A POTENTIAL ROLE OF THE SQUAMATE HARDERIAN GLAND IN VOMEROLFACTION

by

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A thesis submitted for the degree of Doctor of Philosophy at the
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submitted June, 1997

ABSTRACT

The Harderian gland is a large orbital feature, whose structure and function varies among vertebrates. Thus far, scattered anatomical studies have been carried out on disparate squamate (Reptilia) species. In light of recent morphological findings, a role for the gland in vomerolfaction seems its most likely function. The two aims for this present study were: a) To morphologically examine the structure of the Harderian gland within major squamate clades, and b) to infer its potential role as a source of secretion for the squamate VNO. The latter aim was achieved by comparing the secretory capacity of the VNO to an analogous system, the main olfactory system (MOS), and thereby determine the necessity for the extrinsic glandular secretions. By using the comparative method, I was able to identify some evolutionary trends in Harderian gland, olfactory mucosae and VNO structure across a range of taxa. Representative species exhibiting olfactory (Gekkota) or vomerolfactory (Serpentes) specialisation, and generalised nasal chemosensory adaptation (Scincomorpha) were examined. The Harderian glands and chemosensory structures were studied using light microscopic, histochemical and electron microscopic techniques. The Harderian glands were also anaylsed using microchemical (pronase digestion), embryological and autoradiographic techniques. Despite some histological variations, this gland was, in all species, a serous secreting structure associated with the lacrimal apparatus (and thus the VNO). The squamate olfactory mucosae possessed serous secreting submucosal Bowman's glands and mucous secreting sustentacular cells, which led to the formation of the heterogeneous mucus layer covering the sensory epithelium. The source of the serous component of the heterogeneous mucus layer in the VNO was less apparent. By comparing the secretory capacity of both the squamate MOS, and the structurally similar mammalian VNO, I concluded that there should be an extrinsic source of serous secretion for the mucus lining of the squamate VNO. Of the three candidates examined, the Harderian gland seemed most likely. Thus, though there was histological variation among phylogenetic lineages, the association between the serous secreting Harderian gland and the VNO is a consistent feature, and implies that the gland may be the extrinsic serous source for the mucus layer.

DECLARATION

This work contains no material which has been accepted for the award of any other degree or diploma in any university of 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.

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Susan Joyce Rehorek
June 1997

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Rehorek,S.J., Firth,B.T., and Hutchinson,M.N. 1997. Morphology of the Harderian gland of some Australian geckos. J.Morphol.321(3):235-260.

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FUTURE PRESENTATIONS/ PAPERS

Rehorek,S.J., Firth,B.T., and Hutchinson, M.N. Morphological variation of the Harderian gland in gekkonoid lizards. 5th International Congress of Vertebrate Morphologists (July, 1997).

Rehorek,S.J., Firth,B.T., and Hutchinson, M.N. Sources of secretion of the squamate vomeronasal organ. Third World Congress of Herpetology (August, 1997).

Rehorek,S.J., Firth,B.T., and Hutchinson, M.N. The structure of the olfactory mucosa of some squamate reptiles (in prep).

Rehorek,S.J., Firth,B.T., and Hutchinson, M.N. The Harderian gland as a source of secretion for the squamate VNO. (in prep).

Rehorek,S.J., Firth,B.T., and Hutchinson, M.N. The Harderian gland of some Australian pygopods. (in prep).

ACKNOWLEDGMENTS

This study was conducted under the auspices of the Faculty of Medicine, University of Adelaide (PhD scholarship to S Rehorek) and an University Overseas Travel grant.

I would like to thank my supervisors, Dr B.T. Firth and Dr M.N. Hutchinson for their advice throughout the years and the critical analyses of this manuscript. I am also grateful to Professor J Slipka, and Associate Professor A.H. Savitzky for allowing me to view their embryonic material and to both Associate Professor K. Schwenk and Associate Professor Z. Rocek for their advice and insightful ideas during my period overseas. Thanks to Dr N.A. Locket for his criticisms of this manuscript.

Many thanks to Mr. C. Leigh, Mrs G. Hermanis, Ms D. Panuccio, Mr R. Murphy, Mr J. Cecchin and the staff of CEMMSA for their technical assistance, suggestions and willingness to help out. Thanks to the staff of the department of Anatomical Sciences for their support and enthusiasm for my field of research.

I would like to thank my fellow students, Dr L. Soon, Mr R. Moyer, Mr G. Tahmasebi-Sarvestani , Mr M. Teo and Dr C. Roberts, for their support and companionship.

Finally, I would like to thank my family, Mr B.J. McMahon and Ms K. Ogden for their support, enthusiasm and good natured advice given over the years.

CHAPTER 1: INTRODUCTION

1.1 INTRODUCTION

The Harderian gland is a structure found in the orbit of most terrestrial vertebrates. Since its initial description in the deer (*Dama vulgaris*) by Johan Jakob Harder in 1694, the precise function of the Harderian gland has eluded scientists. It is a compound acinar gland generally associated with the nictitating membrane/third eyelid (Sakai, 1981). Other definitions based upon the histological and histochemical properties of the gland led to much confusion with respect to the nomenclature and position of the Harderian and other orbital glands (see Sakai, 1981 for mammals; and Chieffi-Baccari et al., 1992 for turtles). Saint Girons (1988) morphologically defined the orbital glands in reptiles. The Harderian gland was defined as the internal orbital gland which opens into the anterior part of the orbit, whilst the lacrimal glands are the external (anterior and posterior) orbital glands. Payne (1994) defined the Harderian gland (glandula palpebra tertia profundus/deep gland of the nictitating membrane) as a " unitary structure firmly attached to the medial wall of the orbit with a duct normally opening on the inner surface of the nictitating membrane onto the cornea of the eye." In this thesis, I define the Harderian gland as the large gland in the anterior compartment of the orbit, often associated with the inner aspect of the nictitating membrane (diagram 1).

The focus of the majority of the morphological and physiological studies on the Harderian gland has been on rodents, with scattered studies in other tetrapods (see Payne, 1994). In the following section, the structure of the Harderian gland of rodents and other non-reptilian tetrapods will be briefly reviewed, in order to put the scanty results of the reptilian Harderian gland into perspective. Many functions have been put forward for the tetrapod Harderian gland, three of which are pertinent to the squamate reptiles: corneal lubrication, accessory salivary gland, and a role in the vomeronasal system (VNS). The role of the Harderian gland in squamate vomerolfaction is investigated in this study. If the

ANTERIOR

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POSTERIOR

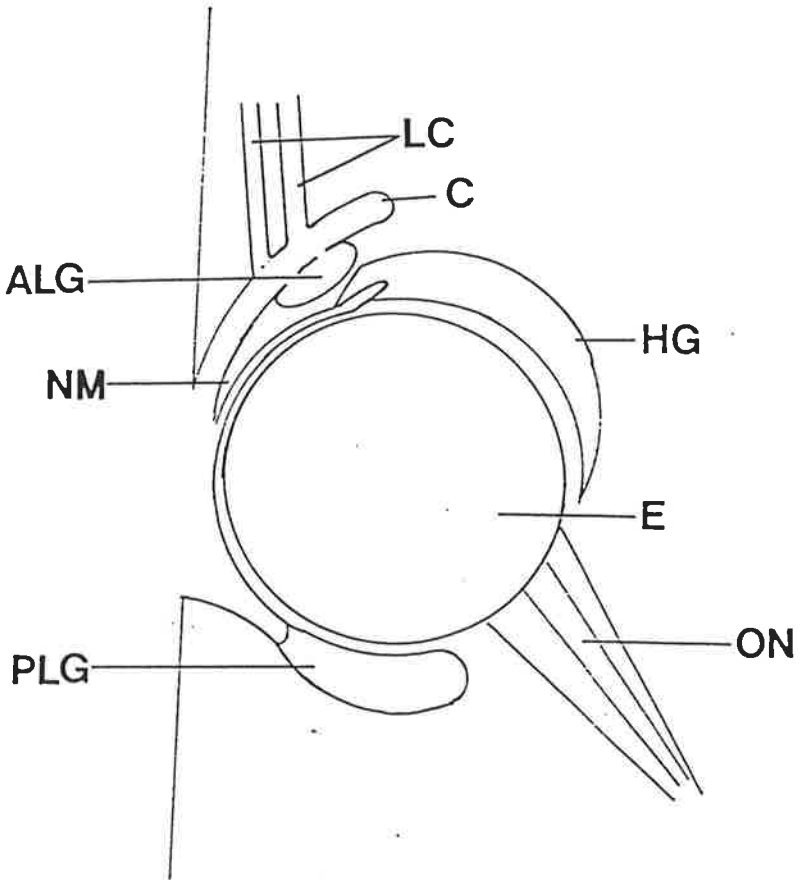


DIAGRAM 1

A schematic diagram of a horizontal section through the orbital region of a reptile. Abbreviations: ALG - Anterior Lacrimal gland; C - Conjunctiva; E - Eye; HG - Harderian Gland; LC - Lacrimal Canaliculi; NM - Nictitating Membrane; ON - Optic nerve; PLG - Posterior Lacrimal gland.

secretions of the Harderian gland flow to the VNO, then the gland may have some function in the lubrication of the VNO. However, little is known about the lubricatory capacity of the squamate VNO. A morphologically and functionally similar nasal chemosensory system, the main olfactory system (MOS) has been studied more extensively. As this is an analogous system to the VNS, it will briefly be described in order to put the relative importance of the lubricatory capacity of the VNO into perspective. Thereafter, the link between the Harderian gland and the VNO is explored, and the potential function of the secretions of the gland in vomerolfaction are explained in detail. Finally, by using the comparative method, I will explain how I intend to test the hypothesis that the Harderian gland functions in squamate vomerolfaction.

1.2 THE HARDERIAN GLAND

The structure and function of the Harderian gland has been described in a variety of tetrapods. Several reviews have compared the Harderian glands of different species (Paule and Hayes, 1958; Sakai, 1981; Saint Girons 1982; Saint Girons, 1985; Olcese and Wesche, 1989; Burns, 1992; Payne, 1994). The present review discusses the structure and function of the Harderian gland generally, followed by a more detailed consideration of the reptile Harderian gland.

1.2.1 NON-REPTILIAN TETRAPODS

There is much variation in the morphology of the non-reptilian Harderian gland (Sakai, 1981; Minucci et al., 1989). It is thus difficult to define a typical, non-reptilian Harderian gland. In this section, I will be briefly describing some of the variations in the Harderian gland morphology, neurovascularisation, secretory product and function among the three non-reptilian tetrapod classes.

1.2.1.1 MORPHOLOGY

There is variation in the presence and relative development of the Harderian and other ocular glands in amphibians. The ocular glands in salamanders consist of a row of compound glands in the mobile lower lid. In some species these glands may be best developed nasally and temporally. In some cases, there are no glandular structures connecting these two glandular aggregations. Thus these two glandular aggregations may be described as the Harderian and lateral lacrimal glands respectively (Walls, 1942). Caecilians possess large Harderian glands compared to other amphibians (Wake, 1985). In anurans (frogs), the Harderian gland is the sole orbital gland (Walls, 1942).

The Harderian gland is present in all birds as a discrete glandular structure. Variations in both number of cells per lobule, as well as number of plasma cells were observed amongst birds (Rothwell et al., 1972; Burns and Maxwell, 1978; Payne, 1994).

The mammalian Harderian gland is well developed in rodents and lagomorphs, and may even be larger than the eyeball itself (Sakai, 1981; 1989; 1992). It appears to be absent in chiroptera, terrestrial carnivores, perissodactyla, primates (Sakai, 1981; Sakai, 1992) and some marsupials (Sakai and van Lennep, 1984). However, due to inadequate preservational techniques, confusing definitions, and lack of histological observations, the Harderian gland of these taxa may require further morphological examination.

1.2.1.2 VASCULATURE and INNERVATION

The Harderian gland of both birds and mammals is vascularised by the ophthalmic blood vessels (Slonaker, 1918; Greene, 1935; Yamashita et al., 1980).

The innervation of the Harderian gland has been mainly investigated in rodents and some birds. In both cases, the orbital gland was found to be innervated by the autonomic

nervous system, from several different nerves including the oculomotor and facial cranial nerves and nerves from the superior cervical ganglion (Slonaker, 1918; Huhtala et al., 1977; Watanabe, 1980; Strum and Shear, 1982; Butler et al., 1984; Walcott et al., 1989). These nerves are often associated with blood vessels (Huhtala et al., 1977; Sakai and Yohro, 1981).

1.2.1.3 TYPE OF SECRETION

The non-reptilian Harderian gland produces a variety of secretions, including lipoidal, mucous and serous granules, as well as porphyrins, melatonin and growth factors, in different species. Rodents are thus far the only groups whose Harderian gland produces only lipid secretory granules (see Nadakavukaren, 1992). Combined mucoid and lipoid secretions are produced by the Harderian glands of a diverse group of species such as the toad, *Bufo viridis*, birds, and the armadillo (Sakai, 1981; Weaker, 1981; Maxwell et al., 1986; Minucci et al., 1989). In the remaining species examined, the Harderian gland produces mucous and/or serous secretory granules (Sakai, 1981). Porphyrins have thus far only been found in the rodent Harderian gland, and at a greater level of production than that of the liver (Cohn, 1955; Spike et al., 1992). The pineal hormone melatonin has been isolated in a variety of species (Vakkuri et al., 1985a and b; Cogburn et al., 1987; Menendez-Pelaez and Buzzell, 1992; Serino et al., 1993). In the guinea pig, the Harderian gland is also a source of growth factors, particularly those pertaining to corneal repair (Yokoyama et al., 1989).

More detailed observations have shown that variations in type of secretory product of both amphibians and mammals. Histological, biochemical and physiological examination has shown that these variations occur seasonally (Di Matteo et al., 1989; Minucci et al., 1990) and can also be sexually dimorphic (Sakai, 1981; Johnston et al., 1985; Buzzell et al., 1989; Minucci et al., 1989; Buzzell and Menendez-Pelaez, 1991; Bodyak and Stepanova, 1994).

1.2.1.4 FUNCTIONS

There are many functions ascribed to the Harderian gland, extensively described by Payne (1994). It is thought to have originally evolved as an orbital lubricatory gland, and may still retain that function in amphibians (Walls, 1942). However, the presence of both other types of secretory products and cell types led to many different types of functions being proposed. Briefly, these can be subdivided into those that deal with the products of either the secretory cells or the non-secretory cells. In the former, the production of mucous (and also in some cases lipid) have been linked to lubricatory roles (Walls, 1942; Cohn, 1955; Lin and Nadakavukaren, 1981; Burns, 1992). Addition functions for lipid include being a solvent for pheromones and other biologically-active substances (Sakai, 1981; Sakai and Yohro, 1981; Yokoyama et al., 1989), having a bactericidal effect (Kuhnel, 1971), and functioning as a pheromone (Johnston et al., 1985; Minucci et al., 1989; Theissen, 1992; Payne et al., 1992). Porphyrins secreted by the Harderian gland, are found exclusively in rodents, and have been ascribed a variety of functions including photoreception (Hugo et al., 1989), protection of the eye (Wetterberg et al., 1970; Hugo et al., 1989), and possibly a role in the retinal-pineal-gonadal chain (Joó and Kahán, 1975; Panke et al., 1979; Hoffman et al., 1985; Spike et al., 1992). The secretion of melatonin has led to the suggestion that the Harderian gland may modulate the gonadal-thyroid axis (Menendez-Pelaez and Buzzell, 1992; d'Istria et al., 1994).

Non-secretory cells, found in the interstitial regions, can also produce and secrete substances into the lumina of the Harderian gland. The presence of immunocompetent cells in the interstitium of the avian and mammalian Harderian gland indicates that it may have a role in the immune system. It is thought to form part of the Head Associated Lymphoid Tissue (HALT) system (Montgomery and Maslin, 1992).

1.2.2 REPTILES

In comparison to non-reptilian tetrapods, the reptilian Harderian gland is a remarkably uniform structure. As a result of the current confusion in terminology of the ocular glands in turtles (Chieffi-Baccari et al., 1992), the turtles will be omitted from the ensuing discussion. The Harderian gland of the remaining reptiles (Lepidosaurs and Crocodilians) has not been exposed to much morphological analysis and little is known of its neurovascularisation. Only three types of secretions and functions have thus far been described in these reptiles.

1.2.2.1 MORPHOLOGY

There are several morphological variations in orbital structures in the three reptilian subclasses, including position of the lacrimal apparatus, presence of other glandular structures, and relative development of the Harderian gland. The morphology of the Harderian gland in squamates, in comparison to other reptiles, is relatively well documented (Saint Girons, 1988). The lacrimal apparatus comprises the lacrimal canaliculi and duct, which connect the Harderian gland to the nasal or vomeronasal areas. In squamates, there is some variation in the level of confluency between the Harderian gland and the anterior end of the lacrimal apparatus (figure 2) (Bellairs and Boyd, 1947; Saint Girons, 1982). Anatomically, the snake Harderian gland is a large structure, often encompassing the eyeball, or even protruding beyond it (Bellairs and Boyd, 1947; Gans, 1974; Savitzky, 1978; McCarthy, 1985; Saint Girons, 1988). The gland is comparatively smaller in lizards and does not differ much from the condition in both *Sphenodon* and the crocodilians (Saint Girons, 1982; 1985).

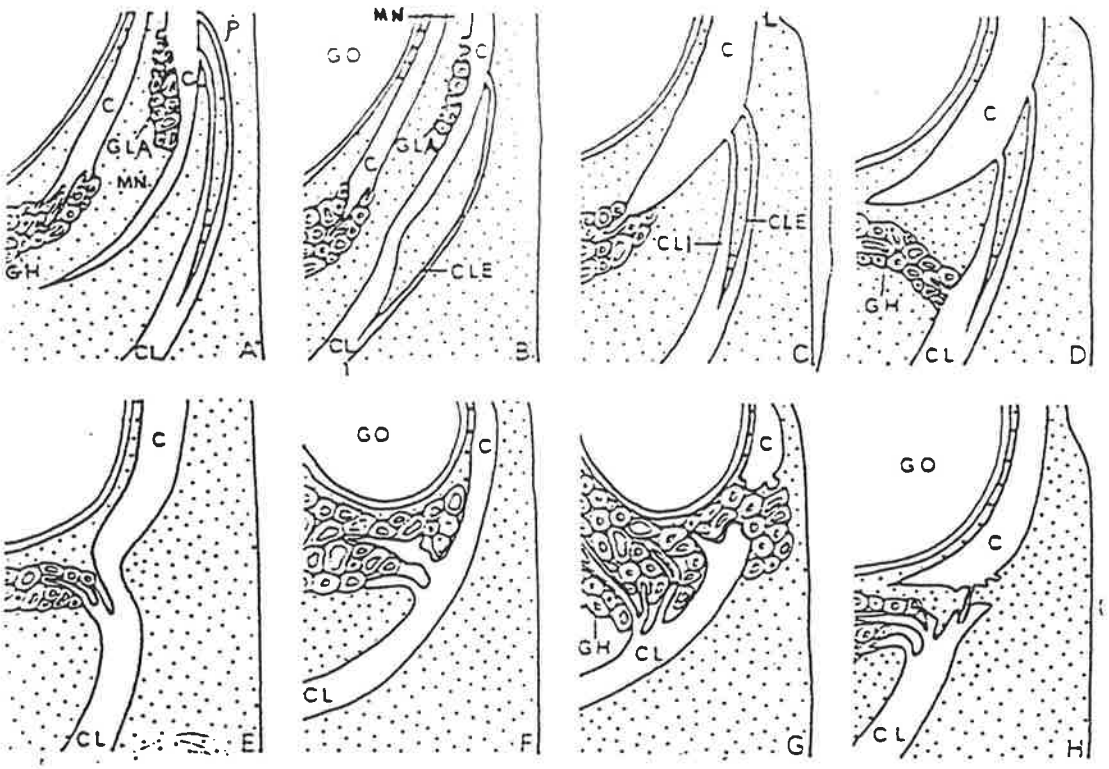


DIAGRAM 2

Schematic transverse sections through the front of the eye of lepidosauria, showing the ducts of the Harderian gland (HG), Lacrimal Canal (CL), and conjunctival space (C). Additional abbreviations: CLE - External Lacrimal Canaliculi ; CLI - Internal Lacrimal Canaliculi; GLA - Anterior Lacrimal gland; GO- eyeball ; L - spectacle; MN - Nictitating Membrane; P - eyelid.

A) lacertids with moveable eyelids, and *Sphenodon*

B) Australian agamids

C) most lizards with a spectacle

D) Pygopods

E) *Brookesia* (other Chameleons?) and Booidea snakes

F) *Feylinia* , Amphisbaeinia, and *Leptotyphlops* (Serpentes)

G) *Typhlops* (Serpentes)

H) Colubroidea snakes

(Taken from Saint Girons, 1982.)

1.2.2.2 VASCULATURE and INNERVATION

The vascularisation of the reptilian Harderian gland is undescribed.

The innervation of the ophidian Harderian gland appears to be derived from the pterygopalatine ganglion (Taub, 1966).

1.2.2.3 TYPE OF SECRETION

The histochemistry of the lepidosaur (Squamate and *Sphenodon*) Harderian gland contains either solely protein secreting cells or a combination of both protein and mucous secreting cells (Saint Girons, 1982; Chieffi-Baccari et al., 1990; Minucci et al., 1992). Mucous cells seem to be restricted to the apical or peripheral regions of the gland (Saint Girons, 1982, Chieffi-Baccari et al., 1990; Minucci et al., 1992). No lipid secretion has been described (Saint Girons, 1982). Ultrastructural analyses have thus far been carried out on only a few squamate species (Chieffi-Baccari et al., 1990 ; Minucci et al., 1992; Rehorek, 1992). In all cases, the protein secreting cells contained previously undescribed composite secretory granules.

The presence of melatonin in the reptilian Harderian gland has been documented in both turtles and squamates (Vivien-Roels et al., 1981). No porphyrins have been described in the reptilian Harderian gland.

1.2.2.4 FUNCTIONS

The combination of various histochemical properties of the Harderian gland and its relationship with the lacrimal canal led Saint Girons (1982) to propose that the Harderian gland in reptiles exhibits the potential for multiple functions. Four possible functions for the

reptilian Harderian gland have been proposed, firstly by Kennedy (1970) and then explained and rationalised by Saint Girons (1982 and 1989). Only three of these are relevant to the condition in squamates.

1) To lubricate the eye: As with non-reptilian tetrapods, it was proposed that the Harderian and the lacrimal glands in squamates would provide abundant lubricant for the corneal surface, with the lacrimal duct draining any excess fluid (Taub, 1966; Saint Girons, 1982).

2) To act as accessory salivary glands: Young and van Lennep (1978) broadly defined the salivary gland as any cell or organ whose secretion discharges into the buccal cavity. They also proposed several general functions for salivary glands. The first two functions associate the salivary glands with digestion, functioning as either a lubricant or as a source of digestive enzymes. The former function was proposed for the Harderian gland in snakes (McDowell, 1969), based on its hypertrophied form (Gans, 1974) and the route of the lacrimal duct (Walls, 1942). Saint Girons (1982) suggested that the Harderian gland produces and secretes digestive enzymes.

3) To serve the vomeronasal organ: The observation of the connection between the Harderian gland and the VNO via the lacrimal apparatus (diagram 3) led Broman (1920) to the hypothesis that the squamate Harderian gland may be involved in vomerolfaction (Saint Girons, 1982; Halpern, 1992). This claim is further supported by the embryological works of Slaby (1979a-c;1981;1982a-c;1984), showing the close relationship of the lacrimal canal and the VNO. There is some variation in the relationship between the opening of the Harderian gland and the lacrimal duct (Bellairs and Boyd, 1947; Saint Girons, 1982 - diagram 2) which may be indicative of both functional and phylogenetic diversity.

The first two hypotheses can be refuted, based on anatomical data and the absence of biochemical evidence. There are at least four observations which refute the lubricatory hypothesis. It was proposed that orbital modifications, which reduce the level of corneal water loss, would affect the structure of a lubricatory gland, which the Harderian gland is

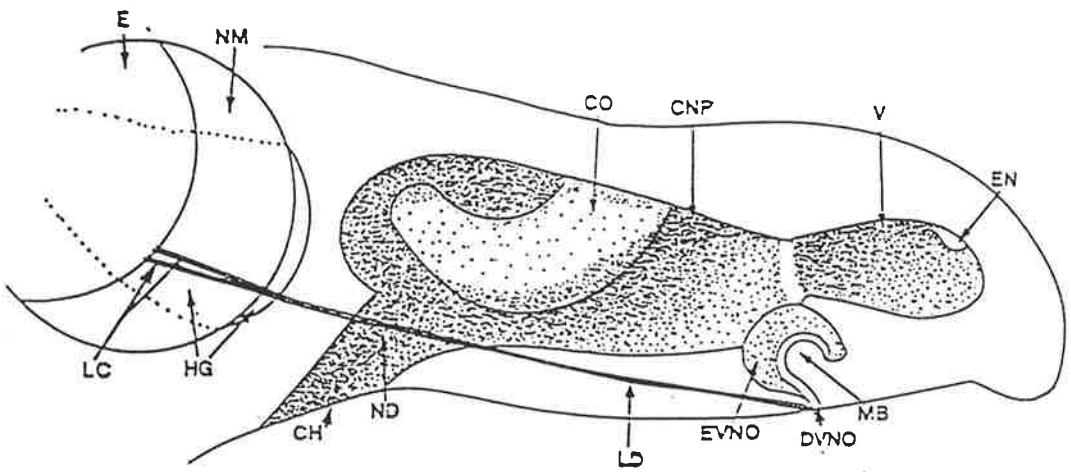


DIAGRAM 3

The ethmoid region of a lizard. Note the three main compartments: the External Nare (EN) open into the Vestibulum (V), Cavum Nasi Proprium (CNP), wherein lies the laterally protruding conch (C) and the Nasopharyngeal duct (ND), opening as the choana (CH) into the oral cavity). The VNO lies below the nasal cavity, and is composed of two parts, the dorso-lateral Vomeronasal epithelium (EVNO) and the ventro-medial Mushroom Body (MB), opening into the oral cavity via the Vomeronasal duct (DVNO). Note the presence of the Lacrimal Duct (LD), which arises from two Lacrimal Canaliculi (LC) from the region of the Nictitating Membrane (NM). The Harderian Gland (HG) is in close proximity. Additional abbreviations : Eyeball (E).

presumed to be. However, neither the presence of an enclosing spectacle, nor a reduced ocular region affects the structure of the Harderian gland in skinks (Rehorek, 1992; Rehorek et al., 1993). Additionally, the connection between the Harderian gland and the orbit is reduced, or even absent, in both snakes and pygopods. In these cases, the Harderian gland connects directly to the lacrimal canaliculi (diagram 2). Finally, the presence of other mucous secreting glands and epithelia suggest that there is an adequate lubricatory system for the squamate orbit, and thus the Harderian gland may have an alternative function. The function of the Harderian gland as an accessory digestive gland is unlikely because of a) the connection between the Harderian gland and the orbit in most lizards (diagram 2) and b) the absence of any corroborating biochemical data. The third hypothesis, that of vomerolfaction, has not been tested. Based on the connection to the VNO via the lacrimal apparatus (diagram 3), the squamate Harderian gland may well function in vomerolfaction. The possible role of the gland in vomerolfaction is unknown, and the answer may lie in the VNO itself. Thus, in the following section, the morphology of the vomeronasal system will be reviewed in an effort to ascertain what could be the possible role of the squamate Harderian gland in vomerolfaction.

1.3 THE NASAL CHEMOSENSORY SYSTEMS

The VNS consists of the vomeronasal organ (VNO) (comprising both sensory and non-sensory mucosae), the neural connections of the sensory epithelium, and a duct which opens into either the nasal cavity, the palate, or into a nasopalatine duct. Although no associated intrinsic glands in the sensory epithelium have been reported in adult squamate reptiles, submucosal vomeronasal glands have been found in various mammals (Kratzing, 1971a; Loo and Kanagasuntheram, 1972; Kratzing, 1984b). The VNO is connected to the nasal and/or oral cavity via the vomeronasal duct or other connecting ducts. In squamate reptiles the VNS may also include the tongue, and both the upper and lower palates

(Schwenk, 1993b; Young, 1993). Thus, the VNS in vertebrates exhibits much variation and may comprise several interlocking components, as seen in squamate reptiles.

The sensory epithelium of the VNO shares a common embryological origin with that of the main olfactory system (MOS). Thus, there are many morphological, and possibly functional, similarities between the two systems. The VNS in some mammals functions solely in a social capacity, such as pheromone detection (Wysocki and Meredith, 1987; Mendoza et al., 1994). In squamates, however, the VNS is also thought to supplement the MOS (Noble and Kumpf, 1936; Cowles and Phelan, 1958, Duvall, 1981). The term vomerolfaction denotes the function of the VNS, as opposed to olfaction of the MOS (Cooper and Burghardt, 1990). This concept has been further extended to include the term 'vomodor', the chemicals which are receptive to the VNS, as opposed to 'odor' for the MOS. Vomerolfaction is poorly understood in comparison to olfaction, based on the level of research each area has received. Thus, morphological comparisons with the MOS may supplement or even explain some of the features observed in the VNS.

Before reviewing the literature of these two nasal chemosensory systems, I will describe the ethmoid region, and give some idea of the proportional distribution of the sensory epithelia in these two sensory systems. The embryology of these chemosensory structures will also be briefly described. This will be followed by a brief discussion of evolution of the VNO in terrestrial vertebrates. Despite the common embryological origin (see Hansen and Zeiske, 1993) and superficial resemblances, there are some differences between the two nasal chemosensory systems, three of which will be described in some detail with special reference to systems in squamate reptiles.

1.3.1 THE ETHMOID REGION

There is much variation in the morphology of the ethmoid region of tetrapods, with respect to the relative development and the position of the epithelia lining the nasal chambers.

In its most generalised form, the ethmoid region of the squamate reptiles is separated into left and right halves by the nasal septum. It consists of a tubular vestibulum which connects the external nares with the main nasal cavity (equipped with lateral projections called conchae) which, in turn, is continuous with the pharynx via the nasopharyngeal duct (Parsons, 1967; Gabe and Saint Girons, 1976 - diagram 3). In reptiles, the choanae also house the body of the external nasal gland, whose ducts open into the nares (Saint Girons, 1988). The main nasal cavity is lined by two different types of epithelia. Whilst the respiratory epithelium lines the ventral and ventrolateral aspects, the dorsal and dorsomedial aspects are lined with ciliated olfactory epithelium, containing the sensory cells (Parsons, 1967; Gabe and Saint Girons, 1976).

The VNO is a paired ventromedial out pocketing of the nasal cavity (Parsons, 1967). It is a dome-shaped, bone encased structure which is situated approximately at the level of the external naris (Gabe and Saint Girons, 1976). Like the conchae of the nasal cavity, there is a hemispherical protrusion, antero-laterally placed, known as the mushroom body (diagram 3) (Gabe and Saint Girons, 1976; Halpern, 1983). The VNO is dorsally lined by the microvillous vomeronasal sensory epithelium, and ventrally, restricted to the mushroom body, by a ciliated non-sensory epithelium (Parsons, 1970; Halpern, 1983; Takami and Hirosawa, 1987).

Although the squamate VNO no longer has a direct connection to the nasal capsule, there are some connecting palatal structures in lizards (diagram 4). From the choanal openings (internal nares) 1 - 2 choanal fissures arise. These are formed by adjacent margins of the maxillary and vomerine elements of the bony palate (Bellairs and Boyd, 1950). Posteriorly, the fissures are quite deep, and are confluent with the nasal cavity. They progress anteriorly, becoming more superficial, and are separated from the nasal capsule initially by mucosal foldings and then later by supporting cartilages of the upper jaw (Bellairs and Boyd, 1950). At this stage, the choanal fissures are termed choanal grooves. These then open into either the palatal duct of the VNO, or close to it (Gabe and Saint Girons, 1976).

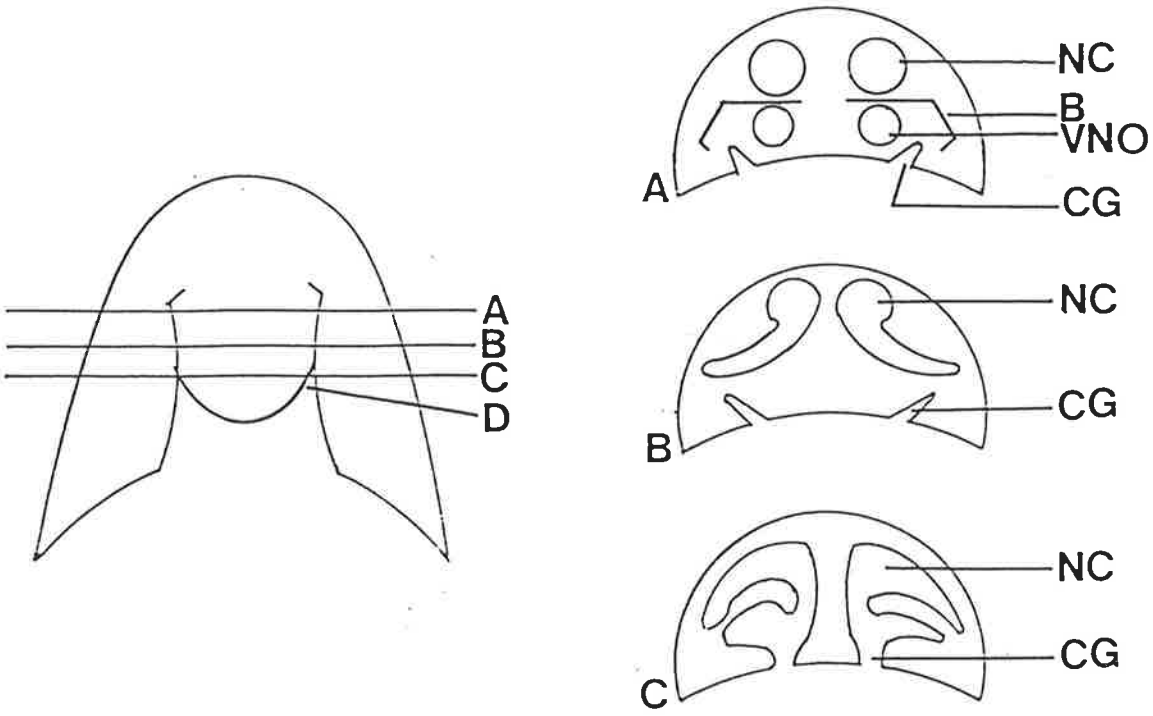


DIAGRAM 4

A schematic diagram of the relationship of the choanal grooves (CG) to the nasal cavity (NC), in a Gekkonid. Section A is through the VNO, and shows the bone (B) which separates the CG from the NC. Section through B shows that the CG is separated by the NC only by some thin connective tissue. Section through C shows that the CG is opens directly into the NC. The Choanal openings would be found at D.

There are at least four basic plans for the arrangement of the choanal grooves on the superficial palate, based on the degree of apposition of the maxillary and vomerine elements (diagram 5). Pratt (1948) suggested that the choanal grooves may act as gutters by which vomodor (chemical substance detected by the VNO), dissolved in palatal and choanal fluids, is passed to the narrow vomeronasal duct. In addition to the variation in palatal morphology, there is morphological variation in tongue structure (Schwenk, 1988) (diagram 5). Only the bifid tongues and reduced choanal grooves, in snakes and some anguimorph lizards, are defined as morphological specialisations for vomerolfaction (Schwenk, 1993a, 1993b; Toubeau et al., 1994). It has been proposed that the bifurcate snake tongue is capable of mediating chemosensory tropotaxis (an ability to distinguish simultaneously the chemical intensity of two disparate points) (Schwenk, 1994).

Another structure associated with the choanal groves is the lacrimal duct (nasal part of the lacrimal apparatus). The lacrimal duct, which has its origins from the orbital region, opens in the vicinity of the VNO in most squamate reptiles (diagram 3). In lizards, the very young embryos possess a lacrimal duct which opens into the VNO or at least very close to it. Subsequent morphogenesis leads to the distal part of the lacrimal duct being incorporated into the spreading outer choanae. Thus, in the adult, the lacrimal duct is shorter, and opens into the choanal groove (Slaby, 1982b).

Thus, the ethmoid region of squamates possesses two morphologically separated and distinct sensory systems: the main (nasal) olfactory organ and the VNO. Within Squamata, some variations were observed in the precise relationship between the VNO duct and both the lacrimal duct and choanal grooves, with potential functional implications. Despite this variation, the lacrimal duct is a conduit between the VNO (or that region) and orbit of all squamates.

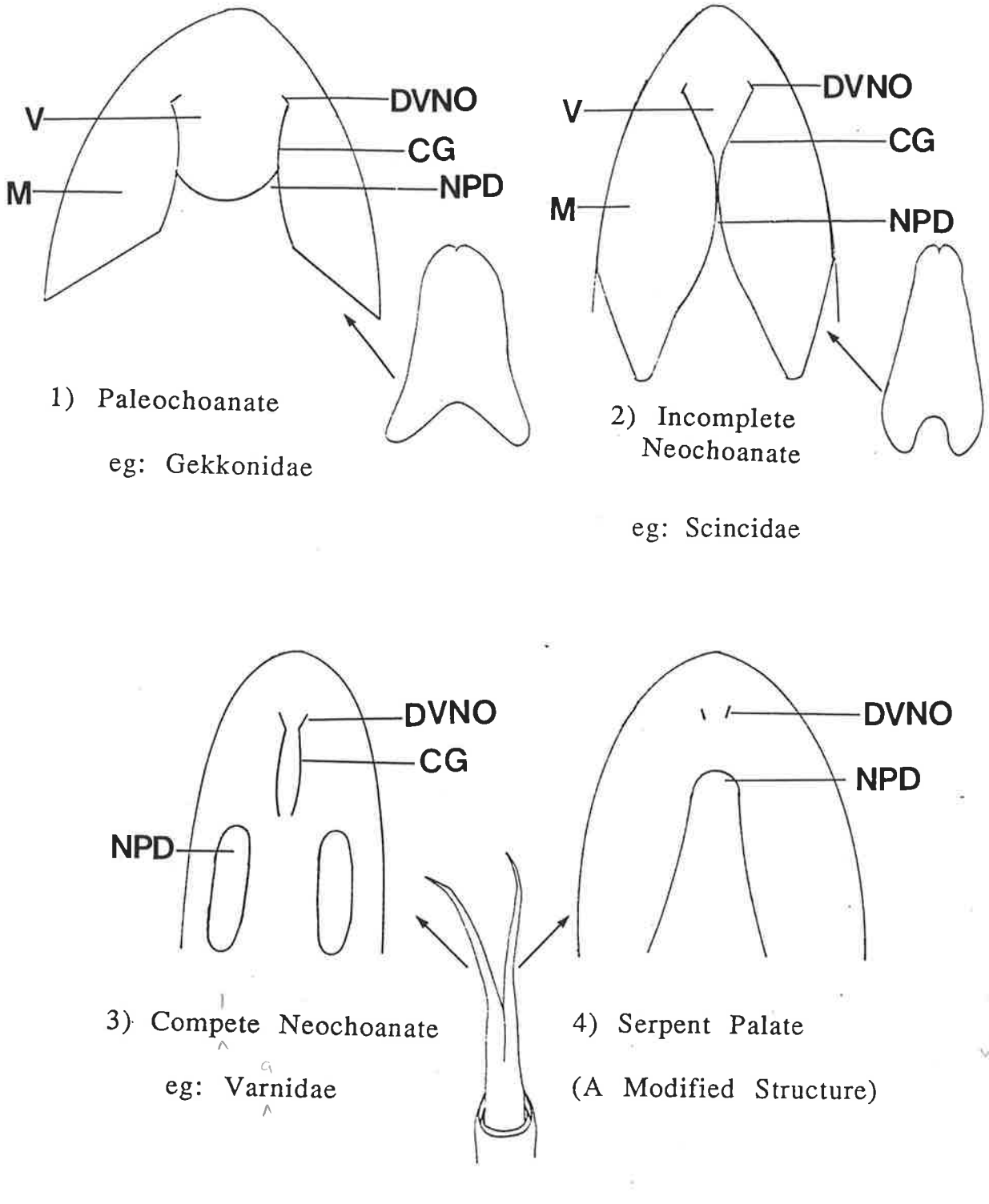


DIAGRAM 5

A schematic diagram showing the relationship between the upper palate morphology and the tongue shape. Note the correlation between the shape of the tongue and the structure of the palate. Classification of the upper palate based on Bellairs and Boyd (1950). Tongues from Schwenk (1993a). Abbreviations: CG - Choanal Grooves; DVNO- Vomeronasal Duct; M- Maxillary cartilage; NPD- Nasopalatine duct; V- Vomerine cartilage

1.3.2 EMBRYOLOGY OF THE NASAL CHEMOSENSORY SYSTEMS

Growth and cellular differentiation of the olfactory zone of the nasal tube in early embryonic stages precedes the primordia of the skeletal elements (Slaby, 1979a). The ectodermal thickenings in the heads of embryos, which give rise to various organs and parts of the peripheral nervous system, are called placodes (see Hansen and Zeiske, 1993), the most rostral pair of which are the olfactory placodes. The vomeronasal epithelium arises from the medial olfactory placode in early stages of foetal development as a separate and anatomically distinct receptor component of the olfactory system (Stensaas et al., 1991). The olfactory placodes consist of two layers, from which the two nasal olfactory cell types (neural receptor and non-neural supporting cells) develop (Cuchieri and Bannister, 1975; Klein and Graziadei, 1983; Hansen and Zeiske, 1993). The origin of the cells in the sensory epithelium of the vomeronasal system (VNS), is thought to be similar. Thus, it can be seen that the MOS and VNS are anatomically distinct at early embryonic stages. The following section will now deal with the origin of the VNS and the level of morphological variation seen in tetrapods.

1.3.3 THE VOMERONASAL SYSTEM IN TETRAPODS

The presence of a vomeronasal system is highly variable within tetrapods, with much conflicting evidence and missing phylogenetic data. From the scattered species whose VNO have been described, there appears to be no pattern.

Though it is thought that the VNO evolved in vertebrates as an adaptation to the terrestrial environment (Bertmar, 1981), there are both morphological and functional lines of evidence to support the view that fish may possess a VNS, or, at least, a precursor to it (Wysocki and Meredith, 1987; Hara, 1992; Eisthen, 1992; Inouchi et al., 1993). In the African Lungfish, the VNO is difficult to identify, and thus a functional VNO seems unlikely (Eisthen, 1992). However, some of the neural connections are present (Derivot, 1984).

In the tetrapods, the VNO (when present) is an anatomically distinct structure, found in the ventral portion of the ethmoid. There is, however, variation in the location and morphology of the VNO (Negus, 1958; Saint Girons, 1976; Scalia, 1976; Kratzing, 1978; Kratzing, 1984b; Gemmell and Nelson, 1988; Schmidt and Wake, 1990; Halpern, 1992; Hatanaka and Matsuzaki, 1993; Eisthen et al., 1994). In eutherian mammals, the presence of the VNO has been correlated with diet specialisations, ecological niches or possibly phylogeny (Haas, 1947; Cooper and Bhatnager, 1976; Mackay-Sim et al., 1985; Harrison, 1987; Gemmell and Nelson, 1988). Squamates show much morphological variation in development of the VNO, as it ranges from being well developed in snakes, to reduced or even absent in some lizard lineages (Haas, 1947; Slaby, 1984; Halpern, 1992; Schwenk, 1993b).

Structurally, the mammalian and lepidosaur VNO share many common, structural features. The VNO is enclosed in vomerine bone/cartilage at the base of the nasal septum (Johnson et al., 1985). This vomerine cartilage is highly variable in position, and is often used as a taxonomic feature (Slaby 1979a-c; 1981; 1982a-c; 1984; Harrison, 1987). A well developed VNO possesses a lateral protrusion into the lumen which is covered by non-sensory epithelium and is comprised of erectile and vascular tissue (Parsons, 1967; Kratzing, 1971a; Adams, 1986).

The VNO of the tuataras, *Sphenodon spp.*, the only extant non-squamate lepidosaur, is a tubular structure, with no luminal projection, and opens into a nasopalatine duct (Parsons, 1970). This is structurally different to both the mammalian and squamate condition, wherein luminar projections are common in well developed vomeronasal systems.

Upon closer examination, there are three main differences between the mammalian and squamate VNO: the position of the duct, the mechanism of vomodor uptake, and rate of development of the two mucosae. First, the VNO of all squamates is connected, via a duct, to the palate (Halpern, 1992). However, in mammals, the position of the duct varies, as it may open into the nasal cavity, the oral cavity or both, via a nasopalatine duct (Broom,

1894; Arnautovic et al., 1970; Kratzing, 1971a/b; 1984; Loo and Kanagasuntheram, 1972; Cooper and Bhatnager, 1976; Adams and Wiekamp, 1984; Hunter et al., 1984; Taniguchi and Mikami, 1985; Kratzing and Woodall, 1988; Harrison, 1987; Wohrmann-Repennig and Ciba, 1989; Moran et al., 1991; Taniguchi et al., 1992).

Second, the difference in duct position is reflected in the mechanism of vomodor uptake. In eutherian mammals, both physiological control of the blood vessels surrounding the lumen of the VNO, as well as behavioural traits (Flehmen) are thought to be mechanisms of vomodor uptake (Müller-Schwarze 1971; Meredith and O'Connell, 1979; Meredith, 1994). Mechanisms of vomodor uptake in the squamates, however, are thought to be propagated by the tongue and the anterior lingual pallets (Noble and Kumpf, 1936; Gillingham and Clark, 1981). Morphological differences and functional dissimilarities in the tongue suggest that the mechanisms for contacting molecules differs between lizards and snakes (Graves and Halpern, 1989). Alternatively, it is thought that the tips of the bifurcate tongues of snakes may not enter the vomeronasal duct itself, but deposit the particles on the anterior lingual pallets instead. When these are elevated, they are directly opposed to the vomeronasal duct (Gillingham and Clark, 1981; Young, 1990).

Third, the rate of development of these two systems differs between squamates and mammals. In comparison to the MOS, the VNS is more fully developed in the mammalian neonate (Brunjes and Frazier, 1986; Gemmell and Nelson, 1988; Garrosa and Coca, 1991). The converse was found to be the case in the garter snake (Holtzman and Halpern, 1991b) as the connections to the neural pathways develop earlier in the MOS than in the VNS (Holtzman and Halpern, 1990; 1991a).

Thus, the structural similarities between the squamate and mammalian VNS imply that vomerolfaction in these two groups essentially works in the same way. The differences observed deal with vomodor uptake (which, in turn, leads to different behavioural adaptations) and rate of development. If there are no structural variations in the vomeronasal chemosensory mucosa of these two vertebrate groups, then these variations are unlikely to

affect the actual function of the VNO. Thus, the structure of the VNS will be more closely examined in the following section, highlighting morphological variations both within the vomeronasal systems and between the VNS and the MOS (whose morphological and functional aspects are more clearly understood).

1.3.4 MORPHOLOGY OF NASAL CHEMORECEPTION

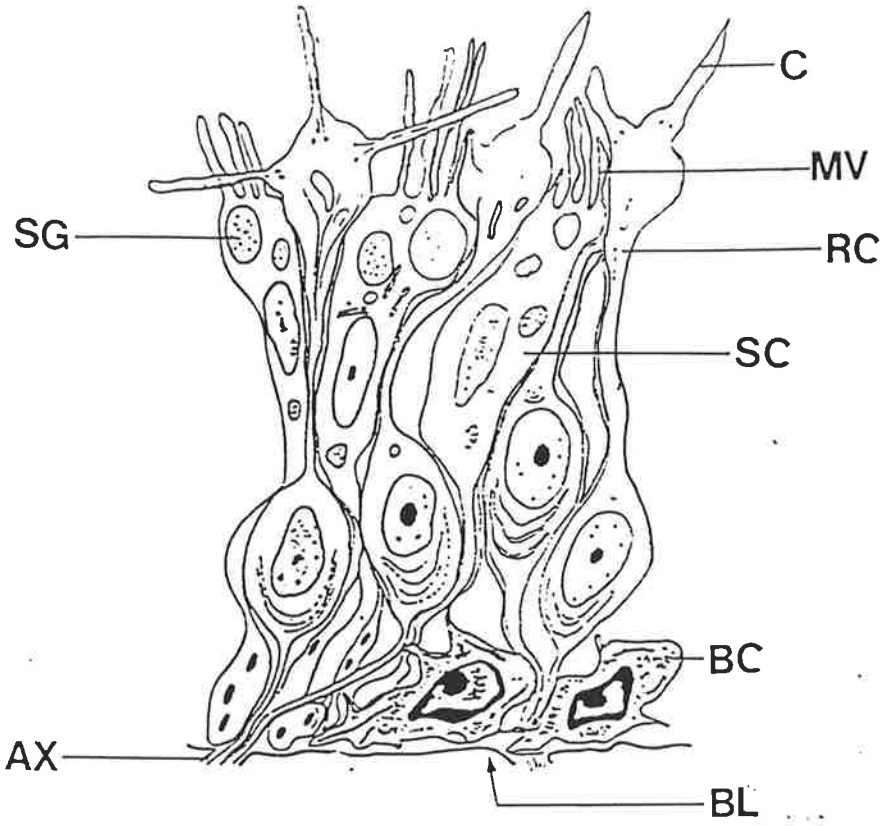
The nasal chemosensory structures consist of two layers: the epithelium and the submucosa (lamina propria). Together, I shall refer to these two layers as the chemosensory mucosa. The pseudostratified chemosensory epithelia of the MOS and VNS are structurally similar, consisting of 3 cell types. Firstly, the receptor cells have dendrites with apical, luminal projections, which are either cilia or microvilli. These bipolar neurons have a central nucleus. The axons pass back through the epithelium, traverse the submucosa of the nasal cavity, to synapse with the second order neurons (mitral cells) in the olfactory bulb. Secondly, the sustentacular cells, which are separate from the bipolar neurons apically, have a nucleus which is closer to the lumen and thin projections which anchor them to the basal lamina. These cells may have apical granules. They are thought to nourish and support the neurons. Finally, the basal cells are undifferentiated cells which are thought to be the precursors of both receptor and sustentacular cells in these epithelia and are capable of cell division and differentiation (Graziadei and Metcalf, 1971).

There are at least two ways of morphologically distinguishing between the VNO and the nasal olfactory organ, including a) neural connections, and b) morphology of the sensory epithelium.

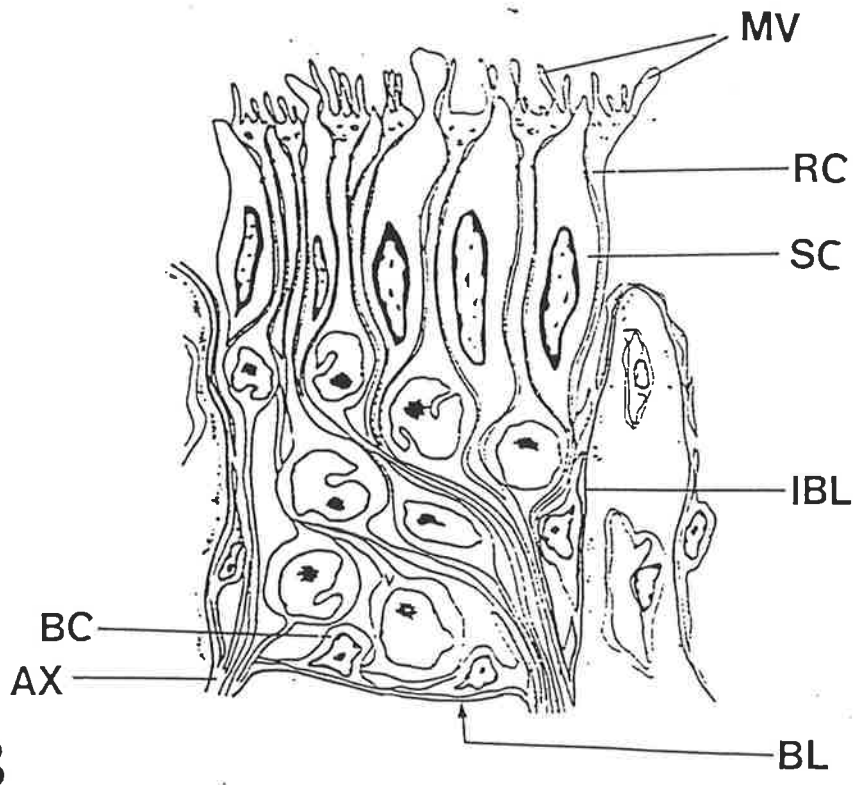
a) Neural connections: The axons of the olfactory and vomerolfactory receptor neurons project and synapse to the mitral cells (second order neurons) of two morphologically distinct regions of the olfactory bulb (Andres, 1970; Halpern, 1976; Meredith, 1991; Halpern, 1992). The nerves from the olfactory epithelium project to the main olfactory bulb (MOB), whilst those of the VNO project to the accessory olfactory bulb (AOB). From there, the mitral axons in the MOB proceed, in reptiles, to several parts of the brain, including the lateral cortex (Halpern, 1983; Lohman and Smeets, 1993). Conversely, the mitral cells from the AOB proceed exclusively onto the limbic system. Thus, the differences between the two systems seem to be restricted to the exact route of the secondary neurons (Lohman and Smeets, 1993). Though further comparative analyses on squamates would prove invaluable, little more can be done until the reptilian limbic system, the presumed site of projection for the second and third order neurons, is further understood.

b) Morphology of the sensory epithelia: Little is known about the reptilian olfactory and vomerolfactory sensory epithelia. Published generalisations appear to be extrapolations based on morphological conservation and a broad phylogenetic comparison of the mammalian olfactory and vomerolfactory epithelia. The olfactory epithelium and vomerolfactory epithelium have the same basic cytological architecture. The three structural differences between the two sensory epithelia include (i) the luminal projections, (ii) the vasculature of epithelium and (iii) the lubricatory capacity.

(i) The dendrites of the olfactory receptor cells are apically constricted by nearby cells. This constriction leads to the formation of olfactory knobs, which protrude above the cellular layer. These olfactory knobs generally house cilia whose size and abundance varies amongst vertebrates (diagram 6A) (Graziadei and Monti-Graziadei, 1976; Menco, 1980; Eisthen, 1992; Eisthen et al., 1994). In some species of birds (Graziadei and Bannister, 1967) and lizards (Kratzing, 1975), the olfactory knobs may also house microvilli. Microvillar receptor cells have also been found in some salamanders and mammals (Okano et al.,1967; Farbman and Gesteland, 1974; Loo, 1977; Kratzing, 1978; Moran et al., 1982a;



A



B

DIAGRAM 6

The olfactory (A) and vomerolfactory (B) epithelia, from Kratzing (1975). Abbreviations: Ax - Axons; BC - Basal Cells; BL - Basal Lamina; C - Cilia; IBL - Invagination of Basal Lamina; MV - Microvilli; RC - Receptor Cell; SC - Sustentacular Cell; SG - Secretory Granule. Taken directly from Kratzing, 1975.

Rowley et al., 1989). The sustentacular cells are non-nervous cells which possess apical microvilli. The presence of junctions connecting sustentacular and receptor cells in the nasal olfactory epithelium implies that there may be some form of communication between the two cell types (Kolnberger, 1971a).

In contrast to the MOS, the VNO receptor neurons bear microvilli in nearly all tetrapods (diagram 6B), except the rabbit (Luckhaus, 1969) and the dog (Adams and Wiekamp, 1984) which possess ciliated receptor neurons. Both receptor cell types have been found in some species of amphibians (Eisthen, 1992). Though some apical protrusions (vomeronasal vesicles) have been described (Kratzing, 1975; Wang and Halpern, 1980a; Bhatnager et al., 1982), they are not as well developed as the olfactory knobs (Ciges et al., 1977). Vomeronasal sustentacular cells also exhibit some variation in apical protrusions. Some amphibians possess short, irregular microprocessess (Eisthen, 1992), whilst other amphibians posses a microvillous apical border (Halpern, 1992). An occasional cilium has been described in the normally microvillous sustentacular cells of Bovines (Adams, 1986), and seems to be more frequent in the frog, *Rana esculenta* (Kolnberger, 1971a). Amphibians and reptiles possess neither a belt of tight junctions connecting receptor and sustentacular cells at their apices, nor any gap junctions (Kolnberger, 1971b).

(ii) As the VNE in some species is thicker than that of the nasal olfactory epithelium, the efficient diffusion of nutrients/wastes to the submucosal blood vessels becomes impossible. Thus, in some squamates and mammals with such thick epithelia, indentations of the basal lamina, which enclose capillaries, are observed (Kratzing, 1975; Wang and Halpern, 1980a). The condition in snakes has developed to such an extent that there is a complex system of these basal laminar indentations leading to a columnar organisation of the VNE (diagram 7). These columns are perpendicular to the overlying sustentacular cell layer (Wang and Halpern, 1980a/b; Takami and Hirosawa, 1987; 1990; Halpern, 1992).

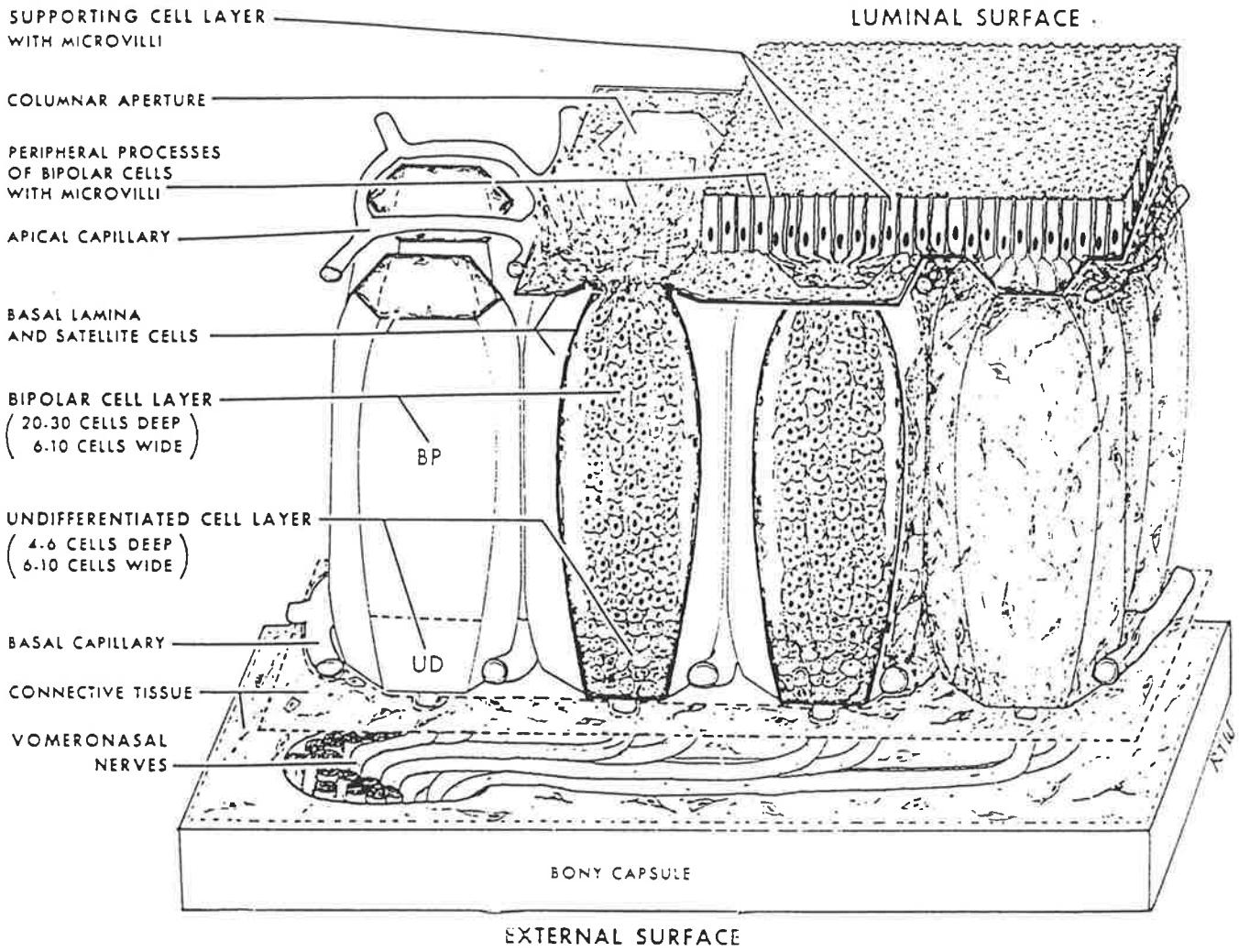


DIAGRAM 7

The Vomeronasal epithelium of the garter snake (*Thamnophis sirtalis*), from Wang and Halpern (1980b). Note the columnar arrangement, supported by invaginations of the basal lamina.

(iii) Intrinsic secretory structures occur within the sensory mucosa itself, while extrinsic structures occur in the non-sensory regions. Before I further discuss the lubricatory capacities of these two chemosensory systems, I will briefly review the functional significance of an adequate lubricatory system in these chemosensory systems.

The surface of the olfactory epithelium in vertebrates is covered by a layer of mucus, wherein the odorant molecules are dissolved prior to reaching the site of olfactory stimulation (Getchell, et al., 1984a/b). In order to interact with the receptor cells (Adams et al., 1981), chemicals must diffuse through the mucus layer, thus implying that the layer acts as a selective barrier, in both a physical and chemical sense. The mucus lining the olfactory epithelium in the nasal cavity is produced in copious amounts by the glandular structures, protecting the underlying epithelium from desiccation and from penetration by foreign particles (Katz and Merzel, 1977).

Although the entire olfactory epithelial surface is covered by this mucus layer, it is only the mucosensory compartments (mucociliary in the MOS, mucomicrovillar in the VNO) which are the sites of receptor-specific and peri-receptor events leading to olfactory transduction (Rama Krishna et al., 1992; Getchell et al., 1993; Takami et al., 1995). In the MOS, there are two intrinsic sources for this mucous layer (the sustentacular cells and the Bowman's glands), whereas in the VNO, some extrinsic sources have also been mentioned.

Microchemical analysis has revealed that the nasal olfactory mucus layer consists of two distinct layers: the thin, outer, watery mucus layer, which is produced by the secretions from the Bowman's glands (Reese, 1965; Andres, 1969) and the inner, more viscous mucus layer, which is produced by the sustentacular cells (Müller et al., 1979). Thus, the two zones have different physicochemical properties (Getchell and Getchell, 1992). There is, however, variation in the secretory capacity, chemical nature and structure of secretory granules of olfactory sustentacular cells in vertebrates, which does not seem to be phylogentically linked (Bannister, 1965; Graziadei, 1966; Thornhill, 1967; Graziadei and Bannister, 1967; Okano et al., 1967; Kleerekoper, 1969; Kratzing, 1970; Graziadei and

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Tucker, 1970; Theisen, 1972; 1973; Graziadei, 1973; Bhatnager and Kallen, 1974; Farbman and Gesteland, 1974; Kratzing, 1975; 1978; Bakhtin, 1976; Yamamoto, 1976; Gabe and Saint Girons, 1976; Graziadei and Monti-Graziadei, 1976; Saint Girons, 1976; Zeiske et al., 1976; 1979a/b; 1992; Yamamoto and Ueda, 1977, 1978a/b, 1979a/b; Menco, 1980; Delfino et al., 1981; Moran et al., 1982a/b; Klein and Graziadei, 1983; Getchell et al., 1984a/b; Theisen et al., 1986; Eisthen et al., 1994; Jones et al., 1994). Bowman's glands are absent in fish and some amphibians (Kleerekoper, 1969; Farbman and Gesteland, 1974). In the remaining vertebrates, these simple, tubular, submucosal glands are strikingly similar (Getchell and Getchell, 1992). Serous or mixed mucous/serous glands have been described in several disparate vertebrate species (Frisch, 1967; Graziadei and Bannister, 1967; Kratzing, 1970, 1975; Bhatnager and Kallen, 1974; Yamamoto, 1976; Menco, 1980; Getchell et al., 1984b; Getchell and Getchell, 1992).

Like the olfactory mucosa, there are two potential intrinsic sources of secretion for the mucus lining the VNO. The presence of secretory granules in the sustentacular cells varies among vertebrates, with little evidence of any phylogenetic correlation (Bannister, 1968; Graziadei and Tucker, 1970: Altner et al., 1970; Loo and Kanagasantheram, 1972; Kratzing, 1975; Gabe and Saint Girons, 1976; Ciges et al., 1977; Wang and Halpern, 1980a; Vaccarezza et al., 1981; Bhatnager et al., 1982;Hatanaka et al., 1982; Adams, 1986; Takami and Hirosawa, 1990; Moran et al., 1991; Adams, 1992; Eisthen, 1992; Bannister and Dodson, 1992; Franceschini et al., 1991; Eisthen et al., 1994). There are generally fewer secretory granules in the sustentacular cells of the VNO than in those of the olfactory epithelium (Kolnberger, 1971a). In addition to this, there is no microchemical evidence of secretions from the sustentacular cells forming part of the mucus lining in the VNS (Takami et al., 1995). Thus, the role of the sustentacular cells in the VNS is at present unknown.

A variety of submucosal mucous or mixed mucous/serous glandular structures have been described associated with the VNO in many non-squamate vertebrates (Parsons, 1970; Kratzing, 1971a; 1984; Loo and Kanagasuntheram, 1972; Bhatnager and Kallen, 1974; Cooper and Bhatnager, 1976; Klaasen and Kuijpers, 1977; Ciges et al., 1977; Vaccarezza et

al., 1981; Adams and Wiekamp, 1984; Adams, 1986; 1992; Kratzing and Woodall, 1988; Franceschini et al., 1991; Taniguchi et al., 1992; Døving et al., 1993; Bhatnager and Wible, 1994; Salazar et al., 1996). These glands are important in vomerolfaction as sources of secretion for the mucus lining. The absence of these glands, in bats at least, is positively correlated to rudimentation of the VNO (Cooper and Bhatnager, 1976). In addition to this, microchemical studies on the rat VNS has shown that the mucus of the mucomicrovillar complex is constituted largely of secretions produced by the subepithelial glandular structures (Takami et al., 1995). No submucosal vomeronasal glands have been described in the VNO of adult squamates (Gabe and Saint Girons, 1976; Kratzing, 1975; Wang and Halpern, 1980a/b; Takami and Hirosawa, 1987). Despite the absence of these glands, it has been demonstrated that the VNO is well developed in most squamates examined and has a layer of mucus carpeting the sensory epithelium. Thus, the importance of the VNO submucosal glands as a source of secretion for the mucus lining, and the absence of such structures in the well developed VNO of squamates, indicates that, in the absence of any intrinsic source of secretion, that there must be an extrinsic one.

There have been several suggestions of such extrinsic sources including the mucous cells in the intermediate and non-sensory areas, oral glands, the lacrimal apparatus, and thus the Harderian gland. Non-sensory epithelia are associated with the VNO of amphibians, squamates, and mammals. The presence of individual goblet and flattened epithelial cells (with few apical secretory granules) in the intermediate and nonsensory areas is a feature common to these species (Arnautovic et al., 1970; Loo and Kanagasantheram, 1972; Ciges et al., 1977; Adams and Wiekamp, 1984; Adams, 1986; Takami and Hirosawa, 1987; Stensaas et al., 1991; Halpern, 1992). Contributions to this mucus layer may come from oral secretory structures, such as salivary glands and the mucous cells on the anterior lingual pallets (Pratt, 1948; Clark, 1981; Ten Eyck and Gillingham, 1985). Another potential source of lubricant for the vomeronasal region, is the lacrimal apparatus (Kratzing, 1975). It is also though to provide some lubricant for the choanal grooves, to enable movement of vomodor to the VNO (Pratt, 1948). Additionally, this lacrimal apparatus may act as a

conduit for orbital secretions (like those from the Harderian gland) to reach the VNO (Bojsen-Møller, 1964; Kratzing, 1984b).

Thus, there are two intrinsic sources of secretion for the heterogenous mucus layer of the MOS. This is in contrast to the squamate VNO, in which the absence of submucosal glands and the improbability of sustentacular granules as components for the mucus layer, leads to the conclusion that there must be an extrinsic source of secretion. Four such sources have been proposed, including individual secretory cells in non-sensory and intermediate regions, oral secretions, and secretions both from the lacrimal apparatus itself and from the glandular structures at the anterior end of the orbit (Harderian gland).

1.4 A COMPARATIVE APPROACH TO THE STRUCTURE AND FUNCTION OF THE SQUAMATE HARDERIAN GLAND

1.4.1 INTRODUCTION

Comparative studies on the structure of the squamate Harderian gland have concluded that it is primarily a protein secreting gland which is associated with the lacrimal apparatus and the nictitating membrane (Saint Girons, 1982; 1988; 1989; Chieffi-Baccari et al., 1990; Minucci et al., 1992; Rehorek, 1992; Rehorek et al, 1993). However, the limited histochemical analyses, the morphological examinations of disparate species, and the unknown function of the squamate Harderian gland make generalisations difficult. By using comparative studies, evolutionary trends can be identified in the comparison of a given variable across a range of taxa (Harvey and Pagel, 1991). If the variable is similar in phylogentically close lineages, and different in phylogenetically distant taxa, then it would be phylogenetically constrained. However, the structure would be adaptive (exhibiting convergent evolution) if either variations are seen among phylogenetically close taxa or phlyogenetically distant taxa have similar structures. Thus, with respect to the squamate

Harderian gland, any morphological variations among major squamate lineages could be correlated to phylogeny. This, in turn, could be correlated with possible functional roles of the gland.

Australian squamate reptiles are remarkably diverse, with representatives from 5 of the major lineages (diagram 8). As they are so diverse, they inhabit many ecological niches, and thus have developed different adaptive responses to the environment. One of these adaptations is the relative development of nasal chemosensory systems. Three of these Australian lineages can be broadly classified according to chemosensory specialisation (Schwenk, 1993b). The snakes (Serpentines) are defined as vomerolfactory specialists, based on several morphological and behavioural adaptations. Such adaptations include the bifurcate tongue and its role in tongue flicking, the columnar structure of the vomeronasal sensory mucosa, and a variety of physiological and behavioural features (see Halpern, 1992 for review). In contrast, Schwenk (1993a) suggested that the gekkotans may be olfactory specialists. This is based on some evidence on the morphology of the brain and tongue, the spread and structure of the olfactory mucosa, and behavioural studies (Gabe and Saint Girons, 1976; Schwenk, 1993a). Finally, Scincomorphs are thought to be an intermediate group with some variation in chemosensory structure and behaviour. Though they have some of the morphological and behavioural elements which are similar to those of snakes (vomerolfactory) and gekkotans (olfactory), they do not possess neither the same combination, nor the same relative development of these elements (Schwenk, 1993b). I shall thus refer to scincomorphs as nasal chemosensory generalists.

If the Harderian gland functions in squamate vomerolfaction, then structural differences are expected to accompany the nasal chemosensory adaptations. Thus, the gland of the snake, a vomerolfactory specialist, would be expected to differ from both those of the gekkotans (potential olfactory specialists) and the scincomorphans (nasal chemosensory generalists). Such variations would imply a potential function of the gland in squamate vomerolfaction.

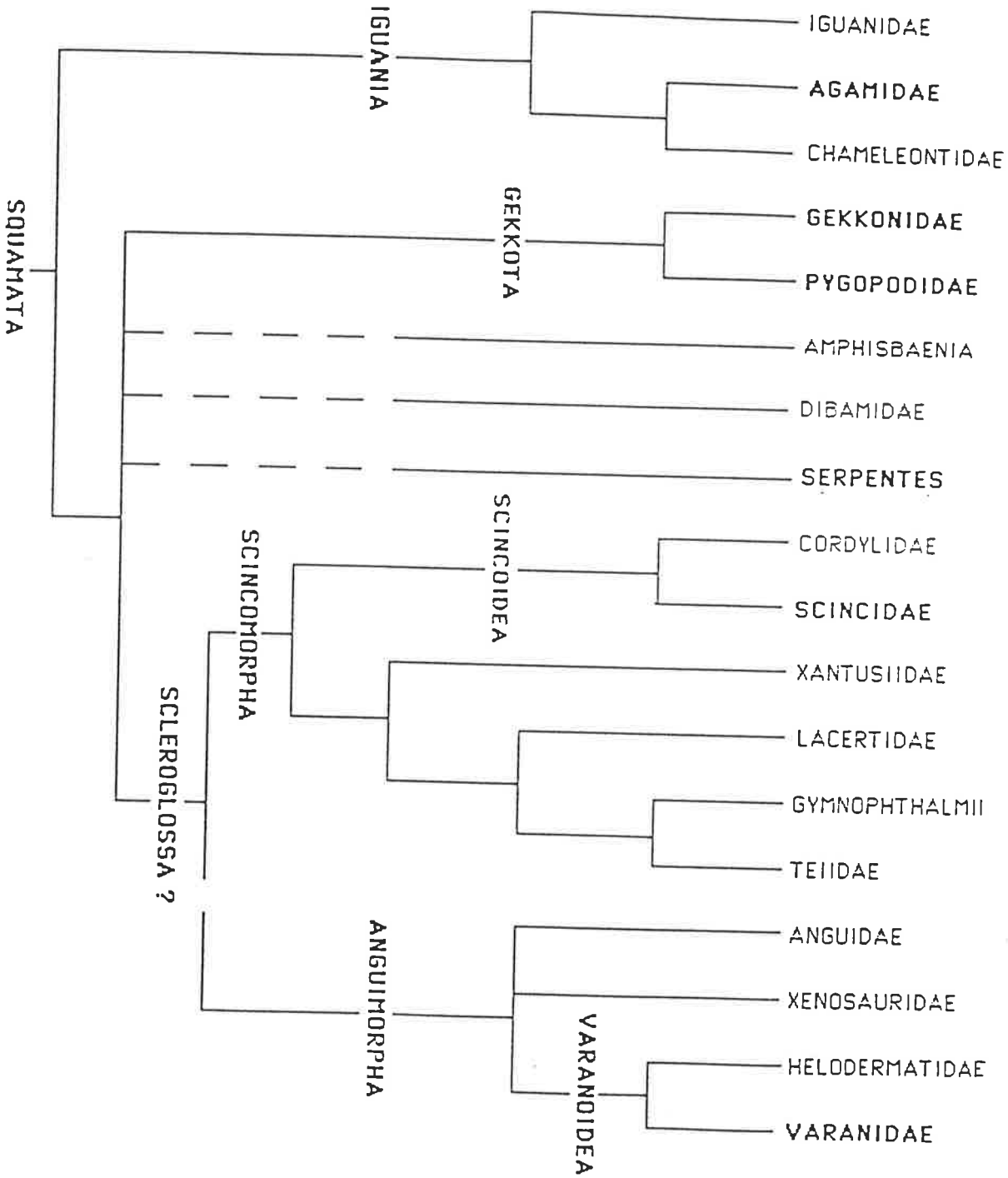

DIAGRAM 8

Phylogenetic relationships within Squamata (from Estes et al., 1988). The 7 groups on bold are those which have representatives in Australia.

1.4.2 PHYLOGENY

The three lineages in question (Serpentes, Gekkota, and Scincomorpha) differ not only in chemosensory adaptations, but also in the level that the Harderian gland and nasal chemosensory structures have been researched. Of these three lineages, the best known is that of the Serpentes (see Halpern, 1992 for review). Snakes have been acknowledged as vomerolfactory specialists (see Halpern, 1992 for review). Most of the research has thus far been centred on the colubroid snakes, and a few disparate studies on other groups. There are three major families within Colubroidea: Viperidae, Colubridae and Elapidae. Only representatives from the latter two families are found in Australia. Thus, the Harderian glands and nasal chemosensory structures of a local elapid species (*Pseudonaja textilis*) were extensively examined in this comparative analysis and used to represent the morphology of a vomerolfactory specialist. However, in order to reduce any bias produced by the singular analysis of just one species of snake (as mentioned above), the Harderian glands of additional species were examined at a more superficial level (ie: only at the light microscopic level). These additional species were chosen from all three colubroid families, as well as from the family Boidae. Thus, at least at the light microscopic level, any variations in Harderian gland structure both within Colubroidea and between Colubroidea and Boidae may provide generalisations about the phylogeny of the gland in advanced snakes.

Less is known about the chemosensory capacity of both the gekkotans and the scincomorphs. Although they have been described as a potential olfactory specialists and nasal chemosensory generalists respectively, there is much less research to substantiate these claims (see Schwenk, 1993b). As they have received little attention, it is difficult to choose a single species from each group as representative of the type of nasal chemosensory adaptation. Thus, a selection of species from both the scincomorph and gekkotan groups will be examined. Only through the examination of multiple, phylogenetically diverse species can the level of variation of both the gland and the chemosensory system in these two lineages be shown.

Within Gekkota, two families have been traditionally recognised: Gekkonidae and Pygopodidae. The relationships of these families are complicated by the subdivision of the Gekkonidae into four separate subfamilies (Kluge, 1987 - diagram 9). Of these five taxa, only three are found in Australia: Gekkoninae, Diplodactylinae and Pygopodidae. As there are little comparative chemosensory data available on the gekkotans, representative species were chosen from each of the three taxa. Furthermore, since there has been no such prior work carried out on gekkonines, diplodactylines or pygopods, two phylogenetically distant species per group were examined in order to be more representative. Thus, by examining a total of six different species, from the three available taxa, any results from the morphological analysis of the Harderian gland and nasal chemosensory systems would be reasonably representative of the Australian gekkotan condition.

Skinks are the only representative of Scincomorpha in Australia. The Australian skinks are further restricted to the subfamily Lygosominae. This family is further subdivided into four separate groups (Greer, 1989 - diagram 10). Like the gekkotans, it is difficult to define a typical skink. Though the skinks are phylogenetically restricted to a single subfamily, they are more diverse at the species level than the gekkotans (Hutchinson and Donellan, 1993). This may be reflected in the chemosensory variation generally observed throughout the Scincomorpha. My data on the lygosomine Harderian gland was restricted to the *Sphenomorphus* group (Rehorek, 1992; Rehorek et al., 1993). In the present analysis, the morphological examination of the Harderian gland and nasal chemosensory structures is expanded to include the *Egernia* and *Eugongylus* groups. This coverage of species from all Australian lygosomine taxa would therefore be expected to provide sufficient data to define the structure of both the Harderian gland and nasal chemosensory systems of the Australian scincid lizards.

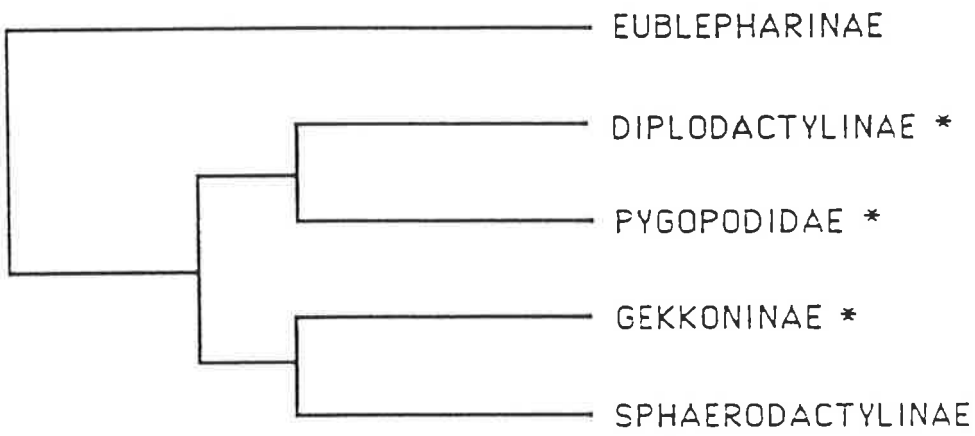

DIAGRAM 9

Phylogenetic relationships within the gekkotan lineage, of which there are only three of the taxa found in Australia (from Kluge, 1987).

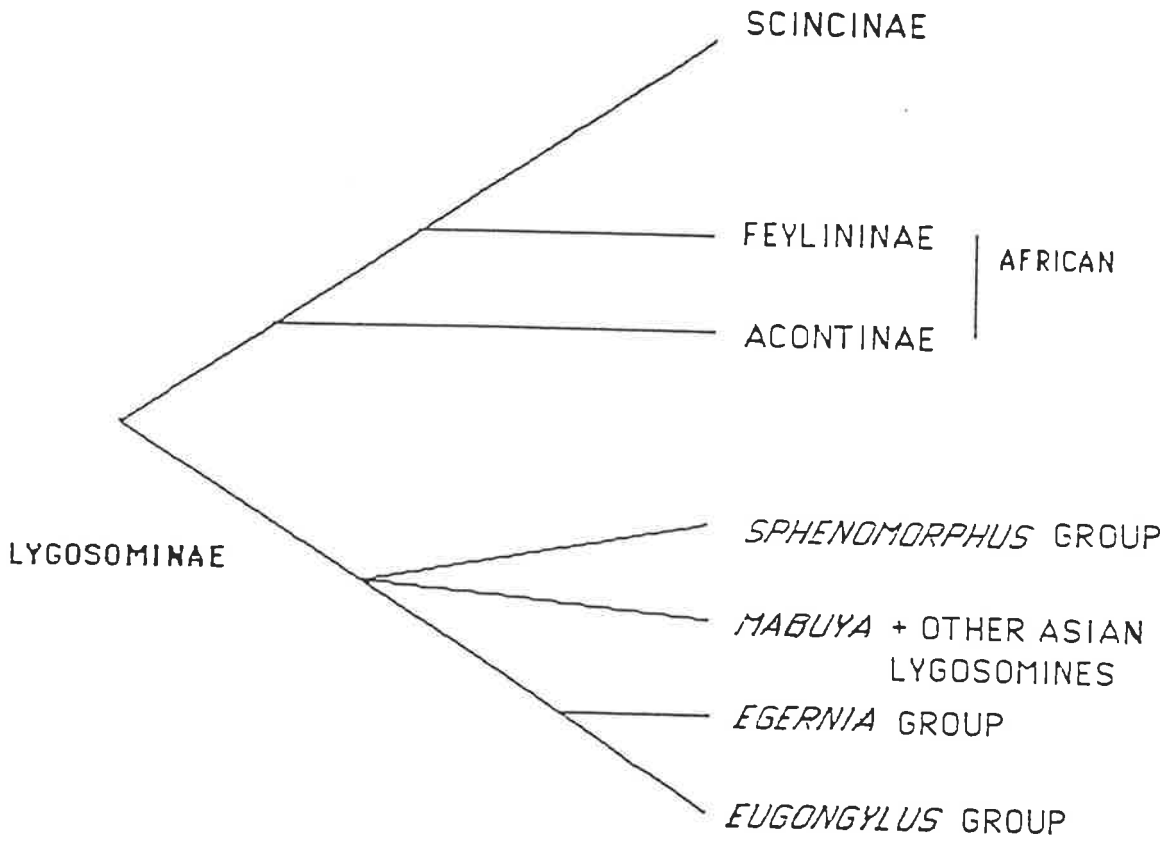

DIAGRAM 10

Phylogenetic relationships of the Scincidae, of which only members of Lygosominae are found in Australia (from Greer, 1989).

In conclusion, three major squamate lineages can be defined by different levels of nasal chemosensory capacity. As comparative morphological examination of both the Harderian gland and nasal chemosensory structures has mainly been conducted on only one lineage, the snakes, which are defined as vomerolfactive specialists, it is justifiable to extensively examine the glands of one species of this lineage, with some comparative analysis at a more superficial level. Conversely, the lack of information from both the gekkotan and scincomorphan lineages necessitates the more detailed examination of multiple, phylogenetically diverse species. Such a comparative phylogenetic analysis would show any trends in the structures of both the Harderian gland and the nasal chemosensory systems in these squamate lineages.

1.5 AIMS AND SIGNIFICANCE

There are two aims of this study. The first aim is to morphologically describe the Harderian glands of Australian squamates from three clades (Serpentes: n=1, Gekkota: n=6, and Scincomorpha: n=3) exhibiting different types of nasal chemosensory adaptation (vomerolfactory, olfactory and generalised nasal chemosensation respectively). The second aim is to examine the likely role of the gland in squamate vomerolfaction, using the data obtained from the first aim.

To meet the first aim, the structure and position of the Harderian gland of representative squamate species was examined using both light and electron microscopic techniques. In addition, the embryogenesis of the Harderian gland of some species was investigated, and autoradiographic studies were conducted on the skink, *Hemiergis decresiensis*, Harderian glands. Thus the comparative technique allowed an identification of any evolutionary trends or possible correlations to the mode of nasal chemosensory adaptation.

To meet the second aim, both functional and comparative approaches were used. Functional analysis entailed the examination of the VNO, in order to determine whether the Harderian gland would function as a source of lubricant for the squamate VNO. Little is known about the morphology and necessity of the lubricatory capacity in the VNO. Thus, the MOS, which is a similar system and has been the subject of more research, was surveyed first in order to determine the necessary level of lubrication in the idealised VNO might be. The comparative analysis assessed the morphology of the nasal chemosensory epithelia of the three clades. This analysis was used to determine whether there were any morphological variations which correspond to the type of nasal chemosensory adaptation. This determined whether the labels of potential olfactory specialist (gekkotans) and nasal chemosensory generalist (skinks) could be morphologically substantiated. Additionally, from both these comparative studies, and those from the Harderian gland, I determined whether the squamate Harderian gland was likely to function in vomerolfaction in the three clades exhibiting different levels of nasal chemosensory adaptation.

CHAPTER 2: MATERIAL AND METHODS

2.1 ANIMALS COLLECTED

A total of 11 species from the three clades were used in this study. The total number of specimens obtained is listed in Appendix 1. All species were collected according to specifications of the National Parks and Wildlife Act of South Australia. Specimens were collected in the Adelaide and Burra regions of South Australia under permit numbers U2338-01, 2, and 3. Additional specimens were obtained from other researchers (see Appendix 1).

The ensuing brief descriptions of these squamates have been taken from Cogger (1992) and Ehman (1992). *Tiliqua rugosa* (formerly *Trachydosaurus rugosa*) (Plate 1A) is a large, slow-moving, diurnal lizard belonging to the *Egernia* group, with an average snout-vent length (SVL) of 30 cm. *Morethia adelaidensis* (Plate 1B) and *M. boulengeri* (Plate 1C) are terrestrial, diurnal skinks, which belong to the *Eugongylus* group. They both have immoveable eyelids and have an average SVL of 6 cm. *Hemiergis decresiensis* (Plate 2A) was used in two parts in this study: autoradiography and embryology of the Harderian gland. They have a semi-moveable eyelid, with a small, transparent disc, and an average SVL of 5 cm. Six species of gekkotans were examined in this study. All gekkotans studied have immoveable eyelids. Both *Christinus marmoratus* (Plate 2B)and *Heteronotia binoei* (Plate 2C) belong to Gekkoninae, and are nocturnal, with SVL's of 6 cm and 8 cm respectively. *Strophurus intermedius* (Plate 3A) and *Nephrurus milii* (Plate 3B) both belong to the subfamily Diplodactylinae and are also nocturnal, and have an average SVL of 7 - 8 cm. Pygopods, commonly known as snake or worm lizards, are the only group of reptiles endemic to the Australian region. *Delma molleri* (Plate 3C) has an average SVL of 11 cm (average total length of 36 cm), whereas *Aprasia pseudopulchella* (Plate 4A) averages 14 cm SVL but is smaller in mass and body diameter (average total length of 23 cm). *Pseudonaja textilis* (Plate 4B) is a large, venomous, elapid snake, with a total length of 220 cm. The hatchling snake (Plate 4C) is approximately the same size, has similar colouration, and often

co-habits with the pygopod, *D. molleri.* Embryos from nine species of snakes were examined from the collection at the Department of Biological Sciences, Old Dominion University, Virginia, USA. Multiple stages and specimens of the following species were examined: Colubridae - *Lampropeltis getulus*, *Coluber constrictor*, *Nerodia sipedon* and *Diadophis punctatus*; Viperidae - *Crotalus horridus*, *Agkistrodon piscivorus* and *A. contortrix*; Boidae - *Python regius* and *P. molurus.*

2.2 HOLDING OF SPECIMENS

All lizards and snakes were obtained during the spring seasons of 1993-1995. All animals used specifically in this research were kept in captivity for a brief time in accordance with the guidelines set by the University of Adelaide Animal Ethics Committee, application number M/58/93.

Lizards obtained from other researchers, had either been involved in behavioural studies (*M. adelaidensis* and *M. boulengeri*) or were going to be used for post-mortem experimentation. Extra samples of all lizard species were also obtained specifically for this study.

Prior to sacrifice, all lizards were stored in plastic boxes, with a piece of thick wire mesh grating, stapled over a square hole in the lid to allow ventilation. Either natural terrain, if freshly caught, or saw dust, was placed in the container. They were watered and fed meal worms *ad libitum.* With the exception of *T. rugosa*, which were housed individually, all lizards were kept in groups of 3-5 per cage. All lizards were sacrificed with an intraperitoneally injected overdose of sodium pentobarbitol (Nembutal). They were then decapitated, and the Harderian glands were dissected out and placed into the appropriate fixative. Sex was determined by dissection.

Due to the toxicity of the venom and the aggressive nature of *P. textilis*, live animals were not kept for long periods, and were sacrificed when received. Adult specimens were cooled, to reduce their activity levels, prior to intraperitoneal injection of a lethal dose of sodium pentobarbitol (Nembutal). Hatchlings were exposed to a chloroform soaked tissue. The snakes were then decapitated, and, in adults, the venom glands and fangs were removed. The tissue was then treated in the same manner as those of the lizards.

2.3 MICROSCOPIC TECHNIQUES USED

2.3.1 LIGHT MICROSCOPY

For light microscopic analysis, specimens were immersed in either Bouins fixative for 24-48 hours or 10% phosphate buffered formalin for at least a week. In order to ascertain the route of the lacrimal duct, entire heads were fixed in 10% phosphate buffered formalin, immersed in decalcifying agent (5% EDTA in 10% phosphate buffered formalin), and then embedded in paraffin. These were then serially sectioned at 7 μm and stained with haematoxylin and eosin.

For all histochemical and ultrastructural analyses a minimum of three specimens per species per technique were used.

Sections of Harderian glands and serial sections of the nasal capsule not previously stained with haematoxylin and eosin were stained with various histochemical stains. The presence of carbohydrates was investigated using the periodic acid-Schiffs (PAS) and PAS / amylase reactions (Drury and Wallington, 1980). A combined alcian blue-alcian yellow technique (Ravetto, 1964) was used for differentiating acid mucosubstances. Mercury bromo-phenol blue (Barka and Anderson, 1965) was used to test for presence of proteins. Bouins-fixed rabbit small intestines were used as controls. The negative control for mercury

bromo-phenol blue required sections which had been collected on gelatin coated slides, exposed overnight to formaldehyde vapour in an oven, and exposed to a 1:1000 aqueous solution of Pronase E (Sigma Chemical Company, no. P-6911) in a 60°C oven for 2 hours.

Formalin fixed, 15 μm thick frozen sections of Harderian glands and nasal conchae were stained with the supersaturated isopropanol method (Lillie, 1954) used to detect oil soluble lipids. Frozen sections of gecko tail were used as the control.

2.3.2 ELECTRON MICROSCOPY

For ultrastructural analysis, the vomeronasal organs (VNO), conchae and Harderian glands were dissected out. The tissue was immediately immersed in 3% formaldehyde/3% glutaraldehyde fixative in 0.1M phosphate buffer pH 7.4 at room temperature for 2-4 hours, postfixed in 1% OsO4 in 0.1M phosphate buffer, dehydrated through a series of alcohols, infiltrated with propylene oxide and embedded in TAAB epoxy resin. Ultrathin sections (0.1 μm) were cut on the ultracut ultramicrotome with a diamond knife. These sections were picked up on copper grids, stained with 2% uranyl acetate and lead citrate, and examined with a Phillips CM 100 transmission electron microscope (TEM).

Enzymatic extraction was used to assess the protein content of the secretory granules observed under electron microscopy. Some thin sections were collected on nickel grids, oxidised in 10% periodic acid and then subjected to enzymatic extraction with Pronase E (Sigma Chemical Company, no. P-6911) for either 24 or 48 hours of incubation at 40°C (Pévet, 1977). These were then stained, as above, and examined with a Phillips CM 100 TEM. The control sections were oxidised in 10% periodic acid and then incubated in distilled water at 40°C.

For scanning electron microscopy (SEM) a few Harderian glands from each species were carefully dissected out, fixed for at least 24 hours in the fixative used for TEM,

postfixed in 1% OsO4 for 1 hour, dehydrated through a series of acetones and critical point dried. Specimens were mounted on metal stands with double sided adhesive tape and sputter-coated with carbon and gold/palladium. They were examined with a Phillips XL 20 scanning electron microscope.

2.3.3 AUTORADIOGRAPHY

Methionine is an amino acid used in the formation of most proteins. By incorporating a radioactive marker (tritium) to this amino acid and injecting it into a biological system, the rate of protein synthesis, involving methionine, can be measured. The solution is injected into the specimen at a non-lethal level, and samples of the tissue are histologically examined at different points in time to ascertain the relative position of the marked amino acids. This entire process is referred to as autoradiography. Specimens of *H. decresiensis* which were used for the autoradiographic experiments, were kept in captivity for the appropriate time in accordance with the guidelines set by the University of Adelaide Animal Ethics Committee, application number S/033/95.

Prior to experimentation, the adult *H. decresiensis* used for the autoradiography, were collected and stored in plastic boxes (as described above), given food and water *ad libitum*. They were kept for two weeks in and environmental chamber, set at typical South Australian spring temperatures (12L; 12D photoperiod and 24^{o}D; 15^{o}N thermoperiod). For autoradiographic analysis, adult *H. decresiensis* were intraperitoneally injected with 0.05 ml of physiological saline containing 10 µCi of L-(Methyl-H3) methionine (Amersham). The lizards were kept in an environmental chamber, and were sacrificed 2, 4, 24, 48 and 69 hours after the initial injection. The Harderian glands were rapidly removed, washed in cold methionine, fixed in Bouins and processed into paraffin. Sections were cut at 7 µm and collected on gelatin coated slides. The slides were immersed in a 50% aqueous dilution of k2 nuclear research emulsion (Amersham) in a darkroom. They were then dried, packed in light-tight boxes and stored at 4^{o}C for the period of exposure. After at least three weeks,

the slides were developed (Kodak D19), washed (5% aqueous acetic acid), and fixed (Ilford Hypam). The slides were then stained in Harris' haematoxylin for 2 minutes, cleared, mounted and then examined with light microscopy.

CHAPTER 3: THE HARDERIAN GLAND

3.1 INTRODUCTION

Little is known about the squamate Harderian gland, with scattered studies on disparate species using a variety of methods. Although present in all squamates, the Harderian gland varies in size, and bears extra orbital portions in some colubrid snakes (Schwarz-Karsten, 1937; Savitzky, 1972; 1978; Saint Girons, 1982; McCarthy, 1985). The works of Saint Girons (1982; 1988; 1989) constitute an introductory survey of the squamate Harderian gland, using a broad phylogenetic base with both histological and histochemical analyses. Further histochemical and ultrastructural analyses have been carried out on a few snake and scincomorph species (Chieffi-Baccari et al., 1990; Minucci et al., 1992; Rehorek, 1992; Rehorek et al., 1993).

In this section, evolutionary trends of the squamate Harderian gland structure were investigated by the comparative method. From the previous scattered studies, the squamate Harderian gland emerged as a serous secreting gland. In this study, I tested this generalisation by examining the Harderian gland of several species using diverse histological techniques. I wanted to determine what was being secreted by the squamate Harderian gland. The connection between the Harderian gland and the lacrimal apparatus, and consequently the VNO, was also analysed in this section. Any morphological variations would therefore imply potential differences in vomerolfactory capacity. Consequently, I hypothesised that if the Harderian gland does function in vomerolfaction, any variations in the connection between the gland and the lacrimal apparatus would indicate differing levels of vomerolfactive capacity.

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3.2 RESULTS

3.2.1 ANATOMY

The squamate Harderian gland consisted of a head, which was connected to the anterior conjunctival region of the orbit, and a tail which extended posteriorly towards the optic nerve. In the skinks examined, the head of the gland was embedded in the corneal aspect of the nictitating membrane. The nictitating membrane was not as distinct a structure in *Morethia* spp. as it was in *T. rugosa*. The ducts of the gland opened directly on to the cornea. These ducts were separated by the nictitating membrane from the lacrimal canaliculi, which open into the anterior border of the orbit (see diagram 1). The shape of the gland in the three skink species examined was similar. In *Morethia* spp., a relatively broad head tapered quickly into the long, tongue-like extension of the tail section (Plate 5A). The tail of the gland passed posteriorly, and terminated in proximity to the optic nerve. However, the Harderian gland of *T. rugosa* had a broader head, and a less abrupt tapering of the tail region. Like that of *Morethia* spp, the tail of the Harderian gland of *T. rugosa* also terminated in close proximity to the optic nerve. Thus, both the shape and relative size of the Harderian gland of these skinks was quite similar.

The one common feature of the Harderian gland of the other species examined was the absence of a nictitating membrane. Thus, the Harderian gland is embedded in the anterior conjunctival epithelium and has the potential to be more closely associated with the lacrimal apparatus. In the case of the gekkos, the Harderian gland was uniform in both structure and relative position to the lacrimal apparatus. Like the skinks, the gekko Harderian gland opened onto the cornea, whilst the lacrimal canaliculi opened into the anterior border of the orbit. Unlike the skinks in this study, there was no nictitating membrane separating the two structures. Thus, the absence of the nictitating membrane in the gekko orbit apparently has not influenced the architecture of the anterior part of the orbit.

Though the gekko Harderian gland was tongue-shaped, it was neither as large, nor as long as that of the skinks (Plate 5B). Furthermore, the Harderian gland of the gekko was dwarfed on the enlarged eyeball. It was confined to the anteromedial aspect of the orbit, with the tail not reaching the optic nerve.

The Harderian gland of both the pygopods and the snake shared certain features, including its relationship to the lacrimal canaliculi and the shape of the gland. In both cases, the absence of a nictitating membrane, and a reduced anterior conjunctival sac, allowed for the formation of a connection between the ducts of the Harderian gland and the lacrimal canaliculi. The ducts of the Harderian gland opened into the lacrimal canaliculi, and not onto the cornea. However, the connection between the lacrimal apparatus and the conjunctival space in the pygopods was through a connecting duct. In the snake, this connection has been reduced to a small opening in one of the lacrimal canaliculi. At least in the snake, the secretions of the Harderian gland have little chance of entering the corneal space.

The shape of the Harderian glands of the snake and the pygopods also showed some convergence. In both cases, the gland was enlarged and had distinct lobes. When compared to the gekkos, the pygopod Harderian gland was much larger. The exact degree of lobulation differed between the two groups. In the case of the pygopod, the Harderian gland was a bottle-shaped structure, with a thinner head and neck. The body of the gland had two expansions, an anterior one pointing dorsally, and a posterior one pointing ventrally (Plate 5C). These lobes were much more pronounced in *P. textilis*, as they increased in size during development. In the hatchling snake, the Harderian gland was only slightly larger than that of the pygopod, with a similar degree of lobulation. In the adult stage, both the gland and the lobes were much larger (Plate 5D). These lobes encompassed the eyeball, barely leaving sufficient space for the optic nerve and muscles (Plate 5E).

3.2.2 LIGHT MICROSCOPY

The lacrimal apparatus, which connects the orbit to the VNO, was lined by simple squamous-cuboidal epithelium. The cells of this epithelium had very few apical secretory granules.

The squamate Harderian gland was a compound tubular gland, surrounded by a connective tissue capsule, whose septa divided the gland into lobules and encompassed the individual glandular tubes. Though it was a compound tubular gland, the cross sectional areas of the tubules will be referred to as acini. The capsular and septal connective tissue consisted of blood vessels, nerves and fibroblasts. The size of these blood vessels was related to the size of the gland, with arterioles present in the Harderian glands of both *P. textilis* and *T. rugosa*, and only capillaries in the other, smaller species. Myoepithelial cells were found between the basal lamina and the secretory epithelia. Variation was observed in the size of the lumina of the acini. In the skinks, pygopods, and *P. textilis*, the lumina were small. This gave the gland a solid appearance. In contrast, the gekko Harderian gland had large lumina, and thus these glands had a spongy appearance (Plate 6A).

There are some histochemical generalisations regarding the squamate Harderian gland. The Harderian glands of all squamates were primarily serous secreting structures, as indicated by the positive reaction to mercury bromo-phenol blue. All serous secretory cells were PAS positive, indicating the presence of neutral mucosubstances (Plate 6B). The negative reaction to all alcian stains suggested that acidic mucosubstances might be absent. However, it is possible that these secretory granules contained some form of mucoprotein whose nature could not be ascertained by the tests used. Because mucosubstances commonly occur as mixtures of mucopolysaccharides and mucoproteins, there is often overlap in the results of mucin staining methods (Drury and Wallington, 1980). As a result, a variety of techniques have to be used and further studies carried out before the precise nature of these proteinaceous mucosubstances found in the these Harderian glands can be

defined. In all cases, there was no evidence of sexual dimorphism in any of the species examined. Other types of secretory products (lipids) and cells (mucous) were observed in the squamate Harderian gland, which varied between taxa. The results of the histochemical analyses are summarised in table 1.

	PAS	PAS/ Amylase	R	BPB	SSIM
Pseudonaja textilis	+ (b) ++ (d)	+ (b) ++ (d)	- (b) Y (d)	++ (b) + (d)	- (b & d)
Morethia adelaidensis + *boulengeri*	+	+	-	++	-
Tiliqua rugosa	+	+	-	++	-
Christinus marmoratus	+	+	-	++	++
Heteronotia bineoi	+	+	-	++	+
Nephrurus milli	+	+	-	++	+
Strophurus intermedius	+	+	-	++	+
Delma molleri	+	+	-	++	+/-
Aprasia pseudopulchella	+	+	-	++	+/-

Table 1: Summary of the results of the histochemical analyses carried out on the Harderian glands of the squamates examined. Abbreviations: "-" = no reaction, "+" = positive reaction, "++" = very positive reaction, b: body of gland, d: ducts of gland, PAS: periodic acid-Schiffs, R: Ravetto's method, Y: yellow, BPB: bromo-phenol blue, SSIM: supersaturated isopropanol method.

In all gekkotan Harderian glands inspected, lipid granules coexist with the protein granules in the secretory cell, seen with the positive reaction to the supersaturated isopropanol method for simple lipids (Plate 6C). The most intense staining occurred in the Harderian gland of the gekkonine gekko, *C. marmoratus*. There was no evidence of any

lipid in any other area of the gekkotan Harderian gland, nor in the gland of other squamates surveyed.

Mucous cells were observed in the Harderian gland of both the snake, *P. textilis* and skinks. In the case of *P. textilis*, the mucous cells were confined to the duct system (Plate 7). This is the only case of a specialised duct system in the squamate examined. These ducts were continuous with the lacrimal canaliculi. The mucous cells of the skinks, especially those of *T. rugosa*, were restricted to the apex of the gland (Plate 8A). In this case, the mucous cells were extensions of the mucous secreting epithelia of the nictitating membrane. These mucous cells did not penetrate deep into the gland.

3.2.3 ULTRASTRUCTURE

The structure of the serous cells was similar in all squamate Harderian glands studied (Plate 8B). The cytoplasmic organelles in these cells were all polarised, with basal nuclei, abundant rough endoplasmic reticulum, and apical secretory granules. The basal nuclei were all active, displaying dispersed nuclear material (euchromatin) and prominent nucleoli. Elongated mitochondria were present throughout the cell. The Golgi complex was rarely discernible and poorly developed. The apical portions of the cells were joined by tight junctions and desmosomes, while the basal portions were connected by interdigitating intermediate junctions. At the apex of the cell, microvillous processes protruded into the lumina.

Ultrastructurally, the only sources of variation among the taxa were observed in the structures of the serous secretory granules and both in the presence and abundance of non-serous granules and cells. With the exception of the gekkotans, such ultrastructural variations were observed between families, and not within.

All serous secretory granules were compartmentalised. However, there was no evidence of any separating intragranular partitions. Only serous cells with a single type of secretory granule were observed in the skinks. In this case, the Harderian gland secretory cells were filled with non-homogeneous secretory granules. These membrane bound granules were usually bipartite, though a third, smaller compartment was observed in *Morethia* spp. (Plate 8C).

Both homogeneous (lipid) and non-homogeneous (protein) granules were observed in the secretory cells of the gekkos. The relative abundance of these two types of secretory granules varied, with apparently more homogeneous granules in *C. marmoratus* (Plate 9A) than those of the other gekkotans (Plate 9B). The close apposition of the endoplasmic reticulum gave the impression that the homogeneous lipid granules (Plate 10A) are membrane bound (Fawcett, 1981). The compartmentalised serous granules, however, showed variation among gekkos, with the presence of singular (Plate 10B) and multiple (Plate 10C) intragranular lamellar crystalline structures in the electron-lucent compartments in gekkonines. No such structures were observed in the diplodactylines (Plate 10D). These lamellar crystalline structures were not affected by pronase digestion (Plate 11A). Like diplodactylines, lipid granules in the pygopod Harderian gland secretory cells were less prevalent (Plate 11B). The lamellar crystalline structures in the compound serous granules (Plate 11C) were not as clearly developed as those of the gekkonine gekkos (Plate 10B). Nevertheless, these lamellar crystalline structures were unaffected by pronase digestion (Plate 11D).

Two different secretory cells were found in the Harderian gland of the snake, *P. textilis* (Plate 12A). The duct was lined by typical mucous cells, with basal nuclei, few discernible organelles and packed with homogeneous, electron-lucent secretory granules (Plate 12B). The serous cells of the body of the gland were filled with non-homogeneous secretory granules (Plate 12C). They exhibited three to four different compartments of varying electron densities (Plate 12D). The most electron-lucent compartment was centrally located in the granule, and formed by "randomly dispersed granules suspended in a

homogeneous matrix" (Minucci et al., 1991). One of these compartments, of intermediate electron-density, contained barely discernible crystalline structures.

3.2.4 AUTORADIOGRAPHY

Instances of secretion into the lumina of the Harderian gland were rarely seen at either the light or electron microscopic levels. There was no evidence of cellular degeneration. Thus the squamate Harderian glands were presumed to use either merocrine or apocrine mechanisms of secretion. This is supported by the autoradiographic evidence. The Harderian glands of *Hemiergis decresiensis* injected with L-(Methyl-H^3) methionine showed that protein was actively being produced during the period of investigation (69 hours). The relative position of the H^3-methionine could be visualised by the silver granules in the cell, visible after autoradiographic treatment. Two hours after injection, a few silver grains were found mainly in the basal part of the Harderian gland secretory cells (Plate 13A). From 4 to 24 hours after injection, there was an increase in the number of silver grains detected, and distributed throughout the secretory cells. There was a dense accumulation of silver grains in both the apex of the cells and in the lumina 24 hours after the injection (Plate 13B). From 24 to 69 hours after injection, the Harderian gland was full of silver granules, which became increasingly concentrated towards the apical portions of the secretory cell lumina. After 69 hours, there were fewer silver grains in the secretory cells and many in the lumina (Plate 13C). Harderian glands extracted from specimens which had not been injected with H^3 - methionine had no silver grains present (Plate 13D).

3.2.5 EMBRYOLOGY

The organogenesis of the Harderian gland and its relationship to the lacrimal duct in the three families of snakes surveyed were similar at the early embryonic stages. At stage 28, the lacrimal duct was first visible. It budded off from the lateral side of the vomeronasal duct, failing to extend to the orbital region. By stage 29, the lacrimal duct reached the orbit. Here the Harderian gland initially appeared as an invagination of the epithelium in close proximity to the opening of the lacrimal duct, and began to develop into a solid tube (Plate 14A). By stage 31, the lacrimal duct proceeded from the lateral to the medial side of the vomeronasal duct. Thereafter, the Harderian gland and its relationship to the lacrimal duct varied among the three families of snake embryos studied.

In the colubrids, at stage 32, the Harderian gland was confluent with the lacrimal duct (Plate 14B). At this stage, the Harderian gland was a semi-solid tube, gradually extending from the conjunctival epithelium into the mesenchymal stroma.

In the viperids, at stage 32, the lacrimal duct largely by-passed the conjunctival space, as it passed deep to the eyeball, and was continuous with the semi-hollow tubules of the Harderian gland. There was a small tubule from the lacrimal duct which opened into the conjunctival space. By stage 33, the Harderian gland was a series of tubes attached to the lacrimal duct. The Harderian gland also opened into the orbit independent of the lacrimal duct. The body of the gland gradually expanded into the mesenchymal stroma deep in the orbit. At stage 34, the lacrimal duct formed an antechamber before reaching the orbital region, sending off a branch to both the conjunctival region and the Harderian gland. The body of the gland gradually expanded into the encapsulated mesenchymal stroma (Plate 14C). At stage 35, the lacrimal duct bifurcated, producing two canaliculi, which opened into the conjunctival space and the Harderian gland respectively. The body of the Harderian gland increased in size as it invaded the mesenchymal stroma (Plate 14D). Some segregation of the acini was seen at this stage. At stage 36, the lacrimal canaliculi were separate, with

the larger medial canaliculus opening into the Harderian gland, and the smaller lateral one opening into the orbit.

In boids at stage 31, the lacrimal canaliculi opened into the Harderian gland. Between stages 33-35, the Harderian gland underwent rapid development. The Harderian gland opened into the conjunctival region independent of the lacrimal canaliculi. By this stage the Harderian gland comprised a series of tubules, with small lumina, extending deep into the orbital region. The acini of the gland were separated by mesenchymal stroma, and the entire gland was encapsulated. At stage 37, the glandular duct cells were distinguished from the cells of the glandular body (Plate 15A), as the lumina could be distinguished in most acini.

In the hatchling elapid, the individual acini of the Harderian gland were separated by a small amount of interacinar connective tissue (Plate 15B). The two lacrimal canaliculi opened directly into the Harderian gland. There was, however, a small connection between the lateral lacrimal canaliculus and the conjunctival space. The Harderian gland filled the oral part of the orbit. The cells of the duct system were histologically distinct from those of the body of the gland.

The Harderian gland of the skink, *Hemiergis decresiensis*, appeared at stage 36 as a thickening of the conjunctival epithelium between the nictitating membrane and the cornea. At this stage, the Harderian gland was a small, solid invagination on the inner aspect of the nictitating membrane (Plate 15C). The lacrimal duct was a thickening of the mesenchymal stroma, connecting the duct of the VNO to the lateral aspect of the orbit. Embryologically, the lacrimal canaliculi were separated from the Harderian gland by the nictitating membrane. This was reminiscent of the adult condition. The adult Harderian gland was a curved, lingually shaped gland which lay in the midline of the orbit, barely reaching the optic nerve. It was a solid gland, with small lumina and little interstitial connective tissue. There was no evidence for a specialised duct system.

3.3 DISCUSSION

In all the squamates inspected in this study, the Harderian gland is a discrete serous secreting structure in the anterior part of the orbit. It is associated with the lacrimal apparatus and is fully developed at birth. Thus, these findings confirm the observations of both Bellairs and Boyd (1947) and Saint Girons (1982; 1988; 1989). Thus, at least superficially, there is consistency in the structure and position of the squamate Harderian gland. The three characteristic features of the squamate Harderian gland (serous secretion, relationship to lacrimal apparatus, and the timing of embryonic development), as well as the absence of sexual dimorphism, will be discussed prior to any exposition of the variations observed in Harderian gland position and structure.

The presence of protein in all squamate Harderian glands in the current investigation indicates that it is primarily a serous secreting structure. Further, the present results from the autoradiographic experiments in the skink show that this protein is being steadily produced and secreted, with a complete turnover in about 69 hours. Thus, the squamate Harderian gland actively produces and secretes protein. Although it is not certain where the secretions flow, the morphology of the orbital region suggests that either the cornea or the lacrimal canaliculi are the most likely destinations for the secretion. Physiological or tracer studies would confirm this.

The association between the Harderian gland and the lacrimal apparatus seems to be a characteristic feature of squamates I examined. Even when the Harderian gland opens into the orbit, the lacrimal canaliculi are still nearby. This observation, together with that showing the development of the lacrimal apparatus prior to Harderian gland inception, suggests a strong link between the Harderian gland and the VNO in squamates. This link is particularly strong in both snakes and pygopods, as in these groups there is little contact between the Harderian gland and the orbit. This confluency, at least in snakes, can be correlated with their vomerolfactory specialisation. Conversely, the separation of the

Harderian gland and the lacrimal canaliculi in both skinks and gekkos implies that they do not exhibit the same level (or type) of vomerolfactory specialisation as snakes.

Though the sequence of maturation of the Harderian gland is similar in all tetrapods, the timing of its development in squamates is different from that of both mammals and amphibians. The rodent Harderian gland develops mainly postnatally (Müller, 1969; Paligova and Pospisilova, 1972; Bucana and Nadakavukaren, 1972, 1973; Vianna et al., 1975a/b; Michael et al., 1988; López et al., 1992). The maturation of this gland in amphibians occurs during metamorphosis (Walls, 1942; Kaltenbach et al., 1980; Shirama et al., 1992; Wake, 1985). This is in contrast to the prenatal development of the Harderian gland in squamates. Thus, the differences in timing of development are due to an earlier ontogenetic development in the squamates, and potentially some of the amphibians, as the sequence of maturation of the gland is similar in all groups.

The Harderian gland exhibits sexual dimorphism in both mammals (see Payne, 1994 for review) and the frog, *Bufo viridis* (Minucci et al., 1989). This is in contrast to the squamates investigated in this study. It is possible, however, that sexual dimorphism may be seasonal, as it is in *Bufo viridis*. As all of the specimens in the present study were collected in one season (spring), supplementary surveyed are required to determine whether this is true of squamates.

The structure of the Harderian gland differs among the four families analysed. The combination of features, including shape, size and histology of the gland, and its relationship to the lacrimal apparatus, corresponds to the type of nasal chemosensory adaptation thought to be exhibited by each of the families. In this study, the combination of a large, serous secreting Harderian gland, whose duct system is lined by mucous cells and confluent with the lacrimal canaliculi, was observed only in the snake, *P. textilis*. Various aspects of these features have been described in other snakes (mainly colubroid) by previous authors (Bellairs, 1947; Saint Girons, 1982; 1988; 1989; Minucci et al., 1991), and thus may be typical of the colubroid snakes examined thus far.

The condition of the Harderian gland is different in the supposedly olfactive gekkos. The gekko Harderian gland is the smallest one examined in this study. Histologically, the large acinar lumina were lined by a single cell type which secretes both lipid and protein. The ducts of the gland were not histologically distinct, and were separated from the lacrimal canaliculi. Though most of these features have been described by Saint Girons (1982; 1988), the presence of lipid has not been previously recorded. Nevertheless, the combination of these features is different to those of the snakes.

Finally, the Harderian glands of skinks, which are thought to be generalised in nasal chemosensation, exhibit another set of features. In this case, the Harderian gland is of an intermediate size, is lined solely by serous cells (with some mucous cells in the anterior portion), and is separated from the lacrimal canaliculi. In this study, the ducts of the skink Harderian gland were separated from the lacrimal canaliculi by the nictitating membrane. Previous observations have shown that even in the absence of the nictitating membrane, the two structures remain in the same position in the orbit (Saint Girons, 1982; Rehorek, 1992). Though the combination of these features is characteristic of lygosomine skinks, some of these features are shared with either the snake or the gekkos. Like the gekkos, the skink Harderian gland is lined by one cell type and is separated from the lacrimal canaliculi. Like the snakes, the serous cells of the skink Harderian gland only produce serous granules and there are some mucous cells associated with the gland. Thus, by sharing certain features with both the snakes and the gekkos, the skink Harderian gland could be described as an intermediate structure.

In contrast to the skinks, there are no features unique to the pygopod Harderian gland, for all the features exhibited occur either in the snakes or in the gekkos. Thus, the pygopod Harderian gland can be described as intermediate between those of the snake and the gekkos. In general, the pygopod Harderian gland resembles that of the snakes macroscopically, whereas it shares many microscopic features with the gekkos. Like snakes, the pygopod Harderian gland is connected to the lacrimal canaliculi, thus allowing

the secretions from the gland to flow into the VNO. Additionally, the gland exhibits a lobular structure, similar to that of the hatchling *P. textilis* . The pygopod Harderian gland is also a solid structure, with small lumina. These features contrast with those of the gekko, wherein there is non-confluency of the Harderian gland ducts and the lacrimal canaliculi, and the gland itself is a much smaller, non-lobular, and histologically spongy structure. Thus, macroscopic evidence suggests that the pygopod Harderian gland is snake-like in nature.

Microscopically, however,, the pygopod Harderian gland differs from that of the snakes. It produces both lipid and serous secretion, and the serous granules are ultrastructurally tripartite with a poorly defined lamellar crystalline structure. This is similar to the condition in gekkos. In contrast to the snakes, the pygopod Harderian gland possesses neither a specialised mucous duct system, nor the typical compound serous granules. Thus, based on these microscopic observations, the pygopod Harderian gland is typical of gekkos. Though the significance of the intermediate nature of the pygopod Harderian gland is unknown, the similarity between the pygopod and snake Harderian glands can be explained in terms of convergent evolution. Since both snakes and pygopods exhibit ecological convergence, this may manifest itself in the type of nasal chemosensory adaptation exhibited. The vomerolfactory nature of the pygopods is supported by behavioural evidence (Schwenk, 1993b). Nevertheless, the pygopod Harderian gland still retains its gekko morphology. Since snakes are defined as vomerolfactory specialists, and if the gekkos are olfactory specialists, then the position of the pygopods, in terms of nasal chemosensation, is difficult to discern at this level of inquiry. Thus, before the significance of the pygopod Harderian gland can be properly discussed, the type of nasal chemosensory adaptation exhibited needs to be examined.

There were other variations among the squamates I inspected, in both a) the rate of development and b) the ultrastructure of serous secretory granules. These cannot be easily explained in terms of nasal chemosensory adaptation.

a) The absence of data on the comparative development of the squamates makes it difficult to draw proper conclusions regarding the rate of Harderian gland development. There is no current uniform staging system for squamate embryos. Thus, the level of discrepancy between scincomorph and snake Harderian gland development is unknown. Nevertheless, the Harderian gland of the snake appears earlier, in stage 29 out of 37 stages (as defined by Zehr, 1962), compared to that of the lacertid *P.s. sicula* (Chieffi-Baccari et al., 1995) and the skink, *H. decresiensis* appearing at stage 36, out of 40 stages (as defined by Dufaure and Hubert, 1961). In both cases, the Harderian gland is mostly developed and potentially functional by birth, with fully developed lumina and fully differentiated cells. Again, this can be correlated to the VNO, which is though to be functional by birth in two species of skinks (Burghart, 1973) and snakes (Holtzman and Halpern, 1990, 1991a/b).

The nictitating membrane is important in the Harderian gland development in *H. decresiensis* (in the present study) and some mammalian species (Paligova and Pospisilova, 1972; Shirama et al., 1992; Michael et al., 1988). In the aquatic frog, *Xenopus laevis*, retardation of Harderian gland development was linked to the absence of the nictitating membrane (Shirama et al., 1992). In this case of the snakes, however, the absence of a nictitating membrane did not lead to retardation of the Harderian gland. Though the earlier development of the snake Harderian gland can be explained in terms of its large size in the adult, further examination of the Harderian glands in both non-snake squamates without nictitating membranes, and snake relatives with a nictitating membrane, would be necessary before any conclusions can be made.

b) There was some variation in the ultrastructure of the serous secretory granules of the Harderian gland, both among and within the squamate lineages. Superficially, the ultrastructure of the serous secretory granules of the species surveyed in this study can be grouped according to the existing phylogenetic organisation (as proposed by Estes et al., 1988). However, the serous granules of the lacertid, *P.s. sicula*, which are similar to those of the gekkonine gekkos (Chieffi-Bacarri et al., 1990), suggest otherwise. At least in scincomorphs, there is variation in the structure of the serous secretory granules between

two families.

Upon closer inspection, variations in both level of compartmentalisation and development of lamellar crystalline structures were observed in the serous granules of all squamate Harderian glands. The significance of this compartmentalisation or lamellar crystalline structure is unknown as the interpretation of the appearance of the compartments of these granules is in dispute. It has been suggested that the compartmentalisation may be due to an artefact of specimen preparation, at the level of fixation, or that it may reflect chemical heterogeneity (Fawcett, 1981; Ghadially, 1988). Though it is known that these crystalline inclusions are proteinaceous, their chemical nature is rarely known (Fawcett, 1981). Crystalline inclusions have been reported in nearly all cellular compartments, including the nucleus, mitochondria, Golgi apparatuses and secretory granules. The analysis of both compartmentalisation and intragranular crystalline structures in other systems of animals may give some clue as to their significance. Compound secretory granules and intragranular crystalline structures occur in a variety of exocrine and endocrine glands. This includes some vertebrate Harderian (Rothwell et al., 1972; Weaker, 1981; Di Matteo et al., 1989; Minucci et al., 1989; Chieffi-Baccari et al., 1992; Bodyak and Stepanova, 1994) and mammalian salivary glands (Leeson, 1967; Tandler and Erlandson, 1972; Zylberberg, 1977; Ichikawa and Ichikawa, 1977; Young and van Lennep, 1978; Donáth et al., 1980; Ichikawa et al., 1980; Herring and Munk, 1994; Tandler et al., 1994), as well as a variety of other glands and glandular structures in both vertebrates (Rhodin, 1975; Adams et al., 1981; Klaasen et al., 1982; Kratzing, 1984; Wells and Widdicombe, 1986; Cormack, 1987; Ghadially, 1988; Brizzi et al., 1991; Gallego-Huidobro et al., 1992) and invertebrates (Kress and Schmekel, 1992; Elofson et al., 1992; Foldi and Lambdin, 1995; Schoeters and Billen, 1995; Heumen et al., 1995; Carcupino, 1996). Thus, two main conclusions can be made about these structures. First, the compartmentalised granule can be found in a variety of glands in a disparate group of animals. Thus, it is difficult to make any functional correlations using such ultrastructural analyses. Second, with the exception of the mucous oesophageal glands of *R. perezi* (Gallego-Huidobro et al., 1992) and the mucous glands of the armadillo Harderian gland (Weaker, 1981), the only uniting feature of the

remaining glands is the presence of a variable amount of protein. As proposed by Zylberberg (1977), the complexity of the ultrastructure of the granules is related to the amount of protein present. In this study, it was found that the squamate Harderian gland was indeed rich in protein. This would explain the presence of the complex secretory granules. Further microchemical and biochemical analyses of these serous secretory granules may reveal the significance of these intragranular structures.

3.4 CONCLUSION

The squamate Harderian gland possesses several structural features which are common to all species examined. In all cases, the Harderian gland is actively secreting a proteinaceous substance, closely associated with the lacrimal apparatus, and is fully developed by birth. Variation in the precise relationship to the lacrimal canaliculi, and the shape and histochemistry of the Harderian gland show unique combinations in snakes, gekkos and skinks and can be correlated to the type of nasal chemosensory adaptation. The pygopods, however, possess features of both the snake and gekko Harderian gland structure and position. Thus, the correlation between Harderian gland structure and nasal chemosensory adaptation is, at this stage, inconclusive in the pygopods. Two other sources of variation, rate of development of the Harderian gland and ultrastructure of the serous secretory granules were discussed. Neither of these could be adequately explained in terms of nasal chemosensory adaptations, and require further developmental and microchemical analyses. Thus, despite these variations, there is some consistency in the structure and position of the Harderian gland in the squamates. In order to determine the significance of these serous granules, presumably travelling down the lacrimal apparatus, the other end of the lacrimal apparatus, the VNO, needs to be studied.

CHAPTER 4: THE OLFACTORY SYSTEM - AN ANALOGY FOR THE VNS

4.1 INTRODUCTION

As discussed in chapter 1, of the three proposed functions for the squamate Harderian gland, only that of vomerolfaction has not been refuted. The possible involvement of the Harderian gland in vomerolfaction is based on its connection to the VNO via the lacrimal apparatus (Bellairs and Boyd, 1947; Saint Girons 1982; 1988; 1989). This morphological connection is further supported by embryological data obtained during this study. The squamate Harderian gland is an active, primarily serous secreting gland (chapter 3). Thus, the secretions of this gland flow either directly (snakes and pygopods) or indirectly (skinks and gekkos) into the lacrimal canaliculi. Microchemical tracing studies would determine how these secretions flow through the lacrimal apparatus, and how exactly the position of the Harderian gland affects the flow of its secretions. Though such a study would explain the mechanism of transfer into the VNO, it would not explain why it would be required in the VNO. In order to answer this question, it is necessary to understand the comparative structure, ultrastructure and histochemistry of the VNO.

Little is known about the intrinsic secretory structures and products of the squamate VNO. Recent microchemical analyses have revealed that the mammalian VNO is lined by a chemically heterogeneous mucus layer (Takami et al., 1995). The MOS, which is a structurally and functionally similar system, is also lined by a heterogeneous mucus layer (Andres, 1969; Müller et al., 1979). Sensory transduction is thought to take place in these mucus layers covering the respective nasal chemosensory epithelia (Rama-Krishna et al., 1992; Getchell et al., 1993; Takami et al., 1995). It is likely that both the structure of this mucus lining and the sources thereof are similar in both systems. The main olfactory organ, which has been studied in greater detail than that of the VNO, has two intrinsic sources of secretion which produce this heterogeneous layer in terrestrial vertebrates. Morphological

analysis of the two sources, the sustentacular cells and the Bowman's glands, has produced mixed results in the scattered tetrapods thus far examined (Getchell et al, 1984b; Getchell and Getchell, 1992). Little is known about the morphology of the lubricatory system in the squamate main olfactory organ. Thus, in this section, the lubricatory capacity of the main olfactory organ will be examined in the three squamate clades that form the subject of this thesis. These results will then be used as a basis for determining the state of the lubricatory systems in the main chemosensory systems (including the less well understood VNO) of terrestrial vertebrates.

4.2 RESULTS

4.2.1 ANATOMY

The anatomy of the squamate nasal capsule was previously described by Parsons (1970). The particular feature I examined here was the nasal conch, a lateral projection from the nasal cavity encompassing the body of the external nasal gland. This gland was located in the centre of the conch, and was overlaid firstly by supportive hyaline cartilage which was then lined dorsally by the olfactory mucosa and ventrally by respiratory epithelium. The size of the conch, and thus the spread of the olfactory mucosa, varied among the squamates examined. The nasal conch of the snake, *P. textilis*, was a small semi-circular projection, encompassing the body of the external nasal gland, but not continuing much beyond it. However, the nasal conch of both the pygopods and skinks was larger. An anterior, semi-circular lateral projection from the nasal wall became progressively isolated from the wall after the termination of the external nasal gland. The conch did not continue much beyond the external nasal gland. In the gekkos, the conch expanded well beyond the end of the body of the external nasal gland, and filled most of the nasal cavity. The crescent-shaped gekko nasal conch was in the posterior aspect of the nasal cavity, and consisted only of

central cartilage covered by olfactory mucosa and respiratory epithelium. The increased size of the nasal conch in this group was accompanied by a comparatively larger surface area of olfactory mucosa in the nasal cavity than either that of the skinks or the snake.

4.2.2 LIGHT MICROSCOPY

The olfactory epithelium consisted of three cell types: receptor, sustentacular and basal cells. Elongate nuclei belonging to the sustentacular cells were more apical than those of the receptor cells. Hair-like protrusions extended from the apical border of the epithelium into the luminal area of the nasal cavity. The submucosa (lamina propria) contained Bowman's glands, blood vessels, nerves and connective tissue. Regional differences in olfactory mucosa morphology were not measured in this study.

Histochemically, the sustentacular cells of all species contained apical granules which stained positively with both PAS and alcian yellow (pH 2.5) of Ravetto's method. The results of the histochemical analyses are summarised in table 2. Individual granules were not clearly identified with mercury bromo-phenol blue, despite the positive staining of the apical region. Variations in number of granules present were observed between species. With the exception of *H. binoei* the gekkotan sustentacular cells contained fewer apical granules than those of other species examined. The Bowman's glands all contained serous secreting cells. The reaction of these granules to both PAS and mercury bromo-phenol blue were more intense in *P. textilis*, than in any other species. There was some lipid detected in the base of the olfactory epithelium in *T. rugosa*.

	PAS	BPB	R	SSIM
Sustentacular cells	++	+/-	Y	-
Bowmans glands	+ *	+ *	-	-

Table 2. Summary of the results of histochemical analyses carried out on the olfactory mucosa of the squamates examined. Abbreviations: "-" = no reaction' "+" = positive reaction, "++" = very positive reaction, PAS: periodic acid-Schiffs, R: Ravetto's method, Y: yellow, BPB: mercury bromo-phenol blue, SSIM: supersaturated isopropanol method.
* ++ in *P. textilis*

4.2.3 ULTRASTRUCTURE

The ultrastructure of the squamate olfactory mucosa differed little from the description by previous authors (see chapter 1). In all cases, secretory granules were observed in both the sustentacular and Bowman's gland secretory cells of all species investigated. The apical border of the sustentacular cells was covered with microvillous projections. Secretory granules were mainly restricted to the supranuclear region of the cell. Elongated mitochondria and lysosomes were observed throughout the cell. The nuclei were at the base of the apical third of the olfactory epithelium. In all cases, the apical portions of both the sustentacular and receptor cells were connected by a series of tight junctions and desmosomes. Tonofilaments were readily identifiable in the sustentacular cells in association with the desmosomes.

The secretory cells of the Bowman's glands were polarised with basal RER and nuclei, and apical secretory granules. Mitochondria were found among the secretory granules, though Golgi complexes could not be readily discerned. Junctional complexes, including desmosomes, were also observed in the apical portion of these cells. All these features are typical of serous secreting cells.

Ultrastructural variations in the secretory capacity and morphology of the granules were observed between a) the sustentacular cells and b) the secretory cells of the Bowman's gland.

a) The sustentacular cells showed variation in the abundance, distribution and ultrastructure of the secretory granules. In general, the granules were homogeneous or showed limited bipartite compartmentalisation. In all gekkotans, secretory granules were only found in the apical portion of the sustentacular cells. The relative abundance of the secretory granules varied among species. In *C. marmoratus*, the spherical bipartite granules were not abundant (Plate 16A) and were not often readily discernible (Plate 16B). The spherical electron-dense secretory granules of *H. binoei*, however, were more abundant (Plate 16C). The membranes surrounding the granules were not always clear (Plate 16D). The abundance of the secretory granules in the sustentacular cells of both diplodactylines and pygopods was intermediate to those of the two gekkonines examined (Plate 17A). These granules were homogeneous structures. However, the granules of *S. intermedius* were elongate (Plate 17B), whereas those of *D. molleri* were spherical (Plate 17C). The sustentacular cells of both the snake and the skinks possessed abundant secretory granules, even more so than *H. binoei*. Some of the granules in both the skinks and *P. textilis* were in the subnuclear region (Plate 18A). At a higher magnification, these secretory granules were spheroid or elongate bipartite structures (Plate 18B), and ultrastructurally resembled the larger bipartite granules of the Bowman's gland (Plate 18C). The secretory granules in the sustentacular cells of the skinks were all homogeneous and of intermediate electron-density (Plate 19A).

b) There were no discernible variations in the abundance and distribution of both the Bowman's glands and the granules in their secretory cells. Variations were noted in the ultrastructure of the serous granules in the Bowman's glands, as their ultrastructure varied from homogeneous to complex structures with lamellar crystalline inclusions. In *P. textilis,* the secretory cells possessed spherical, tripartite granules (Plate 18C). Both spherical and elongate bipartite secretory granules were found in the secretory cells of *T. rugosa* (Plate

19B). The secretory granules of *M. adelaidensis*, however, were irregularly shaped homogeneous structures (Plate 19C), whilst those of *M. boulengeri* were smaller and possessed multiple electron-lucent portions and lamellar structures (Plate 19D). In the gecko, *C. marmoratus*, these granules were clearly partitioned, with multiple electron-lucent portions (Plate 20A), whilst those of *S. intermedius* possessed homogeneous, electron-dense secretory granules (Plate 20B). The secretory granules of the pygopod, D. *molleri*, were also partitioned (Plate 20C), but not as clearly as those of *C. marmoratus*. There was some difference in the ultrastructure of the secretory granules of Bowman's glands in *D. molleri*, as cells containing homogeneous, instead of compartmentalised, secretory granules were observed (Plate 20D). Thus, the Bowman's glands of *D. molleri* possessed both homogeneous and bipartite secretory granules.

4.3 DISCUSSION

The olfactory mucosa of the squamate reptiles investigated possessed many common structural features. In all cases, the olfactory epithelium consisted of the three cell types, and the submucosa (lamina propria) contained blood vessels, nerves and serous Bowman's glands. The apical portions of the sustentacular cells produced both acidic and neutral mucopolysaccharides, based on their reactivity to PAS and alcian yellow (pH 2.5 of Ravetto's method) stains. The apex of the sustentacular cells stained with mercury bromo-phenol blue, thus indicating that protein is present in the apical cytoplasm, but not in the granules. The Bowman's glands produce neutral glycoproteins. Based on the intensity of the protein stain, these granules proved to be rich in protein. This was confirmed ultrastructurally, as the secretory cells of the Bowman's glands are typical serous secreting cells. Thus, the secretions of the sustentacular cells and the Bowman's gland probably form the chemically heterogeneous mucus layer.

Despite these common features, there was still some variation at both the macroscopic and microscopic levels. Variations among squamate species were observed in (a) the spread of the olfactory mucosa throughout the nasal cavity (development of conch), and (b) the distribution of the secretory granules in the sustentacular cells. Both of these variations can, to some extent, be correlated with the level of olfactory specialisation. If gekkos are olfactory specialists, the olfactory mucosa would be expected to have a different structure from those of the skinks and snakes. Thus, these variations will be discussed with reference to the level of nasal chemosensory adaptation.

a) The development of the nasal conch varied between species. Since the conch is dorsally lined by the olfactory mucosa, variations in relative size of the concha would alter the spread of the olfactory mucosa in the nasal cavity. In the case of the gekkos, the conch occupied the entire nasal cavity, whereas that of the other species was much smaller. This confirms the previous observations of the olfactory mucosal spread in squamates (Stebbins, 1948; Gabe and Saint Girons, 1976). Thus in gekkos, the large conch, and the consequently larger area available for the spread of the olfactory mucosa gives support to Schwenk's (1993a) suggestion that, relative to other squamates, gekkonoids may be olfactory specialists.

b) The reduced number of secretory granules produced by the olfactory sustentacular cells in gekkos is also unique to most gekkotans. The converse is found in *P. textilis* and the skinks. The number of secretory granules in the sustentacular cells of *H. binoei* , though abundant by gekkotan standards, were still less abundant in comparison to both the skinks and the snake. The significance of the abundant sustentacular secretory granules in *H. binoei* is unknown, and further investigations of the gekkotan lineage are therefore required.

From the above it can be seen that, the gekkos olfactory mucosa shows a combination of both a generally reduced secretory capacity of the sustentacular cells and a large conch which increases the spread of the olfactory mucosa in the nasal cavity. The

results of this study, and those of Schwenk (1993a), imply that the gekko nasal chemosensory capacity is different from that of the vomerolfactory snakes. Though further morphological, physiological and behavioural studies are required before the gekkos can be described as olfactory specialists, the evidence available from both Schwenk's work (1993a/b) and this study, point towards this conclusion.

The pygopods exhibit features of olfactory mucosa spread and morphology which are intermediate to the gekkos and the skink/snake groupings. Though the conchae are less well developed in pygopods than in the gekkos, the sustentacular cells produce only a few granules. The pygopods resemble non-gekko squamates at the macroscopic level, but are microscopically typical of the gekkos.

Ultrastructural variations in the secretory granules of the sustentacular cells and the Bowman's gland were observed both between and within species. These could not be explained in terms of nasal chemosensory adaptation. In general, the secretory granules of the sustentacular cells were less complex than those of the Bowman's glands, as there were no instances of crystalline inclusions and there were few species with bipartite granules. Apart from that, the secretory granules all showed ultrastructural variations in individual species. Thus it is difficult to define any characteristic features of any group at this stage. Some variation was also observed in the secretory granules of the Bowman's gland between individual *D. molleri*. This may reflect regional variation in morphology of the olfactory mucosa, as sections weren't always taken from the same area of the conch. This type of variation was also reported in the mud puppy (*Necturus maculosus*: Amphibia) (Farbman and Gesteland, 1974). Future studies into the ultrastructures of the squamate olfactory mucosa would thus need to take the possibility of regional variation into account.

4.4 CONCLUSION

The squamate olfactory epithelium is covered by a heterogeneous layer of mucus which is produced by two intrinsic sources: the sustentacular cells and the Bowman's glands. The unique combination of both the distribution of the olfactory mucosa in the nasal cavity and the secretory capacity of the sustentacular cells of the gekkos lend support to Schwenk's contentions that gekkonoids are olfactory specialists. The converse is the case in both *P. textilis* and the skinks. The condition in the pygopods combines the anatomical features of both snakes and skinks and the sustentacular cell morphology of the gekkos. The significance of this will be examined in the next chapter. Despite these variations, the presence of ample intrinsic sources of both the mucous and serous components on the nasal mucus layer are present in all squamates examined. In the following section, the VNO will be analysed, in a similar manner, in order to determine whether the same two intrinsic sources of secretion are responsible for that heterogeneous mucus layer.

CHAPTER 5: THE SQUAMATE VNS

5.1 INTRODUCTION

There have been few morphological analyses of the squamate VNS, especially with reference to its secretory capacity (see chapters 1 and 4). The vomeronasal and olfactory mucosae are thought to be functionally similar based on morphological and embryological observations. A common feature of both sensory epithelia is that they are covered by a heterogeneous layer of mucus (Andres, 1969; Müller et al., 1979; Takami et al., 1995). As discussed in the previous chapter, there are two histochemically distinct intrinsic sources of secretion (sustentacular cells and submucosal Bowman's glands) for the olfactory mucosa in squamates. Thus, if the VNS is morphologically similar to the MOS, then the VNS should have the same two intrinsic sources of secretion. Consequently this chapter examines the morphology of the intrinsic lubricatory structures in the squamate VNO.

5.2 RESULTS

5.2.1 ANATOMY

The squamate VNO was a dome-shaped, bone encased structure, with vomer bones below and the septomaxilla bones above. It had a dorsocaudal sensory, vomeronasal epithelium and an anteroventral, non-sensory epithelium lining the protruding mushroom body (Plate 21A). It was located in the rostral floor of the nasal cavity. The vomeronasal duct opens into the palate. The mushroom body was well developed in all species studied, thus indicating that none of the species had a reduced level of vomerolfactory capacity at this level.

5.2.2 LIGHT MICROSCOPY

Three different mucosae (epithelial and submucosal regions) were identified. These were the sensory vomeronasal mucosa, and the nonsensory mucosae lining the mushroom body and intermediate regions. In all squamates, the vomeronasal epithelium consisted of microvillous bipolar receptor neurons, sustentacular cells and basal cells, in a similar arrangement to the olfactory epithelium. The mushroom body was lined by ciliated, columnar epithelium. Areas of epithelium, displaying intermediate features, separated these two epithelia. The submucosae (lamina propria) associated with each of these areas contained a variable amount of connective tissue, blood vessels and nerves. The submucosa which was associated with the vomeronasal sensory epithelium contained comparatively more blood vessels and nerves than in either of the other regions. No submucosal glandular structures were found in any species studied.

The structure of the vomeronasal epithelium in the elapid snake, *P. textilis*, had a more complex structure than in the lizards studied. Evaginations from the basal lamina extended to just below the sustentacular nuclear layer. This separated the epithelium into columnar structures, with connective tissue and blood vessels in the intervening septa. Less pronounced evaginations were observed in the VNO of the skink, *T. rugosa*. In this case, the septa did not reach the sustentacular nuclear layer, and thus did not separate the epithelium into columns. No evaginations of the basal lamina were observed in any of the other species investigated.

There was very little histochemical variation in the VNO of the squamates surveyed as summarised in table 3. The only areas which consistently showed intense staining with both PAS and alcian yellow (pH 2.5 of Ravetto's method) was the mucus layer covering the VNO and the apical portions of the cells in the intermediate zone (Plate 21B). In all cases, the mucus layer stained positive with all three stains, indicating the presence of both mucous and serous components. The apical portion of the vomeronasal and mushroom

body epithelia in both skinks and *P. textilis* were weakly positive to all stains. Closer inspection revealed that this was due more to content of the cytoplasm, rather than any specific staining for granules.

	PAS	BPB	R
VNE	+	+ / -	Y +
MBE	+ *	+ *	Y +
IE	++	+	Y ++
Mucus layer	++	+ / ++	Y ++

Table 3. Summary of the results of histochemical analyses carried out on the vomeronasal mucosa of the squamates examined. Abbreviations: "-" = no reaction, "+" = positive reaction, "++" = very positive reaction, BPB: mercury bromo-phenol blue, IE: intermediate epithelium, MBE: mushroom body epithelium, PAS: periodic acid-Schiffs, R: Ravetto's method (Y: yellow): VNE: vomeronasal epithelium.
* = ++ in Gekkotans

Fine, intensely PAS (Plate 21B) and mercury bromo-phenol blue (Plate 21C) positive apical granules were observed in the mushroom body epithelium of the gekkotans. This was more readily observable in the gekkos than in the pygopods. A few columnar cells, with strongly PAS positive apical granules, were also observed close to the intermediate regions of the mushroom body.

5.2.3 ULTRASTRUCTURE

5.3.2.1 VOMERONASAL MUCOSA

The vomeronasal mucosa of the squamates examined consisted of a thick epithelial and a much thinner submucosal layer. In the epithelial layer, all three cell types: receptor, sustentacular and undifferentiated basal cells, were identified. Both the receptor and sustentacular cells bore apical microvilli. In all lizards investigated, there were no apical granules in the sustentacular cells (Plate 22A). However, in the snake, *P. textilis*, a few apical, bipartite granules were observed (Plate 22B).

The submucosa (lamina propria) consisted of blood vessels, nerves and connective tissue, wherein fibroblasts, collagen fibres and the occasional mast cell were observed.

5.2.3.2 MUSHROOM BODY

The mushroom body mucosa of all squamates studied consisted of two layers of cells in the epithelium, and a thick submucosal area. The combined layers of the mushroom body mucosa did not equal the thickness of the vomeronasal sensory epithelium. The upper cell layer consisted of columnar cells, with centrally located nuclei (Plate 22C). In all species, apical elongate mitochondria and basal lipid granules were observed. Lysosomes were observed throughout the cell, but most were basally located. Both RER and Golgi complexes were present throughout the cell. Apical desmosomes and tight junctions were replaced by basal interdigitations between cells and the submucosa. At the apex of the cell, both cilia and microvilli were observed (Plate 22D). The nuclei of these cells in *P. textilis* were circular whilst those of the lizards were irregular shaped structures.

The cytoplasm of the cells in the lower cell layer was darker, but still possessed mitochondria, lipid granules, lysosomes, RER and Golgi complexes. The shape of the nuclei were similar to those of the upper layer, exhibiting the same dichotomy in shape between lizards and snakes as described in the upper layer.

The submucosa (lamina propria) of the mushroom body mucosa was continuous with that of the vomeronasal mucosa, and thus contained the same elements. However, the submucosa of the mushroom body contained more fibroblasts, collagen fibres and mast cells. Thus, this layer was thicker than that of the vomeronasal sensory submucosal layer.

There was one unique feature in the gekkotan mushroom body. Small, electron-dense, apical granules were in the mushroom body epithelium of all gekkotans examined. These granules were larger in diplodactylines (Plate 21D) than those either in pygopods or in gekkonine gekkos.

5.3.2.3 INTERMEDIATE MUCOSA

The intermediate mucosa lay between the vomeronasal and mushroom body mucosae and covered a small area of the vomeronasal organ. The thickness of the epithelium and the submucosa varied with respect to their relative position in the VNO. In all species assessed, there were two types of epithelial secretory cells in the intermediate mucosae. Their distribution was correlated with the proximity of the vomeronasal sensory and mushroom body epithelia. Cuboidal secretory cells, with a thin submucosal layer, were found in the larger transition zones. The cells of this epithelium had central nuclei and apical microvilli. A few homogeneous granules were observed in the apical portion of the cell (Plate 23A). Mitochondria, Golgi complex cisternae and a few lysosomes were also observed, spread throughout the cell cytoplasm. The sides of the cells adhered to each other by desmosomes and interdigitating cell walls. Tight junctions were found in the apex of the cell. The second

type of secretory cells were columnar and were observed at the shorter transitional zones. These cells had numerous apical secretory granules, small luminal microvilli, and basal nuclei (Plate 23B).

The submucosal layer was about half the thickness of the epithelium, but increased in thickness as it approached the mucosa of the mushroom body. Though continuous with of the rest of the VNO submucosa, the fibroblasts and collagen fibres were more numerous than in the vomeronasal mucosa, especially in the regions closer to the mushroom body.

5.3 DISCUSSION

5.3.1 SQUAMATE VOMERONASAL MUCOSA

In this study, it was shown that the VNO in these squamates was well developed, and was lined by three different types of mucosae. The epithelia of these mucosae were covered by a chemically heterogeneous mucus layer. The strong reaction to both PAS and alcian yellow (pH 2.5 of Ravetto's method) indicated the presence of acidic mucopolysaccharides (Drury and Wallington, 1980), whilst that of mercury bromo-phenol blue indicated the presence of proteins (Barka and Anderson, 1965). As with the MOS and the mammalian VNO (Takami et al., 1995), the mucus layer in the squamate VNO also consists of two distinct layers, mucous and serous.

At the microscopic level, there was interspecific variation in the thickness of the sensory epithelium and in the distribution of secretory structures in the VNO. The vomeronasal sensory epithelium of the snake was much thicker than that of the lizards. This epithelium was also organised into columns by evaginations from the basal lamina (see

diagram 7, chapter 1). This is one of the morphological features which was used to support the idea of the vomerolfactory specialisation of the snakes (see Halpern, 1992 for review).

The paucity of intrinsic secretory structures is the one overriding feature of the squamate VNO. There are a few intrinsic secretory structures present in the squamate VNO, but they are mainly mucous, and produce few secretory granules. In all species analysed, a few mucous secretory granules were observed in the cuboidal and columnar cells lining the intermediate regions. The results of this study, and the observations of Kratzing (1975) showed that this is the only intrinsic source of secretion in the skink VNO. In all species, there seems to be inadequate sources in intrinsic secretion for the VNO. Though there is a small amount of mucous being produced intrinsically in all species, there is virtually no intrinsic source of serous secretions.

Secretory granules were observed in both the sustentacular cells of *P. textilis*, and the mushroom body epithelium of Gekkotans. Though they differed histochemically, the former being mucous and the latter serous, both secretory granules were only being produced in small quantities. A few apical mucous secretory granules were also observed in the sustentacular cells of other snakes (Bannister, 1968; Altner et al., 1970; Wang and Halpern, 1980a; Takami and Hirosawa, 1990), Anguidae and Anniellidae, and several other disparate species (Altner et al., 1970; Gabe and Saint Girons, 1976). The presence of mucous granules in the sustentacular cells are not unique to the snakes. It is thus unlikely to be a defining morphological feature of snake vomerolfactory specialisation.

The present study confirmed the findings of Gabe and Saint Girons (1976), showing the presence of serous granules in the mushroom body epithelium in gekkotans. Such granules have also been described in some iguanians (Gabe and Saint Girons, 1976). The presence of these granules in the mushroom body implies that their secretion could contribute to the mucus layer of the VNO. These could then be the source of serous secretion in the gekkotan and iguanian VNO. Though Gabe and Saint Girons (1976) proposed that the amount of secretion produced by the mushroom body was sufficient to line

the VNO, there is no supporting physiological or microchemical evidence. Thus, the squamate VNO may not be capable of intrinsically producing large quantities of secretory granules, and is instead restricted to producing a few mucous and possibly serous granules. This is in contrast to the heterogeneous mucus layer of both the VNO and MOS, which contain both mucous and serous portions. Thus, the heterogeneity of the mucus layer and the reduced secretory capacity of the squamate VNO implies that there must be other extrinsic sources of secretion for the mucus lining.

There is some comparative evidence which suggests that two chemically different (mucous and serous) secretory structures are required for the efficient function of the VNS. There are two intrinsic sources of secretion for the VNO in vertebrates: the sustentacular cells and the submucosal glands. Both structurally (see chapter 1) and functionally (Clancy et al., 1984; Halpern, 1987; Wang et al., 1993; Lou et al., 1994; Takami et al., 1995), the VNO of the squamates most closely resembles that of the mammals. The VNO of both squamates and mammals may possess secretory granules in the sustentacular, intermediate and nonsensory cells of the epithelia. However, submucosal glandular structures (akin to the submucosal Bowman's glands of the MOS) have only been found in the mammalian VNO. Thus, the squamate VNO does not possess any vomeronasal/submucosal glands. If the squamate VNO functions in the same manner as that of the mammalian VNO, then the absence of the intrinsic glandular structures, which are thought to be essential in mammalian vomerolfaction (Cooper and Bhatnager, 1976; Takami et al., 1995), implies that there has to be another source for the mucus layer in the squamate VNO (Kratzing, 1975).

5.3.3 CONCLUSIONS

These results show that the squamate VNO possesses a much reduced, and mainly mucous, intrinsic lubricatory capacity. The mammalian VNO, with which the squamate VNO shares many morphological and functional features, possesses ample intrinsic

lubricatory structures, and its relative development was even determined by these structures. Thus, if the squamate VNO is similar to mammals, and since it is covered by a chemically heterogeneous mucus layer, there must be an extrinsic source of secretion for the VNO. In the final chapter, the potential sources of this secretion will be explored.

CHAPTER 6: THE HARDERIAN GLAND AS PART OF THE VNS

6.1. INTRODUCTION

The previous chapters provided a detailed comparative morphological account of the squamate Harderian gland and the secretory structures of both the olfactory and vomeronasal mucosae. The aim of this chapter is to synthesise all of the relevant details, with respect to the potential function of the squamate Harderian gland as a source of lubricant for the VNO. Three separate questions were addressed. 1) How do the two chemosensory mucosae differ in secretory capacity? 2) How important are secretory structures to squamate vomerolfaction? 3) If not intrinsically, from whence do these secretions for the VNO come?

6.2 COMPARISON OF THE MUCOSAE

There were some morphological features of the mucosae which were consistent throughout the clades of squamates surveyed. The olfactory mucosa possessed both secretory sustentacular cells and submucosal Bowman's glands, whilst the VNO consistently had mucous cells in the intermediate regions. However, some level of variation was observed in the abundance and ultrastructure of secretory granules in the olfactory mucosae, and the presence of secretory structures in the VNO. In most cases, these variations were specific to the clades, and thus each clade will be discussed in turn.

In skinks, the olfactory mucosae (MOS) produced adequate amounts of intrinsic secretion from both the sustentacular and Bowman's gland cells. Conversely, only a few

mucous cells were found in the intermediate regions of the VNO. Thus, the combination of the well developed intrinsic secretory system of the olfactory mucosa, and its absence in the VNO is unique to these skinks.

In gekkotans, there are two variations in lubricatory capacity of the nasal chemosensory systems from the skink pattern. The reduced secretory capacity of the sustentacular cells in the MOS is unique to the gekkotans. The significance of this is unknown. The gekkotan VNO also houses unique (compared to the skinks and the snake) secretory feature, in the form of a few apical serous secretory granules in the mushroom body epithelium. The presence of these granules does suggest that the gekkos may be more vomerolfactive than previously thought. This is supported by behavioural studies on eublepharine gekkos (Schwenk, 1993b; Cooper, 1995). Nevertheless, it is still unlikely that there is sufficient mucous (from the intermediate regions) and intrinsically generated serous secretions for the gekkotan VNO to form the chemically heterogeneous mucus layer.

Finally, the secretory structures in the nasal chemosensory structures in the snake differed from those of the skinks and the gekkotans. Though the secretory capacity of the snake olfactory mucosa only differs histochemically from that of the skink, the same cannot be said of the VNO. Like the MOS, the sustentacular cells of the VNO in snakes, and other disparate squamate species (Altner et al., 1970; Gabe and Saint Girons, 1976) contains apical mucous granules. However, the VNO sustentacular cells differ from those of the MOS, in that far fewer mucous granules are produced. Thus, the mucous granules from the intermediate cells and the sustentacular cells are the only intrinsically produced secretions in the VNO of *P. textilis*. There was no evidence for any intrinsic source of protein secretion.

In none of these species was there any evidence of submucosal glands in the VNO. This is in contrast to both the mammalian VNO and the squamate MOS. Thus the squamate VNO is a poorly lubricated structure when compared to the olfactory mucosa.

6.3 IMPORTANCE OF SECRETORY STRUCTURES

As discussed previously, the mucus lining of the olfactory epithelium in vertebrates is heterogeneous, and consists of mucous and serous compartments (Andres, 1969; Müller et al., 1979; Getchell and Getchell, 1992). These are produced intrinsically by the sustentacular and Bowman's gland secretory cells (Andres, 1969; Müller et all, 1979). The results of the present study confirmed the potential for the squamate olfactory mucosae to intrinsically produce both mucous (sustentacular cells) and serous (Bowman's glands) secretions for the mucus lining.

Less is known about the composition and sources of the chemically heterogeneous mucus layer in the VNO. The results of both this study and that of Takami et al (1995) showed that the mucus lining of the VNO is also a chemically heterogeneous structure, with mucous and serous portions. As discussed in chapter 1, the presence of submucosal vomeronasal glands in the bat VNO was positively correlated with the development of the VNO (Cooper and Bhatnager, 1976). However, the source of these secretions for the squamate VNO mucus layer is less well understood. With the exception of the gekkotans, the only intrinsic secretory structures observed in the VNO were mucous. Even in the case of the gekkotans, there is no physiological evidence to suggest that sufficient amounts of protein are intrinsically secreted to form the serous component of the mucus layer. Thus the absence of an adequate, or any source of protein in the VNO indicates that there must be an extrinsic source of protein.

6.4 EXTRINSIC SOURCES OF SECRETION FOR THE SQUAMATE VNO

Two other extrinsic sources for the squamate VNO have been suggested by Kratzing (1975): the salivary glands and the secretions of the lacrimal apparatus. A third source, that of the Harderian gland, is suggested by this study. In this section, I will examine each of these, to see whether they fit the criteria as potential sources of lubricant for the VNO. The ideal candidate would be a serous secretory structure which has access to the VNO.

6.4.1 SALIVARY GLANDS

Though saliva is readily produced by the salivary glands, and secreted into the buccal cavity of the squamates, there has been little evidence linking the salivary fluid in the mouth to the mucus layer of the VNO. Furthermore, the salivary glands of squamates are generally mucous secreting (Saint Girons, 1988). Thus, saliva is an unlikely source of secretion for the mucus layer in the squamate VNO.

6.4.2 LACRIMAL APPARATUS.

The lacrimal apparatus consists of the lacrimal duct and the lacrimal canaliculi. The lacrimal duct opens directly into the duct of the VNO, or in the vicinity thereof, in all squamates with VNOs (Bellairs and Boyd, 1950). Even when the VNO is absent, the lacrimal duct still opens in the same relative region (Bellairs and Boyd, 1950, Slaby, 1984). Thus, Kratzing (1975) proposed that the lacrimal apparatus may be a source of lubricant for the squamate VNO. However, the lacrimal apparatus appears to possess few secretory

granules. Thus, it is unlikely that the lacrimal apparatus itself is a source of secretion for the VNO. However, its ideal location suggests that it may instead be a conduit for Harderian gland secretions to the squamate VNO.

6.4.3 THE HARDERIAN GLAND

The squamate Harderian gland is closely associated with the lacrimal apparatus and produces protein. The association with the lacrimal apparatus as well as the histological structure of the Harderian gland varies among the clades examined. Therefore, the morphological basis for the potential role of the Harderian gland in squamate vomerolfaction will be discussed separately for each clade.

6.4.3.1 SCINCOMORPHA

The ducts of the skink Harderian gland are not continuous with the lacrimal canaliculi. When present, the nictitating membrane acts like a barrier between the two structures. Even in the absence of a nictitating membrane, the two structures retain the same relative position (Rehorek, 1992). The lacrimal canaliculi open into the anterior portion of the conjunctival sac, while the ducts of the Harderian gland open onto the anterior region of the corneal surface. Though this is not a direct connection, and presumably not as efficient as one where there would be a direct connection, it is still possible for the secretory substances from the Harderian gland to reach the lacrimal duct.

A copious amount of protein is produced by the skink Harderian gland. In addition to this, the mucous cells in the apical portion of the Harderian gland, the anterior lacrimal gland of *T. rugosa*, and the anterior compartment of the Harderian gland of the lacertid, *Podarcis s sicula* (Chieffi-Baccari et al., 1990) could also pass into the lacrimal duct. Thus,

the scincomorph Harderian gland, and associated anterior orbital features, are a potential source of both protein and mucous for the VNO mucus layer.

There is variation among the scincomorphs with regard to Harderian gland structure, as suggested by the morphological differences observed between the lygosomine skinks and the lacertid, *Podarcis s sicula*. It is not known whether the condition in *Podarcis* is representative of the lacertids. Thus further examination of other lacertids, as well and other non-lygosomine scincomorphs are required before any generalisations can be made about the scincomorph Harderian gland, and its role in vomerolfaction, can be determined.

6.4.3.2 GEKKOTA

In Gekkotans, the gekkos (gekkonine and diplodactyline) and the pygopods exhibit different orbital anatomy with respect to the Harderian gland and associated features. The Harderian glands of the gekkos are smaller than those of the pygopods. It is therefore possible that the pygopod Harderian gland produces more protein than that of the gekkos. Additionally, the gekko Harderian gland is separated from the lacrimal canaliculi. This is similar to the condition observed in skinks without nictitating membranes. Thus, like the skinks, it is still possible for the secretions from the gekko Harderian gland to reach the lacrimal canaliculi. In pygopods, however, the Harderian gland opens directly into the lacrimal canaliculi, and has restricted access to the orbit via a duct connecting the orbit to the lacrimal canaliculi. Consequently, it is inevitable that the secretions from the pygopod Harderian gland travel down the lacrimal duct and to the VNO.

The functional significance of the lipid produced by the gekkotan Harderian gland is unknown. Though it is a characteristic feature of the Australian gekkotan Harderian gland, an appraisal of gekkos from the other subfamilies, especially those of the behaviourally

more vomerolfactive eublepharine gekkos (Cooper, 1995), are required in order to determine whether this is a feature common to all gekkotans.

6.4.3.3 SERPENTES

The snake Harderian gland, like that of the pygopod, is directly connected to the lacrimal canaliculi. Embryologically, the presence of the lacrimal canaliculi in the orbit precedes the development of the Harderian gland. This suggest a strong link between the Harderian gland and the lacrimal apparatus in snakes. In the case of the colubroids, the connection between the Harderian gland and the orbit is both indirect and minimal, and thus it is expected that the secretions from the gland would flow to the VNO. Furthermore, the Harderian gland produces both serous and mucous secretions, which have the potential of being the sole source for both components in the mucus layer in the VNO.

Morphological investigations of the snake Harderian gland and nasal chemosensory structures have so far been mainly restricted to the Colubroids, with scattered references to the other snake families. Analysis of snake lineages whose vomerolfactory capacity remains largely unstudied (Boiids) and which possess larger Harderian glands (Blind snakes: Typhlopidae) (Saint Girons, 1982), would indicate whether the condition observed in Colubroids is indicative of all snakes. Studies of the closely related Anguimorphs, especially varanids would also prove instructive, because varanids share more vomerolfactive morphological and behavioural features with snakes (Schwenk, 1993b), than the pygopods. Such an analysis would show whether the morphological features of the snake Harderian gland are unique, or whether there are potentially precursors of these structures in Anguimorphs.

6.4.5 CONCLUDING REMARKS

Neither the salivary glands nor the lacrimal apparatus are likely sources of protein for the mucus layer of the VNO in squamates. The Harderian gland, whose secretions pass into the lacrimal apparatus, is the most likely source of extrinsic serous secretion for the mucus layer of the squamate VNO. It is also apparent that there is some variation in both the structure of the squamate Harderian gland and its relationship to the lacrimal apparatus. This level of morphological variation may be correlated with vomerolfactory specialisation, as both the snakes and the pygopods possessed the most efficient connection to the VNO and the largest Harderian glands. Additional behavioural observations support the potentially high vomerolfactory capacity of both pygopods (Schwenk, 1993b). In skinks and gekkos, the structure of Harderian gland, and its less well defined connection to the lacrimal apparatus, implies that the Harderian gland may not be as important in vomerolfaction as it is in the snakes and pygopods. In any case, the Harderian gland is ideally suited to be the sources of protein secretion for the chemically heterogeneous mucus layer of the squamate VNO.

This study also revealed an unexpected degree of variation in the morphology of the intrinsic lubricatory structures of the squamate olfactory and vomerolfactory systems. A few species from three major squamate clades have thus far been examined, each of which had a set of unique intrinsic lubricatory features for their nasal chemosensory systems. It is unknown whether these observations typify the intrinsic secretory capacity of the nasal chemosensory structures in these clades, as only a few members of each clade have been sampled and the morphological comparison is incomplete. Thus, a broader phylogenetic morphological analysis of the chemosensory systems of these three clades is required to confirm the observation of this study.

The morphology of the chemosensory systems in the fourth major squamate lineage, the iguanians, has received little attention. Scattered structural analyses (Haas, 1947; Gabe

and Saint Girons, 1976) and behavioural studies (Duvall, 1982; Cooper, 1989; Cooper and Alberts, 1991) have shown that there is much variation in chemosensory capacity. It is in this clade that there are vomerolfactive (Cooper and Alberts, 1991) and non-vomerolfactive (Haas, 1947) species, as well as the only group of squamates (Chameleons) which lack a VNO (Gabe and Saint Girons, 1976; Slaby, 1984). Thus, a comparative analysis of the chemosensory systems, especially the structure of the Harderian gland in chameleons, would prove instructive in assessing the level of variation thus far observed.

Morphological analyses can only suggest possible functions for the squamate Harderian gland. Its presumptive role in vomerolfaction is based on a series of morphological observations discussed in this thesis. Microchemical studies have revealed the structure of the amphibian and mammalian olfactory (Andres, 1969; Müller et al., 1979; Getchell and Getchell, 1992) and mammalian vomerolfactory (Takami et al., 1995) mucus layers. Such microchemical investigations of the secretory granules in the squamate Harderian gland, and their comparison with the mucus layer of the VNO would determine whether the Harderian gland secretion form part of the mucus layer in the VNO. In conclusion, this study provided evidence for both structural variation in the VNO and the importance of the mucus layer in the squamate VNO. Since the squamate VNO is apparently deficient of adequate intrinsic secretory structures, the Harderian gland is ideally suited to be an extrinsic source of secretion for its mucus layer.

APPENDIX 1

List of species and numbers of specimens examined in morphological and autoradiographic aspects of this thesis.

species	*Tiliqua rugosa*	*Morethia adelaidensis*	*Morethia boulengeri*	*Hemiergis decresiensis*
no. collected	16	9	4	20
additional specimens	a	b	b	c

species	*Christinus marmoratus*	*Heteronotia binoei*	*Strophurus intermedius*	*Nephrurus milii*
no. collected	47	2	5	3
additional specimens	d	d	d	d

species	*Delma molleri*	*Aprasia pseudopulchella*	*Pseudonaja textilis*
no. collected	24	6	18
additional specimens	d		e

TABLE 1:
Additional specimens were obtained from other researchers, under the specifications of the National Parks and Wildlife Act of South Australia. The codes and approrpiate researchers and permit numbers are detailed below.

a) Additional specimens obtained from researcher BT Firth, collected under permit numbers C21465-03 and 4.

b) Additional specimens obtained from researcher MN Hutchinson, collected under permit numbers Q01000-03,4, and 5.

c) Additional specimens obtained from researcher C Leigh, collected under permit number K23749-02.

d) Additional specimens obtained from researcher RW Moyer, collected under permit nos. Y12016-01, 2 and 3.

e) - Courtesy of Venom Supplies, Adelaide and Snake Catchers, South Australia

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PLATE 1: COLOUR PHOTGRAPHS OF SQUAMATES I

Tiliqua rugosa **(A)** is a large skink, at least four times larger than either *Morethia adelaidensis* **(B)** or *Morethia boulengeri* **(C)**.

Scale bar (A) = 4 cm; others = 1 cm.


A


B


C

PLATE 2: COLOUR PHOTOGRAPHS OF SQUAMATES II

Hemiergis decresiensis **(A)** is a slightly smaller skink than either *Morethia* spp. *Christinus marmoratus* **(B)**,and *Heteronotia binoei* **(C)** are distantly related gekkonine gekkos.

Scale bar (C) = 2 cm; others = 1 cm.


A


B


C

PLATE 3: COLOUR PHOTOGRAPHS OF SQUAMATES III

Strophurus intermedius (**A**) and *Nephrurus milii* (**B**) are distantly related diplodactyline geckos. *Delma molleri* (**C**) is a common pygopod, and bears little resemblance to its closest relatives, the diplodactyline geckos.

Scale bars = 2 cm.


A


B


C

PLATE 4: COLOUR PHOTOGRAPHS OF SQUAMATES IV

Aprasia pseudopulchella **(A)** was the other pygopod species examined. This species is distanlly related to *D. molleri,* and is a specalised burrower. The adult of *Pseudonaja textilis* (Elapidae) **(B)** is a large, common snake in the Adelaide region. In its hatchling form **(C)** it superficially resembles and co-habits with *D. molleri.*

Scale bar (A) = 1 cm; (B) = 10 cm; (C) = 2 cm.


A

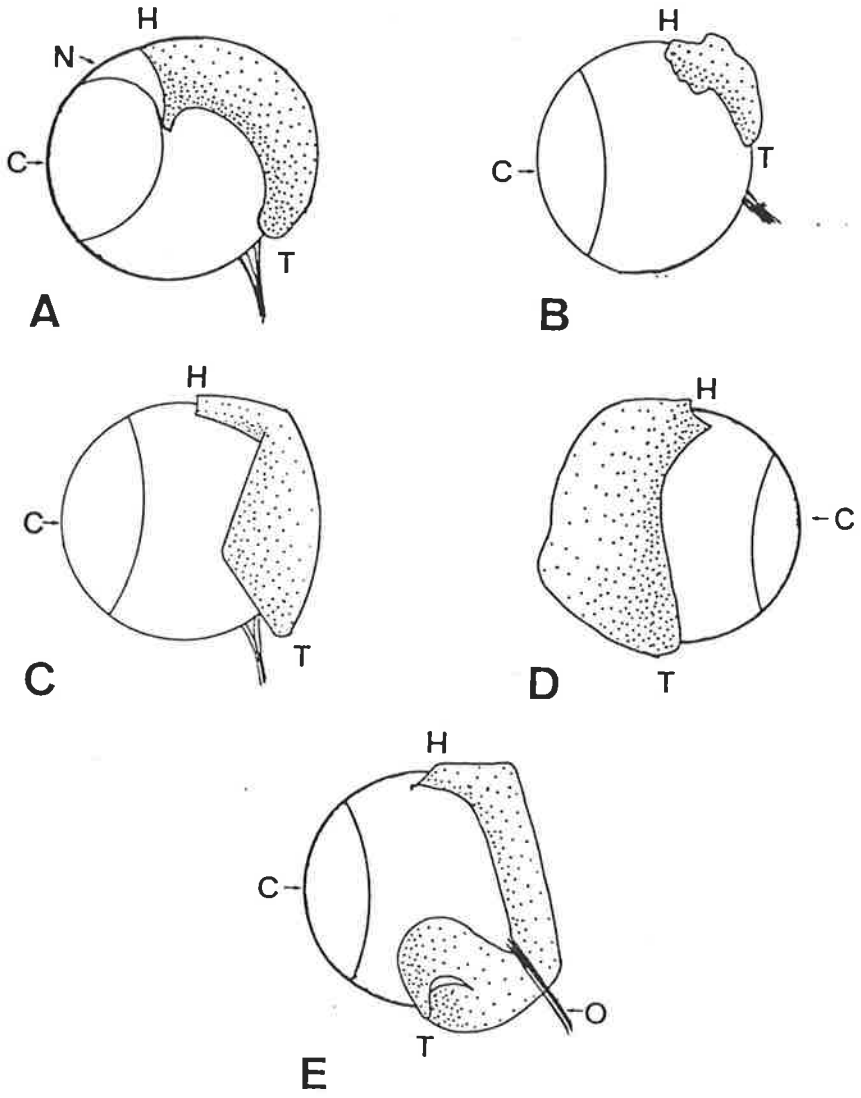

B


C

PLATE 5: SIZE AND SHAPE OF THE SQUAMATE HARDERIAN GLAND

Line diagrams (below) and scanning electron micrographs (opposite) of the Harderian glands, and their position on the eyeball of *M. adelaidensis* **(A)**, *C. marmoratus* **(B)**, dorsal aspects of *D. molleri.* **(C)**, and *P. textilis* : ventropalatal **(D)** and dorsal **(E)** aspects. The nictitating membrane (NM) is present only in the skink, into which the head (H) is embedded. Note the relationships of the head, tail (T) and lobes (L) of the Harderian gland to both the optic nerve (O) and the corneal (C) surface.

Scale bar (B) = 200 µm, others = 500 µm.



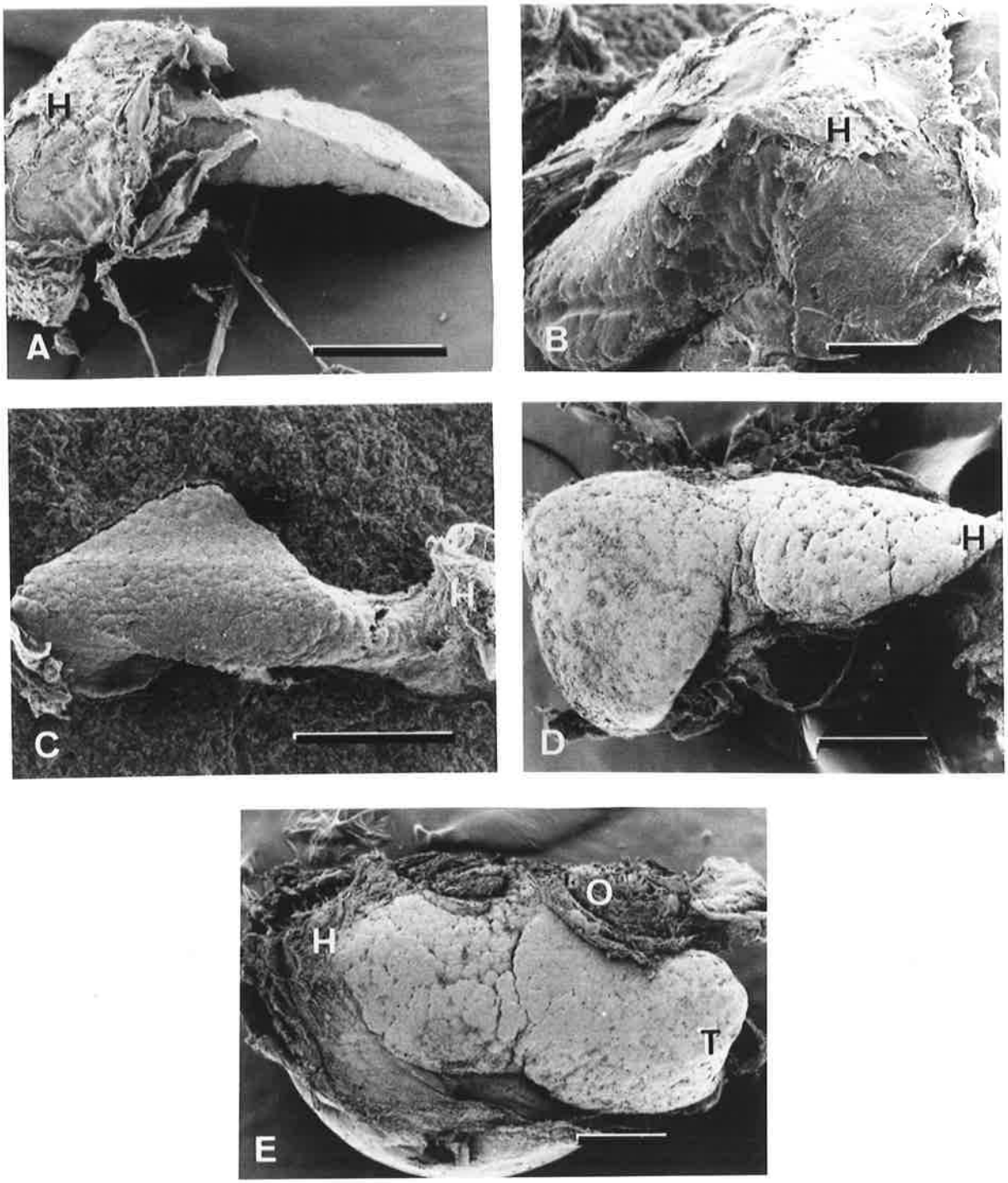
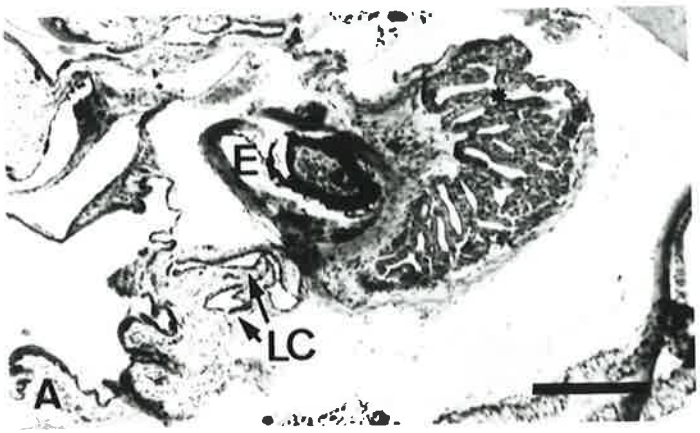
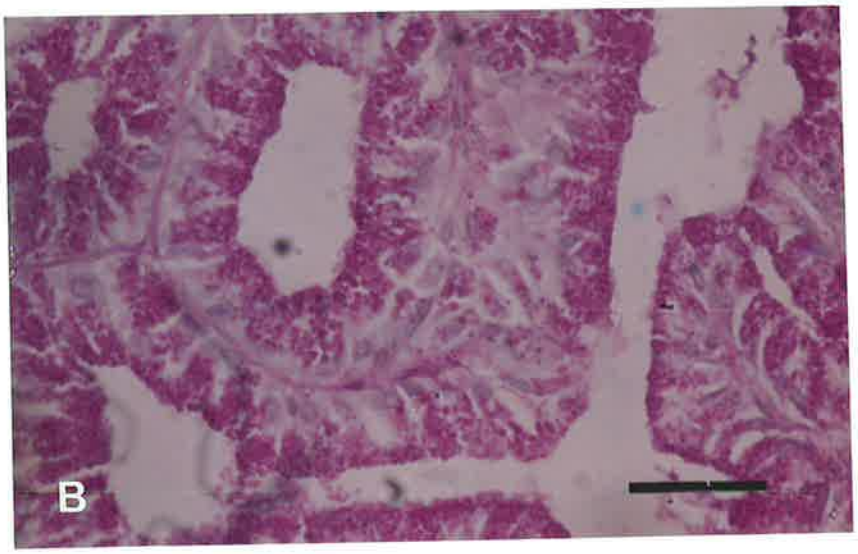

H
A
H
B
H
C
H
D
O
H
T
E

PLATE 6: LIGHT MIOCROGRAPHS OF THE GEKKOTAN HARDERIAN GLAND

Light micrographs of the Harderian gland of *C. marmoratus*, stained with H&E **(A)**, PAS **(B)**, and the supersaturated isopropanol method **(C)**. Note the large lumina (*) and the position of the lacrimal canalciuli (LC) and the eyeball (E).

Scale bar (A) = 200 μm; (B) and (C) = 20 μm.


E
LC
A


B

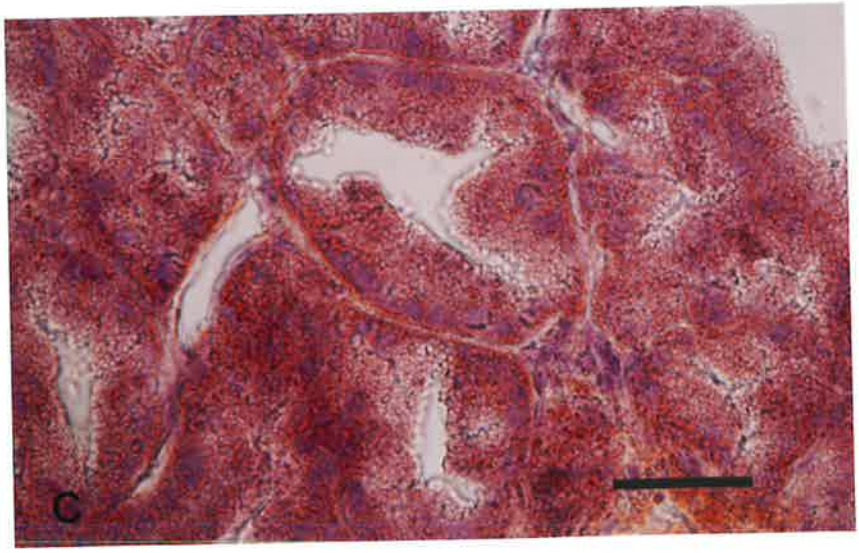

C

PLATE 7: LIGHT MICROGRAPHS OF THE SNAKE HARDERIAN GLAND

The Harderian gland of *P. textilis*, when stained with PAS **(A)** and mercury bromophenol blue **(B)** revealed the presence of mucous ducts (*) in the mainly serous gland.

Scale bar (A) = 100 μm; (B) = 200 μm.

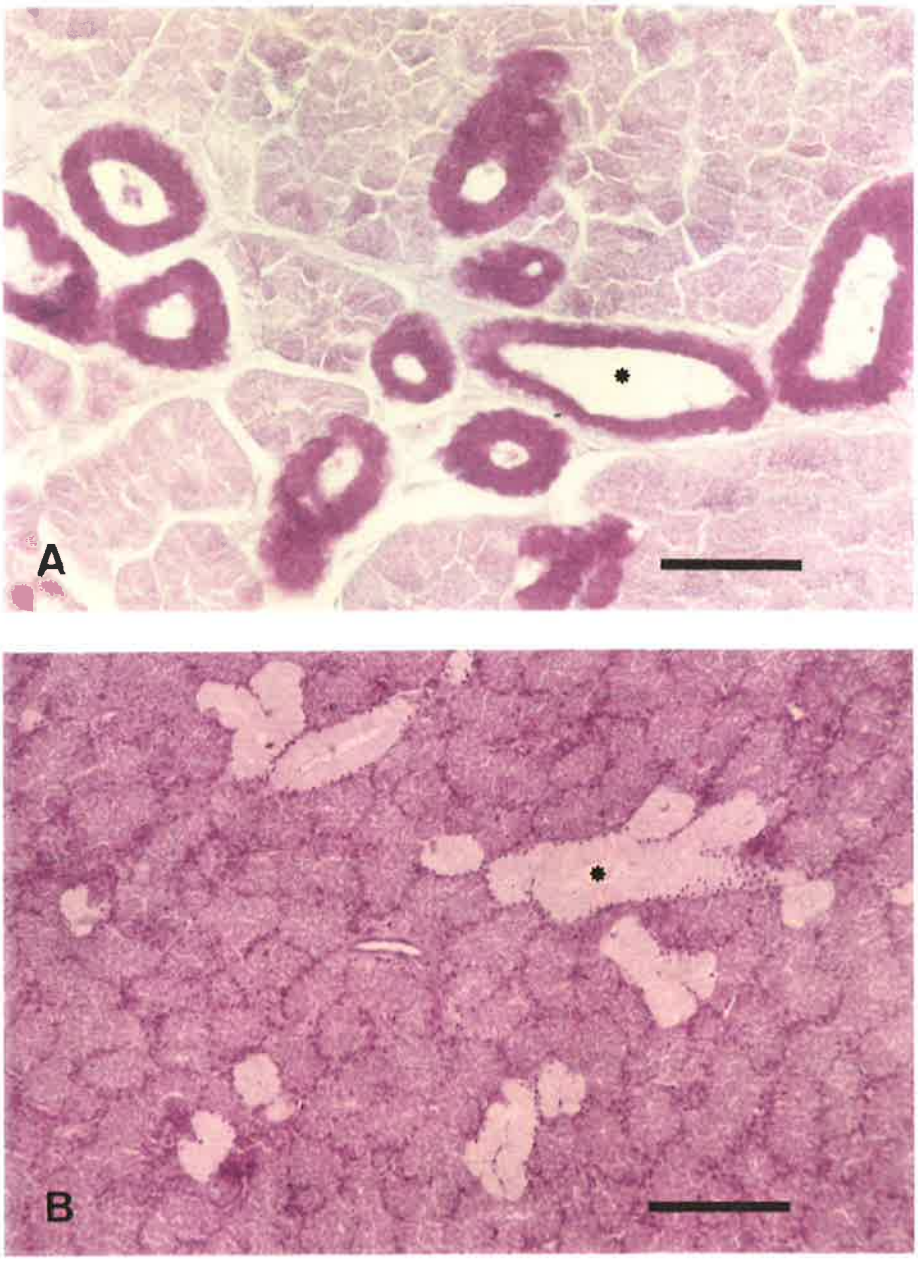

A
B

PLATE 8: MICROGRAPHS OF THE SKINK HARDERIAN GLAND

PAS stained Paraffin section of Harderian gland of *Tiliqua rugosa* (A). Note the association between the head of this gland (H), the Anterior Lacrimal gland (AL), the mucous epithelium of the inner aspect of the nictitating membrane (N) and the conjunctival sac (CS). The limbal and corneal epithelia (CE) are on the inner aspect of the nictitating membrane. Electron micrograph of the serous cells of Harderian gland of *M. boulengeri* (B), reveals the presence of many granules which, upon higher magnification (C), possess a bi- tripartite structure as the compartments are distinctly delineated.

Scale bar (A) = 200 μm; (B) = 2 μm: (C) = 1 μm.

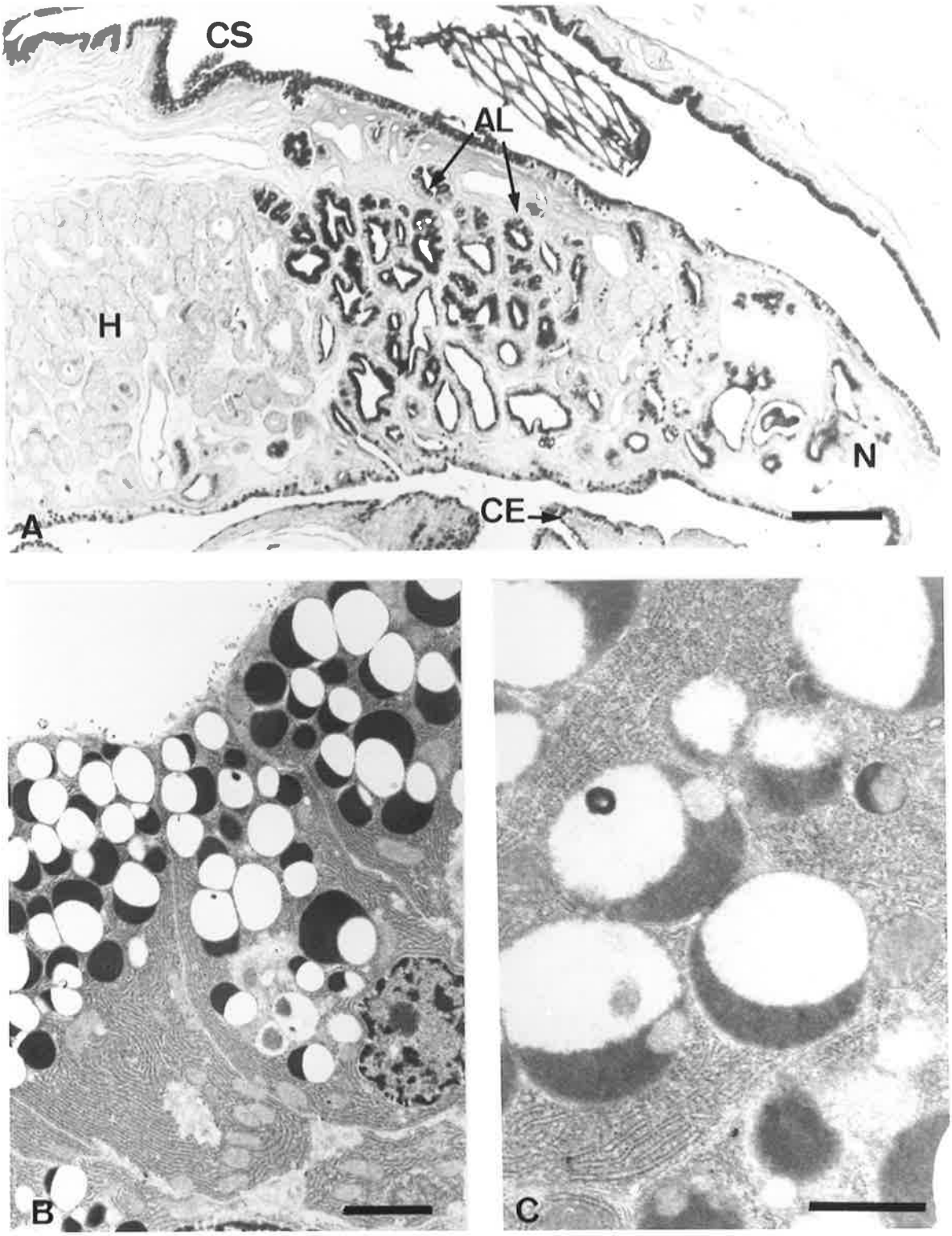

CS
AL
H
N
CE
A
B
C

PLATE 9: ULTRASTRUCTURAL MICROGRAPHS OF THE GEKKOTAN HARDERIAN GLAND I

Electron micrographs of the Harderian gland of *C. marmoratus* **(A)** and *S. intermedius* **(B)**. Note the presence of both compound protein and electron-lucent lipid granules, and the differences in distribution of these granules between the two species.

Scale bars = 5 μm.

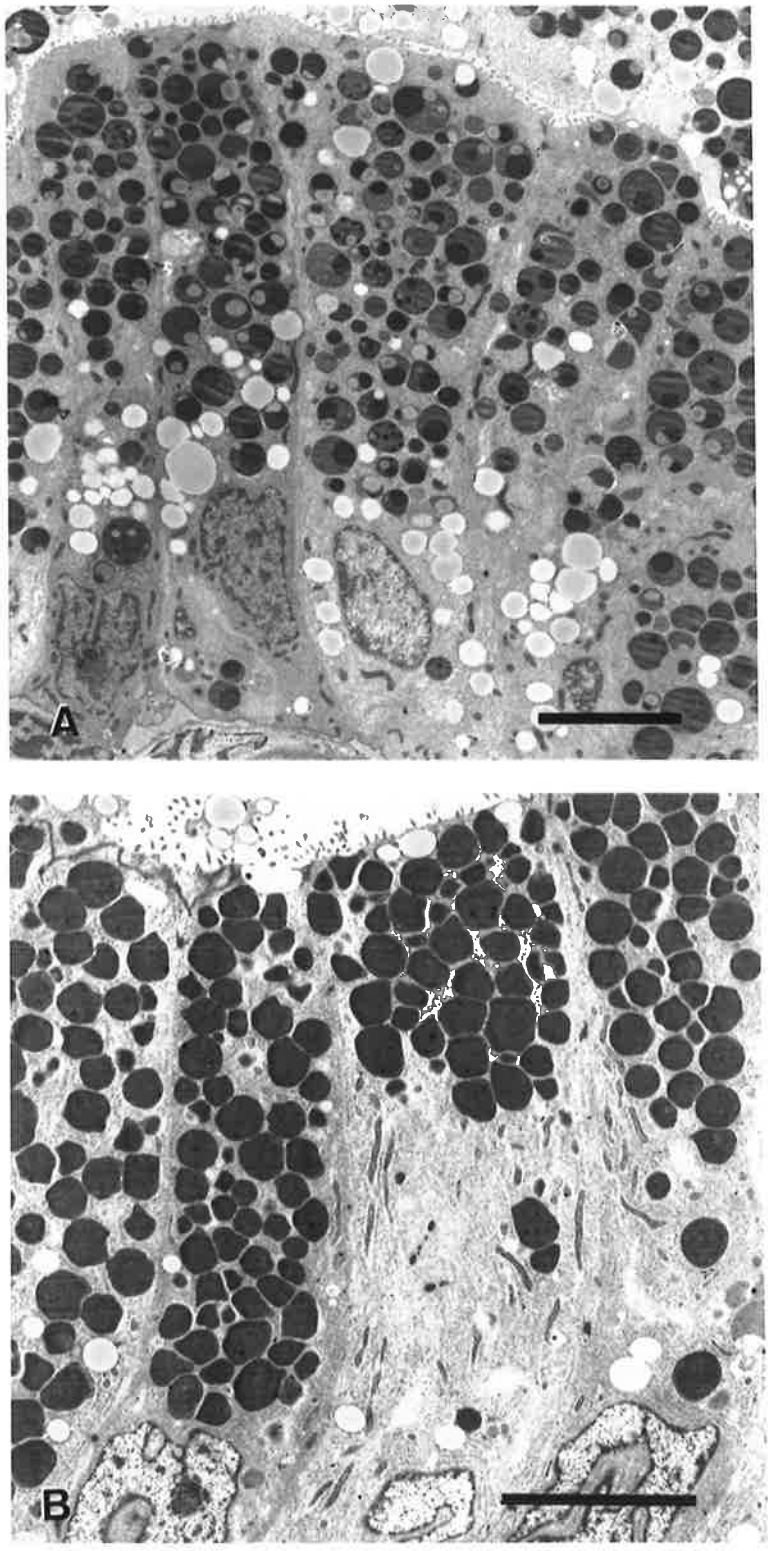

A
B

PLATE 10: ULTRASTRUCTURAL MICROGRAPHS OF THE GEKKOTAN HARDERIAN GLAND II

Electron micrographs of the secretory granules in the Harderian gland of geckos. Note the differences between the electron-lucent lipid granule of *S. intermedius* **(A)**, and the compound protein granules of *C. marmoratus* **(B)**, *H. binoei* **(C)**, and *S. intermedius* **(D)**. Variation in the ultrastructure of these protein granules exists between species at the level of compartmentalisation and the presence of lamellar crystalline structures.

Scale bars = 500 nm.

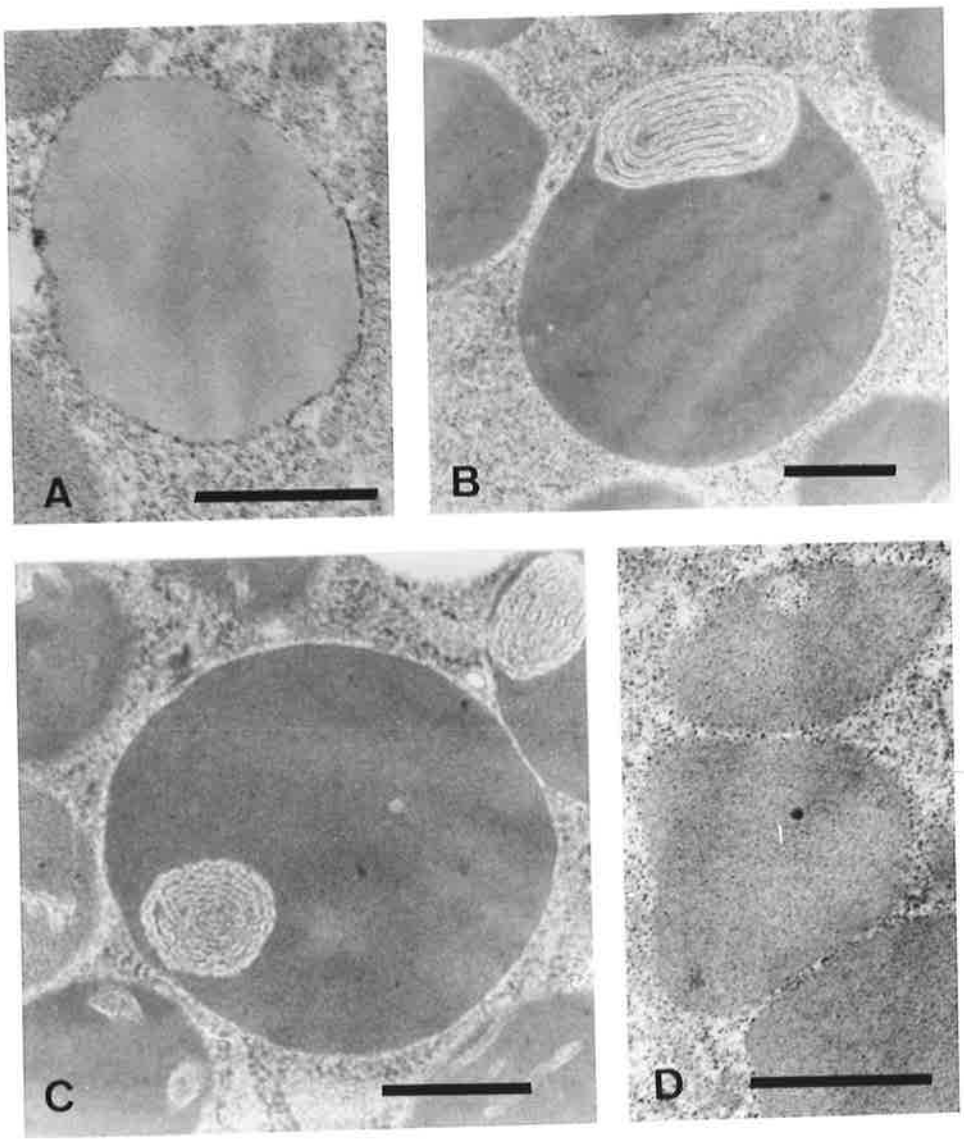

A
B
C
D

PLATE 11: ULTRASTRUCTURAL MICROGRAPHS OF THE GEKKOTAN HARDERIAN GLAND III

48 hours after pronase digestion, the serous granules of *H. binoei* remain largely intact, as the crystalline lamellae are undigested (**A**). Both granule types were also found in the Harderian gland of *D. molleri* (**B**), though the lamellar crystalline structures in the protein granules are not as well developed (**C**), but still withstood pronase digestion (**D**).

Scale bar (B) = 2 μm, others = 500 nm.

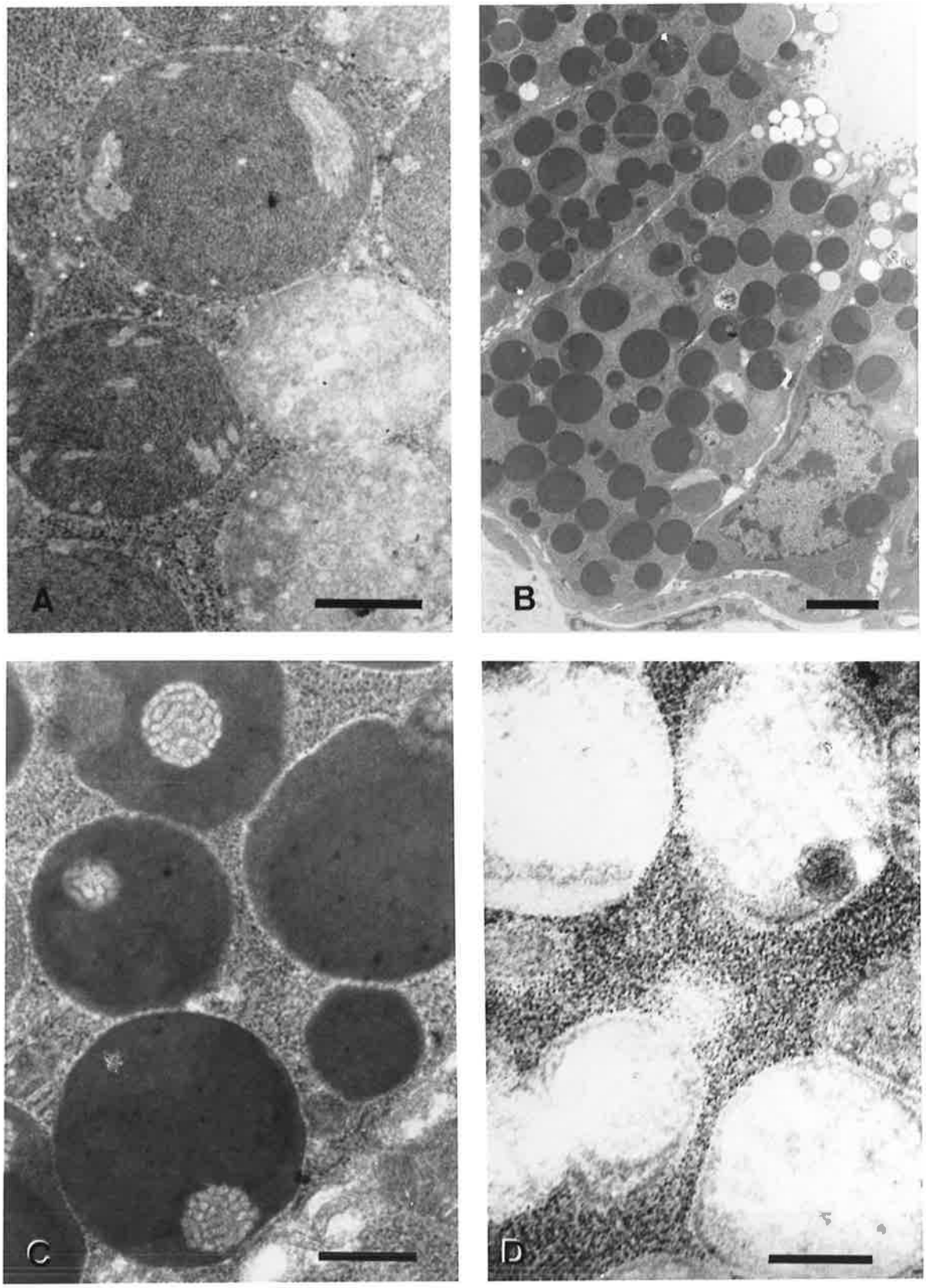

A
B
C
D

PLATE 12: MICROGRAPHS OF THE SNAKE HARDERIAN GLAND

At the light microscopic level (**A**), both mucous (M) ducts and the serous (S) body of the Harderian gland are seen in *P. textilis* . At the electron microscopic level, structural differences of the cells and granules can be seen between the mucous (**B**) and serous (**C**) cells. The secretory granules of the serous cells are multipartite (**D**).

Scale bars (A) = 20 μm; (B) = 5 μm; (C) = 2 μm; (D) = 500 nm.

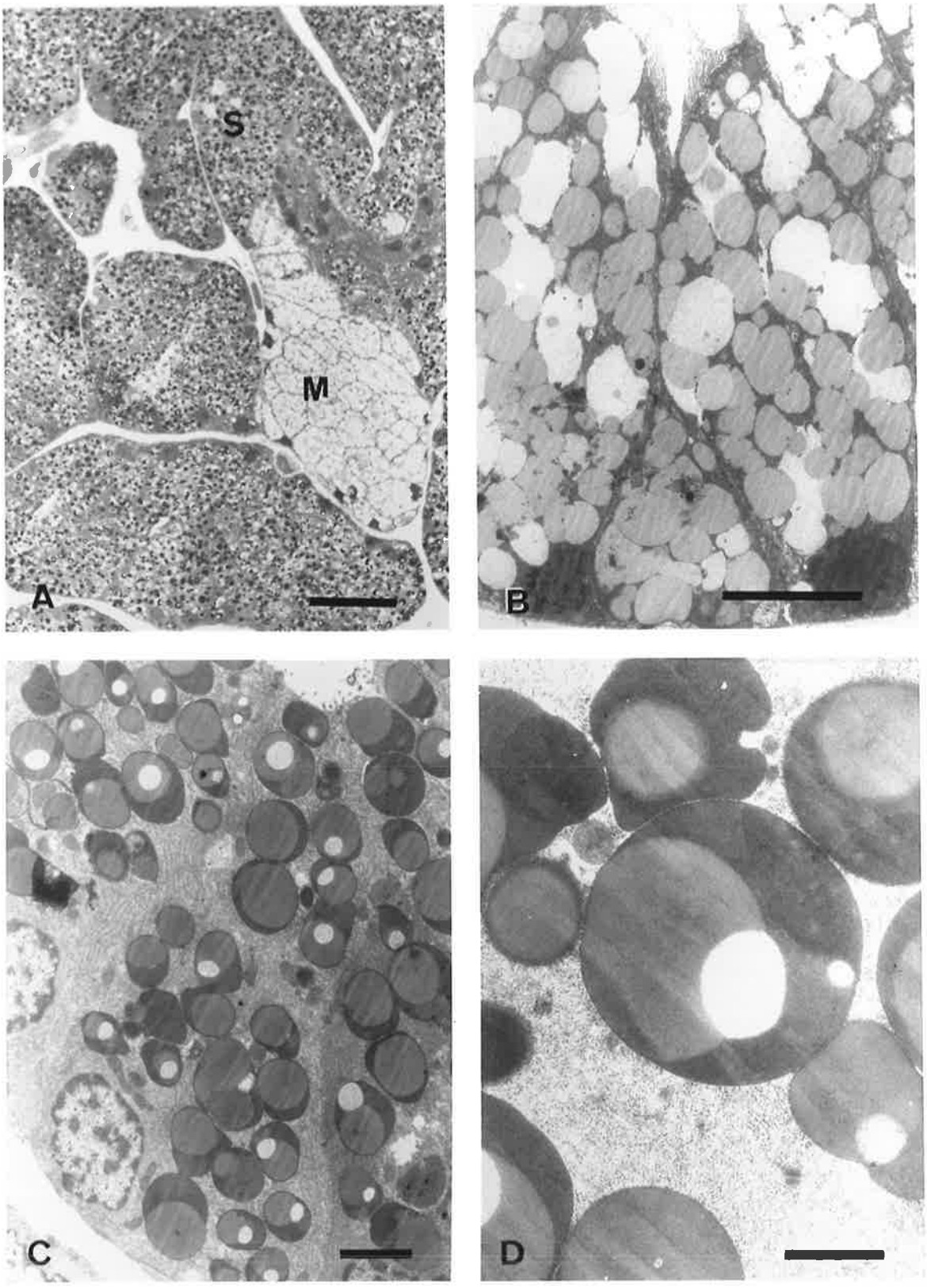

S
M
A
B
C
D

PLATE 13: AUTORADIOGRAPHIC LIGHT MICROGRAPHS

Autoradiographic light micrographs of the skinks, *H. decresiensis* at 2 **(A)**, 24 **(B)**, and 69 **(C)** hours after intial injection with H^3-methionine, and the uninjected control specmen **(D)**. Note the increasing spread of the silver granules, as protein is being produced.

Scale bar (D) = 500 μm; others = 250 μm.

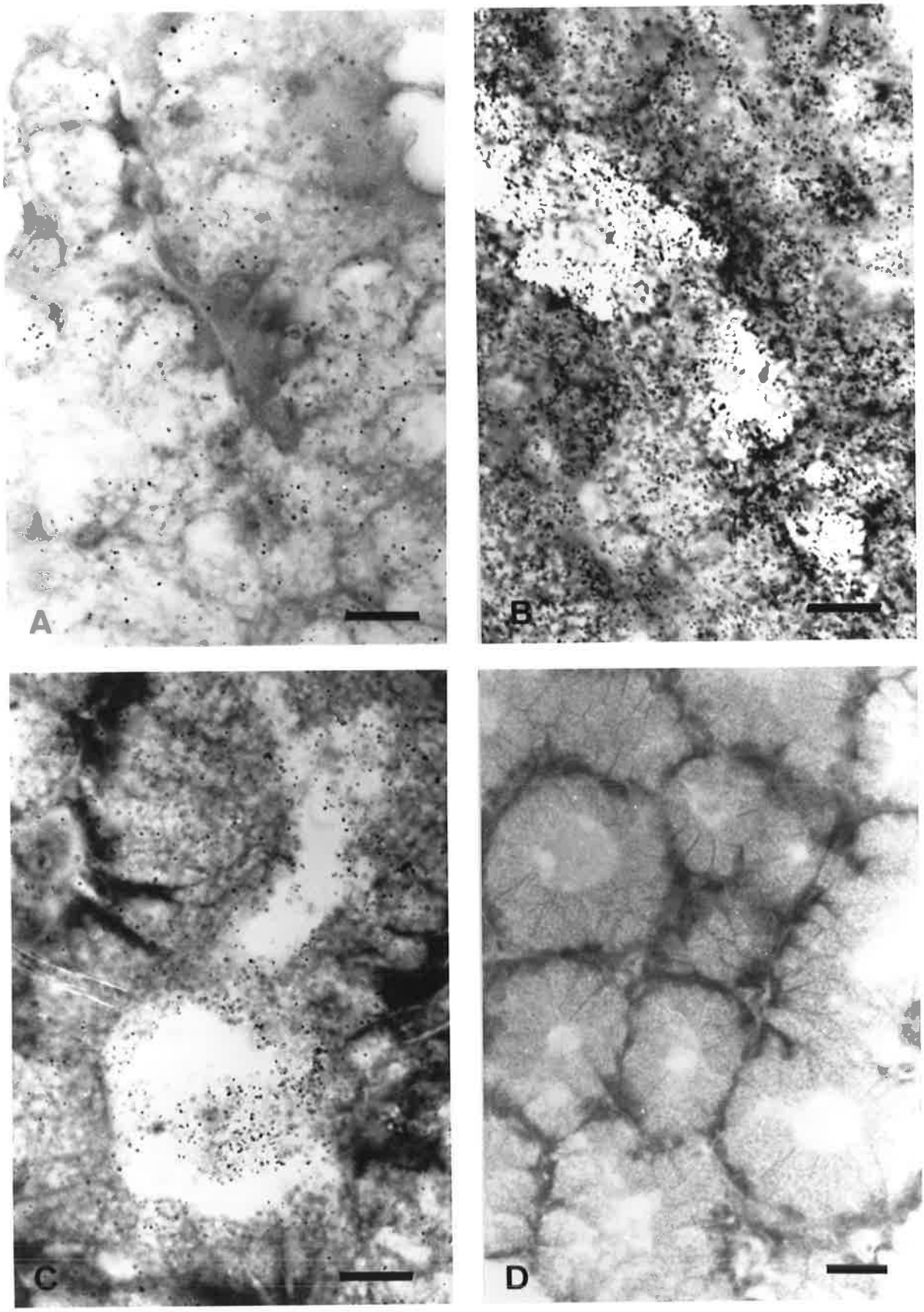

A
B
C
D

PLATE 14: EMBRYOLOGICAL LIGHT MICROGRAPHS OF THE SQUAMATE HARDERIAN GLAND I

Light micrographs of the embryology of the Harderian gland in the snakes *Agkistrodon piscivorus* (Viperidae): stage 29 (**A**), *Diadophis punctatus* (Colubridae): stage 32 (**B**), *Agkistrodon piscivorus* (Viperidae): stage 34 (**C**), and *Agkistrodon piscivorus* (Viperidae): stage 35 (**D**). Note the develpment of the primordia of the gland (P) in the mesenchymal stroma (M) into the maturing Harderian gland (H), and its relationship to the lacrimal duct (LD).

Scale bar (A) = 200 μm; others = 500 μm.

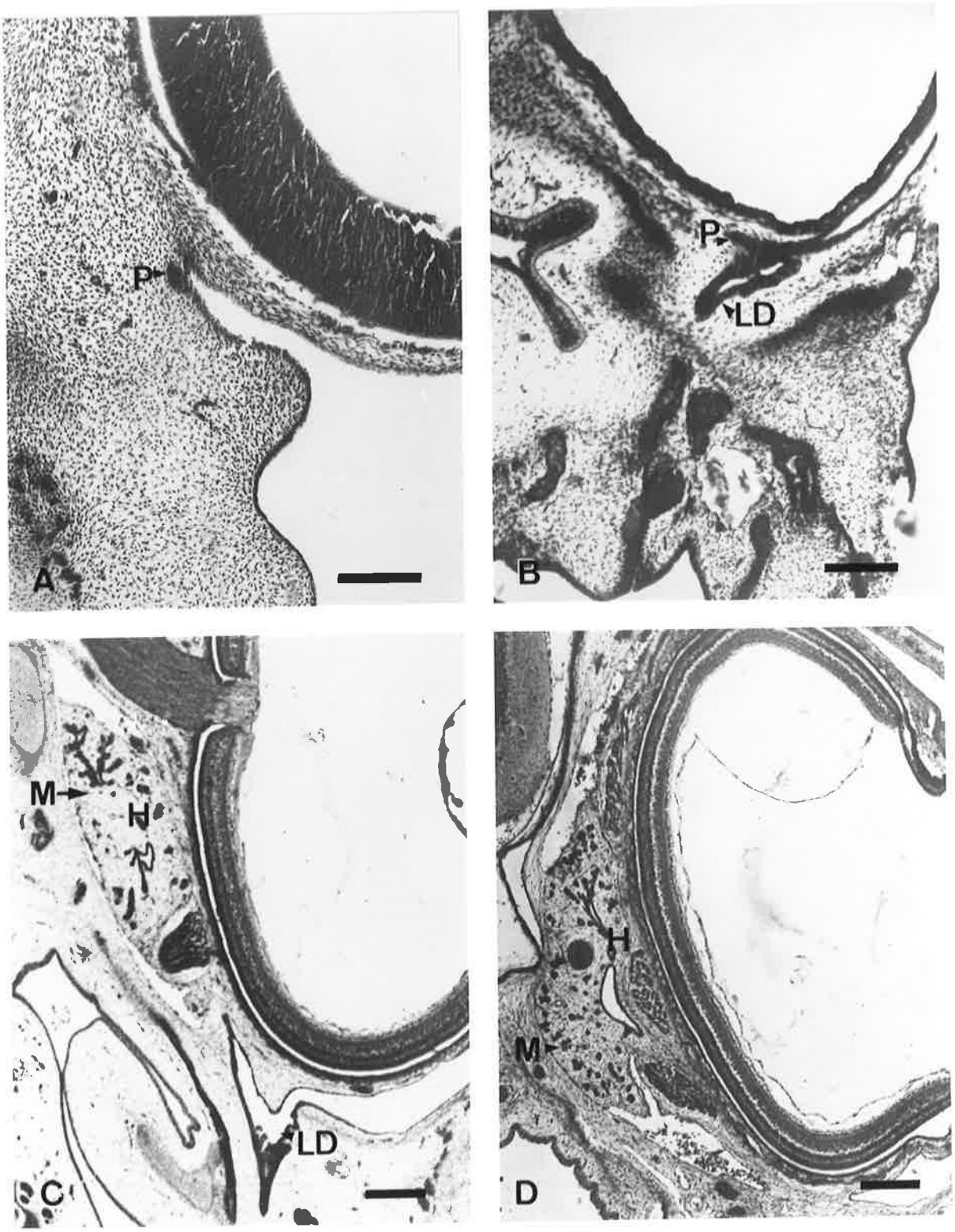
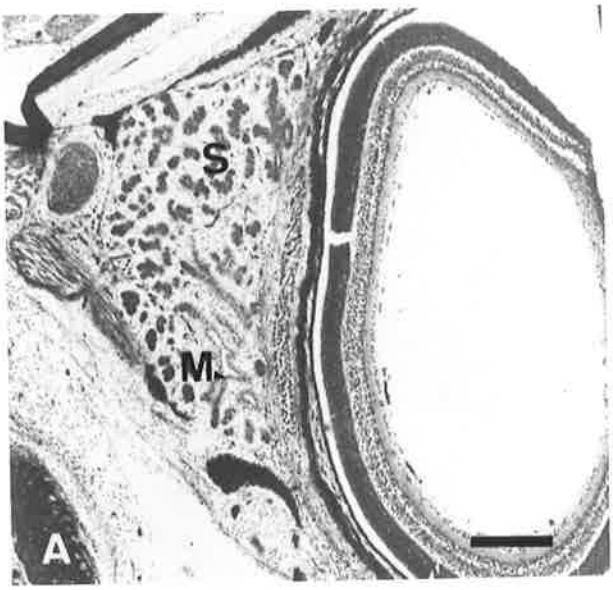
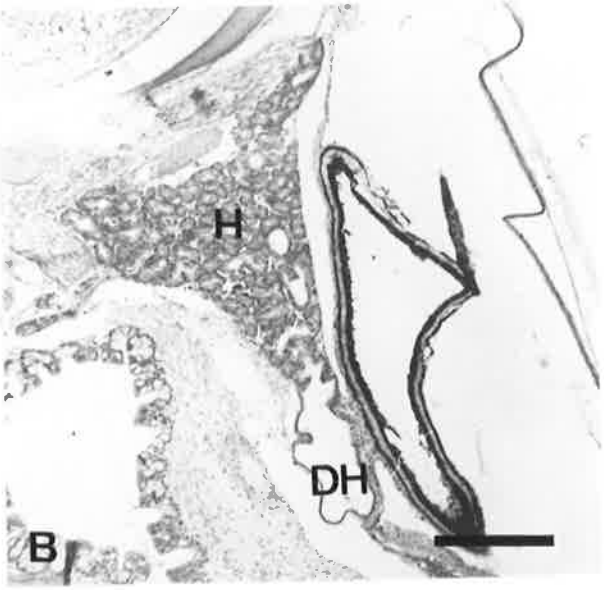

P
A
P
LD
B
M
H
LD
C
H
M
D

PLATE 15: EMBRYOLOGICAL LIGHT MICROGRAPHS OF THE SQUAMATE HARDERIAN GLAND II

At the later stages of development, the snake Harderian glands of *Python regius* (Boidae): stage 37 **(A)** and *P. textilis* (Elapidae): hatchling **(B)** begin to show all the features of the adult gland (H). Identifiable mucous (M) and serous (S) cells, in addition to the confluency of the lacrimal canaliculi with the Harderian hland duct (DH) are observed in the late embryonic stages. This implies that teh snake Harderian gland can potentially be functional at birth. The presumably later inception of the gland in *H. decresiensis* (Scincidae): stage 36 **(C)** the developmental connection of the primordia of the gland (P), the nictitating membrane (N), and the lacrimal duct (LD) distinguishes the development of the Harderian gland in skinks to that of the snakes examined.

Scale bars = 500 μm.


S
M
A


H
DH
B

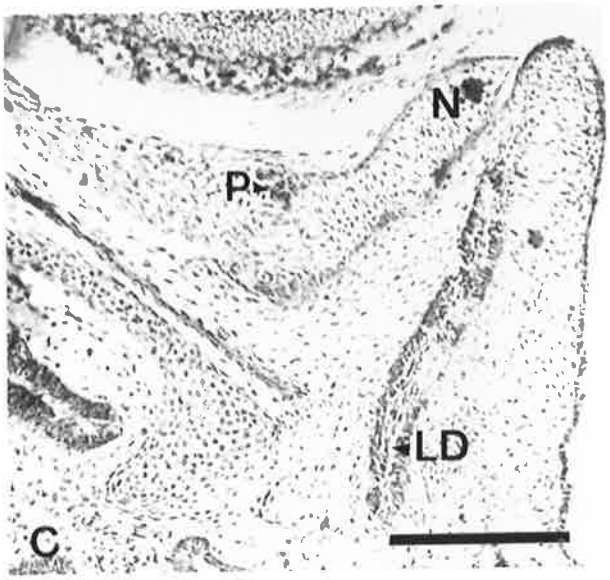

N
P
LD
C

PLATE 16: ULTRASTRUCTURAL MICROGRAPHS OF THE GEKKOTAN OLFACTORY EPITHELIUM I

Both receptor (R) and sustentacular (S) cells are ultrastructurally identifiable in the gecko olfactory mucosa. A few secretory granules were observed in the sustentacular cells of *C. marmoratus* **(A)**. These were all bipartite and elongate structures **(B).** This is in contrast to the numerous granules in the sustentacular cells of *H. binoei* **(C)**, which were homologous and electron-dense **(D).**

Scale bar (A) = 1 μm; (C) = 5 μm; (B) and (D) = 500 nm.

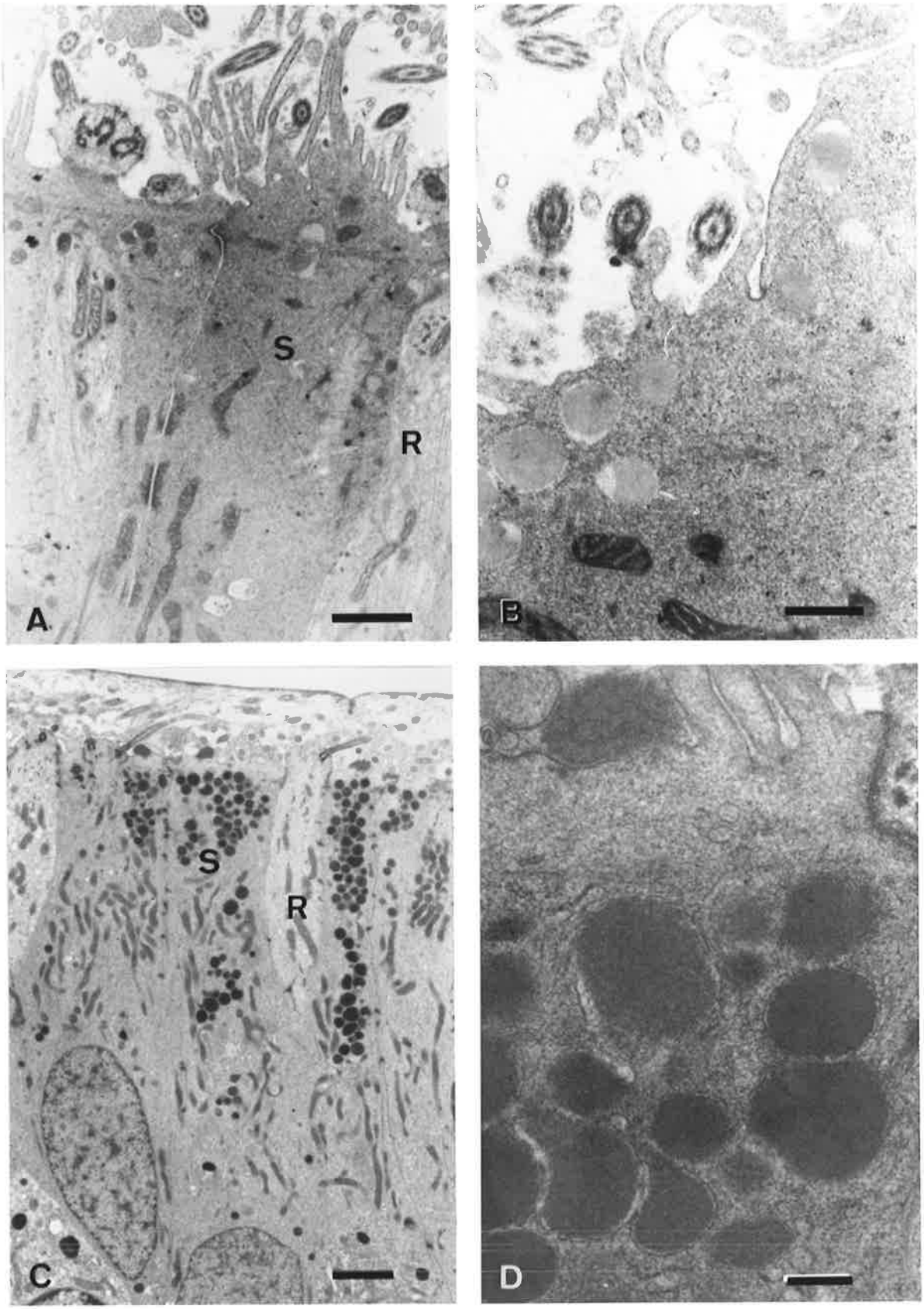
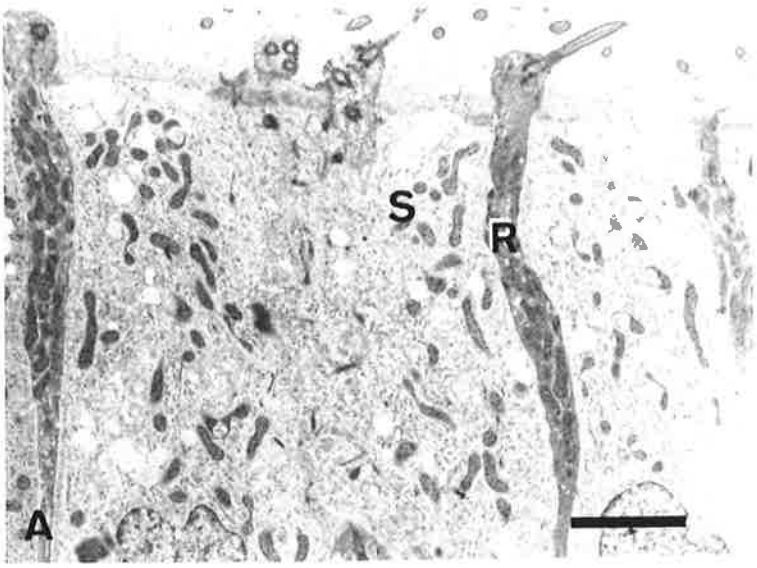
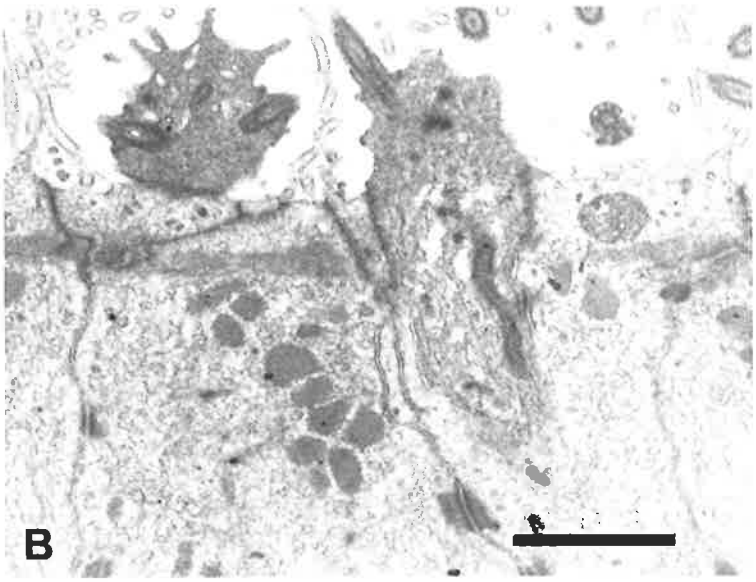

S
R
A
B
S
R
C
D

PLATE 17: ULTRASTRUCTURAL MICROGRAPHS OF THE GEKKOTAN OLFACTORY EPITHELIUM II

Few secretory granules were observed in the sustentacular cells (S) of *D. molleri* (A) and the diplodactylines. Variations were observed in the structure of these granules between *S. intermedius* (B) and *D. molleri* (C). Receptor cells (R) had no such granules.

Scale bar (A) = 2 μm; (B) = 1 μm; (C) = 500 nm.


S
R
A


B

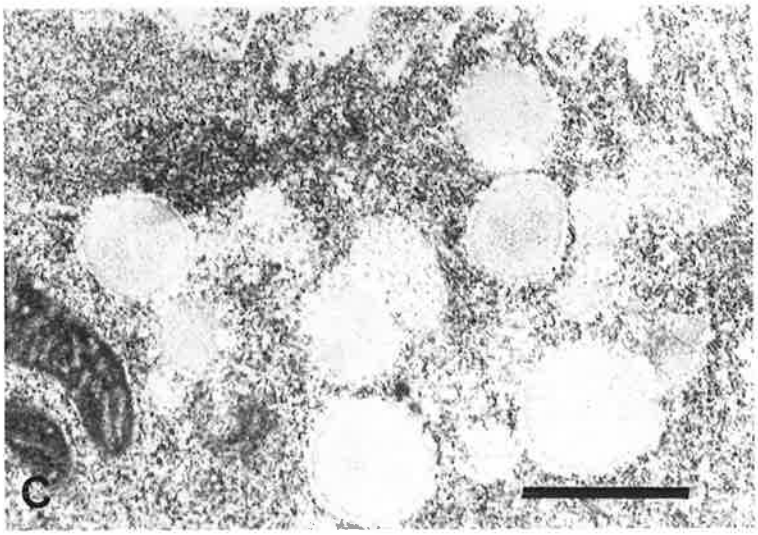

C

PLATE 18: ULTRASTRUCTURAL MICROGRAPHS OF THE SNAKE OLFACTORY MUCOSA

Abundant secretory granules were present in the sustentacular cells (S) of the snake, *P. textilis* **(A)**, which are all bipartite **(B)**. Compartmentalised granules were also observed in the serous cells of the Bowman's glands (C). Receptor cells (R) were agranular.

Scale bar (A) = 2 μm; (B) = 1 μm; (C) = 5 μm.

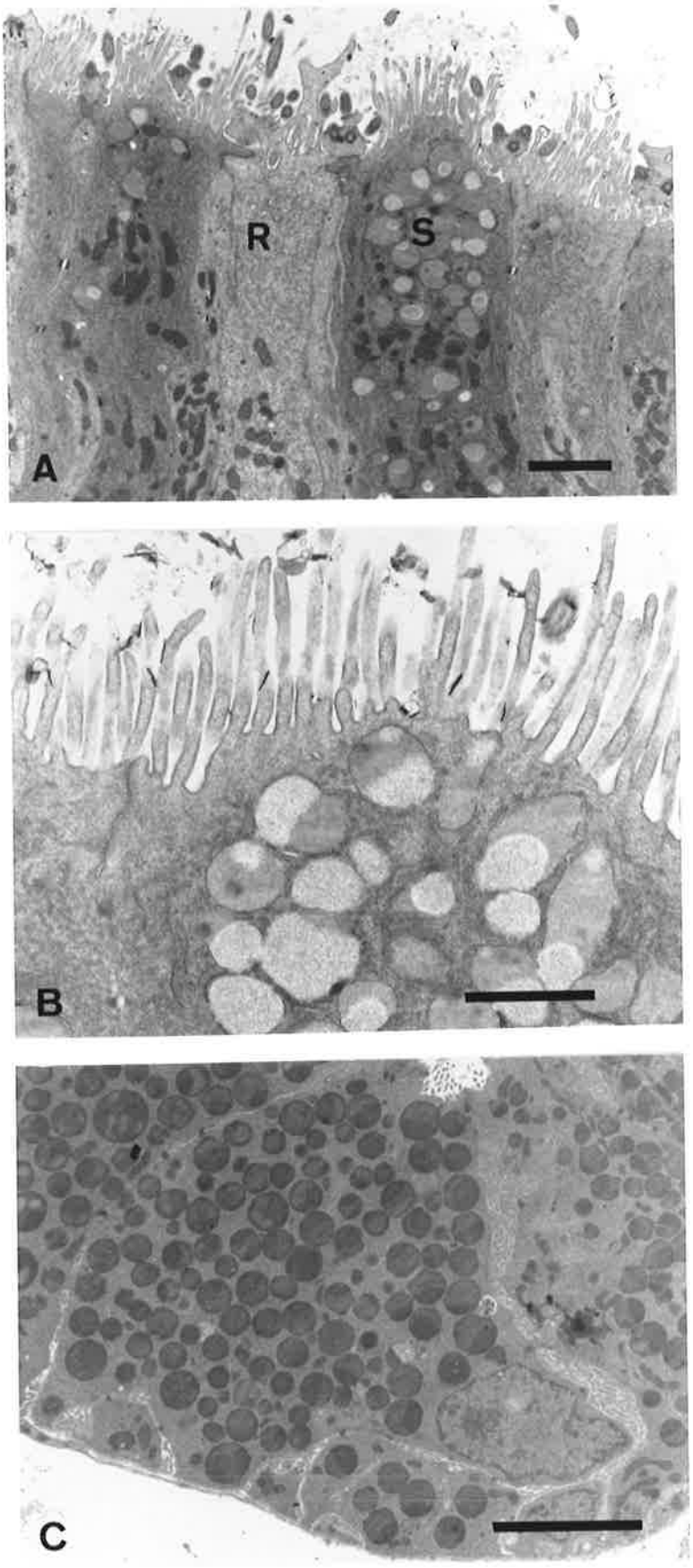

R
S
A
B
C

PLATE 19: ULTRASTRUCTURAL MICROGRAPHS OF THE SKINK OLFACTORY MUCOSA

Electron micrographs of the skink olfactory mucosa, showing the structure of secretory granules in both the sustentacular cells of *M. boulengeri.* **(A)**, and the Bowman's glands of *T. rugosa.* **(B)**, *M.adelaidensis* **(C)**, and *M.boulengeri* **(D)**. Abundant secretory granules in the olfactory epithelium are confined to the sustentacular (S) and not the receptor (R) cells. Some ultrastructural variation in these granules, especially between those of the Bowmans glands, are apparent.

Scale bar (D) = 500 nm; others = 1 μm.

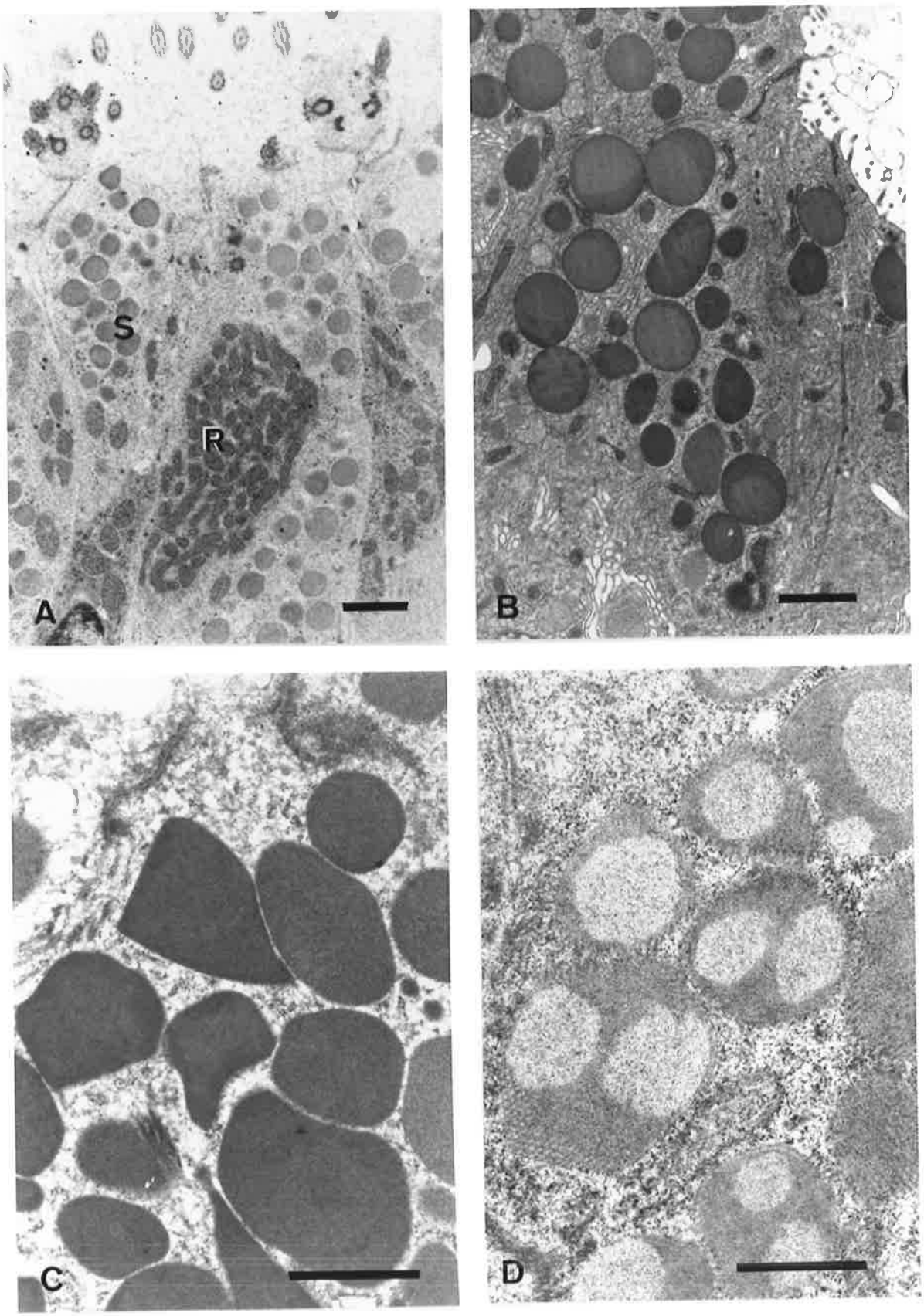

S
R
A
B
C
D

PLATE 20: ULTRASTRUCTURAL MICROGRAPHS OF THE GEKKOTAN BOWMAN'S GLANDS

Much variation was seen in the ultrstructure of the secretory granules of the Bowmans gland in gekkotans. The compartmentalised granules in *C. marmoratus* **(A)** and homogeneous ones of *S. intermedius* **(B)** are typical of the structural variation. Ultrastructural variations were also observed within the same species, as seen in *D. molleri* **(C)** and **(D)**.

Scale bars (A) = 1 μm; (B) = 2 μm; (C) and (D) = 500 nm.

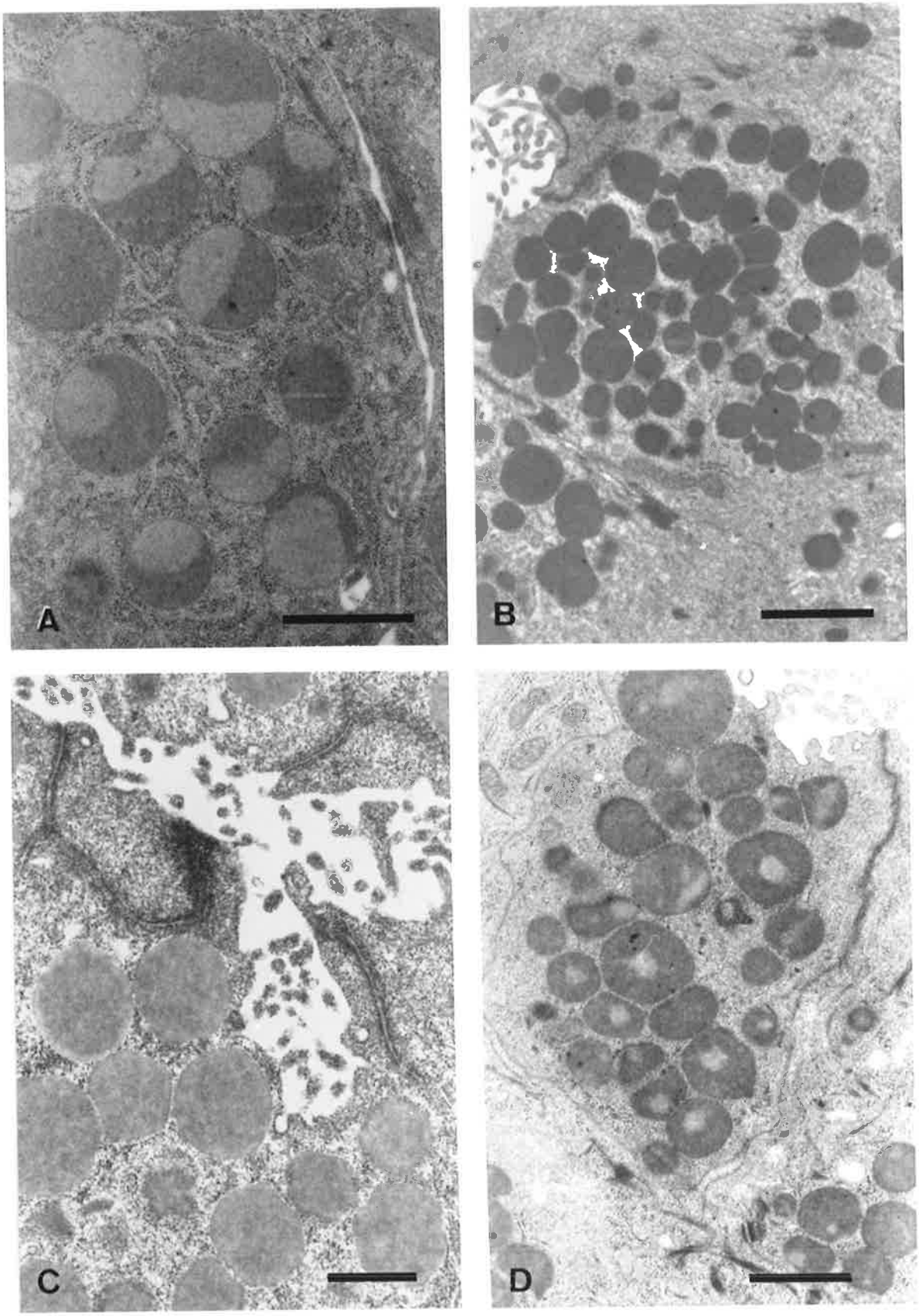
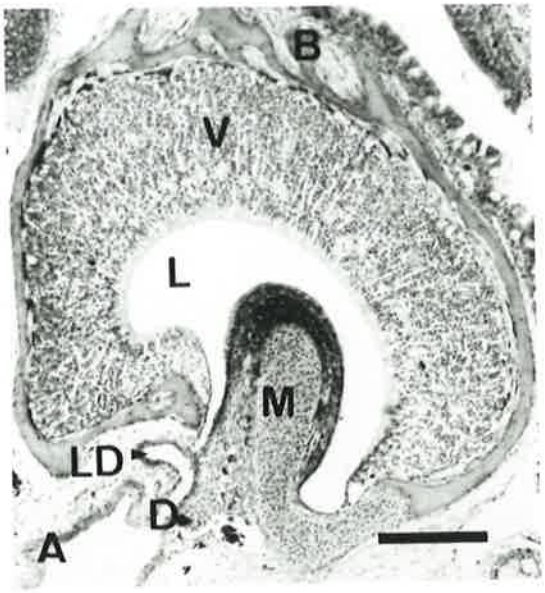
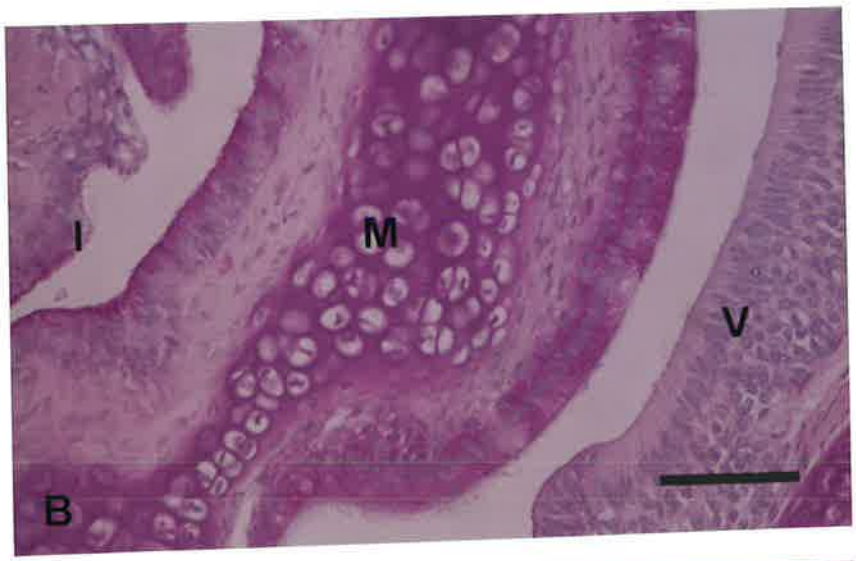

A
B
C
D

PLATE 21: LIGHT MICROGRAPHS OF THE SQUAMATE VNO

Note: the medial side of the VNO is on the left hand side of the micrograph. The VNO of the *P. textilis*, like that of other squamates, consists of vomeronasal sensory epithelium (V), which is encapsulated by bone (B). It is connected to the palate by a duct (D), which itself is associated with the lacrimal duct (LD). A ventro-medial projection, the mushroom body (M), reduces the available space in the lumen (L). Intermediate epithelium (I) is found between the sensory vomeronasal and the non-sensory mushroom body epithelium (A). Staining with PAS (B) and mercury bromo-phenol blue (C) of the VNO of *C. marmoratus*, revealed the presence of mucins in the intermediate region (I) and protein in the mushroom body epithelium (M).

Scale bar (A) = 200 μm; others = 100 μm.


B
V
L
M
LD
D
A


I
M
V
B

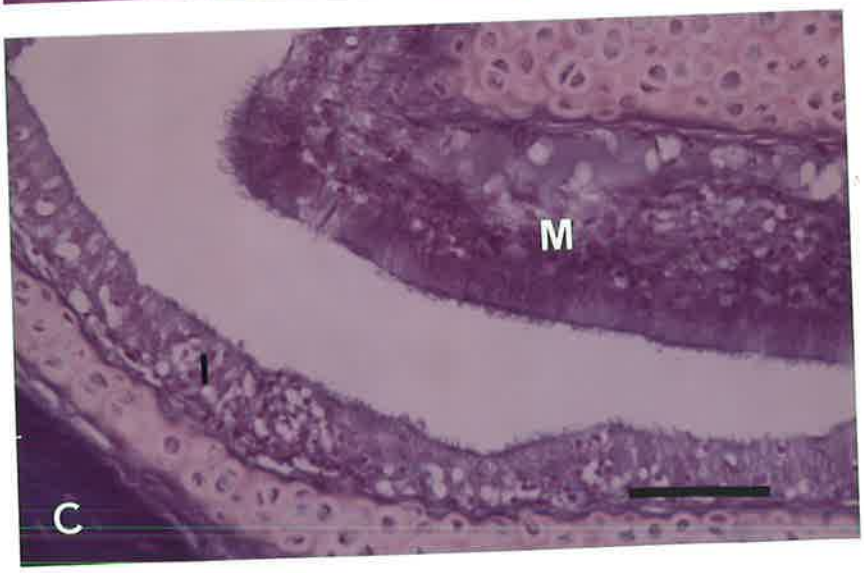

M
I
C

PLATE 22: ULTRASTRUCTURAL MICROGRPAHS OF THE SQUAMATE VNO I

Neither the sustentacular nor the receptor cells in the lizard VNO sensory epithelium possessed secretory granules, as seen in *C. marmoratus* (**A**). This contrasts to the condition in the snake, *P. textilis*, wherein the microvillous sustentacular cells contained a few apical secretory granules (**B**). The columnar cells of the mushroom body of *C. marmoratus*, contained apical cilia and mitochondria, irregularly shaped nuclei and some basal lysosomes (**C**). At higher magnifications, the gekkotan mushroom body epithelium differed from the other squamates examined. As seen in *S. intermedius* both apical cilia and microvilli as well as a modest amount of electron-dense granules and mitochondria are found in the gekkotan mushroom body epithelium (**D**).

Scale bar (C) = 2μm; others = 1 μm.

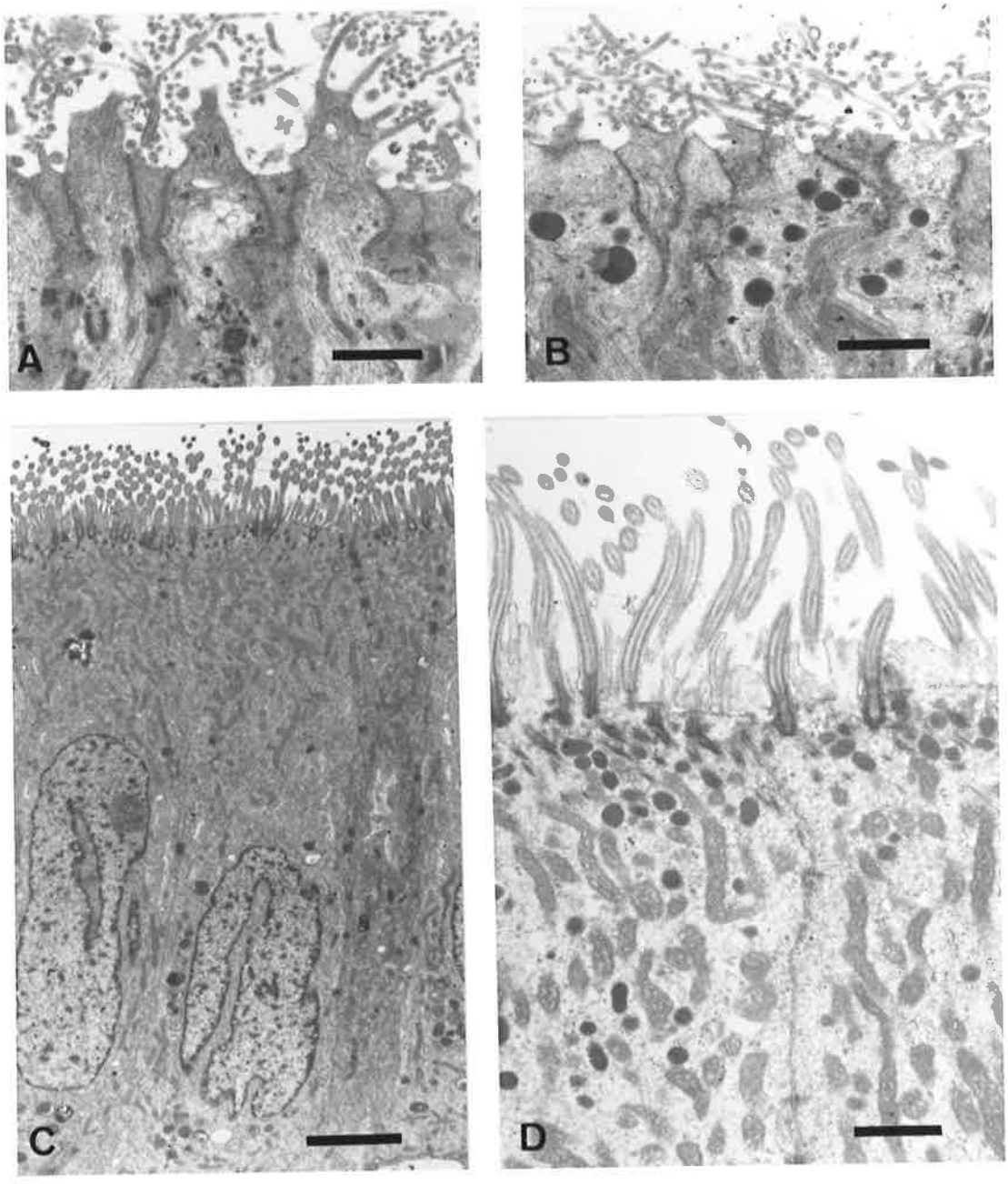
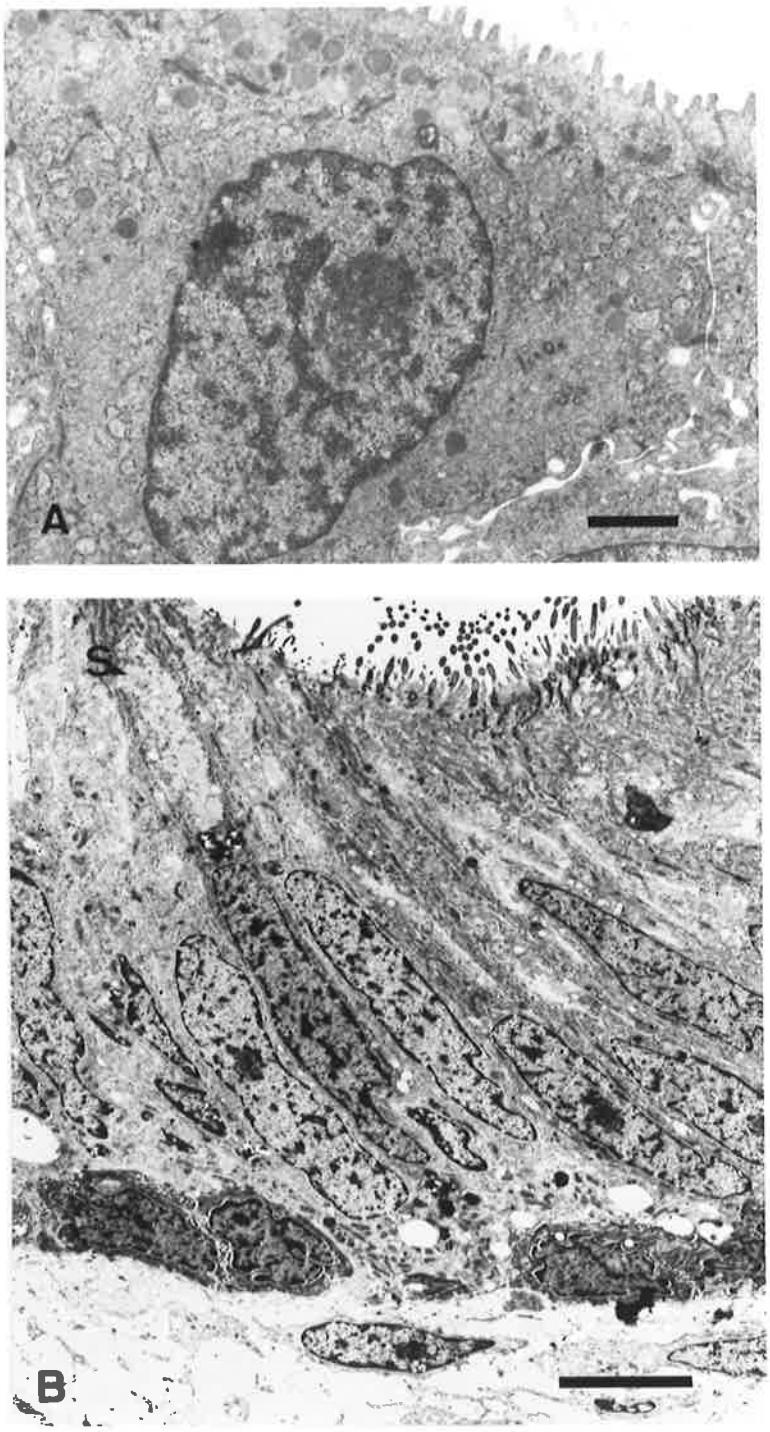

A
B
C
D

PLATE 23: ULTRASTRUCTURAL MICROGRAPHS OF THE SQUAMATE VNO II

Two types of intermediat cells were in the VNO of all squamates examined. The intermediate cells leading to the vomeronasal duct were cuboidal and possess few apical secretory granules, as seen here in *P. textilis* (A). The intermediate cells between the mushroom body and the vomeronasal epithelium, however, were columnar, with more apical secretory granules, as seen here in *C. marmoratus* (B).

Scale bar (A) = 1 μm; (B) = 5 μm.


A
S
B

Rehorek, S.J., Firth, B.T., and Hutchison, M.N., (1997) Morphology of the Harderian gland of some Australian geckos.
Journal of Morphology, v. 231 (3), pp. 253-259, March 1997

THE ANATOMICAL RECORD 0:1–6 (1997)

Squamate Harderian Gland: An Overview

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ABSTRACT ***Background:*** **The Harderian gland is an orbital feature found in most terrestrial vertebrates. Although there have been several reports on the structure of the squamate Harderian gland, there has been little recent discussion as to its potential function. This article reviews both the recent morphological observations and their implications on the potential functions of the squamate Harderian gland.**

Methods: **Literature on the gross structure, histochemistry, and ultrastructure of the squamate Harderian gland and associated structures was reviewed. These observations were then used to assess morphologically the likelihood of the proposed functions.**

Results: **A high level of morphological variation was found in the squamate Harderian gland. Three functional hypotheses, including roles in orbital lubrication, digestion, and vomerolfaction, were considered. Both morphology of the squamate Harderian gland and the presence of alternate secretory sources suggest that it is unlikely to function in orbital lubrication. There is little evidence to suggest a function in digestion. Both the presence of the connecting lacrimal apparatus and the reduced intrinsic secretory capacity of the vomeronasal organ suggest that the Harderian gland may function in vomerolfaction.**

Conclusions: **The most likely role of the squamate Harderian gland seems to be in vomerolfaction. Morphological variations observed in the Harderian gland may mirror the different degrees and mechanisms of vomerolfaction. Further studies, including comparative morphological, experimental, and microchemical analyses, are required to test this hypothesis. Anat. Rec. 000:00–00, 1997.** © 1997 Wiley-Liss, Inc.

Key words: Harderian gland; squamates; lacrimal duct; vomeronasal organ; olfaction

The Harderian gland was originally described by Harder (1694, cited by Kennedy, 1970) in the deer, *Dama dama*. Since then, there has been much controversy surrounding this orbital gland and its putative presence in various tetrapods (Sakai, 1981; Sakai and van Lennep, 1984; Chieffi Baccari et al., 1992). The vast majority of work concerning the Harderian gland has been carried out on rodents, with scattered references to other tetrapods (Payne, 1994).

Little is known about the squamate Harderian gland, with scattered studies on disparate species using a variety of methods. The structure of the squamate Harderian gland has been reviewed (Chieffi et al., 1992). The present review focuses on recent morphological observations that suggest a functional relationship between the gland and nearby chemosensory structures.

Squamate Harderian glands are often overlooked in the literature, not only with respect to their morphology but also in their relationship to other orbital features and the significance thereof. In reptiles, there are at most three orbital glands, including two lacrimal glands and the Harderian gland (Saint Girons, 1982; Fig. 1). The reptilian Harderian gland has been defined by using anatomical criteria based on its position in the anterior portion of the orbit and its association with the nictitating membrane (Cowan, 1970; Saint Girons, 1982, 1985; Payne, 1994). The relationship of the lacrimal apparatus, which includes the lacrimal duct and canaliculi, to both the Harderian gland and the vomeronasal organ (VNO; Fig. 2), was originally described by Bellairs and Boyd (1947, 1949), leading both Saint Girons (1988) and Halpern (1992) tentatively to link the Harderian gland to the vomeronasal chemosensory system. This and two other functional hypotheses are discussed after a brief review of the squamate Harderian gland.

REPTILIAN HARDERIAN GLAND

The works of Saint Girons (1982, 1985, 1988) constitute an introductory survey of the reptilian Harderian

Contract grant sponsor: University of Adelaide.
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Received 26 January 1996; accepted 7 February 1997.

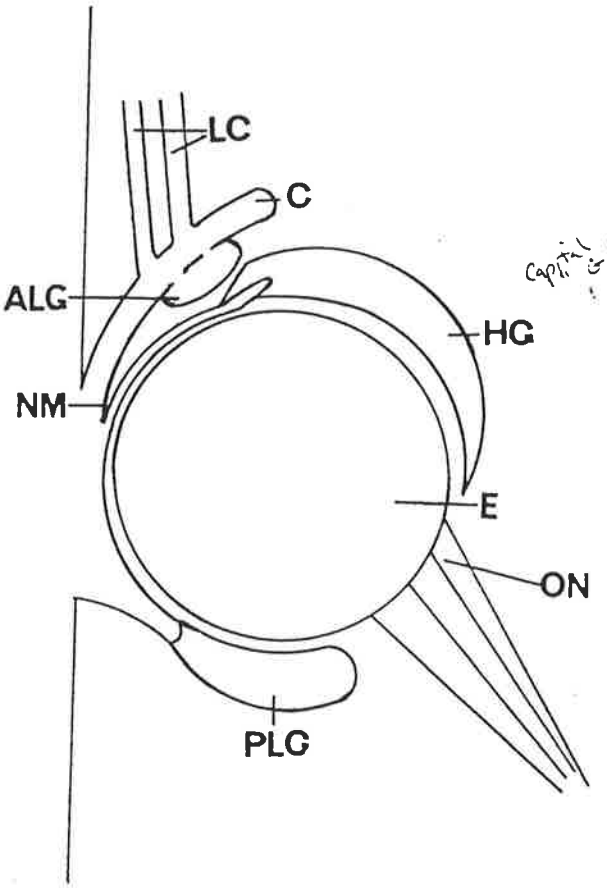

Fig. 1. A schematic diagram of a horizontal section through the orbital region of a reptile, showing the relationships between the various orbital structures. ALG, anterior lacrimal gland; C, conjunctiva; E, eyeball; HG, Harderian gland; LC, lacrimal canaliculi; NM, nictitating membrane; ON, optic nerve; PLG, posterior lacrimal gland.

gland, using a broad phylogenetic base with both histological and histochemical analyses. The Harderian gland is present in all squamates, but the size of the gland is much larger in some anguimorph lizards and most snakes, filling most of the orbital space (Dullemeijer, 1959, Figs. 1-54; McDowell, 1969, Figs. 1-9, Plates 1-2; Saint Girons, 1988). In both viperid snakes and skinks, the size of the orbit seems to limit the relative development of the Harderian gland in these groups (Dullemeijer, 1959; Rehorek, 1992). However, even when there is ample space, there seems to be a functional limit that the Harderian gland does not exceed (Rehorek et al., 1993). Nonetheless, in many colubrid snakes, mainly the cryptozoic and fossorial species, the Harderian gland has an extraorbital portion (Schwarz-Karsten, 1937; Savitzky, 1972, 1978; McCarthy, 1985). This position has led to the modification of the arrangement of both the venom glands and associated musculature (Savitzky, 1978). The significance of this variability in size is unknown at present.

Histochemical analyses of the squamate Harderian gland have shown that it produces mucous or serous secretions (Saint Girons, 1982) or a combination of the two (Schwarz-Karsten, 1937; Saint Girons, 1982; Minucci et al., 1992). Chieffi-Baccari et al. (1990) observed dual secretions in *Podarcis sicula* (Lacertidae) but defined the mucoid region as the anterior lacrimal gland. There are instances of solely mucoid Harderian glands in reptiles, but these species appear to be phylogenetically distantly related (*Sphenodon*, *Xenopeltis* snakes, *Caiman* and amphisbenians; Saint Girons, 1982). In some anguid and lacertid lizard species, an intermediate zone has been described showing histochemically both mucous and serous reactions (Saint Girons, 1982; Chieffi-Baccari et al., 1990).

Recent histochemical analyses of some Australian Gekkota have revealed the presence of both lipid and protein in the Harderian gland secretory cells (Rehorek et al., 1996, 1997). Lipid granules in the Harderian gland have not been described in any other reptiles thus far, although their presence has been noted in amphibians (Minucci et al., 1989), birds (Maxwell et al., 1986), and mammals (Sakai, 1981). A variety of functions have been ascribed to the lipid produced in the Harderian gland of these three classes. Thus, it is unlikely that the lipid produced by the gekkotan Harderian gland has a function similar to any of the other classes.

Ultrastructural studies of the Harderian gland have only been carried out on a few squamate species. Within Scincomorpha, the strucutre of the Harderian gland exhibited much variation between the two families examined. The Harderian gland of *Podarcis sicula* (Lacertidae) was shown histochemically to possess three zones: anterior mucous and intermediate and posterior serous. Ultrastructural observations of the serous area by Chieffi Baccari et al. (1990) revealed an unusual compound secretory granule, which they termed "special secretory granules," with an apparently laminar crystalline portion within one of the zones. In the five skink species (Scincidae) studied thus far, the serous secreting Harderian gland also possesses a compound secretory granule but without the crystalline structures (Rehorek, 1992).

Variations in the ultrastructure of the Harderian gland protein secretory granules were also observed in the gekkotans. Gekkonine gekkoes possessed compound secretory granules that were similar to the "special secretory granules" of *Podarcis s. sicula*, but those of diplodactyline gekkoes were less complex (Rehorek et al., 1997). The protein secretory granules of pygopods had an intermediate structure, with less well-developed lamellar crystalline structures (Rehorek et al., 1996).

In the colubroid snakes *Coluber viridiflavus* (Colubridae) (Minucci et al., 1992) and *Pseudonaja textilis* (Elapidae) (personal observation), it was found that the mucous and serous cell types are located in two anatomically distinct areas. Ultrastructural analyses also showed an unusual tripartite secretory product in the serous secretory cells but without the unusual crystalline arrangement. Thus, the ultrastructure of the protein secretory granules in the squamates thus far examined has revealed enormous variation. Ultrastructural complexity of secretory granules implies chemical heterogeneity (Fawcett, 1981) and may be related to the amount of protein present (Zylbeberg, 1977). However, the molecular biology and function of the secretory granules in the squamate Harderian gland are unknown.

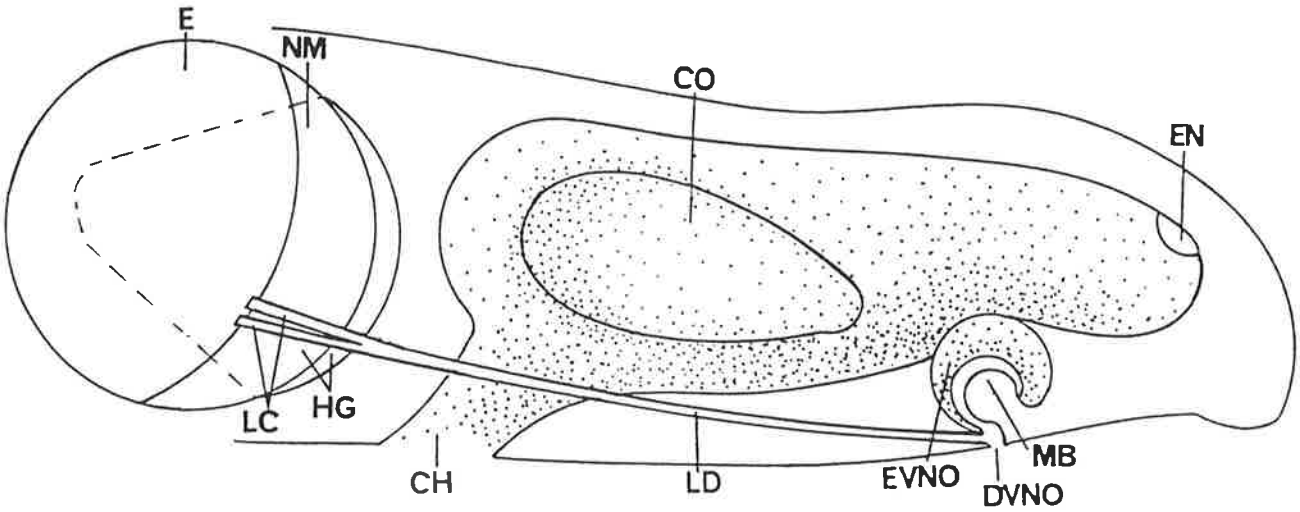

Fig. 2. A schematic diagram of the lateral view of a squamate nasal capsule, showing the relationship among the Harderian gland (HG), the lacrimal duct (LD), and the duct for the vomeronasal organ (DVNO). CH, choana; CO, conch; E, eye; EN, external nares; EVNO, epithelium of the VNO; LC, lacrimal canaliculi; MB, mushroom body; NM, nictitating membrane.

HARDERIAN GLAND AND LACRIMAL DUCT CONNECTION

The VNO and the Harderian gland in squamates are connected by the lacrimal duct (Pratt, 1948; Parsons, 1970; Fig. 2). There seems to be much morphological variation concerning the relationship among the duct, the orbit, and the Harderian gland (Bellairs and Boyd, 1947; Saint Girons, 1982). When present, the nictitating membrane physically separates the lacrimal duct and the Harderian gland, allowing the secretion from the Harderian gland to enter the orbital environment (Fig. 1). This situation was found in lizards with moveable eyelids, including agamids (Bellairs and Boyd, 1947). The lacrimal duct divides into two lacrimal canaliculi before reaching the orbit. The loss of the nictitating membrane allows the lacrimal canaliculi to be closer to the openings of the Harderian gland, as seen in gekkos. In pygopods, amphisbenians, and snakes, the Harderian gland opens directly into the lacrimal duct or into one of the lacrimal canaliculi, which itself has a reduced connection to the orbital region. A similiar condition was found in *Brookesia* (Chameleonidae) (Saint Girons, 1982).

LACRIMAL DUCT AND VNO CONNECTION

The relationship between the lacrimal duct and the VNO also shows considerable morphological variation. The structure of the lacrimal duct of squamates is well documented with both morphological (Bellairs and Boyd, 1947, ~~1949~~; Saint Girons, 1982) and embryological (Slaby, 1979a-c, 1981, 1982a-c, 1984) observations showing the close relationship between it and the VNO. The lacrimal duct opens into the oral cavity in the vicinity of the duct of the VNO in most squamate reptiles (Fig. 2). In all lizards studied, very young embryos possess a lacrimal duct ~~that~~ opens laterally into the vomeronasal duct or at least very close to it. Subsequent morphogenesis leads to the distal part of the lacrimal duct being incorporated into the spreading outer choanae. Thus, in the adult, the lacrimal duct is shorter and opens into the choanal grooves, which are choanal fissures connecting the choanae to the vomeronasal duct (Slaby, 1982b). In chameleons, in ~~which~~ the VNO is absent in the adult (Gabe and Saint Girons, 1976), the lacrimal duct opens directly into the oral cavity (Slaby, 1984). The closure of the choanal groove in embryonic varanids (Slaby, 1979c) and teiids (Slaby, 1982a) results in the lacrimal duct opening directly into the lateral side of the vomeronasal duct. Similarly, in snakes, the lacrimal duct opens directly into the vomeronasal duct, although on the medial aspect (Bellairs and Boyd, ~~1949~~). 1950

Thus, the Harderian gland is connected to the VNO, or the palate, by the lacrimal apparatus in all squamates. The morphological variations in the relationships of these structures suggest functional differences. Based on this morphological connection, Broman (1920) proposed that the squamate Harderian gland functions in vomerolfaction. The evidence for this and two other functional hypotheses will be discussed in light of the recent observations in the following section.

FUNCTION OF THE HARDERIAN GLAND IN REPTILES

The combination of various histological properties of the Harderian gland and its relationship with the lacrimal duct led Saint Girons (1982) to propose that the Harderian gland in reptiles exhibits the potential for multiple functions. Four possible functions for the reptilian Harderian gland have been proposed, first by Kennedy (1970) and then with further elaboration by Saint Girons (1982, 1989). Three of these seem relevant to the condition in squamates, and the fourth seems to apply only to crocodilians.

As with nonreptilian tetrapods, it was proposed that the Harderian and the lacrimal glands in squamates would provide abundant lubricant for the corneal surface, with the lacrimal duct draining any excess fluid (Taub, 1966; Saint Girons, 1982). However, there are at

least three observations that appear to refute this hypothesis.

The evolutionary modification of the lower eyelid has led to the development of a clear scale, or spectacle, in some species. This spectacle has been found in squamates that inhabit arid zones and may have evolved to reduce corneal water loss (Greer, 1983). In skinks, intermediate stages of spectacle development have been observed, and, hence, the development of the spectacle can be observed in a single family. Both gekkonine and diplodactyline geckos and snakes possess complete spectacles. The third eyelid, or nictitating membrane, which also has a protective function, is made redundant by the presence of the spectacle. In all squamates with a spectacle, the nictitating membrane is much reduced or absent. Comparative morphological analyses have revealed that these orbital variations do not alter the structure of the squamate Harderian gland (Saint Girons, 1982; Rehorek, 1992; Rehorek et al., 1993). This observation implies that the squamate Harderian gland does not function in corneal lubrication.

Skinks with a sand-swimming type of fossoriality have undergone skull miniaturization. This compaction mainly occurs in the lateral regions, including the nasal and ocular regions, for the brain, which cannot be reduced beyond a certain functional limit (Rieppel, 1984). Besides an inevitable reduction in orbit size due to allometry, eye diameter becomes further reduced in the more derived burrowers as vision becomes less important. If the Harderian gland were to function in corneal lubrication, then the reduced corneal surface would lead to consequent structural changes in the Harderian gland of the more extreme burrowers within a lineage. Morphological comparsion of the Harderian gland of *Lerista spp.*, including species with skull compaction (*Lerista edwardsae*), spectacles (*Lerista muelleri*), and unmodified orbital region (*Lerista bougainvilli*), revealed no significant variation in relative size or structure of the Harderian gland (Rehorek, 1992; Rehorek et al., 1993). Thus, the structure of the eyelid and the degree of fossoriality seem to have littel impact on the structure of the Harderian gland.

Yet another source of variation is the relationship between the Harderian gland and the lacrimal duct. In both snakes and pygopods, the Harderian gland is unable to contribute directly to corneal lubrication because it has little contact with the orbit, opening directly into the lacrimal canal duct.

Finally, there are other glandular structures present in the orbital region of squamates that could serve the function of corneal lubrication, including the lacrimal mucous glands and the mucous epithelia (Fig. 1; Saint Girons, 1988; Rehorek, 1992). Thus, the squamate Harderian gland appears to be superfluous as a source of corneal lubricant.

The squamate Harderian gland may function as an accessory salivary gland. Young and van Lennep (1978) broadly defined the salivary gland as any cell or organ whose secretion discharges into the buccal cavity. They also proposed several general functions for salivary glands. The first two associate the salivary glands with digestion, functioning as either a lubricant or a source of digestive enzymes. The former function was proposed for the Harderian gland in snakes (McDowell, 1969) based on its hypertrophied form (Gans, 1974) and the route of the lacrimal duct (Walls, 1942). Saint Girons (1982) suggested that the Harderian gland produces and secretes digestive enzymes. However, this hypothesis lacks corroborating biochemical data. It also fails to take into account the observation that in squamates (with the exception of snakes and pygopods) the Harderian gland empties its contents directly into the orbital space (Bellairs and Boyd, 1947; Saint Girons, 1982).

The relationship among the Harderian gland, the lacrimal duct, and the VNO, originally observed by Broman (1920), suggest that the secretion from the squamate Harderian gland has a role in vomerolfaction (Savitzky, 1972; Saint Girons, 1982; Halpern, 1992). Although a morphological connection exists between the Harderian gland and the VNO (Fig. 2), the precise function of the Harderian gland in the vomeronasal system is speculative. The Harderian gland may produce a solvent for the choanal grooves to enable movement of odorant molecules to the VNO (Pratt, 1948). Another possibility is that the solvent for the VNO arises from the lacrimal duct itself (Pratt, 1948; Kratzing, 1975), implying that the lacrimal duct also is capable of producing some form of secretion. However, there is no histological evidence for this notion.

Another suggestion is that the Harderian gland produces an enzyme that facilitates vomeronasal transduction of nonvolatile chemicals (Saint Girons, 1989). The ability to perceive nonvolatile substances is one of the features that, in squamates, distinguishes vomerolfaction (Cooper and Burghardt, 1990) from nasal olfaction (Schwenk, 1995). The link between the Harderian gland and a chemosensory system has been described in at least one anamniote. Wake (1985) concluded that the cecilian Harderian gland functions as a source of lubrication for the tentacle. Thus, the connection between the squamate Harderian gland and the VNO may be analogous to the connection between the Harderian gland and the chemosensory tentacle in caecilian amphibians (Wake, 1985).

The role of the Harderian gland in squamate vomerolfaction is supported further by morphological observations of the VNO and its similarity to the olfactory system. Molecular transduction of odor molecules at receptor cells in the olfactory epithelium requires their dissolution in a fluid medium. There are two sources of fluid for the olfactory epithelium: the secretory cells lining the sensory epithelium and the submucosal Bowman's glands (Kratzing, 1975). The vomeronasal epithelium is generally thought to have little instrisic secretory capacity (Halpern, 1992). Submucosal vomeronasal glands, whose position and relative development varies among species (Adams, 1992), have been described in the VNO of mammals. The absence of these glands has been linked to the rudimentation of the VNO in bats (Cooper and Bhatnager, 1976). No such submucosal glandular structures have been reported in the well-developed squamate VNO (Kratzing, 1975). Thus, the necessity of a fluid medium and the absence of an intrinsic source of secretion suggest that there should be an extrinsic source of fluid in the squamate VNO. A possible extrinsic source of lubricant for the squamate VNO is the Harderian gland via the lacrimal duct.

Saint Girons (1989) suggested that the Harderian gland may in fact have several functions in the same organism. It is possible that the secretions may somehow be segregated, with mucous secretions (when present) lubricating the eye and serous secretions flowing down to the VNO (Minucci et al., 1992). As yet, there is no evidence to support this idea. In all cases, the explanations are poorly substantiated because of few observations, poor taxonomic scope, and the absence of experimental, microchemical, and ultrastructural analysis. Little is also known about the lacrimal apparatus.

In conclusion, the squamate Harderian gland exhibits more morphological variation than other investigators have indicated. It has a variable relationship with nearby structures such as the lacrimal apparatus and the VNO. At present, its most likely functional role seems to be as a facilitator of vomerolfaction as a source of enzymes for sensory transduction, a source of lubricant, or both. Both behavioral and morphological studies imply that there are differing degrees and mechanisms of vomerolfaction in squamates (for reviews, see Halpern, 1992; Schwenk, 1995). If the Harderian gland is part of the vomeronasal system in squamates, then the large interspecific variation observed in Harderian gland structure may parallel these different mechanisms. It would be advantageous to complete a more comprehensive histological and morphological study of the squamate Harderian gland before attempting to ascribe a function to it. Although the Harderian gland has not been linked experimentally to the vomeronasal system, the obvious morphological connection suggests that it should be fruitful to compare morphologically the vomeronasal/Harderian gland system to the olfactory system in squamate reptiles.

ACKNOWLEDGMENTS

I thank B.T. Firth, M.N. Hutchinson, and K. Schwenk for critical review of this manuscript. This work was supported by a University of Adelaide postgraduate scholarship to the author.

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