

**COMPARATIVE ANALYSIS OF THE OLFACTORY BULBS
OF THE NILE CROCODILE (*Crocodylus niloticus*), ROCK
MONITOR LIZARD (*Varanus albigularis*) AND NILE
MONITOR LIZARD (*Varanus niloticus*)**



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LIST OF ABBREVIATIONS:

AOB- Accessory Olfactory Bulb

EPL- External Plexiform Layer

CR- Calretinin

CB- Calbindin

GCL- Granule Cell Layer

GLCL- Glomerular Cell Layer

IPL- Internal Plexiform Layer

MCL- Mitral Cell Layer

MOB- Main Olfactory Bulb

MOE- Main Olfactory Epithelium

MOS- Main Olfactory System

NS- Nucleus Sphericus

ONL- Olfactory Nerve Layer

ORN- Olfactory Receptor Neuron

PV- Parvalbumin

SVZ- Sub-Ventricular Zone

V- Ventricle

VNO- Vomeronasal Organ

VNS- Vomeronasal System

DECLARATION

I, Marcel Elijah Rappetti, declare that this dissertation is my own, unaided work. It is being submitted for the Degree of Master of Science (Medicine) by research, at the University of Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.



(Signature of candidate)

08th day of August 2024 in Johannesburg, South Africa

DEDICATION

To my parents, Eugene Alec Rappetti and Umbritha Rappetti

ABSTRACT

Introduction/Background: Reptilian brains have been poorly studied historically, which lead to many scientific misconceptions. Over the last few decades more research has been dedicated to these taxa demystifying some of these earlier misconceptions. One area of interest that requires clarity relates to the reptilian olfactory system and its connection to the vomeronasal system (VNS). To understand these systems better we require a comprehensive anatomical account of the structures involved. By understanding the structure more accurately, inferences can be made about the function and presence of the associated underlying systems. Crocodilians are suggested to lack a vomeronasal system, but contradictory findings have indicated the presence of a putative accessory olfactory bulb which forms part of the vomeronasal system. No study provides a complete anatomical account of the olfactory bulb to confirm or negate these findings for crocodilians. Squamates present with a well-established vomeronasal system and is the ideal comparator to identify the structural components of the olfactory bulbs that are involved in the VNS. Most studies defining the anatomy of the olfactory bulbs are confined to snakes with few studies focused on lizards.

Aim: The aim of the current study was to identify and describe the chemoarchitecture of the olfactory bulbs in one of the Squamate groups where the vomeronasal/accessory olfactory system is apparently well-established, monitor lizards (*Varanus albigularis* and *Varanus niloticus*) and in a Crocodilian group where the VNS is suspected to be absent, Nile Crocodiles (*Crocodylus niloticus*).

Methods: The olfactory bulbs of three reptile species (Nile Crocodile, Rock Monitor and Nile Monitor lizard) were sectioned and stained for Nissl and immunohistochemically stained for Calbindin, Calretinin, Parvalbumin, NMDA receptor 2B and $G\alpha_0$ protein. The resultant sections were viewed under a low power stereomicroscope and the relevant cytoarchitectural boundaries were traced using a camera lucida. Immuno-labelled sections were viewed under a low power dissecting microscope and the relative cellular density profiles were determined by observation and stereology.

Results: The Monitor Lizards presented both the main olfactory bulb (MOB) and accessory olfactory bulb (AOB). The Nile Crocodile presented a MOB and a unique region we believe to be a putative accessory olfactory bulb.

Discussion/Conclusion: This unique region lacks the classical features of the AOB described for squamates. However, it is independent and separated from the MOB with clear cell layers and cellular boundaries. This structure may be an evolutionary remnant of an AOB that has remained in the olfactory bulb of the Nile Crocodile. Functional and behavioural studies would need to be performed to determine the role of this region in olfaction and its possible association with VNS.

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1. INTRODUCTION AND BACKGROUND INFORMATION

Carl Linnaeus, the father of modern taxonomy, is known for his disgust and dislike of reptiles (Linnaeus, 1797), a theme that seems to have extended into the history of biology and contributed to the reluctance of studying these taxa. Moreover, reptiles were perceived as less intelligent in comparison to other species (Kemp, 2019). However, recent studies continue to change popular misconceptions such as those expressed by Linnaeus (1797) by focussing on reptilian behaviour, social interactions, and life history (Amiel, 2013; Matsubara *et al.*, 2017; Eagan, 2018; Divers and Stahl, 2019). Today it is common knowledge that reptiles are complex creatures that rely heavily on sensory input for their survival (Naumann, 2015). Therefore, analysing major sensory modalities, such as olfaction, used by reptiles may provide useful insights into how they might perceive the world that can improve conservation strategies (Hoover, 2010).

1.1.1. *The olfactory system*

Olfaction is the perception of environmental chemical stimuli which is achieved by specialised sensory neurons that are located in the nasal cavity (Doty, 2001; Gong, 2012). The ability to perceive chemical information assists different species of animals in chemo-communication and plays an important role in their sexual and social behaviours (Van Hartevelt and Kringelbach, 2015; Boesveldt and Parma, 2021; Bakker *et al.*, 2022). Olfaction occurs through the main olfactory system (MOS) (which exists in all vertebrate species) and vomeronasal (accessory) olfactory system (VNS) (only in certain vertebrate species) (Jacobs, 2012). The MOS detects a variety of environmental chemical stimuli including volatile odour molecules whilst the VNS detects mostly non-volatile, pheromonal stimuli (Burghadt, 1980; Luo and Katz, 2004).

1.1.2. *Main Olfactory System Vs Vomeronasal Olfactory System*

The main olfactory system (MOS) has been extensively researched in amniotes (such as mammals, reptiles and birds) and numerous articles have been published discussing both the structure and function of this system (Roper, 1999; Sanchez-Andrade and Kendrick, 2009; Ennis *et al.*, 2015; Tufo *et al.*, 2022). The vomeronasal system (VNS), on the other hand, has only been described in certain vertebrate

species (in reptiles such as lizards and snakes, some mammals and in certain amphibian species) (Burghadt, 1980; Mucignat-Caretta *et al.*, 2012; Woodley, 2014). The MOS and VNS possess different sensory epithelium such that the expression of the different receptor types within those different sensory epithelium are used to distinguish between the MOS and VNS (Luo and Katz, 2004, Fig. 1).

In amniotes, the MOS consists of a main olfactory epithelium (MOE) that possesses olfactory receptor neurons (ORNs) (Roper, 1999; Sanchez-Andrade and Kendrick, 2009; Taniguchi *et al.*, 2011; Taniguchi and Taniguchi, 2014). These ORNs synapse in a structure situated towards the most rostral region of the brain called the main olfactory bulb (MOB) and project further to various regions in the brain (Sanchez-Andrade and Kendrick, 2009; see Fig. 1). The VNS consists of a chemosensory organ called the vomeronasal organ (VNO – also known as Jacobson's organ) that possesses vomeronasal sensory neurons (Baum and Cherry, 2015). These neurons contains two types of vomeronasal receptors that are characterised by the expression of G-protein coupled receptor subunits (Spehr and Munger, 2009). Type 1 vomeronasal receptors (V1R) are associated with the G-protein α -i2 subunit (G_{i2}), type 2 receptors are associated with the G-protein α -0 subunit ($G_{\alpha 0}$) (Kondoh *et al.*, 2022). These vomeronasal receptors relay signals to the accessory olfactory bulb (AOB) and project further to the amygdaloid complex in amniotes (Lanuza and Halpern, 1998; Mucignat-Caretta *et al.*, 2012; see Fig. 1).

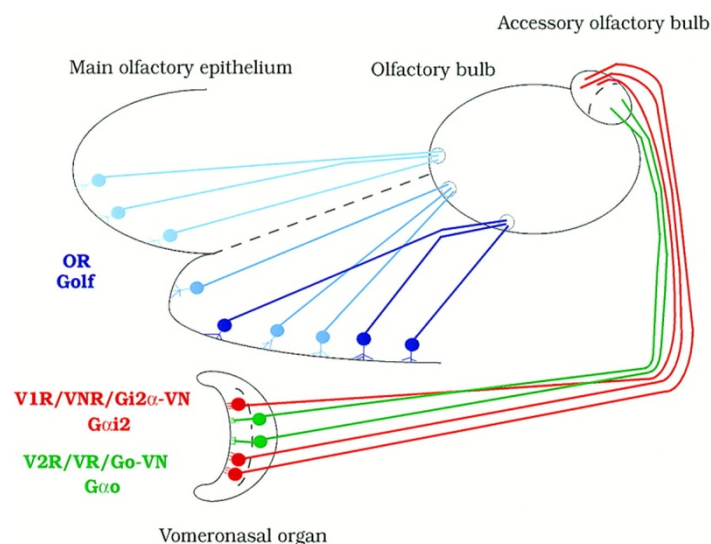


Figure 1: The schematic provides an outline of the expression of the different receptors for the ORNs between the MOB and AOB in amniotes (Abbreviations: OR- olfactory receptor neuron, V1R- Type 1 vomeronasal receptor, V2R- Type 2 vomeronasal

receptor, $G\alpha_0$ - G-protein α -0 subunit, $G\alpha_{i2}$ - G-protein α -i2 subunit and VN- Vomeronasal sensory neurons). The neurons projecting to the MOB are scattered throughout the MOE. These neurons from the MOE converge in the MOB as indicated by the light and dark blue lines. The VNO projects neurons that converge in the AOB. The red line indicates the movement from the V1 receptors and the green line indicates the movement from the V2 receptors. (Image Source: Bargmann, 1997).

The olfactory bulbs (both main and accessory) process the significant sensory information (Sanchez-Andrade and Kendrick, 2009; Mucignat-Caretta *et al.*, 2012). In general, the MOB is comprised of six major cell layers with each layer playing a specific role in interpreting significant sensory information (Fig. 2). In the MOB, there are multiple types of excitatory and inhibitory neurons that play a crucial role in neurotransmission (Sanchez-Andrade and Kendrick, 2009; Tufo *et al.*, 2015). The MOB and AOB possess the same major olfactory cell layers with similarities in the expression of the different olfactory cell types/receptors and overall organisation in amniotes (Bargmann, 1997; Eisthen, 1997; Dulac and Wagner, 2006; Bruce, 2009).

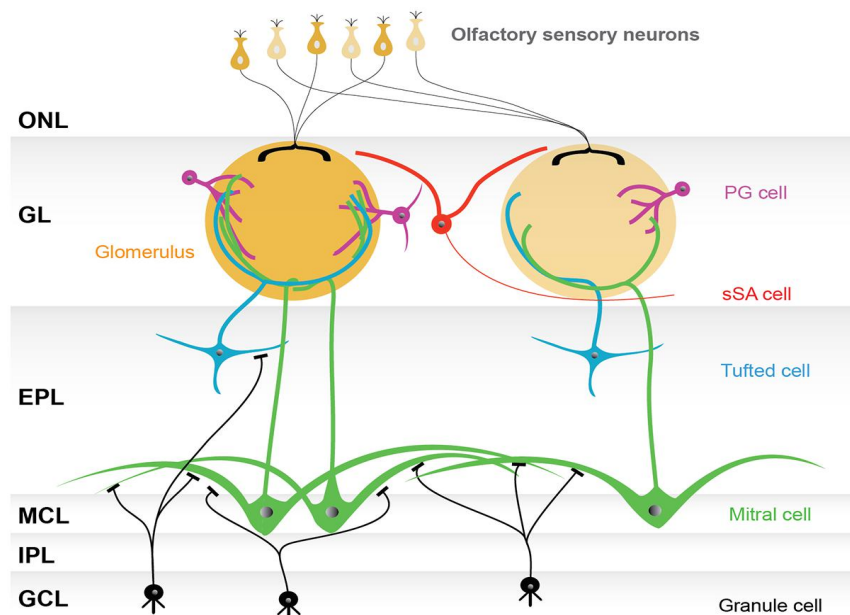


Figure 2: Schematic representation of the MOB in amniotes (Abbreviations: GCL-granule cell layer, IPL-internal plexiform layer, MCL-mitral cell layer, EPL-external plexiform layer, GLCL-glomerular cell layer and ONL- olfactory nerve layer). The ONL receives the inputs from the olfactory receptor neurons (ORNs) and the axons of ORNs synapse with dendritic processes of mitral and tufted cells to form glomeruli in the GLCL. The plexiform layers (IPL and EPL) are additional layers of synaptic processing. Mitral cells in the MCL form major output channels that send afferent information to the brain and the GCL regulates output of neurons to the brain. (Image Source: Martínez Marcos, & Halpern, 2014).

1.1.3. Phylogenetic relationships

Amniotes are limbed vertebrates that belong to the clade Amniota that is comprised of Reptilia, Mammalia and Birds (Gans and Bell, 2001; Morris and Abzhanov, 2021). Amniotes are defined as organisms whose embryos develop within an amnion (Tizard, 2023). Over 300 million years ago, amniotes divided into two separate clades namely: Synapsid (i.e. mammals) and Sauropsida (reptiles and birds) (Striedter and Northcutt, 2019). The taxonomic class reptilia is broad and diverse comprising of testudines (turtles and tortoises), snakes, lizards, tuataras, birds and crocodilians (Kemp, 2019). Phylogenetically, reptiles can be subdivided into the following taxonomic groupings which include Lepidosaurs (which include tuatara and Squamates i.e., lizards and snakes) and Archosaurs (such as birds, testudines, and crocodilian) (Gans and Bell, 2001). Crocodilians are subdivided further into three families namely: Crocodylidae (Crocodiles), Alligatoridae (Alligators and Caiman) and Gavialidae (Gharial) (Conley and Shilton, 2018). Lepidosaurs differ genetically and morphologically from archosaurs (Gans and Bell, 2001) resulting in considerable structural and functional variation in the olfactory systems between these phylogenetic groups (Bestwick *et al.*, 2020).

Lepidosaurs have a well-established olfactory system and possess the MOS and VNS encompassing both the MOB and an AOB (Okuyama and Sasaki, 2021; Crowe-Riddell and Lillywhite, 2023). Archosaurians, on the other hand, seem to either lack the VNS completely or possess a redundant or different version of this system (Eisthen, 1992; Abdali *et al.*, 2020). Testudines are known to possess the MOS (including the MOB) and the VNS (Nakamuta *et al.*, 2016). Despite the lack of a structural VNO in the testudines, many of the functions typically ascribed to a fully developed VNO has been shown to be undertaken by cells within the olfactory epithelium (Abdali *et al.*, 2020). Birds which are the closest known living relative of crocodilians (Chiappe, 2004) are known to possess the MOS (specifically the MOB) but no indication of a VNS has been demonstrated structurally or functionally in this taxonomic group (Roper, 1999). However, the olfactory system in crocodilians have yielded conflicting ideas concerning the VNS, thus the aim of the current study was to address some of this conflict by providing a comprehensive neuroanatomical account of the crocodilian olfactory bulb as well as two squamates (with established VNS) that

have not been described before.

Most studies investigating the olfactory system in reptiles focus primarily on squamate reptiles (Burghadt, 1980; Holtzman and Halpern, 1990; Filoramo and Schwenk, 2009; Mason and Parker, 2010; Kondoh *et al.*, 2013a). Squamates are the most diverse and species rich reptiles whose morphology and genetic makeup serve as model organisms for research on evolutionary patterns between mammals and other vertebrates (Blackburn, 2006; Daza, 2014; Blackburn, 2018). The olfactory system in squamates such as snakes is highly specialized, and the MOS and VNS are well established (Døving and Trotier, 1998). The VNS in snakes comprises of a vomeronasal sensory epithelium that is thick and is coupled with a large well-defined VNO (often referred to as snake-VNO) (Halpern and Kubie 1980). The cellular organisation and delineation of the cell layers of the olfactory bulbs (MOB and AOB) are well defined in snakes (Iwahori *et al.*, 1989; Holtzman and Halpern, 1990; Kaczmarek and Rupik, 2021; Peng *et al.*, 2022; Assis and Souza, 2023). The VNS in other squamates such as lizards share many similarities with the olfactory system of snakes (Gregorovicova *et al.*, 2012; Ruiz–Monachesi *et al.*, 2020; Okuyama and Sasaki, 2021; Torres and Sanchez-Quinteiro, 2023). Lizards such as *Varanids* (i.e., monitor lizards) are known to possess a well-developed olfactory system that encompasses both the MOS and the VNS (Crosby and Humphrey, 1939). Whilst the olfactory systems in *Varanids* are well-established, there has been no comprehensive neuroanatomical account of the olfactory systems of the Rock Monitor (*Varanus albigularis*) and the Nile Monitor lizards (*Varanus niloticus*). Comparing the clearly defined and well established main and vomeronasal olfactory systems in a reptile such as *Varanids* to the poorly-understood olfactory system of the Nile Crocodile would provide useful information regarding both the differences and similarities between them.

1.1.4. The olfactory system of Nile Crocodiles

Crocodylians are semi-aquatic creatures whose morphology has sparked the interest of researchers for many years (Salisbury and Frey, 2000; Somaweera and Shine, 2013; Green *et al.*, 2014; Fleming, 2014; Darlim *et al.*, 2020). These reptiles play an important role in conserving ecosystems by providing important ecological benefits

such as regulating the aquatic population in the environment which aids in future regeneration of marine life (Lourenço-de-Moraes *et al.*, 2023). Due to their dominance, superior morphology and their efficient predatorial nature, crocodilians adapt relatively easy to their environments enabling them to occupy a large territories of fresh water (such as rivers and lakes) and salt water surroundings (Summers, 2015).

The use of the olfactory system in crocodilians is reported to be primarily involved in detecting food and to aid in hunting (Scott and Weldon, 1990; Eisthen, 1997). Crocodilians are able to locate decaying prey and carcasses in land or water over long distances which indicates a chemoattraction to food (Weldon and Ferguson, 1993). Olfaction in crocodilians is amplified by a movement known as gular pumping (Weldon *et al.*, 1992). Gular pumping is a process whereby the entire throat expands and contracts in order to pump air through the nasal cavity (Weldon and Ferguson, 1993). Crocodilians are known to increase the action of gular pumping when introduced to various scents of food indicating the ability to detect food particles through olfaction (Pooley, 1962; Weldon *et al.*, 1992; Weldon and Ferguson, 1993). The use of the olfactory system in crocodilians is fascinating because these reptiles are known to hunt prey in both aquatic and terrestrial environments (Pooley, 1962).

With regard to the neuroanatomy and functioning of the olfactory system of crocodilians, there is a lack of information addressing specific structures and their associated functions. From the limited research available, basic descriptions of the olfactory system and the associated target locations for olfactory signals has been reported (Hansen, 2007; Taniguchi *et al.*, 2011; Taniguchi and Taniguchi, 2014). Studies by Scalia *et al.*, (1969) and Hansen (2007) reported that the olfactory systems in caimans and alligators possess an established main olfactory system that is comprised of structures such as a large MOB, a main olfactory epithelium and olfactory tracts that project olfactory signals to various target locations such as the anterior olfactory nucleus, piriform lobe cortex, hippocampal cortex, and various other regions in the brain (Scalia *et al.*, 1969; Hansen, 2007). The general description of the MOB and its associated cell layers in crocodilians is consistent with other descriptions of the MOB in all vertebrates (Crosby, 1917; Scalia *et al.*, 1969; Jacobs, 2012).

The lack of a VNS in crocodilians is demonstrated through missing anatomical

structures that make up the system. In early embryology, the vomeronasal organ of the VNS is known to degenerate prior to hatching (Parsons, 1970). However, in crocodilians it may be possible that these reptiles possess the VNS. Through studies, the following components have been identified in the Nile Crocodile: the accessory olfactory bulb (Ngwenya *et al.*, 2017) and the nucleus sphericus (Ngwenya *et al.*, 2017; Billings *et al.*, 2020a). These components identified are all structural elements of the vomeronasal system in reptiles. The presence of these structures indicate the possible presence of a VNS in crocodilians.

1.1.5. Chemoreception in Reptiles and its potential role in pheromonal communication in Crocodilians

Processing of chemical cues in the vomeronasal system has been shown to be crucial for pheromonal communication (Burghadt, 1980; Burghadt 1993; Schwenk, 1993; Cooper, 1997; Teshera and Clark , 2021; Crowe-Riddell and Lillywhite, 2023). It is well documented that the VNS (in response to pheromonal cues) is known to play a pivotal role in regulating many animals' behavioural traits and characteristics in squamates (Burghadt, 1980; Mason and Parker, 2010; Mendyk *et al.*, 2020; Ruiz–Monachesi *et al.*, 2020; Van Moorlegghem, 2020; Teshera and Clark, 2021). The ability to process chemosensory information enables these reptiles to have successful social interactions (Ruiz-Monachesi *et al.*, 2020; Gillingham and Clark, 2023). Chemoreception is one of the main sensory modalities utilised by squamates to navigate their environment, detect prey and communicate with conspecifics (Cooper, 1989; Cooper, 1997; Pathak *et al.*, 2015; Glaudas *et al.*, 2019). Chemoreception is enhanced in squamates by the action of tongue flicking (a behavioural trait exhibited by these reptiles in response to chemical signals) (Cooper Jr and Habegger, 2001). The difference in the rates of tongue flicking between different species of snakes and lizards indicates intra-species variation in response to different pheromonal cues (Cooper, 1997; Schwenk and Thewissen, 2008; Pathak *et al.*, 2015).

Chemoreception in crocodilians and the integration of chemosensory information is poorly understood (Pooley, 1962; Morpurgo *et al.*, 1993; Weldon *et al.*, 1993; Martín and López, 2011; Naumann, 2015; Soares and Bierman, 2017). Crocodilians appear to only possess a main olfactory system and the evidence for this is mainly behavioural

studies (Weldon *et al.*, 1992; Weldon and Ferguson, 1993). However, it is possible based on the presence of glands and secretion of pheromonal stimuli in these reptiles, crocodilians may possess a VNS (Weldon and Ferguson, 1993; Garcia-Rubio *et al.*, 2002).

Crocodilians were reported to possess glands that are capable of producing and secreting pheromones that are involved in mating and nesting activities (Weldon *et al.*, 1990; Weldon and Ferguson, 1993; Whyte *et al.*, 1999; Bauer and Reep, 2022). The ability to produce and secrete different pheromonal cues suggests that pheromonal communication is indeed possible in crocodilians (Garcia-Rubio *et al.*, 2002). In juvenile crocodilians, the action of gular pumping was enhanced in response to glandular secretions (Weldon and Ferguson, 1993). The enhanced rate of gular pumping seen suggests the role of olfaction in processing and interpreting these secretions. However, very little is known about behavioural responses such as the increased rate of gular pumping in response to glandular secretions in crocodilians to ascribe pheromonal properties (Weldon and Ferguson, 1993). The use of pheromones in crocodilians for mating and sexual behaviours remains poorly studied (Martín and López, 2011; Wyatt, 2017).

1.1.6. Re-evaluating the olfactory system in the Nile Crocodile

Previous studies present some inconsistencies when discussing the presence of the VNS in crocodiles (Parsons, 1970; Eisthen, 1992; Torres *et al.*, 2023). The VNS in reptiles consists of a VNO that contains sensory epithelium (which is made up of both sensory neurons and chemoreceptors) that use different transduction mechanisms that will selectively process chemical stimuli and is located in the anterior-inferior portion of the nasal septum (Luo and Katz, 2004; Eisthen and Wyatt, 2006). Chemical signals detected by the sensory neurons and chemoreceptors of the VNO extend to different regions of the accessory olfactory bulb (AOB) (Mucignat-Caretta *et al.*, 2012). From the accessory olfactory bulb, the efferent projections target a specialised structure found in reptiles, the nucleus sphericus (Lanuza and Halpern, 1998). The VNO, AOB and nucleus sphericus collectively form the main structural elements of the VNS in reptiles (Lanuza and Halpern, 1998). Structural elements of the VNS in reptiles such as the VNO are known to degenerate prior to hatching in crocodiles (Pooley,

1962; Parsons, 1970; Lanuza and Halpern, 1998). It is unclear what causes this structure to degenerate in early embryology. The majority of studies that identify the absence of a VNS in crocodilians are fixated on the same study by Parsons (1970) that shows that the VNO degenerates before hatching (Bertmar, 1981; Gauthier *et al.*, 1988; Weldon and Ferguson, 1993; Senter, 2002; Heiss *et al.*, 2023). Since the study done by Parsons (1970), there has been no current research that has tried to understand if crocodilians possess the vomeronasal organ or not. The VNS is made up of other structures some of which have been identified putatively for e.g. a putative AOB was identified in the Nile Crocodiles (Ngwenya *et al.*, 2017) and a unique amygdaloid nucleus directly tied to the AOB and subsequently the VNS in reptiles, the nucleus sphericus (Ngwenya *et al.*, 2017; Billings *et al.*, 2020a). To date, the only study that suggests a putative AOB in crocodilians is Ngwenya *et al.*, (2017). As such one of the main goals of the present study was to provide a complete neuroanatomical account of the crocodilian olfactory bulbs to identify if an AOB does in fact exist within these animals.

The ability to secrete chemical signals that are involved in mating and nesting in crocodilians suggests that there is a system that these reptiles possess in order to interpret this sensory information (Parsons, 1970; Garcia-Rubio *et al.*, 2002). Behavioural responses such as the gular pumping may provide insights into how the olfactory system is possibly utilised in order to interpret these pheromonal cues (Weldon and Ferguson, 1993; Lanuza and Halpern, 1998). However, the ability to interpret pheromonal stimuli in crocodiles could potentially indicate that crocodiles might be using some version of a VNS or that crocodiles do not depend on the VNS for pheromonal communication (Garcia-Rubio *et al.*, 2002). If crocodilians do in fact possess an AOB, there is a strong possibility that it might have some form of a VNS that hasn't been identified.

In squamates, there are many shared similarities between their vomeronasal systems (Gregorovicova *et al.*, 2012). This includes the overall organisation of the VNO specifically looking at the distribution of cells in the vomeronasal sensory epithelium and the position of the VNO in the nasal cavity (Ubeda-Bañon *et al.*, 2011; Taniguchi and Taniguchi, 2014). The cellular organisation, delineation of the cell layers and the olfactory tracts of the central and peripheral parts of the vomeronasal (accessory)

olfactory system are well established in squamates (Iwahori *et al.*, 1989). It is also evident that squamates possess a well-developed AOB that is anatomically distinct from the MOB (Nieuwenhuys, 1966; Drakory, 2011). Anatomical descriptions of the crocodilian MOS have been restricted to caimans and alligators (i.e. Alligatoridae). Studies report that both species possess a large MOB with long olfactory tracts (Scalia *et al.*, 1969; Hansen, 2007). In both cases, the presence of a clear vomeronasal organ or an accessory olfactory bulb was not found. However, earlier studies indicate that either crocodilians did possess components of the VNS such as the VNO in early embryology (Parsons, 1970). The presence of this structure in early embryology suggests that at some point in time, the VNO was a utilised sensory organ. In the VNS, the VNO relays signals to be processed in the AOB (Lanuza and Halpern, 1998). This means that if the VNO was present in crocodilians, there must have been a processing centre (i.e., an AOB) that received chemical signals from the VNO.

In summary, when considering the Sauropsida, the Lepidosaur (specifically squamates) possess a well-established olfactory system with a main olfactory system and a vomeronasal olfactory system identified to varying degrees in these species (Burghadt, 1980). However, when we focus on the archosaurian group, birds only possess the main olfactory system and no vomeronasal olfactory system, testudines present a main olfactory system and a different type of vomeronasal system and crocodilians are suggested to have a vomeronasal system based on the identification of a putative AOB (Ngwenya *et al.*, 2017) and nucleus sphericus (Ngwenya *et al.*, 2017; Billings *et al.*, 2020a). By investigating the olfactory bulbs anatomically, the current study will be able to add to a growing body of knowledge to understand if the VNS is present in crocodilians and if so, to what degree.

1.2. AIM

The aim of the study was to identify and describe the chemoarchitecture of the olfactory bulbs in two different reptile taxonomic groupings - one in the Squamate group where the vomeronasal/accessory olfactory system is apparently well-established, monitor lizards (*Varanus albigularis* and *Varanus niloticus*) and one in a Crocodilian group where the VNS is suspected to be absent, Nile Crocodiles (*Crocodylus niloticus*).

1.3. OBJECTIVES

- 1.3.1. To create a neuroarchitectural outline and provide a description of the immunohistochemical organisation of the olfactory bulbs in the Nile Crocodile, the Rock Monitor and Nile Monitor lizards.
- 1.3.2. To draw a comparison between the cellular organisation of the olfactory bulbs of the Nile Crocodile and the Rock and Nile Monitor lizards.
- 1.3.3. To identify anatomically if the AOB is present in the Nile Crocodile.

2. METHODOLOGY

2.1. Ethical clearance

Ethical clearance (appendix A) was obtained from the Wits University animal research ethics committee (AREC22-10-002A) for the use of the Rock Monitor lizards in this study. An adaption or modification and extension to experiments (AM&E) application was requested thereafter for the inclusion of Nile Monitor lizards to also be used in the study (appendix B). This study also utilized previously collected materials from 2 Nile Crocodiles (AREC clearance waiver: # 2011/14/2B; appendix C) that were sourced from the Thaba Kwena crocodile farm in Limpopo, South Africa.

2.2. Specimen Acquisition

The monitor lizard heads were sourced from Hoedspruit Reptile Centre (HRC), Limpopo, South Africa. Individuals that were injured were collected in Hoedspruit by the HRC veterinarian (Dr. Jessica Briner, SAVC registration no: D14/13120) and given necessary medical attention; when it was deemed that the injuries were too extensive, the animals were euthanized (see Appendix D). All necessary permits for the transport of the tissue were obtained (see Appendix E).

2.3. Sample demographics

Three monitor lizards (two *Varanus albigularis* and one *Varanus niloticus*) and two Nile Crocodiles (*Crocodylus niloticus*) were used in this study (refer to Table 1).

Table 1: Details for each of the Nile Crocodiles and Monitor lizards that were used in this study (Croc-Nile Crocodile, VA- Rock Monitor lizard and VN- Nile Monitor lizard).

Brain number (n=5)	Sex	Body weight(g)	Brain weight (g)	Snout-to- tail length (cm)
Croc 1	Male	3260	4.168	96.5
Croc 2	Male	28080	5.908	190
VA 1	Male	645	1.2	70
VA 2	Male	2980	2.3	162
VN 1	Female	1550	1.8	115.1

2.4. Sample preparation

Following euthanasia, the monitor lizards were decapitated and the eyes and all soft tissue (e.g. skin, muscle, tendons and ligaments) were carefully removed, exposing the skull. The heads were then immersion fixed in 4% paraformaldehyde (PFA) in 0.1M phosphate buffer (PB) solution and stored at 4°C. Following this, the tissue was then transported to the School of Anatomical Science, Health Science Faculty, University of the Witwatersrand, Johannesburg for the removal of the brain. The brain was dissected out of the skull and post-fixed at 4°C for 24 hours in 4% PFA before being cryoprotected in a solution of 30% sucrose in 0.1M phosphate buffer at 4°C for at least 72 hours and stored in an antifreeze solution consisting of ethylene glycol and glycerol at -20°C until use.

2.5. Dissection Procedures

Removal of the brain:

The posterior dorsal skin was excised using a scalpel to allow for the base of the skull and spinal column to be visible. In order to expose the brain, the membranes (i.e. the meninges) covering the gap between the skull and spinal cord were excised using a scalpel and a pair of scissors. The skin on the dorsal surface of the head was removed by cutting through the postorbital bone and into the eyes using scissors. Once the skin was removed, the muscle tissue on the sides of the skull were removed. There was no bone protecting the sides of the brain so extreme caution was used. The posterior portion of the skull was then removed. Rongeurs were then used to break apart the upper surface of the skull of the lizard to expose the brain. (see Fig. 3)



Figure 3: The “exposed” Rock Monitor lizard brain during dissection protocols. The arrow indicates the brain.

Removal of the olfactory bulbs:

The frontal bone covering the olfactory bulbs and tracts were carefully removed. The olfactory bulbs are positioned anterior to the eyes, therefore extreme caution was used when exposing the olfactory bulbs and tracts. The dura mater was removed from the brain. The olfactory bulbs were then carefully removed from the vomeronasal and main olfactory nerves. The ventral nerves, the optic chiasm (which resides on the inferior aspect of the brain) and the pituitary gland of the brain were then severed using scissors which allowed the brain to be separated fully from the head. Both olfactory bulbs were used from the Rock Monitor and Nile Monitor lizard.

2.6. Tissue processing

The olfactory bulbs were dissected from the rostral aspect of the telencephalon, removed from the freezer, and placed in 30% sucrose at 4°C for approximately 3 days; after which the tissue was frozen and sectioned at 50 μ m thickness using a cryostat microtome in the sagittal (specimen VA1, VN1 and Croc 2) and coronal planes (specimen VA1, VA2, VN1 and Croc 1) (Leica Biosystems, CM1860 cryostat, 2016). A total of six series was collected for immunohistochemical detection and Nissl staining for all specimens (see Table 2).

2.7. Immunohistochemistry

A total of five series was collected for immunohistochemical detection (Table 2). For immunohistochemistry, the sections were treated with an endogenous peroxidase inhibitor (1.6% of 30% H₂O₂, 49.2% methanol: 49.2% 0.1M Phosphate Buffer (PB)) followed by three 10-minute rinses in 0.1 M PB. The sections were incubated in a blocking buffer solution that contained Triton X 0.25% in PB (0.1M), 3% of the normal goat serum and 2% bovine serum albumin (BSA) for 2 hours at room temperature. The sections were then transferred to a primary antibody solution that contained the appropriate dilution (see table 2) of the primary antibody in the blocking buffer solution and incubated for 48 hours at 4°C (Billings et al., 2020). Following this, the sections were rinsed 3 times in 0.1 M PB (10 min each) and incubated at room temperature for 2 hours in a solution of 0.1M PB, 3% normal goat serum, 2% BSA and 1:1000 dilution of the appropriate biotinylated secondary antibody. Following further rinses, these sections were then incubated in an avidin-biotin solution (Vectastain® Elite ABC Kit, Vector Laboratories, Burlingame, Ca) for 1 hour at room temperature. The sections were then rinsed a further 3 times (10 min each) before chromogenic precipitation using 3,3'- Diaminobenzidine (DAB) substrate kit (ab64238; Abcam). Once the desired staining intensity had been achieved, the reaction was stopped by placing the sections in PB. Thereafter, the sections were mounted on 0.5% gelatin-coated glass slides and were then dehydrated through a graded series of alcohol and cleared in xylene before being coverslipped with Depex mounting medium.

Negative controls were performed by omission of either the primary or secondary antibody.

The final series collected was stained for Nissl substance. The sections were mounted on 0.5% gelatine coated slides and allowed to dry for at least 72 hours. Sections were put in a defatting solution consisting of 50% methanol and 50% chloroform overnight, before being rehydrated in a graded series of alcohol and stained with a 1% cresyl violet solution. The stained sections were then differentiated in 70% alcohol with 0.2% acetic acid until the desired contrast was reached, following which they were dehydrated in a graded series of alcohols, cleared in xylene and cover slipped using depex mounting medium.

Table 2: Table showing the different antibodies used in the immunohistochemical procedures at the appropriate dilutions.

Target Protein	Species	Immunogen	Supplier	Lot number	References	Dilution
CR (Rabbit anti Calretinin)	Rabbit	Produced in rabbits by immunization with recombinant human calretinin containing a 6-his tag at the N-terminal.	Swant	7697	Schwaller <i>et al.</i> , (1993) Hendry <i>et. al.</i> , (1989)	1:2500
CB (Rabbit anti-Calbindin D-28k)	Rabbit	Recombinant rat calbindin D-28k.	Swant	CB-38a	Airaksinen <i>et al.</i> , (1997) Hendry <i>et. al.</i> , (1989)	1:2500
PV (Straight anti-serum classic)	Rabbit	Recombinant rat parvalbumin.	Swant	PV 27	Celio and Heizmann, (1981) Hendry <i>et. al.</i> , (1989)	1:2500
Gα_0 (A2)	Mouse	Raised against G α_0 of bovine origin.	Santa Cruz biotechnology	sc-13532	Lau and Cherry, (2000) Spehr and Munger, (2009)	1:1500
NMDAR2B	Rabbit	Fusion protein from the C-terminus of the NR2B-subunit of rat NMDA Receptor	R&D Systems	PPS013	Grosshans and Browning, (2008)	1:750
Secondary Antibodies: 1) goat anti mouse	Mouse	Other immunogen type corresponding to Mouse IgG	Abcam	ab150113	Liu <i>et al.</i> , (2023)	1:1000
2) goat anti rabbit	Rabbit	N/A	Abcam	ab150077	Zhang <i>et al.</i> , (2023)	1:1000

2.8. Data Analysis

The Nissl-stained sections were examined using a low power stereomicroscope and the cytoarchitectural boundaries of each section were traced using a camera lucida. In the final step, the drawing was scanned and redrawn with the Canvas 8 (Deneba) drawing programme. The immuno-labelled sections were viewed under a low power dissecting microscope and the relative cellular density profiles were determined by observation (qualitative analysis) and stereology (quantitative analysis).

2.9. Stereological analysis

A quantitative analysis of the sections was performed to calculate the cellular density for calretinin, calbindin, parvalbumin and NMDA receptor 2B immunoreactive cells throughout the whole olfactory bulb of one individual from each of the species of interest. Three-dimensional virtual tissue sections were scanned using MBF Bioscience (Colchester, VT) system with a three - plane motorized stage, Zeiss.Z2 vario axiomager, and thereafter analysed using Stereo Investigator software (MBF,; 64-bit). The optical fractionator probe was used to count the cells and the counting grids/frames were optimised in order to ensure a coefficient of error below 0.1 (King *et al.*, 2002). Parameters were set to ensure a standardised unbiased cell count of the immunoreactive cells (Table 3).

Table 3: Stereological parameters used to estimate the number of cells for calretinin and calbindin the AOB of the monitor lizards.

<i>Stain</i>	<i>Brain</i>	<i>Sectioning plane</i>	<i>Counting frame (μm)</i>	<i>Grid size(μm)</i>	<i>Disector height (μm)</i>	<i>Coefficient of error</i>	<i>Measured defined mounted thickness (μm)</i>	<i>Section evaluation interval</i>	<i>No. of sections</i>
<i>CR</i>	VA 1	Coronal	200 X 200	200 X 200	14	0.05	33.4	16	7
	VN 1	Coronal	200 X 200	200 X 200	14	0.03	31	16	7
<i>CB</i>	VA 1	Coronal	200 X 200	200 X 200	14	0.04	30.1	16	7
	VN 1	Coronal	200 X 200	200 X 200	14	0.06	29.7	16	7

3. RESULTS

3.1. Key Findings

The Nissl, calcium binding proteins, NMDA receptors and G proteins activated successfully within all three reptile species. Neuroanatomical outlines of the olfactory bulbs were produced from Nissl staining for the Nile Crocodile and both monitor lizards. The main olfactory bulb was clearly seen in all three species and the accessory olfactory bulb was seen in the Monitor Lizards. A putative accessory olfactory bulb is present in the Nile Crocodile. The density profiles for the calcium binding proteins and NMDA receptors were produced for all three reptile species.

3.2. Rock Monitor and Nile Monitor lizards olfactory bulbs

In lizards, the olfactory bulbs are generally located in the nasal cavity with the AOB situated caudomedially to the MOB (Bruce, 2009). In the case of both monitor lizards, the AOB was much larger than the MOB (Fig. 4).



Figure 4: Image demonstrating the ventral view of the Rock Monitor lizard brain. The olfactory bulbs reside in the rostral aspect and the spinal cord is positioned caudally. (Abbreviations: MOB- Main Olfactory Bulb and AOB- Accessory Olfactory Bulb).

3.3. Rock Monitor Lizard olfactory bulb

The Rock Monitor (*Varanus albigularis*) olfactory bulbs presents with both the MOB and an AOB (Fig. 4 and 7). In cross-section the MOB is easily identified by the centrally positioned ventricle with all the olfactory bulb cell layers circumventing the ventricle

(Fig. 5). The layers described from the most external to internal aspects of the MOB include the following: The Olfactory Nerve Layer, Glomerular Cell Layer, External Plexiform Layer, Mitral Cell Layer, Internal Plexiform Layer, Granule Cell Layer and Sub-Ventricular Zone (Fig. 5 and 8). The olfactory nerve layer resides on the ventro-medial aspect of the olfactory bulb (Fig. 5 and 7A). The vomeronasal nerve is also seen on the medial aspect of the main olfactory bulb (Fig. 5). This vomeronasal nerve does not form part of the main olfactory bulb. The glomerular cell layer contains large glomeruli that forms a band throughout the olfactory bulb (See Fig 5). The plexiform layers are very thin cell layers in the MOB (Fig. 5 and 8) However, the external plexiform layer (which is adjacent to the glomerular cell layer) appears to be thicker than the internal plexiform layer (which is adjacent to the granule cell layer) in the MOB (see Fig. 5 and 8A). As the series progresses from rostral to caudal in the MOB, the external plexiform layer appears to decrease in thickness (Fig. 8A and 8F). The granule cell layer has a granular appearance with small, densely packed cells, while the mitral cell layer is characterised by large loosely arranged mitral cells (see Fig. 5). The granule cell layer appears to be one of the thickest cell layers in the MOB (Fig. 5 and 8E). In the MOB, apart from the plexiform layers, the rest of the cell layers do not appear to have any considerable changes in the thickness from rostral to caudal (Fig. 8). The MOB increases in size as the series progresses from rostral to caudal. (Fig. 7A-7C). The olfactory ventricle increases in size as the series progresses from the most rostral aspect to the most caudal aspect of the MOB (Fig. 7A-7C).

There is a rapid change in shape of the olfactory ventricle in the olfactory bulb at the end of the MOB series. The olfactory ventricle starts transforming into a crescent shaped structure and is gradually displaced to the lateral aspect of the olfactory bulb (Fig. 7D-7F). This change in shape of the ventricle indicates the transition from the main olfactory bulb to the accessory olfactory bulb.

The AOB is positioned further caudally to the MOB (see Fig. 4). The AOB appears to be considerably larger than the MOB in size (Fig.4). The AOB and MOB display the same major olfactory bulb cell layers (Olfactory Nerve Layer, Glomerular Cell Layer, External Plexiform Layer, Mitral Cell Layer, Internal Plexiform Layer, Granule Cell Layer and the Sub-ventricular Zone) and the differences seen between the cell layers of the two bulbs are minimal (Figure 5 and 6). In the MOB, the cell layers circumvent

the ventricle whereas in the AOB the cell layers reside adjacent to the laterally displaced olfactory ventricle (See Fig. 5 and 6, 8, 9 and 10). The cell layers in the AOB appear to be more loosely organised with less clear cellular boundaries separating the different cell layers (Fig. 6). The relative organisation of the cell layers differ between the MOB and AOB. The olfactory nerve layer in the AOB forms a large part of the bulb compared to the olfactory nerve layer in the MOB which is a thin layer encasing the entire bulb (Fig. 5 and 6). The VNN stems from the rostral part of the MOB and runs along the medial aspect of the MOB and then invaginates into the AOB (Fig. 7C, 7G, 7I and 9D). Similarly to the MOB, the glomerular cell layer contains large glomeruli that forms a band throughout the olfactory bulb (See Fig 6). The internal plexiform layer is much thicker than the external plexiform layer in the AOB than in the MOB (see Fig. 8A and 10D). The mitral cell layer is less defined in the AOB where the cells are more loosely organised in comparison to the MOB (see Fig. 5 and 6). The granule cell layer looks like the most superficial layer in the AOB whereas in the MOB it is the innermost layer (Fig. 5, 6, 8A, 9C and 10A). The shape of the olfactory ventricle in the AOB differs to the MOB where the ventricle in the AOB is crescent shaped and is positioned towards the lateral region of the olfactory bulb (Fig. 7A and 7G).

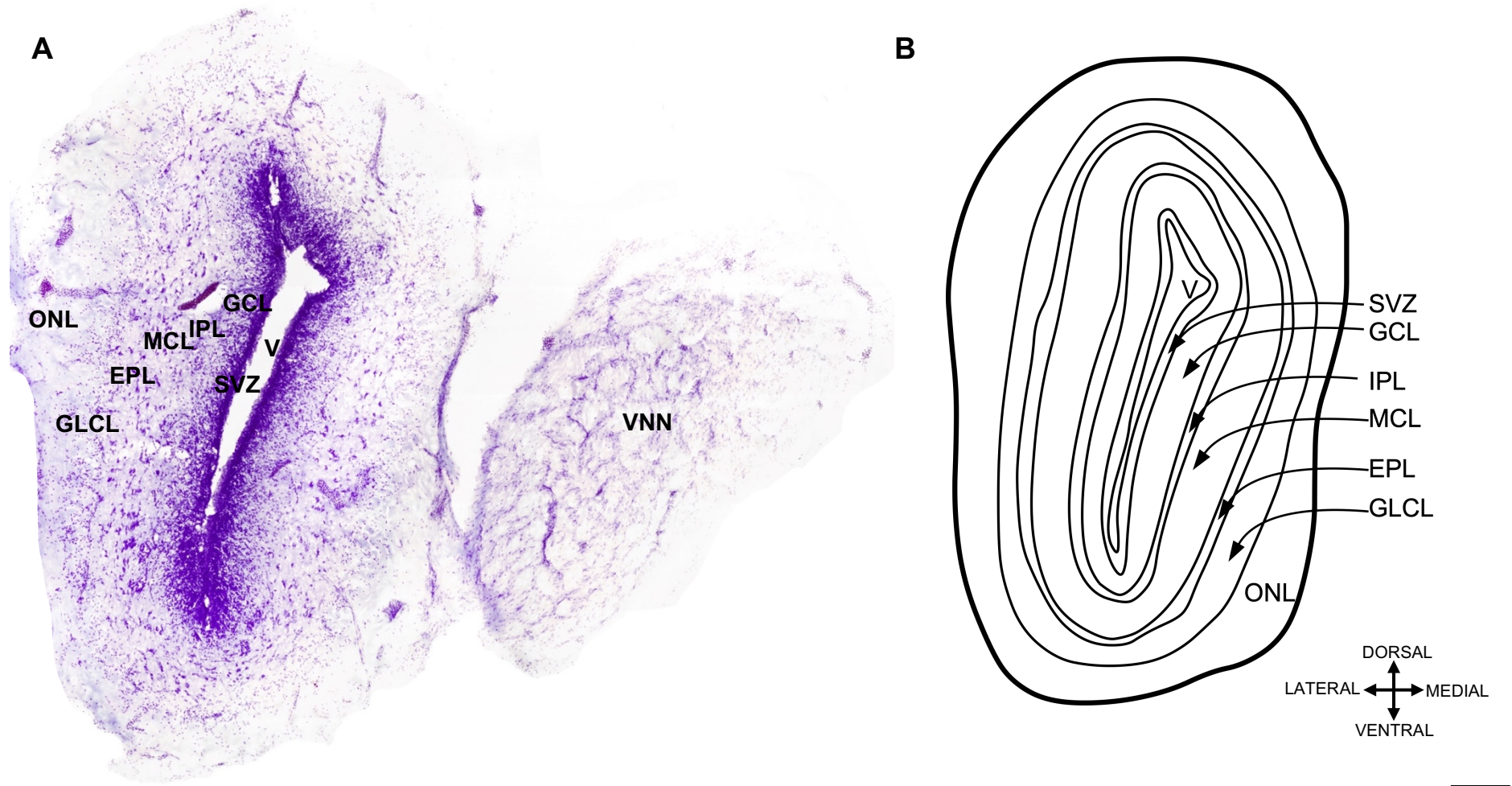


Figure 5: Coronal section of the Main Olfactory Bulb (MOB) of the Rock Monitor Lizard as produced by **A)** Nissl Staining at 20X Magnification and **B)** Line Drawing. (Abbreviations: Ventricle-V, Subventricular Zone-SVZ, Olfactory Nerve Layer-ONL, Glomerular Cell Layer-GLCL, External Plexiform Layers-EPL, Mitral Cell Layer-MCL, Internal Plexiform Layer-IPL and Granule Cell Layer-GCL). Scale Bar = 500 μ m.

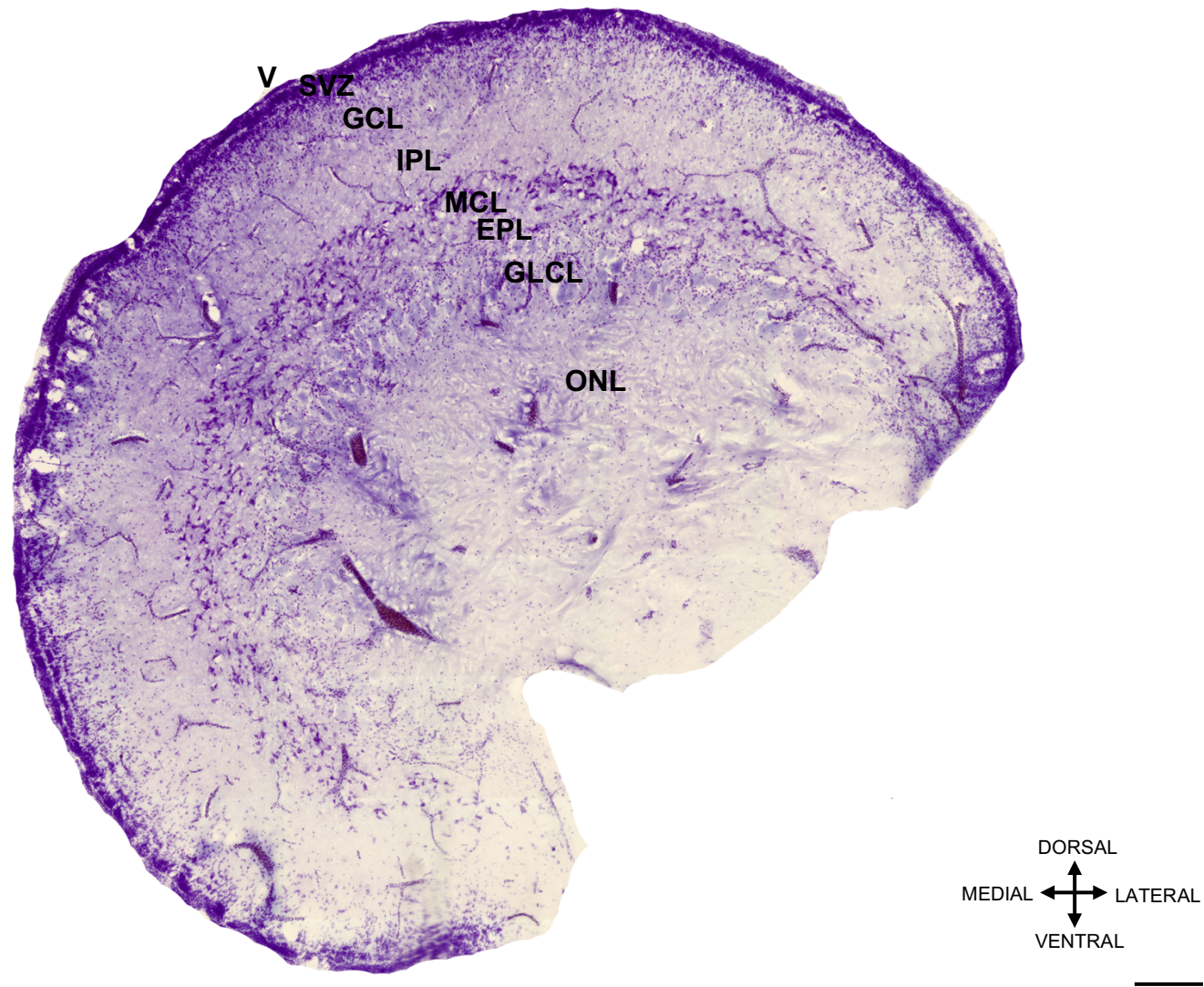


Figure 6: Coronal section of the Accessory Olfactory Bulb (AOB) of the Rock Monitor Lizard as produced by Nissl Staining at 20X Magnification. (Abbreviations: Ventricle-V, Subventricular Zone-SVZ, Olfactory Nerve Layer-ONL, Glomerular Cell Layer-GLCL, External Plexiform Layers-EPL, Mitral Cell Layer-MCL, Internal Plexiform Layer-IPL and Granule Cell Layer-GCL). Scale Bar= 500 μ m.

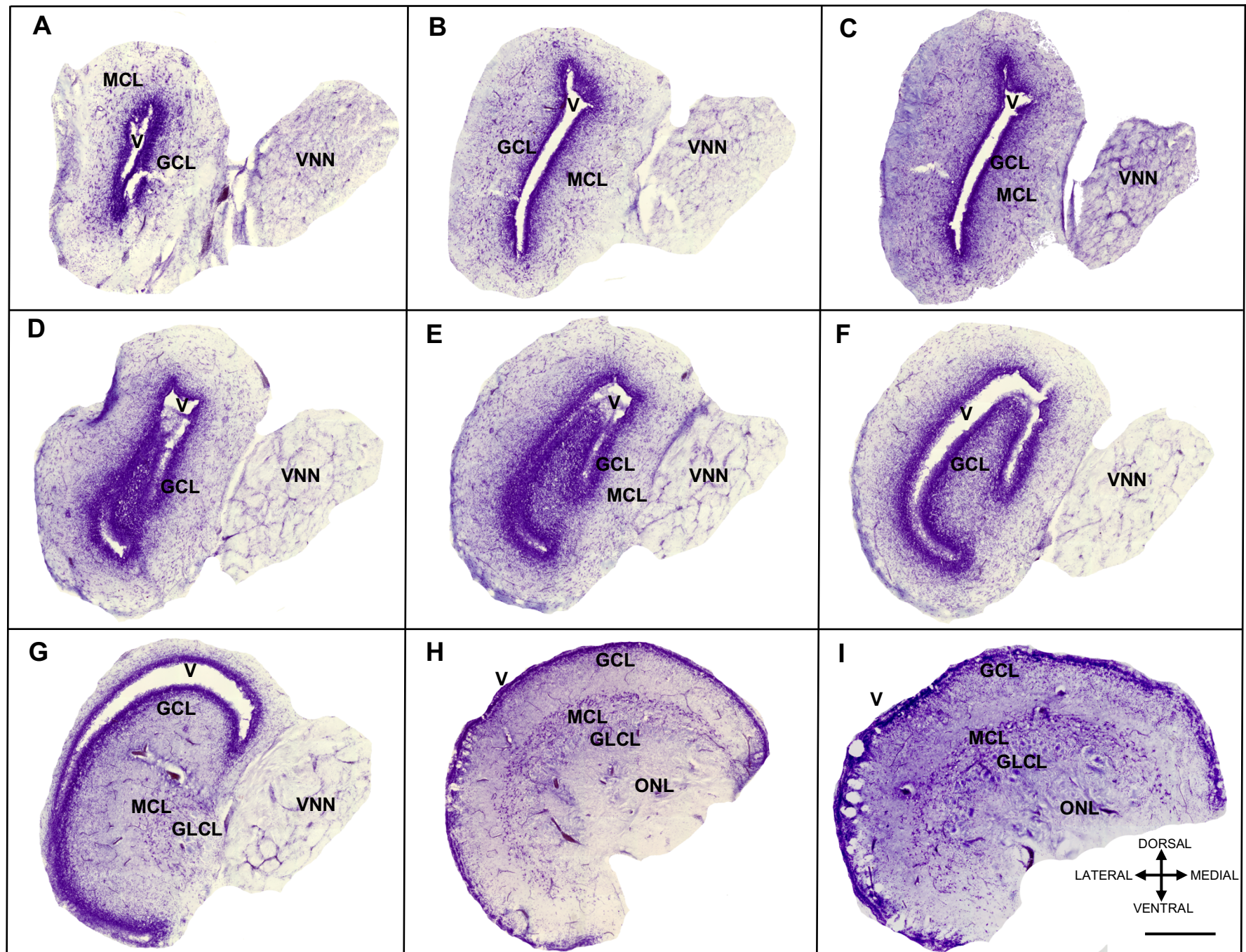


Figure 7: Coronal sections of the Rock Monitor lizard olfactory bulbs showing the change of shape of the olfactory ventricle from the MOB to the AOB as produced from Nissl staining at 20X magnification.

(A-C): Represents the main olfactory bulb. **A)** Most rostral aspect, **B)** Middle region and **C)** Most caudal aspect. As the series progresses in the main olfactory bulb, the olfactory ventricle becomes larger.

(D-F): Demonstrates the transition between the main olfactory bulb and the accessory olfactory bulb. In this series, the olfactory ventricle is forming a crescent shape and is gradually being displaced to the lateral region of the olfactory bulb.

(G-I): Represents the accessory olfactory bulb. **G)** Most rostral aspect, **H)** Middle region and **I)** Most caudal aspect. The olfactory ventricle is laterally displaced with all the olfactory cell layers residing adjacent to the ventricle.

(Abbreviations: GCL-Granule cell layer, MCL-Mitral Cell layer, GLCL-Glomerular cell layer, ONL-Olfactory nerve layer, V-Ventricle and VNN- Vomeronasal Nerve). Scale Bar = 500µm.

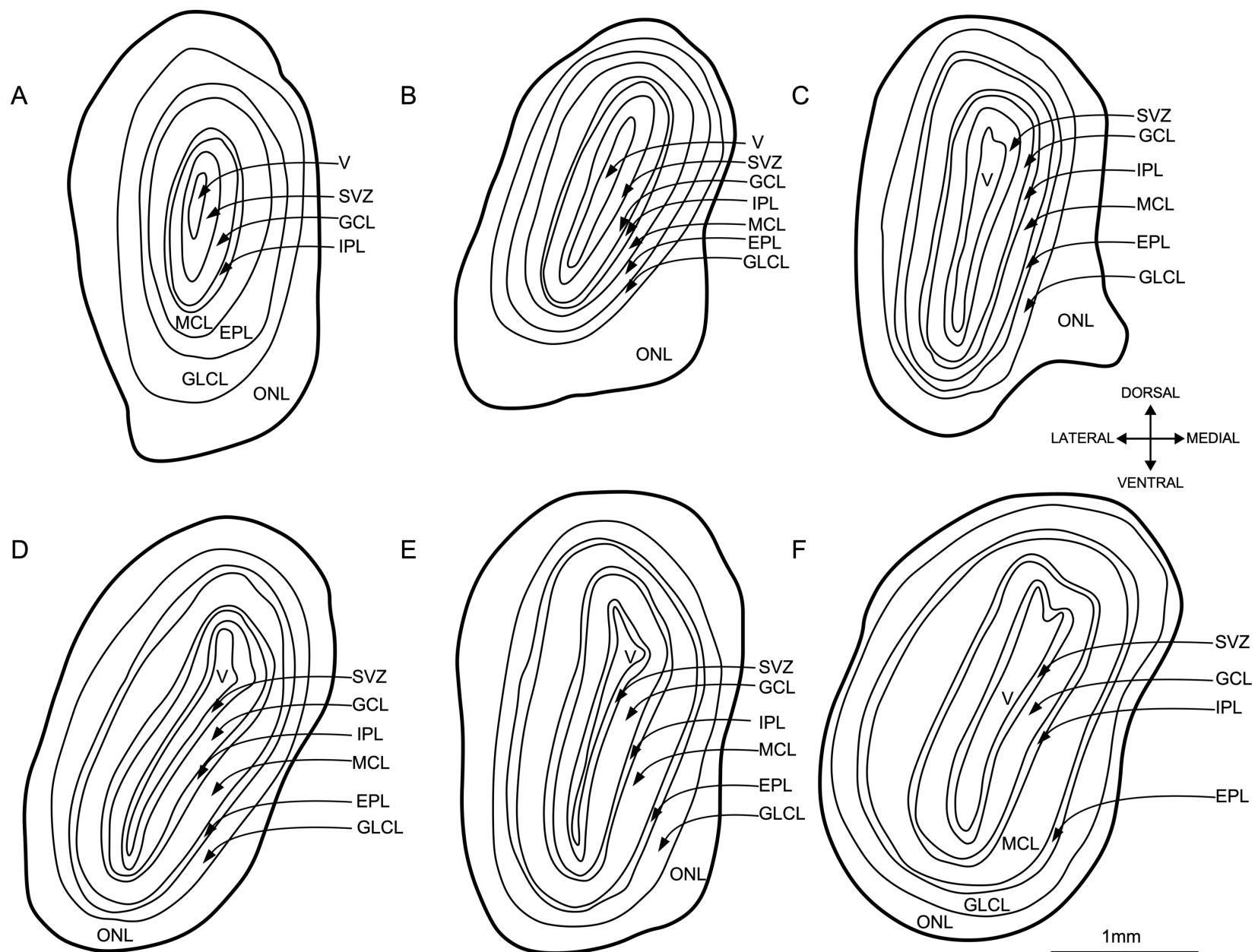


Figure 8: Neuroanatomical outlines of coronal sections of the Rock Monitor MOB (A-F: most rostral to most caudal) produced from Nissl stains at 5X magnification (abbreviations: GCL- Granule cell layer, IPL-Internal plexiform layer, MCL-Mitral Cell layer, EPL-External plexiform layer, GLCL-Glomerular cell layer, ONL-Olfactory nerve layer and SVZ-Subventricular Zone).

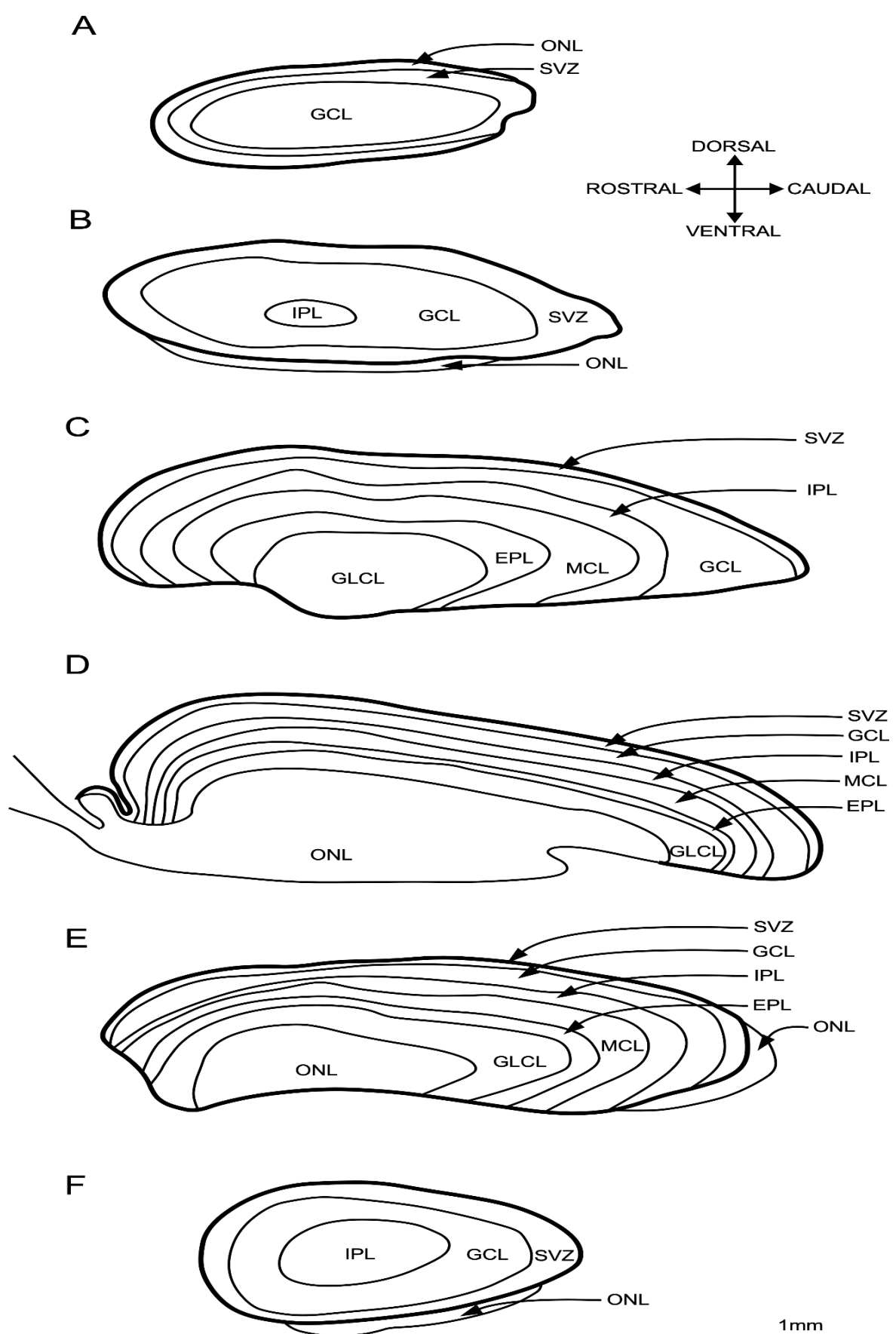


Figure 9: Neuroanatomical outlines of sagittal sections of the Rock Monitor AOB (A-F: most lateral to most medial) produced from Nissl stains at 2.5X magnification (abbreviations: GCL-Granule cell layer, IPL-Internal plexiform layer, MCL-Mitral Cell layer, EPL-External plexiform layer, GLCL-Glomerular cell layer, ONL-Olfactory nerve layer and SVZ- Subventricular Zone).

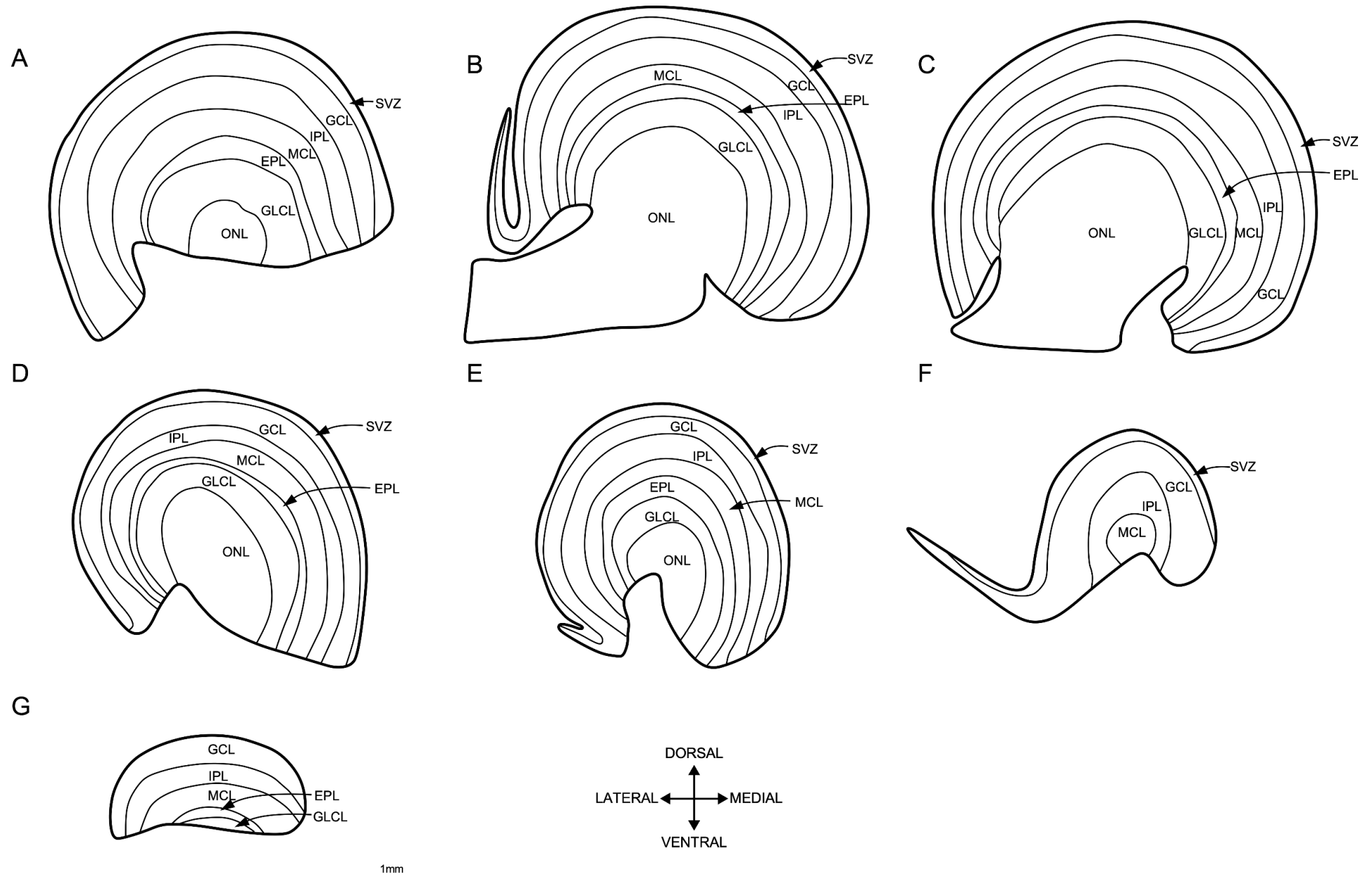


Figure 10: Atlas of the Rock Monitor AOB coronal sections (A-G: most rostral to most caudal) produced from Nissl stains at 6.3X magnification (abbreviations: GCL- Granule cell layer, IPL-Internal plexiform layer, MCL-Mitral Cell layer, EPL-External plexiform layer, GLCL-Glomerular cell layer, ONL-Olfactory nerve layer and SVZ-Subventricular Zone).

3.4. Nile Monitor Lizard olfactory bulb

The Nile Monitor Lizard (*Varanus niloticus*) accessory olfactory bulb is almost identical to the accessory olfactory bulb of the Rock Monitor lizard. The AOB possess the same major olfactory bulb cell layers with minimal differences seen in the expression of these layers between the Rock Monitor and Nile Monitor lizard (Fig. 7,8, 9 and 10). The cell layers in the AOB of the Nile Monitor Lizard are clearly demarcated with clear cell boundaries delineating the different cell layers (Fig. 9 and 10). The cell layers seen from the external aspect to internal aspect of the olfactory bulb are the ONL, GLCL, EPL, MCL, IPL, GCL and the SVZ (Fig. 9 and 10). The ONL resides ventromedially to the olfactory bulb and the GCL appears to be the thickest cell layer throughout the olfactory bulb (see Fig. 9 and 10).

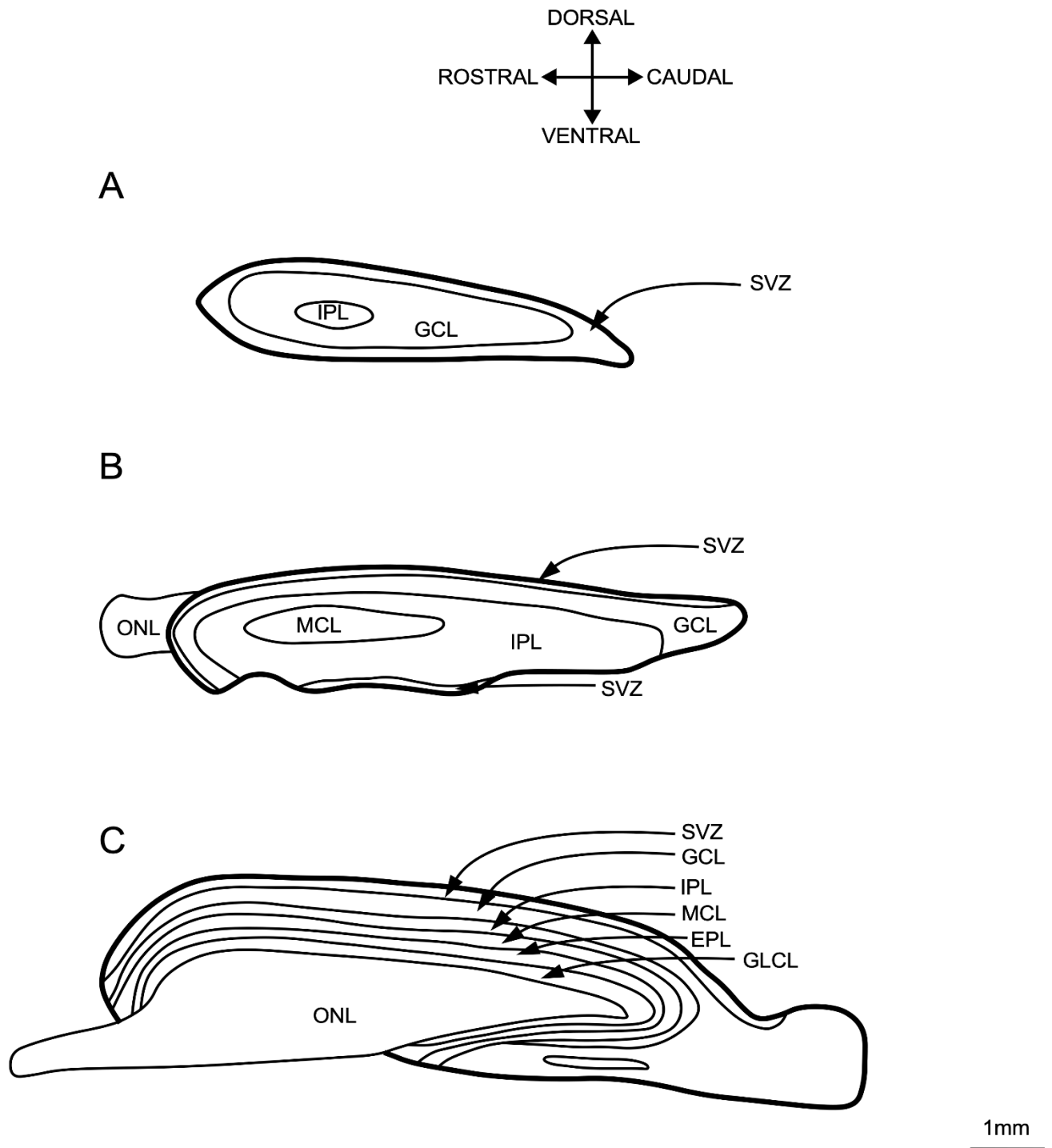


Figure 11: Neuroanatomical outlines of the sagittal Sections of the Nile Monitor accessory olfactory bulb (A-C: lateral to medial) produced from Nissl stains at 1.6X magnification (abbreviations: GCL- Granule cell layer, IPL-Internal plexiform layer, MCL-Mitral Cell layer, EPL-External plexiform layer, GLCL-Glomerular cell layer, ONL- Olfactory nerve layer and SVZ- Subventricular Zone).

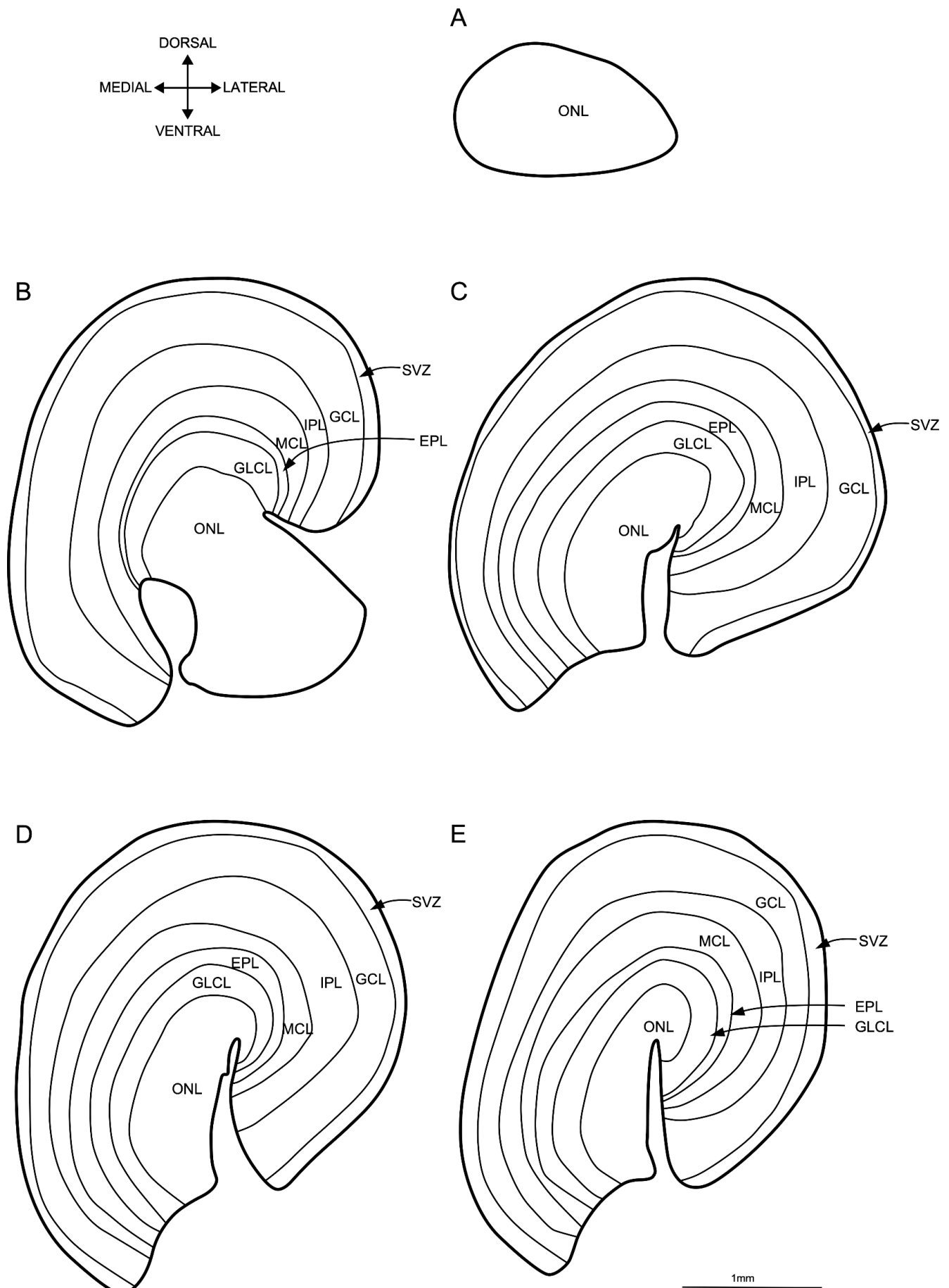


Figure 12: Atlas of the Nile Monitor accessory olfactory bulb coronal sections (A-E: rostral to caudal) produced from Nissl stains at 6.3X magnification (abbreviations: GCL- Granule cell layer, IPL-Internal plexiform layer, MCL-Mitral Cell layer, EPL- External plexiform layer, GLCL-Glomerular cell layer, ONL-Olfactory nerve layer and SVZ-Subventricular Zone).

3.5. Nile Crocodile olfactory bulb

The Nile Crocodile (*Crocodylus niloticus*) olfactory bulb displays clearly demarcated boundaries between cell layers (see Fig. 13, 14 and 15). Throughout the series, all the olfactory cell layers are easy to identify as they circumvent the centrally positioned ventricle (Fig. 11 and 12). All major olfactory cell layers are observed in these series (namely: the Olfactory Nerve Layer, Glomerular Cell Layer, External Plexiform Layer, Mitral Cell Layer, Internal Plexiform Layer and the Granule Cell Layer). (Fig. 13, 14 and 15). The SVZ is also evident in the Nile Crocodile olfactory bulb. However, this layer is relatively thinner in comparison to the other cell layers (Fig. 13). The olfactory nerve layer is the outermost layer of the olfactory bulb that encases the entire bulb (Figure. 13, 14). The external plexiform layer is much thicker than the internal plexiform layer (Fig. 14K). This is similar to what is seen in the MOB of the Rock Monitor lizard (Fig.8A). The internal plexiform layer eventually forms part of the olfactory tract in the Nile Crocodile (see Fig. 14H). The mitral cell layer forms a thin band around the olfactory bulb and separates the plexiform layers from each other (Fig.13). The GCL appears to be the thickest cell layer and is a cell dense layer that resides on the internal aspect of the olfactory bulb adjacent to the olfactory ventricle (Fig. 13 and 14E). It is also clear that the internal plexiform layer eventually forms part of the olfactory tract (Fig. 14G-14J). The MOB of the Nile Crocodile is very organised and is similar to the MOB of the Rock Monitor Lizard (Fig. 16). The cell layers in both species circumvent the olfactory ventricle and the same cell layers are seen in the same order from the external aspect (i.e., the olfactory nerve layer) to the internal aspect (i.e., the granule cell layer) (Fig. 16). The olfactory bulb in the Nile Crocodile appears to be homogenous (Fig. 13 and 14). We do not see similar structural changes occurring in the Nile Crocodile as seen in the Rock Monitor lizard.

There is an invagination that occurs on the medial aspect of the olfactory bulb (it appears as if the cell layers of the MOB are being pulled towards the olfactory ventricle) (Fig.15C and 18A). As the series continues this invagination turns into layers of its own (Fig. 15D and 18B). This invagination eventually turns into an independent set of layers that are clearly seen and is separate from the MOB cell layers (Fig. 15E, 15F and 18C). This independent set of layers eventually becomes fully encased in the ventricle (see figure 15F,15G, 17). The cell layers of the tissue encased in the ventricle

are very distinct with clear boundaries separating each cell layer (see Fig. 15E-15H and 17). This region we believe is a putative accessory olfactory bulb. Interestingly, in an older crocodile, there also appears to be a suspended piece of tissue that resides in the olfactory ventricle (14G-14I). In the series, after this invaginated tissue is fully encased in the ventricle, the rest of the olfactory bulb seems to return to its normal cellular structure with each cell layer forming its distinct cellular boundaries between them (see Fig. 15I-15J).

There is a possibility that a putative AOB does exist based on the fact that there is a unique region being identified that has its own set of cell layers identical to the MOB (Fig. 17).

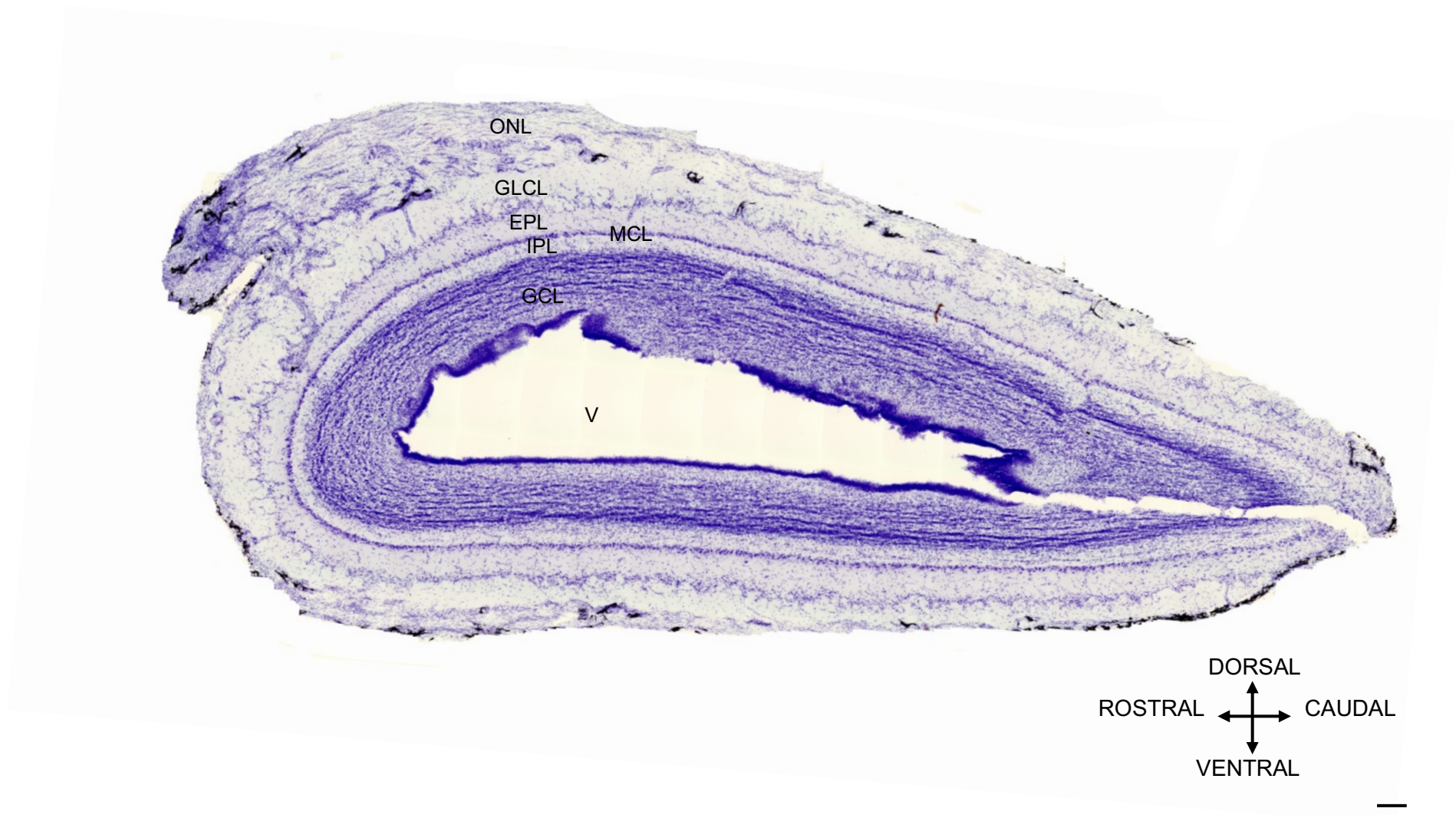
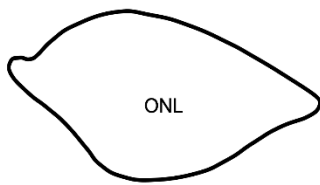
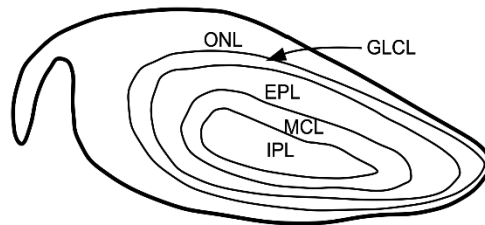


Figure 13: Nissl image of the sagittal section of the olfactory bulb in the Nile Crocodile at 20X magnification. (Abbreviations: Ventricle-V, Olfactory Nerve Layer-ONL, Glomerular Cell Layer-GLCL, External Plexiform Layers-EPL, Mitral Cell Layer-MCL, Internal Plexiform Layer-IPL and Granule Cell Layer-GCL). Scale Bar= 500 μ m

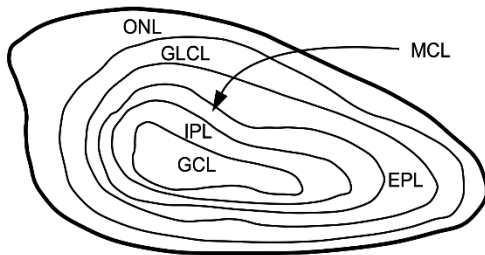
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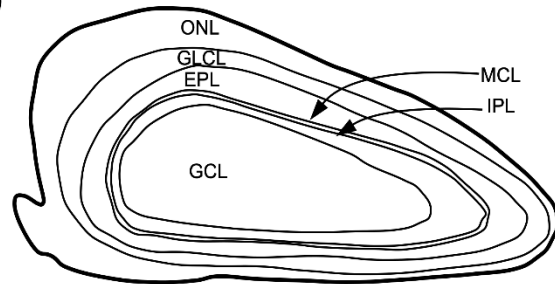
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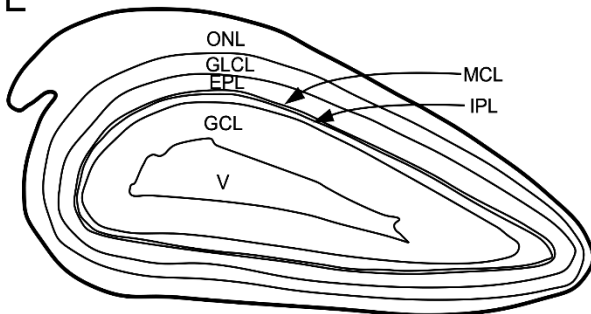
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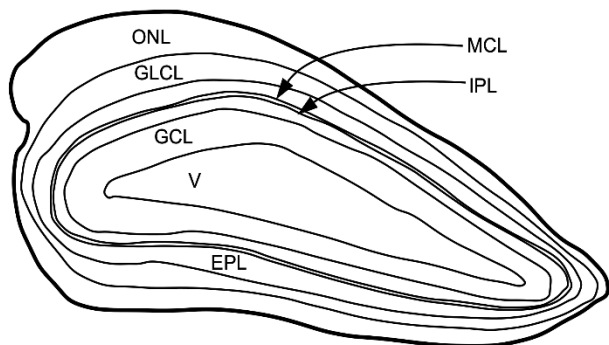
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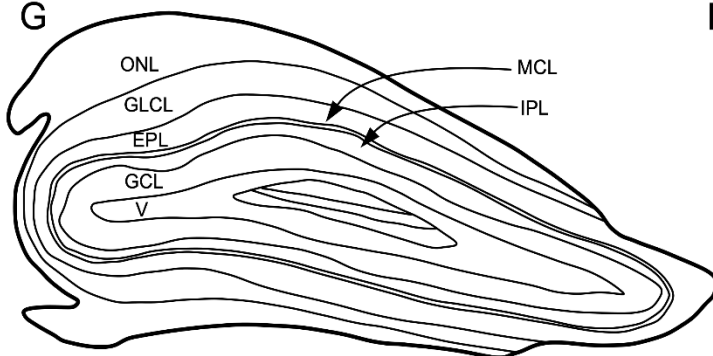
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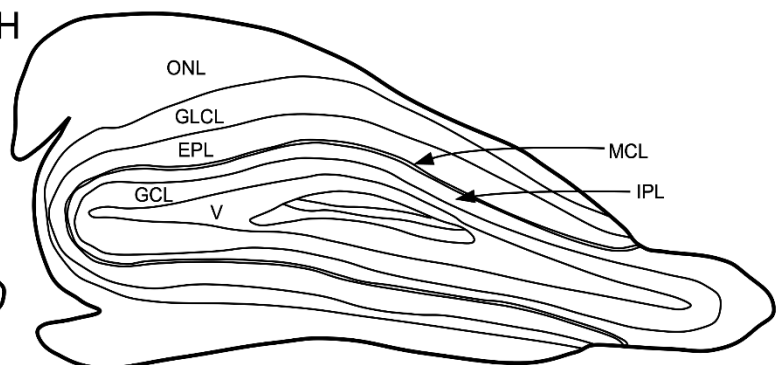
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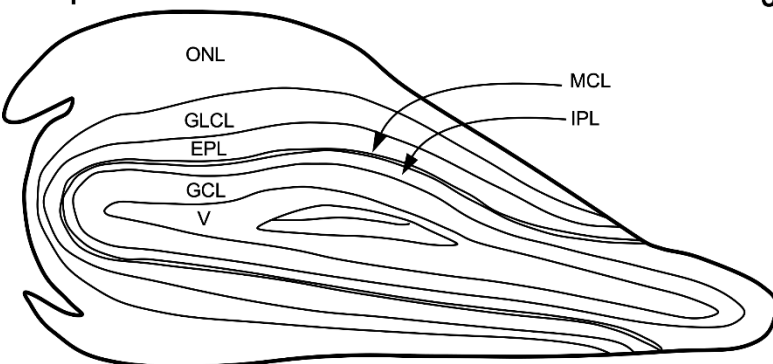
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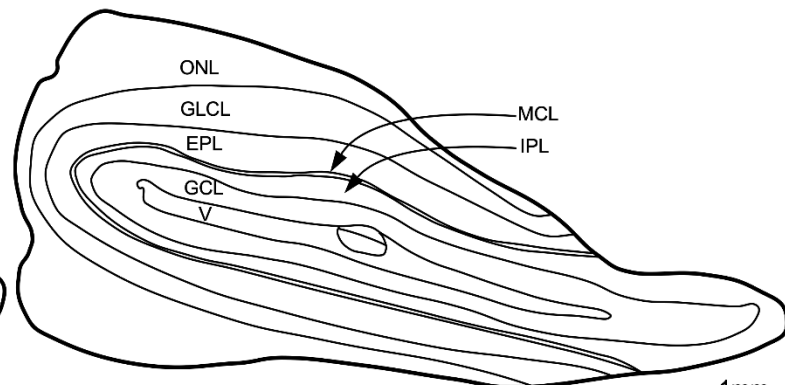
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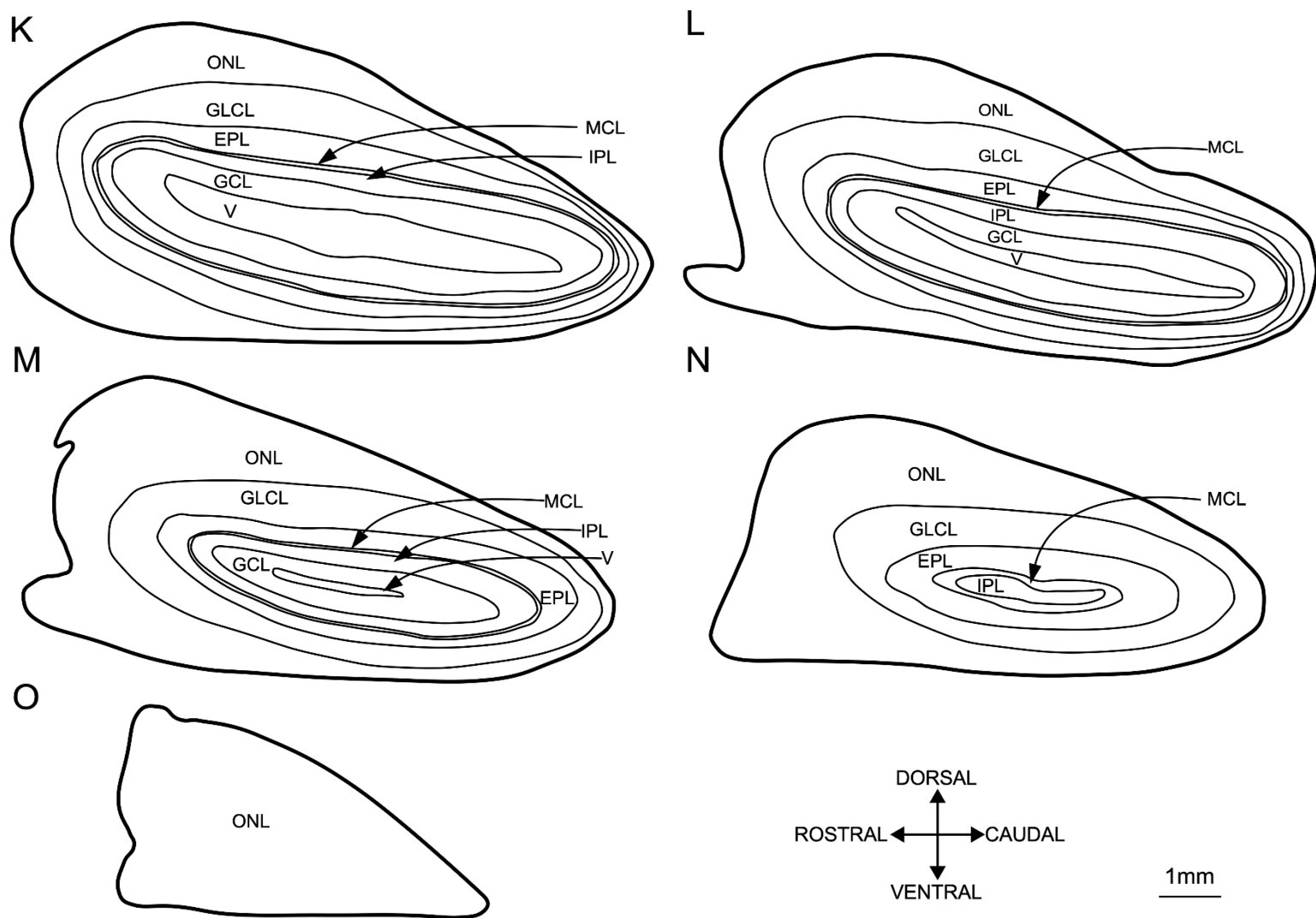
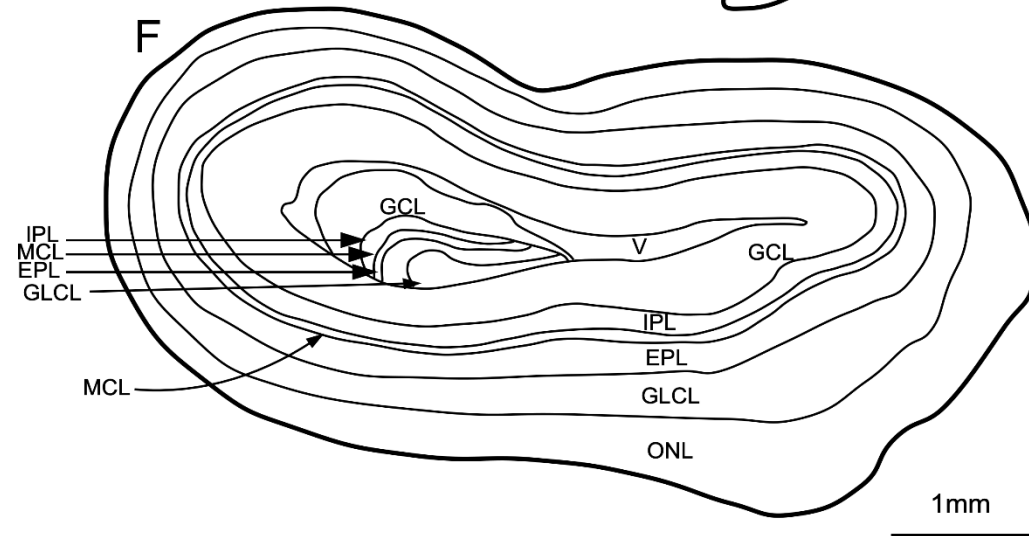
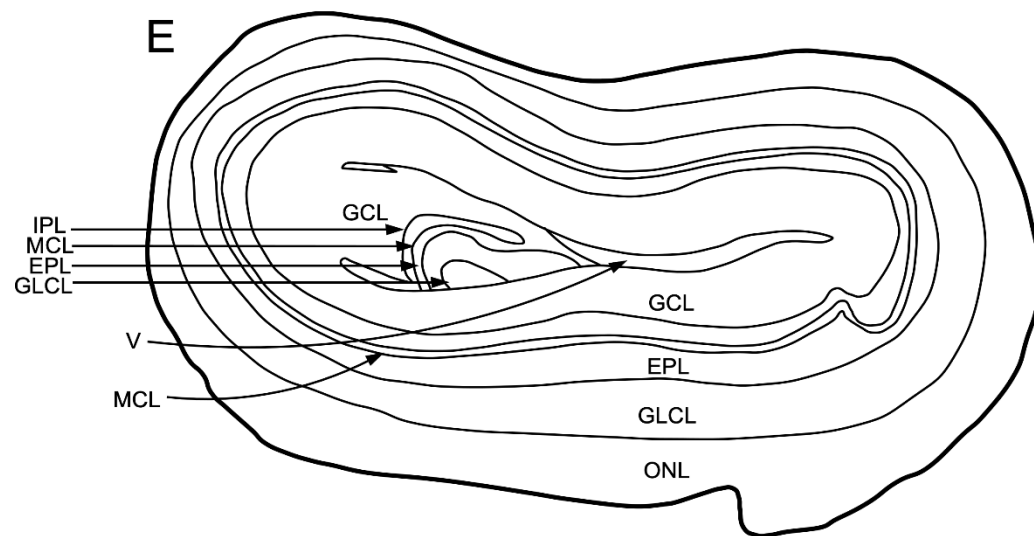
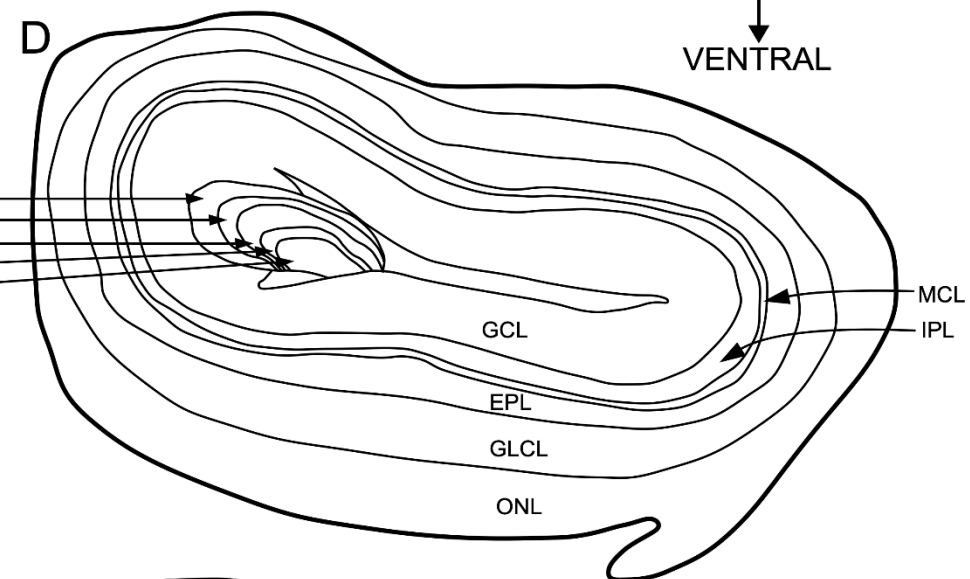
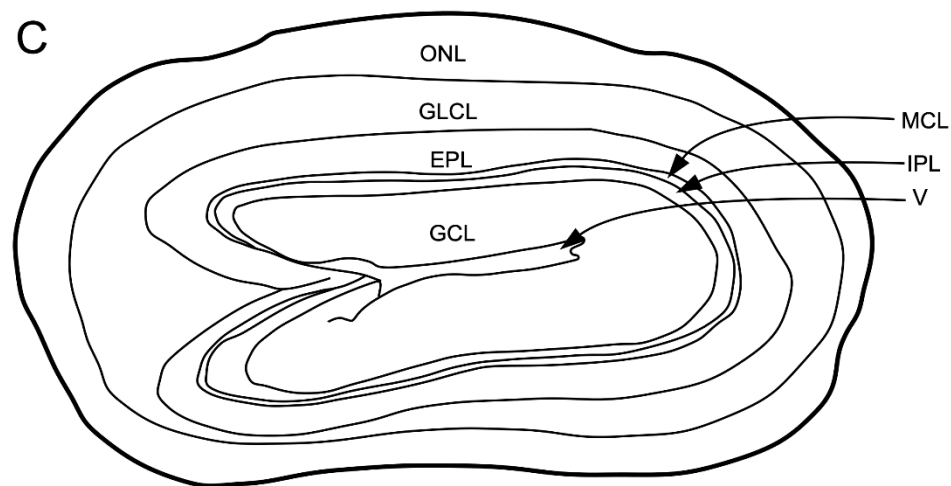
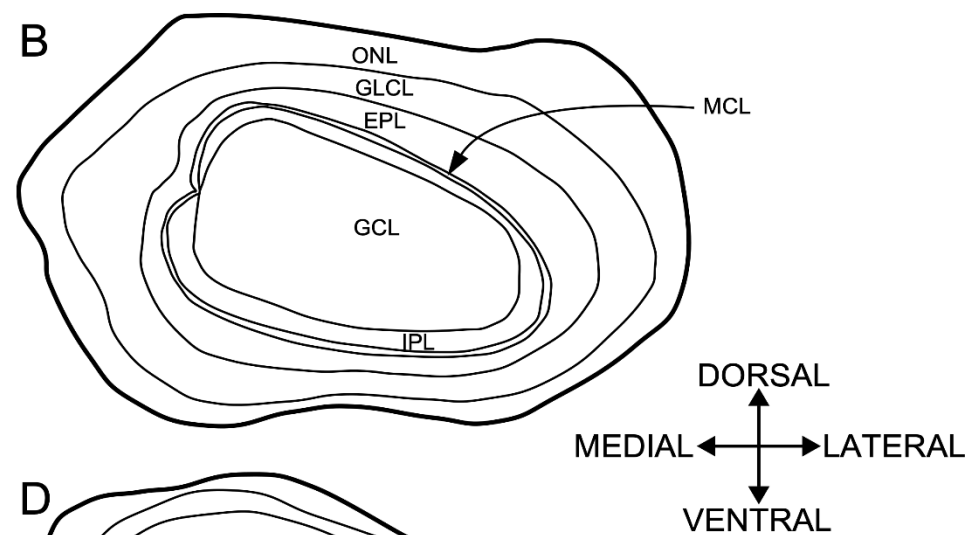
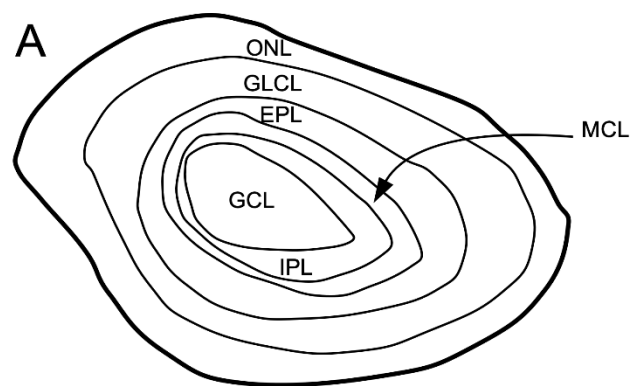


Figure 14: Neuroanatomical outlines of the sagittal Sections of the Nile Crocodile olfactory bulb (A-O: lateral to medial) produced from Nissl stains at 2X magnification (abbreviations: GCL- Granule cell layer, IPL-Internal plexiform layer, MCL-Mitral Cell layer, EPL-External plexiform layer, GLCL-Glomerular cell layer, ONL-Olfactory nerve layer and V-Ventricle).



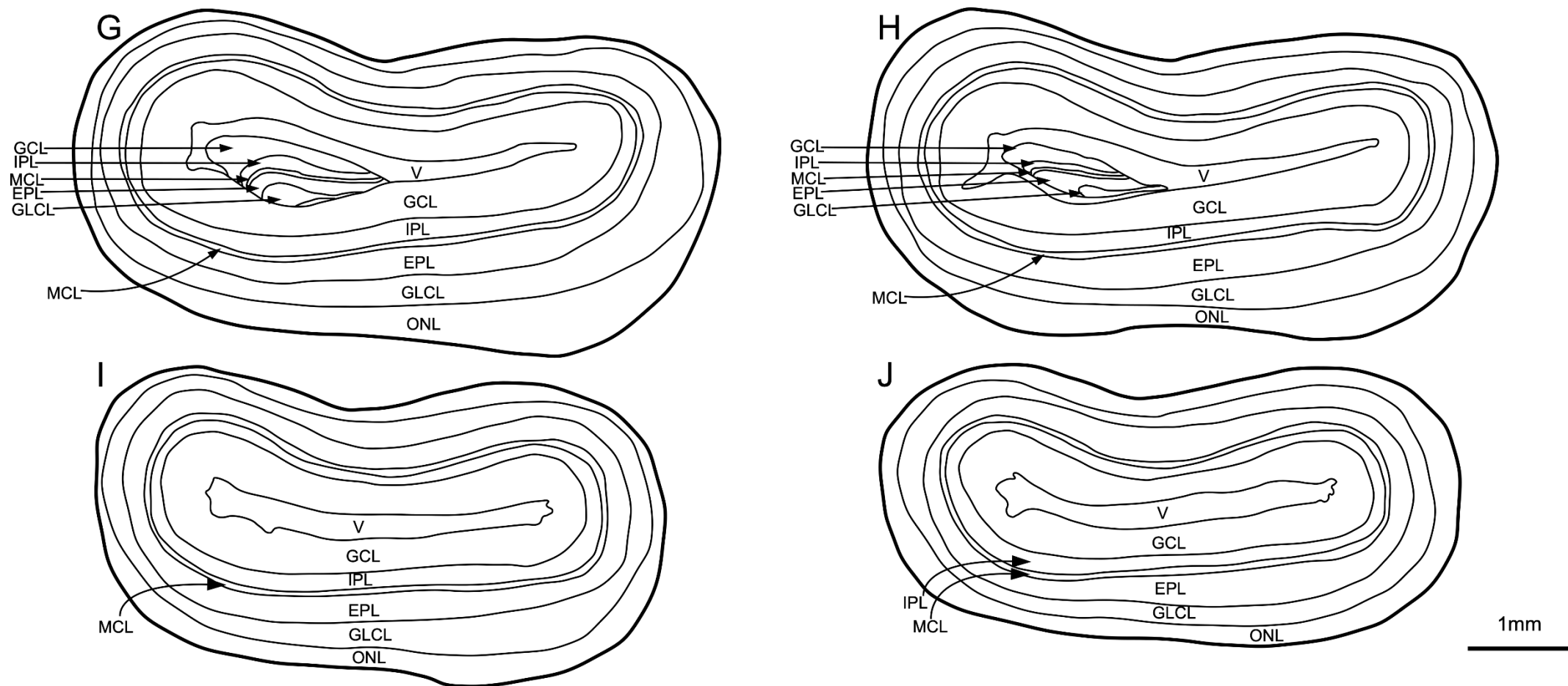


Figure 15: Atlas of the Nile Crocodile olfactory bulb coronal sections (A-J: rostral to caudal) produced from Nissl stains at 4X magnification (abbreviations: GCL- Granule cell layer, IPL-Internal plexiform layer, MCL-Mitral Cell layer, EPL-External plexiform layer, GLCL-Glomerular cell layer, ONL-Olfactory nerve layer and V-Ventricle).

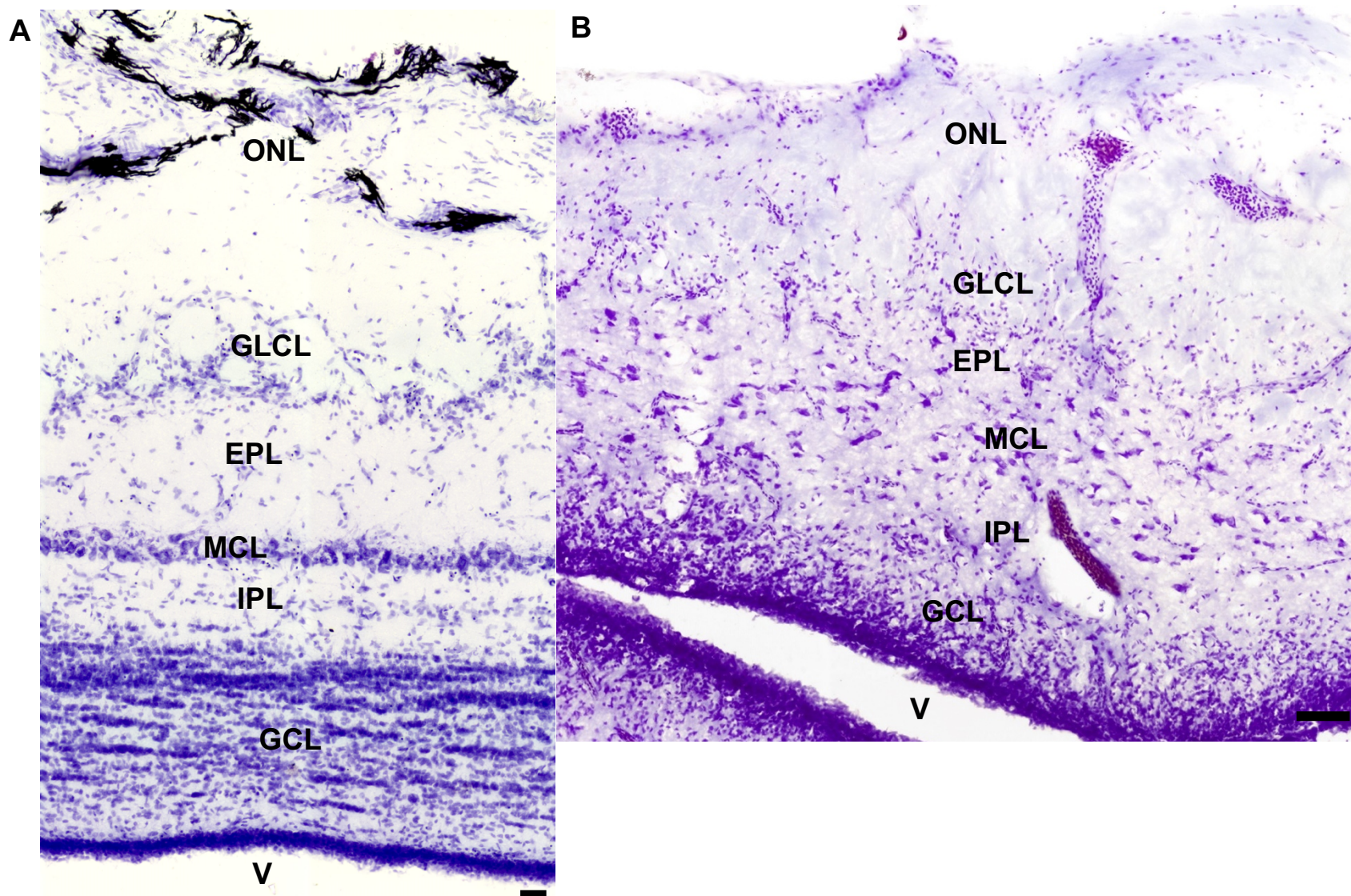


Figure 16: Comparison of the cellular organisation of Main olfactory bulb of the Nile Crocodile and Rock Monitor Lizard. **A)** Nile Crocodile Main olfactory bulb, **B)** Rock Monitor lizard main olfactory bulb. The cell layers appear to be more organised in the Nile Crocodile as opposed to the Rock Monitor lizard. (Abbreviations: Ventricle-V, Olfactory Nerve Layer-ONL, Glomerular Cell Layer-GLCL, External Plexiform Layers-EPL, Mitral Cell Layer-MCL, Internal Plexiform Layer-IPL and Granule Cell Layer-GCL). Scale Bar= 125µm at 20X Magnification.

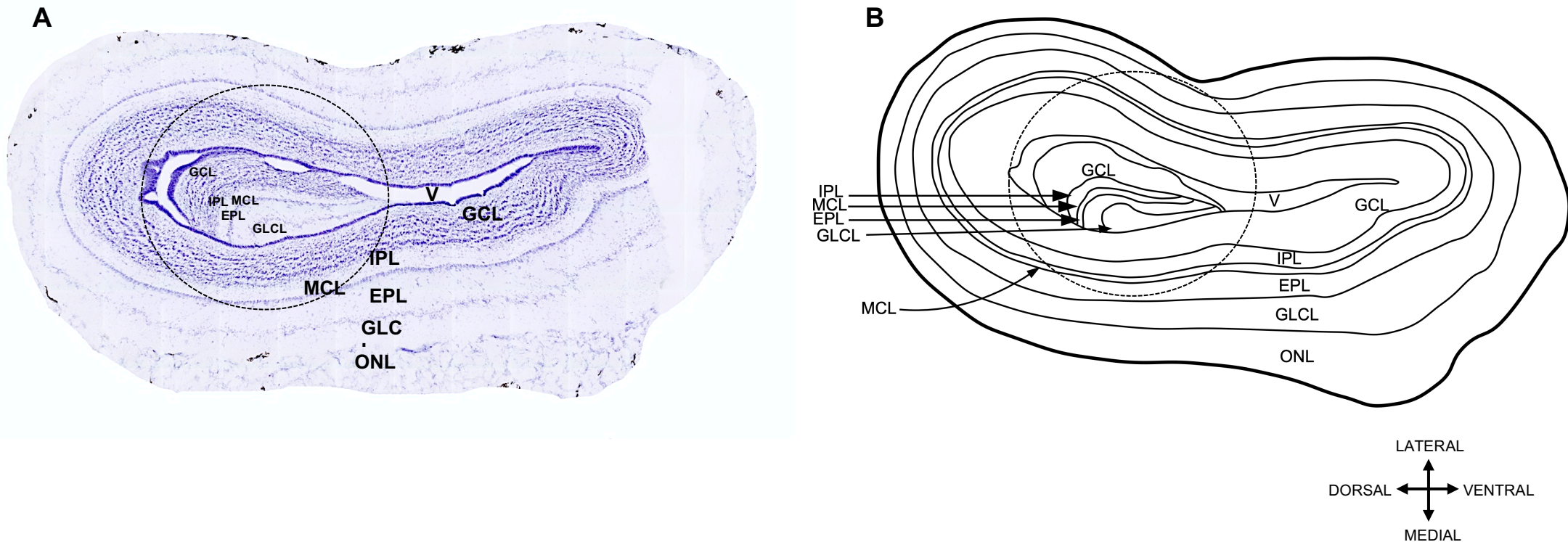


Figure 17: Coronal sections demonstrating the putative accessory bulb located within the ventricle of the Main olfactory bulb of the Nile Crocodile. **A)** Nissl Staining at 20X Magnification **B)** Line Drawing. The circled region in both **A** and **B** indicates the putative accessory bulb structure (Abbreviations: Ventricle-V, Olfactory Nerve Layer-ONL, Glomerular Cell Layer-GLCL, External Plexiform Layers-EPL, Mitral Cell Layer-MCL, Internal Plexiform Layer-IPL and Granule Cell Layer-GCL). Scale Bar= 500 μ m at 20X Magnification.

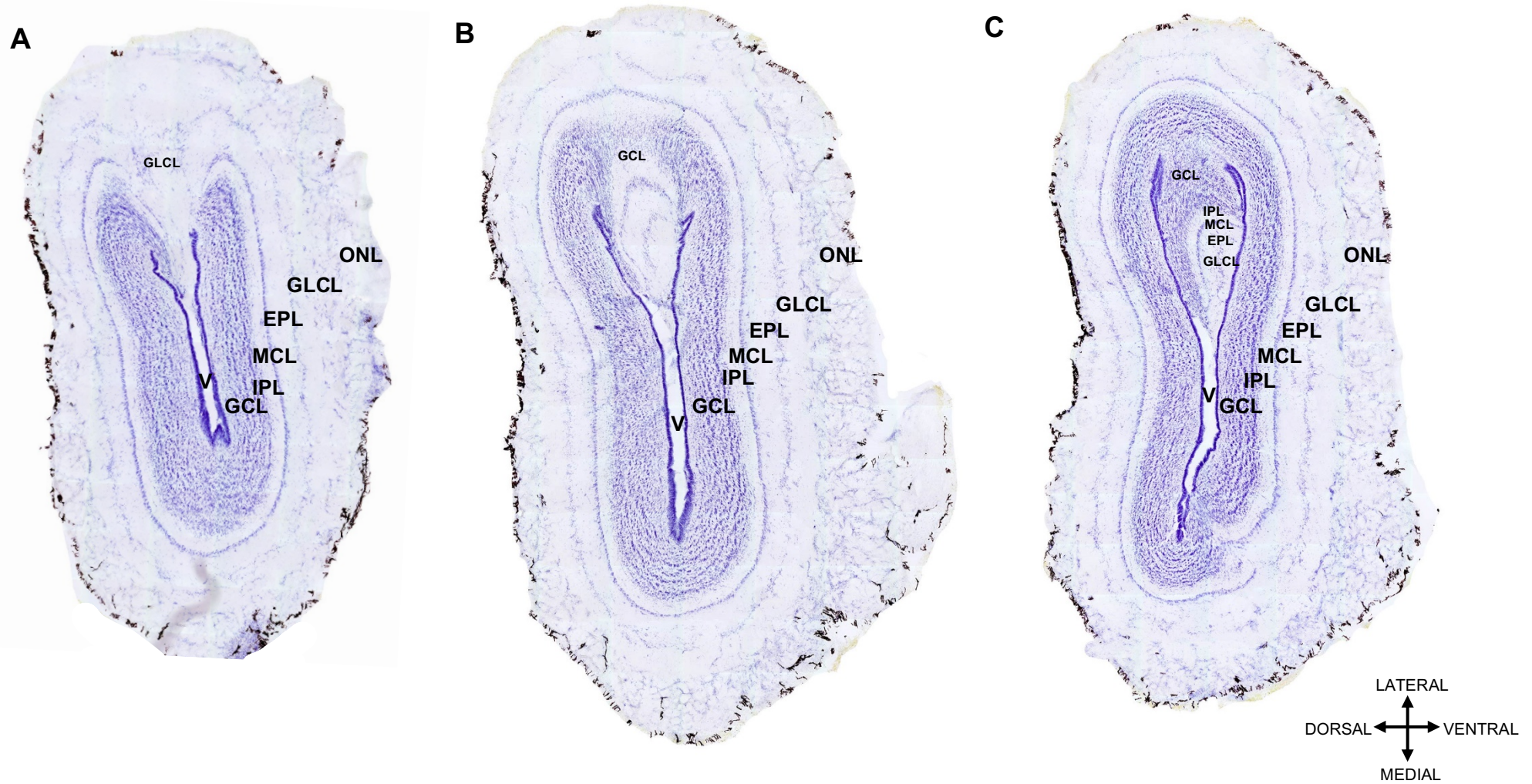


Figure 18: Series demonstrating the invagination that occurs in the Main olfactory bulb of the Nile Crocodile at various points of the bulb **A)** Most rostral aspect, i.e., the beginning of the invagination, **B)** The middle region of the invagination and **C)** Most Caudal region of the invagination prior to the tissue being fully encased in the ventricle. The invagination forms very clear and organised cell layers as it progresses towards the ventricle (Abbreviations: Ventricle-V, Olfactory Nerve Layer-ONL, Glomerular Cell Layer-GLCL, External Plexiform Layers-EPL, Mitral Cell Layer-MCL, Internal Plexiform Layer-IPL and Granule Cell Layer-GCL). Scale Bar= 500 μ m at 20X Magnification.

3.6. *Immunohistochemical analysis*

Both a qualitative analysis (density profile tables) and a quantitative analysis (stereological cell counts) were done in order to determine the expression levels of specific proteins in the olfactory bulbs of the Nile Crocodiles and the accessory olfactory bulbs of the Rock and Nile Monitor lizards. In general, the greatest expression of calretinin and calbindin was seen in the GCL of the olfactory bulbs for all species (MOB and AOB for the monitor lizards) (Table 5; Fig. 20).

3.7. *High concentration of inhibitory and excitatory interneurons in the GCL*

Calretinin and calbindin immunoreactive cells appeared to be rounded and homogenous in all species (The Nile Crocodile and Monitor lizards) (Fig. 19). These cells assumed a diffuse arrangement in the GCL of the MOB in the Nile Crocodile and the AOB of the Monitor Lizards (see Fig. 19 and 20). With the CB staining, there appears to be axons terminals/fibres in the form of bouton en passant (Fig. 19). Parvalbumin (PV) immunoreactive cells were not visible throughout the olfactory bulbs of the monitor lizards. In the crocodiles, PV immunoreactive cells were extremely low density but were visible and assumed a diffuse expression throughout all the layers of the olfactory bulb (see Table 5 and Fig. 20). The NMDA receptor 2B (NMDAR 2B) subunit was expressed in the cytoplasm of cells, which had a homogenous distribution across all the olfactory bulb cell layers in all species (Fig. 19 and 20). The Nile Crocodiles had high levels of expression of NMDAR 2B when compared to the monitor lizards, (Table 5; Fig. 20). The NMDAR2B immunopositive cells were mainly confined to the GCL in the MOB of the Nile Crocodile with some cells scattered throughout all the cell layers (Fig. 20). G protein α_0 subunit ($G\alpha_0$) expression was more evident in the Rock and Nile Monitor lizard in the olfactory nerve and glomerular cell layers of the AOB (Fig. 21). In the Nile Monitor Lizard, the $G\alpha_0$ protein expression looks to be homogeneously expressed, but is likely due to high background. It is clear that the glomeruli show high levels of expression in both lizard species (Fig. 21). These $G\alpha_0$ proteins suggest the presence of the V2R receptor expressing axons in the AOB of the monitor lizards. In the Nile Crocodile, $G\alpha_0$ expression was not evident throughout the entire olfactory bulb (Fig. 21).

Stereological analysis revealed a greater density of CR and CB immunolabelled cells in the accessory olfactory bulb of the Rock Monitor Lizard than the accessory olfactory bulb of the Nile Monitor Lizard (see Table 4).

Table 4: Cell density calculated for each calretinin and calbindin using the estimated population per measured volume in each of the accessory olfactory bulbs for the Monitor lizards.

Calcium Binding Proteins	BRAIN	ESTIMATED POPULATION USING MEAN SECTION THICKNESS	MEASURED VOLUME (mm³)	DENSITY (per mm³)
CR Calretinin	Rock Monitor	134 597, 50	18,393	7317,866
	Nile Monitor	91 455, 78	16,721	5469,52
CB Calbindin	Rock Monitor	96 217, 10	17,756	5515,05
	Nile Monitor	80 659, 66	20,400	3953.90

Table 5: Cellular density profiles of the calcium binding proteins and NMDA in the olfactory bulb of the Nile Crocodile and the AOB of the Monitor lizards (abbreviations: GCL- Granule cell layer, IPL-Internal plexiform layer, MCL-Mitral Cell layer, EPL- External plexiform layer, GLCL-Glomerular cell layer, ONL-Olfactory nerve layer).

<i>Stain</i>	<i>Cell Layer</i>	<i>Nile Crocodile</i>	<i>Rock Monitor</i>	<i>Nile Monitor</i>
CR	GCL	<i>moderate</i>	<i>moderate</i>	<i>moderate</i>
	IPL	<i>low</i>	<i>low</i>	<i>low</i>
	MCL	<i>low</i>	<i>low</i>	<i>low</i>
	EPL	<i>low</i>	<i>low</i>	<i>low</i>
	GLCL	<i>low</i>	<i>low</i>	<i>low</i>
	ONL	<i>low</i>	<i>low</i>	<i>low</i>
CB	GCL	<i>moderate</i>	<i>moderate</i>	<i>moderate</i>
	IPL	<i>low</i>	<i>low</i>	<i>low</i>
	MCL	<i>low</i>	<i>low</i>	<i>low</i>
	EPL	<i>low</i>	<i>low</i>	<i>low</i>
	GLCL	<i>low</i>	<i>low</i>	<i>low</i>
	ONL	<i>low</i>	<i>low</i>	<i>low</i>
PV	GCL	<i>low</i>	<i>low</i>	<i>low</i>
	IPL	<i>low</i>	<i>low</i>	<i>low</i>
	MCL	<i>low</i>	<i>low</i>	<i>low</i>
	EPL	<i>low</i>	<i>low</i>	<i>low</i>
	GLCL	<i>low</i>	<i>low</i>	<i>low</i>
	ONL	<i>low</i>	<i>low</i>	<i>low</i>
NMDAR-2B	GCL	<i>high</i>	<i>low</i>	<i>low</i>
	IPL	<i>low</i>	<i>low</i>	<i>low</i>
	MCL	<i>low</i>	<i>low</i>	<i>low</i>
	EPL	<i>low</i>	<i>low</i>	<i>low</i>
	GLCL	<i>low</i>	<i>low</i>	<i>low</i>
	ONL	<i>low</i>	<i>low</i>	<i>low</i>

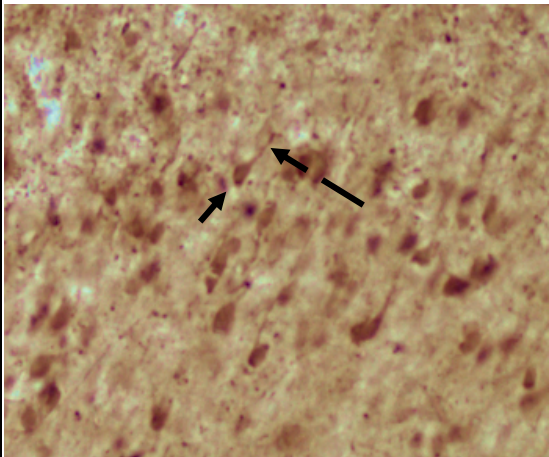
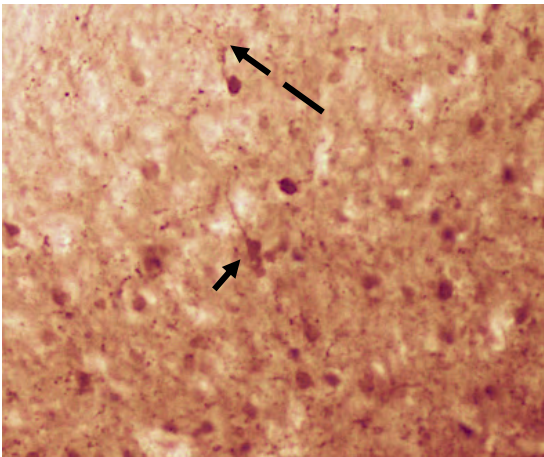
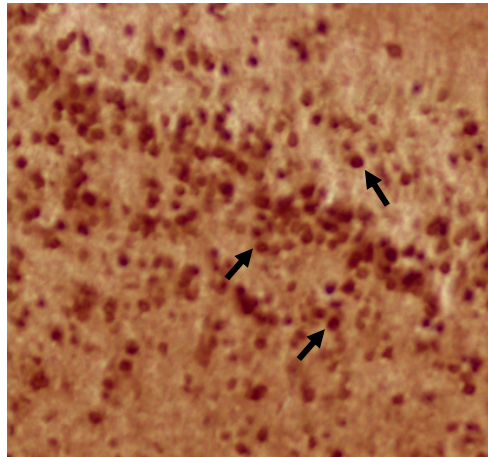
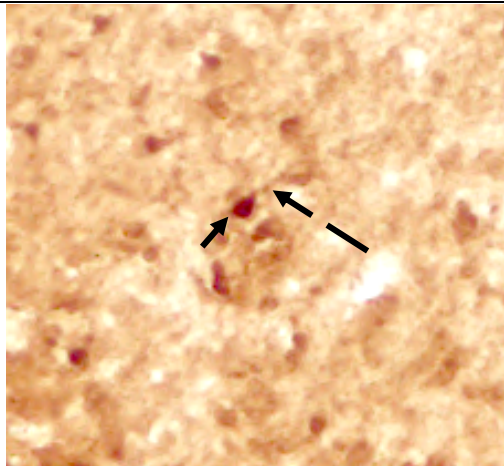
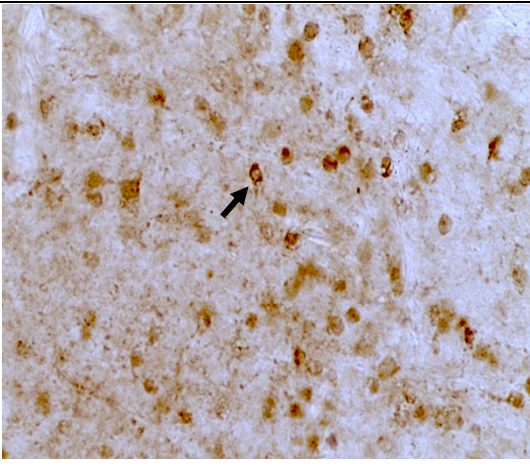
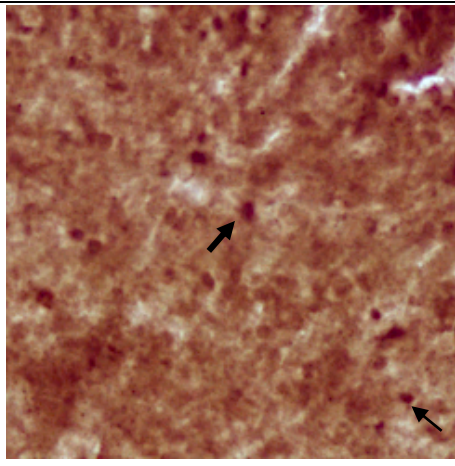
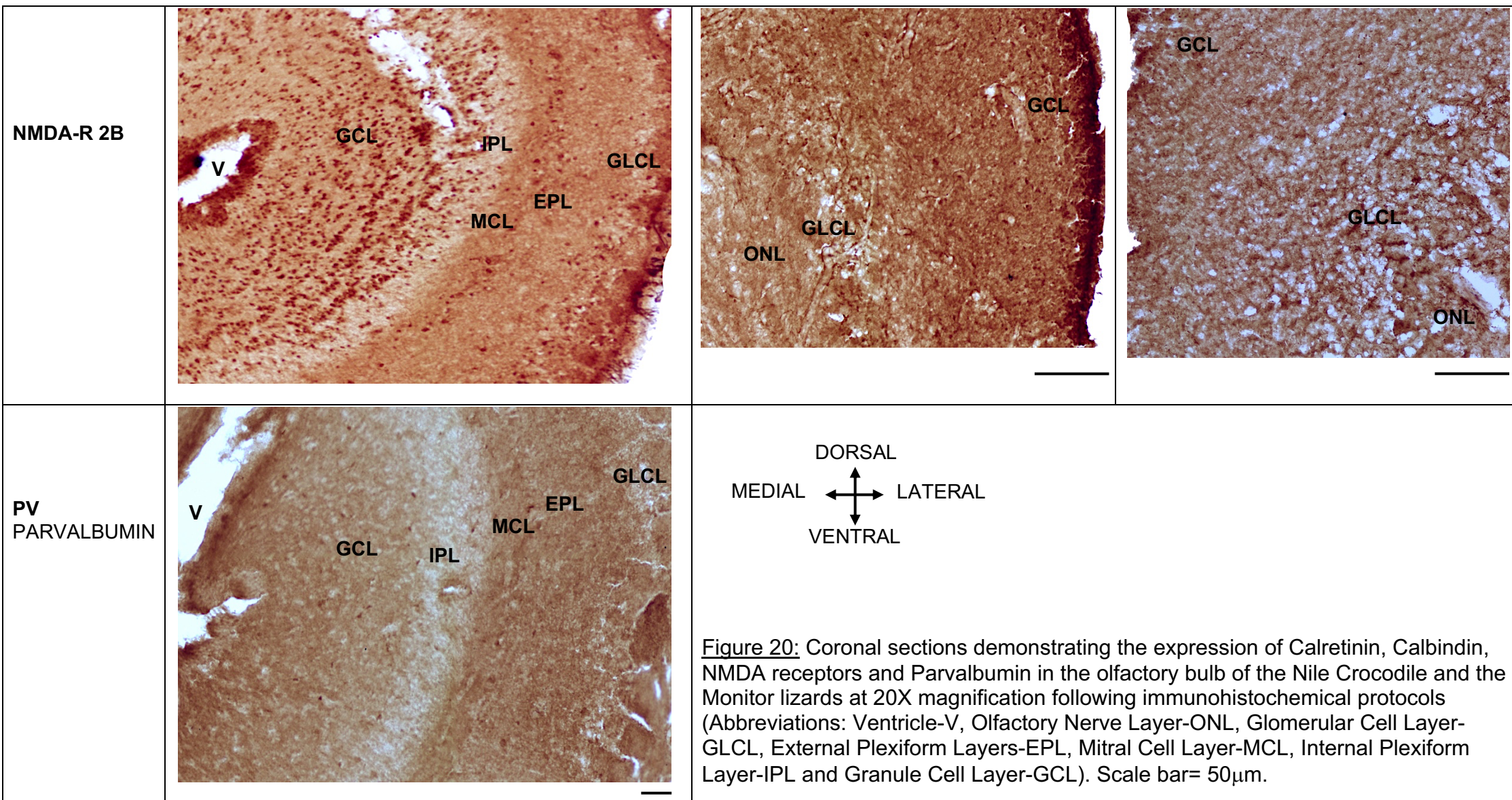
	CR CALRETININ	CB CALBINDIN	NMDA Receptor subunit 2B (NMDA R2B)
NILE CROCODILE			
MONITOR LIZARD			

Figure 19: Images of the different cells and cell types present in the olfactory bulbs of the Nile Crocodile and the Monitor Lizards at 20X magnification. The solid arrows indicate a single cell and the dashed arrow represent the dendritic extensions. Scale bar= 50µm.

	NILE CROCODILE	ROCK MONITOR	NILE MONITOR
CR CALRETININ			
CB CALBINDIN			



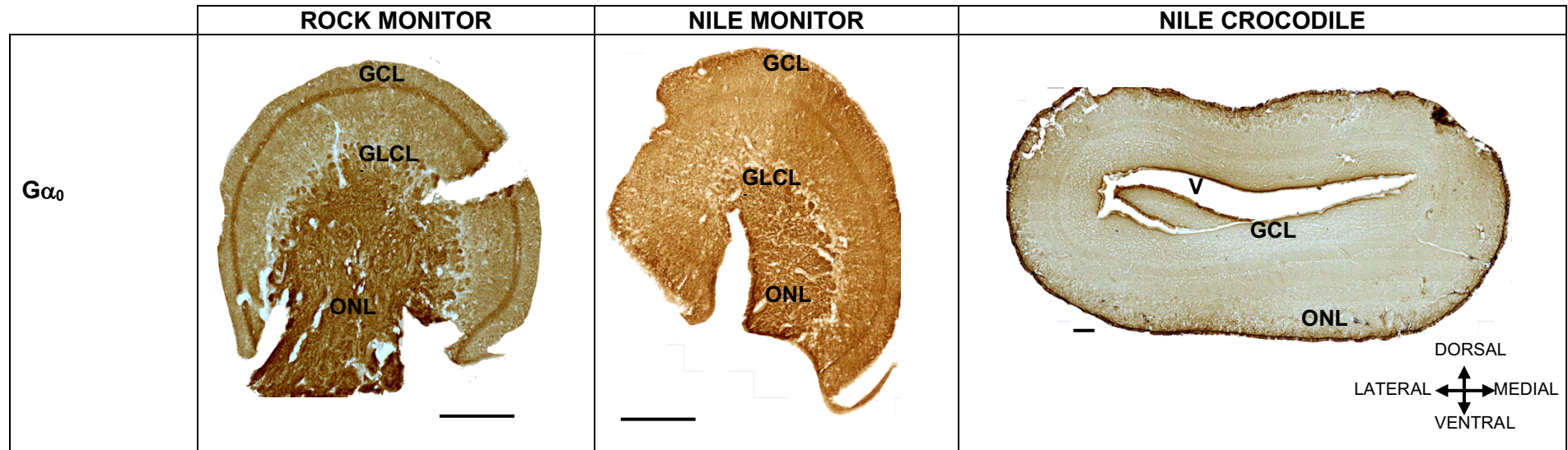


Figure 21: Coronal sections showing the expression of $G\alpha_0$ protein in the AOB of the Rock and Nile Monitor lizard and in the main olfactory bulb of the Nile Crocodile at 20X magnification following immunohistochemical protocols. The expression of the $G\alpha_0$ protein appears to be restricted to the Olfactory Nerve Layer and Glomerular Cell Layer of the accessory olfactory bulbs in both the Monitor lizards. It is clear that the glomeruli show high levels of expression in both lizard species. The $G\alpha_0$ protein does appear to express in the olfactory bulb of the Nile Crocodile (abbreviations: ONL-Olfactory nerve layer, GLCL-Glomerular Cell Layer and V-Ventricle). Scale Bar = 500 μ m.

4. DISCUSSION

In line with the aims of the current study a comprehensive immunohistochemical account of the olfactory bulbs of the Nile Crocodile (*Crocodylus niloticus*), Rock Monitor (*Varanus albigularis*) and Nile Monitor (*Varanus niloticus*) lizards was completed. The olfactory bulbs of the Rock and Nile Monitor lizards are similar with subtle differences identified in their respective neuroarchitectures. Both monitor lizards possessed a clearly and well defined MOB and an AOB, with the only difference noted in the relative density of calretinin and calbindin immunolabelled neurons. The neuroarchitecture of the MOB in the Nile Crocodile is very similar to that of the monitor lizards however, an AOB was not clearly defined but a unique region of interest was highlighted as a putative AOB.

4.1. The neuroanatomy of the olfactory bulbs

The Rock Monitor lizard possessed both the MOB and an AOB. The neuroanatomical divisions of the MOB and AOB in the Rock Monitor lizard seem to be consistent when compared to the olfactory bulbs in other squamates (snakes and lizards) (Crosby and Humphrey, 1939; Nieuwenhuys, 1966; Llari and García-Verdugo, 1989; Meisami and Bhatnagar, 1998; Sanchez-Andrade and Kendrick, 2009; Nagayama *et al.*, 2014; Taniguchi and Taniguchi, 2014; Hunyadi *et al.*, 2020; Imamura *et al.*, 2020; Lodovichi, 2021). The AOB of the Rock Monitor and Nile Monitor lizard are similar (see Fig. 10 and 12). Despite not having a MOB of the Nile Monitor lizard in the current study, we can infer that it undergoes a similar structural change as the Rock Monitor lizard. The differences between the MOB and AOB in the Rock Monitor lizard seen was the change in the shape of the olfactory ventricle, the change in the orientation of the olfactory cell layers and the change in the thickness of the plexiform layers (see Fig. 5,6, 7A and 7G). These differences noted is consistent to what is seen amongst other squamate olfactory bulbs (Crosby and Humphrey, 1939; Nieuwenhuys, 1966). Varanids are diverse family of reptiles that can adapt to a number of habitats (from terrestrial to aquatic) (Pacini and Harper, 2008). These squamate reptiles are known to rely heavily on chemoreception in order to survive (Burghardt, 1980). The changes seen in the olfactory bulbs (the transition between the MOB to the AOB) of these reptiles is suggestive of the reliance on the VNS in interpreting non-volatile,

pheromonal cues and its ability to enable these squamate reptiles to adapt to their different environments.

The neuroanatomy of the main olfactory bulbs in the Rock Monitor lizard and Nile Crocodile are similar in the current study. The MOB in the Nile Crocodile possesses the same major olfactory cell layers as the Rock Monitor lizard with minor differences noted between these cell layers (Fig. 5, 13A and 16). One of the differences noted was the varying thickness of the mitral and granule cell layer of the MOB. The mitral cell layer is thinner and the granule cell layer is thicker in the MOB of the Nile Crocodile (see Fig. 16). Whereas in the MOB of the Rock Monitor lizard, the mitral cell layer is a thicker cell layer and the granule cell layer is a thinner cell layer (see Fig. 16). The MOB of the Nile Crocodile also possessed a subventricular zone. However this cell layer is extremely thin in comparison to the subventricular zone in the MOB of the Rock Monitor lizard (see Fig. 16). The MOB functions to interpret a vast range of chemical signals (Shepherd *et al.*, 2004). The similarities seen between the MOB of the Rock Monitor lizard and the Nile Crocodile suggests that the MOB of these different reptiles would function the same due to them being anatomically similar. The cellular organisation and cell layer thickness of the MOB in the Nile Crocodile and Rock Monitor lizard does not differ much with previous descriptions of the MOB of other amniotes (Meisami and Bhatnagar, 1998; Roper, 1999).

The AOB in the Monitor lizards has six layers identical to the MOB and receives a thick nerve from the rostral-medial aspect of the bulb known as the vomeronasal nerve and this nerve eventually leads into and forms part of the olfactory nerve layer in the AOB (Kondoh *et al.*, 2013a). The olfactory bulbs (MOB and AOB) of the Rock Monitor lizard undergoes structural changes which results in disruptions to the cellular organisation of the olfactory bulbs (see Fig. 7). In the Nile Crocodile, the olfactory bulb does not appear to undergo a similar structural change but rather appears to be a homogenous structure (see Fig. 13). These structural changes are classical features that distinguish the MOB and AOB in squamate reptiles (Crosby and Humphrey, 1939).

Immunohistochemical analysis revealed that both the AOB of the Monitor lizards and the olfactory bulbs of the Nile Crocodiles possessed both excitatory and inhibitory interneurons, primarily located in the granule cell layer (refer to Fig. 20). The presence

of these neurons indicates that there is a transfer of information. There are no major differences in the organisation and chemical makeup of the AOB of the Monitor lizards and the MOB of the Nile Crocodile. Analysis of the G_{a0} protein revealed that the Nile Crocodile olfactory bulb lacked the expression of this protein and as expected, the AOB of the Monitor lizards expressed this protein (Fig. 21). This G-protein is used to indicate the presence of the vomeronasal receptors in order to determine the presence of the AOB (Chamero *et al.*, 2011). The change in the cellular organisation of the olfactory bulbs, the shape and position of the olfactory ventricle and the presence of specific G-protein coupled receptors are common features shared in the AOB of squamates (Halpern, 1976; Iwahori *et al.*, 1989a; Iwahori *et al.*, 1989b; Jia and Halpern, 1996).

4.2. *Do Nile Crocodiles possess an AOB?*

The Nile Crocodiles does not appear to possess some of the distinguishing classical features of the AOB described above in Squamates. Despite the absence of these common AOB features, there are regions of interest that provide a unique insight that are suggestive of the potential presence of the AOB or remnants of it (see Fig. 17 and 18). This invagination of the cell layers formed an independent structure within the ventricle that is separate from the MOB (see Fig. 15G). The cell layers of this structure are identical in number but separated from the MOB, a feature described for the squamate AOB. The cell layers within this region are as distinct and clearly defined as those in the main olfactory bulb (see Fig. 17). Given that the layers of the AOB are typically less well defined and the cells of the AOB are more diffusely arranged than those of the MOB, it is difficult to definitively conclude that this region performs functions distinct from the MOB.

A secondary aspect relating to this region of interest seems to indicate a movement of cells that resembles a pattern of cell migration. However, based on the process of the rostral migratory stream, the abundance of cells formed during neurogenesis emanates from the subventricular zone to the olfactory bulb (Bayer and Altman, 2004). Whereas the neuroanatomical outline indicates that the cell movement is from the outer layers of the olfactory bulb (specifically the glomerular layer) towards the ventricle until this cluster of cells form a distinct independent anatomical region within

the ventricle (Fig. 15C-15G, 17, 18A and 18C). Once the tissue is suspended within the olfactory ventricle, the olfactory bulb cell layers return to their original layout as the series continues once the tissue is fully encased in the olfactory ventricle (see Fig. 15F-15H). In the current study there is a representation of a young and older Nile Crocodile (age was determined by weight and length). The suspended piece of tissue similar to that of the invagination in the younger crocodile was also seen in the older Nile Crocodile (see Fig. 14I-14J). Based on the presence of this structure in both a younger and older (adult) Nile Crocodile it is difficult to ascribe age as a confounding factor for the development of this tissue.

This region of the olfactory bulb is poorly understood and will require developmental studies tracking this phenomena through an ontogenetic series describing the crocodilian bulb at different stages. What we do know is that the crocodilian, did at some stage possess a VNO, Parsons, (1970) identifies this structure developmentally and suggests that it degenerates prior to hatching. The VNO forms a direct connection with the AOB (Martínez-Marcos and Halpern, 2004). If a VNO was present in the crocodiles, the associated AOB may have also existed in these reptiles. Based on this premise, the AOB may be present, or degenerates developmentally as the VNO does or some remnant of the AOB might still be within this species. We can only speculate on whether this region of interest is the AOB based on the neuroanatomical description provided in this study. However, as mentioned, developmental studies as well as functional studies might shed light on what the structure and its associated function might be. Studies would need to be done where parts of the olfactory bulb (i.e., this region of interest) are lesioned and any behavioural deficits exhibited are observed. Studies that also analyse the fibre patterns originating from the olfactory bulbs should be done in order to determine which regions of the brain receive these chemical signals and also what type of chemical signals are processed in this region of the bulb (Halpern, 1987).

The VNS in amniotes is made up of structural elements such as the VNO (which contains the sensory epithelium) and the AOB (the primary information processing centre) which projects further to the amygdaloid complex of the brain (Halpern, 2003). In reptiles, the AOB projects further to a unique reptilian structure called the nucleus sphericus (Lanuza and Halpern, 1998).

The crocodilians have been described to not possess a VNS due to a lack of the VNO (Parsons, 1970). However putative accounts have been highlighted in the literature indirectly suggesting that some of the anatomical components might be present. Ngwenya *et al.*, (2017) described the inclusion of an AOB and Billings *et al.*, (2020a) and Ngwenya *et al.*, (2017) suggest that a nucleus sphericus might be present. Considering that these structures were described to be cryptic, they may not necessarily comply with the anatomical representation of these structures identified in squamates. For example, the nucleus sphericus (NS) seems to be present but it does not match exactly what we see in squamates (snakes specifically) (Martínez-Marcos *et al.*, 2002; Ngwenya *et al.*, 2017). It should be noted that the author is cognisant that the putative structures described (i.e. AOB and NS) have been identified in the Nile Crocodile only and more studies in other crocodilians is required and in many regards a revision of some of the earlier anatomical work that was conducted on Alligatoridae in particular (Hansen, 2006). Few studies if any focus on the crocodilian Posterior Dorsal Ventricular Ridge (PDVR) where the NS has been identified so we require more anatomical atlases of the entire telencephalon in a variety of croc species to identify if these putative structure are present for all crocodilians and in what form and to what degree (Billings *et al.*, 2020b; Ngwenya *et al.*, 2017).

The other alternative is that these structures might either be vestigial. It is a possibility that these structures are lost during the process of evolution (either because some other system has taken over their function) and this is why they don't match exactly the anatomy in squamates. An example of this is the degeneration of the human tail (Ahmed *et al.*, 2020). As humans began to walk (as bipedal) the need for a tail for balance and locomotion declined forming a remnant of the tail bone i.e., the coccyx (Ahmed *et al.*, 2020). On the other hand, if these VNS structures were not lost during the process of evolution, they might present themselves as anatomically different compared to other amniotes whilst performing the same function. Crocodilian are not squamates but rather archosaurs i.e., the phylogenetic relationship is why the structure might present differently so the VNS could have developed differently in the different taxa (see Fig. 22). For example, the wings of the birds and insects performs the same function. However, the structure of the wings between birds and insects differ (e.g. insect wings lack bones) (Alexander, 2015). This is an example of parallel evolution where similar traits develop in species that are not closely related (Orr,

2005).

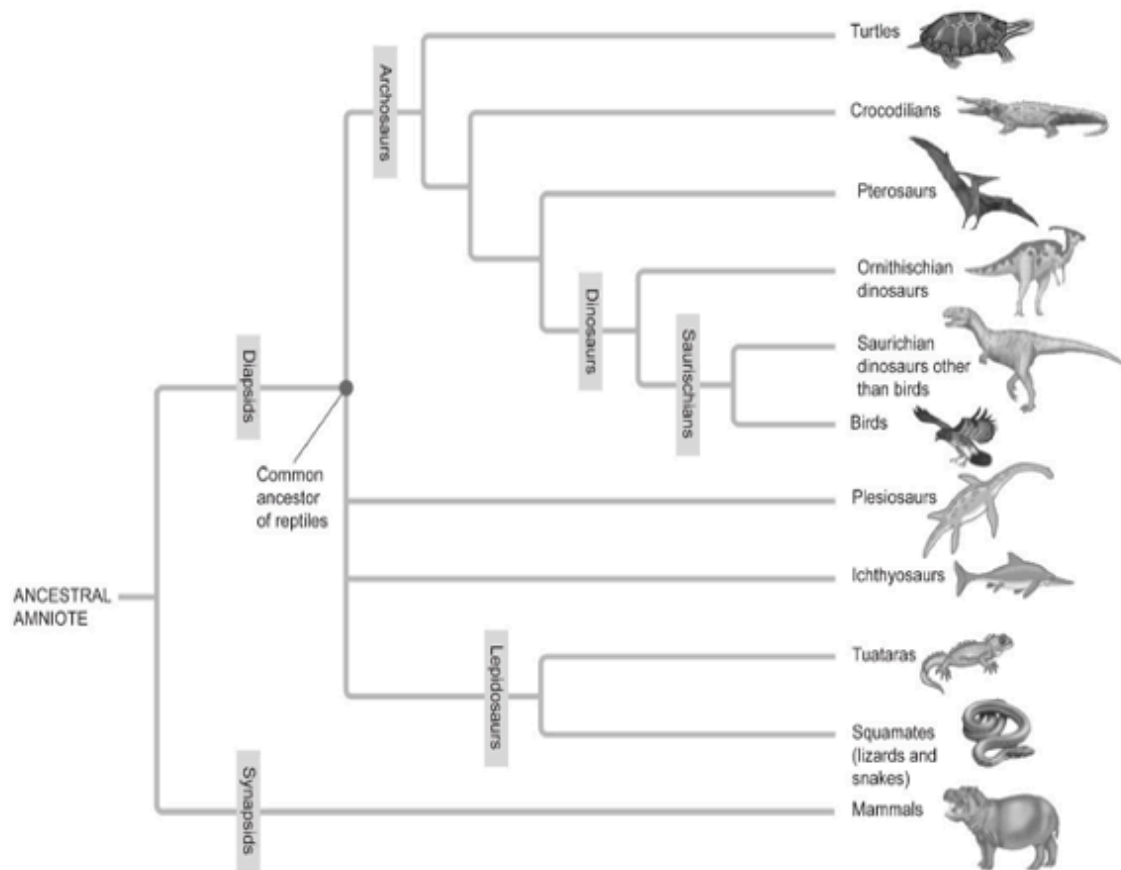


Figure 22: Phylogenetic tree showing the relationships between reptiles, mammals and birds (Urry *et al.*, 2020).

Whilst the VNS is suggested to be absent in the crocodilians, other archosaurians such as testudines possess a unique olfactory system that encompasses structural features of the VNS (Shohi and Kurihara, 1991). The features of the VNS in testudines may differ anatomically when compared to the VNS in squamate reptiles. However, these features of the VNS in testudines share similar functional roles as the squamate VNS (Taniguchi and Taniguchi 2014, Abdali *et al.*, 2020). Testudines possess structures that resemble parts of the VNS such as the VNO (Wakabayashi *et al.*, 2007; Nakamuta *et al.*, 2016; Abdali *et al.*, 2020). The presence of vomeronasal like sensory epithelium (such as microvillous ORNs) was found in *Chelydra serpentina* (snapping turtle) (Taniguchi and Taniguchi, 2014). The components of the VNS do not have to be anatomically similar across different species of reptiles in order to determine the presence or absence of structures.

Based on the phylogenetic relationship of crocodilians and birds, it stands to reason that crocodilians (like birds) would not possess a VNS (Taniguchi *et al.*, 2011)(see Fig. 22). However, crocodilians did possess a VNO at some point in time (Parsons, 1970). Over time, as this semi-aquatic species evolved with its environment, the VNO may have degenerated. Gene mutations and environmental influences can impact the degeneration of organs (Smith and Wright, 2022). Like in humans, the VNO is identified in neonates (Bruintjes and Bleys, 2023). However, in adults, the VNO appears to be non-functional and is therefore a vestigial organ (Bruintjes and Bleys, 2023). The VNO may be a structure that is not necessary for survival in humans and as a result of genetic mutations and the environment, the VNO has become ineffective in humans. Something similar to this may be occurring in the Nile Crocodile. If the putative accessory olfactory bulb identified in the current study is proved to have functional roles similar to that of the AOB in squamates, it would suggest the presence of some version of a VNS in the Nile Crocodile.

Generally, reptiles are known to possess differences in their olfactory systems based on their environment and the presence or absence of parts of the VNS (Caroline Crosby and Humphrey, 1939). The most primitive reptile species (Tuatara: *Sphenodon*) is known to possess a vomeronasal system (Schwenk, 1986). Crocodilians share a common ancestor with Tuatara (see Fig. 22). The presence of the VNO in early embryology of crocodilians suggests that there are also genetic factors/traits for the VNS that are conserved throughout the evolutionary history of these reptiles (Modesto and Anderson, 2004). The development of the olfactory system occurs early in embryogenesis where the olfactory placode differentiates giving rise to olfactory receptor neurons (ORNs) and the olfactory sensory epithelium (Suárez *et al.*, 2012). The structures involved in the main olfactory system and the VNS such as the MOB and AOB derived from chemical cues given by the telencephalon of the specific species (Treloar *et al.*, 2010).

4.3. *Pheromonal communication is not solely dependent on the VNS*

In squamates, behavioural traits as a result of the VNS in response to pheromones are well documented (Burghardt, 1980; Burghardt 1993; Schwenk, 1993; Cooper, 1997; Recio *et al.*, 2020; Teshera and Clark , 2021). Pheromones are chemicals that are

secreted by organisms aiding in intraspecies communication (Fan and Ting, 2014). Whilst it is known that the VNS is primarily responsible for interpreting pheromonal cues, some pheromones can be detected by removing parts of the VNS (Swaney and Keverne, 2009). For example, in mammals such as rabbits, the VNO was removed and the specific pheromone responsible for detecting the nipples during suckling was still functioning (Hudson and Distel, 1986). The absence of the VNO in this study did not disrupt the pheromonal effects of the pheromone. So the absence of a component of the VNS did not mean that there was a lack of the VNS in rabbits from the study (Hudson and Distel, 1986). This just demonstrates that the VNS can still function without associated parts (Brennan, 2001). Crocodilians are reported to be capable of producing pheromonal secretions (Whyte *et al.*, 1999). So, despite the lack of the VNO in crocodiles, it is possible that regions like the putative AOB may still be involved with processing pheromonal stimuli. However, further functional and behavioural studies would need to be carried out to validate this.

4.4. *Influence of the environment on the development of the VNS*

The absence of the VNS in most aquatic wildlife (including, fish and marine mammals) and in air-borne vertebrates such as birds and bats, indicates that the VNS may not be necessary for chemo-communication in the marine environment and is of very little use to birds and bats in the air (Bertmar, 1981; Grus and Chang, 2006; Taniguchi *et al.*, 2011). Some testudines (for example, the red-eared slider) and crocodilians are semi-aquatic (Semenov, 2010; Marn and Kooijman, 2022). Crocodilians, being semi-aquatic in nature, may present similarities neuroanatomically and functionally to the olfactory system of testudines.

In summary the current study does not provide absolute existence of the AOB and it still remains unclear whether crocodilians possess an accessory olfactory system. The results of the study do lend useful insights that can be further tested using a combination of anatomical and functional neuroscientific tools to challenge a theory that has been accepted for decades that crocodilians do not possess a VNS and that the VNO degenerates prior to hatching (Parson, 1970).

4.5. *Limitations*

The limitations of the study included the lack of G alpha i2 specificity of the antibody, the loss of the MOB in the Nile Monitor lizard specimens (only one Nile Monitor lizard was available for use in the study and as such when the MOB was lost during experimental procedures, only the AOB was available for analysis), and luxol blue optimisation failures. The luxol fast blue stain was meant to be used in order to identify the fibre tracts in the olfactory bulbs. However, despite performing a number of trials, the luxol blue optimisation continuously failed. This led to the omission of the luxol fast blue stain from the research due to the limited amount of tissue available for the actual research study. The limited amount of tissue was due to the fact that this study utilised Monitor lizards deemed unfit for survival and previously collected Nile crocodile specimens. The facility that provided the Monitor lizards for the study (Hoedspruit Reptile Centre) could only provide those destined for death due to accidents. These animals had to be euthanised which limited the sample size, statistical variations and also variations in morphology.

5. CONCLUSION

In conclusion, this study provides a neuroanatomical outline of the *Varanus* species olfactory bulbs (between the Rock Monitor and Nile Monitor lizard) and they are very similar with subtle differences seen. The Nile Crocodile does not have an AOB that matches what has been described for squamates or Lepidosaurians. However, a unique region of interest has been identified that might be a putative equivalent or analogue. Further studies analysing the specific chemical stimuli processed in this region would need to be performed in order to validate these claims. Crocodiles are an important part of biodiversity, ecology and they phylogenetically occupy an interesting position which potentially allows for the study of the evolution of the brain. Therefore, it is important to develop a greater understanding of their brains and how they function to understand evolutionary patterns and improve conservation strategies.

6. REFERENCES

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7. APPENDIX

A. ETHICS CLEARANCE CERTIFICATE

ANIMALS RESEARCH ETHICS COMMITTEE (AREC)



STRICTLY CONFIDENTIAL

CLEARANCE CERTIFICATE NUMBER: AREC22-10-002A

APPLICANT: Mr M Rappetti

School: School of Anatomical Sciences; Department: ; Location: Hoedspruit Reptile Centre and School of Anatomical Sciences

PROJECT TITLE: Comparative analysis of the olfactory system in the Nile Crocodile (*Crocodylus niloticus*) and Monitor Lizard (*Varanus albigularis*)

Category: A; Species and Numbers involved: 4X, Sub adults/Adults that are approximately one metre in size, Male or Female, Monitor Lizard (*Varanus Albigularis*)

Approval is hereby given for the use of animals for the research project named above and described in the application reviewed by a quorate meeting of the AREC held on 28 Feb 2023. This approval remains valid until 25 Apr 2025 and is conditional to the following (if blank there are no special conditions):

Condition 1	Condition 2	Condition 3	Condition 4

All material changes to the approved research must be reported to the AREC before they are implemented. Failure to do so will invalidate this clearance certificate.

An annual progress report must be provided to the AREC.

The use of these animals is subject to AREC guidelines on the use and care of laboratory animals, is limited to the procedures described in the application and is subject to additional conditions listed below:

I, the Chair of the AREC (or my designated representative) am satisfied that the proposed research is ethical as judged by local law, international standards and University policy.

Signed:  Date: 26/04/2023
(Chairperson of the AREC)

I am satisfied that the persons listed in this application are competent to perform the procedures described in the application, in the context of Section 23 (1) (c) of the veterinary and Para-veterinary Professions Act (19 of 1982).

CC: Student supervisor: «Title1» «Initials1» «Supervisor_surname»
Director Wits Research Animal Facility (WRAF): Dr Kim Jardine

B. AM&E CERTIFICATE



AREC 2022 AM&E

Please note that only electronically completed applications will be accepted.

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG ANIMAL RESEARCH ETHICS COMMITTEE

registered as Animal Research Ethics Committee (AREC) with the South African National Health Research Ethics Council (NHREC) of the National Dept. of Health, reg. no. AREC -101210-002

APPLICATION FOR ADAPTATIONS OR MODIFICATIONS AND EXTENSIONS TO EXPERIMENTS

- a. Principal Investigator (PI) Name: **Marcel Elijah Rappetti**
- b. School / Department: **Anatomical Sciences**
- c. Email Address: **1672214@students.wits.ac.za**

- d. Experiment to be modified / extended

AREC NO

Original AREC Clearance Certificate Number	AREC22	10	002A
Clearance Certificate Expiry date	25 April 2025		
Number of previous AM&Es	0		

- e. Project Title: **Comparative analysis of the olfactory system in the Nile Crocodile (*Crocodylus niloticus*) and Monitor Lizard (*Varanus albigularis*)**

	No.	Species
f. Number and species of animals <u>originally approved</u>	4	<i>Varanus albigularis</i>
g. Number of additional animals previously <u>approved on AM&Es</u>	-	-
i. Number of animals used and currently involved in the study to date	3	<i>Varanus albigularis</i> (2) and <i>Varanus niloticus</i> (1)

- j. Specific details of Adaptations/Modification/ Extension:

Inclusion of another species of monitor lizard, Nile Monitor Lizard (*Varanus niloticus*) to be used in the study alongside the Rock Monitor Lizard (*Varanus albigularis*). In addition to this, we wish to increase the number of specimens (monitor lizards) to be used for this study from four (4) to ten (10).

- k. Motivation for Adaptations/Modification/ Extension:

The institute that is providing the specimens for the study (Hoedspruit Reptile Centre (HRC)) indicated that they can provide Rock Monitor Lizards (*Varanus albigularis*) that were destined for sacrifice due to injuries incurred from car accidents and attacks from dogs etc. However, as we enter the warmer summer months such injuries, and the subsequent need for euthanasia, are not limited to Rock monitor lizards. The HRC has recently had to euthanize a Nile monitor lizard (*Varanus niloticus*) as a result of injury. The brain tissue was collected in the same manner as that of the rock monitor lizard and is available for analysis. Both the Nile and Rock monitor lizard are applicable and useful for the current study as both species present an established vomeronasal system, which is the focus of the study. We are therefore requesting permission to use both the Rock monitor (original application) and the Nile monitor lizards (i.e., the inclusion of an additional species based on availability). Both

Date: 04 October 2023

PI Signature:



RECOMMENDATIONS:

Approval for an increased in the number of Rock Monitor Lizard (*Varanus albigularis*) specimens to be collected (Total n=10).

Approval for the inclusion of the Nile Monitor Lizard (*Varanus niloticus*) species in the study and for samples collected by the HRC to be used by the researchers.

Date: 3 November 2023

Signature:



Deputy Chairperson, AREC

C. ETHICS WAIVER



ANIMAL RESEARCH ETHICS COMMITTEE

Registration number: AREC-101210-002

Date: 21/10/2022

Certificate reference: Waiver 21-10-2022-O

Category: O

Applicant: Marcel Elijah Rappetti

Department: School of Anatomical sciences (WITS)

Tel: 011 717 2660; **Email:** 1672214@students.wits.ac.za

Re: Waiver from the Animal Ethics Research Committee of the University of the Witwatersrand

This letter is to confirm that Marcel Rappetti, MSc student (#1672214) under the supervision of Drs Brendon Billings and Ayanda Ngwenya (School of Anatomical Sciences), does not require full Animal Ethics Research Committee clearance to undertake the work titled **“Comparative analysis of the olfactory system in the Nile Crocodile (*Crocodylus niloticus*) and monitor lizard (*varanus albigularis*)”**.

Reason for waiver

The project is using already collected materials from 2 crocodiles (AREC clearance # 2011/14/2B).

Details of the study

The aim of the present study is to identify and describe the chemoarchitecture of the olfactory system in two different reptiles - one in the squamate group where the VNS is apparently well-established, monitor lizards (*Varanus albigularis*) and one in an archosaurian where the VNS is suspected to be absent, Nile Crocodiles (*Crocodylus niloticus*).

Paraffin-embedded crocodilian nasal epithelium will be serially sectioned in coronal and sagittal sections at 5µm and stained with H&E. Comparisons of microstructure (the cell types, olfactory receptor neurons and the olfactory fibre tracts) will be made using light microscopy to identify similarities with monitor lizard epithelium. The sections will also undergo immunohistochemistry for antibodies (calcium binding proteins (calbindin, parvalbumin and calretinin); tyrosine hydroxylase and vesicular glutamate transporter 2; and Gai2 and Ga0 subunits of the vomeronasal receptors type I (V1R) and II (V2R)). Finally, the remaining 2 series of sections will be stained for Nissl substance and myelin. The sections will be viewed with a low power stereomicroscope and the cytoarchitectural boundaries of each Nissl and myelin-stained section will provide a framework that will be traced using a camera lucida, the expression of immunopositive cells and fibres will be indicated within the borders.

The individuals covered by the waiver are Marcel Rappetti, Drs Brendon Billings and Ayanda Ngwenya (School of Anatomical Sciences).

Please contact me should you require further information.

Yours sincerely

A handwritten signature in blue ink, appearing to read 'Frederic Michel'.

Prof Frederic Michel
Chair: Animal Research Ethics Committee
University of the Witwatersrand

D. HOEDSPRUIT REPTILE CENTRE SOP FOR EUTHANASIA OF ANIMALS



Humane Euthanasia of Reptiles

Introduction

- Euthanasia refers to the act of killing an animal to relieve suffering.
- All staff involved in euthanasia are to be fully trained and a planned, rehearsed and coordinated approach is to be taken.
- Euthanasia is not necessarily an act of veterinary surgery and in most circumstances can be carried out by anyone provided that is done so humanely, and that person has received adequate training. Veterinarians do, however, have access to specific drugs that non-veterinarians are not legally permitted to access.

Purpose

- The primary purpose of euthanasia should be to relieve suffering.
- The decision to euthanise should be based on an assessment of multiple factors including but not limited to:
 - i. The extent of injury/nature of disease
 - ii. Available treatment options
 - iii. Prognosis
 - iv. Quality of life (free of pain, ability to interact with others, ability to eat/drink, ability to move, ability to behave normally etc.)
 - v. Welfare of the animal
 - vi. Age

Method

1. Injectable agents
 - a. Sodium pentobarbital
 - i. Sodium pentobarbital can be administered through venous access, intracardiac, intracoelomic, subcutaneous lymph spaces or directly into the brain.
 - ii. Prior sedation is strongly recommended – this can be achieved through inhalation anaesthetics or injectable agents.
 - iii. Only licenced veterinarians can utilize this method as only veterinarians are allowed to handle scheduled drugs.
2. Freezing
 - a. Freezing can be used to euthanize reptiles.
 - b. Reptiles must be placed in a suitable container with newspaper or paper towel.
 - c. The container can then be placed in an appropriate freezer.
 - d. This technique should not be used for species that have adapted freeze tolerance strategies.

3. Cervical dislocation

- a. Spinal cord severance followed by destruction of brain tissue is an acceptable method of euthanasia in reptiles.
- b. Severing of the head must be done swiftly and smoothly and followed by pithing of the brain.
- c. Operators must be trained and skilled in this procedure.



Chris Cooke

Owner/Director



Jessica Briner

Veterinarian

E. SECTION 20 PERMIT



agriculture, land reform & rural development

Department:
Agriculture, Land Reform and Rural Development
REPUBLIC OF SOUTH AFRICA



Directorate Animal Health, Department of Agriculture, Land Reform and Rural Development Private Bag X138, Pretoria 0001
Enquiries: Ms Mama Laing • Tel: +27 12 319 7532 • E-mail: MamaL@daird.gov.za Website: www.daird.gov.za
Reference: 12/11/1/1/20 (5022NT)

Mr. Marcel Elijah Rappetti
School of Anatomical Sciences
Wits Medical School
University of the Witwatersrand
2193
1672214@students.wits.ac.za

Dear Mr. Rappetti,

RE: PERMISSION TO DO RESEARCH IN TERMS OF SECTION 20 OF THE ANIMAL DISEASES ACT, 1984 (ACT NO 35 OF 1984)

Title of research project / study: "Comparative analysis of the olfactory system in the Nile crocodile (*Crocodylus niloticus*) and monitor lizard (*Varanus albigularis*)"

Your application dated 6 June 2023, as received on 21 July 2023, requesting permission under Section 20 of the Animal Disease Act, 1984 (Act No. 35 of 1984) to perform a research project or study, refers. I am pleased to inform you that permission is hereby granted to perform the following study, with the following conditions:

Conditions:

1. This permission does not relieve the researcher of any responsibility which may be placed on him by any other act of the Republic of South Africa;
2. The research project is approved as per the application form dated 6 June 2023 and the correspondence thereafter. Written permission from the Director: Animal Health must be obtained prior to any deviation from the conditions approved for this



Department of Agriculture, Land Reform and Rural Development - Departement van Landbou, Grondervorming en Landelike Ontwikkeling - Mkhazha wa zwa Vhuleni, Mavedzozo ya Mavu na Mvekedzozo ya Mahayani, uMnyango Wezokimo, izinguquko Kwezomhlaba Nokuthuthukiswa Kwezindawo Zasemakhaya - Ndzawulo ya Vutini, Antswiso wa Mtshezi na Nkhuvukiso wa Matikodokaya - Litiko Letekulima, Tingucuko Kutemhlaba Nokuthuthukiswa Kwezindawo Zasemaphandleni - Umnyango wazokulima, ukubuyiselwa kweNtshukiswa kwezindawo zamaKhaya - Kgoro ya Temu, Peakanyoswa ya Naga le Tihobolo ya Dinaga - magae Letapha le Temothuo, Kabobotha ya Naha le Tihobolo ya Dibaka tsa Mahae - Letapha le Temothuo, Pusetso dinaga le Tihobolo ya Metsemagae - ISebe lazoLimo, uBuyekazo lwemhlaba noPhuhliso lamaPhandle

Page 1

research project under this Section 20 permit. Please apply in writing to Mamali@dairnet.gov.za.

3. If required, an application for an extension must be made by the responsible researcher at least one month prior to the expiry of this Section 20 permit. Please apply in writing to Mamali@dairnet.gov.za.
4. No part of this research project may begin until the valid ethical approval has been obtained in writing from the relevant South African authority.
5. All biological or potentially infectious material must be packaged and transported in accordance with the International Air Transport Association (IATA) requirements and/or the National Road Traffic Act, 1996 (Act No. 93 of 1996);
6. Only the following may be utilized in this research project:
 - 6.1. Monitor lizard (*Varanus albigularis*) heads sourced from the Hoedspruit Reptile Centre, Mpumalanga, and for which a state veterinary letter has been obtained
 - 6.2. Rabbit polyclonal antibodies:
 - 6.2.1. Rabbit anti Calbindin D-28k, Rabbit anti Calretinin and Anti-parvalbumin PV27 from Swant, Switzerland
 - 6.2.2. Rabbit anti glial fibrillary associated protein (GFAP) antibody ab7260, Anti-Glutamate Receptor 1 (AMPA subtype) antibody ab31232, Anti-NMDAR1 antibody - Neuronal Marker ab17345, Anti-NMDAR2B antibody ab65783, Anti-VGLUT1 antibody ab77822, Anti-VGLUT2 antibody ab217943 and Anti-MAP2 antibody - Neuronal Marker ab32454 from Abcam, United Kingdom
 - 6.2.3. GluR2 antibody, GluR4 antibody, NMDAR2A Polyclonal Antibody, NMDAR2C Polyclonal Antibody and NMDAR2D Polyclonal Antibody from Thermo Fisher, United States of America
 - 6.2.4. GluR1 from Bio-Rad, United States of America
 - 6.2.5. GRIN2B/NMDAR2B antibody from R&D Systems, North America
 - 6.2.6. AIF-1/Iba1 Antibody NBP2-19019 from Nevus Biologicals, United States of America
7. Veterinary import permits in terms of Section 6 of the Animal Diseases Act 1984 (Act no 35 of 84) must be obtained for the importation of all rabbit polyclonal antibodies to be utilized in this study as per point 6.2 above prior to importation thereof;
8. Only histopathology and immunohistochemistry may be performed on the samples outlined in point 6.1 above at the University of the Witwatersrand Neuroscience Laboratory;
9. All potentially infectious material utilised or generated during or by the research



SUBJECT: PERMISSION TO DO RESEARCH IN TERMS OF SECTION 20 OF THE ANIMAL DISEASES ACT, 1984
(ACT NO. 35 OF 1984) 12/11/1/1/20 (5022NT)

project is to be destroyed and disposed of by a waste disposal company registered to remove biohazardous waste at completion of the research project with the exception of mounted monitor lizard olfactory bulbs which must be kept under access control at the University of the Witwatersrand Neuroscience laboratory;

10. Any incidence or suspected incidence of a controlled or notifiable disease in terms of the Animal Diseases Act, 1984 (Act No 35 of 1984), must be reported immediately to the responsible state veterinarian;
11. It is the responsibility of the researcher and relevant laboratory or facility managers to ensure that the human safety aspects of this research project are adequately addressed;
12. Records must be kept for five years for auditing purposes.

Title of research/study: Comparative analysis of the olfactory system in the Nile crocodile (*Crocodylus niloticus*) and monitor lizard (*Varanus albigularis*)

Researcher: Mr. Marcel Elijah Rappetti

Institution: School of Anatomical Sciences, Wits Medical School, University of the Witwatersrand, Gauteng

Permit Expiry Date: 31 August 2026

Our ref: 12/11/1/1/20 (5022NT)

Kind regards,



DR.MP A

DIRECTOR: NIMAL HEALTH

Date:

2023-08-14

