae of eyes. The absorption of light by visual pigments initiates a complex phototransduction cascade within photoreceptors, leading to the conduction of information across the various layers of the retina, and ultimately causing neuronal impulses to be transmitted from the retina to the brain. Diagram modified from Lamb *et al.* (2007) and Fu (2015).

Study group: marine snakes

Evolution and ecological diversity

Marine hydrophiine snakes are represented by two groups within the elapid snakes that have made recent, secondarily-aquatic transitions. Elapids are front-fanged venomous snakes with more than 350 species distributed throughout Africa, the Americas, Asia and the Australo-Pacific

regions (Lillywhite, 2014). Hydrophiinae are a subfamily of elapid snakes that comprise the marine lineage of sea kraits that form the sister clade to the Australo-Paupan terrestrial elapids (*e.g.* taipans, mulgas, death adders) plus marine sea snakes (Figure 1.2A) (Keogh *et al.*, 1998; Pyron *et al.*, 2013; Scanlon and Lee, 2004; Slowinski and Keogh, 2000). Based on fossil-dated Bayesian phylogenetic analyses using nuclear and mitochondrial sequences, sea kraits (genus *Laticauda*) are estimated to have diverged from terrestrial, Afro-Asian elapids (*e.g.* cobras, mambas, coral snakes) between 23 and 13 million years ago, and sea snakes diverged from terrestrial Australo-Paupan hydrophiines between 8 and 6 million years ago (Lee *et al.,* 2016; Sanders and Lee, 2008; Sanders *et al.*, 2008). Sea kraits and sea snakes share many synapomorphies with their terrestrial relatives including pterygoid teeth and neurotoxic venom (McCarthy, 1985), but also have derived traits specific to the transition to marine habitats such as laterally-compressed bodies and paddle-shaped tails for swimming and cutaneous gas exchange for increasing submergence times (Dunson, 1975; Heatwole, 1975; Rasmussen *et al.*, 2011). Sea kraits are oviparous and best described as 'amphibious' because they rely on both marine and terrestrial habitats: hunting on coral reefs and returning to the land to mate, lay eggs, drink freshwater and digest prey (Rasmussen *et al.*, 2011). In contrast, sea snakes are viviparous with most of the approximately 62 species living entirely in aquatic habitats.

Over the last century, substantial morphological and genetic data have contributed to our current understanding of species diversity and phylogenetic relationships among sea snakes. Sea snakes comprise 47 species within the rapidly-speciating and ecologically diverse *Hydrophis* clade (Lee *et al.*, 2016) and nine species within the *Aipysurus–Emydocephalus* clade (*sensu* Voris, 1977). Nested within these fully-marine lineages are three semi-aquatic species, in the monotypic genera *Hydrelaps, Ephalophis* and *Parahydrophis,* that form sister clade to the *Hydrophis* plus *Microcephalophis gracilis* (Figure 1.2B). Reciprocal monophyly among the two major clades of sea snakes, *Hydrophis* and *Aipysurus–Emydocephalus*, is supported by both morphological and genetic data (Lukoschek and Keogh, 2006; Sanders *et al.*, 2013; Smith, 1926; Voris, 1977). Both major clades show convergent adaptations to fully-aquatic habits including different tissues to exclude seawater from the mouth and different vertebral bones to form the paddle-shaped tail (Rasmussen, 2002; Sanders *et al.*, 2012). Vacant aquatic niches were thought to drive the adaptive radiation of sea snakes (Lillywhite *et al.*, 2008), but recent molecular phylogenetic analyses challenge this assumption by showing that increased speciation rates do not coincide with early invasions of marine habitats (Lukoschek and Keogh, 2006; Sanders *et al.*, 2013). Instead, most of the species richness is attributed to recent (~2.5 million years ago) explosive speciation of the *Hydrophis* clade, their estimated speciation rate is three times greater than background estimates for elapids based on Bayesian methods and sampling 50% of elapid taxa with correction for differential sampling across clades (Lee *et al.,* 2016). The anomalous speciation rates in *Hydrophis* likely

reflects complex historical vicariance throughout the Indo-Pacific and genetic and/or developmental propensity to exploit ecological opportunities (Lee *et al.*, 2016; Lukoschek and Keogh, 2006; Nitschke *et al.*, 2018; Sanders *et al.*, 2010; Ukuwela *et al.*, 2016). The accelerated speciation has resulted in short internode branch lengths in *Hydrophis* phylogeny (Figure 1.2B) and explains much of the taxonomic uncertainty that has plagued nomenclature of sea snakes, such that 10 to 16 previously recognised monotypic or paraphyletic genera (Rasmussen, 1994; Smith, 1926; Voris, 1977) have now been synonymised with *Hydrophis* (Rasmussen *et al.*, 2014; Sanders *et al.*, 2013). Finally, the full extent of species diversity and distribution is still the topic of active research; recent studies have discovered cryptic species, hybridisations and new populations of presumed extinct species (D'Anastasi *et al.*, 2016; Sanders *et al.*, 2015, 2014, Ukuwela *et al.*, 2012, 2013).

Sea snakes have evolved distinct ecologies and associated morphologies ('ecomorphs'), successfully colonising a range of shallow-water habitats throughout the warm waters of the Indian and Pacific Oceans. Given their phylogenetically nested position within Australian hydrophiines, sea snakes are thought to have evolved in Australian waters and subsequently colonised South-East Asian, Indian and Pacific Oceans (Ukuwela *et al.*, 2016). Sea snakes typically forage for benthic prey (*e.g.* eels, gobies) and as such most species occupy marine habitats shallower than 100 m deep including coral reefs, seagrass beds, estuaries and lagoons (Rasmussen *et al.*, 2011; Udyawer *et al.*, 2018). Notable exceptions to these broad ecological preferences are: 1) pelagic sea snakes (*Hydrophis platurus*) that ambush pelagic fish in open-ocean currents, contributing to their widespread distribution throughout the Pacific and Indian oceans, 2) some freshwater species that dwell in lakes (*e.g. Hydrophis semperi*, *Hydrophis sibauensis*), and 3) three semi-aquatic species (genera *Hydrelaps, Parahydrophis, Ephalophis*) that forage in intertidal mangrove habitats of northern Australia (Lillywhite *et al.*, 2017; Rasmussen *et al.*, 2011). The species in the *Aipysurus–Emydocephalus* clade show some novel feeding strategies (*e.g.* egg-eating, Voris, 1966), but the *Hydrophis* clade is the most ecologically diverse and includes repeated origins of extreme reductions in head-size and body proportions among 'microcephalic' species that specialise on burrowing eel prey (Figure 1.5) (Glodek and Voris, 1982; Sherratt *et al.*, 2018; Voris and Voris, 1983). The recent marine transition and variety of ecologies among sea snakes provides an opportunity to study how sensory systems changes in response to ecological transitions.

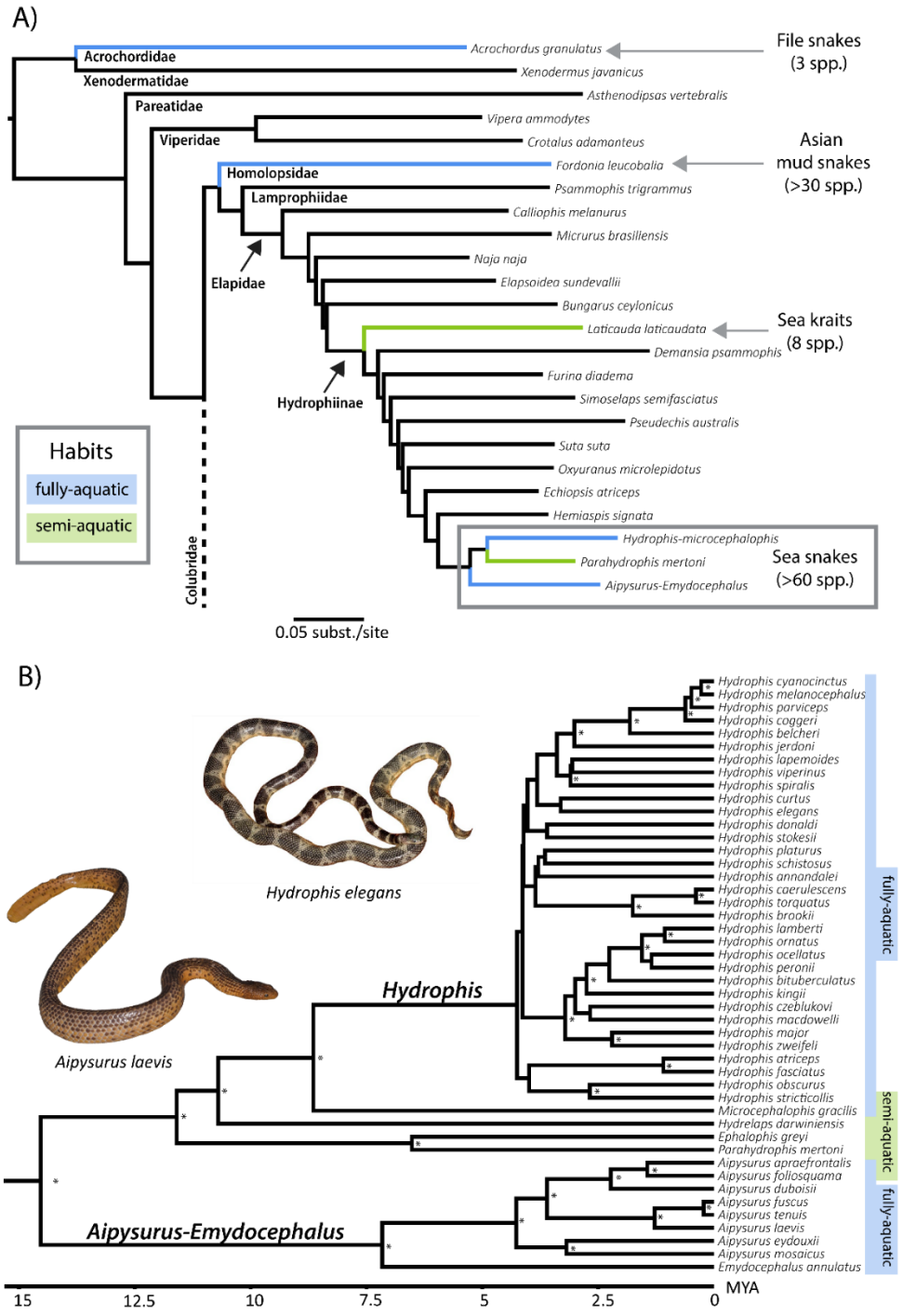

FIGURE 1.2. Major adaptive radiations to aquatic habitats in snakes. A) Phylogenetic tree with representative lineages from the major families of snakes; life-history habits are shown by branch colours, which illustrates multiple independent transitions from terrestrial (black branches) to semi-aquatic and/or fully-aquatic habits; Maximum likelihood tree based on 12 genes from Pyron *et al.* (2013), modified with permission from Udyawer *et al.* (2018). B) Bayesian tree showing relationships among viviparous sea snakes with aquatic and semi-aquatic habits indicated by taxa colours (branch tips); axis represents time in millions of years ago (MYA); posterior probabilities greater than 0.90 are indicated by asterisks (*) at nodes. Inset images show species representative of the major clades of sea snakes, *Hydrophis elegans* (*Hydrophis* clade) and *Aipysurus laevis* (*Aipysurus-Emydocephalus* clade). Tree modified from Sherratt *et al.* (2018) and images of snakes from Mirtschin *et al.* (2017) with permission.

Vision, hearing and chemoreception are important senses for terrestrial snakes, but how the shift to aquatic habitats has influenced these sensory capabilities in marine snakes has yet to be fully realised. Based on morphological studies of the retinae, marine snakes are thought to have lower visual acuity compared to their terrestrial relatives (Hart *et al.*, 2012;; Hibbard and Lavergne, 1972; Kordi and Shabanipour, 2014), but behaviorual observations suggest that vision is still important for foraging, predator avoidance and mate recognition (Heatwole, 1999; Karthikeyan *et al.*, 2008; Shine *et al.,* 2005). Recent work on functional mutations within opsin gene sequences suggests that spectral sensitivities in sea snakes have shifted to match the light qualities of marine habitats; *e.g.* ancestral UV sensitive short-wavelength sensing photoreceptors have shifted to violet or blue spectral regions (Simões *et al.*, unpublished data). Olfactory receptors in the olfactory tract are important for sea kraits that still rely on terrestrial habitats, but many olfactory genes are absent or pseudogenes in sea snakes, perhaps because valvular nostrils are sealed during submergence (Figure 1.3) (Kishida and Hikida, 2010). In contrast, chemoreception via the vomeronasal organ is probably important for both sea kraits and sea snakes, which have been recorded tongue-flicking underwater presumably aiding close-range detection during foraging and courtship behaviours (Figure 1.3B) (Guinea, 1986; Kutsuma *et al.*, 2018; Shine, 2005; Shine *et al.*, 2004). Adaptations of chemosensory receptors to the divergent properties of chemical stimuli in the water *versus* air (*e.g.* water-solubility, rapid dilution) have yet to be investigated in marine snakes. Finally, preliminary electrophysiological studies in a limited number of species suggest that sea snakes have auditory sensitivities to low-frequency sound, likely transduced by mechanoreceptors in the inner ear and/or skin (Chapuis, *et al.,* unpublished data; Westhoff *et al.*, 2005). These studies paint an emerging picture of the sensory ecology of marine snakes by studying the functional changes in sensory genes within a phylogenetic context and in combination with behavioural, electrophysiological and ecological data. Similar approaches can be applied to explore cutaneous senses in marine snakes.

The skin as a sense organ

Mechanoreception

The skin is the primary organ for the sense of touch, which is the ability to sense physical displacement known as 'mechanoreception' (Dehnhardt and Mauck, 2008b). Cutaneous mechanoreceptors respond to direct deformation of the skin and can relay information about the modality (*e.g.* vibration, texture), location, intensity and duration of stimuli (Hao *et al.*, 2014). Mechanoreceptors that respond to innocuous stimuli are termed 'low-threshold mechanoreceptors' (LTMRs) and are categorised according to their rate of adaptation or 'neural responsiveness': rapidly adapting (RA) LTMRs such as free nerve endings, Meissner corpuscles

and Pacinian corpuscles; and slowly adapting (SA) LTMRs such as Merkel cells and Ruffini corpuscles. Irrespective of their rate of adaptation and type of information transduced, LTMRs consist of similar ultrastructure components: sensory axon terminals and neuronal components that contact surrounding cells within the skin (Zimmerman *et al.*, 2014) (Figure 1.4).

Mechanoreception is a significant, albeit comparatively overlooked, sensory modality for snakes (Young, 1997). Being in intimate contact with the substrate over or in which they are moving, snakes use tactile cues to explore and navigate (Keathley, 2004; Young and Morain, 2003), discriminate prey types (Aota, 1940; Nishida *et al.*, 2000) and engage in courtship behaviours (Carpenter, 1977; Noble, 1937). In terrestrial snakes, mechanoreception is facilitated by numerous cutaneous mechanoreceptors, the most conspicuous of which are termed 'scale sensilla' (also referred to as 'papillae' or 'tubercles') that are present all over the body but are concentrated on the head (Figure 1.4) (von Düring and Miller, 1979; Jackson, 1977; Landmann, 1975; Povel and VanDerKooij, 1997; Underwood, 1967). Scale sensilla in terrestrial snakes resemble Meissner corpuscles (Figure 1.4) identified in mammalian skin and electrophysiology studies indicate that they are sensitive to similar physical displacement thresholds to mammals (Jackson and Doetsch, 1977b, 1977a; Proske, 1969). However, how snakes integrate information from multiple scale sensilla located in various regions of the head and body to form their perception of touch remains poorly understood.

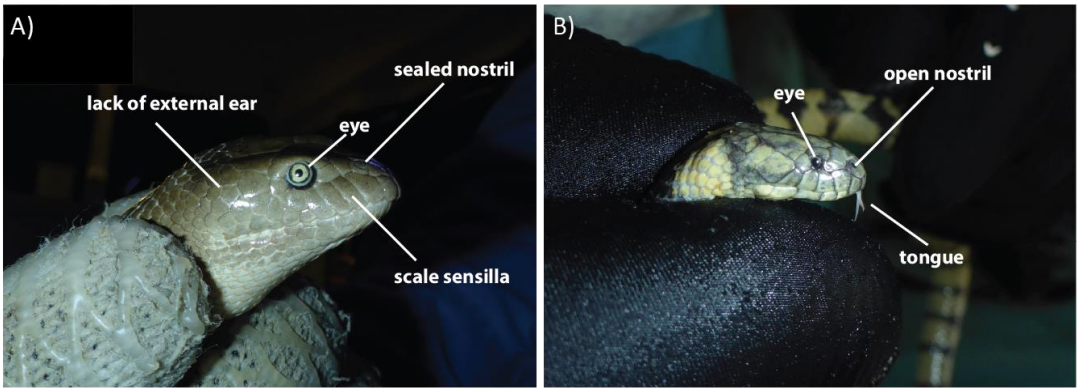

FIGURE 1.3. Cephalic sensory organs in sea snakes. A) Image of *Hydrophis stokesii* shows a large eye, sealed nostrils and small scale organs termed 'sensilla'. B) Image of *Hydrelaps darwiniensis* showing a small eye, open nostril, and forked tongue that delivers chemicals to the vomeronasal or Jacobson's organ. Like some other aquatic snakes, marine snakes have valvular nostrils to exclude seawater while submerged, which likely impacts chemoreception via the olfactory bulb. Like terrestrial snakes, sea snakes lack external ear openings but likely transduce sound via bone conduction. Scale sensilla are highly variable in size among species, e.g. sensilla in *H. stokesii* are discernible by the naked eye, while those in *H. darwiniensis* can only be identified under magnification. Images by D. Gower and used with permission.

Scale sensilla in marine snakes are presumptive mechanoreceptors (Figure 1.3A) that are thought to have been co-opted for enhanced sensitivity in the marine environment. Hydrodynamic reception is a ubiquitous sense among aquatic taxa (Kalmijn, 1988) and convergently evolved in aquatically-foraging animals via co-option of cutaneous mechanoreceptors—*e.g.* vibrissae in pinnipeds, push receptors in platypus, and corpuscles in shorebirds (reviewed in Dehnhardt and Mauck, 2008a; Schneider *et al.*, 2016). Similarly, the function of scale sensilla in marine snakes was thought to be co-opted for hydrodynamic reception, purportedly allowing sea snakes and sea kraits to detect water motion caused by potential predators, prey and mates, or pressure changes caused by weather events (Heatwole, 1999; Karthikeyan *et al.*, 2008; Lillywhite *et al.*, 2009; Liu *et al.*, 2010; Westhoff *et al.*, 2005; Young, 2003). Two independently-aquatic lineages of snakes (genera *Acrochordus, Erepeton*) have highly derived cutaneous mechanoreceptors that are highly sensitive to water motion generated by fish-prey (Catania *et al.*, 2010; Povel and VanDerKooij, 1997). However, the morphological characteristics and diversity of scale sensilla in hydrophiine snakes has not been quantified, precluding meaningful comparisons with other hydrodynamic receptors.

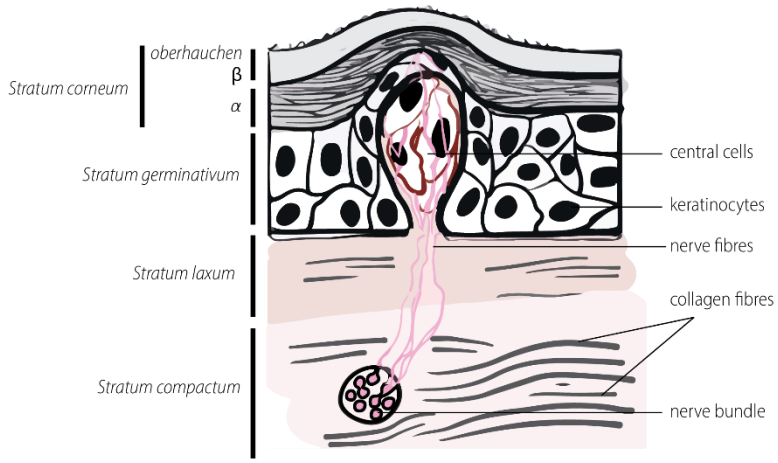

FIGURE 1.4. Schematic diagram illustrating structure of skin and scale sensilla in terrestrial snakes. Hardened epidermal scales are comprised of both living and dead epidermal cells (keratinocytes) that reside in heterogenous layers: the inner layer of living cells (*stratum germinativum*), and the outer layer of non-nucleated, cornified keratinocytes (*stratum corneum*) that also gives rise to patterns of micro-sculpturing on the surface of the scale (*oberhaütchen*). Underlying the epidermis is the dermis composed of two layers: a loosely arranged layer of connective tissue (*stratum laxum*) and a denser layer of collagen fibres (*stratum compactum*). The typical structure of a scale sensillum in a terrestrial snake resembles low-threshold mechanoreceptors of mammals, comprising central cells derived from the dermis (*stratum laxum*) and nerve fibres that together form an 'bump' in the outer epidermis (*stratum corneum*). Diagram modified from Landmann (1975, 1986) and Jackson and Doetsch (1977a).

To test for various adaptive functions for scale sensilla, we must first describe extant morphological variation (*i.e.* sensilla traits) and account for the effects of allometry and phylogeny

on that variation. Changes in organismal traits are correlated with changes in body size due to ontogeny and phylogeny (*i.e.* allometry, Gould, 1966). The allometric relationship between body size and morphology, therefore, has important implications for trait evolution including sensory evolution, *e.g.* plasticity in neural investment and constraints on organ size (Fox and Wilczynski, 1986; Lee *et al.*, 2014; Niven and Laughlin, 2008), but these effects have been overlooked in studies of scale sensilla despite great variation in head and body sizes (Figure 1.5). Furthermore, sensilla traits are expected to be similar among closely-related species simply because of their shared ancestry (*i.e.* phylogeny) (Pagel, 1999; Rezende and Diniz-Filho, 2012). Thus, it is necessary to account for the phylogenetic non-independence of sensilla traits among hydrophiine taxa. Finally, determining the function role of scale sensilla has been further hindered by the sheer morphological diversity of scale modifications among snakes, especially among sea snakes that have scales with central longitudinal ridges (keels), spines (*e.g. Hydrophis curtus, Emydocephalus annulatus,* Guinea 1996) and 'rugosities' (*sensu* Avolio *et al.*, 2006a, 2006b). These scale modifications are often morphologically overlapping in size and shape, and can co-occur on the same scales or body regions as scale sensilla (pers. obs). It is important to consider various non-sensory functions for these scale modifications, *e.g.* enhanced structure for skin colour (Spinner *et al.*, 2013) and/or friction during locomotion or mating (Avolio *et al.*, 2006a). Thus, to test for links between ecological traits (i.e. selection) and sensilla traits, we need to characterise the morphology and diversity of scale sensilla while also accounting for the effects of allometry and phylogeny.

FIGURE 1.5. Variation of head and body size in sea snakes illustrating the importance of allometry in analyses of trait evolution in marine hydrophiine snakes. Typical head-body proportions are shown by a subadult, male *Hydrophis stokesii* (specimen on the left, WAMR174251); reduced head size and fore *versus* hind-body girths are shown by a subadult, male *Microcephalophis gracilis* (specimen on the right, FMNH201933). The 'microcephalic' ecomorph has evolved in multiple, independent lineages within the *Hydrophis* clade and is correlated with specialist feeding on burrowing eels (Sherratt *et al.,* 2018). Image taken by the author.

Dermal photoreception

In organisms that lack fur or feathers, the skin is a key site for 'dermal' photoreception, which is the ability to sense light independently of photoreceptive structures and cells that are found in the eye (reviewed in Kelley and Davies, 2016). Among squamate reptiles, dermal photoreception has been linked to thermoregulatory behaviours in wall lizards (*Podarcis muralis*, Tosini and Avery, 1996) and colour change in the skin of Moorish geckos (*Tarentola mauritanica*, Fulgione *et al.*, 2014), but olive sea snakes (*Aipysurus laevis*) are the only squamate to show dermal phototaxis, which is the movement, including the entire or part of the body, away from light (Zimmerman and Heatwole, 1990).

Although dermal phototaxis is common in marine invertebrates (reviewed in (Ramirez *et al.*, 2011; Wolken, 1988), only a few vertebrate lineages are known to use dermal phototaxis (lamprey, hagfish, aquatic amphibians and sea snakes), responding to light on their elongate hind-bodies and/or tails by withdrawing under cover to avoid detection by predators (Alder, 1976; Baker *et al.*, 2015; Patzner, 1978; Pearse, 1910; Reese, 1906; Ronan and Bodznick, 1991; Steven, 1955; Young, 1935). The convergent evolution of dermal phototaxis in these divergent lineages may be driven by the same selection pressure and even underpinned by parallel molecular pathways. Despite the ostensible importance of dermal phototaxis in vertebrates, the ecological drivers and molecular basis for this convergent phototactic trait has not been investigated.

Our understanding of vertebrate photoreception has been primarily informed by studies of the eye, a complex organ that houses photoreceptive cells (*e.g.* rods, cones) containing G-protein coupled receptors known as opsins that are rendered light-sensitive due to the binding of a chromophore moiety, a derivative of vitamin A. Opsins contain binding sites for 'retinal', the chromophore which, in the presence of light, changes from *cis-* to *all-trans* configuration causing a conformational change in the opsin and initiating a complex phototransduction cascade (Fu, 2015; Invergo *et al.*, 2013), ultimately leading to the sense and sensation of vision (Figure 1.1). The visual cycle is complete when retinal is regenerated from *all-trans* to a *cis-* form and by-products are metabolised (Saari, 2012). Most amniotes (mammals and reptiles) have four or five visual opsins expressed within their photoreceptors (reviewed in Simões and Gower, 2017). Snakes retain only one to three visual opsins, expressing these within retinal photoreceptors of diverse morphologies that suggest transmutation processes proposed by Walls (1942), supported by Simões *et al.* (2016). In addition to visual opsins, a plethora of 'non-visual' opsins have been identified in vertebrates, some of which are linked to various light-mediated behaviours and physiological processes (*e.g.* pinopsin in pineal gland or parietal opsin in parietal eye) but many others have labile protein-expressions and diverse or unknown functions (Davies *et al.*, 2015; Peirson *et al.*, 2009; Porter, 2016). Thus, the molecular pathways of dermal photoreception in sea

snakes might have evolved via co-option of existing visual machinery of the eye, or through novel elaboration of non-visual pathways.

Broad aims

I aimed to address two questions around two cutaneous senses in hydrophiines: 1) How has the sense of touch evolved in the transition from land to sea? and 2) How has a novel phototactic trait arisen in sea snakes? These aims were addressed using a comparative approach that integrated morphological, behavioural, molecular and ecological data.

Aim 1

How has the sense of touch evolved in the transition from land to sea?

Marine snakes are thought to detect water motion (*i.e.* hydrodynamic reception) and pressure changes (*i.e.* hydrostatic baroreception) using purported scale mechanoreceptors ('sensilla') but these hypotheses have not been explicitly tested. If sensilla have acquired an enhanced mechanosensory function in marine snakes, then we might expect the outer and underlying morphology of these scale organs to be have changed in the marine lineages compared to terrestrial lineages. For example, modified mechanoreceptors in crocodilians and other aquatic snakes protrude from the surface of the scale and have specialised sensory cells and nerve connections underlying these sensory organs.

To test the hypothesis that scale sensilla have been co-opted for enhanced mechanoreception, I use morphological techniques (quadrate sampling, scanning and transmission electron microscopy, immunohistochemistry and histology) to qualitatively and quantitatively access sensilla traits in terrestrial and marine snakes. I also account for the effects of allometry and phylogeny on sensilla traits and use Bayesian analyses to reconstruct ancestral sensilla traits in sea kraits and sea snakes. I also investigate scale protuberances in a single species of sea snake, *Emydocephalus annulatus*, to explore the role of scale modifications in the unique tactile courtship of this species.

Aim 2

How has a novel phototactic trait arisen in sea snakes?

Dermal phototaxis of the paddle-shaped tail was hypothesised as an adaptation to avoid detection by predators and is often considered to be ubiquitous among sea snakes and potentially sea kraits and burrowing lineages of snakes (Young and Morain, 2003; Zimmerman and Heatwole, 1990). However, Despite the discovery of tail phototaxis in a single species of sea snake (*Aipysurus laevis*) over two decades ago, the ecological drivers, molecular basis and evolutionary origin of this novel phototactic trait has not been investigated further in sea snakes.

1. Evolutionary origin

   A major aim is to understand whether this trait has evolved in only a single species or whether it shared by all sea snakes and thus an adaptation that evolved early in the transition to aquatic habitats. I conducted behavioural tests in eight species of sea snakes and mapped the taxonomic distribution of tail phototaxis ability onto the phylogeny. I use parsimony in developing a preliminary hypothesis of the evolutionary origin of this novel phototactic trait.

2. Ecological drivers

   To test the hypothesis that this trait is an adaptation to avoid predators, I quantify tail damage in museum specimens of two phototactic and two non-phototactic species. I use these data to test whether the probability of sustaining tail injuries is influenced by tail phototactic ability in snakes. I expected that phototactic species might have lower bite rates indicating greater protection from attacks or have increased bite rates due to an intrinsically higher vulnerability to predation.

3. Molecular basis and evolutionary novelty

   Because the dermal photoreceptive structures and genes involved in tail phototaxis are entirely unknown, I comprehensively profiled genes related to visual and non-visual photoreceptors in whole transcriptomes of tail and body skin, eye and other available organs. I expected that the molecular basis of phototaxis might be based on a novel expression of existing visual pathways typically used in vision-formation of the eye or co-option of non-visual pathways present in the skin. These results are used to develop a candidate list of light-sensing genes and pathways, which can be used to understand the parallel evolution of this trait among vertebrates.

Thesis outline

Chapter 2

The evolution of scale sensilla in the transition from land to sea in elapid snakes

I use Bayesian analyses to infer ancestral and derived sensilla traits (number, density, area, coverage) among 19 species of terrestrial and marine elapid snakes. Sensilla traits were quantified using morphological techniques (quadrate sampling, scanning electron microscopy) that accounted for the effects of allometry (head size) and phylogeny (shared descent). I found that there is substantial variation in the overall coverage of sensilla (0.8 to 6.5%) among aquatic and terrestrial lineages of elapid snakes. However, only the aquatic taxa sampled had protruding, dome-shaped sensilla and exceptionally higher overall coverage was found in a only a few of the fully-aquatic species of sea snakes. These results suggest that changes in sensilla traits do not

coincide with the transition to aquatic habitats in sea kraits or sea snakes, but rather that sensilla traits might be correlated with specific ecologies of a few species of sea snakes. This chapter was developed from the topic of my honours research with new data collected (quadrate sampling of postocular scales) and more analyses conducted (repeatability, phylogenetic corrections) during my PhD candidature and is hence included in this thesis.

Chapter 3

Ultrastructural evidence of a mechanosensory function of scale 'sensilla' in sea snakes (Hydrophiinae)

Building on data from Chapter 2, this chapter reveals the underlying cell types and nerve connections of scale sensilla in sea snakes. By integrating multiple morphological techniques (histology, immunohistochemistry and transmission electron microscopy), I examine the ultrastructural resemblances of scale sensilla on the head and tail of two sea snakes, *Aipysurus laevis* and *Hydrophis stokesii*, and compare these results with data available for terrestrial snakes. Taken together with data from Chapter 2, these results provide the first evidence for a mechanosensory function for scale sensilla among sea snakes, suggesting that these scale organs may have a role in hydrodynamic reception in sea snakes.

Chapter 4

Up close and personal: the role of enlarged scale organs in tactile foreplay of turtle-headed sea snakes (*Emydocephalus annulatus*)

Because many lineages of sea snakes have conspicuous scale protuberances in addition to scale sensilla (*e.g.* spines, rugosities), I investigated the morphology of these structures in a single species of sea snake, *Emydocephalus annulatus*. After determining that scale protuberances are present only in males, I examined the ultrastructure (histology, scanning electron microscopy) of scale protuberances in males. These results suggest that scale protuberances are mechanosensory organs under sexual selection in this species indicating that the evolution of scale modifications may be correlated with unique ecological and life history traits. .

Chapter 5

Phototactic tails: Evolution and molecular basis of a novel sensory trait in sea snakes

I significantly extend previous studies by testing for dermal phototaxis in eight species of sea snakes. Based on the results, I was able to map tail phototaxis onto the phylogeny of sea snakes and hypothesize the evolutionary origin of this trait in a clade of six *Aipysurus* species. To test the hypothesis that tail phototaxis is an adaptive trait used to aid in concealment from predators, I quantified tail damage in museum specimens and estimated probabilities of sustaining tail injuries

in two phototactic and non-phototactic species. I then identified candidate phototaxis genes and signalling pathways using gene profiling of skin transcriptomes taken from phototactic species.

Chapter 6

Summary and conclusions.

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Chapter 2

The evolution of scale sensilla in the transition from land to sea in elapid snakes

Chapter 2 The evolution of scale sensilla in the transition from land to sea in elapid snakes

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Abstract

Scale sensilla are small tactile mechanosensory organs located on the head scales of many squamate reptiles (lizards and snakes). In sea snakes and sea kraits (Elapidae: Hydrophiinae), these scale organs are presumptive scale sensilla that purportedly function as both tactile mechanoreceptors and potentially as hydrodynamic receptors capable of sensing the displacement of water generated by movement (*e.g.* of potential predators, prey or mates). We combined scanning electron microscopy, silicone casting of the skin, and quadrate sampling with a phylogenetic analysis to assess morphological variation in sensilla on the postocular head scale/s across four terrestrial, 13 fully-aquatic and two semi-aquatic species of elapids. Substantial variation exists in the overall coverage of sensilla (0.8 to 6.5%) among the species sampled and is broadly overlapping in aquatic and terrestrial lineages. However, two observations suggest a divergent, possibly hydrodynamic sensory role of sensilla in sea snake and sea krait species. Firstly, scale sensilla are more protruding (dome-shaped) in aquatic-associating species than in their terrestrial counterparts. Secondly, exceptionally higher overall coverage of sensilla is found only in the fully-aquatic sea snakes, and this attribute appears to have evolved multiple times within this group. Our quantification of coverage as a proxy for relative 'sensitivity' represents the first analysis of the evolution of sensilla in the transition from terrestrial to marine habitats. However, evidence from physiological and behavioural studies is needed to confirm the functional role of scale sensilla in sea snakes and sea kraits.

KEYWORDS: sea snake, sensilla, mechanoreceptor, hydrodynamic, sensory, elapid

Introduction

Evolutionary transitions from terrestrial to aquatic habitats provide important insight into how organismal traits respond to major adaptive shifts. Unfortunately, opportunities to examine such inferences are limited because many secondarily aquatic taxa lack living, phylogenetically close, terrestrial relatives. An important exception are the front fanged hydrophiine snakes (Elapidae), which comprise approximately 100 species of Australo-Melanesian terrestrial snakes, 60 species of fully-aquatic viviparous sea snakes, and eight species of semi-aquatic oviparous sea kraits (*Laticauda*). The whole group is estimated to share a common ancestor dated between 14 and 26 million years ago (Mya); the semi-aquatic sea kraits form the sister lineage to the terrestrial plus viviparous marine species, and the viviparous marine clade diverged independently from within the terrestrial group only 6 to 8 Mya (Sanders *et al.*, 2008). Thus, hydrophiines are excellent candidates for studying the evolution of organismal traits resulting from transitions between land and sea.

Our understanding of how selection pressure shapes morphological and physiological evolution in aquatic hydrophiines has advanced in several areas, particularly in traits relating to locomotion (Aubret and Shine, 2008; Brischoux *et al.*, 2010; Graham *et al.*, 1987; Shine and Shetty, 2001), gas exchange (Dunson and Stokes, 1983; Graham, 1974; Heatwole, 1977; Lillywhite *et al.*, 2009), diving (Heatwole and Seymour, 1975; Heatwole *et al.*, 1979; Seymour, 1974) and osmotic balance (Brischoux *et al.*, 2012; Lillywhite *et al.*, 2014). A number of studies have also sought to understand the evolution of hydrophiine sensory systems associated with the transition to marine life (*e.g.* hearing, Westhoff et al. 2005; vision, Hart et al. 2012; pressure detection, Liu et al. 2010; chemoreception, Shine 2005). Nonetheless, the roles of mechanoreception and hydrodynamic reception in the marine environment remain understudied.

Mechanoreception of the external environment is a sensory modality found across diverse taxa. Most terrestrial animals rely on direct touch with solid surfaces. In contrast, the high density and viscosity of water allows many marine organisms to sense the displacement of water using specialised hydrodynamic receptors (Denny, 1993; Thewissen and Nummela, 2008). Hydrodynamic reception allows the detection of water movement from both biotic sources (*e.g.* prey, predators and mates) and abiotic sources (*e.g.* turbulence caused by water currents deflected past physical objects) (Dehnhardt and Mauck, 2008b). Strong selection pressure to evolve hydrodynamic reception is suggested by its ubiquitous presence in fish and cephalopods, both of which have a well-developed lateral line system (Budelmann and Bleckmann, 1988; Coombs *et al.*, 1987; Kalmijn, 1988). In addition, many secondarily aquatic tetrapods have evolved hydrodynamic receptors, in some cases via exaptation of tactile mechanoreceptors (*e.g.* the whiskers of pinnipeds; Dehnhardt et al. 1998; Dehnhardt et al. 2001).

This study examines the putative sensory organs concentrated on the head scales of terrestrial and aquatic elapid snakes. Here we refer to these organs as 'scale sensilla', but they are variously termed 'sensillae', 'corpuscles', 'tubercles' and 'papillae' in the literature (von Düring and Miller, 1979; Jackson, 1977; Povel and VanDerKooij, 1997; Underwood, 1967; Westhoff *et al.*, 2005). In terrestrial elapids, scale sensilla are present on the head in large numbers (~6000 per snake) where they function as tactile mechanoreceptors used for sensing the surrounding substrate by direct contact (von Düring and Miller, 1979; Jackson, 1977; Jackson and Doetsch, 1977a, 1977b; Povel and VanDerKooij, 1997; Proske, 1969b, 1969a). In aquatic elapids the function of scale sensilla remains uncertain due to the hitherto limited number of physiological and morphological studies. Auditory brainstem responses to water movement have been recorded in the sea snake *Hydrophis (Lapemis) curtus,* but direct extracellular electrophysiological recordings of individual scale sensilla were unsuccessful (Westhoff *et al.*, 2005). A comparative morphological study that included *H. curtus* found markedly more protruding sensillum ultrastructure in aquatic compared to terrestrial snakes (Povel and VanDerKooij, 1997). These studies, as well as reports of sea snakes and sea kraits responding to vibrations and pressure changes (Heatwole, 1999; Liu *et al.*, 2010), and the limited role of vision for prey capture in some species (Hart *et al.*, 2012; Karthikeyan *et al.*, 2008), point to the potential importance of scale sensilla for hydrodynamic reception in aquatic elapid snakes. However, the literature on scale sensilla lacks both quantitative (size and coverage) and descriptive (ultrastructure) analysis across terrestrial and aquatic species (Dehnhardt and Mauck, 2008a; Young, 2003), making it difficult to draw comparative conclusions of sensilla function.

This study is the first to quantify the traits of scale sensilla in an ecologically and phylogenetically broad sample of snakes, and to analyse these traits within a phylogenetic framework. We begin with a qualitative assessment of the ultrastructure of sensilla on the nasal scale, before undertaking a quantitative examination of the numerical-density of sensilla, the mean size of individual sensilla, and the overall coverage of sensilla on the postocular scale/s of 13 fully-aquatic, four terrestrial and two independently semi-aquatic species of elapids. We discuss our findings in relation to the hypothesis that scale sensilla have been co-opted from a tactile mechanoreceptor in the terrestrial elapids to a hydrodynamic receptor in the sea snakes and sea kraits.

Materials and methods

Specimens

Traits of scale sensilla were examined in 44 individuals from 19 species in the family Elapidae (Table 2.1). Preserved specimens were obtained from the South Australian Museum, the Western Australian Museum and the Field Museum of Natural History. Specimens collected from the

same locality were used where possible to minimise intraspecific variation over geographical ranges. Only adult male specimens were used to control for the effects of ontogeny and sexual dimorphism (Supplementary material 1, Table S2.1, for specimen list and location).

This paper follows the most recent nomenclature for sea snakes by using *Hydrophis* as the currently accepted genus-level synonym to include species previously in the genera *Pelamis, Enhydrina, Astrotia, Thalassophina, Lapemis* and *Disteira* (Rasmussen *et al.*, 2014; Sanders *et al.*, 2013). Taxa are categorised into terrestrial, fully-aquatic or semi-aquatic according to field observations (Cogger, 2000; Wilson and Swan, 2013). The sea snake *Hydrelaps darwiniensis* is phylogenetically nested within the fully-aquatic species as sister lineage to *Hydrophis* but relies on both marine and terrestrial habitats and is therefore grouped here with the other semi-aquatic taxon, *Laticauda*.

Qualitative analysis

High depth of field photographic images of whole-snake heads were composed for six representative elapid species comprised of one terrestrial species (n = 1), four fully-aquatic species (n = 4 individuals) and one semi-aquatic species (n = 1) from the subfamily Hydrophiinae (Supplementary material 1, Table S2.2, for details of photography and specimens). In addition, high-magnification images of sensilla ultrastructure on the nasal scale (Figure 2.1) were captured using scanning electron microscopy (SEM) for a subset of elapid taxa, comprised of one terrestrial species (n = 1), five fully-aquatic species (n = 5 individuals) and one semi-aquatic species (n = 1) from the subfamily Hydrophiinae (Table 2.1). The posterior part of the nasal scale was dissected from museum specimens that had been frozen, fixed in 10%-formalin and stored in 100%-ethanol. These samples were rinsed in a phosphate buffered saline solution containing 4% sucrose (pH 7.2), before immersion in a consecutive series of ethanol solutions (70, 90, 100%), followed by immersion in Hexamethyldisilazane. Samples were then left to air-dry for 5 min before being mounted with an epoxy resin on carbon or platinum coated aluminium stubs. The coated samples were then viewed with a high-vacuum, 10 kV SEM (XL30, Philips, Japan). In addition to the nasal scale, the first sublabial, third supralabial, postocular and parietal scales from the sea snakes *Hydrophis major* and *Hydrophis stokesii* were examined directly in environmental SEM (450 Quanta, FEI, USA).

TABLE 2.1. Taxonomy, ecology and sample size of the elapids analysed in the present study.

Taxonomy					*Ecology*[*]		*Sample size*	
Subfamily	*Genus*	*Species*	*Synonyms*	*Authority*	*Habitat*	*Foraging area*	*Qualitative*[†]	*Quantitative*[‡]
Hydrophiinae	*Aipysurus*	*duboisii*		Bavay (1869)	Fully-aquatic	Generalist	1	1
		fuscus		Tschudi (1837)	Fully-aquatic	Coral reef specialist		1
		laevis		Lacépède (1804)	Fully-aquatic	Generalist		1
		eydouxii			Fully-aquatic	Sandy-bottoms		1
	Emydocephalus	*annulatus*		Krefft (1869)	Fully-aquatic	Coral reef specialist	1	2
	Hydrophis	*curtus*	*Lapemis curtus, Lapemis hardwicki*	Shaw (1802)	Fully-aquatic	Generalist	1	5
		cyanocinctus		Daudin (1803)	Fully-aquatic	Generalist		3
		donaldi		Ukuwela *et al.* (2012)	Fully-aquatic	Turbid estuaries		1
		major	*Disteria major*	Shaw (1802)	Fully-aquatic	Generalist	1	3
		platurus	*Pelamis platura*	Linnaeus (1766)	Fully-aquatic	Pelagic		4
		schistosus	*Enhydrina schistosa*	Daudin (1803)	Fully-aquatic	Turbid estuaries		4
		stokesii	*Astrotia stokesii*	Gray (1846)	Fully-aquatic	Generalist	1	1
		viperinus	*Thalassophina viperinia*	Schmidt (1852)	Fully-aquatic	Generalist		3
	Hydrelaps	*darwiniensis*		Boulenger (1896)	Semi-aquatic	Tidal mudflat/mangroves		3
	Laticauda	*colubrina*		Laurenti (1768)	Semi-aquatic	Coral reefs/rocky intertidal	1	2
	Notechis	*scutatus*		Peters (1861)	Terrestrial	Varied, coastal habitats		3
	Pseudonaja	*textilis*		Duméril *et al*. (1851)	Terrestrial	Varied, arid habitats	1	1
	Vermicella	*annulata*		Gray (1946)	Terrestrial	Varied, burrowing		1
Elapiniiae	*Naja*	*kaouthia*		Lesson (1768)	Terrestrial	Varied, burrowing		4
						Total	7	44

*Summarised from Wilson and Swan (2013) and Cogger (2000). †Scanning electron microscopy analysis. ‡Silicone cast analysis

Quantitative analysis

Silicone casting

Quantitative sensilla morphology was examined on the postocular scale/s (Figure 2.1) of three terrestrial species (n = 5 individuals), 13 fully-aquatic species (n = 30) and two semi-aquatic species (n = 5) from the subfamily Hydrophiinae, and one terrestrial species (n = 4) from the subfamily Elapiinae (Table 1). Following similar methods used for fossilised leaf cuticles (Moisan, 2012; Rigby and Clark, 1965), each snake head was cast in a silicone mould using a two-component, low-viscosity, vinylpolysiloxane and black polymer (Pinkysil©, Barnes, Australia), which was applied in a series of layers at 30-min intervals. Layering produced casts with an adequate final thickness (ca. 3 mm) and reduced the incidence of bubbles. Fully cured casts (ca. 3 – 4 h) were peeled off and glued onto cardboard.

Table 2.2. Morphological parameters quantified from the postocular scale/s using silicone cast analysis.

Parameter	*Description*	*Units*	*Symbol*
Number of sensilla	Total number of sensilla sampled		$N_{(s)}$
Total sensilla area	Total area of sensilla sampled	mm^2	$A_{(s)}$
Total grid cell area	Total area of grid cells sampled	mm^2	$A_{(c)}$
Numerical-density of sensilla	Number of sensilla per unit area of postocular scale/s	mm^{-2}	$N_{A(s,\,c)}$
Mean sensillum size	Mean area of individual sensilla on the postocular scale/s	μm^2	$\bar{A}_{(s)}$
Overall coverage of sensilla	Total area of sensilla relative to total area of the postocular scale/s	%	$A_{A(s,\,c)}$

Imaging and quadrate sampling

Silicone casts of the postocular scale/s from each specimen were illuminated with a fluorescent flash and two fibre-optic lights (Studio Dynolite 2000, Dynalite Inc, USA) coupled to a diffuser to reduce specular reflexions from the cast. A high depth of field photographic image was composed for each cast (Supplementary material 1, Table 2.2) and a 1 mm scale bar was added using imaging software (Adobe Photoshop CS5 Extended, Adobe Systems, USA). Sensilla were quantified from the images using a quadrate sampling method and a script developed with analytical software (MatLabR2015a v8.5, Mathworks, USA). The script automatically superimposed ~100 grid cells over the postocular scale/s. Sensilla within a systematically random selection of 10 grid cells were then manually identified. Any grid line that crossed a sensillum on the top or right edge of the cell was excluded. The following measurements were then obtained from the images and analysed: total number of sensilla located within the grid cells ($N_{(s)}$), total area covered by the sensilla located within the grid cells ($A_{(s)}$, mm^2) and total area of sampled grid cells ($A_{(c)}$, mm^2). Measurements of $A_{(s)}$ and $A_{(c)}$ were facilitated by the script which automatically detected the scale bar and provided a pixel-to-area conversion. The numerical-density of sensilla

($N_{A(s,c)}$, mm^{-2}), the mean sensillum size ($\bar{A}_{(s)}$, μm^2) and the overall coverage of sensilla as a percentage ($A_{A(s,c)}$, %) on the postocular scale/s were then calculated for each specimen given $N_{(s)}$, $A_{(s)}$ and $A_{(c)}$ (Table 2.2).

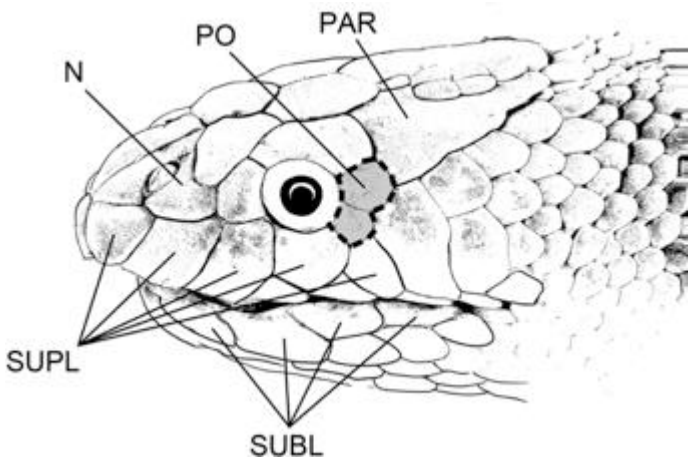

FIGURE 2.1. Scale sensilla terminology used in the presentstudy. Nasal (N), supralabials (SUPL), sublabials (SUBL), postoculars (PO) and parietal (PAR). Sampling region for quantitative silicone cast analysis of scale sensilla indicated by dashed line around the postocular scale/s.

Allometry

To account for the potential effects of head size, $N_{A(s,c)}$, $\bar{A}_{(s)}$ and $A_{A(s,c)}$ were scaled against a proxy estimate of head volume (V_h, mm^3), which was calculated for each specimen as the product of mean head linear measurements (length × width × height). We also tested for the potential effects of $N_{A(s,c)}$ on $\bar{A}_{(s)}$, and $A_{A(s,c)}$ because we predicted that the density of organs within the postocular scale/s would limit the size and coverage of individual sensilla. We used the 'pic' function in the 'ape' library in R to generate phylogenetic independent contrasts of $\log_{10}$-transformed trait data. A linear regression analysis of these data was performed using the 'lm' function in the package 'lme4' (Bates *et al.*, 2015; Paradis *et al.*, 2004). F-tests were used to determine whether the exponent for each trait on head size was significantly different from zero. Because $\bar{A}_{(s)}$ was found to strongly correlate with $N_{A(s,c)}$, $A_{A(s,c)}$ was used for reconstruction of ancestral states.

Phylogenetic analysis

Sequence data, model selection and data partitioning

DNA sequence data were obtained from Genbank for all 19 elapid lineages. The alignment comprised 3818 base pairs from the mitochondrial genes, cytb (cytochrome b), 16S rRNA and 12S rRNA, and the nuclear coding genes, RAG-1 and RAG-2 (recombination reactivating gene 1 and gene 2) and c-mos (oocyte maturation factor). These genes have previously been found to provide sufficient resolution to reconstruct elapid phylogeny and divergence times (39, 48-52).

Because DNA sequences were unavailable for *Vermicella annulata* sampled in the morphological analysis, we substituted this species with DNA data from the closely related congener *V. intermedia* in the molecular analysis. Sequences were checked for ambiguities, and alignments were assembled from consensus sequences of forward and reverse reads in Geneious Pro v5.1.7 (Kearse *et al.*, 2012). The appropriate partitioning schemes and best-fit models were selected using Partition Finder v1.1.1 (Lanfear *et al.*, 2012) under the Bayesian Information Criterion with branch lengths linked and the greedy search algorithm (Table 2.3).

Table 2.3. Partition schemes and models applied to elapid sequence data and log-transformed traits of sensilla.

Partition	*Locus/Trait*	*Model*
1	RAG-1 (2nd fragment-2), RAG-2 1, C-mos 1	TrN
2	ND2 (1,2,3), RAG-1 (1st fragment-1-2-3-4), RAG-1 (2nd fragment-3), RAG-2 2, C-mos-2	HKY + G
3	RAG1 (2nd fragment-1), RAG2 3, C-mos 3	HKY
4	16S, ND4 1, cytb 1	GTR + G
5	ND4 2, cytb 2	HKY + I + G
6	ND4 3, cytb 3	TrN + G
7	Coverage of sensilla; %	Brownian

Elapid phylogeny and reconstruction of ancestral traits of sensilla

Time calibrated phylogenies were reconstructed for the concatenated alignment using Bayesian analysis implemented in BEAST v1.8.1, which uses a Markov Chain Monte Carlo approach to simultaneously estimate topology, divergence times and ancestral character states (Drummond and Rambaut, 2007). The analysis was run with the six-partition scheme and substitution models selected by Partition Finder (Table 2.3). Substitution model parameters were unlinked across partitions, and clock models were linked across partitions. A Yule tree model prior with a uniform distribution was applied. A relaxed clock was used with an uncorrelated and log-normally distributed model of branch rate variation (Drummond *et al.*, 2006). Because fossils are currently unavailable within Elapidae, two secondary node age priors were obtained from previous molecular dating studies to calibrate divergence times (Sanders *et al.*, 2008). Prior age distributions were applied to: 1) the split between *Naja* (Elapiinae) and all remaining taxa (Hydrophiinae), using a normal distribution with a mean of 24 million years ago (Mya) and 95% confidence intervals of 15 Mya-32 Mya; and 2) the split between *Laticauda* and all other remaining hydrophiine taxa, using a normal distribution with mean 15 Mya and 95% confidence intervals of 9 Mya-22 Mya.

The distributions of ancestral states were estimated for the log-transformed $A_{A(s,c)}$. This parameter was treated as a continuous trait under the default Brownian model of character evolution, which allows trait changes to move at a constant and non-directional rate, and is appropriate in the present analysis because traits of sensilla are not yet sufficiently sampled to test alternative (*e.g.* directional) models of trait evolution (Martins and Hansen, 1997). The Markov Chain Monte Carlo was run for 50,000,000 generations with parameters sampled every 5000 generations. Effective sample sizes for all estimated parameters were assessed using Tracer v1.4 (Rambaut and Drummond, 2007) and the first 20% of sampled trees were excluded as burn-in. The remaining 8000 trees were used to find the sampled tree with the highest sum of node support values (maximum credibility tree) using Tree Annotator v1.7.1 (Drummond *et al.*, 2012). Tree graphics were adjusted using FigTree v1.4.2 (Rambaut, 2014).

Results

Qualitative traits of sensilla

High depth of field photographic images of elapid heads showed scale sensilla that resembled round bumps protruding from the epidermis (Figure 2.2). Scale sensilla were typically concentrated towards the anterior and lateral sides of the head and became sparser towards the neck and body. The sensillum ultrastructure imaged under SEM showed that the terrestrial species *Pseudonaja textilis* had numerous flat, elliptical scale sensilla (major axis length ~25-30 μm; minor axis length ~15-20 μm), whereas the aquatic-associating species had rounder, dome-shaped scale sensilla that protruded prominently from the surrounding epidermis (Figure 2.3). The diameter of sensilla varied greatly between the aquatic-associating species, with the smallest in *Laticauda colubrina* (~20 μm), *Hydrophis curtus* (20-30 μm) and *Emydocephalus annulatus* (30 μm), and the largest in *Aipysurus duboisii* (70 μm), *Hydrophis major* (65-75 μm) and *Hydrophis stokesii* (70 μm). In general, the size and shape of sensilla did not vary within an individual.

Quantitative traits of sensilla

Interspecific variation in traits of sensilla

Numerical-density of sensilla ($N_{A(s,c)}$) ranged from 2.8 mm^{-2} in *H. stokesii* to 91 mm^{-2} in *Vermicella annulata* (Figure 2.4). Mean sensillum size ($\bar{A}_{(s)}$) overlapped among aquatic-associating and terrestrial species. Nonetheless, exceptionally large sensilla were found in five fully-aquatic sea snakes: *A. duboisii* (17,000 μm^2), *E. annulatus* (11,700 μm^2), *H. major* (11,000 μm^2), *H. stokesii* (8500 μm^2) and *Aipysurus laevis* (7000 μm^2). In comparison, the smallest sensilla were found in the following terrestrial and semi-aquatic species: *Notechis scutatus* (800 μm^2), *Hydrelaps darwiniensis* (400 μm^2) and *V. annulata* (200 μm^2). Overall coverage of sensilla ($A_{A(s,c)}$) also tended to be higher in fully-aquatic species, particularly in the sea snakes, *A. duboisii* (6.5%), *E. annulatus* (3.8%), *A. laevis*

(3.8%), *Hydrophis schistosus* (4.4%) and *H. major* (3.9%), compared to the lowest found in the terrestrial *Naja kaouthia* (0.8%). The semi-aquatic species had relatively smaller $\bar{A}_{(s)}$ and lower $A_{A(s,c)}$ compared to fully-aquatic species: *Hydrelaps darwiniensis* ($\bar{A}_{(s)}$ = 400 μm^2, $A_{A(s,c)}$ = 1.5%) and *Laticauda colubrina* ($\bar{A}_{(s)}$ = 1000 μm^2, $A_{A(s,c)}$ = 1.2%).

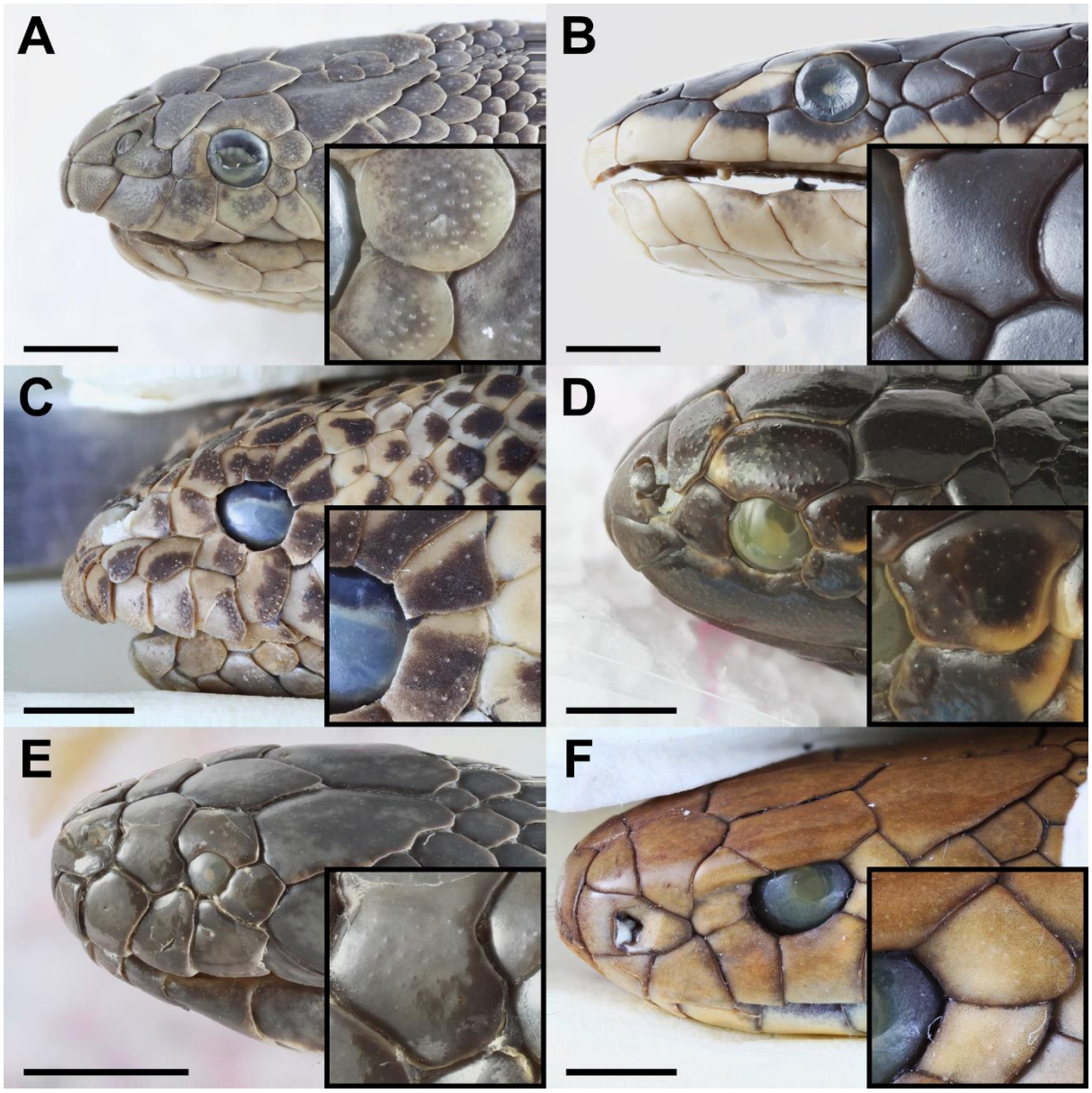

FIGURE 2.2. High depth of field photographs of the heads of six elapid species (A) *Hydrophis schistosus*, (B) *Hydrophis platurus,* (C) *Aipysurus duboisii*, D) *Emydocephalus annulatus,* (E) *Hydrelaps darwiniensis* and (F) *Pseudonaja textilis.* Species are representative of fully-aquatic (A, B, C, D), semi-aquatic (E) and terrestrial (F) ecologies. Insets show sensilla within the postocular scale/s. Scale bar = 3 mm.

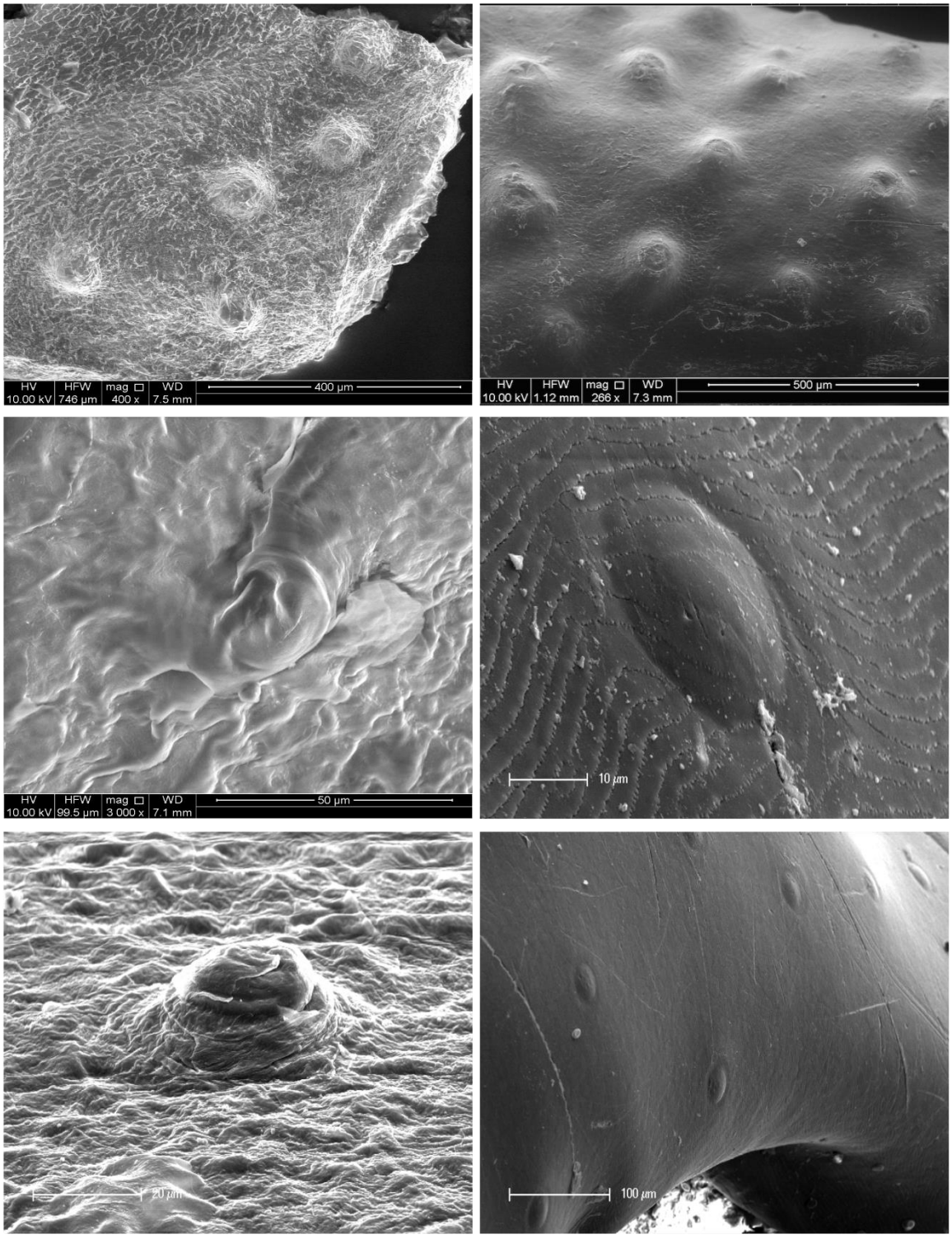

FIGURE 2.3. Sensilla viewed under scanning electron microscope on the nasal scale of five species (A) *Aipysurus duboisii*, (B) *Hydrophis major*, (C) *Laticauda colubrina*, (D/F) *Pseudonaja textilis* and (E) *Hydrophis curtus*. Species are representative of fully-aquatic (A, B, E), semi-aquatic (C) and terrestrial (D/F) ecologies. Scale bars are indicated for each image (note the variable magnifications).

Regressions of independent contrasts yielded non-significant relationships between traits of sensilla ($N_{A(s,c)}$, $\bar{A}_{(s)}$, and $A_{A(s,c)}$) and head volume (V_h, mm^3) (Table 2.4). Nonetheless, a significant relationship was found between $\bar{A}_{(s)}$ and $N_{A(s,c)}$ ($F_{1, 16} = 13.4$, $p = 0.02$) with $\bar{A}_{(s)}$ decreasing as $N_{A(s,c)}$ increases (Figure 2.5, Table 2.4). However, $A_{A(s,c)}$ was found to be independent of $N_{A(s,c)}$ ($F_{1, 16} = 0.0002$, $p = 0.99$). Because the terrestrial *Vermicella annulata* is an outlier for head volume, we repeated the regression analyses with this species excluded; this did not change the outcome of our results (not shown).

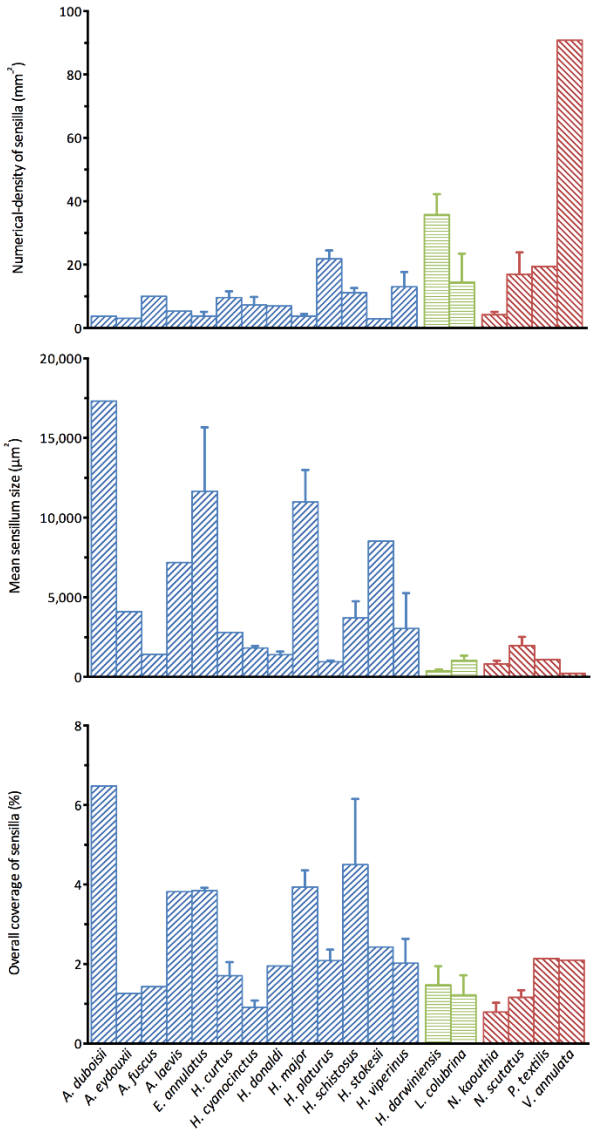

FIGURE 2.4. Numerical-density of sensilla, mean sensillum size and overall coverage of sensilla quantified from the postocular scale/s of 13 fully-aquatic species (blue), two semi-aquatic species (green) and four terrestrial species (red). Data are means ± s.e.m. calculated from 1 – 6 individuals per species (n = 44 individuals in total).

TABLE 2.4. Allometric relationship between head volume (V_h) and numerical-density of sensilla ($N_{A(s,c)}$), mean sensillum size ($\bar{A}_{(s)}$) and overall coverage of sensilla ($A_{A(s,c)}$) across 19 elapid species. Also shown is the relationship between $N_{A(s,c)}$ and $\bar{A}_{(s)}$, and between $N_{A(s,c)}$ and $A_{A(s,c)}$. Phylogenetic independent contrast linear regressions generated from mean data calculated from 1 – 6 individuals per species (n = 44 individuals in total). Equations are in the form $y = a\ X^b$, where y is the trait of sensilla, a is the coefficient (elevation), b is the exponent (slope) and X is either V_h (mm^3) or $N_{A(s,c)}$ (mm^{-2}).

Traits of sensilla, y	x	Coefficient, a	Exponent, b	95% CI	r^2	df	F
$N_{A(s,c)}$ (mm^{-2})	V_h	365	-0.13	0.67	0.04	1,16	0.63
$A_{A(s,c)}$ (%)	V_h	1.60	0.11	0.70	0.02	1,16	0.47
$\bar{A}_{(s)}$ (μm^2)	V_h	45	0.25	1.02	0.06	1,16	1.09
$\bar{A}_{(s)}$	$N_{A(s,c)}$	29,800	-1.04	1.21	0.45	1,16	13.4*
$A_{A(s,c)}$	$N_{A(s,c)}$	2.80	-0.01	1.11	0.16	1,16	0.01

* $P < 0.01$

Elapid phylogeny and reconstruction of ancestral coverage of sensilla

The BEAST maximum credibility tree (Figure 2.6) is consistent with previous studies in topology, posterior support values and divergence times (Lukoschek and Keogh, 2006; Kate L Sanders *et al.*, 2013; Sanders *et al.*, 2008). The sea snakes are nested within the terrestrial snakes, with *N. scutatus* being their closest terrestrial relative. *Naja kaouthia* (Elapiinae) is sister to all other sampled taxa (Hydrophiinae) and the sea krait *L. colubrina* is the earliest diverging lineage within Hydrophiinae. The most recent common ancestor of the sea snakes is dated at approximately 9 Mya. The two major clades of sea snakes (*Aipysurus* and *Hydrophis*) are recovered as monophyletic sister clades with a most recent common ancestor dated at approximately 7 Mya. As in previous studies, the semi-aquatic *Hydrelaps darwiniensis* is sister to *Hydrophis* and interspecific relationships among the rapidly radiating *Hydrophis* remain largely unresolved (Lukoschek and Keogh, 2006; Kate L Sanders *et al.*, 2013).

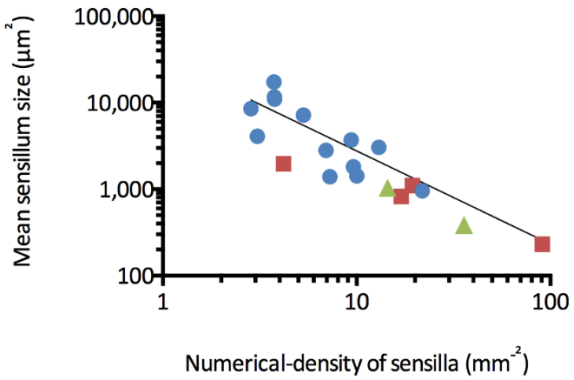

FIGURE 2.5. Relationship between mean sensillum size and the numerical-density of sensilla quantified from the postocular scale/s of 13 fully-aquatic species (blue), two semi-aquatic species (green) and four terrestrial species (red). Data are means calculated from 1 – 6 individuals per species (n = 44 individuals in total).

The BEAST ancestral state reconstruction for $A_{A(s,c)}$ is shown using branch width and dark to light colour hues (Figure 2.6). Unusually high $A_{A(s,c)}$ was found only in sea snakes and appears to have evolved multiple times in the fully aquatic *Aipysurus* (*A. duboisii*, 6.5%; *E. annulatus*, 3.8%) and *Hydrophis* (*H. schistosus*, 4.5%; *H. major*, 3.7%) groups. Estimates of ancestral $A_{A(s,c)}$ were consistently higher within these fully-aquatic clades (1.9-2.8%) compared to within the semi-aquatic and terrestrial lineages (1.5-1.9%). However, $A_{A(s,c)}$ was only slightly higher in the common ancestor of sea snakes (2%) than in sampled terrestrial taxa.

Discussion

Vision, chemoreception and hearing are important senses for terrestrial snakes, but these stimuli have different characteristics underwater, thus altering the selective pressures on sensory systems in sea snakes and sea kraits that have adapted to aquatic living (Nummela and Thewissen, 2008). It is reasonable to expect that other sensory organs might compensate for the reduced sensory cues in a transition from land to sea. In particular, we hypothesise that the purported scale sensilla located on the head of sea snakes and sea kraits might function as enhanced tactile mechanoreceptors sensitive to direct contact with solid surfaces, as well as hydrodynamic receptors sensitive to the displacement of water generated by its motion. In this study, we quantify the overall coverage of sensilla as a proxy for relative 'sensitivity' in 19 species of elapids encompassing terrestrial, fully- and semi-aquatic ecologies, which we have analysed within a phylogenetic framework.

Our results show substantial variation in the overall coverage of sensilla among elapid species, ranging from 0.8% in the terrestrial cobra *Naja koauthia* to 6.5% in *Aipysurus duboisii*. Variation in coverage of sensilla is broadly overlapping in the sampled terrestrial, semi-aquatic and fully-aquatic lineages. However, higher overall coverage of sensilla over the postocular scale/s is found in only five (of 13 sampled) fully-aquatic sea snakes. In contrast, all of the four terrestrial and two semi-aquatic taxa sampled have consistently lower overall coverage of sensilla. Images under SEM reveal that the sensillum ultrastructure is markedly more protruding (dome-shaped) in the six aquatic-associating hydrophiines that we sampled, in contrast to the flatter sensilla of the single terrestrial species sampled here and the terrestrial species reported in previous SEM studies (Jackson, 1977; Jackson and Doetsch, 1977a; Jackson and Sharawy, 1980; Povel and VanDerKooij, 1997). These results are discussed below in relation to methodological considerations and the hypothesis that scale sensilla have both a tactile mechanoreceptor function as well as a derived hydrodynamic function in sea snakes and sea kraits.

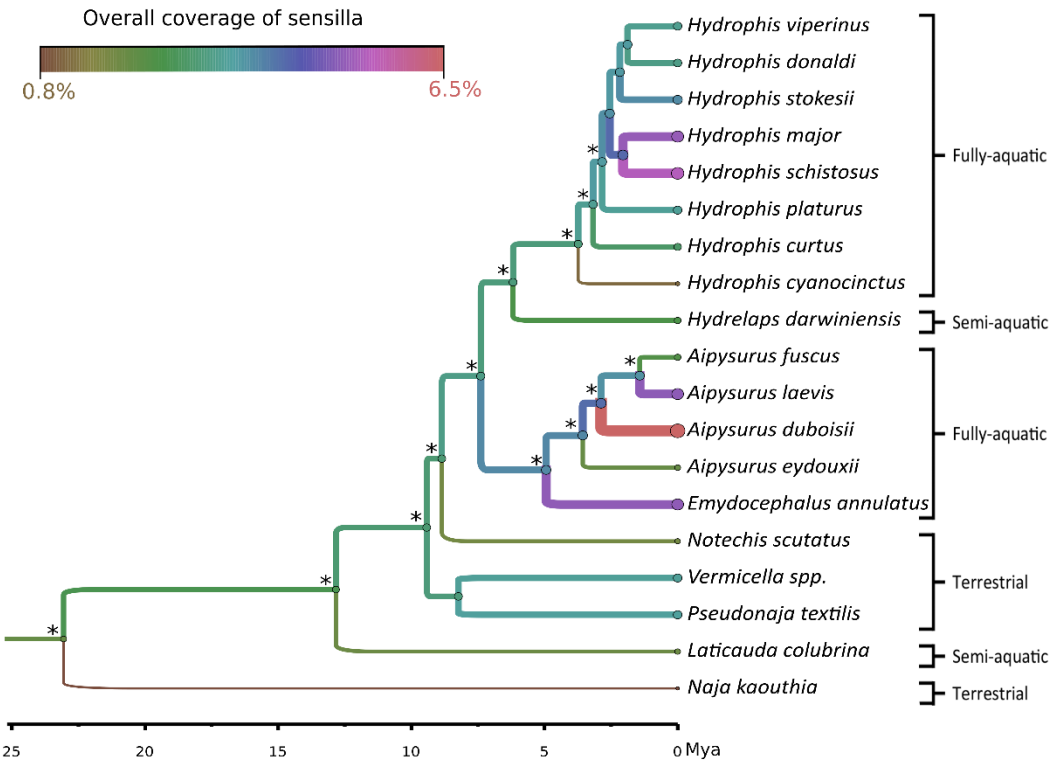

FIGURE 2.6. BEAST maximum clade credibility tree representing phylogenetic relationships and morphological data for 19 elapid species. The horizontal axis indicates timescale in millions of years ago. Node posterior probabilities >0.9 are indicated by asterisks (*). The overall coverage of sensilla (%) is depicted using colour gradient and line weight on the branches. Because DNA sequences were unavailable for *Vermicella annulata*, DNA data from the closely related congener *V. intermedia* were used in substitute.

Allometric effect of head size on traits of sensilla

Allometric scaling showed that the relationship between the traits of sensilla, $N_{A(s,c)}$, $\bar{A}_{(s)}$ and $A_{A(s,c)}$, and head volume were all non-significant after accounting for phylogenetic effects (Table 2.4). Nonetheless, there appears to be a trend for a trade-off between mean sensillum size (μm^2) and numerical-density of sensilla (mm^{-2}) among the species examined (Figure 2.5). However, overall coverage of sensilla (%) is invariant of numerical-density (Table 2.4). Scale organ counts have been estimated in other squamates (*e.g.* Agamidae, Gekkonidae, Iguanidae, Colubridae, Elapidae, Leptotyphlopidae, Uropeltidae), but these studies do not account for allometric effects, precluding meaningful comparison with our results (Jackson, 1977; Matveyeva and Ananjeva, 1995; Povel and VanDerKooij, 1997; Underwood, 1967).

Phylogeny and ancestral reconstruction of the overall coverage of sensilla

BEAST ancestral state reconstruction yielded estimates of overall coverage of sensilla that were only slightly higher for the common ancestor of the fully-aquatic sea snakes (2%) than for preceding nodes in the terrestrial elapids (1.5 – 1.9%) (Figure 2.6). *Hydrelaps* and *Laticauda*, which have convergent semi-aquatic habits, also have relatively lower overall coverage, close to values for the terrestrial taxa. Thus, quantitative traits of sensilla do not appear to have undergone dramatic shifts coinciding with transitions to marine habits. However, our analysis reveals independent origins of exceptionally higher overall coverage of sensilla in the fully-aquatic *Aipysurus* and *Hydrophis* groups, indicating a divergent, possibly hydrodynamic, sensory role in at least some aquatic lineages.

Multiple increases in overall coverage of sensilla in different species of sea snakes may reflect a shifting of mechanoreceptor sensitivity in response to differing ecologies. The increase in overall coverage of sensilla found in *Hydrophis major* (3.9%) and *Hydrophis schistosus* (4.4%) might reflect increased selection pressure to develop a hydrodynamic sense because both species specialise on active prey and often hunt in waters with low visibility (Heatwole and Cogger, 1993; Voris and Voris, 1983). However, higher overall coverage of sensilla in *Emydocephalus annulatus* (3.8%) and *A. duboisii* (6.5%) is less easily explained by their ecology. *Emydocephalus annulatus* usually inhabits clear waters on coral reefs where it specialises on sessile fish eggs (Voris, 1966). *Aipysurus duboisii* is thought to share similar habitat preferences and foraging habits with closely related *Aipysurus laevis* (Heatwole and Cogger, 1993), a species that our results indicate has considerably lower overall coverage of sensilla (3.8%) than *A. duboisii*. It is possible that an ecological or behavioural factor that has yet to be discovered in *A. duboisii*, such as nocturnal hunting or mate searching, could explain its unusually higher overall coverage of sensilla compared to all other sampled species.

It is also unclear how sensilla might function in semi-aquatic elapid snakes. The two semi-aquatic lineages sampled here have very different ecologies: *Laticauda* hunts crevice-sheltering prey in clear coral reefs, whereas *Hydrelaps* occupies inshore waters with low visibility but hunts in burrows at low tide (Wilson and Swan, 2013). Abrasion during terrestrial locomotion might impose a cost on larger sensilla or higher overall coverage of sensilla. Alternatively, terrestrial life may require particular sensory adaptations to maintain function on land, and evolution of sensilla may be less constrained in fully-aquatic snakes. Detailed comparative analysis of the many convergent and divergent ecological specialists within sea snakes and sea kraits (Sanders et al. 2013; Voris & Voris 1983) is needed to shed light on the sensory role of scale sensilla in marine environments.

Comparison of the sensillum ultrastructure

Our qualitative results suggest morphological convergence between scale sensilla on aquatic-associating hydrophiines and the facial organs found in crocodilians and other aquatic snakes. Scanning electron microscopy revealed protruding dome-shaped structures in all of the five sea snakes sampled and single sea krait, whereas comparably flat (two-dimensional) sensilla were observed in the closely related terrestrial species examined here (Figure 2.3) and the eight terrestrial species from the families Colubridae, Xenopeltidae, Cylindrophiidae and Letotyphlopidae that were examined in previous SEM studies (Jackson, 1977; Jackson and Doetsch, 1977a; Jackson and Sharawy, 1980; Povel and VanDerKooij, 1997). The dome-shaped ultrastructure is possibly better suited to receiving stimuli from multiple directions, as would be the case for fluid displacement in aquatic habitats (Dehnhardt and Mauck, 2008b). Indeed, the sensillum ultrastructures for the six aquatic-associating species are remarkably similar to the dome-shaped papillae of crocodilians, which are sensitive to disturbances on the surface of the water (von Düring and Miller, 1979; Jackson *et al.*, 1996; Soares, 2002). Three-dimensional hydrodynamic organs are also found in two non-elapid aquatic snake lineages: the tentacled snake, *Erpeton tentaculatum* (Homalopsidae), and the three species of file snakes in the genus *Acrochordus*. *Erpeton* has large and densely innervated tentacle-like organs on its head that are used for detecting the characteristic escape response of its fish prey (Catania, 2010; Catania *et al.*, 2010). In *Acrochordus*, each head and body scale bears dense tufts of fine hair-like protrusions (Povel and VanDerKooij, 1997). Although the dome-shaped scale sensilla of sea snakes and sea kraits are subtler than the mechanoreceptors of non-elapid aquatic snakes, they might provide greater sensitivity in aquatic habitats compared to the two-dimensional sensilla found in closely related terrestrial species.

Methodological considerations and caveats

There are various methodological hurdles when attempting to compare sensilla across divergent and ecologically diverse taxa. We used a silicone casting technique to make sensilla easily identifiable and minimise taxonomic differences in scale pattern and pigmentation. We also devised a software script to enable quadrate sampling within the postocular scale/s. This approach allowed us to compare traits of sensilla among multiple elapid species, and also generate the first estimate for surface area of sensilla both as the mean sensillum size and overall coverage. Future comparative analyses should aim to expand sampling within species, and include additional taxa (especially terrestrial) to better support statistical testing of the relationships between overall coverage of sensilla and ecological transitions.

Another important caveat is the lack of physiological and behavioural studies supporting a sensory role of scale sensilla, both as a tactile mechanosensory or derived hydrodynamic, in sea

snakes and sea kraits. Hence, we cannot exclude the possibility of other functional roles. For example, scale sensilla may be electromagnetic receptors used to guide migration or position in the water column. Alternatively, sensilla may not be sensory organs at all; higher overall coverage of sensilla might aid in skin shedding, swimming performance, gripping prey and/or mates, or avoiding algae fouling (Dean and Bhushan, 2010; Fish *et al.*, 2011). Furthermore, implicit in our interpretations is the assumption that sensilla surface area is a good indicator of sensilla 'sensitivity', but this has yet to be empirically tested. Further physiological and behavioural experiments are necessary before we can conclusively link morphological changes in overall coverage of sensilla with a sensory function in sea snakes and sea kraits.

Conclusions

Our study devised a novel approach to quantifying the traits of scale sensilla, which enabled meaningful comparison across a broad sample of elapid snakes. In particular, our estimates of overall coverage of sensilla provided a proxy for putative mechanoreceptor sensitivity and allowed the first analysis of sensilla evolution in the transition from terrestrial to marine habits in snakes. Our results indicate multiple increases in overall coverage of sensilla within the fully-aquatic sea snakes, in addition to a more dome-shaped sensillum ultrastructure in fully- and semi-aquatic lineages compared to terrestrial lineages. These findings are consistent with a derived, possibly hydrodynamic, sensory role for scale sensilla in sea snakes and sea kraits, but rigorous testing of this hypothesis will ultimately require behavioural and physiological studies. The novel methodological approach presented here is easily transferable to other reptilian lineages that have undergone adaptive shifts.

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Supplementary materials

Tables

Table S2.1 List of museum specimens used in this study.

Table S2.2 Methods for photographing specimen silicone casts and specimens used in Figure 2.2.

Table S2.3. GenBank accession numbers for four mitochondrial and three nuclear genes sampled for 19 sampled elapid taxa in this study.

Electronic files

File ES2.1 Morphological species data calculated in MatLab using images of specimen silicone casts used in this study (excel)

File ES2.2 Matlab code for quantifying sensilla traits from specimen silicone casts (pdf).

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Chapter 3

Ultrastructural evidence of a mechanosensory function of scale 'sensilla' in sea snakes (Hydrophiinae)

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Jenna M. Crowe-Riddell, Ruth Williams, Lucille Chapuis, Kate L. Sanders

Abstract

The evolution of epidermal scales was a major innovation in lepidosaurs, providing a barrier to dehydration and physical stress, while functioning as a sensitive interface for detecting mechanical stimuli in the environment. In snakes, mechanoreception involves tiny scale organs ('sensilla') that are concentrated on the surface of the head. The fully marine sea snakes (Hydrophiinae) are closely related to terrestrial hydrophiine snakes but have substantially more protruding (dome-shaped) sensilla that often cover a larger portion of the scale surface. Various divergent selection pressures in the marine environment could account for this morphological variation, including enhanced detection of mechanical stimuli (used in either direct contact with stimuli or indirect contact via water motion, *i.e.* 'hydrodynamic reception'), or co-option for alternate sensory or non-sensory functions. We addressed these hypotheses using immunohistochemistry and light- and electron microscopy to describe the cells and nerve connections underlying scale sensilla in two sea snakes, *Aipysurus laevis* and *Hydrophis stokesii*. Our results show ultrastructural features in the cephalic sensilla of both marine species that closely resemble the mechanosensitive Meissner-like corpuscles that underlie terrestrial snake sensilla. We conclude that the sensilla of marine hydrophiines have retained a mechanosensory function, but future studies are needed to examine whether they are sensitive to hydrodynamic stimuli.

KEYWORDS: sea snake, sensilla, cutaneous, mechanoreceptor, skin, ultrastructure, transmission electron microscopy

Introduction

Hardened epidermal scales are a characteristic trait of snakes (and other lepidosaurs: lizards and tuatara) that facilitate defensive signalling, camouflage, water retention, and locomotion (Cheng *et al.*, 2010; Lillywhite and Maderson, 1988; Maderson *et al.*, 1998). The epidermal scales also provide the primary surface for mechanoreception, which is the ability to sense mechanical stimuli that result from pressure or physical displacement (vibration) (Dehnhardt and Mauck, 2008). Scale 'sensilla' ('tubercles' *sensu* Underwood, 1967) are small mechanoreceptors that protrude from the surface of epidermal scales of the head and body of snakes. Snakes are likely to use these mechanosensory organs to explore and navigate substrate (Keathley, 2004; Young and Morain, 2003), discriminate prey types (Aota, 1940; Nishida *et al.*, 2000) and engage in courtship behaviours (Noble, 1937), but the anatomy and neurophysiology of scale sensilla are conspicuously understudied in comparison to other sensory organs, *e.g.* eyes (Simoes *et al.*, 2016), auditory structures (Young, 2003), vomeronasal organ (Shine *et al.*, 2004) and heat-sensing pits (Gracheva *et al.*, 2010).

In terrestrial snakes, scale sensilla are concentrated on the head and are highly sensitive to mechanical stimulation, particularly moving stimuli (Jackson and Doetsch, 1977a; Proske, 1969c, 1969a, 1969b). The underlying ultrastructure of cephalic scale sensilla consists of an innervated cluster of dermal cells (dermal capsule) that displaces the surrounding epidermis to create round skin elevations (von Düring and Miller, 1979; Jackson, 1977; Jackson and Sharawy, 1980). These underlying features of scale sensilla have been likened to 'Meissner corpuscles', which are low-threshold mechanoreceptors (LTMRs) sensitive to innocuous ('light touch') stimuli in the glabrous (hairless) skin of mammals (Roudaut *et al.*, 2012; Zimmerman *et al.*, 2014). Scale sensilla on the body of snakes are less specialised in their underlying ultrastructure: they lack dermal capsules and the outer skin elevations are instead caused by a superficial thickening of the epidermis (Avolio *et al.*, 2006a; Noble, 1937). These ultrastructural differences between head and body scale sensilla, and the concentration of sensilla on the head, are thought to reflect the role of the head as the primary tactile interface in snakes (Jackson and Doetsch, 1977a; Underwood, 1967).

Snakes exhibit substantial variation in the size, shape, density and distributions of their scale sensilla. Enlarged and/or high densities of sensilla have been reported in fossorial snakes and some sea snakes (Hydrophiinae, Chapter 2), whereas in other colubroid snakes sensilla are small and/or sparse (*e.g.* Dipsadinae) or even absent in some species (*e.g.* Viperidae) (Jackson, 1977; Underwood, 1967; Wallach and Ineich, 1996; Young and Wallach, 1998). Interspecific differences in sensilla traits likely relate to aspects of species' environment, ecology, and phylogeny.

However, our understanding of the adaptive diversity of snake sensilla is hindered by a lack of comparative data describing differences in external sensilla traits and underlying ultrastructure.

Hydrophiine snakes (Elapidae) provide a useful comparative framework to investigate the evolution of scale sensilla in response to major ecological transitions (Crowe-Riddell *et al.*, 2016). The fully marine, viviparous sea snakes comprise a clade of more than 60 species that evolved within the terrestrial Australian hydrophiine radiation (tiger snakes, death adders, taipans) approximately 9 to 18 million years ago (Lukoschek and Keogh, 2006; Sanders *et al.*, 2008; Sanders *et al.*, 2013). Previous work has found that the cephalic sensilla of sea snakes are substantially more protruding (dome-shaped) compared to their terrestrial counterparts, and in some lineages cover a much larger proportion of the scale surface (> 6% *versus* < 2.5% in sampled taxa, Chapter 2; Crowe-Riddell *et al.*, 2016). This divergence in external sensilla morphology might reflect divergent selection pressures in the marine environment. However, the hitherto lack of data on the ultrastructure of scale sensilla in sea snakes precludes meaningful comparisons with terrestrial snakes.

In their external appearance, the dome-shaped sensilla of sea snakes closely resemble the integumentary scale organs (ISOs) of crocodilians, which are cephalic mechanoreceptors with elaborate Merkel-cell neurite complexes and sensitivity to water motion (Jackson *et al.*, 1996; Leitch and Catania, 2012; Soares, 2002). A dome-shaped scale organ provides increased surface area for stimuli to be received from multiple directions, possibly enhancing hydrodynamic sensitivity in an aquatic habitat (Dehnhardt and Mauck, 2008). Indeed, two independently aquatic snakes, *Erpeton* and *Acrochordus*, are distantly related to hydrophiine sea snakes but have protruding organs that are sensitive to water motion generated by the movement of fish prey (*i.e.* hydrodynamic stimuli) (Catania *et al.*, 2010; Povel and VanDerKooij, 1997). It is also plausible that sensilla have been co-opted in sea snakes for a different sensory modality, such as dermal photoreception (found in *Aipysurus* sea snakes (Zimmerman and Heatwole, 1990; see Chapter 5), or electromagnetic sensing for navigation (Lillywhite, 2014). Alternatively, scale sensilla may have been co-opted for a non-sensory function such as enhanced friction for gripping during mating, or disruption of the skin boundary layer to increase swimming performance (analogous to the denticles of shark skin or tubercles on the fins of whales, Avolio *et al.*, 2006b; Dean and Bhushan, 2010; Fish *et al.*, 2011).

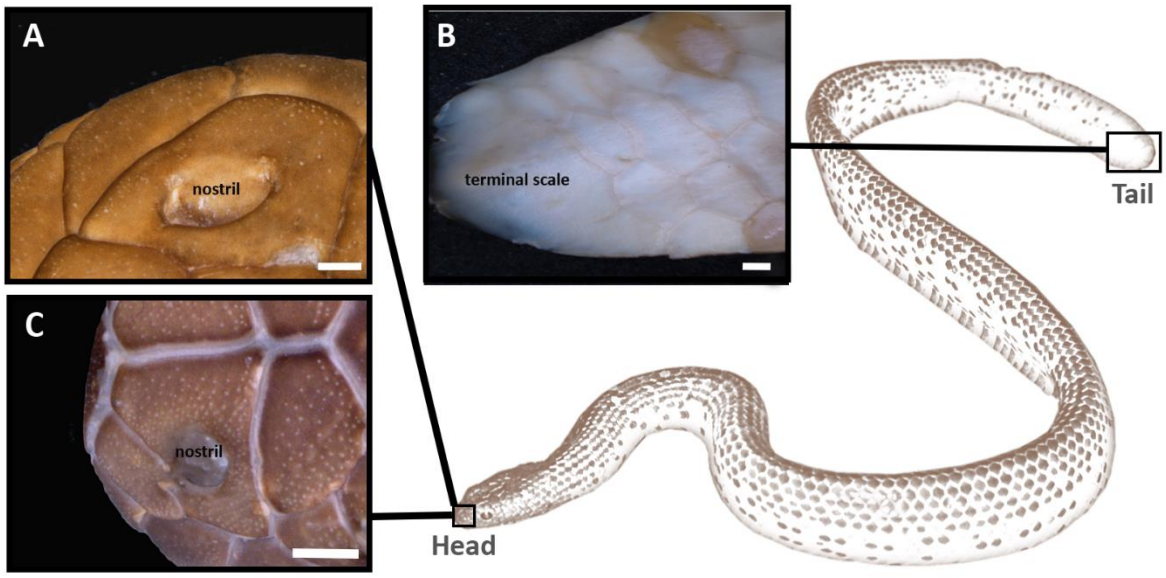

FIGURE 3.1. Gross morphology of the skin in sea snakes illustrating small, unpigmented scale organs ('sensilla'). Line drawing of sea snake indicates sampling region of available skin samples: nasal scales from the head (*Aipysurus laevis* and *Hydrophis stokesii*), and supralabial scales from the head and caudal scales from the tail (*A. laevis* only). A) Gross morphology of scale sensilla on the nasal scale of *A. laevis*. B) Gross morphology of the caudal scales of *A. laevis* illustrating sparse scale sensilla. C) Gross morphology of scale sensilla on the nasal scale of *H. stokesii*. Stereomicroscope images were taken from museum specimens: A, B) WAMR174260 and C) FMNH202826. Scale bars represent 1 millimetre. Line drawing based on image of *A. laevis* from (Mirtschin *et al.*, 2017) and modified with permission.

We aimed to better understand the evolution of scale sensilla in sea snakes by describing their ultrastructure in two fully-aquatic species (*Aipysurus laevis* and *Hydrophis stokesii*) using immunohistochemistry, and light and electron microscopy. If sea snake sensilla are modified for enhanced mechanosensory roles, either tactile or hydrodynamic, we would expect them to have retained the ultrastructure described in terrestrial snakes. Co-option for alternative sensory roles would be implicated if different cell types are present, such as the Merkel-cell neurite complexes of crocodilian ISOs. Finally, if dome-shaped sensilla provide a non-sensory (*e.g.* structural) function, we would expect their elevation from the skin surface to be created by superficial thickening of the epidermis with no associated neuronal or receptive cells.

Materials and methods

Specimens and tissue sampling

Two museum specimens of the sea snake species *Aipysurus laevis* (one individual) and *Hydrophis stokesii* (one individual) were used for gross morphological observations (Table 3.1). Fresh specimens of these species (two individuals of *A. laevis*; one individual of *H. stokesii*) were collected 1–10 km offshore from the coast of Broome in June 2015 and September 2016.

Animals were collected in accordance with a Department of Parks and Wildlife of Western Australia licence to take fauna for scientific purposes (Permit #SF010002). Animals were euthanised by an injection of barbiturate (Pentobarbital), which was carried out in accordance with the guidelines of the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (National Health and Medical Research Council, 2013), under Animal Ethics Committee protocols from the University of Adelaide (S-2015-119) and the University of Western Australia (RA/3/100/1369).

Immediately after euthanasia, cephalic scales were sampled from all three sea snakes, and tail scales were sampled from the posterior dorsal surface and ventral tip of the tail in a single *A. laevis* (because this species exhibits tail phototaxis linked to dermal photoreception, see Chapter 5). Entire scales were dissected to sample the whole skin from epidermis to subcutaneous tissue. The specimen details and locations of sampled scales are shown in Table 3.1 and Figure 3.1. A single specimen of *Oxyuranus scutellatus* (the Australian taipan) was sourced from a captive breeding population (Venom Supplies Pty Ltd, South Australia) to sample brain tissue for antibody controls (see below) because this species is closely related to the sea snakes (Sanders *et al.*, 2008). All samples were fixed by immersion in either 4% paraformaldehyde for immunohistochemistry, or 1.5% glutaraldehyde and 4% paraformaldehyde for electron-microscopy. After immersion in fixative for 24 hours, samples were washed and stored in phosphate buffered saline (PBS; pH 7.4) with sucrose, before being transferred into phosphate buffer with 0.05% sodium azide.

Stereo and light microscopy

The outer skin morphology of museum specimens was examined using a stereomicroscope with a mounted camera (SMZ25, Nikon Inc., Japan). Specimens were submerged in water and illuminated by a ring of light-emitting diodes (P2-FIRL LED Ring Illumination Unit, Nikon Inc., Japan) to reduce specular reflections from the scales. A high-depth-of-field photographic image was composed using imaging software (NIS-Elements Advanced Research v5.10, Nikon Inc., Japan).

The general cellular morphology of the skin samples was examined using light microscopy. Samples were dehydrated by successive immersion in alcohol, then paraffin-embedded for serial sectioning (10 μm). Slides were stained with hemotoxylin-eosin or Gomori's One-Step (Gomori, 1950), scanned using a digital slide scanner (Nanozoomer, Hamamatsu Photonics, Japan) and measurements taken using imaging software (Nanozoomer Digital Pathology v2.6, Hamamatsu Photonics, Japan). We measured the height (thickness) of the epidermis located above scale sensilla, and at adjacent areas of skin that did not contain sensilla. Because the outer layer of hardened skin (beta layer) sometimes became artificially separated from surrounding layers during

tissue processing, we measured only the living (nucleated) epidermal layer (*stratum germinativum*). The diameter and height of dermal capsules (papilla) and other dermal structures were measured and the ratio of diameter:height calculated.

TABLE 3.1. Taxonomy, life stage, museum accession or field numbers and sample size of two species of sea snakes (Hydrophiinae) used in this study. Time until last shed was deduced for captive specimens by the presence of shed skins. Tissue samples were collected from captive specimens for various microscopy analyses: stereomicroscopy (SM), light microscopy (LM), transmission electron microscopy (TEM) and immunohistochemistry (IHC). Museum specimens were sourced from the Western Australian Museum (WAM) and the Field Museum of National History, Chicago (FMNH).

Taxonomy		*Specimen information*			*Tissue samples*	
Genus	*Species*	*Museum / Field number*	*Life stage / sex*	*Time to last shed (days)*	*Scale location*	*Microscopy analyses*
Aipysurus	*laevis*	KLS0690	Adult male	18	6th supralabial (right side)	LM
					Posterior tip of tail (right side)	LM
	laevis	#AL270916	Adult male	> 128	Nasal scale (right side)	TEM
	laevis	WAM R174260	Subadult male	Unknown	Gross morphology of skin	SM
Hydrophis	*stokesii*	#HS270916A	Adult male	107	Nasal scale (right side)	LM & IHC
					Nasal scale (left side)	TEM
	stokesii	FMNH 202826	Juvenile Unknown sex	Unknown	Outer morphology of cephalic skin	SM

Statistics

We used the two-sample t–test (unpaired) to examine differences in epidermal thickness between scale organs and adjacent skin that did not contain scale organs. Before statistical analyses, we checked that data were normally distributed using Bartlett's test. Statistical analyses were performed using base packages in R v3.5.1 (R Core Team, 2017).

Immunohistochemistry

Immunohistochemistry was performed on paraffin-embedded serial sections (10 μm) for a neuronal marker, protein gene product 9.5 (PgP9.5). Briefly, slides were blocked for endogenous peroxidase with 0.5% hydrogen peroxide in methanol at room temperature for 30 minutes (min). Slides were rinsed in PBS and processed in 10 mM sodium citrate (pH 6.0) for heat-induced epitope retrieval. Slides were washed twice in PBS, before blocking in 3% normal horse serum

(NHS) in PBS for 30 min. Sections were incubated with mouse monoclonal anti-PgP9.5 antibody (dilution 1:2000 with 3% NHS) at room temperature overnight. Sections were then washed twice in PBS and incubated with a peroxidase-conjugated secondary antibody (IgG anti-mouse, 1:500 diluted in PBS with 3% NHS) for 30 min, then incubated with streptavin peroxide (dilution 1:1000 with 3% NHS) for 1 hour. Binding sites were revealed using a red chromogen (NovaRed Peroxidase Substrate Kit, Vector, USA) according to manufacturer instructions and incubated for 2 to 3 min. Slides were washed in distilled water for 5 min before counterstaining in Harris hematoxylin for 30 to 60 seconds and allowed to air dry. A primary antibody control was performed using the above protocol on snake (taipan) brain tissue; a secondary antibody control was performed using the above protocol, with the primary antibody incubation step omitted, on snake brain and cephalic skin tissue. Slides were imaged using an optical microscope (BX51, Olympus, Australia) and the saturation and hue of images was adjusted using imaging software (Adobe Photoshop v2017.1.1, Adobe Systems Inc., USA). Note that due to preservation issues we were unable to perform immunohistochemistry on these cephalic skin sections in *A. laevis*.

Electron microscopy

To view ultrastructure, skin samples were prepared for electron microscopy. Samples were post-fixed in 2% osmium tetroxide solution, then dehydrated in ascending series of ethanol and infiltrated in epoxy resin. Resin blocks were then polymerised overnight at 70 degrees Celsius. Semi-thin (1 μm) sections were cut and stained with toluidine blue to locate an individual scale sensillum under light microscopy. Ultra-thin (70 nm) sections were cut and stained with uranyl acetate and lead citrate. Sections were placed on nickel coated mesh grids and viewed at 100 kV under a transmission electron microscope (Tecnai G^2 Spirit TEM, FEI Company, USA).

Results

Several epidermal layers were identified using light microscopy: the nucleated layer (*stratum germinativum*) was non-keratinised and consisted of a basal layer of elongate or columnar cells and one to three layers of round, loosely arrange keratinocytes; the non-nucleated layer (*stratum corneum*) consisted of keratinised α and β cells. According to definitions from Cheng *et al.*, (2010) and Maderson *et al.*, (1998), skin samples that were viewed under light microscopy (Table 3.1) were in the resting phase of epidermal shedding cycle; skin samples viewed under the electron microscope (Table 3.1) appeared to be in pre-renewal phase.

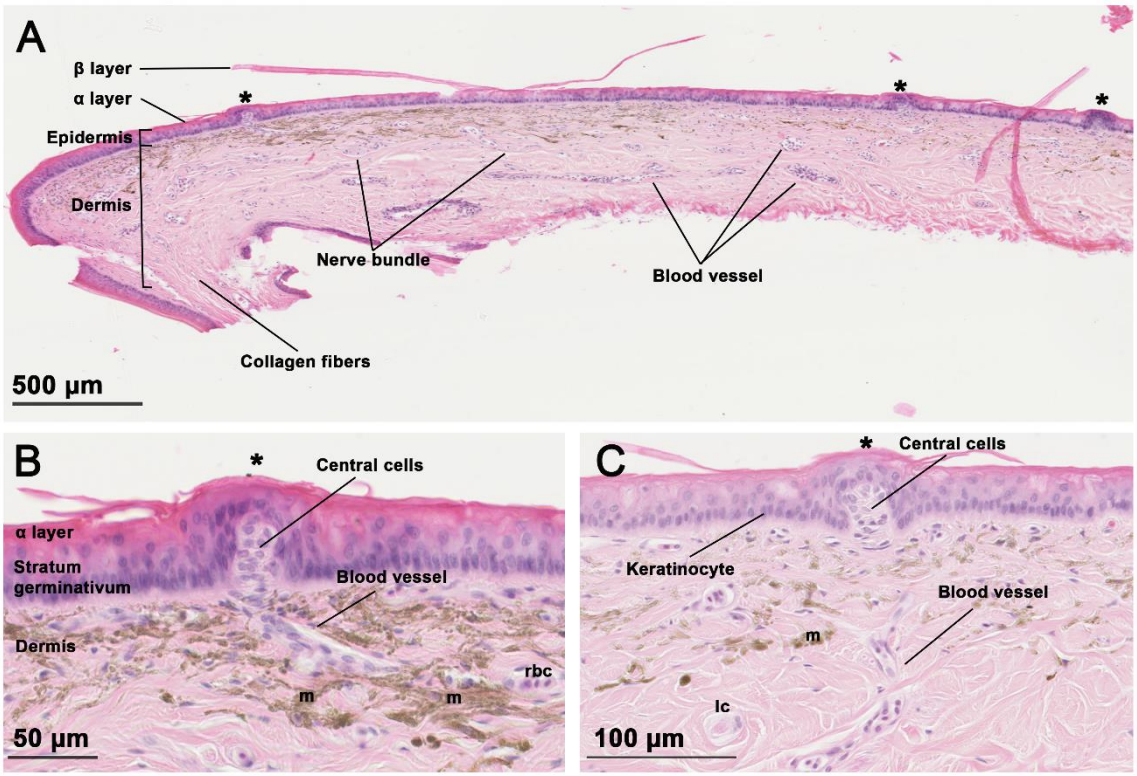

FIGURE 3.2. Light micrographs of cephalic skin (supralabial scale) from *Aipysurus laevis*. A) Scale sensilla (*) are skin elevations (bumps) created by dermal papilla (dermal capsule); other features of the dermis are clearly visible including nerve bundles, blood vessels and collagen; note that the beta layer has artificially separated from alpha layer. B-C) Higher magnification of transverse cross-section of scale sensilla (*) that show central cells within the dermal capsule, which displace the *stratum germinativum* of the epidermis; dermal capsules are vascularised by blood vessels; note the red blood cells (rbc), lamellar corpuscles (lc) and melanophores (m) within the dermis. Slides were stained with hemotoxylin-eosin and magnified at A) ×5.5, B) ×20 and C) ×30.

Cephalic scale organs

Observed under a stereomicroscope, the cephalic scale sensilla appeared as unpigmented external elevations ('bumps') of outer skin (Figure 3.1). Observed under light microscopy, the cephalic scale sensilla of *A. laevis* (Figure 3.2) and *H. stokesii* (Figure 3.3) shared a similar structure that consisted of a cluster of 9 to 11 cells ('central cells'), which were horizontally arranged, originated in the dermis and evaginated the epidermis to create a dermal capsule ('papilla'). The ratio of length to diameter of the dermal capsule was approximately 1:1 for both *A. laevis* and *H. stokesii* (ES File3.2). The dermal capsule was occasionally tapered at its basal end in *H. stokesii* (Figure 3.3A), but remained expanded in *A. laevis* (Figure 3.2B, C). In some dermal capsules we were able to identify a blood vessel leading to (and thus presumably vascularising) the central cells (Figure 3.2B). In *H. stokesii,* the Gomori's One Step stain revealed collagen fibres interspersed between central cells and often separated the dermal capsule from keratinocytes within the epidermis

(Figure 3.3C). In both species, the dermal capsule displaced surrounding epidermal layers so that the columnar cells of the *stratum germinativum* were positioned above the dermal capsule, causing the bumps of the outer skin surface (Figure 3.2; Figure 3.3). In *A. laevis*, the epidermis above the dermal capsule ('cap cells') was approximately 50% thinner than the epidermis of the surrounding regions of skin that did not contain sensilla (17 μm; $t = -11.16$, 110 d.f., $P < 0.001$) and in *H. stokesii,* it was approximately 15% thinner than the adjacent flat epidermis (28 μm; $t = -2.19$, 67 d.f., $P = 0.03$).

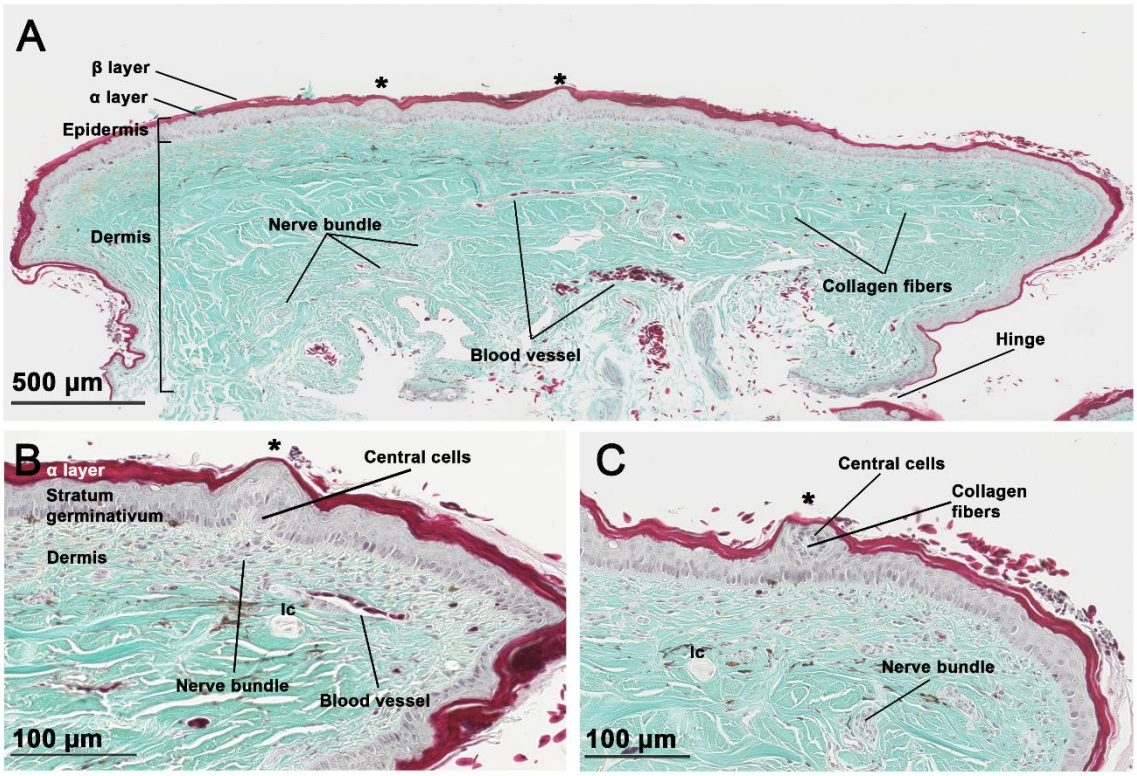

FIGURE 3.3. Light micrographs of cephalic skin (nasal scale) from *Hydrophis stokesii*. A) Scale sensilla (*) are skin elevations (bumps) created by dermal papilla (dermal capsules); other features of the dermis are clearly visible including nerve bundles, blood vessels and collagen fibres, and hinge region of the scale. B) Higher magnification of transverse cross-section of scale sensillum (*), the central cells within the dermal capsule displace the *stratum germinativum* of the epidermis. C) Traverse cross-section of edge of scale sensillum shows a small bundle of collagen fibres surrounded by central cells. Note the lamellar corpuscles (lc) within the dermis. Slides were stained with Gomori's one-step and magnified at A) ×6.2, B) ×22.8 and C) ×23.

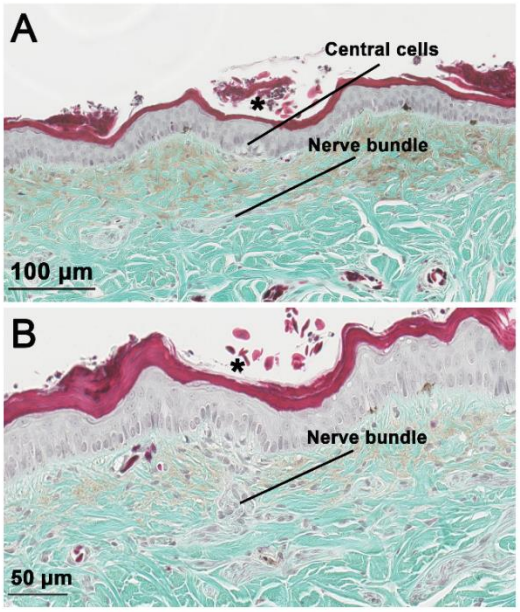

FIGURE 3.4. Light micrographs of a transverse cross-section of cephalic skin (nasal scale) of *Hydrophis stokesii* showing dermal capsules not associated with external skin elevations (bumps). A-B) Central cells of a dermal capsule (*) displace surrounding *stratum germinativum* of the epidermis, but do not result in skin elevations. Nerve bundle are closely associated with base of the dermal capsule. Slide was stained with Gomori's one-step and magnified at A) ×20 and B) ×40.7

There was a second type of dermal capsule on the cephalic scales in *H. stokesii* that contained approximately 10 central cells and displaced the surrounding epidermis but, in contrast to the cephalic scale sensilla, did not result in a distinctive bumps in the outer skin surface (Figure 3.4). These smaller scale sensilla were more variable in shape compared to typical sensilla (ratio length:diameter 1.7; ES File3.2) and often located at the base of depressions on the outer surface of the skin (Figure 3.4). The epidermis above the dermal capsule was 25% thinner than adjacent flat epidermis (25 µm, $t = -2.76$, 26 d.f., $P = 0.01$; approximately same height as cap cells of other cephalic sensilla, $t = 0.85$, 12 d.f., $P = 0.41$).

The cephalic dermis and epidermis of *H. stokesii* were immunoreactive for PGP9.5 (Figure 3.5). Specificity of immunoreactions were confirmed by antibody controls (Figure S3.1) and by the localised staining of nerve bundles that had previously been identified under light microscopy (Figure 3.2A; Figure 3A). Dermal axons travelled to the scale sensilla (Figure 3.5C), then meandered through the central dermal capsule before innervating the outer epidermis and often terminating as distinct discoid endings in the alpha layer (Figure 3.5A, B). These discoid endings were primarily located above the dermal capsule but were also present in flat epidermis that did not contain sensilla (Figure 3.6A). Unfortunately, the second type of dermal capsules in *H. stokesii* (described above; Figure 3.4) were not present in the sections stained for immunohistochemistry.

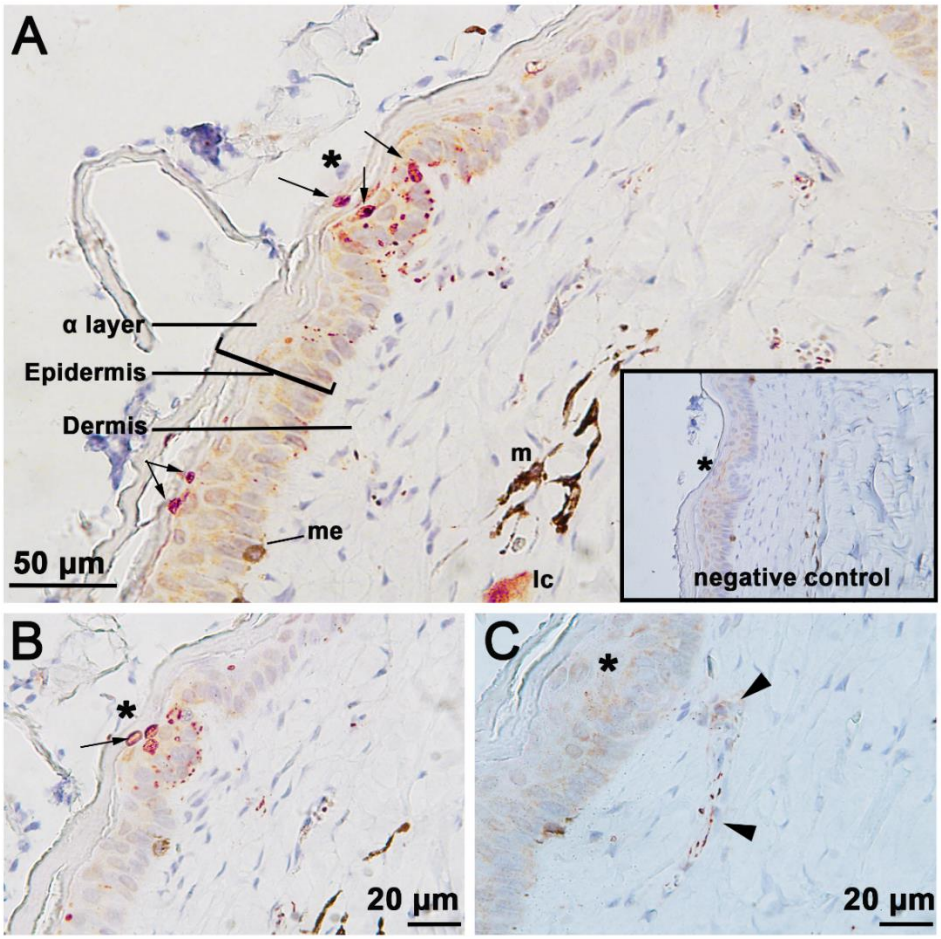

FIGURE 3.5. Immuno-reactivity of a neuron specific protein (PGP9.5) on cephalic skin (nasal scale) of *Hydrophis stokesii*; reactive protein appears dark pink. A) Transverse cross-section of scale sensillum (*) with neuronal-positive stain within the dermal capsule, as well as within the epidermis and alpha layer above the dermal capsule. Several neuronal-positive, discoid endings (arrows) are present within the *stratum germinativum* and alpha layers of the epidermis. Lamellar corpuscles (lc) within the dermis are also immuno-positive and can be distinguished from melanocytes (me) and dispersed melanophores (m), which have a dark brown colouration. B) Deeper cross-sections of a scale sensillum showing neuronal-positive discoid endings (arrows). C) A trail of neuronal-positive stain (arrow heads) leading to a forming scale sensillum (*). Negative control was conducted by omitting primary antibody. Slides were counter stained with Harris hematoxylin and magnified at A) ×30, B) ×50, C) ×50.

Immunoreactions were also localised to ovoid structures within the cephalic dermis of *H. stokesii* (Figure 3.6). These structures corresponded to lamellar cells that were ovoid in shape and resembled small Pacinian-like corpuscles (mean length 29 ± 15 µm and mean diameter of 22 ± 12 µm; ES File3.2)). The location of these 'lamellar corpuscles' in *H. stokesii* ranged from 61 to 124 µm (mean 93 µm) depth from the basal layer of the epidermis. Lamellar corpuscles were also identified in the cephalic dermis of *A. laevis* that were a similar ovoid shape (mean length 37 ± 26 µm and mean diameter 25 ± 5 µm; Figure 3.2C) to those found on the cephalic dermis of *H. stokesii*. The location of the lamellar corpuscles in *A. laevis* ranged from 53 to 168 µm (mean 118 µm; ES File3.2) depth from the basal layer of the epidermis. Although the lamellar corpuscles

were dispersed throughout the dermis (*stratum laxum*), they were often subjacent to scale sensilla (Figure 3.2C; Figure 3.3B,C). Unfortunately, due to preservation issues we were unable to perform immunohistochemistry on these cephalic skin sections in *A. laevis*.

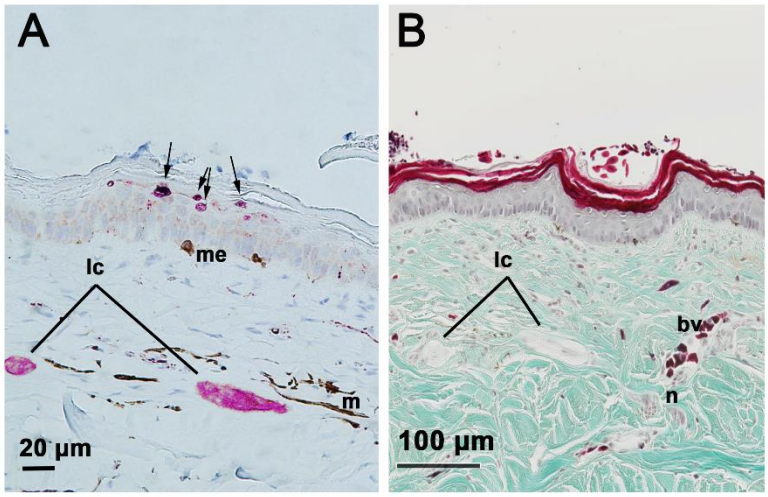

FIGURE 3.6. Immuno-reactivity of a neuron specific protein (PGP9.5) of lamellar corpuscles (lc) in the cephalic dermis (nasal scale) of *Hydrophis stokesii*. The location within the dermis, and co-localisation of immuno-staining with lamellar structures suggests that they are Pacinian-like corpuscles. A) Immuno-reactivity of PGP9.5, reactive protein appears dark pink, showing immuno-positive stain localised to lamellar corpuscles (lc) in the dermis and discoid endings (arrows) in the epidermis. These structures can be distinguished from melanocytes (me) and dispersed melanophores (m), which have a dark brown colouration. B) Deeper cross-sections of the skin showing structure of lamellar corpuscles and an associated blood vessel (bv) and nerve bundle (n). Slides were stained and magnified: A) Harris hematoxylin, ×30, and B) Gomori's One Step, ×50.

The dermal capsule of a scale sensillum was observed in *A. laevis* using electron microscopy (Figure 3.7). High magnification images showed a cluster of central cells within the dermal capsule (Figure 3.7B). These central cells were distinguished from surrounding keratinocytes by their round shape and lack of tonofilaments (Figure 3.7B inset two). Tonofilaments were present in the intracellular space of keratinocytes throughout the epidermis (Figure 3.7B). Tight junctions (desmosomes) and associated tonofibrils can be seen between central cells and keratinocytes (Figure 3.7B inset two). In the intercellular domain, small bundles of transverse collagen fibres and a single, small putative nerve axon were present at base of dermal capsule (closer to dermis; Figure 3.7B inset one). Small phospholipid inclusions were also present (Figure 3.7B inset one). Unfortunately, we were unable to image the putative axon at higher magnification so could not confirm the presence of neuronal elements (*e.g.* lamellar arrangement of Schwann cells, neurofilaments).

Scale organs on the tail of *Aipysurus laevis*

Two scale structures were identified in the tail skin of *A. laevis*. Although, we were unable to discern bumps in the outer tail skin surface using a stereomicroscope (Figure 3.1B), several skin elevations were identified in cross-sections of the skin under light microscopy (Figure 3.8). The epidermal elevations of the tail ('tail scale sensilla') lacked the dermal capsules associated with the cephalic scale sensilla, the outer bumps were instead created by thickening of the epidermis (Figure 3.8A), which was 57% thicker than adjacent flat epidermis (47 μm, $t = 14.18$, 86 d.f., $P < 0.001$) and 17 μm (65%) thicker than cap cells of cephalic sensilla ($t = -14.26$, 18 d.f., $P < 0.001$). Tail scale sensilla also lacked the collagen fibres and blood vessels that were associated with cephalic scale sensilla. A second scale structure identified in the tail skin of *A. laevis* consisted of a small dermal capsule of approximately 10 central cells with a ratio of length and diameter of 1:1 (ES File3.2; Figure 3.8B). Although the dermal capsule displaced the surrounding epidermal layer (including the columnar cells of the *stratum germinativum*) this did not result in elevations of the outer epidermis (Figure 3.6B). The epidermis above the dermal capsule was 42% thinner than adjacent epidermis that did not contain dermal capsules (11 μm; $t = -4.65$, 86 d.f., $P < 0.001$) and slightly thinner (5.5 μm) than the cap cells of cephalic sensilla ($t = 2.59$, 18 d.f., $P = 0.02$). Subjacent to these tail dermal capsules, collagen fibres in the dermis (*stratum laxum*) were dispersed and melanosomes could not be seen (Figure 3.8B). Unfortunately, due to preservation issues we were unable to perform immunohistochemistry on these tail sections.

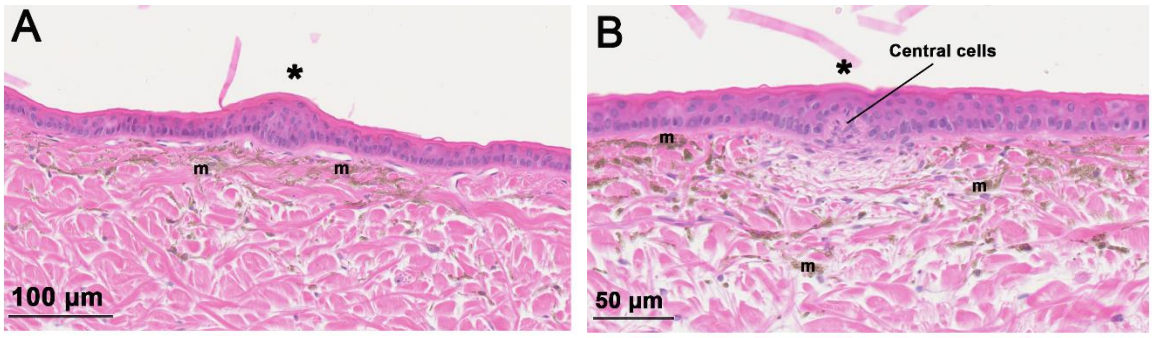

FIGURE 3.8. Light micrographs of transverse cross-sections of tail skin (posterior caudal scales) of *Aipysurus laevis*. A) A scale sensilla (*) in the tail are skin elevations created by a thickening of underlying epidermis. B) Unknown dermal capsule (*) consists of central cells that displaces surrounding *stratum germinativum* of the epidermis, but does not result in skin elevations. Note that the dermis immediately underlying dermal capsule consists of loosely arranged collagen fibres devoid of melanophores (m). Slides were stained with hemotoxylin-eosin and magnified at A) ×16, B) ×17.2

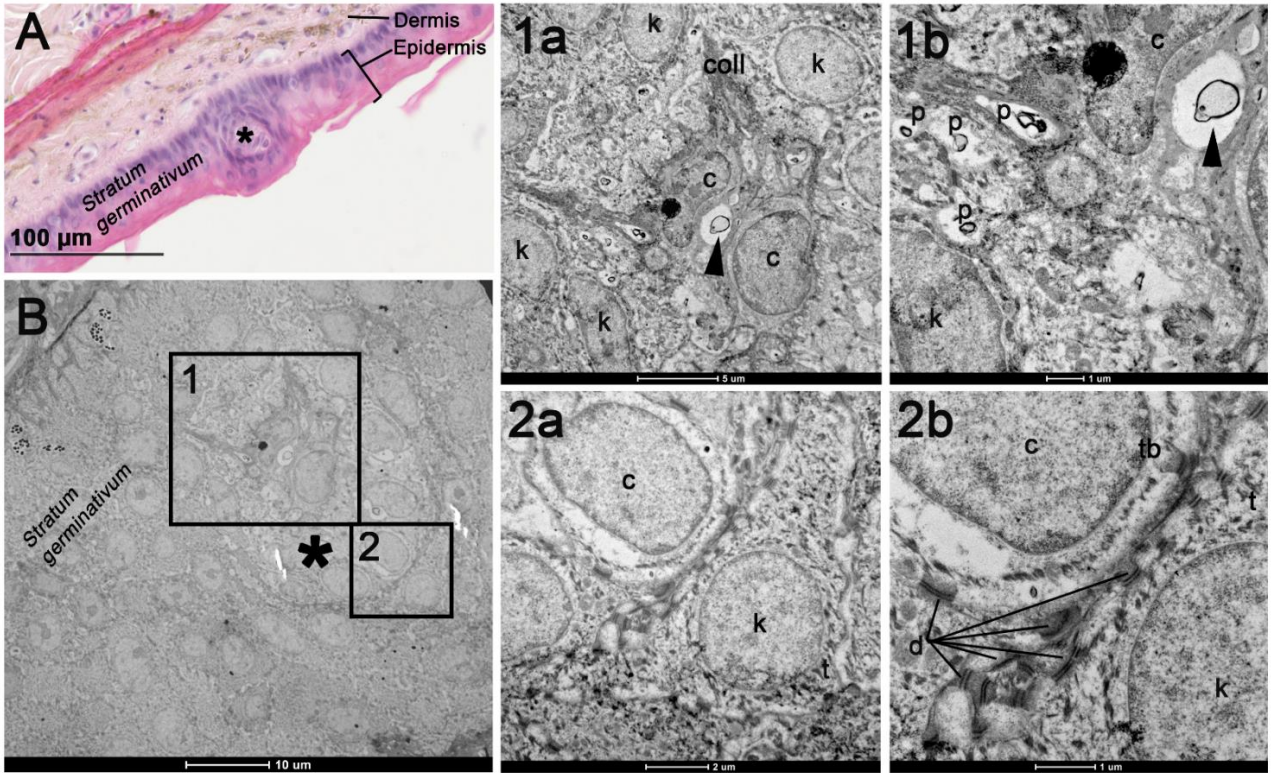

FIGURE 3.7. Light micrograph and transmission electron micrographs (TEM) of cross sections of cephalic scale sensilla in sea snakes. A) Transverse cross-section of scale sensilla (*) in *Aipysurus laevis* showing the dermal capsule within the epidermis. B) Higher magnification of dermal capsule (*) in *A. laevis*. First inset shows nuclei of central cells (c) and epidermal cells (keratinocytes; k), and collagen fibres (coll), a structure typically found within the dermis, in the intercellular domain of the dermal capsule. A putative myelinated axon (arrow heads) is present in the intercellular domain of the central cells; small phospholipid (p) inclusions are also present. Inset two shows intercellular junctions (desmosomes; d) at the membrane of central cells (c) and the keratinocytes (k). Note the fine keratin filaments (tonofibrils; tb) associated with the desmosomes and large aggregations of keratin filaments tonofilaments (t) in the intracellular domain of the keratinocytes. Light micrograph slide was stained with hemotoxylin-eosin and magnified at A) ×34.1; TEM: B) ×1900, 1a) ×4800, 1b) ×6800; 3a) ×9300 and 3b) ×18,500.

Discussion

Cephalic scale organs

Scale sensilla and dermal capsules

Previous work found that the cephalic sensilla of sea snakes are substantially more protruding and often cover a larger proportion of the scale surface than the sensilla of terrestrial hydrophiine snakes (Chapter 2; Crowe-Riddell *et al.*, 2016). The present study shows that, despite these differences, sensilla in sea snakes have retained a similar underlying ultrastructure to their terrestrial counterparts. The sensilla examined in *Aipysurus laevis* and *Hydrophis stokesii* are characterised by a dermal capsule that consists of an aggregation of central cells with collagen fibres, blood vessels and nerve axons in the intercellular domain that together displace the surrounding epidermis (Figure 3.2-7). A similar underlying structure has been reported for the cephalic scale sensilla of ten terrestrial species representing the major phylogenetic groups of snakes (henophidians, scolecophidians and colubroids) and lizards (agamids, iguanids and varanids) (Ananjeva *et al.*, 2010; von Düring and Miller, 1979; Jackson, 1977; Jackson and Sharawy, 1980; Landmann, 1975; Matveyeva and Ananjeva, 1995; Noble, 1937; Stovall, 1985). In terrestrial snakes and sea snakes, the epidermis above the dermal capsule is comprised of columnar keratinocytes (*i.e. stratum germinavatum*) that form a layer that is 15% to 50% thinner than the epidermis of adjacent flat skin. The columnar keratinocytes above the dermal capsule have been described as 'cap cells' and suggested to provide protection against abrasion or aid in transducing mechanosensory stimuli (Jackson, 1977).

We discovered that sea snake skin contained free nerve axons that extend from the dermis and terminate within the alpha layer (epidermis) as distinct discoid structures (Figure 3.3). In terrestrial colubroid snakes, these structures have variously been described as 'discoid receptors' (von Düring and Miller, 1979), 'end bulbs' (Proske, 1969b) and 'button-like' (Noble, 1937) nerve endings. In the sea snake skin, we found discoid receptors distributed throughout the epidermis, but aggregated above dermal capsules (Figure 3.5A, B) deriving from axons at the base of the dermal capsule (Figure 3.5C). This adds evidence for a sensory function of scale sensilla in sea snakes.

Our images from transmission electron microscopy provide the first high resolution ultrastructure data of a cephalic scale sensillum in a snake. Inspection of Figure 3.7B shows that the central cells within dermal capsule are clearly differentiated from surrounding keratinocytes by their lack of tonofilaments. Tonofilaments are keratin formations that provide structural integrity to the epidermis (Cross and Mercer, 1993). Although lacking in tonofilaments, central cells maintain contact elements with surrounding keratinocytes via multiple tight junctions (desmosomes) (Figure 3.7B, inset two). A putative axon was also identified in the intercellular

domain of the dermal capsule (Figure 3.7B, inset one), which may represent the 'terminal receptors' or myelinated axons previously identified in lizards (Landmann, 1975). We did not find synaptic contacts between axons and central cells, which is consistent with light microscopy studies of other colubroid snakes (*e.g. Elaphe,* Jackson and Sharawy, 1980) and suggests that the central cells (and associated dermal capsule) have a structural role rather than functioning as a direct transducer of stimuli.

In addition to dermal capsules associated with cephalic scale sensilla, we detected capsules typically (but not always) located at the bottom of depressions in the outer skin in *H. stokesii* (Figure 3.4). These dermal capsules consisted of approximately 10 central cells (Figure 3.5) and displaced surrounding keratinocytes but, in contrast to the ultrastructure we describe for cephalic scale sensilla, did not result in a skin elevation (bump). Putative nerve structures leading to the dermal capsule were identified using Gomori's One Step stain under light microscopy (Figure 3.4B), but we were unable to conduct antibody staining of neuronal markers. It is unclear whether these dermal capsules are distinct scale structures or merely undeveloped or damaged scale sensilla.

Lamellar corpuscles

We detected lamellated, ovoid cells in the deeper dermis of cephalic skin in both species examined and demonstrated that these lamellar corpuscles were neuronal-positive in *H. stokesii* (Figure 3.6). These structures resemble the 'non-encapsulated lamellated receptors' identified in snakes (*e.g. Boa, Elaphe,* Aota, 1940; von Düring and Miller, 1979; Nishida *et al.*, 2000) and lizards (*e.g. Iguana, Agama,* von Düring, 1973). The location deeper in the dermis and ovoid shape of these receptors resemble small, Pacinian-like corpuscles (Lumpkin *et al.*, 2010; Roudaut *et al.*, 2012). Pacinian (Vater-Pacini) corpuscles are rapidly adapting LTMRs that are sensitive to skin indentation and vibratory ('deep touch') stimuli of high frequencies (peak 250 Hz, range 40 to 800 Hz), and they are present in glabrous skin of mammals (Roudaut *et al.*, 2012). Pacinian corpuscles consist of connective tissue and fibroblasts lined by flat neuronal 'Schwann' cells; the lamellar structures identified in sea snakes tested immuno-positive for the neuronal maker PgP9.5 suggesting that these are indeed modified neuronal cells. The sensitivity of these receptors has not been targeted in previous electrophysiological tests in snakes.

Ancestral and derived sensory functions for cephalic scale organs

The ultrastructural features described above for cephalic sensilla of terrestrial and marine snakes represent all of the components of Meissner-like corpuscles. Meissner corpuscles are rapidly adapting LTMRs present in the dermal papilla of mammal glabrous skin (Lumpkin *et al.*, 2010). Electrophysiological experiments of cranial nerves in colubroid snakes found that they are rapidly adapting LTMRs with receptive fields that overlap with Meissner corpuscles (*i.e.* 12 mm^2, Jackson

and Doetsch, 1977b). Our finding that the cephalic sensilla of sea snakes share a very similar ultrastructure with their terrestrial relatives (and appear to lack novel or specialised cell types) provides evidence that marine lineages have retained the ancestral mechanosensory role for these organs.

The dome-shape and often high coverage of scale mechanoreceptors in sea snakes suggests divergent selection on these organs in marine environments, either for retained mechanosensitivity for direct contact or a derived sensitivity to indirect contact (*i.e.* water motion or 'hydrodynamic stimuli'). There is no obvious reason that sea snakes should require a heightened tactile sense compared to terrestrial species. Sea snakes forage in benthic habitats, frequently probing burrows and crevices as do terrestrial snakes on land (Sherratt *et al.*, 2018). It seems more likely that sea snakes have experienced selection pressures for sensitivity to hydrodynamic stimuli (Crowe-Riddell *et al.*, 2016). Observations of the sea snake *Hydrophis* (*Pelamis*) *platurus* approaching and biting a vibrating object (Heatwole, 1999) provides some behavioural evidence that sea snakes are responsive to hydrodynamic stimuli. Evoked potentials have been recorded from the midbrain of the sea snake *Hydrophis* (*Lapemis*) *curtus* in response to a vibrating sphere (50 to 200 Hz, peak sensitivity at 100 Hz), but no nervous response was successfully recorded directly from a scale sensillum of this species (Westhoff *et al.*, 2005). However, more recently, auditory evoked potentials were recorded (from the midbrain) of *A. laevis* and *H. stokesii* in response to tone bursts from 40 to 600 Hz (peak sensitivity at 60 Hz) (Chapuis *et al.,* unpublished data). These preliminary investigations showed that some species of sea snakes are capable of detecting low amplitude water motion and pressure and/or particle motion caused by sound stimuli. Moreover, although these studies were not able to discern whether hydrodynamic stimuli were being received by scale organs in the skin or hair-cells in the inner ear, the peak sensitivities to the mechanical stimuli broadly overlap with peak sensitivities of Meissner (10 to 50 Hz) and Pacinian (200 to 300 Hz) corpuscles.

Hydrodynamic reception allows the detection of water motion, usually caused by water disturbances or animal movement, and is characterised by very low frequency components (peak at 10 Hz, maximum 50 Hz, Bleckmann *et al.*, 1991; Kalmijn, 1988). It has evolved repeatedly in aquatic organisms (*e.g.* the lateral line systems in fish, cephalopods and amphibians) wherein hydrodynamic stimuli are transduced by cutaneous mechanoreceptors (Budelmann and Bleckmann, 1988; Coombs *et al.*, 1987). Cutaneous mechanoreceptors have also been co-opted for hydrodynamic reception in aquatically-foraging animals including mammals (*e.g.* star-nosed moles (Catania, 1995), platypus (Pettigrew *et al.*, 1998), birds (*e.g.* ducks, geese, ibis (Cunningham *et al.*, 2010) and reptiles (Schneider *et al.*, 2016).

Among snakes, two independently aquatic taxa (that are distantly related to hydrophiines) have evolved highly derived scale mechanoreceptors to sense the water motions generated by the

movement of prey. Scale sensilla in file snakes (*Achrochordus*) are vascularised like sea snake sensilla but instead of a dermal capsule they consist of specialised epidermal cells that underlie highly-derived bristles that protrude from the skin (Povel and VanDerKooij, 1997). Tentacled snakes (*Erpeton tentaculum*) have the largest mechanoreceptors among vertebrates with two cephalic tentacles (2 to 3 millimetres) made up of dermis, epidermis and free nerve endings (Catania *et al.*, 2010; Winokur, 1977). These snake lineages represent older aquatic transitions, and their mechanoreception is linked to specialised ambush-predator strategies for hunting in turbid freshwater habitats of low visibility (Catania, 2012). In contrast, sea snakes have recent marine origins and are ecologically very diverse: species variably occupy blue water reefs or turbid inshore habitats, are diurnal or nocturnal, and specialise on active or sedentary prey. The turtle-headed sea snake, *Emydocephalus annulatus*, is notable in having the second highest scale coverage of sensilla (3.8%) while being diurnally active and specialising on sessile fish eggs in clear water reefs (Chapter 2, Crowe-Riddell *et al.*, 2016; Voris, 1966). This suggests that optimal foraging may not be the primary selection pressure for hydrodynamic sense in sea snakes.

Tail scale organs

Based on cellular morphology, there is a clear distinction between cephalic and posteriorly located scale sensilla in sea snakes. Scale sensilla present on the tail skin of *A. laevis* do not contain dermal capsules; skin elevations are instead created by a thickening of the epidermis (Figure 3.8A). These 'simplified' sensilla structures have been reported in studies of the body skin of the sea snake *E. annulatus* (Avolio *et al.*, 2006b) and the tail skin of terrestrial snakes (Noble, 1937). Many functional roles have been proposed for body scale sensilla in sea snakes, including mechanoreception, sex recognition, and enhanced friction for improved swimming performance, gripping and/or ecdysis (Avolio *et al.*, 2006a, 2006b; Crowe-Riddell *et al.*, 2016). We were unable to stain for the presence of free nerve endings in tail scale sensilla, however, nerve staining of 'supracloacal tubercles' in *Thamnophis sirtalis* and *Nerodia rhombifer* (formally *Natrix rhombifera*) found that they were innervated in a similar pattern to cephalic scale sensilla, and thought to be important for sensory feedback to position during copulation (Noble, 1937; Pisani, 2012). Thus, these posteriorly located scale sensilla are clearly differentiated from cephalic scale sensilla by their ultrastructure and likely have a mechanoreceptive and/or structural function in sea snakes.

The ultrastructural differences of cephalic scale sensilla compared to posteriorly located sensilla may reflect variances in mechanoreceptor sensitivity in the head compared to those on the rest of the body. Research in mammals suggests that the structure of the skin organ may be just as important as the neurons that carry the electrical impulse – collagen can provide physical tethering, structural integrity, or aid in propagating or modulating the sensation of force (Zimmerman *et al.*, 2014). Thus, the absence of a dermal capsule for scale sensilla on the body and tail skin might indicate a less specialised mechanoreceptor with differential sensitivity to

cephalic mechanoreceptor. Furthermore, cephalic cutaneous receptors are innervated by specialised cranial nerves (*e.g.* trigeminal ganglion), while the rest of the body is innervated by peripheral nerves of the spinal cord (*i.e.* dorsal root ganglion) (Dehnhardt and Mauck, 2008). These neural pathways are thought to reflect differences in somatosensory processing wherein the head harbours specialised tactile receptors that are used to actively seek stimuli in the surrounding environment, in contrast to the body, which passively receives information (Dehnhardt and Mauck, 2008; Schneider *et al.*, 2016). Expanding on neurophysiological data in terrestrial snakes, our results suggest that the head of sea snakes is the prime exploratory organ for actively seeking mechanical stimulation. Future studies should investigate the neural pathways and compare electrophysical responses underlying scale mechanoreceptors distributed on the head and body of snakes.

Dermal photoreception and other cutaneous sensory modalities

The skin provides a primary interface for receiving multiple stimuli, creating an opportunity for multi-modal cutaneous receptors. Indeed, molecular and electrophysiological studies of ISOs in crocodiles indicate multi-modal sensitivity to mechanical stimuli and thermal and pH gradients (Brooks and Jackson, 2007; Di-Poï and Milinkovitch, 2013). In addition to previous studies using electron microscopy (Chapter 2, Crowe-Riddell *et al.*, 2016; Povel and VanDerKooij, 1997), our study demonstrates that snake scale sensilla are devoid of pores and so a chemosensory function is highly unlikely. Salinity is an important predictor of sea snake distribution (Brischoux *et al.*, 2012) because most species require access to freshwater for hydration (Lillywhite *et al.*, 2012), but pH receptors are more likely to be located in papillae in the mouth (Burns, 1969; Nishida *et al.*, 2000). Thermal sensitivity of scale sensilla has been investigated in *Elaphe* colubroid snakes, which found that although some cutaneous nerves are exclusively sensitive to heat, mechanoreceptive fibres are not responsive to either heating or cooling (Jackson and Doetsch, 1977a, 1977b). Dermal photoreceptors in the tail skin of *Aipysurus* sea snakes mediate phototactic behaviour in these species (Zimmerman and Heatwole, 1990; Chapter 5). Although we did not detect candidate photoreceptive structures (*e.g.* photoreceptors, lenses) in the tail skin of *A. laevis,* we did find 'simplified' scale sensilla (described above) and other small dermal capsules (Figure 3.8B). Given that cutaneous receptors have been linked with both mechano- and photoreception in amphibians (Baker *et al.*, 2015) and marine invertebrates (Pei *et al.*, 1996), these scale organs merit further investigation for their putative role in photoreception. Finally, an electro-magneto-sense has been proposed for scale sensilla in snakes (Povel and VanDerKooij, 1997), but our understanding of the spatial ecology, and thus long-range navigation abilities, of sea snakes is limited.

Conclusions

Our study shows that the ultrastructure of cephalic sensilla in sea snakes closely resembles the mechanosensitive Meissner-like corpuscles that underlie sensilla in terrestrial snakes. This provides evidence that the sensilla of marine hydrophiine lineages have retained an ancestral mechanosensory function. Our findings provide the basis for future research into the sensitivity of cutaneous receptors in sea snakes including mechano-, hydro- and photo-sensory modalities. Our study highlights that snakes are an important group for understanding the evolution of mechanoreception in vertebrates, particularly in response to shifting sensory landscapes.

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Supplementary materials

Figures

Figure S3.1 Primary and secondary antibody controls for PGP9.5 in taipan (*Oxyuranus scutellatus)* brain tissue and sea snake (*Hydrophis stokesii*) cephalic skin tissue.

Electronic files

File ES3.1 Supplementary data are interactive digital scans of slides (ndp.view files), this electronic file is available at Figshare, https://adelaide.figshare.com/s/f029519e4f6304b34565

File ES3.2 Skin measurements for *A. laevis* and *H. stokesii.* (.xlxs).

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Chapter 4

Up close and personal: The role of enlarged scale organs in tactile foreplay of turtle-headed sea snakes (*Emydocephalus annulatus*)

Chapter 4 Up close and personal: the role of enlarged scale organs in tactile foreplay of turtle-headed sea snakes (Emydocephalus annulatus)

Jenna M. Crowe-Riddell, Chris J. Jolly, Kate L. Sanders

Abstract

Tactile communication is used in many intraspecific interactions, but its role in mating systems has been comparatively overlooked. Here, we investigate the sensory roles of scale protuberances in the mating system of a fully-marine reptile, turtle-headed sea snakes (*Emydocephalus annulatus,* Hydrophiinae). We tested for sexual dimorphism in scale protuberances in museum specimens (n = 59), finding that in addition to the previously noted rostral spine (RS) on the snout and rugosities near the cloaca ('anal knobs', AKs), male *E. annulatus* have large scale organs located on the chin (termed here 'genial knobs', GKs). Histological data of scale protuberances indicate that the RS and GKs are comprised primarily of 'soft' dermal layers, whereas thickened epidermis underlies the rugosities and AK of the body. In combination with behavioural observations of wild *E. annulatus*, our morphological results suggest that scale protuberances in males are cutaneous mechanoreceptors that serve specific functions during courtship and mating. The RS and GK contrasts expectations for corneous spines/claws that serve only to mechanically stimulate female during courtship but may also give sensory feedback for the male (*i.e.* tactile foreplay), whereas the AK are likely to be used in the final stages of mating (*e.g.* body and cloacal alignment). This study demonstrates that tactile communication is under sexual selection in male *E. annulatus* and may play a role in the mating systems of other sea snakes.

KEYWORDS: mating systems, sea snake, tactile, sexual selection, female choice

Introduction

Tactile communication is used in a range of intraspecific interactions such as mate recognition, competition over resources and reinforcement of social bonds (reviewed in Hill, 2001; Sparks, 1967). In mating systems where female choice operates, tactile signalling can increase fertilisation success for both sexes. Males use tactile cues during courtship to stimulate interest in mating (*e.g.* foreplay), coordinate copulatory behaviours and induce oocyte growth in mates (*e.g.* vitellogenesis, Wallace, 1985). Females can use tactile cues to recognise conspecific mates and choose the most attractive suitors. Tactile communication can involve indirect cues (*e.g.* vibration, Hill, 2001), direct manipulation of the body and appendages, ritualistic displays (*e.g.* titillation sequence in freshwater turtles, Liu *et al.*, 2013) and elaborate secondary sexual morphologies (*e.g.* anal appendages in damselflies, Robertson and Paterson, 1982). However, the role(s) of tactile signalling in mating systems has predominately been overlooked in favour of visual and acoustic signals, which bias the literature towards the subset of taxa that use such cues (*e.g.* birds, fishes, frogs and insects, Coleman, 2009).

Snakes are not widely appreciated for their intimate interactions, but they use tactile signals in courtship (Carpenter, 1977) as well during defensive displays (Carpenter and Ferguson, 1977), male-male combat (Carpenter, 1984; Carpenter *et al.*, 1978), parental care (Greene *et al.*, 2002), and the formation of social hierarchies (Carpenter, 1984; Yeager and Burghardt, 1991). Fertilisation success in snakes is dependent on tactile signals because these are essential to induce neuroendocrine changes and receptive behaviours in the female (Mendonça et al. 2003; Mendonça & Crews 1990; Mendonça & Crews 2001), as well co-ordinate intromission for both partners (Harrison, 1933; Noble, 1937; Pisani, 2012). Pre- and post-copulatory behaviours in diverse snake taxa involve entwinning of the body, biting, chin-rubbing and muscular contractions (Phase I); lifting of the tail by the male and lifting of the anal scale by the female known as 'cloacal gaping' (Phase II); followed by intromission and coitus (Phase III) (Carpenter, 1977). In addition to synchronising physiological and behavioural processes central to fertilisation, these behaviours are likely to stimulate interest in mating (*i.e.* foreplay) by direct contact of cutaneous mechanoreceptors located in regions of frequent contact such as the chin and tail (see Noble, 1937). Indeed, elaborate secondary morphologies associated with tactile foreplay are found in pythons and boas, in which corneous pelvic spurs—articulated by vestigial hind limbs—are used by males to stroke and lift the female's scales to prod the soft interstitial skin, which initiates cloacal gaping (Gillingham and Chambers, 1982; Stickel and Stickel, 1946). The only other snakes that are known to use a corneous protuberance in courtship and mating are the fully-marine, turtle-headed sea snakes in the genus *Emydocephalus* (Hydrophiinae).

Turtle-headed sea snakes are a species-complex that occupy shallow-water habitats from the Sea of Japan and East China Sea (*E. ijimae,* Stejneger, 1898) to the Timor and Coral Seas of the Indo-Pacific (*E. annulatus,* Krefft, 1869). In addition to cephalic cutaneous mechanoreceptors (CMs, 'sensilla' *sensu* Crowe-Riddell *et al.,* 2016, Chapter 2), *Emydocephalus* species have a modified rostral scale that protrudes from the tip of the snout forming a 'rostral spine' (RS; Figure 4.1). This structure was initially thought to aid in these species' specialised strategy of feeding entirely on fish eggs (McCarthy, 1987; Voris, 1966). Observations of wild *E. annulatus*, however, have revealed that the RS is present only in males and is used to nudge and/or prod the neck and back of females during courtship and mating (Guinea, 1996). Male *E. annulatus* also have numerous scale bumps ('rugosities' *sensu* Avolio *et al.,* 2006a; 2006b) along the body, which may have a role in mechanoreception and/or a structural function during mating. These scale protuberances and associated prodding behaviours result in direct mechanical stimulation and are thought to induce receptivity in females (*e.g.* cessation of swimming, cloacal gaping) and thus should show ultrastructural resemblances to corneous claws (*e.g.* of pelvic spurs in pythons and boas). However, these protuberances might also provide sensory feedback to the male to enhance his motivation in mating, in which case these structures should show ultrastructural resemblances to CMs.

Here, we generate morphological and histological data to explore the adaptive significance of scale protuberances in *E. annulatus* including the RS, rugosities on the anal scales ('anal knobs', AK), and undescribed, yet conspicuous, structures on the underside of the chin (termed here 'genial knobs', GK). Reassessment of behavioural data in the literature considering these results suggest that these conspicuous scale structures are used in intersexual tactile signalling. Our results demonstrate that the RS and large GKs are present only in adult males and are comprised primarily of dermal components. This contrasts expectations for hard corneous spines/claws (*e.g.*, pelvic spurs in pythons and boas) that serve only to stimulate the female and suggests that these protuberances may also provide sensory feedback to the male. The AK differ from these cephalic protuberances in being comprised primarily of a thickened living epidermal layer that closely resemble the scale rugosities previously described on the ventral scales of this species.

Material and methods

We examined 59 museum specimens including 23 adult females, 32 adult males, a single juvenile female and three juvenile males that were collected from Ashmore Reef and nearby Hibernia Reef in the Timor Sea, Australia. Approximately half of these specimens had been collected during the winter while individuals were observed courting and mating (n = 30; May 1992; M. L. Guinea, pers. comm.) and the other half during summer, outside of the mating season (n = 29; December to January 1974) (Table S4.1). We measured specimen snout-vent

length (SVL) and tail length, and determined their sex by palpating hemipenes and/or calculating the tail to SVL ratio since male snakes have proportionally longer tails than females (Shine *et al.*, 1999). Specimens were photographed (Canon EOS 7D and Canon EF-S 60mm f/2.8 Macro USM Lens) and presence or absence of scale projections were scored for the 'rostral spine' (RS), 'genial knobs' (GK) and 'anal knobs' (AK). Genial knobs were identified by their presence on the first six genial scales and by their large size (Figure 4.1), which is approximately 0.5 mm in diameter (*c.f.* to cephalic mechanoreceptors (CMs) that rarely exceed 0.2 mm in diameter; Table 4.2). The approximate length of the RS was estimated by measuring images of specimens using imaging software (Photoshop, Adobe Systems, USA), with a one-millimetre reference scale bar. To account for the potential effects of body size on length of RS, this trait was scaled against a proxy estimate of body size (SVL, mm) and an tail length as an indicator sexually maturity (TailL, mm; King 1989; Shine et al. 1999). A linear regression analysis of $\log_{10}$-transformed trait data was conducted using the 'lmodel2' package v1.7 (Legendre 1998) in R v3.5.1 (R Core Team, 2017). We examined the ultrastructure of a rostral, genial and anal scale in a male *E. annulatus* specimen using electron- and light-microscopy. These scales had been previously fixed in 10% formalin and stored in 70% ethanol and were viewed directly at 10 kV under an environmental scanning electron microscope (450 Quanta eSEM, FEI, USA). Scales tissues were then prepared for histology. Briefly, the scales were dehydrated by successive immersion in alcohol, then paraffin-embeded and serial sections (10 μm) were stained with Gomori's One-Step (Gomori, 1950). Slides were scanned using a digital slide scanner (Nanozoomer, Hamamatsu Photonics, Japan) and measurements taken using imaging software (Nanozoomer Digital Pathology v2.6, Hamamatsu Photonics, Japan).

We measured the height (thickness) of the epidermis, which comprised the nucleated layer of living cells (*stratum germinativum*) and the overlaying hardened, non-nucleated α and β layers (*stratum corneum*). We also measured the underlying dermal layers, including the loose connective tissue (*stratum laxum*) and the deeper densely packed collagen (*stratum compactum*). We used the two-sample t–test (unpaired) to examine variation in tissue thickness between protuberances and adjacent, flat skin. Before statistical analyses, we checked that data were normally distributed using Bartlett's test, if data were not normally distributed a Welch two sample t-test was used instead. Statistical analyses were performed using base packages in R.

Results and discussion

Sexual dimorphism

The adult males had pronounced rostral spines (RSs), anal knobs (AKs) and genial knobs (GKs), all of which were absent in sampled females and juveniles (Table 4.1; Figure 4.1; Figure S4.1). Sexually dimorphic RSs and AKs have previously been described in this species but our study is

the first to report the presence of GKs on the chin. Our results indicate that sexual dimorphism in scale protuberances develops at sexual maturity and, unlike ventral scale 'spines' in other sea snakes (*e.g. Hydrophis curtus*, Avolio et al. 2006a), they are not shed at the end of the mating season. All specimens examined had numerous cutaneous mechanoreceptors (CMs) on the head (rostrum and genial scales) and body (anal scales), irrespective of life stage, sex or mating season (Table 4.1; Figure 4.1; Figure S4.1), consistent with the view that these receptors are used for non-sexual functions related to various behaviours throughout the individual's lifetime (Crowe-Riddell et al., 2016).

TABLE 4.1. The presence of scale projections in *Emydocephalus annulatus* in adult males (n = 32) and females (n = 23), and juvenile males (n = 3) and females (n = 1). Note: in the two male specimens that lacked genial knobs, both the rostral spine and anal knobs were absent, which indicates that these individuals may not yet reached sexual maturity.

	Adult				Juvenile			
	Male		Female		Male		Female	
Scale projections	No.	%	No.	%	No.	%	No.	%
Cephalic mechanoreceptors	32	100	23	100	3	100	1	100
Rostral spine	30	94	0	0	0	0	0	0
Genial knobs	30	94	0	0	0	0	0	0
Anal knobs	30	94	0	0	0	0	0	0

Rostral spine and genial knobs

The RS consisted of a laterally flattened projection of smooth skin that protruded 0.56 to 2.26 mm (mean = 1.2 mm) from the rostral scale and tapered into one to four blunt points (mean = 2; Figure 4.1; Figure 4.2A; ES File4.1). Length of RS was not significantly correlated with SVL ($-1.06 \times SVL^{+0.40}$, $r^2 = 0.07$) or tail length ($-1.01 \times TailL^{+0.51}$, $r^2 = 0.11$). The GKs consisted of a round projection of smooth, pale skin that was located at the centre of the chin scale and was approximately three times larger (mean = 300 μm x 378 μm [diameter x length]) than adjacent scale mechanoreceptors (mean = 94 μm x 95 μm; Figure 4.1B; Figure 4.2).

Given the structure of other cornified scales in squamates, *e.g.* claws of lizards and presumably of spurs of pythons and boas (we expected that the scale protuberances of *E. annulatus* would be comprise primarily the 'hard' *stratum corneum* (Calvaresi *et al.*, 2016; Cheng *et al.*, 2010; Lillywhite, 2014). However, while the *stratum corneum* was thickened in RS, we found that the underlying structures of both the RS and GKs comprise primarily 'soft' dermal layers (Figure 4.2; Table 4.2). The *stratum laxum* of both the RSs and GKs was significantly thicker than adjacent flat skin (3.5× in RS, 1.3× in GK), and was devoid of pigment cells (melanophores) in both

structures, but contained loose connective tissue, fibroblasts, blood vessels and nerve axons (Figure 4.2B; Table 4.2; ES File4.2). The *stratum germinativum* was thicker in the RS than adjacent skin and comprised 10 layers of immature α cells *(c.f.* typical 5-6 cell layers) and the *stratum corneum* was approximately 3× thicker than in adjacent, flat skin (Table 4.2; ES File4.2). The tapered points or 'spines' of the RS were created by an extension of the *stratum laxum* and thinning of the overlaying *stratum germinativum,* and an aggregation of dermal cells were present at the base of each point (Figure 4.2A, inset). In GKs, the *stratum germinativum* and α layer of the *stratum corneum* were of the same thickness as adjacent, flat skin (ES File4.2). A hemispherical skin elevation was located on top of GKs under scanning electron microscopy (SEM), which resembled a CM but was slightly larger (121 μm x 113 μm) than CMs found on adjacent flat skin (Figure 4.2C; Figure S4.1). However, these elevations could not be located in cross-section under light microscopy. The β layer of the *stratum corneum* was highly reduced or absent at the centre of the GK, similar to adjacent CMs (Figure S4.1).

A gland was located in the rostral scale (1 mm deep from the epidermis; Figure S4.2) but did not contain ducts leading to the surface of the skin; hence this likely represents the rostral expansion of the supralabial gland that has previously been identified in *Hydrophis* sea snakes but is of unknown function (Burns & Pickwell 1972). Finally, although protruding GKs were absent in females and juvenile males, some specimens had small, pale patches of skin in approximately the same location as GKs in adult males (Figure 4.1C; Figure S4.1). These patches were typically flat, but occasionally elevated to approximately half the size of the mean GKs of males and 2.5× times larger than adjacent CMs (Table 4.2).

Rugosities and anal knobs

Two large AKs were located under SEM, which showed that they were similar in size (mean = 298 μm x 342 μm) to the GKs found in the same specimen. The knobs had one to two hemispherical skin elevation(s) in their centre (Figure 4.2E) that resembled a CM (77 μm x 83 μm) and were similar in size to CMs found on cephalic skin. In contrast to the GKs, the underlying structure of AKs was formed by a thickening of the *stratum germinavitum* and *stratum corneum* (α layer) of the epidermis (note that the β layer could not be measured because it had artificially separated from the tissue during processing; Figure 4.2F; ES File4.2). The hemispherical skin elevations were formed by a small dermal projection that displaced the epidermis, causing it to bend caudally to form the elevation on top on the knob (Figure 4.2F). Therefore, AKs and other sexually dimorphic rugosities present on the body scales in *E. annulatus* (Avolio *et al.*, 2006b) share an ultrastructural resemblance with the 'supracloacal tubercles' described in terrestrial snakes, which are thought to aid in cloacal alignment during Phase II of courtship (Harrison, 1933; Noble, 1937).

The cephalic protuberances of *E. annulatus* (RS and GKs) are located in regions of frequent contact during Phase I and II of tactile courtship (*e.g.* snout prodding and chin rubbing, Guinea, 1996), and both are formed by an extension of the soft dermal tissue (Figure 4.2B, D). In contrast to the cephalic protuberances, the rugosities on the body and around the cloaca (AKs) receive contact during Phase II and III (*e.g.* tail lifting, cloacal alignment) of tactile courtship, and are primarily comprised of cornified epidermis (Figure 4.2F; Guinea, 1996). This suggests differing functions for these scale organs, with cephalic protuberances used in tactile foreplay, while the body and anal rugosities (AKs) are used to coordinate intromission and/or provide enhanced grip during mating.

TABLE 4.2. Results of two sample t-test (unpaired) to examine differences in epidermal and dermal thickness between scale projections, rostral spine (RS), genial knobs (GKs) and anal knobs (AK), compared to adjacent, flat skin. Before statistical analyses, data were checked for normal distribution using Bartlett's test; if data were not normally distributed, we used a Welch two sample t-test[1].

	Rostral spine				
	Beta layer	*Alpha layer*[1]	*Germinavitum*[1]	*Laxum*[1]	*Compactum*
t	-5.5	-12.8	-3.2	-24.3	-1.2
df	20	10	11	11	20
p	2.12E-05**	9.60E-08**	8.62E-03*	4.55E-11**	0.25
lower CI	-36.8	-83.2	-55.7	-455.5	-338.7
upper CI	-16.6	-56.7	-10.2	-380.1	72.6
mean flat	22.6	26.2	65.3	167.0	915.7
mean RS	49.4	97.2	98.2	584.8	1038.7
	Genial knob				
	Beta layer	*Alpha layer*	*Germinavitum*	*Laxum*	*Compactum*
t	8.1	-0.4	-0.8	-2.6	-0.8
df	14	14	14	14	14
p	1.26E-06*	0.72	0.48	2.12E-02**	0.45
lower CI	8.5	-4.1	-8.2	-31.3	-44.2
upper CI	14.7	2.9	4.0	-3.0	20.6
mean flat	13.2	12.7	36.5	55.8	216.9
mean GK	1.7	13.3	38.6	72.9	228.7
	Anal knob				
	Beta layer	*Alpha layer*[1]	*Germinavitum*[1]	*Laxum*	*Compactum*
t	*NA*	2.9	12.3	-2.2	-4.7
df	*NA*	14	15	22	15
p	*NA*	1.17E-02*	2.40E-09**	3.51E-02*	3.12E-04**
lower CI	*NA*	1.3	41.2	-133.1	-101.6
upper CI	*NA*	8.6	58.5	-5.3	-37.9
mean flat	*NA*	7.3	93.9	233.9	128.4
mean AK	*NA*	12.2	44.1	164.7	58.9

NA = beta layer was not measured due to artificial separation from tissue during processing

[1]Welch two-sample t test *p < 0.05 **p <0.001

The cephalic protuberances of *E. annulatus* may provide an enhanced sensory feedback for males that has been a previously overlooked aspect of tactile foreplay in this species. Phase I courtship in many snake taxa typically involves chin rubbing or vertical vibratory movements of the head by the male (Carpenter, 1977). Experiments that taped different regions of the body in captive *Thamnophis* and *Nerodia* snakes (Colubridae) found that males with obscured chins that had courtship behaviours ceased briefly after chin-rubbing (Noble, 1937). In contrast, male snakes with an obscured anal scale engaged in long bouts of courtship but were unable to achieve cloacal alignment and intromission (Noble, 1937). Based on these behavioural experiments, stimulation of CMs on the chin are thought to maintain the male's sexual motivation, whereas AKs or CMs near the cloaca are important for tactile coordination (Harrison, 1933; Noble, 1937; Pisani, 2012). The GKs of *E. annulatus* have an ultrastructural resemblance to adjacent CMs (*i.e.* are composed of extended dermal tissue with a thin β-layer; Figure 4.2D inset) but are 3.5× larger than adjacent CMs, and more conspicuous than those found on the chins of adult male *Thamnophis* and *Nerodia* (J. Crowe-Riddell pers. obs.). Thus, GKs may be derived from CMs and be similarly mechanosensitive but have specialised functions in courtship and mating. The rostral prodding of male *E. annulatus* is similar to the pelvic spur prodding used during courtship displays of pythons and boas, except that *E. annulatus* males prod the anterior rather than posterior dorsal region of the female, and 'scale-lifting' by male *E. annulatus* has not been reported. Also contrasting expectations of pelvic spurs is that the ultrastructure of the RS is primarily 'soft' dermis in contrast to the 'hard' cornified epidermis of claws. In combination with behavioural data, our histological data suggests that the enlarged mechanoreceptors on the chin (GKs) and the extended tissue of the snout (RS) provides both mechanical stimulation for the female and sensory feedback to the male.

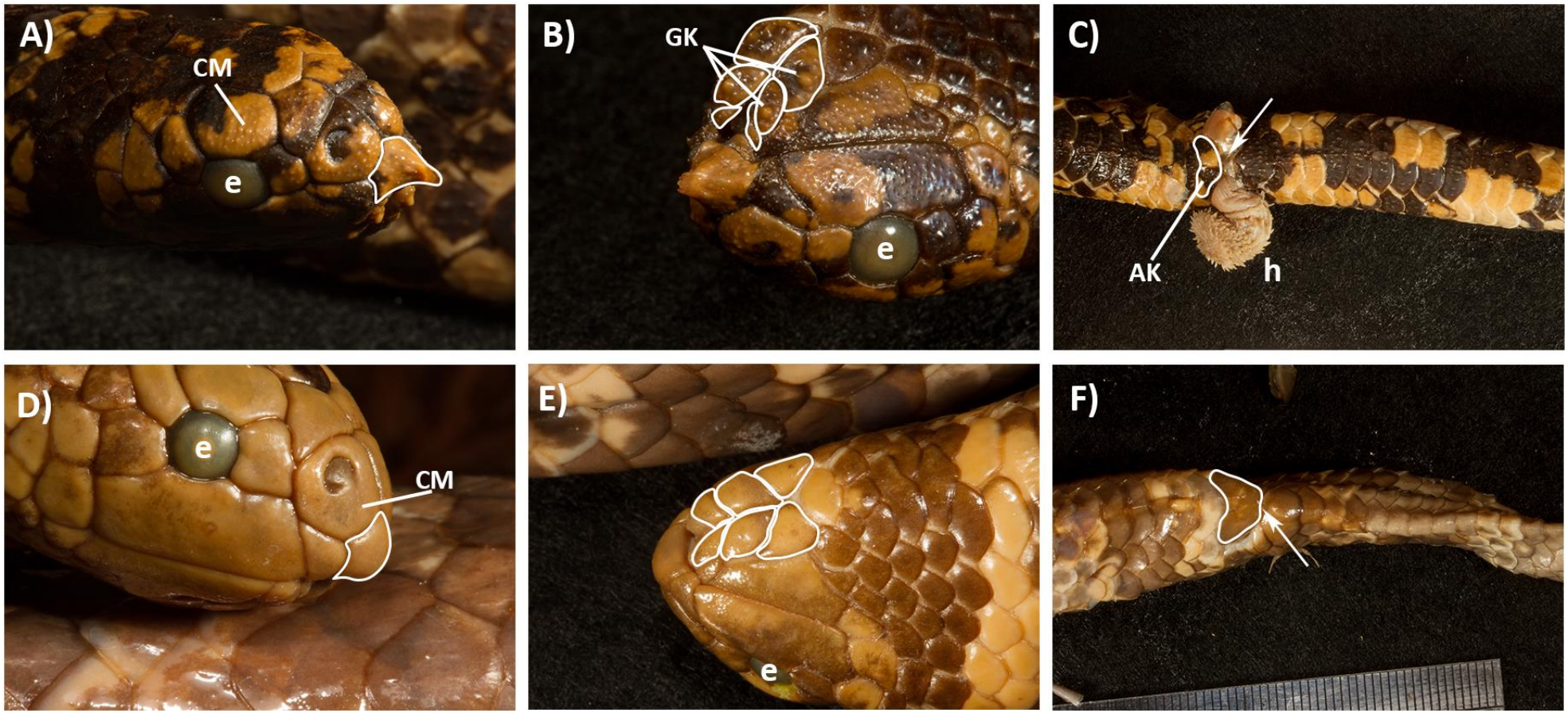

FIGURE 4.1. Sexual dimorphism of scale projections in *Emydocephalus annulatus*. Images of an adult male illustrating the A) rostral spine, B) genial knobs (GK) and C) anal knobs (AK). D-F) Images of an adult female illustrating the absence of scale projections in regions corresponding to the males (indicated by a black outlines). The eye (e), cloaca (arrows), hemipene (h) and scale mechanoreceptors (CM) are also indicated.

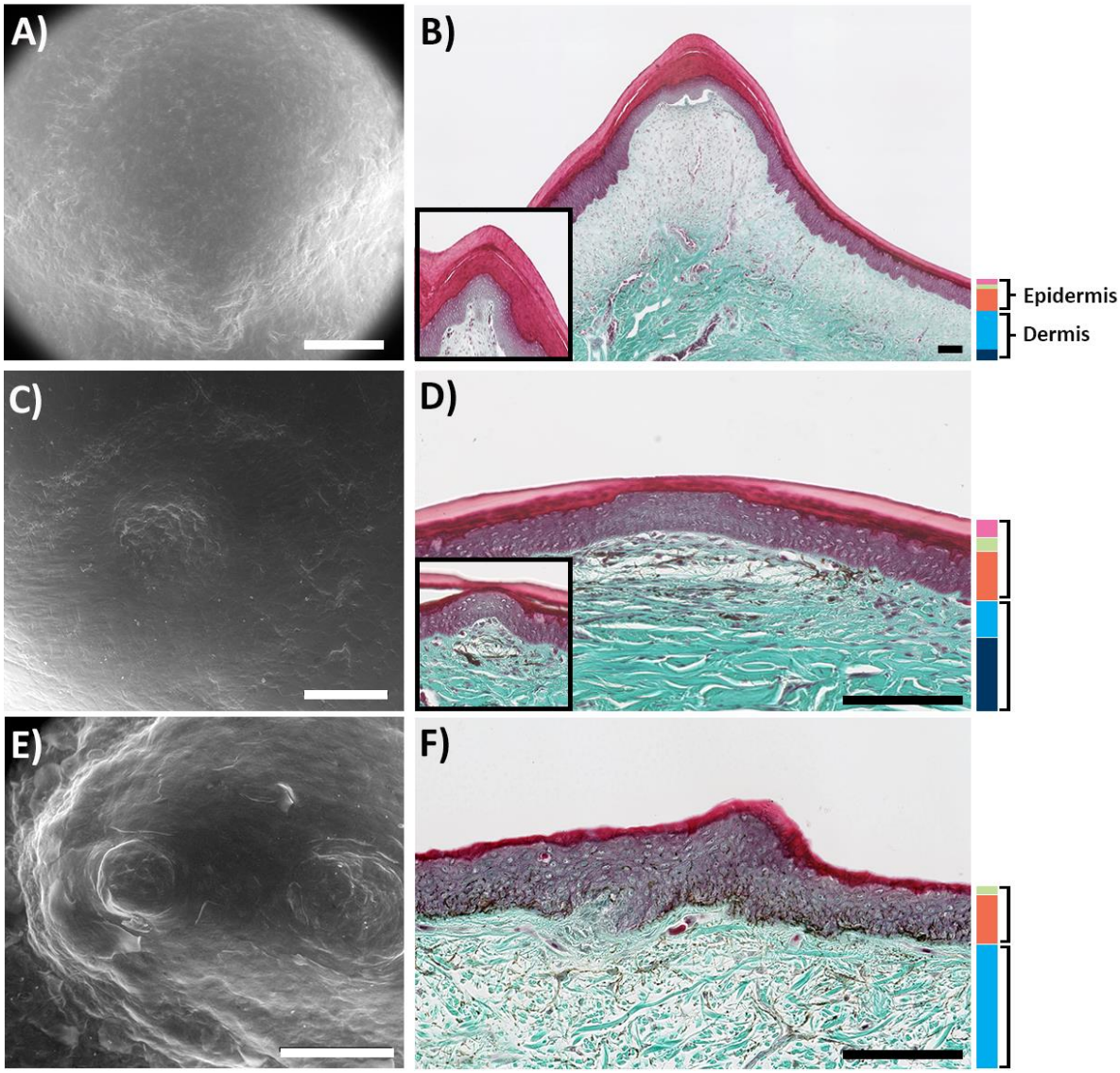

FIGURE 4.2. Scale protuberances in male *Emydocephalus annulatus* illustrating the outer and underlying morphology a A-B) rostral spine, inset shows structure of blunt point; C-D) genial knob, inset shows an adjacent scale mechanoreceptor, and E-F) anal knob. Note the variable proportions of the skin that comprise each scale protuberance: the epidermal layers of the *stratum corneum* (α layer, pink; β layer (green) and the *stratum germinativum* (orange); the dermal layers of the *stratum laxum* (light blue) and *stratum compactum* (dark blue). All scale bars are 100 µm, histological sections were stained with Gomori's One Step, and magnification was A) 295×, B) 5.22×, inset 19.1×, C) 1200×, D) 30×, inset 29.5×, E) 841× and F) 37.8×.

Sensory drive and bias

Sea snakes evolved from terrestrial elapid snakes approximately 9 to 18 million years ago and the ecological shift to an aquatic lifestyle has clearly influenced their sensory and signalling capabilities (Cummings and Endler, 2018; Sanders *et al.*, 2008; Udyawer *et al.*, 2018). In extant terrestrial and semi-aquatic snakes, pheromone trails left by receptive females are used to attract conspecific mates, allowing males to assess body condition and track females over kilometre

distances (Guinea, 1986; Mason *et al.*, 2000; Shine *et al.*, 2003). In contrast, pheromone signals in the sea are ephemeral due to water currents and rapid dilution, resulting in strong selection for alternative sensory modalities (*e.g.* tactile) in fully-marine snakes (Shine, 2005). The buoyancy force and three-dimensional quality of aquatic habitats is likely to reduce some somatosensory cues (*e.g.* males cannot apply pressure by lying on top of females), while also reducing abrasion costs associated with developing large CMs. Thus, the evolution of scale protuberances in *Emydocephalus* may be the result of an increased selection for tactile cues in courtship and mating behaviour, in combination with relaxed selection of CM size and/or coverage. More broadly, the reduction of somatosensory cues in aquatic habitats may have influenced the evolution of sexually dimorphic scale rugosities reported in sea snakes, *e.g.* spine-bellied sea snakes, *Hydrophis curtus* (Avolio *et al.,* 2008a). Further research should examine the ultrastructure and density of rugosities, but preliminary data suggests that these scale protuberances are highly variable across sexes, populations and species of sea snakes (Avolio *et al.,* 2008a; pers obvs.). This suggests that scale protuberances might be linked to shifts in mating strategies in the transition from land to sea in fully-marine snakes.

The sensory-bias hypothesis predicts that sexual displays that arise in males are influenced by pre-existing sensory traits of both males and females (Fuller *et al.*, 2005). Previous work found that *E. annulatus* have the second-largest CMs recorded among sampled elapids, covering 3.8% of the scale's surface (19 species and only males sampled, Chapter 2, Crowe-Riddell *et al.*, 2016). The foraging strategy of *E. annulatus* likely relies on a combination of chemosensory, visual and tactile cues–snakes meander in search of nests of fish eggs (visual) and, once located (chemoreception), they scrape the eggs off the substrate using a fused second supralabial scale (touch) (Guinea, 1996; Kutsuma *et al.*, 2018; Shine *et al.*, 2004). Additionally, spatial distribution studies suggest that *E. annulatus* form social associations and individuals are known to probe each other with their rostrum when they come into contact (Guinea, 1996; Shine *et al.*, 2005). Therefore, it is plausible that changes in CM traits initially arose due to selection for optimal foraging or intraspecific recognition, but CMs may have since developed in scale protuberances as a result of sexual selection.

Sexual conflict

The relationship between female choice and male coercive tactics (*i.e.* sexual conflict) has been debated in many mating systems especially those that feature elaborate tactile displays and so it merits discussion here (see Arnqvist and Rowe, 2005; Eberhard, 2004). During mating seasons, male *E. annulatus* cease feeding to actively pursue females and the courtship behaviours of the male could be interpreted as coercive mating tactics: they chase and incessantly prod females to induce stress responses including motionless behaviour and cloacal gaping in the female (Goiran

et al., 2013; Guinea, 1996). However, more typical signs of stress such as rapid or erratic swimming have not been observed in female *E. annulatus* (C. Goiran, pers. comm.) and females tend to exhibit foraging behaviour during the mating seasons (Goiran *et al.*, 2013). Furthermore, female control in *E. annulatus* is supported by sexual size dimorphism (females are larger) and the high rate at which males loose contact with females during courtship (up to 60% of the time) (Goiran *et al.*, 2013). Ultimately, it is likely that an interplay of female choice and sexual conflict that has influenced the evolution of secondary sexual characteristics and ritualised courtship in *E. annulatus*. Future studies should aim to test whether female *E. annulatus* discriminate tactile stimulation (both pre- and post- copulatory) from rival males as a component of mate choice and, if this is demonstrated, the physiological and behavioural basis underpinning this specialised sensory trait.

Conclusions

Our study exemplifies how data on morphological and behavioural traits can be integrated to inform our understanding of the adaptive significance of novel phenotypes. Further studies are needed to uncover the developmental and physiological mechanisms underlying mating systems in *E. annulatus*. We posit that aquatic snakes are a rich system for furthering knowledge of understudied tactile sensory modalities in snake evolution.

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Supplementary materials

Tables

Table S4.1. List of 59 specimens of *Emydocephalus annulatus* with sexual dimorphism in scale protuberances scored.

Figures

Figure S4.1 Gross morphology of scale structures in juvenile *Emydocephalus*

Figure S4.2 Cross-section of a gland in rostrum in male *Emydocephalus annulatus*

Electronic Files

ES File4.1 Length (mm) of scale protuberances, rostral spine, genial knobs, anal knobs and cephalic mechanoreceptors) based on low magnification images in a *Emydocephalus annulatus*. (.xlxs).

ES File4.2 Measurements of skin thickness based on cross-sections (histology) of scale projections in a male *Emydocephalus annulatus* (R129834) (.xlxs).

The following electronic files are available at Figshare, https://adelaide.figshare.com/s/137bf5c9113b1902a123

ES File4.3 Images showing morphology of scale protuberances in *Emydocephalus annulatus*. Images were taken of 59 museum specimens from the Australian Museum (AM), the Museum and Art Gallery of the Northern Territory (MAGNT) and Western Australian Museum (WAM) (jpegs, zipped).

ES File4.4 Interactive digital scans of slides of scale projection in a male *Emydocephalus annulatus* (R129834) (ndp.view, zipped)

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Chapter 5

Phototactic tails: Evolution and molecular basis of a novel sensory trait in sea snakes

CHAPTER 5 Phototactic tails: Evolution and molecular basis of a novel sensory trait in sea snakes

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Abstract

Dermal phototaxis has been reported in a few aquatic vertebrate lineages spanning fish, amphibians and reptiles. These taxa respond to light on the skin of their elongate hind-bodies and/or tails by withdrawing under cover to avoid detection by predators. Here, we investigated tail phototaxis in sea snakes (Hydrophiinae), the only reptiles reported to exhibit this sensory behaviour. We conducted behavioural tests in 17 wild-caught sea snakes of eight species by illuminating the dorsal surface of the tail and mid-body skin using cold white, violet, blue, green and red light. Our results confirmed phototactic tail withdrawal in the previously studied *Aipysurus laevis*, revealed this trait for the first time in *A. duboisii* and *A. tenuis,* and suggested that tail photoreceptors have peak spectral sensitivities between blue and green light (457–514 nm). Based on these results, and an absence of photoresponses in five *Aipysurus* and *Hydrophis* species, we tentatively infer that tail phototaxis evolved in the ancestor of a clade of six *Aipysurus* species (comprising 10% of all sea snakes). Quantifying tail damage, we found that the probability of sustaining tail injuries was not influenced by tail phototactic ability in snakes. Gene profiling showed that transcriptomes of both tail skin and body skin lacked visual opsins but contained melanopsin (*opn4x*) in addition to key genes of the retinal regeneration and phototransduction cascades. This work suggests that a non-visual photoreceptor (*e.g.* Gq rhabdomeric) signalling pathway underlies tail phototaxis, and provides candidate gene targets for future studies of this unusual sensory innovation in reptiles.

KEYWORDS: extraocular, dermal phototaxis, sea snakes, dermal photoreception, melanopsin

Introduction

Most organisms use non-visual light detection to regulate essential physiological and behavioural functions (Wolken, 1995). Prominent roles of non-visual photoreception include colour changes in the skin that facilitate camouflage, communication or thermoregulation, phototactic orientation and movement, and the circadian and seasonal timing of key biological events (Foster and Soni, 1998; Peirson *et al.*, 2009). Various cephalic or 'extraocular' tissues have been linked to non-visual photoreception, such as the parietal organ and pineal complex (Foster and Soni, 1998). In organisms lacking fur or feathers, the skin also provides a primary site for non-visual photoreception (Kelley and Davies, 2016).

Dermal photoreception or the 'dermal light sense' mediates dermal phototaxis, defined here as the movement, including the whole body or a body part of an organism, towards or away from light (Kelley and Davies, 2016; Millott, 1968; Steven, 1963). This sensory modality is best known among marine invertebrates, of which many species migrate along vertical light gradients and show abrupt withdrawal responses to sudden changes in light intensity (reviewed in Wolken 1988; Ramirez et al. 2011). Among vertebrates, dermal phototaxis have been described in lampreys (Ronan and Bodznick, 1991; Steven, 1950; Young, 1935), hagfish (Patzner, 1978; Steven, 1955), aquatic salamanders, and a single frog (*Xenopus laevis* tadpole) (Alder, 1976; Baker *et al.*, 2015; Pearse, 1910; Reese, 1906; Sayle, 1916). Olive sea snakes (*Aipysurus laevis*) are the only reptiles reported to show dermal phototaxis (Zimmerman and Heatwole, 1990), but this species' phototactic behaviour is strikingly similar to that of the other elongate, aquatic vertebrates.

Sea snakes, lampreys, hagfish and aquatic salamanders all exhibit dermal photosensitivity that is most pronounced at the dorsal tips of their tails and stimulates negative phototaxis. Lamprey larvae and hagfish respond to tail illumination by deflecting their tails, swimming and/or burrowing to conceal themselves in river and lake beds (Binder and McDonald, 2008; Deliagina *et al.*, 1995; Patzner, 1978; Steven, 1955; Ullén *et al.*, 1993; Young, 1935). Resting olive sea snakes and aquatic salamanders respond with localised tail movements, often retracting their tail-paddles under reef or rock overhangs.

The convergent innovation of phototactic tails in elongate aquatic taxa that diverged relatively early in the >400 million-year evolutionary history of vertebrates suggests similar selection for concealment from predators. These selection pressures may be particularly strong in animals with vulnerable hind-bodies and tail paddles that are anatomically remote from the concentration of sensory organs on the head. Sea snakes have various predators, such as sharks and marine mammals, and specimens often have bite injuries to their tails, sometimes resulting in partial loss of the paddle (Heatwole, 1975; Masunaga *et al.*, 2008). Tail paddles are vital to efficient underwater locomotion so tail damage must impact feeding, mating success and vulnerability to

predation (Aubret and Shine, 2008). Zimmerman and Heatwole (1990) demonstrated that captive *A. laevis* sea snakes concealed their tails under artificial reef during daylight more often than night, when tails were more likely to be protruding while the rest of the body was concealed. Hence, phototactic responses are expected to provide protection during daytime (and possibly dim-light) resting periods.

The genetic and physiological mechanisms underlying dermal phototaxis remain largely unknown for any vertebrate taxon (Kelley and Davies, 2016). Hindering research progress is a conspicuous absence of photoreceptive structures such as stacked membranes or lenses within photoreceptive skin (Ramirez *et al.*, 2011). However, gene expression studies have revealed visual opsins in colour-changing cells within the skin of cephalopods (Kingston *et al.*, 2015; Mäthger *et al.*, 2010; Ramirez and Oakley, 2015), teleosts (Ban *et al.*, 2005; Chen *et al.*, 2013; Schweikert *et al.*, 2018) and gekkonid lizards (Fulgione *et al.*, 2014). This shows that the dermal photoreceptors involved in colour change likely evolved by co-opting existing visual photoreceptor pathways of the eye, despite lacking structures found in classical photoreceptors (Kingston and Cronin, 2016; Ramirez *et al.*, 2011). Other studies in teleosts (Bertolesi and McFarlane, 2018) and amphibians (Moriya *et al.*, 1996; Provencio *et al.*, 1998) have identified a role for non-visual opsins in colour change, implying that independent, non-visual photoreceptor pathways underlie dermal photoreception in these diverse taxa.

In this study, we sought to better understand the evolution and molecular basis of tail phototaxis in sea snakes. We first used behavioural tests of tail (caudal) phototaxis in wild-caught sea snakes from eight species with the aim to better resolve the evolutionary origin of the trait. We then screened for candidate phototaxis genes expressed in the skin of two phototactic species. Because the dermal photoreceptive structures and genes involved in phototaxis are entirely unknown, we comprehensively profiled genes related to visual and non-visual photoreceptors in whole transcriptomes of tail and body skin, eye and other available organs. Finally, we quantified injuries on the tails of species with and without phototactic abilities, with the expectation that phototactic species might have lower bite rates indicating greater protection from attacks or have increased bite rates due to an intrinsically higher vulnerability to predation.

Materials and methods

Specimens

Sea snakes are fully marine squamate reptiles that are phylogenetically nested within the Australo-Papuan terrestrial front-fanged snakes (Elapidae: Hydrophiinae). Phototactic responses were measured in 17 wild-caught, captive individuals of eight species that spanned all major lineages of sea snakes: *Aipysurus laevis* (the only sea snake previously tested for phototactic

behaviour), three other *Aipysurus* species, three *Hydrophis* species and one semi-aquatic species *Hydrelaps darwiniensis* (Supplementary materials; Table S5.1; ES File 5.1). Tail injuries were recorded for a total of 111 museum specimens from two phototactic species, *A. laevis* (*n* = 39) and *A. duboisii* (*n* = 12), and two non-phototactic species *H. major* (*n* = 45) and *H. stokesii* (*n* = 15) (ES File 5.2). The examined specimens were chosen from the same collection locality (Gulf of Carpentaria, Queensland, Australia) to minimise the effect of geographic variation in predation pressures; specimen information (snout-vent length, weight, sex and age) was available from (Fry G, personal communication).

Experiments and euthanasia were conducted in accordance with the Animal Ethics Committee of University of Adelaide (S-2015-119) and University of Florida (201502798) and specimens were collected and transported in accordance with Department of Parks and Wildlife of Western Australia licences to take fauna for scientific purposes (Permit #SF010002) and export fauna interstate (Permit #EA007665), Department of Environment, Water and Natural Resources of South Australia import permit (Permit #I12978) and from the Area de Conservación Arenal Tempisque (ACT) del Sistema Nacional de Areas de Conservación (SINAC), Costa Rica (No. ACT-OR-DR-055-17).

Behavioural experiments

Experimental set up

During experiments on *A. duboisii*, *A. laevis, A. tenuis* and *H. major* at the University of Adelaide, a snake was transferred from the seawater holding tank (24−28°C, 450L volume, 35 ppm, 12 h:12 h day: night) to a round, black plastic behavioural arena (60 cm diameter × 60 cm height, 50 L volume) filled with seawater (24−28°C, 35 L volume, 13 cm depth) and covered by a mesh net. The arena was housed in a dark room lit by a single florescent red globe positioned 1 m above the arena (Figure S5.7). A lid was placed over the arena for 1–2 h to allow the snakes to adapt to the arena before initiating trials. Trials were recorded with a camera (GoPro Hero3+, Go Pro Inc., USA; 29.97 fps; 1920 × 1080) positioned above the behavioural arena. During experiments on *A. laevis, A. mosaicus*, *A. tenuis, H. darwiniensis*, *H. major, H. stokesii* and *H. platurus* at field sites, snakes were transferred to a rectangular, black plastic behavioural arena (66 cm length × 44 cm width × 23 cm height, 60 L volume) filled with freshwater (29 L, 10 cm depth) and covered by a mesh net. A lid was placed over the arena as described in experiments at the University of Adelaide, and trials were recorded directly by the observer and (where possible) with a camera (GoPro Hero3+, Go Pro Inc., USA; 29.97 fps; 1920 × 1080) positioned above the behavioural arena.

Light source

The light stimulus was delivered to localised areas of skin (Figure 5.4B) using a hand-held flashlight (UltraFire SH98 3-mode white light zooming, WhaFat Technological, Hong Kong) that incorporated a light-emitting diodes (LEDs) bulb that emitted white light with a spectral range of 300-900 nm. To test phototactic responses to different wavelengths of light, a hand-held flashlight (UltraFire 4-in-1 1-mode light) with interchangeable coloured LED bulbs was used to emit four colours: violet, blue, green and red of wavelengths of 393 nm, 457 nm, 514 nm and 623 nm, respectively. The flashlights were powered by two 7.4 volts rechargeable batteries (Fenix ARB-L3, Fenixlight, USA) that were re-charged after 6−12 trials to maintain a near-constant light output. At the start of each trial, the relative flashlight irradiance was measured using a PM100 digital optical power meter (Thorlabs, USA) and S210A UV-NIR thermal power head held 30 cm below the flashlight. Spectral and relative irradiance measurements are in Supplementary materials (Figure S5.1).

Behavioural trials

Trials commenced after the snake had been inactive for at least 2 min. Experiments consisted of 2−4 sets of six trials, each trial being separated from the next by intervals of at least 1 h. Each snake was subjected to a mean of 17 trials over the course of the experiment. White light was shone on the dorsal surface of the tail skin ($T_{(s)}$) for duration of 5.3 s (± 1.30 s) at a distance of approximately 30cm. To control for the possibility that snakes responded to scattered light reaching the eyes, or the sight or sound of approaching experimenter, a white light was also shone on the dorsal surface of the mid-body skin ($B_{(s)}$) (Figure 5.1; Figure S5.7). Presentation of light was alternated between $T_{(s)}$ and $B_{(s)}$, and the order of presentation was reversed every set of 6 trials, for *A. duboisii* (n = 1), *A. laevis* (n = 4), *A. mosaicus* (n = 1), *A. tenuis* (n = 2), *H. major* (n = 1) and *H. platurus* (n = 2). In separate experiments, white light was presented only on $T_{(s)}$ in a single individual each of *A. laevis, A. tenuis, A. mosaicus, Hydrelaps darwiniensis, H. major* and *H. stokesii* (Table S5.1). *Aipysurus tenuis* (n = 2) appeared to be responsive to illumination of the body in addition to the tail, thus a new experiment was performed to test for phototactic response to body illumination in this species using an individual from *A. laevis* (n = 1) as a control. Experiments consisted of 6 sets of 5 trials in which white light was shone on the dorsal surface of the body at four locations (Table S5.2); light was presented sequentially along the body and the order of presentation was reversed between each trial set. A final experiment was conducted to test for sensitivity to different colours of light (violet, blue, green and red) in *A. tenuis* (n = 2) and *A. laevis* (n = 1).

Consistent with previous behavioural testing (Zimmerman and Heatwole, 1990), a response was considered as 'negative phototaxis' if the part of the skin illuminated moved away from the light within 10 s and no other part of the snake moved. Behavioural responses were converted to

a phototactic score to indicate whether a negative phototaxis occurred (Table 5.1) and mean response per species was calculated as a percentage (%) of trials in which phototaxis was observed. Latency to response was determined by viewing video footage of the phototactic response frame-by-frame (*i.e.* at 33.4 ms intervals) in GoPro Studio software v2.5.12 (CA, USA), and calculated as the difference between the time at which the light stimulus was switched on and the time at which the first phototactic movement of the snake occurred.

TABLE 5.1: Categories of behavioural responses to light on the skin. Phototactic scores were negative phototaxis = 1 and no phototaxis = 0.

Category	*Description of behaviour*	*Phototactic score*
CW	Tail withdraws completely out of light within 10 s and no other part of snake moves	1
W	Tail withdraws away from light within 10 s and no other part of snake moves	1
TL	Tail tilts from dorsal plane to sagittal plane within 10 s and no other part of snake moves	0
TJ	Sudden movement of tail only	0
BJ	Sudden movement of body only	0
B	Body undulates as in swimming movement	0
HT	Head moves to location of tail, tail may or may not withdraw	0
BW	Body withdraws away from light within 10 s and no other part of snake moves	1
NR	No response, body and tail do not change position	0

We mapped phototactic behaviour as a binary character (tail phototaxis absent or present) onto an existing phylogeny for sea snakes (Sherratt *et al.*, 2018). Using these data, the most parsimonious interpretation of the origin of phototaxis in sea snake evolution was inferred by eye based on the assumption that gains and losses of this trait are rare and equally likely.

Transcriptome profiling

Tissue collection

Because the cellular structures responsible for light-sensing in the tails of sea snakes are unknown, we were unable to target specific locations in the skin for differential gene expression. Instead, we sampled the whole skin tissue (dermis, epidermis, beta layer) from three regions (two tail and one body) in two phototactic species and used whole transcriptome profiling to identify phototaxis genes. Seven skin samples were taken for RNA-sequencing: the photoreceptive tail tip of two *A. laevis* and one *A. tenuis*, putatively non-photoreceptive anterior ventral surface of the tail of a single *A. laevis* and *A. tenuis,* and the variably photoreceptive dorsal surface of the hind-

body a short distance anterior of the vent of a single *A. laevis* and one *A. tenuis* (Figure 5.4B). In addition to the skin samples, we assessed the tissue-specificity of expression of genes related to photoreception by also sampling four non-skin tissues available from other projects: whole eye of *A. laevis,* and heart, testis and liver of the olive-headed sea snake *H. major* (Table S5.3).

Details of RNA extraction, sequencing, filtering and assembly

Tissues were homogenised using mortar and pestle in lysis buffer and grinder with liquid nitrogen before extracting total RNA (Roche Tissue RNA extraction kit). Library preparation and transcriptome sequencing for six skin tissues was performed by the Queensland Brain Institute Centre for Brain Genomics (QBI, Brisbane, Australia), for the eye by Beijing Genomics Institute (BGI, Shenzhen, China), and for one skin, testis, heart and liver by Australian Genome Research Facility (AGRF, Adelaide, Australia). Following RNA extraction (Roche Tissue RNA Extraction Kit) and quality control, dual indexed TruSeq libraries were generated and sequenced on an Illumnina HiSeq2000 machine (Illumina Inc., San Diego, CA) using V4 chemistry, producing 125 and 150 bp paired-end sequencing reads.

The quality of the raw reads was assessed using FastQC v0.11.4 (Andrews, 2010), QUAST v 4.5. (Quality Assessment Tool for Genome Assemblies; (Gurevich *et al.*, 2013), and using *ngsReports* v.0.99 (Ward *et al.*, 2018) package in R. v3.4.2 (Team, 2017). Adapter sequences and low-quality reads were trimmed using AdapterRemoval v2.1.7 (Schubert *et al.*, 2016) applying default quality parameters and a minimum sequence length of 20 bp. To reconstruct transcriptomes, *de novo* assembly was carried out the Trinity v2.5.1 pipeline (Grabherr *et al.*, 2011; Haas *et al.*, 2013) with default settings and a minimum contig length of 200 bp. Following transcript assembly, protein-coding regions were determined using TransDecoder v3.0.1. (Haas *et al.*, 2013). Finally, assemblies were assessed for completeness, both by assessing the RNA read representation of the assemblies by aligning the trimmed reads back to their respective assemblies using Bowtie2 v2.2.9 (Langmead and Salzberg, 2012) and by examining the presence of full-length protein-coding genes in the assemblies by searching against the SwissProt protein databases (The UniProt Consortium, 2017) using BLAST+ (Camacho *et al.*, 2009).

Unsupervised clustering of tissue samples was carried out using multi-dimensional scaling plots in R v3.4.2 (R Core Team, 2017) using the *edgeR* package v3.20.1 (Robinson *et al.*, 2009) and log counts per million, with gene selection set to 'pairwise' for the top 500 genes. The intersections of expression levels among tissue samples were explored using the *UpSetR* package v.1.3.3 (Lex *et al.*, 2016).

TABLE 5.2. Statistical summary of sequencing.

Species	*ID*	*Phototactic region*	*Paired end raw reads*	*% Removed*	*Trimmed reads*	*Reads mapped*	*% Align*
Aipysurus laevis	KLS0459	Eye	41,960,313	0	41,960,313	37,485,594	89.3
		Skin photoreceptive tail tip (dorsal)	30,343,697	37.9	18,839,010	18,377,480	97.6
	KLS0656	Skin photoreceptive tail tip (dorsal)	16,098,369	43.6	9,082,453	8,060,311	88.7
		Skin non-photoreceptive tail (anterior)	17,696,227	28.8	12,601,077	11,943,910	94.8
		Skin non-photoreceptive body near vent (dorsal)	27,845,728	36.8	17,588,152	16,791,799	95.5
Aipysurus tenuis	KLS0654	Skin photoreceptive tail tip (dorsal)	20,397,990	52.0	9,798,407	8,841,047	90.2
		Skin non-photoreceptive tail (anterior)	31,707,833	32.2	21,486,415	20,745,575	96.6
		Skin photoreceptive body near vent (dorsal)	16,828,408	32.1	11,428,795	10,375,249	90.8
Hydrophis major	KLS0460	Heart	31,189,072	41.0	18,392,676	17,928,884	97.5
		Liver	31,187,724	39.8	18,782,654	18,265,069	97.2
		Testis	27,457,192	40.4	16,371,706	15,700,688	95.9

Abundance estimates of genes

Estimated transcript abundances were generated using Salmon v8.2 (Patro *et al.*, 2017), a pseudo-alignment program that quantifies gene expression without the need for direct genome alignments. RNA reads were mapped to a pitviper (*Protobothrops mucrosquamatus*) transcriptome (Aird *et al.*, 2017), which was the best-annotated and closely related transcriptome currently available, and quantified reads were normalised using fragments per kilobase of transcript per million mapped reads (FPKM). To compare transcript abundance of genes related to photoreception among tissue samples, FPKM counts were filtered by reference sequence gene categories (O'Leary *et al.*, 2016) for predicted mRNA that are known to be involved in phototransduction and retinoid metabolism pathways of squamate reptiles (Schott *et al.*, 2017) (Table S5.4). FPKM counts (Table S5.5) for visual genes were then log-transformed and a heatmap generated in R using the *pheatmap* package v1.0.8 (Kolde, 2012) (ES File 5.3).

Verifying the presence of genes related to visual and non-visual photoreceptors

Many vertebrate visual genes are part of large gene families that have high sequence similarity but include genes with non-visual functions (Porter *et al.*, 2011). To verify the sequence identity of quantified transcripts with putative visual functions, we assessed the phylogenetic position of

assembled sequences within maximum likelihood trees of visual genes from representative vertebrate groups (*Python molurus, P. mucrosquamatus, Thamnophis sirtalis, Pogona vitticeps, Gekko japonicus, Anolis carolinensis, Homo sapiens*). Transcripts nested within clades of vertebrate visual genes were considered to be verified visual genes. Conversely, if transcripts were recovered inside a clade of related genes with non-visual functions, these were considered to be erroneously mapped reads and indicated as such on the FPKM heatmap. Briefly, putative visual transcripts were located by custom nucleotide BLAST searches (Altschul *et al.*, 1990) of assembled tissue transcriptomes (ES File 5.4) with visual genes from representative vertebrate groups: squamates (*P. molarus, P. vitticeps, T. sirtalis, G. japonicus*), birds (*Gallus gallus*) and mammals (*Homo sapiens*), obtained from GenBank (Coordinators, 2016). Significant nucleotide BLAST search hits (E-value < 1e-02; bit score > 200) were extracted from transcriptomes and aligned with representative vertebrate visual genes in Geneious v9.1.8 (Kearse *et al.*, 2012) using a MUSCLE translation alignment v3.4 (Edgar, 2004). Aligned sequences were checked for ambiguities and a maximum likelihood tree for each gene was built using RAxML v7.2.8 (Stamatakis, 2006). We used an unpartitioned GTR GAMMA substitution model and the "rapid bootstrapping and search for best-scoring ML tree" algorithm with 1000 replicates. Trees were rooted by con-familial genes or, if tree was for a single gene only, a mammal gene sequence.

Quantifying tail injuries

Tail condition was recorded in sea snake museum specimens (Figure S5.8). To evaluate the prevalence of tail injuries among the sampled species we used a hurdle model to examine 1) the presence of tail damage, and conditional upon damage occurring, 2) the number of tail injuries. The presence of damage was modelled assuming a binomial variance and logit link, and the count of tail injuries component of the model assumed a truncated Poisson variance and log link. We included the interaction between snout-vent length (cm) and species in our models because older (typically larger) snakes are expected to have more tail injuries and this relationship may differ between species. Snout-vent length (svl) was mean-centred for analysis. Other explanatory variables (sex, weight) were assessed using likelihood ratio tests. The likelihood of tail damage seen in non-phototactic species (*H. major* and *H. stokesii*) was compared to that observed in phototactic species (*A. laevis* and *A. duboisii*) using planned contrasts (Torsten *et al.*, 2008). These analyses were conducted in R using additional packages *multcomp* v.1.4.8 (Bretz and Westfall, 2014) and *countreg* v.0.2 (Zeilies *et al.*, 2008).

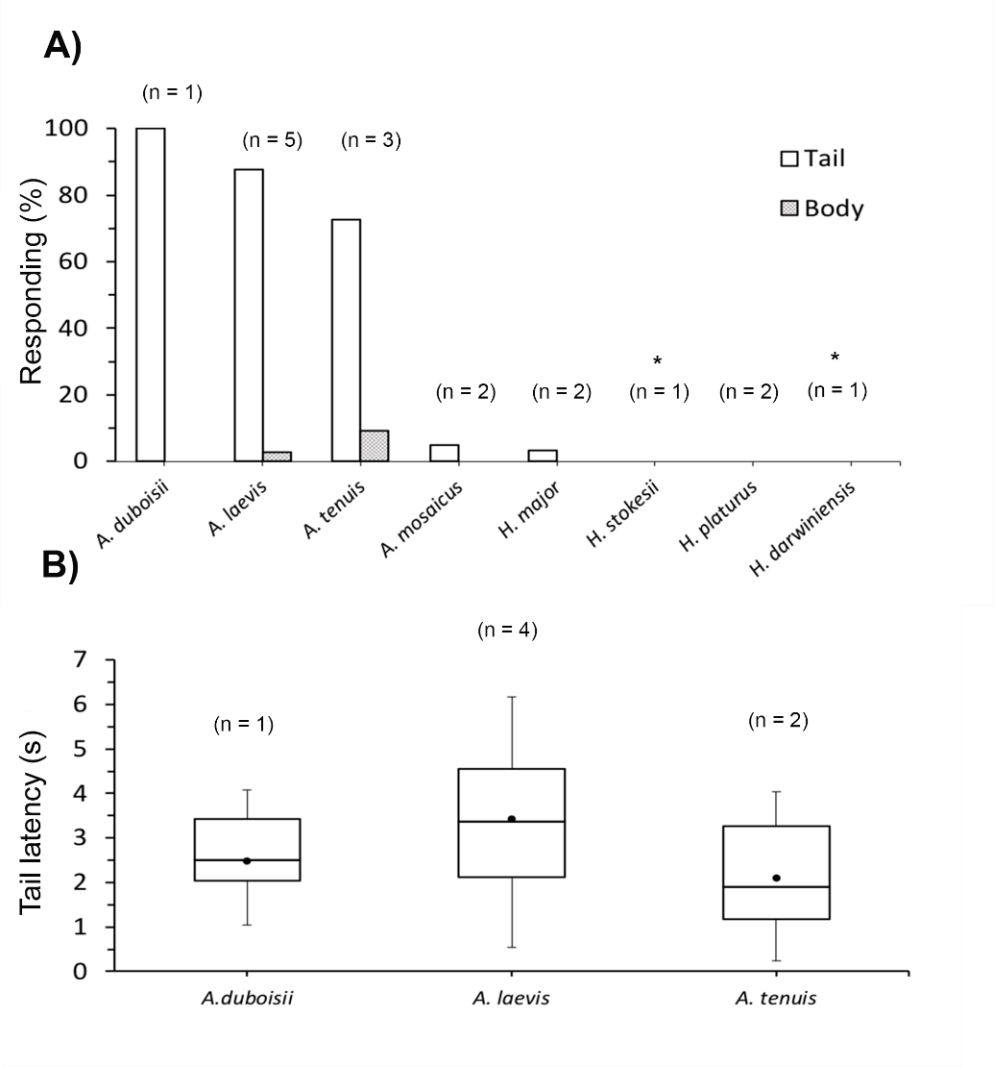

FIGURE 5.1. Negative phototaxis in response to white LED light on the dorsal surface of skin in sea snakes. 'Negative phototaxis' was recorded if the illuminated region moved away from the light within 10 s and no other part of the snake moved. A) Response (%) to light on tail skin and body skin in eight species; asterisks indicate species in which light was shone on the tail skin only. B) Tail latency in the phototactic species; box plots represent median (middle solid horizontal line), mean (black dots) and range (dotted line) of latencies across a mean of 6 trials per individual.

Evidence for dermal phototaxis in eight species and evolutionary origin in sea snakes

Our behavioural tests provided evidence of negative tail phototaxis in all individuals of *Aipysurus laevis, A. tenuis* and *A. duboisii* (tail withdrawals in response to white LEDs, 100%, 87% and 70% of trials, respectively), but not in any of *A. mosaicus* (5%), *Hydrophis major* (3.2%), *Hydrelaps darwiniensis*, *Hydrophis stokesii* or *H. platurus* (Figure 5.1A). Consistent with previous observations in *A. laevis* (Zimmerman and Heatwole, 1990), tail phototaxis in individuals of *A. laevis, A. duboisii* and *A. tenuis* was a stereotyped movement of the tail towards the centre of body mass and away

from the light stimulus (Table 5.1; ES File 5.5). Tail latencies recorded for the three phototactic species showed that tails moved within 7 seconds (s) of illumination. Mean response times were 2.1 s for *A. tenuis*, 2.5 s for *A. duboisii* and 3.4 s for *A. laevis* (Figure 5.1B), and the shortest tail latencies recorded for each species were 0.25 s for *A. tenuis*, 0.55 s for *A. laevis* and 1.05 s for *A. duboisii* (Figure 5.1B). To control for the effects of the experimenter and to test for phototactic responses to scattered light reaching the eyes, trials of tail response were alternated with trials of white light shone on the mid-body (instead of tail). This was done for 11 individuals of six species and yielded no phototactic responses to mid-body illumination in *A. duboisii, A. mosaicus*, *H. major* and *H. platurus*, and low response rates in *A. tenuis* (9% of 11 trials, $n = 2$) and *A. laevis* (2.8% of 36 trials, $n = 5$) (Figure 5.1A).

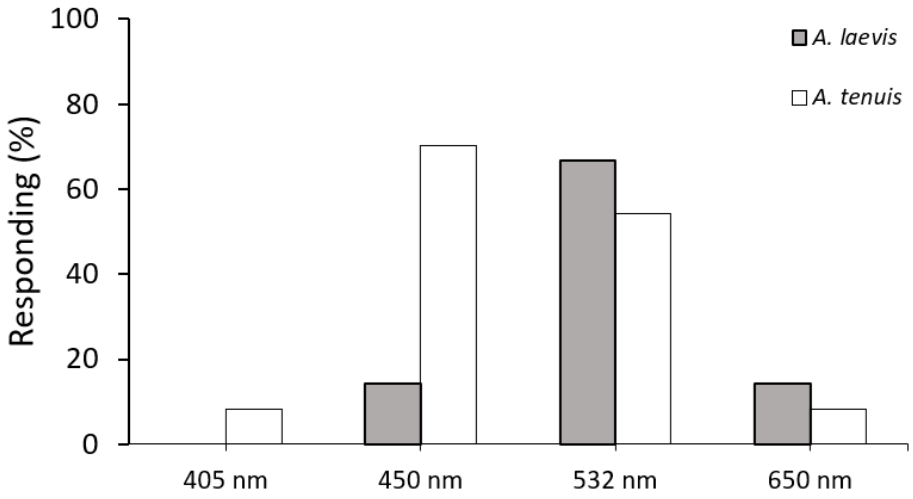

FIGURE 5.2. Negative tail phototaxis in response to four coloured LED lights, violet (392 nm), blue (347 nm), green (514 nm) and red (623 nm), in three captive individuals from two species, *Aipysurus laevis* (n = 1) and *A. tenuis* (n = 2), across a mean of 6 trials per individual. 'Negative phototaxis' was recorded if the illuminated region moved away from the light within 20 s and no other part of the snake moved.

To test preliminary observations of phototactic responses to hind-body illumination in *A. tenuis*, a separate experiment was performed on *A. tenuis* ($n = 2$) and *A. laevis* ($n = 1$). Here, phototaxis was also recorded in response to white LED light shone on four dorsal regions along the body axis. *Aipysurus tenuis* showed a stereotyped withdrawal (movement of the illuminated part of the body towards the centre of body mass and away from the light stimulus; ES File5.6) in response to illumination of the dorsal skin on the hind-body (pre-vent, 66.7% of 12 trials), posterior mid-body (16.7% of 12 trials), anterior mid-body (16.7% of 12 trials) and neck region (22.1% of 13 trials), but at a comparatively lower rate compared to tail illumination (100% of 12

trials; ES File 5.6). In contrast, no phototactic responses were recorded in any of the four regions of body skin in *A. laevis*.

For phototactic species *A. tenuis* and *A. laevis,* we compared latencies of responses to tail illumination from four different wavelengths of light produced by LEDs having approximately equal intensities. The results suggest peak sensitivities of tail photoreceptors between 457 and 514 nm (Figure 5.2); but this pilot experiment did not allow us to generate full response curves for spectral sensitivity or latency of tail movement because only four wavelengths were tested. Relative irradiance measurements for the white, violet, green, blue and red light are shown in Figure S5.1.

Based on the results of our behavioural tests, and an expectation that evolutionary gains and losses of phototaxis are rare and equally likely, the most parsimonious inference is that this sensory modality evolved in the ancestor of a clade of six *Aipysurus* species: *A. laevis, A. fuscus, A. tenuis, A. duboisii, A. foliosquama* and *A. apraefrontalis* (Figure 5.3). An alternative scenario under which phototaxis evolved in the common ancestor of all *Aipysurus* and was lost on the lineage leading to *A. mosaicus* involves one additional step. Hence, pending future studies of additional *A. mosaicus* individuals and key taxa such as *Emydocephalus* and *Ephalophis-Parahydrophis* (indicated by asterisks on Figure 5.3), we tentatively infer a single origin of phototaxis within *Aipysurus*, and an absence of this trait in all other sea snakes (*i.e.* 90% of species, including the ~50 *Hydrophis* species).

TABLE 5.3. Summary statistics of Trinity assemblies.

Specimen			*Contigs*	*Length*			*Contigs >= 1000 bp*		*Contigs >= 5000 bp*		*Contigs >= 10000 bp*		
Spp.	ID	Tissue	No. assembled	Total length (bp)	Longest contig (bp)	N50 contig length (bp)	No.	%	No.	%	No.	%	GC %
A. laevis	KLS0459	Eye	230,892	80,614,082	18,092	2,806	24,256	10.5	2,721	1.2	130	0.06	42.3
		Skin photoreceptive tail tip (dorsal)	130,893	70,608,010	21,767	2,164	24,074	18.4	1,163	0.9	21	0.02	42.7
	KLS0656	Skin photoreceptive tail tip (dorsal)	49,779	5,155,121	8,160	814	1,223	2.5	11	0.0	0	0.00	43.6
		Skin non-photoreceptive tail (anterior)	90,353	23,528,511	10,860	1,121	7,786	8.6	50	0.1	2	0.00	44.7
		Skin non-photoreceptive body near vent (dorsal)	159,456	63,237,696	14,787	1,784	21,986	13.8	708	0.4	20	0.01	44.4
A. tenuis	KLS0654	Skin photoreceptive tail tip (dorsal)	76,189	7,419,783	8,560	780	1,643	2.2	4	0.0	0	0.00	42.7
		Skin non-photoreceptive tail (anterior)	118,552	44,671,944	10,895	1,478	16,550	14.0	144	0.1	4	0.00	44.4
		Skin photoreceptive body near vent (dorsal)	92,552	14,741,471	10,723	892	4,120	4.5	21	0.0	2	0.00	44.4
H. major	KLS0460	Heart	124,657	68,064,584	51,061	1,995	23,763	19.1	820	0.7	18	0.01	42.9
		Liver	125,968	49,361,792	19,451	1,595	18,496	14.7	178	0.1	8	0.01	42.8
		Testis	200,649	92,040,799	11,282	1,743	32,250	16.1	784	0.4	5	0.00	43.1

Expression of genes related to visual and non-visual photoreceptors

Assembled transcriptomes for sea snake eye, heart, liver, testis and seven skin tissues were profiled for genes relating to visual and non-visual photoreceptors (Table S5.3; Table S5.4). Five vertebrate phototransduction genes were not detected in the eye transcriptome (*sws2*, *rho2, grk1, gnat1, gucy2F*, *pde6a, pde6h*). This is consistent with previous genomic and transcriptomic studies that suggest these genes are missing in snake genomes, hence they were not profiled in the remaining tissue transcriptomes. Summary statistics for sequencing, assembly and transcript completeness are given in Table S5.2, Table S5.3 and Figure S5.2 of the Supplementary materials. Multidimensional scaling plots and overall expression profiles for tissue transcriptomes are also given in Supplementary Results (Figure S5.3-5).

Opsins

Three genes encoding for visual opsins (*opn1sw, rho1, opn1lw*) were detected in *A. laevis* eye, and a single visual opsin (*opn1sw*) was detected in *H. major* testis (Figure 5.4). Genes for two non-visual opsins were also expressed: *xenopus-like* melanopsin (*opn4x*) was detected in *A. laevis* eye, *H. major* testis and two skin transcriptomes each from *A. laevis* and *A. tenuis*. Neuropsin (*opn5*) was expressed in *A. laevis* eye, *H. major* testis and a single skin transcriptome from *A. laevis* (Figure 5.4).

Phototransduction

A total of 24 genes related to phototransduction in visual photoreceptors of vertebrates (*i.e.* ciliary genes) were detected in the *A. laevis* eye, 17 in *H. major* testis, nine in *H. major* heart, seven in *H. major* liver and 13 across *Aipysurus* skin tissues (Figure 5.4). There was no discernible co-expression pattern between putatively photoreceptive skin and variably or non-photoreceptive skin (Figure 5.4). Phototransduction genes detected in the majority (four or more) of skin tissue samples were *arrb2, gna11, guca1b, pdc-like, pdc-likeb1, pdc-likeb3, pde6d,* and *pde6g* (Figure 5.4). Genes *grk7-like* and *grk5* were detected in a single skin transcriptome each from *A. laevis* and *A. tenuis,* and *sag* in a single skin transcriptome from *A. tenuis* (Figure 5.4). Phototransduction genes *grk5, guca1a/c, pdc, gnat2*, *sag,* and *rcvrn* were detected in the skin using FPKM levels, but gene tree analyses indicated that these are most likely homologous with *grk5-like*, *guca1b, pdc-like2/3, gnai2/3, arrestin C-like*, and *hippocalcin-like* (*hpcl-like),* respectively (ES File5.7). The following 11 phototransduction genes were not detected in skin transcriptomes: *cnga3, cngb1, cngb3, gnat2, guca1a, guca1c, gucy2d-like, pdc, pdc-like2, pde6b-like, pde6c* and *slc24a2* (Figure 5.4; ES File5.7). Genes related to phototransduction in non-visual photoreceptors (*e.g.* intrinsically-photoreceptive retinal ganglion cells; ipRGCs) and invertebrate visual photoreceptors (*i.e.* rhabdomeric genes), *gna11,*

plcb1, *plcb3*, *plcb4,* were also profiled and found to be widely expressed across all organs including skin (data not shown).

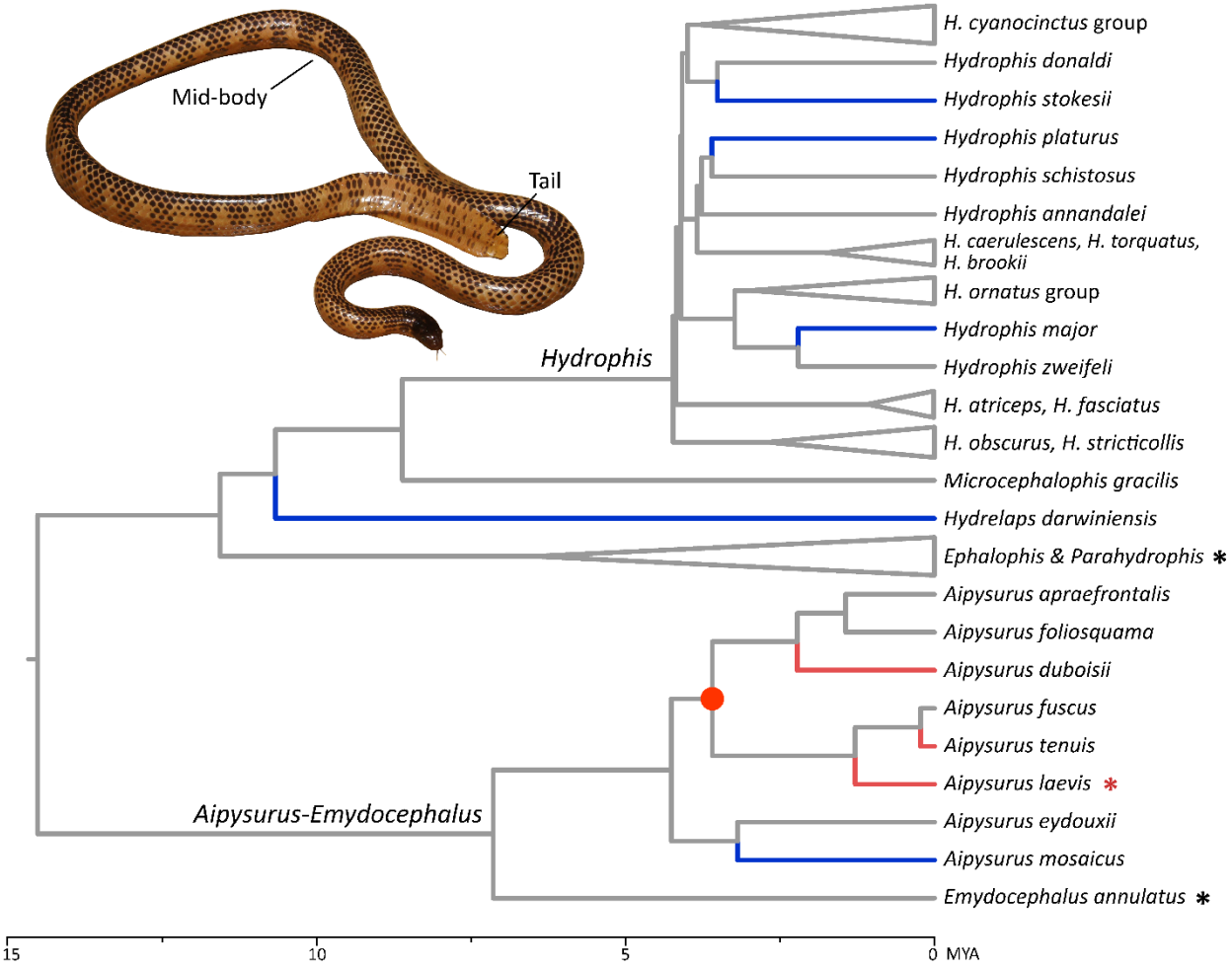

FIGURE 5.3. Phylogenetic tree of sea snakes showing distribution of tail phototaxis: red branches represent species that showed phototactic responses to localised white light on the tail but not the mid-body, blue branches represent species that were unresponsive to localised white light on both the tail and mid-body, and untested species are shown as grey branches. Based on these currently available data (17 individuals of 8 species), the most parsimonious inference is that tail phototaxis evolved in the ancestor of a clade of six *Aipysurus* species (the node marked with a red dot). The only previously studied species, *Aipysurus laevis,* is indicated by red asterisk. Tree modified from Sherratt et al., (2018); legend is in millions of years ago (MYA); image of *Aipysurus tenuis* shows regions that were tested for phototactic responses, taken with permission from Mirtschin, Rasmussen, & Weinstein (2017).

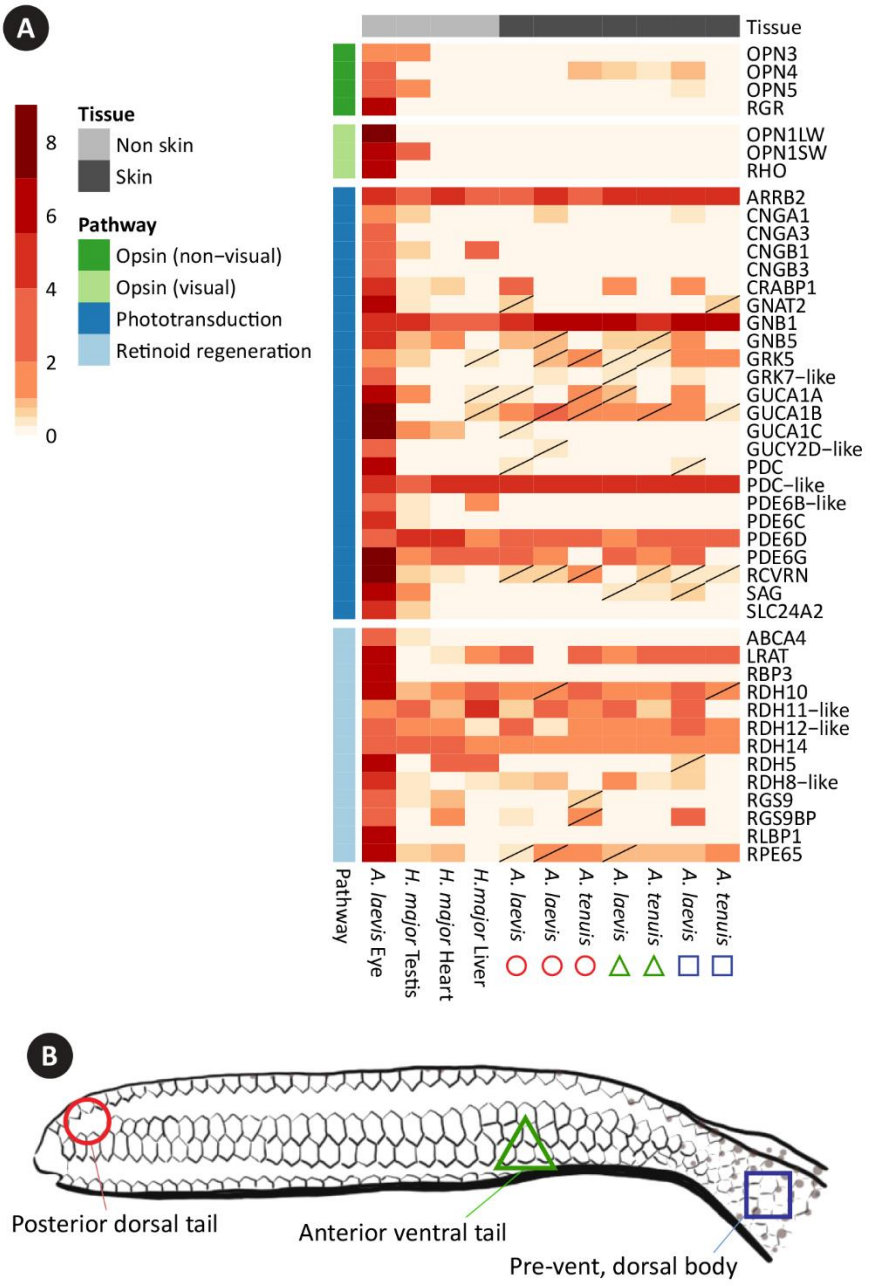

FIGURE 5.4. Gene profiling of tissue transcriptomes from *Aipysurus laevis, A. tenuis* and *Hydrophis major* sea snakes. A) Heatmaps show normalised expression levels of genes for visual pigments (opsins), phototransduction cascades related to visual photoreceptors and retinoid regeneration. RNA reads were quantified by pseudoalignment to a pitviper (*Protobothrops mucrosquamatus*) transcriptome; fragments per kilobase of transcript per million mapped reads (FPKM) were log-transformed for visualising in heatmap; strikethrough cells indicate transcripts whose visual function could not be verified by nucleotide BLAST searching and phylogenetic analysis (ES File 5.7). B) Schematic diagram of tail showing where skin tissues were collected from phototactic species (*A. laevis* and *A. tenuis*). Putative dermal light sensitivity is indicated by red circle (photoreceptive), green triangle (non-photoreceptive) and blue square (photoreceptive in *A. tenuis* only). Diagram modified from Zimmerman and Heatwole (1990).

A total of 13 genes related to retinoid regeneration were detected in the *A. laevis* eye, eight in *H. major* testis, nine in *H. major* heart, seven in *H. major* liver, and eight across *Aipysurus* skin tissues (Figure 5.4). There was no discernible co-expression pattern between putatively photoreceptive skin and variably or non-photoreceptive skin (Figure 5.4). Genes detected in the majority (more than four) skin tissues were *lrat, rdh8-like, rdh10, rdh11-like, rdh12-like, rdh14,* and *rpe65*, and the gene *rgs9bp* was detected in two skin tissues from *A. laevis* (Figure 5.4). The identity of some retinoid regeneration genes that were detected in the skin tissues using FPKM levels (*rhd5* and *rgs9*) could not be verified by custom nucleotide BLAST searches and phylogenetic analysis (ES File5.7). The following retinoid regeneration genes were not detected in skin transcriptomes: *abca4, rbp3, rdh5, rgr and rgs9*.

The relationship between tail damage and phototactic ability

Phototactic species *A. laevis* and *A. duboisii* had slightly higher proportions of damaged tails (67% and 58 %, respectively) compared to non-phototactic species *H. major* (47%) and *H. stokesii* (40%) from the same geographic location (Gulf of Carpentaria, Australia) (Table S5.6). We predicted that phototactic ability (*i.e.* species) would explain differences in the likelihood of tail damage occurring. However, there was no effect of species on likelihood of tail damage ($\chi^2 = 2.5$, $P = 0.47$; Table S5.7). There was a positive relationship between body length (measured from the snout to the vent: svl) and the probability of tail damage; 10cm increases in svl nearly doubled the likelihood of tail damage (1.97-fold increase, 95% confidence interval 1.26-3.27; $\chi^2 = 10.9$, $P = 0.001$; Table S5.7; Figure S5.6). This relationship was consistent across all the species sampled (*i.e.*, no species *svl interaction; $\chi^2 = 1.4$, $P = 0.69$). We therefore found no evidence for our *a priori* hypothesis of differences in the likelihood of tail damage between non-phototactic (*H. major* and *H. stokesii*) and phototactic species (*A. laevis* and *A. duboisii*; Table S5.8). Furthermore, conditional on damage occurring, there was no evidence for differences in the number of injuries between species ($\chi^2 = 5.4$, $P = 0.14$) or associated with size (*i.e.*, svl; $\chi^2 = 0.05$, $P = 0.9$; Table S5.7).

Discussion

Our study presents substantial new data on a novel sensory trait that is underexplored in vertebrates. The difficulties inherent in collecting and housing live sea snakes meant that we were unable to extensively replicate behavioural experiments. However, our tests of 17 individuals of eight species yielded highly consistent results with low variability both within individuals and among individuals within species. These results confirm the phototactic ability of the only previously studied species of reptile (the olive sea snake, *Aipysurus laevis*) and reveal this trait for

the first time in *A. duboisii* and *A. tenuis* (Figure 5.1; ES File5.5). We recorded phototactic responses of the hind-body in *A. tenuis* that have not previously been reported and may be linked to the elongate body form of this species (thus increased distance between the hind-body and cephalic sensory organs). All other species tested showed little or no response to light on the body skin, which suggests that photoreceptive regions are primarily located in the tail skin.

We found that snakes were most responsive to blue and green light, and least responsive to violet and red. Considering the narrow bandwidth and the approximately balanced in light output (at least, in energy terms) of the colored LEDs, we suggest that dermal photoreceptors have spectral sensitivities between 457 and 514 nm (the peaks of the blue and green LEDs). Such a spectral location is consistent with the spectral sensitivities of other dermal photoreceptors such as chromatophores in cephalopods (470–480 nm; Ramirez and Oakley 2015) as well as that of our candidate non-visual opsins (*e.g.* melanopsin; Díaz et al. 2016; Bertolesi and McFarlane 2018). However, our pilot experiment lacks the necessary spectral resolution that would allow us to distinguish melanopsin-based photoreception, with a peak sensitivity typically around 480nm, from that of rhodopsins (with peak sensitivities generally around 500 nm). Latencies recorded for sea snake tails were comparable to hagfish and lampreys, *i.e.* between one and six seconds (Newth and Ross, 1954; Steven, 1955).

Based on an expectation that losses and gains of phototaxis are rare, we offer a preliminary hypothesis that this sensory modality originated in the ancestor of a clade of six *Aipysurus* species (Figure 5.3). To better resolve the origin of phototaxis, future studies will be needed to increase individual sampling (particularly of putatively non-phototactic *Aipysurus*, *i.e.* the *A. mosaicus* species complex), and target key lineages such as *Emydocephalus* and *Ephalophis-Parahydrophis*. Nevertheless, the absence of phototactic responses in six individuals from four species that are widely distributed in the large *Hydrelaps-Hydrophis* clade suggests that most of the >60 known sea snake species lack phototactic tails, prompting the question of why only some sea snakes have evolved (or retained) this sensory behaviour.

Numerous species traits must influence vulnerability to predation and/or the locomotory costs of tail damage, including diel and spatial activity patterns, preferred habitat and depth, and size of body and tail. *Aipysurus* species have smaller geographic ranges and stronger patterns of mitochondrial geographic structure compared to *Hydrophis* (Nitschke *et al.*, 2018); these observations indicate lower dispersal propensities in *Aipysurus*, which might result in slower swimming speeds and thus stronger selection for strategies for crypsis such as tail phototaxis. However, there is no particular trait, or combination of traits, that solely characterizes the species shown (or inferred) in our study to have phototactic tails. All sea snakes have paddle-shaped tails used for locomotion, and all species are active foragers that rest at times during the day, often

under coral or rocky overhangs (Rasmussen *et al.*, 2011). Furthermore, we found no difference in the likelihood of tail damage or in the total number of injuries sustained by phototactic *A. laevis* and *A. duboisii* compared to the non-phototactic *H. stokesii* and *H. major,* which suggests that there is no intrinsically higher (or lower) vulnerability to predation in the phototactic populations sampled. The *Aipysurus-Emydocephalus* and *Hydrophis* clades, however, show notable differences in their adaptations to marine habits, including use of different tissues to seal the mouth and different vertebral processes to support their tail paddles (Sanders et al. 2012). Hence, a recent origin of tail phototaxis in just the *Aipysurus-Emydocephalus* clade might be best explained by historical contingency, rather than an absence of similar selection pressures in other sea snakes.

Candidate genes underlying dermal phototaxis

The conspicuous absence of classical visual photoreceptor structures in the skin of phototactic sea snakes, lampreys, hagfish and aquatic amphibians poses a significant challenge to research on vertebrate dermal photoreception. Based on our expectation that tail phototaxis could be mediated by independent or novel genetic pathways, we decided to screen whole skin transcriptomes for genes related to visual and non-visual photoreceptors. This approach yielded genes of interest in the eye of sea snakes and low expression abundance and variably non-specific patterns of expression across tissue types (Figure 5.4; Table S5.5). Below we discuss a putative role for these candidate genes in a non-visual photoreceptor pathway in sea snake skin.

Light detection pathways begin with light-absorbing pigments such as the visual opsins that are expressed in the classical retinal photoreceptors, rods and cones, and are also implicated in dermal photoreception in cephalopods (Kingston and Cronin, 2016; Ramirez and Oakley, 2015), teleosts (Chen *et al.*, 2013; Schweikert *et al.*, 2018) and gekkonid lizards (Fulgione *et al.*, 2014). Absorption of light by opsins initiates a complex phototransduction cascade in which the chromophore retinaldehyde (vitamin A) bound with the opsin must photoisomerize from a *cis* to an *all-trans* conformation. In visual opsin systems, photoisomerization then activates phosphodiesterase-6 (PDE6) through coupling with a heterotrimer G protein 'transducin' (GNAT), producing a hyperpolarising current by the opening of cyclic-nucleotide gated channels (CNG) in the photoreceptor membrane (Figure 5.5A).

As expected from transcriptomic and genomic studies of vision in snakes, several phototransduction genes (*grk1, gnat1, gucy2F*, *pde6a, pde6h*) and two visual opsin genes (*sws2* and *rho2*) were absent in the *A. laevis* eye transcriptome (Bhattacharyya *et al.*, 2017; Davies *et al.*, 2009; Hart *et al.*, 2012; Hauzman *et al.*, 2017; Schott *et al.*, 2017, 2015; Simões *et al.*, 2016). All three of the visual opsins found in snakes *(opn1lw, opn1sw* and *rho1*) were detected in the *A. laevis* eye, but none were detected in the skin of *A. laevis* or *A. tenuis* (Figure 5.4). Consistent with the absence of a visual opsin to absorb light, only a few vertebrate phototransduction genes were present in the

skin, and together these genes form an incomplete phototransduction cascade for image-forming vision (Figure 5.5A). Importantly, we did not detect transcripts for GNAT (*gnat2*), PDE6 rod-specific units (*pde6b*) and regulator of G-protein signalling 9 (*rgs9*). However, 13 visual phototransduction genes were found to be expressed in the skin of sea snakes (Figure 5.4), providing to a shortlist of genes that might be involved in independent, non-visual photoreceptor pathways (Figure 5.5).

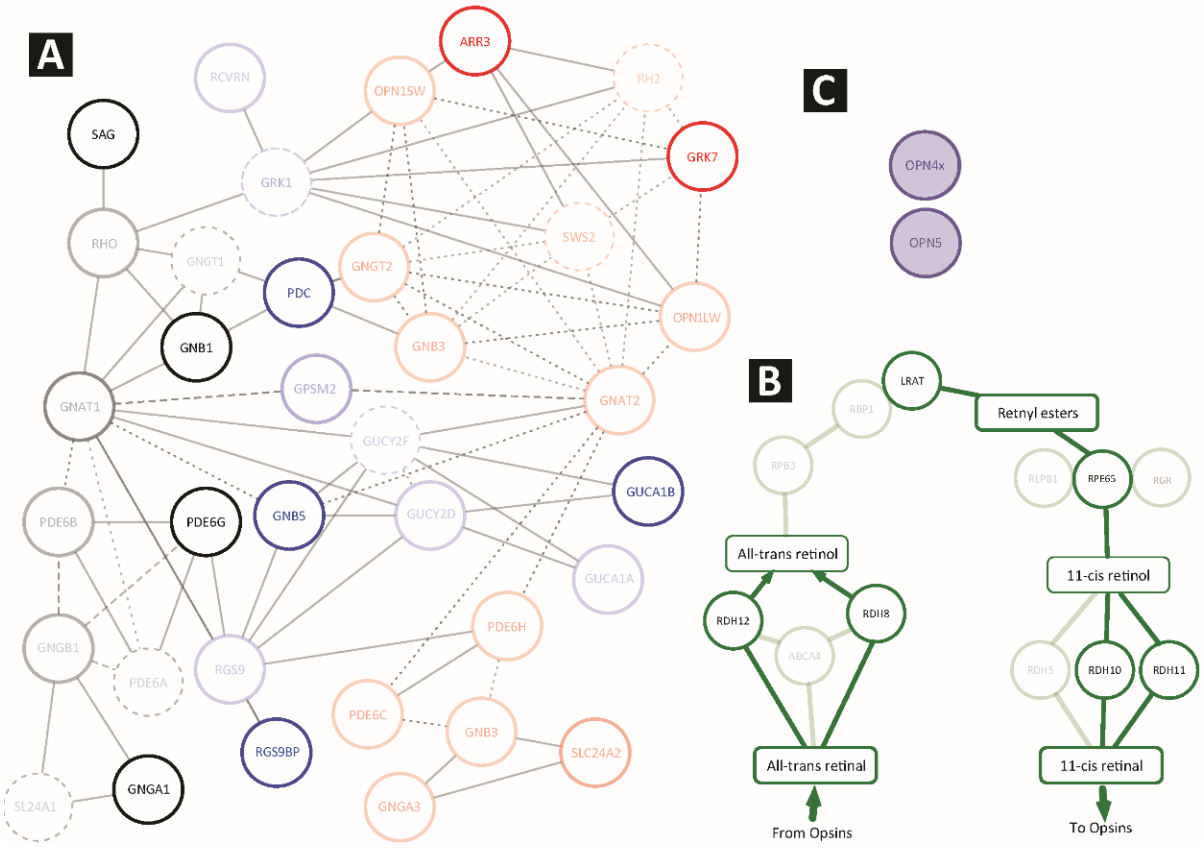

FIGURE 5.5. Visual and non-visual phototransduction pathways; highlighted genes are expressed in the sea snake skin tissue. A) Vertebrate phototransduction pathways specific to rod photoreceptors (black circles), cone photoreceptors (red circles) and both rods and cones (blue circles). Genes absent in snake genomes are also indicated (dashed line) and the visual genes present in eye but absent in skin transcriptomes are faded. B) The retinoid regeneration pathway (green circles). C) Non-visual opsins found in both putative phototactic and non-phototactic sea snake skin (shaded purple). Diagrams modified from Fu (2015); Invergo, Montanucci, Laayouni, & Bertranpetit (2013); Saari (2012).

We detected in the skin two candidate light-absorbing pigments for initiating tail phototaxis in sea snakes: 'xenopus-like' melanopsin (*opn4x)* and neuropsin *(opn5)* (Figure 5.5C) are vertebrate genes associated with a range of non-visual protein functions and patterns of tissue-specific expression. Neuropsin is present in the brain and skin of vertebrates, and is thought to play a role in retinal photoentrainment, changes of skin colour in fish (Buhr *et al.*, 2015; Schweikert *et al.*, 2018) and dermal phototaxis in *Xenopus* tadpoles (Currie *et al.*, 2016). The 'mammal-like' class of

melanopsin (*opn4m*) is present in the ipRGCs of the eye (Bellingham *et al.*, 2006; Provencio and Warthen, 2012) and some cranial nerves (Matynia *et al.*, 2016), and has a range of photosensory functions including photoentrainment of molecular clocks, local pupil light reflex, DNA repair and melatonin synthesis (reviewed in Peirson et al. 2009; Bertolesi and McFarlane 2018). The role of *opn4x* is understudied but it is expressed in a wide range of tissues including the brain and eye of fish, amphibians, reptiles, turtles and birds (reviewed in Davies et al. 2014). Because *opn4x* is expressed in dermal melanophores of fish and amphibians (Bertolesi and McFarlane, 2018; Oshima, 2001; Provencio *et al.*, 1998) and neuromasts of the lateral line system in *Xenopus* tadpoles (Baker *et al.*, 2015) it is a good candidate pigment for non-visual light detection pathways in non-mammalian vertebrates such as sea snakes (Kelley and Davies, 2016).

The pathways interacting with *opn4x* are incompletely known, but the gene is similar in DNA sequence and function to opsins that use phototransduction pathways of invertebrate photoreceptors (*i.e.* rhabdomeric) (Díaz *et al.*, 2016; Graham *et al.*, 2008; Isoldi *et al.*, 2005). Following photoisomerization, melanopsin is thought to activate a G-protein Gq/11 (GNAQ / GNA11) and phospholipase C (PLC) second messenger cascade, producing depolarizing currents by the activation of TRP-like channels (TRP) (Díaz *et al.*, 2016). We detected genes that encode the primary proteins in the putative melanopsin pathway, GNAQ (*gnaq*) and PLC beta (*plcb1*, *plcb3, plcb4),* across a range of sea snake tissues including skin (data not shown), suggesting that some type of Gq rhabdomeric signalling pathway is possible for melanopsin-based dermal photoreception in sea snakes. However, these genes are also integral to a range of cellular pathways, so further molecular studies are needed to confirm their role in tail phototaxis. If *opn4x* is indeed responsible for mediating phototaxis in sea snakes, previous studies of *opn4x* expression (Baker et al., 2015; Davies et al., 2014; Provencio et al., 1998) would suggest three candidate cell types that may be associated with dermal photoreceptors: 1) dermal melanophores involved in colour change, 2) dermal mechanoreceptors, and 3) peripheral nerve endings in the epidermis that may or may not be associated with dermal mechanoreceptors. Given that dermal phototaxis is not linked to colour change in sea snakes, we suggest that future studies are most likely to find photoreceptive structures in either peripheral nerve endings in the epidermis or dermal mechanoreceptors, or a combination of both.

The phototransduction cascade is completed with the regeneration of *all-trans*-retinaldehyde to supply new *cis*-retinaldehyde to the opsin, which involves retinal pigment epithelium 65 Da (RPE65), lecithin retinol acyltransferase (LRAT) and various retinol dehydrogenase (RDH) proteins (Figure 5.5B). We detected seven genes involved in retinal regeneration that were widely expressed across *Aipysurus* skin and *Hydrophis* tissues (Figure 5.5B), including *rpe65*, the expression of which is generally thought to be restricted to the retinal pigment epithelium and cone

photoreceptors of the eye (Wright *et al.*, 2015). *Rpe65*, in conjunction with *lrat* (also expressed in the tail skin), has a key role in isomerization of the opsin chromophore (Saari, 2012; Wright *et al.*, 2015). Although the *opn4m* has an intrinsically photoisomerizing (*i.e.* bistable) function, light stability in *opn4x* is variably monostable or bistable depending on the isoform and/or taxon (Díaz *et al.*, 2016; Tu *et al.*, 2006). Significantly, associated retinal regeneration proteins of the eye*, rlbp1* and *rgr,* are absent from *Aipysurus* skin tissues. Although the operation and interaction of *opn4x* and/or *opn5* with *rpe65 and lrat* (and other retinal regeneration genes) within the skin is not entirely clear, a role in the regeneration of opsin chromophore in dermal photoreceptors would seem likely.

Conclusions

Our sea snake skin transcriptomes yielded non-visual opsins (and an absence of visual opsins), in addition to several phototransduction and retinal cycle genes, providing preliminary evidence that tail phototaxis may be mediated by genes related to non-visual photoreceptors that do not involve image-forming vision but rather provide information on overall light levels in the environment. Although future studies are needed to confirm a functional role of our candidate genes in mediating tail phototaxis and uncover the precise location of photoreceptive structures in sea snake skin, these findings highlight the utility of gene expression profiling as a first step in identifying the molecular mechanisms underlying sensory evolution. Dermal phototaxis may be more prevalent in vertebrates than currently recognised. We suggest that it is likely to be particularly important for aquatic or burrowing taxa with elongate bodies and/or tails that are anatomically remote from the concentration of sensory organs on their heads. Transcriptome profiling studies in other reptiles (including putatively non-phototactic sea snakes) should target skin to identify patterns of taxon- and tissue-specific expression of genes related to visual and non-visual photoreceptors.

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Supplementary materials

Tables

Table S5.1. Specimen information

Table S5.2. Specimens tested for body phototaxis

Table S5.3. Specimen and transcriptome details

Table S5.4. List of phototaxis genes

Table S5.5. FPKM heatmap for phototaxis genes

Table S5.6. Prevalence of tail injuries in museum specimens

Table S5.7. Summary of hurdle model for tail damage

Table S5.8. Planned contrasts for tail damage

Figures

Figure S5.1. Normalised spectral curves for light source

Figure S5.2. Transcript coverage

Figure S5.3. MDS plot for transcriptomes

Figure S5.4. Normalised expression levels

Figure S5.5. Upset plots expression profiles

Figure S5.6. Probability of tail damage

Figure S5.7. Experimental set up

Figure S5.8. Types of tail injuries in museum specimens

Electronic files

Raw RNA-seq reads are available at NCBI Sequence Read Archive: SUB4931382

The following electronic files are available at Figshare, doi: 10.25909/5c1ade89814b0

ES File 5.1: Beh-exp.xlsx containing raw and summary data for behavioural experiments of 17 individuals from eight species of sea snake (.xlxs).

ES File 5.2: Tail-beh-injuries.xlsx containing raw data of tail damage in museum specimens of sea snakes (.xlxs).

ES File 5.3: FPKMheatmap.zip containing R script for generating MDS plot & FPKM heatmap, TPM matrix for each sea snake transcriptome created using Salmon (zipped).

ES File 5.4: TranscriptomeAssemblies-PhototacticTails-skin-heart-liver-eye-testis.zip containing transcriptomes for sea snake tissues assembled using Trinity pipeline. (zipped)

ES File 5.5: Video containing examples of tail phototaxis in sea snakes (.mov).

ES File 5.6. Video containing examples of body phototaxis in sea snakes (.mov).

ES File5.7. RAXML-gene-trees.zip containing maximum likelihood gene trees in fasta and nexus format shows relationship among putative phototaxis sea snake transcripts and phototaxis genes from representative vertebrate lineages. (zipped)

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Chapter 6

Discussion

CHAPTER 6 Discussion

Thesis overview

Two independently marine lineages are nested within terrestrial elapid snakes (subfamily: Hydrophiinae): the oviparous sea kraits (8 species) that diverged approximately 18 million years ago and the viviparous sea snakes (>60 species) that diverged approximately six million years ago (Chapter 1, Figure 1.2). In this thesis, I explore how two cutaneous senses have evolved in marine hydrophiine snakes: mechanoreception via direct touch and indirect 'hydrodynamic reception', and dermal photoreception underpinning tail phototaxis. Although these cutaneous senses are thought to be important for many aspects of marine snake ecology, studies on the evolutionary significance of these traits are limited due to dearth of data on presumptive scale mechanoreceptors (*i.e.* scale sensilla) and photoreceptive structures in tail skin. I investigated two aspects of these cutaneous senses in marine snakes: 1) How the sense of touch has evolved in the transition from land to sea, and 2) How a novel phototactic trait has arisen in sea snakes. These aims were addressed using phylogenetic comparative methods and integrated molecular, morphological, behavioural, phylogenetic and ecological data.

In this final chapter, I summarise previous chapters in the context of these two broad aims, outlining the narrative that has driven my curiosity throughout this research as well as future avenues of research in the sensory systems of snakes. In closing, I advocate for the power of integrative approaches in addressing complex problems in biology, especially the evolution of novel traits, as well the role of science communication for progress in science and society.

How has the sense of touch evolved in the transition from land to sea?

Morphological diversity and adaptive role of scale sensilla

Chapter 2 and Chapter 3 investigated the morphological diversity of scale sensilla to better understand the ancestral and derived functions of these traits in marine snakes. In Chapter 2, I quantified the morphological variation in scale sensilla (number, density, area, coverage) across 19 elapid species (terrestrial, semi-aquatic, fully-aquatic). After accounting for the effects of allometry and phylogeny, I used Bayesian methods to reconstruct ancestral sensilla traits. These results showed that the overall coverage of sensilla (*i.e.* total area of the scale covered in sensilla) was not significantly altered in the common ancestors of sea kraits or sea snakes. However, there was great variation in overall coverage of sensilla among extant terrestrial and aquatic lineages, with higher overall coverage only found in the fully-aquatic sea snakes (>6% in fully-aquatic *c.f.* <2.5% in terrestrial). Furthermore, both sea snakes and sea kraits had strikingly protruding (dome-shaped) sensilla compared to terrestrial snakes.

Various divergent selection pressures in aquatic habitats might account for the morphological variation in scale sensilla in aquatic lineages, such as enhanced detection of mechanical stimuli, or co-option for alternate sensory or non-sensory functions. I addressed these hypotheses in Chapter 3 by examining the ultrastructure underlying scale sensilla in two species of sea snakes: *Aipysurus laevis* and *Hydrophis stokesii*. Histological data revealed that scale sensilla in these sea snakes closely resemble the mechanosensitive 'Meissner-like' corpuscles that underlie sensilla in terrestrial snakes, providing the first evidence that sensilla have retained an ancestral mechanosensory role in marine snakes. Describing the underlying morphology in Chapter 3, I also discovered that the structure of sensilla is variable depending on their location on the head or body. To further explore whether scale sensilla have been co-opted to detect hydrodynamic stimuli, future research is needed to investigate behavioural and electrophysiological responses to water motion in sea snakes. Our results suggest that marine snakes are a rich system for increasing knowledge of an understudied sensory modality in snake evolution.

Life history strategies and sense of touch

The morphological diversity of scale sensilla, especially the fine-scale differences of sensilla traits found on different parts of the body within individuals (Chapter 3), prompted the question of how life-history strategies have influenced the evolution of scale modifications in sea snakes. I explored this question in Chapter 4 by focusing on the role of enlarged scale organs in the mating system of a single species of sea snake, *Emydocephalus annulatus*. Sexual dimorphism in scale modifications on the snout ('rostral spine') and sensilla-like bumps on the body and anal scale ('anal knobs') had previously been described in this species, but I discovered that males also have chin organs ('genial knobs') that resemble enlarged scale sensilla. Following this, I described the ultrastructure of genial knobs, rostral spines and anal knobs and considered these results in the context of known courtship and mating behaviours for this species. Based on these results, I speculate that the cephalic organs have self-stimulatory sensory functions in males (sensory feedback) and females (mechanical stimulation), while the anal knobs of males may be more important for the final stages of mating (*e.g.* co-ordinating body and cloacal position). Further work is needed to determine the how mechanical stimuli is transduced and integrated by individuals. Chapter 4 illuminates how the marine transition might have caused divergent selection pressures on mechanoreception, resulting in conspicuous scale structures such as scale protrusions in *E. annulatus* as well as scale 'rugosities' and spines in other species of sea snakes. These results also reinforce how the unique ecologies among sea snakes may have a larger influence on the evolution of tactile sense than the initial invasion of aquatic habitats *per se*.

Future research directions for sense of touch in hydrophiines

Chapters 2 to 4 yielded insights into the trait variation and potential adaptive roles of scale mechanoreceptors in hydrophiine marine snakes, which can form the basis of future research on how mechanoreception has evolved during aquatic transitions and ecological adaptations. Previous research has shown that many sea snakes have conspicuous scale rugosities (Avolio *et al.*, 2006a, 2006b) and various sensory and non-sensory hypotheses have been proposed regarding their adaptive function (*e.g.* grip, swimming performance, mechanoreception). Although these rugosities may be superficially similar in their outer morphology, data generated in this thesis suggests that rugosities have distinct underlying ultrastructure that varies with their location on the body (Chapter 3), sex and/or other life history traits (Chapter 4). Therefore, these data show a complex evolutionary history for the various scale modifications reported in sea snakes (including sensilla, rugosities, protrusions, spines, etc.) and demonstrate the value of using a combination of morphological, allometric and phylogenetic methods. The methods used in Chapter 2 could also be used to study mechanoreception in a range of squamate lineages that have experienced multiple adaptive transitions and display variability in their scale mechanoreceptors (*e.g. Anolis* and *Varanus* lizards; other terrestrial snakes; pers. obs.). Data generated in these chapters can be used to inform the neurological basis of mechanoreception in snakes and a range of techniques (*e.g.* soft tissue CT-scanning, *in situ* hybridisation, RNA-sequencing) can be used to elucidate the precise nerve connections and molecular mechanisms underpinning this cutaneous sense. Future studies might also seek to correlate changes in cranial nerves or brain regions with sensilla traits (coverage, size, density) because increased neural investment often underlies sensory shifts (George and Holliday, 2013; Leitch *et al.*, 2015; Muchlinski, 2010, 2008).

These chapters demonstrate that cutaneous senses are an important component of the sensory ecology of marine snakes and this new knowledge has implications for conservation. Over the last few decades, sea snake populations have been experiencing sharp declines in regions of oil and gas exploration (*e.g.* Northwest Shelf of Western Australia, Lukoschek *et al.*, 2013), and there is concern that noise from seismic surveys may be having an impact on vulnerable populations (Chapuis *et al.,* in prep). Seismic exploration uses loud, low-frequency bursts of sound emitted by air-guns to locate oil and gas reserves and has major impacts on the biology of many aquatic animals (reviewed in Carroll *et al.*, 2017; Richardson, 2008). Preliminary electrophysiological studies suggest that sea snakes are sensitive to low-frequency sound between 40 to 600 Hz with peak sensitivity between 60 and 100 Hz (Chapuis *et al.*, in prep; Westhoff *et al.*, 2005). These studies have limited sample sizes and were unable to differentiate whether stimuli were transduced by mechanoreceptors in the skin or inner ear. The taxonomic distribution and

morphological descriptions of scale sensilla described in this thesis provide the ground work for future studies on the neurophysiological basis of mechanoreception. For example, I demonstrated that ultrastructure of scale sensilla varies in different regions of the body (Chapter 3; Chapter 4), which might indicate differing levels of neural responsiveness within individuals. Furthermore, the large variation in sensilla among marine snakes (Chapter 2) emphasizes the importance of sampling across the hydrophiine phylogeny to ensure that electrophysiology studies capture the variation in mechanoreception among species with differing ecologies. Improving our understanding of how marine snakes sense vibration underwater is relevant to management policies that aim to limit seismic surveys in marine regions.

How has a novel phototactic trait arisen in sea snakes?

In investigating the morphological diversity of scale mechanoreceptors, I was struck by the presence of several traits that appear to be unique to some species or clades of sea snakes (*i.e.* apomorphies, *e.g.* large mechanoreceptors, Chapter 2 and Chapter 3; enlarged scale organs, Chapter 4). This prompted the question of whether other sensory innovations have evolved in the transition from land to sea in hydrophiines–leading me to a short note describing the first record of cutaneous (dermal) photoreception in reptiles (Zimmerman and Heatwole, 1990). The phototactic tails of sea snakes were discovered in a single species of sea snake (*Aipysurus laevis*) nearly three decades ago but received little to no attention in the intervening years, which has limited our knowledge of the taxonomic distribution, ecological drivers and genetic and physiological basis of this novel sensory trait. Chapter 5 provides an overview of dermal photoreception, suggesting that the evolution of phototactic tails is a convergent adaptation in hagfish, lamprey, aquatic amphibians and sea snakes, used to avoid predation among aquatic animals with secretive habits, elongate bodies and paddle-shaped tails. Dermal photoreception in vertebrates is a challenging system to study because the skin lacks 'classical' visual photoreceptor structures (*e.g.* lens, stacked membranes). I met this challenge by integrating multiple data (*e.g.* behavioural, transcriptome) within an ecological and phylogenetic context to yield insights into the evolution and molecular basis of tail phototaxis in sea snakes.

The evolution and molecular basis of tail phototaxis

In Chapter 5, I conducted behavioural tests on eight species of sea snakes, replicating the findings of the previous study on *A. laevis* (Zimmerman and Heatwole, 1990), and revealed phototactic responses for the first time in *A. duboisii* and *A. tenuis* (including on the hind-body of the latter species). Based on these behavioural experiments, I offer a preliminary hypothesis that tail phototaxis originated in the ancestor of just six *Aipysurus* species (Figure 5.3). I then used transcriptome profiling of the skin in phototactic species to give a candidate list of phototaxis

genes in sea snakes, which suggested that a melanopsin-based, Gq signalling pathway underpins tail phototaxis in sea snakes (Figure 5.5). Finally, I characterised the relationship between tail damage and tail phototactic ability in museum specimens, finding that there was no intrinsically higher (or lower) predation pressure in the populations of phototactic species examined (*i.e. A. laevis* and *A. duboisii*).

Future research directions for dermal photoreception

In presenting new data on a novel sensory trait in sea snakes, Chapter 5 substantially expands knowledge of dermal photoreception in reptiles and invites future research on the evolutionary importance of this trait among vertebrates. To resolve the evolutionary origin of tail phototaxis, future studies should increase intra- and inter-specific sampling at key lineages across sea snake phylogeny, especially *Emydocephalus* and *Ephalophis Parahydrophis* genera, and independently marine sea kraits. Future studies might also consider testing for phototactic tails in other elongate vertebrates that burrow, such as caecilians, amphisbaenians and eels. Building on our hypothesis for the evolutionary origins of tail phototaxis, future studies can explore the ecological conditions that influence the evolution of tail phototaxis in *Aipysurus* such as water depth and activity patterns. Chapter 5 also informs the physiology of tail phototaxis, I identified broad spectral sensitivity for two species between peaks of the blue and green light (Figure 5.2), but further experiments are needed to detect responses–both behavioural and electrophysiological–to different intensities and spectral distributions of light. Future studies can use other molecular techniques (*e.g.* differential gene expression, *in situ* hybridisation) to target candidate gene pathways in peripheral nerves and/or mechanoreceptors (see Chapter 3, Figure 3.8) to reveal the cellular structures responsible for dermal photoreception. Finally, the parallel evolution of tail phototaxis among vertebrates that diverged more than 500 million years ago (*i.e.* lamprey and hagfish, Benton and Donoghue, 2007) and reptile lineages (*i.e.* sea snakes) that diverged less than 10 million years ago (Sanders *et al.,* 2008)) provides an opportunity to test for convergent molecular pathways underpinning dermal photoreception. Our candidate opsin, melanopsin, is known to initiate an 'invertebrate' Gq signalling cascade for light-detection, which suggests that the genetic resources for tail phototaxis predate the divergence of vertebrates. Thus, molecular studies on phototactic lineages may identify parallel evolution of underlying molecular and developmental mechanisms among vertebrates.

Integrative approaches for solving complex problems in evolution

Sensory ecology and levels of biological organisation

A major challenge in biology is how to integrate knowledge related to differing levels of biological organisation, from molecular to morphological to behavioural traits. This problem is

particularly salient in studies of sensory ecology, which seek to link ecological innovations (*e.g.* secondary invasions of aquatic habitats) with physiological and molecular mechanisms (Dangles *et al.*, 2009). In this thesis, I approached these challenges by integrating multiple data from various levels of biological organisation (*e.g.* transcriptomic, cellular, morphological, behavioural, phylogenetic), which yielded major insights on how cutaneous senses have evolved in marine snakes. Despite this range of approaches, this thesis remains limited in scope because it focuses on individual senses in isolation from other sensory systems and neural networks. In understanding how senses evolve, it is important to acknowledge that sensory systems do not operate or evolve independently of each other, sensory stimuli are detected by multiple sensory organs and/or cells with outputs that are then filtered, processed and integrated by higher neural pathways (Thewissen and Nummela, 2008). As our knowledge of individual senses and underlying neural networks in hydrophiine snakes expands, future research must strive to integrate these data to build a more complete model of sensory evolution. One approach to address this in hydrophiines might use whole-genome comparisons to track how large gene families (and/or regulatory elements) underlying multiple sensory modalities change over time (Perry *et al.*, 2018). Future studies of sensory evolution must combine data from multiple perspectives while using integrative approaches and transdisciplinary collaborations.

Evolutionary novelty

A major focus of this thesis was to understand how novel sensory pathways can evolve in response to major adaptive shifts. Early definitions of evolutionary novelty link novel traits directly to ecological innovation, for example, Mayr defines novel traits as *"any newly acquired structure or property that permits the performance of a new function, which in turn, will open a new adaptive zone"* (1963, p. 603). Fitting with this definition, sea snakes and sea kraits have evolved many novel traits that accommodated major adaptive transitions to aquatic habitats such as paddle-shaped tails, tissues to seal the mouth and nostrils, salt glands and cutaneous gas exchange (Dunson, 1975; Sanders, Rasmussen and Elmberg, 2012). These morphological and physiological adaptations are likely to have appeared early in the evolution of these clades, clearly linked to aquatic habits as evidence by their homoplasy with other secondarily-aquatic animals (*e.g.* paddle-shaped tail of mosasaurs, or salt glands in sea birds). In contrast, the cutaneous senses investigated throughout this thesis—mechanoreception and dermal photoreception—show highly derived traits found in only a few nested lineages within the sea snakes, and thus uncorrelated with the initial evolutionary invasion of aquatic habitats (see Chapter 2, Figure 2.5; Chapter 5, Figure 5.3). Furthermore, accelerated speciation rates are prominent in the *Hydrophis* clade, a radiation that postdates the invasions of aquatic habitats in sea snakes (see Chapter 1; Lee *et al.*,

2016; Sanders *et al.*, 2010). How might I explain these patterns of novel trait evolution in sea snakes?

Expanding on Mayr's definition, Pigliucii (2008) emphasizes that although novelties are coupled with ecological functions they are also built on existing variation, *i.e.* novel traits use pre-existing structures in new ways. This conceptualisation of novelty describes how new functions are created by co-opting existing molecular processes. Such processes are thought to drive the evolution of photoreception (Nilsson, 2009; Plachetzki and Oakley, 2007) including in snakes, wherein it is related to morphological transmutation of retinal photoreceptors (Bhattacharyya *et al.*, 2017; Schott *et al.*, 2015; Simões *et al.*, 2016; Walls, 1942). These examples of sensory evolution in snakes exemplify how sensory pathways are labile and subject to historical contingency, *i.e.* genetic mutations and expression can occur by chance events influenced by pre-existing molecular and developmental processes (Blount *et al.*, 2008; Gould, 1989). Chapter 5 also explores these concepts, with gene profiling suggesting that the rare evolution of tail phototaxis in 10% of sea snakes is likely created by co-opting pre-existing pathways (*i.e.* non-visual melanopsin and Gq signalling) in distinct patterns of expression (*i.e.* the skin). Furthermore, innovations in mechanoreception and dermal photoreception are uncoupled with initial invasions of aquatic habitats in marine snakes. These findings highlight the importance of distinguishing selection pressures from historical contingency due to phylogeny and plasticity in molecular pathways.

Science and society

The focus of my research has been clearly directed towards the fundamental biology of snakes, with outputs primarily directed towards academics and the primary research literature. Nevertheless, I believe that this needs a broader context, particularly to extend the reach of the work beyond academia, to engage the public in science, and to establish discussion with the people who, ultimately, fund academic endeavour. In addition, I believe that those engaged in fundamental research should be aware of potential uses for their research, and its scope for impact. Research on novel sensory traits has the potential to capture public attention and curiosity, especially for species that are not typically considered 'charismatic' such as venomous snakes (see Albert *et al.*, 2018). Engaging people with evolutionary phenomena can also foster empathy, curiosity and sense-of-place in the natural world (see Wilson, 1984). Although, at least historically, the communication of science has perhaps been considered peripheral to the obligations of scientists, elements of my work throughout my PhD were dedicated to communicating research through non-academic channels such as public databases, museums, art, blog posts and social media. These interactions led to direct academic outputs, for example, posts on an online sea snake group facilitated the publication of a short natural history note that

substantially extends previous records of diving behaviour in sea snakes (Appendix A: 'First records of sea snakes diving to the mesopelagic zone'). Communicating science is also essential to achieving conservation goals (see Martín-López *et al.*, 2007) and stimulating general scientific literacy (Appendix B: Blog post, 'What went wrong in communicating the Tasmanian tiger genome'). Thus, science communication is an important tool for scientists because it is by sharing passion and inviting curiosity that we might improve science and society.

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Supplementary material

Supplementary material for Chapter 2

TABLE S2.1. List of museum specimens used in this study: Western Australian Museum 'WAM', South Australian Museum 'SAMA', and Field Museum of Natural History 'FMNH'.

Genus	*Species*	*Museum*	*Tag number*	*Locality*
Aipysurus	*duboisii*	WAM	R156216	Exmouth Gulf, Western Australia
	eydouxii	SAMA	R22569	PulauUbin, Singapore
	fuscus	WAM	R129815	Ashmore reef, Western Australia
	laevis	WAM	R174246	Broome, Western Australia
Emydocephalus	*annulatus*	WAM	R129824	Shark Bay, Western Australia
	annulatus	WAM	R165708	Ashmore reef, Western Australia
Hydrelaps	*darwiniensis*	SAMA	R2270D	Darwin, Northern Territory
	darwiniensis	WAM	R22973	Port Headland, Western Australia
	darwiniensis	WAM	R43390	Port Headland, Western Australia
Hydrophis	*curtus*	FMNH	202019	Jahore, West Malaysia
	curtus	FMNH	202021	Jahore, West Malaysia
	curtus	FMNH	202030	Jahore, West Malaysia
	curtus	FMNH	202032	Jahore, West Malaysia
	curtus	FMNH	201910	Jahore, West Malaysia
	cyanocinctus	FMNH	201399	Jahore, West Malaysia
	cyanocinctus	FMNH	201569	Jahore, West Malaysia
	cyanocinctus	FMNH	201572	Jahore, West Malaysia
	donaldi	SAMA	R66274	Weipa, Queensland
	major	WAM	R174252	Broome, Western Australia
	major	WAM	R174253	Broome, Western Australia
	major	WAM	R36550	Carnarvon, Western Australia
	platurus	FMNH	16927	Ecuador
	platurus	FMNH	41591	Piura, Peru
	platurus	FMNH	171674	Costa Rica
	platurus	FMNH	171688	Costa Rica
	schistosus	FMNH	198486	Jahore, West Malaysia
	schistosus	FMNH	206655	Jahore, West Malaysia
	schistosus	FMNH	206657	Jahore, West Malaysia
	schistosus	FMNH	206725	Jahore, West Malaysia
	stokesii	WAM	R174251	Broome, Western Australia
	viperinus	FMNH	201476	Jahore, West Malaysia
	viperinus	FMNH	201578	Jahore, West Malaysia
	viperinus	FMNH	201594	Jahore, West Malaysia
Laticauda	*colubrina*	SAMA	R48012	Babeldaob, Palau
	colubrina	SAMA	R56928	Solomon Islands
Naja	*kaouthia*	SAMA	R63789	Captive, South Australia
	kaouthia	SAMA	R63791	Captive, South Australia
	kaouthia	SAMA	R63792	Captive, South Australia
	kaouthia	SAMA	R63793	Captive, South Australia
Notechis	*scutatus*	SAMA	R18601	Eyre Peninsula, South Australia
	scutatus	SAMA	R25143	Mt Remarkable, South Australia
	scutatus	SAMA	R30505	Williams Is., South Australia
Pseudonaja	*textilis*	SAMA	R18833	Alexandrina, South Australia
Vermicella	*annulata*	SAMA	R13318	Flinders ranges, South Australia

TABLE S2.2. High depth of field photographic images of whole-snake heads were composed for six representative hydrophiine species using a series of multi-focus photographs, acquired with a digital DSLR camera (EOS 5D, Canon, Japan) with macro lens (Canon MP-E 65mm, f/2.8 set to magnify 1.4×) and on mount with flashlights (Visionary Digital BK+ Lab Imaging System, Dun, Inc., USA). Resulting images were stacked into a single output using designated imaging software (Zerene Stacker v1.04; Zerene Systems, USA). These photography methods were also utilised for silicone casts of whole-snake heads in Quantitative analysis. Museum specimens from Western Australian Museum 'WAM', South Australian Museum 'SAMA', and Field Museum of Natural History 'FMNH'.

Species	*Museum*	*Tag number*
Aipysurus duboisii	WAM	R156216
Emydocephalus annulatus	WAM	R165708
Hydrelaps darwiniensis	SAMA	R22973
Hydrophis platurus	FMNH	41951
Hydrophis schistosus	FMNH	201569
Pseudonaja textilis	SAMA	R18833

Supplementary material for Chapter 3

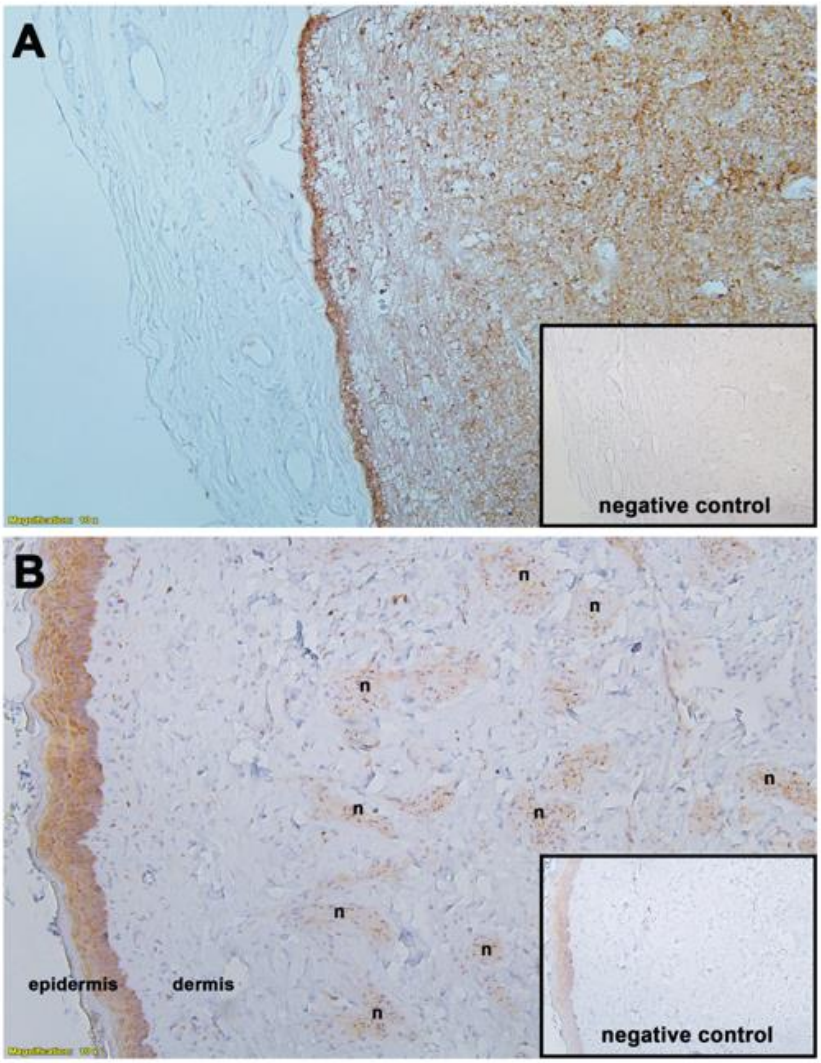

FIGURE S3.1. Primary and secondary antibody controls for PGP9.5 in A) taipan (*Oxyuranus scutellatus)* brain tissue and B) sea snake (*Hydrophis stokesii*) cephalic skin tissue. Negative controls were performed by omitting primary antibody incubation step. Note the generalised staining of the epidermis in snake skin, likely resulting from cross-reactivity of secondary and / or tertiary antibodies during immunohistochemistry procedure; nerve bundles (n) do not show same cross-reactivity

Supplementary material for Chapter 4

TABLE S4.1. List of 59 specimens of *Emydocephalus annulatus* with presence of scale projections, rostral spine (RS), genial knobs (GK), anal knobs (AK) and cephalic mechanoreceptors (M), and length of rostral spine (RSL, mm) and snout-vent length (SVL, mm). Other life history traits, accession numbers, collection locality and dates are reported. Specimens were examined from the Australian Museum (AM) and the Museum and Art Gallery of the Northern Territory (MAGNT). 'Mating season' *E. annulatus* were collected during the winter months (June to July) and 'non-mating' season specimens were collected during summer (November to December). Note that the specimens collected during winter were directly observed courting and mating prior to collection (M L Guinea, pers. comm.).

Museum	*ID*	*Age*	*Sex*	*RS*	*RSL*	*GK*	*AK*	*CM genial*	*SVL*	*Locality*	*Date*	*Season*
AM	R40486	adult	f	no	na	no	no	yes	930	Cartier Reef	8/11/1973	Non-mating
AM	R41513	adult	f	no	na	no	no	yes	599	Ashmore Reefs	11/02/1974	Non-mating
AM	R41515	adult	f	no	na	no	no	yes	548	Ashmore Reefs	11/02/1974	Non-mating
AM	R41517	adult	f	no	na	no	no	yes	497	Ashmore Reefs	11/02/1974	Non-mating
AM	R41536	adult	f	no	na	no	no	yes	500	Ashmore Reefs	11/02/1974	Non-mating
AM	R41540	adult	f	no	na	no	no	yes	614	Ashmore Reef,	11/02/1974	Non-mating
AM	R41541	adult	f	no	na	no	no	yes	545	Hibernia Reefs	11/02/1974	Non-mating
AM	R42702	adult	f	no	na	no	no	yes	555	Scott Reef		Non-mating
AM	R42704	adult	f	no	na	no	no	yes	556	Hibernia Reef	13/12/1974	Non-mating
AM	R44468	adult	f	no	na	no	no	yes	457	Ashmore Reefs	21/11/1974	Non-mating
AM	R44573	adult	f	no	na	no	no	yes	608	Ashmore Reefs	25/11/1974	Non-mating
AM	R44585	adult	f	no	na	no	no	yes	575	Ashmore Reefs	25/11/1974	Non-mating
AM	R33358	adult	m	yes	1.57	yes	yes	yes	581	Ashmore Reefs	9/02/1973	Non-mating
AM	R33359	adult	m	yes	0.70	no	no	yes	396	Ashmore Reefs	9/02/1973	Non-mating
AM	R40484	adult	m	yes	0.98	yes	yes	yes	481	Ashmore Reefs	8/11/1973	Non-mating
AM	R40485	adult	m	yes	na	yes	yes	yes	538	Ashmore Reefs	8/11/1973	Non-mating
AM	R40495	adult	m	yes	1.70	yes	yes	yes	612	Ashmore Reefs	8/11/1973	Non-mating
AM	R41512	adult	m	yes	1.03	yes	yes	yes	561	Ashmore Reefs	11/02/1974	Non-mating
AM	R44433	adult	m	yes	1.22	yes	yes	yes	557	Ashmore Reef	21/11/1974	Non-mating

AM	R44466	adult	m	yes	1.31	yes	yes	yes	467	Ashmore Reefs	21/11/1974	Non-mating
AM	R44471	adult	m	yes	na	yes	yes	yes	553	Ashmore Reefs	21/11/1974	Non-mating
AM	R44472	adult	m	yes	1.21	yes	yes	yes	508	Ashmore Reefs	21/11/1974	Non-mating
AM	R44641	adult	m	yes	na	yes	yes	yes	597	Hibernia Reef	26/11/1974	Non-mating
AM	R44645	adult	m	no	na	yes	yes	yes	558	Ashmore Reefs	26/11/1974	Non-mating
AM	R45050	adult	m	yes	1.29	yes	yes	yes	513	Unknown		
AM	R44432	juvenile	f	no	na	no	no	yes	305	Ashmore Reefs	21/11/1974	Non-mating
AM	R44474	juvenile	m	no	na	no	no	yes	261	Ashmore Reefs	21/11/1974	Non-mating
AM	R44485	juvenile	m	no	na	no	no	yes	257	Hibernia Reef	21/11/1974	Non-mating
AM	R44487	juvenile	m	no	na	no	no	yes	280	Ashmore Reefs	21/11/1974	Non-mating
MAGNT	R17755	adult	f	no	na	no	no	yes	917	Hibernia Reef	8/05/1992	Mating
MAGNT	R17759	adult	f	no	na	no	no	yes	636	Hibernia Reef	8/05/1992	Mating
MAGNT	R17762	adult	f	no	na	no	no	yes	610	Hibernia Reef	8/05/1992	Mating
MAGNT	R17763	adult	f	no	na	no	no	yes	696	Hibernia Reef	8/05/1992	Mating
MAGNT	R17784	adult	f	no	na	no	no	yes	551	Hibernia Reef	14/05/1992	Mating
MAGNT	R21255	adult	f	no	na	no	no	yes	737	Hibernia Reef	11/05/1992	Mating
MAGNT	R21256	adult	f	no	na	no	no	YES	383	Hibernia Reef	12/05/1992	Mating
MAGNT	R21259	adult	f	no	na	no	no	yes	462	Hibernia Reef	12/05/1992	Mating
MAGNT	R21265	adult	f	no	na	no	no	yes	424	Hibernia Reef	13/05/1992	Mating
MAGNT	R21267	adult	f	no	na	no	no	yes	559	Hibernia Reef	13/05/1992	Mating
MAGNT	R21268	adult	f	no	na	no	no	yes	614	Hibernia Reef	13/05/1992	Mating
MAGNT	R17754	adult	m	yes	1.35	yes	yes	yes	806	Hibernia Reef	8/05/1992	Mating
MAGNT	R17756	adult	m	yes	1.16	yes	yes	yes	828	Hibernia Reef	8/05/1992	Mating
MAGNT	R17757	adult	m	yes	0.92	yes	yes	yes	759	Hibernia Reef	8/05/1992	Mating
MAGNT	R17758	adult	m	yes	1.71	yes	yes	yes	769	Hibernia Reef	8/05/1992	Mating
MAGNT	R17760	adult	m	yes	1.27	yes	yes	yes	824	Hibernia Reef	8/05/1992	Mating
MAGNT	R17761	adult	m	yes	1.17	yes	yes	yes	752	Hibernia Reef	8/05/1992	Mating
MAGNT	R17764	adult	m	no	na	no	no	yes	612	Hibernia Reef	8/05/1992	Mating
MAGNT	R17765	adult	m	yes	0.73	yes	yes	yes	728	Hibernia Reef	8/05/1992	Mating
MAGNT	R17766	adult	m	yes	1.07	yes	yes	yes	680	Hibernia Reef	8/05/1992	Mating

MAGNT	R17767	adult	m	yes	1.24	yes	yes	yes	725	Hibernia Reef	8/05/1992	Mating
MAGNT	R17768	adult	m	yes	0.78	yes	yes	yes	643	Hibernia Reef	8/05/1992	Mating
MAGNT	R17769	adult	m	yes	1.20	yes	yes	yes	782	Hibernia Reef	8/05/1992	Mating
MAGNT	R17771	adult	m	yes	1.21	yes	yes	yes	776	Hibernia Reef	8/05/1992	Mating
MAGNT	R21254	adult	m	yes	1.20	yes	yes	yes	548	Hibernia Reef	11/05/1992	Mating
MAGNT	R21257	adult	m	yes	0.56	yes	yes	yes	469	Hibernia Reef	12/05/1992	Mating
MAGNT	R21258	adult	m	yes	1.52	yes	yes	yes	555	Hibernia Reef	12/05/1992	Mating
MAGNT	R21263	adult	m	yes	0.80	yes	yes	yes	535	Hibernia Reef	13/05/1992	Mating
MAGNT	R21264	adult	m	yes	2.26	yes	yes	yes	614	Hibernia Reef	13/05/1992	Mating
MAGNT	R21266	adult	m	yes	1.13	yes	yes	yes	572	Hibernia Reef	13/05/1992	Mating

FIGURE S4.1. Gross morphology of scale structures in juvenile *Emydocephalus annulatus* showing that both sexes lack rostral spines, genial knobs and anal knobs. A) Underside of the head in a juvenile male, B) anal scales in a juvenile male, C) underside of the head in a juvenile female, and D) anal scales in a juvenile female. Specimens are from the Australian Museum: A) & B) R44485 and C) & D) R44432.

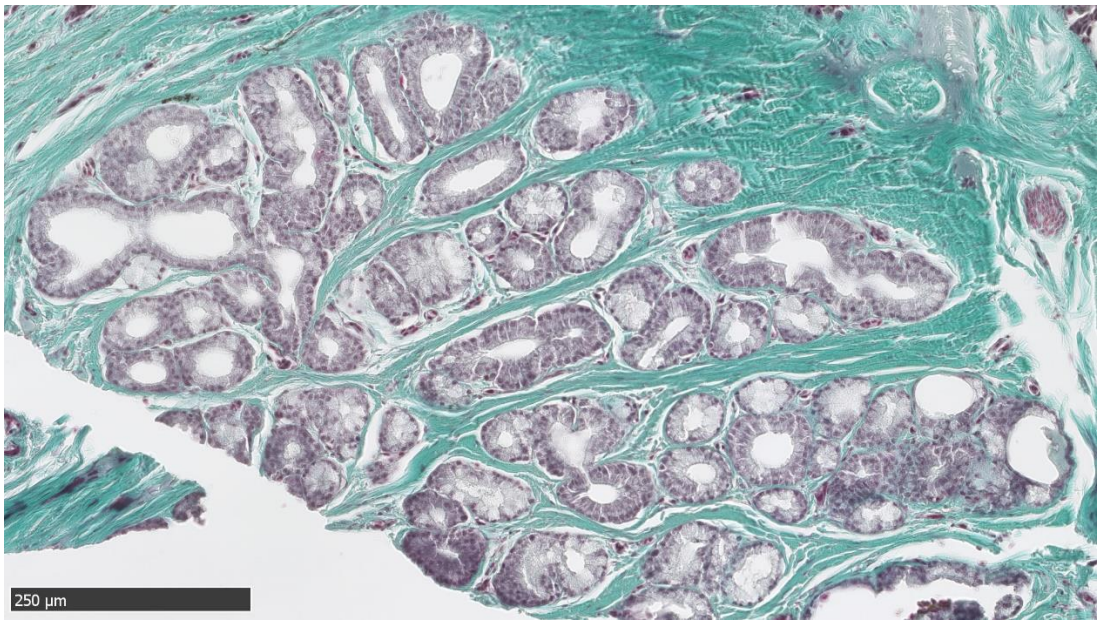

FIGURE S4.2. Cross-section of a gland in rostrum in male Emydocephalus annulatus (WAMR129824). Slide was stained with Gomori's One Step and magnified at 16.4×.

Supplementary material for Chapter 5

Specimen information

Most species were collected 1−10 km offshore from the coast of Broome, Western Australia, in June 2015 and September 2016, but *Hydrophis platurus* was collected offshore from Coco Beach, Guanacaste, Costa Rica, in June 2017. A total of eight snakes from four species were transported from Western Australia to the University of Adelaide, South Australia for behavioural experiments. Nine additional snakes from seven species were kept overnight for behavioural experiments at the Australian or Costa Rican field locations and released after experiments near their sites of capture (Table S5.1).

Light source measurements

To understand the spectral properties of the flashlights, spectral irradiance measurements of the white and coloured LED flashlights were made by holding the flashlights 30 cm (the distance used to illuminate the snakes' tails) from the end of a bare-ended 600 μm diameter fibre optic (Ocean Optics P-600-2-UV/VIS; Ocean Optics Inc., USA) connected to a cooled CCD spectrometer (Ocean Optics QEPro) with measurements made in a darkroom using OceanView ver. 1.3.4 software (Ocean Optics Inc, USA) running on a MacBook Pro computer. Wavelength calibration was checked using a HG-1 (Ocean Optics Inc., USA) mercury-cadmium line emission spectrum lamp and no significant differences between observed and calibrated line spectral positions were noted. Although calibrated against a standard light (Ocean Optics LS1-CAL) to provide absolute irradiance measurements, these were found to be highly dependent on the exact alignment of flashlight and fibre as well as the adjustment of the flashlight focus. In consequence, only measurements of flashlight relative spectral irradiances are reported (Figure S5.1).

The relative irradiance due to the coloured LED bulbs was lower than that produced by the white LED bulb. The irradiance ratios measured during field experiments averaged 47.7: 45.5: 47.9: 51.3: 115 for the UV:Blue:Green:Red:White LED flashlights, respectively. Neutral-density spectral filters of nominal 15% and 30% transmission were used to maintain the relative irradiance approximately equal (in energy terms) for the coloured LED bulbs. Laboratory experiments to determine the relative spectral irradiances of the flashlights showed that that rank order for irradiance was unchanged whether expressed in energy or photon irradiance, and we

can therefore conclude that the rank order of irradiance for the flashlights, in photon irradiance terms, during experiments, was White<<Blue<Violet≅Green<Red.

Statistics for sequencing, assembly, sample clustering

We obtained > 292 million paired-end reads from skin, testis, heart, liver and eye tissues. After the trimming of short and low-quality reads, ~196 million paired-end reads (67%) were used for the de novo assembly of tissue transcriptomes. Mapping reads back to respective assemblies resulted high (mean of 94%) alignment rates (Table 5.2). Statistics for transcriptome assemblies, including contig lengths, N50 and GC content, are given in Table 3. Assembled transcripts were aligned against proteins in SwissProt database showing high completeness, with 4000 to 6000 transcripts reaching 100% coverage per tissue (Figure S5.2). Although skin samples of *A. tenuis* and one of *A. laevis* had lower number of transcripts (<2500) that reached 100% coverage per tissue compared to other samples (Figure S5.2). Tissue transcriptomes clustered on the multidimensional scaling plot by tissue type, with the seven skin transcriptomes forming a separate cluster from the testis, heart, eye and liver transcriptomes (Figure S5.3). Within skin samples, there was no evidence of clustering based on dermal photoresponses previously identified in behavioural experiments (Figure 5.4; Figure S5.3).

Expression levels and gene profiling

Based on mRNA read quantification, a large amount (47%) of genes are lowly expressed (<1 FPKM) and only 4% of genes are highly expressed (>100 FPKM) across tissue transcriptomes (Figure S4). Expression levels are similar with few differences between tissue types and species. However, it is important to note that identification and quantification levels are likely affected by the reference transcriptome, a pitviper (*Protobothrops mucrosquamatus*), which is a relatively distantly related taxon (Viperidae) compared to the taxa studied here (Hydrophiinae).

Upset plots show the expression profiles that characterise each tissue transcriptome and species (Figure S5.5). In *Hydrophis major*, 35% of genes are conserved across liver, heart and testis tissues; the testis had a highly distinct expression profile with >3000 (29%) unique genes (Figure S5.5A). In *Aipysurus laevis*, 27% of genes are conserved across skin and eye tissues; the eye has ~2700 (26%) unique genes; only 3% of genes are conserved across all four skin samples (Figure S5B). Differences in expression profiles among skin tissues in *A. laevis* may be due to sampling two individuals at distinct life-history stages (juvenile and adult) and / or temporal differences in skin shedding cycle. In *Aipysurus tenuis*, 60% of genes are conserved across the three skin tissues; there are <700 unique genes in any one tissue, which is to be expected given each sample is from the same tissue type within the same individual (Figure S5.5C).

TABLE S5.1. Taxonomy, life stage, museum accession numbers and sample size of eight species of wild caught, captive sea snakes (Hydrophiinae) used in behavioural experiments. During experiments to test phototaxis, white light was shone on the dorsal surface of the either the tail skin ($T_{(s)}$) or midbody skin ($B_{(s)}$) of each snake as indicated. "Location" specifies whether specimens were transported for behavioural experiments to the University of Adelaide (UoA), South Australia, or kept overnight at field locations in Broome (B), Western Australia, or in Coco Beach, Costa Rica (CR).

Taxonomy		*Specimen information*					*Tested*	
Genus	*Species*	*Field numbers*	*Museum*	*Sex*	*Life stage*	*Location*	*N*	*Skin*
Aipysurus	*duboisii*	KLS0664	NA	M	Subadult	UoA	1	$T_{(s)}$ $B_{(s)}$
	laevis[a]	KLS0691	NA	M	Adult	UoA	5	$T_{(s)}$ $B_{(s)}$
		KLS0690	NA	M	Adult	UoA		$T_{(s)}$ $B_{(s)}$
		KLS0689	NA	F	Adult	UoA		$T_{(s)}$ $B_{(s)}$
		KLS0656[b]	NA	M	Juvenile	UoA		$T_{(s)}$ $B_{(s)}$
		KLS0463	NA	M	Juvenile	B		$T_{(s)}$
	mosaicus	KLS0785	NA	M	Adult	B	2	$T_{(s)}$ $B_{(s)}$
		KLS0587	R174528	F	Adult	B		$T_{(s)}$
	tenuis	KLS0657	NA	M	Adult	UoA	3	$T_{(s)}$ $B_{(s)}$
		KLS0654[b]	NA	M	Adult	UoA		$T_{(s)}$ $B_{(s)}$
		KLS0461	NA	Unk	Juvenile	B		$T_{(s)}$
Hydrelaps	*darwiniensis*	KLS0599	R174537	F	Adult	B	1	$T_{(s)}$
Hydrophis	*major*	KLS0662	NA	Unk	Juvenile	UoA	2	$T_{(s)}$ $B_{(s)}$
		KLS0460[b]	R174542	M	Adult	B		$T_{(s)}$
	platurus	#1	NA	M	Adult	CR	2	$T_{(s)}$ $B_{(s)}$
		#2	NA	M	Adult	CR		$T_{(s)}$ $B_{(s)}$
	stokesii	KLS0483	NA	Unk	Juvenile	B	1	$T_{(s)}$
Total	8						17	

[a]Non-visual phototaxis previously recorded in this species.

[b]Tissues collected for RNA sequencing

TABLE S5.2. Details of specimens tested for phototaxis in response to illumination of the body. Body intervals are measured using percentages of the total count of ventral (belly) scales: pre-vent ($B_{(s1)}$), posterior body (66% of ventral scales from the head: $B_{(s2)}$), anterior body (33% of ventral scales from the head: $B_{(s3)}$) and base of the head ($B_{(s4)}$). Snout-vent length = svl.

Specimen					*Number of ventral scales from the vent*			
Individual ID	Species	svl	Ventral count	$T_{(s)}$	$B_{(s1)}$	$B_{(s2)}$	$B_{(s3)}$	$B_{(s4)}$
KLS0656	A. laevis	48cm	135	NA	135	89	46	1
KLS0657	A. tenuis	78cm	187	NA	187	123	62	1
KLS0654	A. tenuis	92cm	180	NA	180	119	59	1

TABLE S5.3. Details of samples used for transcriptomic analyses. Organs collected from *Aipysurus* and *Hydrophis* specimens. Skin tissues were collected from *Aipysurus* including the photoreceptive tail tip, putatively non-photoreceptive anterior end of the tail, variably photoreceptive dorsal surface of the end of the body a short distance anterior of the vent.

Individual ID	Species	Sex	Life stage	Organ	Tissue region	Assembly ID
KLS0459	*A. laevis*	Female	Adult	Eye	Whole eye	Eye
				Skin	Photoreceptive tail tip (dorsal)	Skin_tailA1
KLS0656*	*A. laevis*	M	Juvenile	Skin	Photoreceptive tail tip (dorsal)	Skin_tailA2
				Skin	Non-photoreceptive tail (anterior)	Skin_tailB1
				Skin	Non-photoreceptive body near vent (dorsal)	Skin_body1
KLS0654*	*A. tenuis*	M	Adult	Skin	Photoreceptive tail tip (dorsal)	Skin_tailA3
				Skin	Non-photoreceptive tail (anterior)	Skin_tailB2
				Skin	Photoreceptive body near vent (dorsal)	Skin_body2
KLS0460*	*H. major*	M	Juvenile	Heart	NA	Heart
				Liver	NA	Liver
				Testis	NA	Testis

*Same individual was tested for tail phototaxis in behavioural experiments

TABLE S5.4. Genes involved in vertebrate phototransduction and retinoid regeneration pathways used in transcriptome profiling of skin and non-skin sea snake tissues. Gene selection informed from previous studies and availability in reference transcriptome for the pitviper *Protobothrops mucrosquamatus* For full description of tissue codes refer to Table S5.3.

Full name	*Gene name*	*Pathway*
Arrestin beta 2	Arrb2	Ciliary Phototransduction
Cellular retinoic acid binding protein 1	crabp1	Ciliary Phototransduction
Cyclic nucleotide gated channel alpha 1	Cnga1	Ciliary Phototransduction
Cyclic nucleotide gated channel alpha 3	Cnga3	Ciliary Phototransduction
Cyclic nucleotide gated channel beta 1	Cngb1	Ciliary Phototransduction
Cyclic nucleotide gated channel beta 3	Cngb3	Ciliary Phototransduction
Guanine nucleotide binding protein G protein 1	gnat1	Ciliary Phototransduction
Guanine nucleotide binding protein G protein 2	gnat2	Ciliary Phototransduction
G-coupled receptor kinase 7-like	grk7-like	Ciliary Phototransduction
G-coupled receptor kinase 5	grk5	Ciliary Phototransduction
Guanine-nucleotide-binding protein subunit beta-1	gnb1	Ciliary Phototransduction
Guanine-nucleotide-binding protein subunit beta-5	gnb5	Ciliary Phototransduction
Guanylate cyclase activator 1A	guca1a	Ciliary Phototransduction
Guanylate cyclase activator 1B	guca1b	Ciliary Phototransduction
Guanylate cyclase activator 1C	guca1c	Ciliary Phototransduction
Phosducin	pdc	Ciliary Phototransduction

Phosducin-like	pdc-like	Ciliary Phototransduction
Phosducin-like 2	pdcl2	Ciliary Phototransduction
Phosphodiesterase 6B (like)	pde6b-like	Ciliary Phototransduction
Phosphodiesterase 6C	pde6c	Ciliary Phototransduction
Phosphodiesterase 6D	pde6d	Ciliary Phototransduction
Phosphodiesterase 6G	pde6g	Ciliary Phototransduction
Phosphodiesterase 6H	pde6h	Ciliary Phototransduction
Retinal guanylyl cyclase 1-like	Gucy2d-like	Ciliary Phototransduction
Recoverin	rcvrn	Ciliary Phototransduction
S-antigen arrestin	sag	Ciliary Phototransduction
Solute carrier family 24 member 2	slc24a2	Ciliary Phototransduction
Guanine nucleotide binding protein G protein alpha	gna11, gnaq	Rhabdomeric Phototransduction
Phospholipase C beta-1	plcb1	Rhabdomeric Phototransduction
Phospholipase C beta-3	plcb3	Rhabdomeric Phototransduction
Phospholipase C beta-4	plcb4	Rhabdomeric Phototransduction
ATP binding cassette subfamily A member 4	abca4	Retinoid regeneration
Lecithin retinol acyltransferase	lrat	Retinoid regeneration
Regulator of G-protein signalling 9	rgs9	Retinoid regeneration
Regulator of G-protein signally 9 binding protein	rgs9bp	Retinoid regeneration
Retinol binding protein 1	rbbp1	Retinoid regeneration
Retinol binding protein 3	rbp3	Retinoid regeneration
Retinol dehydrogenase 5	rdh5	Retinoid regeneration
Retinol dehydrogenase 8 (like)	rdh8-like	Retinoid regeneration
Retinol dehydrogenase 10	rdh10	Retinoid regeneration
Retinol dehydrogenase 11 (like)	rdh11-like	Retinoid regeneration
Retinol dehydrogenase 12 (like)	rdh12-like	Retinoid regeneration
Retinol dehydrogenase 14	rdh14	Retinoid regeneration
Retinal pigment epithelium-specific protein 65Da	rpe65	Retinoid regeneration
Rhodopsin	rho	Visual opsin
Short-wavelength cone pigment	opn1sw	Visual opsin
Long-wave cone pigment	opn1lw	Visual opsin
Encephalopsin	opn3	Non-visual opsin
Melanopsin xenopus-like	opn4x	Non-visual opsin
Neuropsin 5	opn5	Non-visual opsin
Retinal G protein coupled receptor	rgr	Non-visual opsin

TABLE S5.5. Fragment per kilobase of transcript per million mapped reads (FPKM) for visual genes across sea snake organ transcriptomes. FPKM were log-transformed for viewing as a heatmap in manuscript. Refseq category for each gene name correspond to *Protobothrops mucrosquamatus* reference transcriptome . For full description of tissue codes refer to Table S5.3.

			Non skin				*Skin*						
Gene name	*Refseq category*	*Pathway*	*A.laevis Eye*	*H.major Testis*	*H.major Heart*	*H.major Liver*	*A.laevis TailA1*	*A.laevis TailA2*	*A.tenuis TailA3*	*A.laevis TailB1*	*A.tenuis TailB2*	*A.laevis Body1*	*A.tenuis Body2*
OPN3	XM_015816690.1	Opsin (non-visual)	2.05	2.14	0.10	0.00	0.00	0.00	0.00	0.00	0.08	0.00	0.00
OPN4x	XM_015815691.1	Opsin (non-visual)	7.83	0.07	0.00	0.00	0.00	0.00	1.22	0.71	0.41	1.20	0.00
OPN5	XM_015812351.1	Opsin (non-visual)	23.44	4.66	0.00	0.10	0.00	0.00	0.00	0.00	0.00	0.59	0.00
RGR	XM_015823421.1	Opsin (non-visual)	561.45	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
OPN1LW	XM_015812260.1	Opsin (visual)	2651.29	0.11	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
OPN1SW	XM_015825841.1	Opsin (visual)	257.72	36.73	0.12	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
RHO	XM_015823472.1	Opsin (visual)	333.69	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
ARRB2	XM_015813192.1	Phototransduction	78.21	51.39	99.17	37.93	36.51	60.14	41.50	72.05	87.18	73.91	95.42
CNGA1	XM_015828517.1	Phototransduction	5.71	0.78	0.00	0.00	0.00	0.84	0.00	0.00	0.00	0.50	0.00
CNGA3	XM_015829790.1	Phototransduction	34.03	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
CNGB1	XM_015812080.1	Phototransduction	13.51	1.06	0.05	7.87	0.07	0.00	0.00	0.09	0.11	0.06	0.00
CNGB3	XM_015813093.1	Phototransduction	32.88	0.18	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
CRABP1	XM_015816355.1	Phototransduction	94.55	0.51	0.67	0.14	8.33	0.00	0.00	3.30	0.28	4.71	0.00
GNAT2	XM_015821725.1	Phototransduction	732.92	0.61	0.00	0.00	0.82	0.00	0.00	0.00	0.00	0.00	0.75
GNB1	XM_015819597.1	Phototransduction	121.38	56.30	29.51	15.27	71.89	311.91	452.90	323.66	155.15	304.56	386.99
GNB5	XM_015823706.1	Phototransduction	124.35	1.46	3.09	0.26	1.58	1.34	0.00	1.02	0.86	4.93	0.00
GRK5	XM_015817244.1	Phototransduction	3.95	0.70	0.17	0.54	0.18	1.21	1.82	0.57	0.58	2.32	4.09
GRK7-like	XM_015821098.1	Phototransduction	24.86	0.00	0.06	0.05	0.17	0.45	0.00	0.34	0.00	0.45	0.00
GUCA1A	XM_015821339.1	Phototransduction	700.37	4.42	0.00	0.62	0.37	0.00	2.61	1.41	0.00	1.83	0.00
GUCA1B	XM_015821340.1	Phototransduction	1589.51	0.00	0.00	0.82	2.78	7.86	4.57	1.88	3.74	2.44	0.49
GUCA1C	XM_015822416.1	Phototransduction	1212.72	2.95	1.32	0.00	0.61	0.00	0.00	0.00	0.00	0.00	0.00
GUCY2D-like	XM_015826485.1	Phototransduction	31.69	0.04	0.00	0.00	0.00	0.57	0.00	0.00	0.00	0.05	0.00

PDC	XM_015813313.1	Phototransduction	363.77	0.00	0.00	0.12	0.31	0.00	0.00	0.00	0.00	0.35	0.00
PDC-like	XM_015822152.1	Phototransduction	122.21	47.12	87.58	57.69	75.62	118.11	71.01	150.16	119.73	150.73	122.95
PDE6B-like	XM_015828048.1	Phototransduction	29.32	0.38	0.00	3.27	0.00	0.00	0.00	0.00	0.00	0.00	0.00
PDE6C	XM_015822696.1	Phototransduction	80.25	0.57	0.00	0.00	0.00	0.00	0.00	0.26	0.00	0.00	0.00
PDE6D	XM_015825389.1	Phototransduction	51.34	78.30	134.38	5.19	6.85	6.48	43.88	6.27	33.43	17.32	25.82
PDE6G	XM_015815331.1	Phototransduction	1672.29	3.99	7.25	6.63	15.57	4.48	0.00	25.51	2.01	22.34	0.00
RCVRN	XM_015810942.1	Phototransduction	3084.44	0.84	0.55	0.12	0.85	1.07	3.10	0.00	0.80	0.36	0.57
SAG	XM_015813710.1	Phototransduction	625.65	1.77	0.00	0.08	0.00	0.25	0.00	0.39	0.57	0.75	0.00
SLC24A2	XM_015825096.1	Phototransduction	74.78	0.68	0.00	0.00	0.09	0.00	0.00	0.00	0.00	0.16	0.00
ABCA4	XM_015829247.1	Retinoid regeneration	27.01	0.43	0.00	0.05	0.02	0.00	0.00	0.00	0.00	0.06	0.00
LRAT	XM_015816562.1	Retinoid regeneration	742.72	0.00	0.39	2.09	23.17	0.00	19.24	5.64	9.54	12.83	9.91
RBP3	XM_015823138.1	Retinoid regeneration	265.78	0.06	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
RDH10	XM_015817157.1	Retinoid regeneration	272.76	1.61	4.11	7.43	3.79	3.40	7.74	5.96	4.75	8.44	4.49
RDH11-like	XM_015826948.1	Retinoid regeneration	5.84	15.18	1.15	84.87	1.09	13.33	2.99	21.50	0.88	32.57	0.00
RDH12-like	XM_015814101.1	Retinoid regeneration	35.04	3.77	4.57	0.57	15.82	0.50	2.44	4.36	4.61	8.03	4.17
RDH14	XM_015813058.1	Retinoid regeneration	27.23	11.85	7.22	2.67	4.07	2.27	5.71	2.08	3.71	3.73	5.82
RDH5	XM_015824195.1	Retinoid regeneration	411.95	0.00	7.13	14.01	0.20	0.00	0.00	0.00	0.00	1.02	0.00
RDH8-like	XM_015823256.1	Retinoid regeneration	223.28	0.35	0.00	0.44	1.06	1.32	0.00	2.04	0.28	0.88	0.00
RGS9	XM_015810365.1	Retinoid regeneration	44.50	0.57	1.44	0.00	0.00	0.00	0.91	0.00	0.13	0.10	0.00
RGS9BP	XM_015821677.1	Retinoid regeneration	39.87	0.19	2.69	0.00	0.29	0.00	4.21	0.00	0.16	7.08	0.00
RLBP1	XM_015815287.1	Retinoid regeneration	497.78	0.24	0.00	0.00	0.09	0.00	0.00	0.00	0.00	0.00	0.00
RPE65	XM_015829586.1	Retinoid regeneration	891.90	0.82	1.17	0.06	0.56	5.74	2.09	1.46	1.28	1.52	5.97

TABLE S5.6. Prevalence of tail injuries and level of tail damage in sea snakes. Most common type of injuries were small wounds < 0.5cm (50.7%) and punctures (29.7%), followed by large wounds > 0.5cm (15.9%) and scars (3.6%).

	Uninjured tails		*Injured tails*	
Species	*No.*	*%*	*No.*	*%*
A. laevis	13	33.3	26	66.7
A. duboisii	5	41.7	7	58.3
H. major	24	53.3	21	46.7
H. stokesii	9	60.0	6	40.0

TABLE S5.7. Summary of hurdle model showing relationship between (conditional on tail damage occurring) total number of tail injuries and snout-vent length (svl), and whether the relationship differed between the species *Aipysurus laevis, A. duboisii, Hydrophis major* and *H. stokesii*. CI = confidence interval.

Count model (truncated Poisson with log link)					*95% CI for OR*	
	Estimate	*Std. error*	*z value*	*P value*	*lower*	*upper*
Intercept (A. laevis)	0.79	0.17	4.77	1.84e-06**	0.47	1.12
A. duboisii	0.18	0.30	0.61	0.54	-0.40	0.77
H. major	-0.25	0.26	-0.98	0.33	-0.75	0.25
H. stokesii	-0.93	0.57	-1.63	0.10	-2.04	0.19
svl	0.001	0.01	0.12	0.90	-0.02	0.02

Zero hurdle model coefficients (binomial with logit link)					*95% CI for OR*	
	Estimate	Std. error	z value	P value	lower	upper
Intercept (A. laevis)	0.49	0.37	1.337 0.1814	0.37	-0.22	1.23
A. duboisii	0.18	0.74	0.24	0.81	-0.55	1.99
H. major	-0.42	0.49	-0.86	0.39	-0.55	0.70
H. stokesii	-0.87	0.66	-1.32	0.19	-1.51	0.68
svl	0.07	0.03	2.98	0.003*	0.03	0.13

**Indicates significant P value <0.001 *Indicates significant P value <0.05

TABLE S5.8. Planned contrasts comparing the scale of log-odds of tail damage for phototactic species (*Aipysurus laevis* & *A. duboisii*) and non-phototactic species (*Hydrophis major* & *H. stokesii*). Multiple contrasts were based on generalised linear models. CI = confidence interval.

					95% CI for Estimate	
Planned contrasts	*Estimate*	*Std. error*	*z value*	*P value*	*lower*	*upper*
Non-phototactic (*H. major* vs *H. stokesii*)	0.45	0.63	0.72	0.84	-1.05	1.96
Non-phototactic vs *A. laevis*	-0.64	0.48	-1.31	0.44	-1.80	0.51
Non-phototactic vs *A. duboisii*	-0.82	0.70	-1.16	0.58	-2.49	0.85

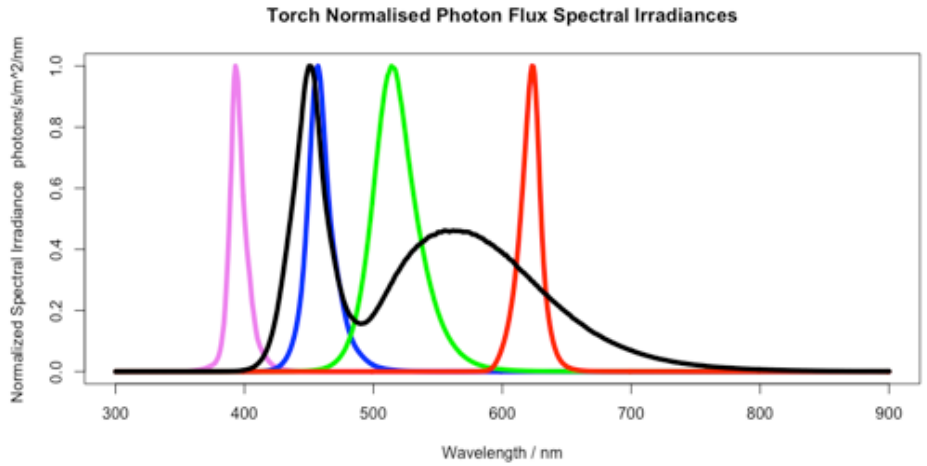

FIGURE S5.1. Normalised spectral irradiance in photons for the violet, blue, green, red and white LED torches. Peak emissions for the coloured LEDs were located at 393, 457, 514 and 623 nm.

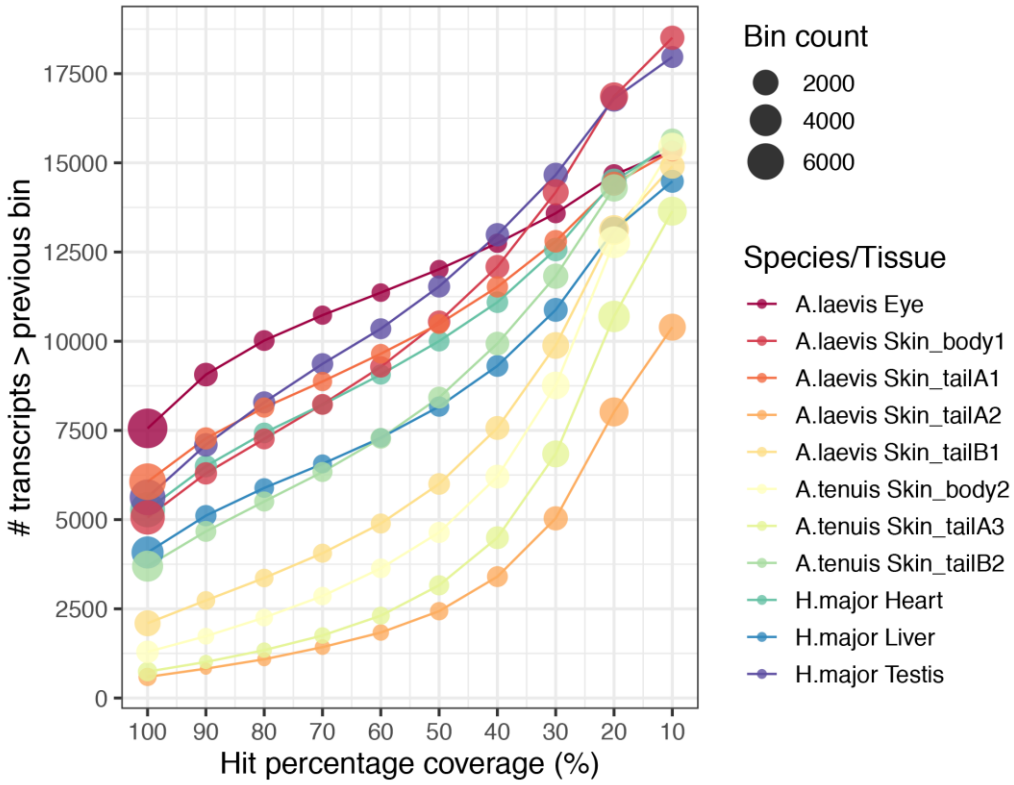

FIGURE S5.2. Coverage of full-length-transcripts using BLAST+ of SwissProt database, showing total number of proteins (size of circles) that were counted per bin for each sample. Note: single transcripts that had multiple hits to the same protein were collapsed into one representative hit. For full description of tissue codes refer to Table S5.3.

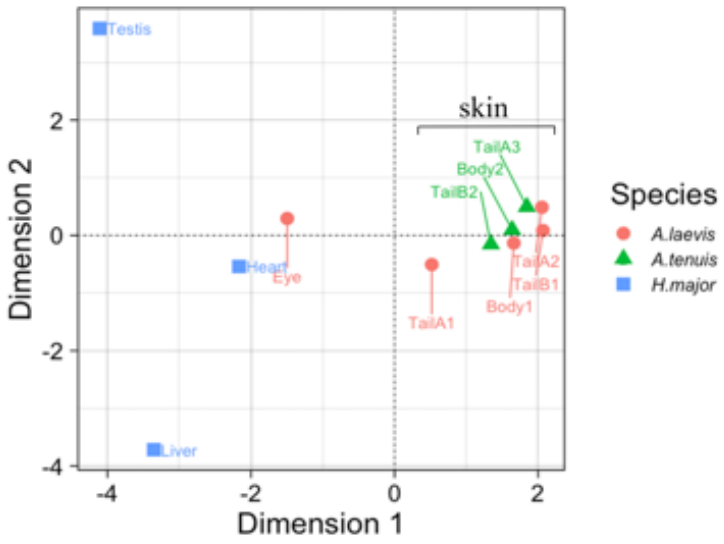

FIGURE S5.3: Multi-dimensional scaling plot showing separation of samples based on gene abundance estimates log-counts-per-million (CPM). Distances on the plot represent leading log2-fold-change between the samples for the top 500 genes that distinguish those samples. For full description of tissue codes refer to Table S3.

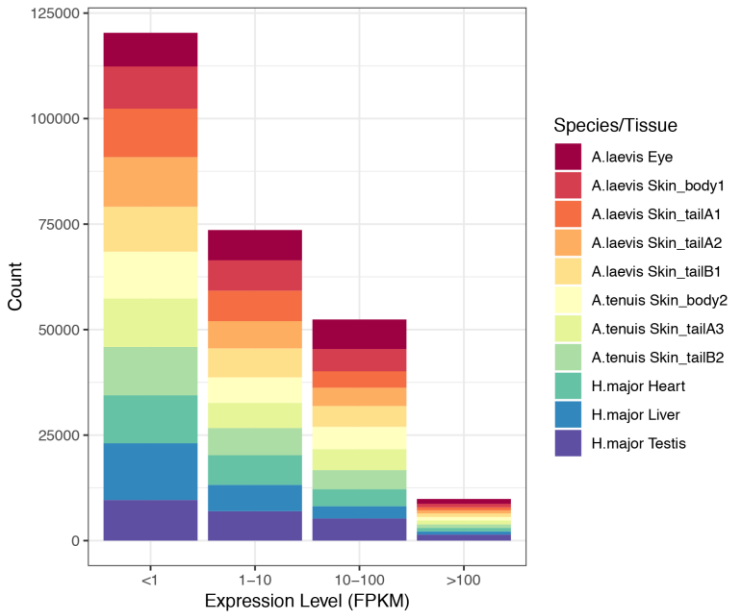

FIGURE S5.4. Normalised expression levels for tissue transcriptomes from *Hydrophis major*, *Aipysurus laevis* and *A. tenuis*. RNA reads were quantified by pseudoalignment to a pitviper (*Protobothrops mucrosquamatus*) transcriptome and normalised using fragments per kilobase of transcript per million mapped reads (FPKM). For full description of tissue codes refer to Table S5.3.

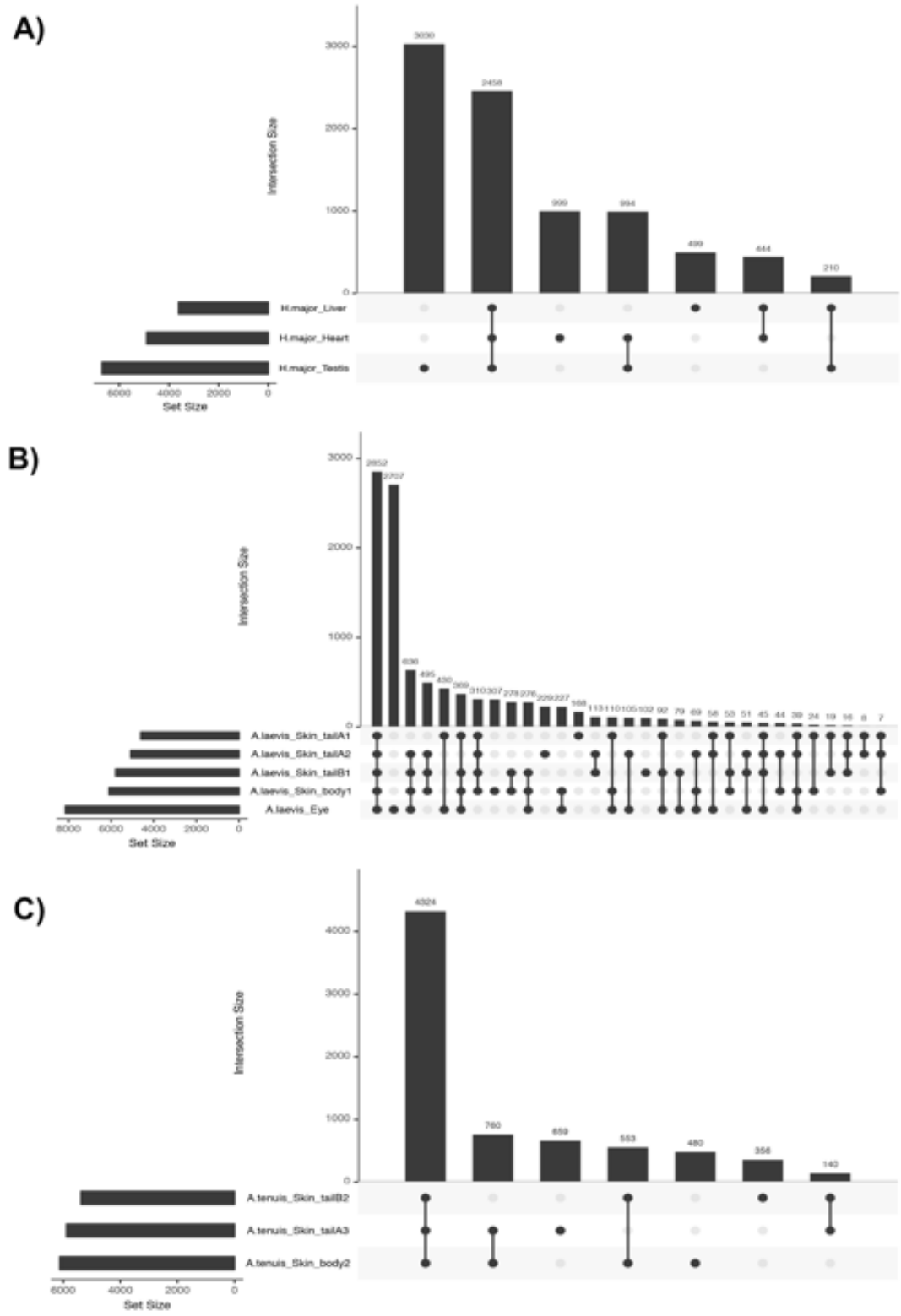

FIGURE S5.5. Upset plots of expression profiles that characterise each tissue transcriptome per species: A) *Hydrophis major*, B) *Aipysurus laevis*, and C) *A. tenuis*. RNA reads were quantified by pseudoalignment to a pitviper (*Protobothrops mucrosquamatus*) transcriptome and normalised using fragments per kilobase of transcript per million mapped reads (FPKM). For full description of tissue codes refer to Table S5.3.

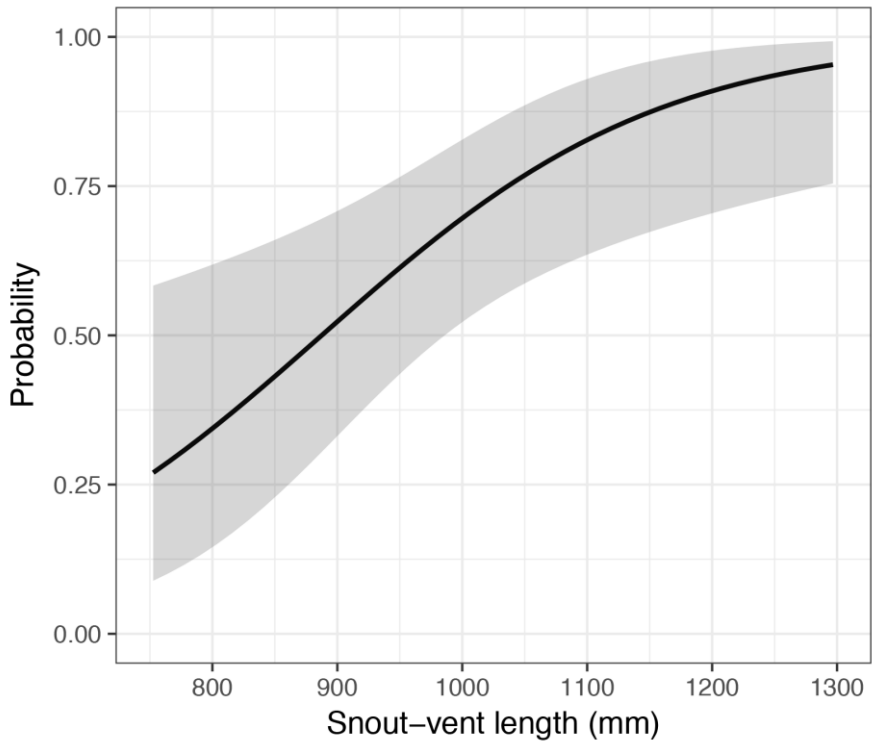

FIGURE S5.6. Probability of tail damage given snout-vent length in sea snakes. Probability estimated using a generalised linear model assuming binomial variance and logit link (damage ~ 0 + species + svl).

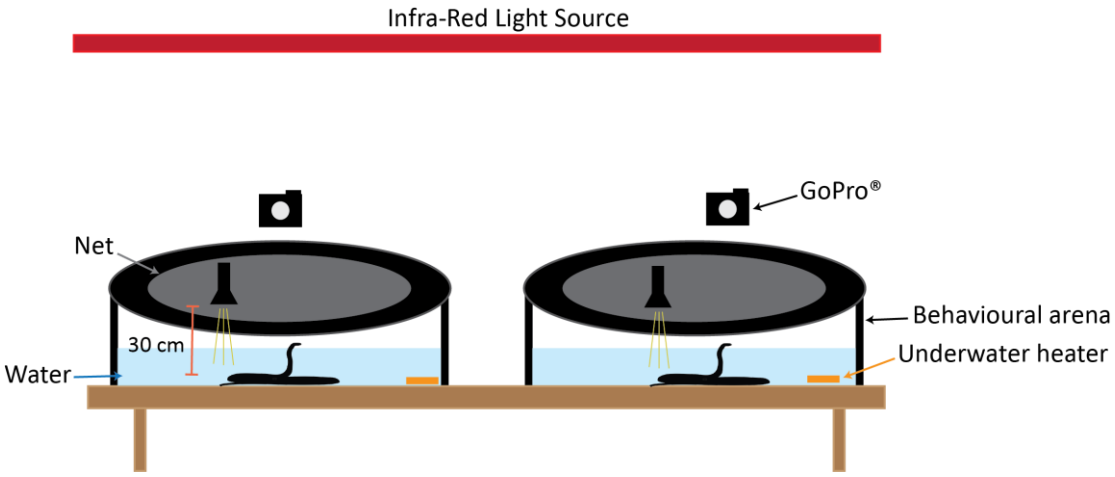

FIGURE S5.7. Experimental set up for behavioural experiments. Snakes were transferred from holding tanks to round behavioural arenas (60 cm diameter × 60 cm height, 50 L volume) containing an underwater heater and seawater (24-28°C, 35 L volume, 13 cm depth), covered by a mesh net. Areas were housed in dark soom lit by a single florescent red globe positioned 1 m above the arena. Localised areas of skin were illuminated using a flashlight held at approximately 30 cm distance.

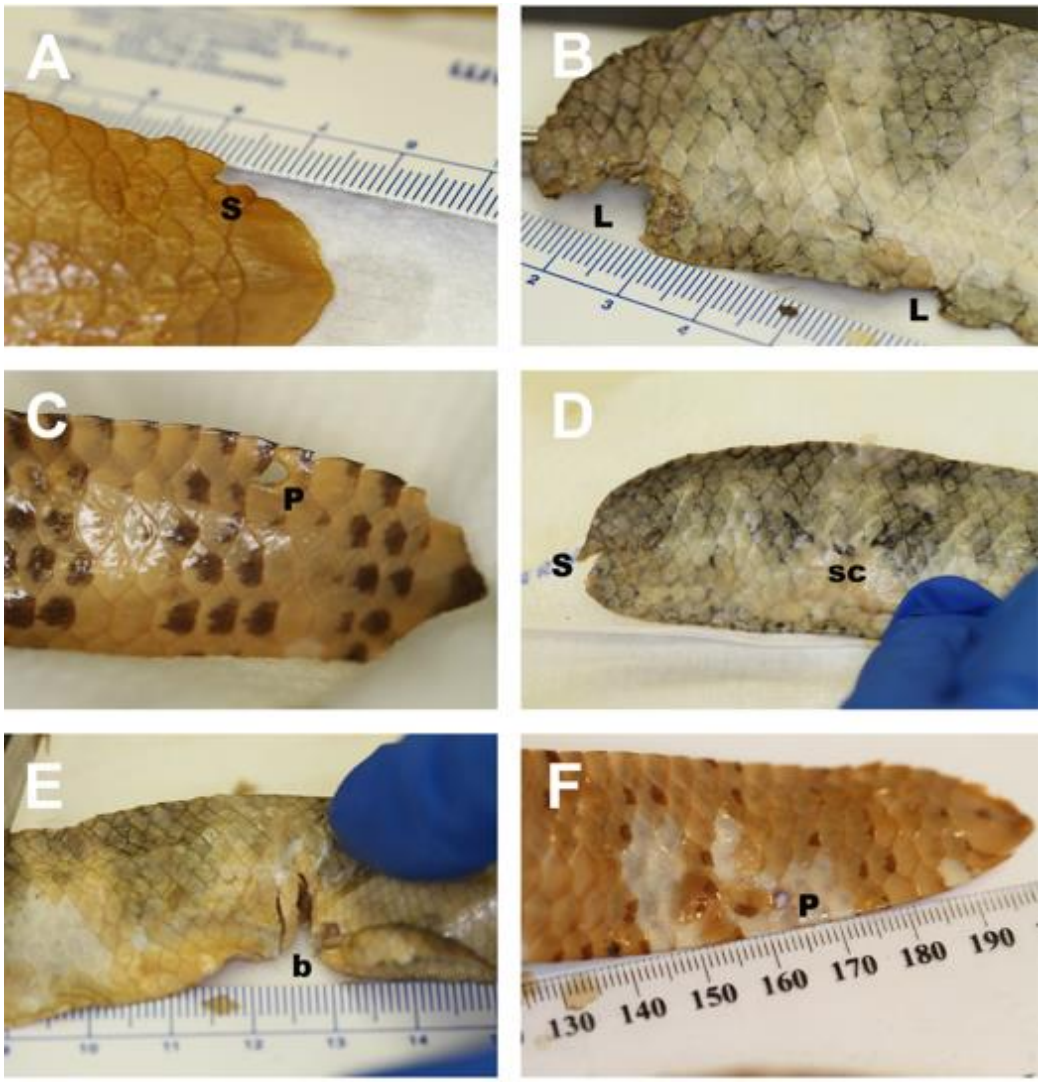

FIGURE S5.8. Types of injuries recorded in museum specimens of sea snakes: A) Small injury (S) with < 0.5cm tissue missing also known as 'tail nicks', B) bites that cause a large injury (L) with > 0.5cm tissue missing, C) Puncture wounds (P) potentially caused by a tooth, D) scar (sc) potentially inflicted by a bite, E) post-mortem injury likely caused by boat propeller (b) as evidenced by exposed muscle and connective tissue, and F) perfectly round puncture wounds (P) most likely caused by barnacle ectoparasites. Specimens from the species *Aipysurus laevis, A. duboisii, Hydorphis major* and *H. stokesii* were photographed (Canon EOS7D, Canon, Japan) with a macro lens (100 mm, f/2.8 ultrasonic, Canon, Japan). Species and museum numbers for specimens: A) *Aipysurus laevis* QMJ81232, B) *Hydrophis major* QMJ83345, C) *A. duboisii* QMJ83776, D) *H. major* QMJ82897, E) *H. major* QMJ83581 and F) *A. duboisii* AMR140886. Images by J. Crowe-Riddell.

Appendices

Appendix A: First records of sea snakes diving to the mesopelagic zone

The following pages contain a natural history observation I wrote during my PhD. It is not part of my thesis but is related to the general biology of sea snakes.

Crowe-Riddell, JM, D'Anastasi, BR, Nankivell, JH, Rasmussen, AR, Sanders KL (in review) First records of sea snakes (Elapidae: Hydrophiinae) diving to the mesopelagic zone (>200 metres). *Austral Ecology* x: xx-xx

First records of sea snakes (Elapidae: Hydrophiinae) diving to the mesopelagic zone (>200 metres)

Jenna M. Crowe-Riddell, Blanche R. D'Anastasi, James H. Nankivell, Arne R. Rasmussen, Kate L. Sanders

Abstract

Viviparous sea snakes (Elapidae: Hydrophiinae) are fully-marine reptiles distributed in the tropical and subtropical waters of the Indian and Pacific Oceans. Their known maximum diving depths range between 50 and 100 metres (m), which is thought to limit their ecological ranges to shallow habitats. We report two observations, from industry-owned remote underwater vehicles (ROVs), of hydrophiine sea snakes diving and foraging at depths of approximately 250 m in the Browse Basin on Australia's Northwest Shelf, in 2014 and 2017. These observations show that sea snakes are capable of diving to the low-light, cold-water (14.5ºC) mesopelagic zone, also known as the 'twilight' zone. These record-setting dives raise new questions about the thermal tolerances, diving behaviour and ecological requirements of sea snakes. In addition to significantly extending previous diving records for sea snakes, these observations highlight the importance of university-industry collaboration in monitoring understudied deep-sea habitats.

KEYWORDS: sea snakes, diving behaviour, depth, Northwest Shelf, mesopelagic zone, remotely operated vehicles (ROVs), deep sea, industry collaboration

Main text

Viviparous sea snakes (Elapidae: Hydrophiinae) are a recent, secondarily marine radiation of venomous snakes that have many physiological and anatomical adaptations to marine life, including valvular nostrils, paddle-shaped tails, sublingual salt glands and cutaneous gas exchange (Dunson & Stokes 1983; Dunson 1975; Rasmussen et al. 2011). Of the 62 recognised sea snake species, only the pelagic *Hydrophis platurus* is known to hunt at the sea surface; all other sea snakes are benthic foragers that hunt close to the sea floor, typically by probing crevices and burrows (Rasmussen et al. 2011). Sea snakes are thought to supplement up to 23% of their oxygen requirements while submerged by using cutaneous gas exchange, but must also periodically swim to the sea surface to breath air (pulmonary oxygen uptake), which limits the duration and depth

of dives (Seymour 1974; Heatwole & Seymour 1975; Udyawer et al. 2016). The known depth distributions of most species are shallower than 40–50 m depth (Heatwole & Seymour 1975) and there are only a few records of sea snakes at depths greater than 100 m. A snake identified by a diver as *Aipysurus laevis* (B. Sheils pers. comm.) was observed at 133 m at the Goodwin oil platform on the Northwest Shelf off Karratha, Western Australia (Greer 1997). In 2006, the Galathea III expedition collected a *Hydrophis elegans* at the sea surface above depths of 145 m offshore from Broome, Western Australia; immediately after capture the snake regurgitated a benthic eel species indicating that it had been foraging near the sea floor (A.R. Rasmussen, pers. obs.). The deepest record from a demersal trawl vessel, at 93–103 m, is also from Western Australia and the specimen was identified as *Hydrophis czeblukovi* (formerly *H. geometricus*) (Smith 1986).

The maximum depths and diving limits have been difficult to determine for sea snakes because underwater observations are typically limited to shallow water habitats that are easily surveyed (*e.g.* coral reefs, seagrass beds, coastal bays), and the logistical challenges of tagging individual snakes means that remote tracking efforts have been restricted to a few species in coastal localities (Udyawer et al. 2018). However, remotely operated vehicles (ROVs) and baited remote underwater video stations (BRUVS) provide an effective way to observe diving behaviour at greater depths (Udyawer et al. 2014; Macreadie et al. 2018).

Here, we report the deepest dives ever recorded for sea snakes, substantially extending current knowledge of the diving capabilities and ecological requirements of these marine reptiles. The two observations were video-recorded on ROVs in 2014 and 2017 in the Browse Basin on Australia's Northwest Shelf. On the 16th of November 2014, a sea snake was filmed swimming at 245 m depth (Figure 1A). The second snake was filmed on the 18th of July 2017 at 239 m and appeared to be foraging by swimming close to the sandy sea floor and stopping in several places to briefly probe the substrate with its head (Figure 1B); the ROVs' temperature probe recorded 26.5^{o}C (degrees Celsius) at the sea surface and 14.5^{o}C at the time the snake was video-recorded. Oceanic depths between 200 and 1000 m encompass the mesopelagic ('twilight') zone characterised by low-light penetration and a cold-water thermocline. The mesopelagic zone of the Browse Basin ranges from approximately 14^{o}C and 21 atmospheric pressure (atm) at 200 m to 8^{o}C and 51 atm at 500 m (Rayson 2011).

The two snakes were provisionally identified as *Hydrophis* species due to their distinctive head and body proportions; both have small heads and narrow fore-body relative to hind-body girths that are typical of the many *Hydrophis* species that specialise on burrowing prey (Sherratt et al. 2018). The snakes appear to belong to the same species because of their very similar head-

body proportions and colour patterns (between 40 and 45 dark bands in both specimens). However, based on the images available, it was not possible to identify these snakes to species level or exclude the possibility that they belong to a presently unrecognised species.

Figure 1. New depth records for sea snakes, observed from video-recordings from remotely operated underwater vehicles (ROVs), on Australia's Northwest Shelf. A) Image of an unidentified sea snake species swimming at a depth of approximately 245 m on 16th of November, 2014. B) Image of an unidentified sea snake foraging at a depth of approximately 239 m on 18th of July, 2017. The snakes appear to belong to the same species because of their very similar head-body proportions and colour patterns (45 dark bands in both the 2014 and 2017 specimens).

The new records of sea snake activity at depths of up to 245 m significantly extend the known depth range for sea snakes, prompting questions about the physiological mechanisms that allow them to function at cooler waters and higher pressures. Extended dives to deep-sea habitats are likely achieved by a bimodal gas exchange: an increased level of cutaneous gas exchange might relieve the higher pressures of internal gases (*i.e.* 'the bends'), and cooler temperatures might decrease total oxygen consumption– reducing the frequency of trips to the sea surface to breathe and thus extending total submergence times (Udyawer et al. 2016; Seymour 1974). However, further studies are needed to understand the interaction between metabolism, bimodal oxygen uptake and activity levels across temperature gradients for deep-diving sea snakes (Udyawer et al. 2016). Thermal tolerance estimates for sea snakes are predominately based on laboratory studies of *H. platurus* that indicate an ideal thermal range of 20–37°C, cessation of feeding, locomotion and orientation at temperatures below 18 °C, and lower lethal limits of 14–17°C (Dunson & Ehlert 1971; Graham et al. 1971). The present study reports two records of sea snakes at 14.5°C and describes foraging behaviour at this temperature (Figure 1B), indicating a higher range for thermal tolerance than previously recorded for sea snakes. Sea surface temperatures are a major determinant of the current geographic distribution of sea snakes (Heatwole et al. 2012; Lillywhite et al. 2017), but how oceanic temperatures affect the vertical distribution (*i.e.* diving ranges) is a comparatively understudied aspect of the spatial ecology of sea

snakes (Udyawer et al. 2018). Finally, these observations raise questions about sensory adaptations of deep-diving sea snakes, such as how they might orient and navigate in this light-reduced habitat.

The record-setting dives reported here challenge widely held assumptions of the limits to sea snake behaviour, physiology and ecology. Further observations (using *e.g.* animal tracking, ROV surveys) are needed to determine whether such deep dives are an unusual or typical behaviour of the species recorded in the present paper, and other sea snakes more generally. Finally, these new dive records emphasise the importance of collaborations between research and industry organisations to survey previously overlooked deep-water habitats for sea snakes.

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Appendix B: What went wrong in communicating the Tasmanian tiger genome?

The following pages contain a blogpost I posted on the 10th of January 2018. It is not part of my thesis but is demonstrates my commitment to communicating science.

Crowe-Riddell, JM, (2018) What went wrong in communicating the Tasmansian tiger genome? Available at: www.jennacroweriddell.com/jblog/thylacine-scicomm

What went wrong in communicating the Tasmanian tiger genome?

January 10, 2018 *Jenna Crowe-Riddell*

My earliest memory of confronting extinction was in primary school – growing up in Australia in the 1990s, a kid's education show 'Behind the News' covered a story on cloning the Tasmanian tiger. Prominent in my mind is an image of a shrivelled pup floating in a jar with clinical writing scrawled across a label tied to its paw. We were told that DNA could be extracted from this specimen and used to re-animate the species. Over 20 years later, and despite official efforts to clone the tiger being scrapped in 2005, it seems we are still captivated by the idea of de-extinction.

The publishing of the thylacine genome received a lot of media attention (rated in the top 5% of all research outputs scored by Altmetric) and along with it came a revival of the cloning story. However, if you take a look at the abstract in the thylacine genome paper published in Nature Ecology and Evolution, it does not mention the prospective cloning as an application of sequencing the genome. In fact, a lot of the information in the media coverage is conspicuously absent from the original publication. In addition to claims of the thylacine genome sequencing bringing us 'one-step closer to cloning the tiger', there have been more pernicious claims that the species' was already 'on the way out' long before the colonial invasion of Australia.

Communication breakdown

The science communication of the thylacine genome paper broke down in several ways and ultimately led to the dispersal of inaccurate and misleading information. To help clear up the SciComm mess around the reporting of the thylacine sequencing paper, I spoke to several scientists that work in ancient DNA, including an author on the paper, evolutionary biologist Dr Kieren Mitchell, and Dr Lauren White, a wildlife geneticist and expert in population genetics of Tassie tigers.

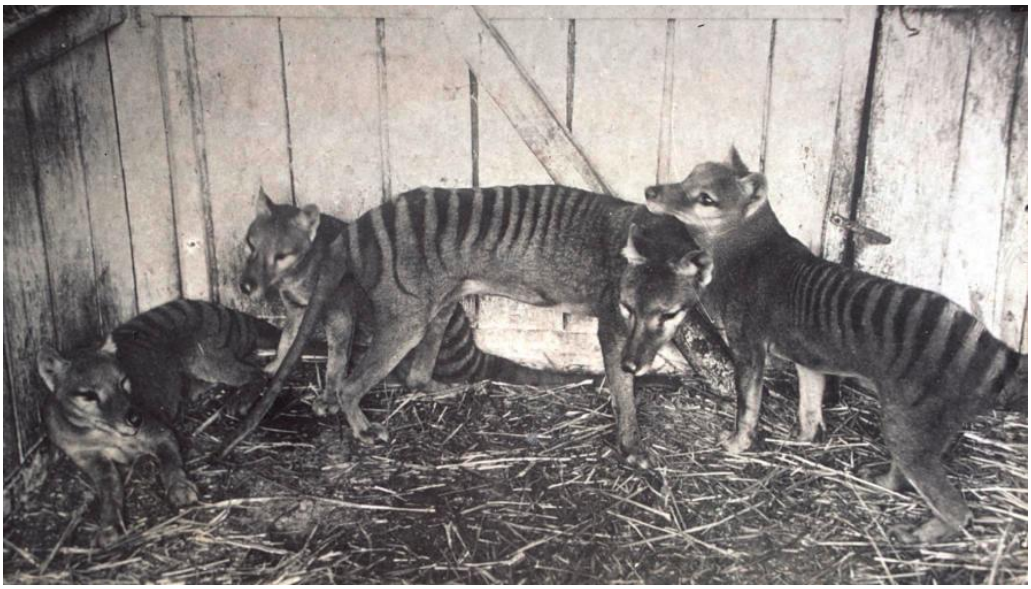
Does sequencing the genome make it easier to clone the Tassie tiger?

This is a relatively straightforward SciComm mistake because cloning the tiger looms fairly large in the cultural zeitgeist but it is not mentioned *anywhere* in the paper, thus this misinformation is most likely a case of sensationalism by the media. When I asked White about whether sequencing

the genome is the first step to cloning, she said, *"Technically yes, having the genome is a pre-requisite for cloning. But it's such a tiny baby step that it's not worth mentioning."*

"The thylacine would be a terrible candidate for de-extinction, even if we had the know-how"

Turns out cloning a species and the wider process of 'de-extinction' is a huge undertaking and that, as with issues in cloning the Tassie tiger in the past, there are many, *many* other problems that we would need to overcome. White makes the point that *"the current ideas about how to go about de-extinction cloning (none of which have actually been successful, even on living animals) require a close living relative of the extinct species. The thylacine's closest living relative is the numbat, hardly a good surrogate. So, the thylacine would be a terrible candidate for de-extinction, even if we had the know-how."*



The loss of the Tassie tiger has become an emblem of Australia's mammal extinction crisis, but sequencing the thylacine genome will not allow us to reanimate the species. Photo credit: TMAG Tasmanian Museum and Art Gallery

The problem with such zealous reporting on de-extinction is that 1) Tassie tigers are terrible candidates to clone, and 2) it could jeopardise existing conservation efforts and funding for living species. Cloning extinct species for the purpose of de-extinction is an area that has been criticised as pseudoscience and would swiftly lead us into a murky ethical bog, so it's a topic beyond the scope of this blog post*.

Ultimately, it's a shame that the de-extinction angle of the media coverage appears to be at the expense of the other interesting, and biologically important, findings of the paper. Speaking of which,

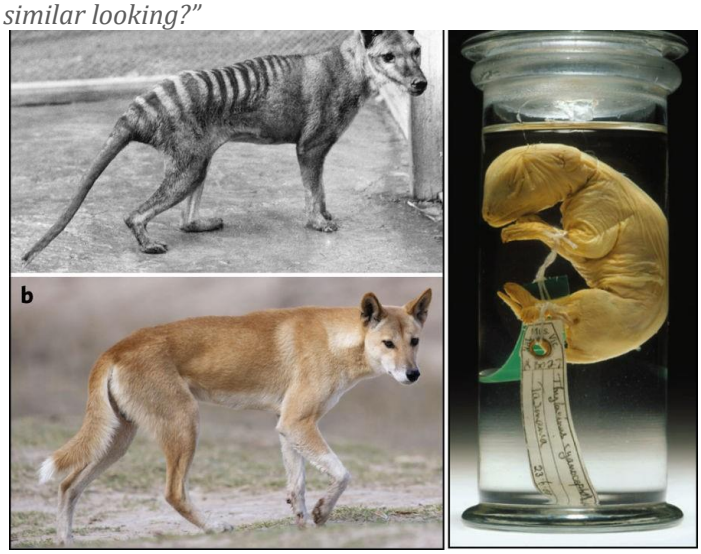
if the genome wasn't sequenced to help clone Tassie tigers, why did scientists do it? The answer to that brings me to the next SciComm mistake.

Why are Tassie tigers being compared to dogs?

Media coverage has emphasized the finding that thylacines are not closely related to dogs, which is true, but by no means a surprise to any biologist. Tassie tigers are marsupial mammals, whereas a dog is a eutherian or placental mammal. Dividing mammals into these broad groupings is based on many biological traits, but a simple way to think about the difference is to compare their reproduction: marsupials give birth to 'undeveloped young' that usually spend time in a pouch, whereas eutherian mammals have longer gestation periods and no pouch. Scientists have known for a long time that marsupials and eutherians are only distantly related - they are estimated to have last shared a common ancestor over 160 million years ago, an age when mammals were fur balls at the feet of dinosaurs. So, what did the paper actually find?

> *"[So] How then can two organisms separated by as much independent evolution as the thylacine and wolf be so similar looking?"*

Basically, marsupial Tassie tigers are an example of convergent evolution with carnivorous eutherian mammals (*e.g.* dogs, wolves, foxes). That is, these mammals are only distantly related to each other and yet they look *extremely similar* in body size and skull shape because they are both predators. It's a kind of paradox in genomics and evolution, as Mitchell explains, *"We generally expect closely related organisms to have more similar genomes than more distantly related organisms, but our genome is also the "blueprint" that determines our physical characteristics. [So] How then can two organisms separated by as much independent evolution as the thylacine and wolf be so similar looking?"*



The first figure from the thylacine genome paper clearly invites comparison between the two, distantly related mammal groups, making it easy to see how journalists may have mis-understood the paper.

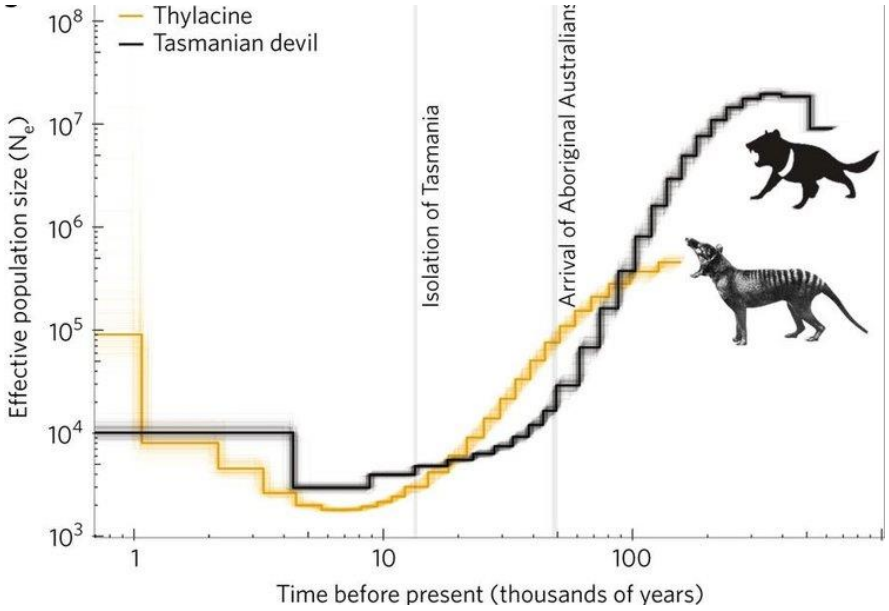
The bulk of the genome paper set out to address this very question. Mitchell says *"One possible solution to this apparent paradox is that closely related organisms may have more similar genomes* ***on average*** *than more distantly related organisms, but that small and important segments of the genome could be very similar between distantly related organisms"*. They compared the genes inside the genome to see "*if there were any genes that were more similar between the thylacine and wolf than expected, but didn't really find anything"*, says Mitchell. They suggest it could instead be the 'non-gene' parts of the genome, which regulate when genes are switched on or off, that might be responsible for the convergent evolution.

What the media should have emphasised was that the paper was comparing genes that were shared by both thylacines and dogs that may explain why they look so similar. However, just by skimming the abstract and main figures, it's easy to see how a non-expert might misunderstand the nuance of the genomics evolution paradox. Furthermore, if you relied on the media release alone then you'd be in trouble too: *"Secrets from beyond extinction: Tasmanian tiger was a kangaroo in wolf's clothing*". Unfortunately, the title of the press release reflects the ambiguities within it.

So, what's the SciComm lesson to take away? Most SciComm is an interaction between scientists, communications team at the university or research institution, and journalists. Most journalists do not have specialised training in Science, thus it's an essential job for scientists to work closely with their Comms team to accurately communicate the findings of their paper while also being vigilant of possible misinterpretations. To be fair, this is not a trivial task, but it does highlight a prominent issue facing SciComm today.

Thylacine genome shows that the population was already in trouble

This is by far the most dangerous idea to have been circulated as a result of the media coverage on the thylacine genome paper. Surprisingly, this misinformation probably began with a piece in The Conversation by Prof Andrew Pask who's the last author on the thylacine genome paper. Although he concedes in the first sentence that there's no doubt that humans killed off the Tassie tigers, the inflammatory headline contends otherwise.



The second figure from the thylacine genome paper shows effective population size through time: showing a steady decline in Tassie tigers and devils over thousands of years. However, populations appear to have stabilised and even increased approx. 6,000 years ago.

Pask, and other media coverage, point to the 'low genetic diversity' as proof of the Tassie tiger's predisposition to extinction. However, the thylacine genome paper did not analyse genetic diversity, instead the authors analysed the genome to measure the effective population size. This showed that Tassie tiger populations were actually on the rise before colonial invasion of Tasmania. Although effective population size can be indirectly linked to genetic diversity there are far better ways to *directly* measure it. White points out that *"There are analyses that can be done on single genomes that look at the number of putatively deleterious mutations or the amount of inbreeding, but these were not presented in the paper."*

"The fact is, thylacines could have had adequate genetic diversity to remain perfectly healthy and the population size decrease we observed might just have been part of natural (survivable) population fluctuations." – Mitchell

The main issue with this misinformation is that, based on the results of the paper, you cannot come to the conclusion that Tassie tigers were 1) at risk of extinction, or 2) more susceptible to diseases or other ill effects of having low genetic diversity.

Final thoughts

"I think a really good opportunity was missed… [to communicate] the process of evolution to the public." - Mitchell

Despite some excellent coverage of the thylacine paper (*e.g.* Nature), the overwhelming focus was of de-extinction cloning. This diminished the opportunity to convey the fascinating main findings of the paper.

As a scientist, I think there's always a risk that your results will be misunderstood, but it is our role to effectively and accurately communicate them to a non-specialist Comms team or journalist. Hopefully, as scientists, journalists and general Science communicators, we can learn from the mistakes in the media coverage of the thylacine genome paper.

What did you think of the media coverage on the paper? Could it have been handled better? Do scientists have to get better at SciComm? Or should there be greater emphasis on Science training for journalists? Interested in hearing your thoughts in the comments!

*Maybe the topic for my next blog post, but for now here's some further reading within the murky ethical bog of de-extinction: The Hunter by Julia Leigh, Rise of the Necrofauna by Britt Wray, and Imagining Extinction by Ursula K. Heise