International Journal of Advanced Research in Biological Sciences
ISSN: 2348-8069
www.ijarbs.com
SJIF Impact Factor: 5.142 Volume 3, Issue 3 – 2016

Research Article

SOI: http://s-o-i.org/1.15/ijarbs-2016-3-3-9

Development of eye regions and Harderian glands in young *Chameleo chameleon*

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Abstract

The pattern structure of retina, cornea and Harderian gland of *Chameleo chameleon* is still not clearly investigated. Young chameleons were collected and categorized in four groups according to the morphological analysis (G1, G2, G3 and G4). Chameleons were determined by morphological criteria of head diameter and length of fore and hind limbs. The eye cups were dissected. The cornea, retina and Harderian gland were separated. Morphological, morphometric, histological and immunohistochemical reaction of both GFAP and iNOS were investigated. Macroscopic observations were carried out of eye pattern shape. Histological observations of cornea revealed that it is composed of multicellular corneal epithelium, compact stroma infiltrated by keratocytes and endothelium. The whole retinal thickness was decreased with the advancement of growth. The retinal cell layers are photoreceptors, thin outer nuclear and outer plexiform layer, thick inner nuclear and inner plexiform layer & ganglion and nerve fiber layer. The photoreceptors are composed of single and double cones only characteristic of diurnal life. Harderian glands were composed of follicles lined with epithelium, the follicular lumen filled with hyaline secretion in G4. Immunohistochemically, GFAP and iNOS show moderate to intense immunoreaction in the different groups reflecting innervation and blood supply. The authors concluded that there is integration between corneal, retinal and Harderian gland functions to ensure good vision highly adapted for chameleon predation.

Keywords: *Chameleo chameleon*, Cornea, Retina, Harderian gland, Light microscopy, Immunohistochemistry.

Introduction

Chameleons are small to mid-size reptiles having characteristics of rapid change of their color. These species are mostly diurnal and solitary living. Chameleons are specialized for their ability to prey with highly adapted mobile eyes [1,2]. The chameleons switched to a different oculomotor behaviour during prey. The amplitudes of the synchronous saccades were usually different in the two eyes. It has existence of two independent premotor neuronal circuits for left and right eye saccadic motor control in the chameleon. The eyes move independently of one another [3,4]. The cornea is a transparent structure covering the front of the eye. Chameleons have a specialized convex cornea. The increased power of the cornea improves sight resolution in a narrow field of vision [5,6]. Pcheliakov [7] reported a poor differentiation of epithelium and endothelium in grass-snake, lizard and turtle. At cytological level, a great number of mitochondria compactly arranged in the middle part in the epithelial cells. The photoreceptor cells composed mainly of two types; rods and cones, which work at low and intensive light, respectively. Nocturnal species possessed mainly rods, meanwhile diurnal ones have high numbers of cones and rods are few or missing [8]. The visual cell layer is made up of single and double cones, No rods are present. The internal nuclear layer is thicker than the external nuclear layer. The nuclei and ganglion cells of light- and dark-

adapted animals do not show any differences in staining. A prominent conus papillaris and a fovea are present. The epithelial pigment layer and the cones do not undergo any photomechanical changes. It was not possible to condition the chameleons to yield any behavioral responses [9].

The Harderian gland and ocular function is of great importance and no available information about its exact role in young *Chameleo chameleon*. The gland present in terrestrial vertebrates including anuran amphibia, reptiles, birds and mammals[10]. The Harderian gland is located medioventrally to the eyeball in aves [11-13]. According to Sakai [14], Harderian gland produces a mucous secretion in amphibians, serous or sero-mucous secretion in reptiles and lipid secretion in mammals. In snakes, the gland wholly ensheathed the eye and optic nerve. It is located deep to the postorbital bone and inferior to the optic nerve and open into the the lacrimal duct [15]. In squamates, the gland ducts open to the anterior portion of the orbit [16,17]. The gland is the most source of serous secretion for the fluid in the squamate vomeronasal organ [18]. In *Pseudonaja textilis* (Elapidae) and *Thamnophis sirtalis* (Colubridae), the Harderian gland is large and its secretions pass directly into the vomeronasal organ via the nasolacrimal duct [19]. In *Alligator mississippiensis*, the gland takes a tongue-shaped structure, located deep in the rostroventral portion of the orbit, and is associated with the deep aspect of the nictitating membrane [20].The function of the Harderian gland is lubrication of the cornea and third eyelid, synthesis of hormones (androgens, estrogens), production of pheromones and growth factors– particularly in birds– providing salt in turtles, osmoregulation and thermoregulation in some rodents [12,21,22].

The present study aimed to illustrate the structural and developmental aspects of ocular regions of *chameleo chameleon* and their correlation with their function.

Materials and Methods

Juvenile individuals of *Chameleo chameleon*, Linnaeus (1758) (Class: Reptilia; Order, Squamata; Suborder Sauria; Family, Chameleonida; Genus, *Chameleo*; Species, *chameleon*) were collected from Abou−Rawash desert, Giza Governorate, Egypt. The age of specimens were categorized into four stages (n=6) according to the variations of total body length, head length, tail length, body width and limbs length as represented in table (1) and figure (1).

Histological investigation

The Harderian gland and eyecups of the selected stages were dissected and isolated. The cornea and retina of each eye were separated and fixed in 10% phosphate buffered formalin (pH 7.4). They were dehydrated in 60, 70, 80, 90& 100% of ethyl alcohol, cleared in xylene and mounted in molten paraplast at 58-62ºC. Five micron sections were carried out, stained with hematoxylin and eosin and investigated for histopathological changes under a bright field light microscope. The thickness of whole retina and their layers were measured by linear ocular micrometer and recorded.

Immunohistochemical staining of GFAP and iNOS:

The Harderian gland and eyes were dissected, fixed 72h in 4% paraformaldehyde. Then, embedded in paraffin blocks, sectioned in (5 µm thick), deparaffinized with xylene and rehydrated through descending grades of alcohol. The blocking of endogenous peroxidase activity was carried out by incubating specimens in 2% hydrogen peroxide for 5 minutes. Antigen retrieval of formalin-fixed sections was carried out by microwaving the sections in 10 mM citrate buffer (pH 6.0) at 95–100^{O}C for 10 min. The slides were then incubated overnight at 4^{O}C in a humidified chamber with the primary antibodies of GFAP (Cat sc-33673, 1:400, mouse, Santa Cruz) and iNOS (Cat. sc-7271, 1:500, mouse, Santa Cruz). After rinsing with a phosphate-buffered saline solution for 15 minutes, the specimens were incubated in biotinylated secondary antibody for 50 minutes at room temperature. After an incubation step with Avidin–Biotin– horseradish peroxidase conjugate for 30 minutes, sections were stained with 0.04% 3,3'-diamino-benzidine tetrahydrochloride and counterstained with Hematoxylin. Omission of the primary antibody served as a negative control. The intensity of immuno-reaction was expressed as + for weak, ++ for moderate and +++ for intense immunoreaction.

Statistical analysis

Data were presented as mean ± standard error. The statistical analysis was performed with multi-variant analysis of variance (ANOVA) and post hoc analysis using SPSS (version 18) software package for windows.

Results

Gross morphology:

Table (1) illustrates the morphological parameters of growing youngs of *Chameleo chameleon.* The age of individuals categorized according to morphological criteria of head length, body width, length of tail and limbs. Macroscopic observations revealed that, the eyes protrude laterally from the head taking a cone-shaped structure. The eyelids encircles the pupil and fuse with it, leaving only a small part exposed. During the eye opening, a circular to oblique ocular opening appeared meanwhile during the eye closure a longitudinal slit of ocular region was detected (Fig. 1).

Tab (1): Morphometric analysis of G1, G2, G3 and G4 of *Chameleo chameleon* in (*mm*).

	TBL	HL		SVL	TL	BW	FLL	HLL
		Antero-posterior	Dorso-ventral					
G1	7.88±0.86*	1.70±0.11*	0.9±0.11*	4.65±0.31*	3.45±0.18*	1.73±0.14*	1.24±0.32*	1.66±0.38*
G2	10.33±0.65*	2.72±0.20*	1.87±0.16*	6.08±0.57*	4.90±0.21*	2.69±0.10*	2.78±0.33*	3.37±0.32*
G3	13.69±0.78**	3.14±0.17**	2.22±0.24*	7.71±0.77**	6.02±0.46*	3.12±0.09**	3.16±0.17*	3.73±0.25**
G4	17.56±0.56**	3.48±0.44**	2.98±0.21**	10.51±0.65**	7.96±0.54**	3.52±0.22**	4.17±0.29**	4.95±0.17**

Each result represents the mean±SE (n=6). Abbreviations; *****, Non significant; ****** Significant at P <0.05; **BW**, Body width (in the middle of trunk); **FLL**, Fore limb length; **HL**, Head length (from tip of snout to the corner of the neck); **HLL**, Hind limb length; **SVL**, Snout vent length; **TBL**, Total body length; **TL**, Tail length.

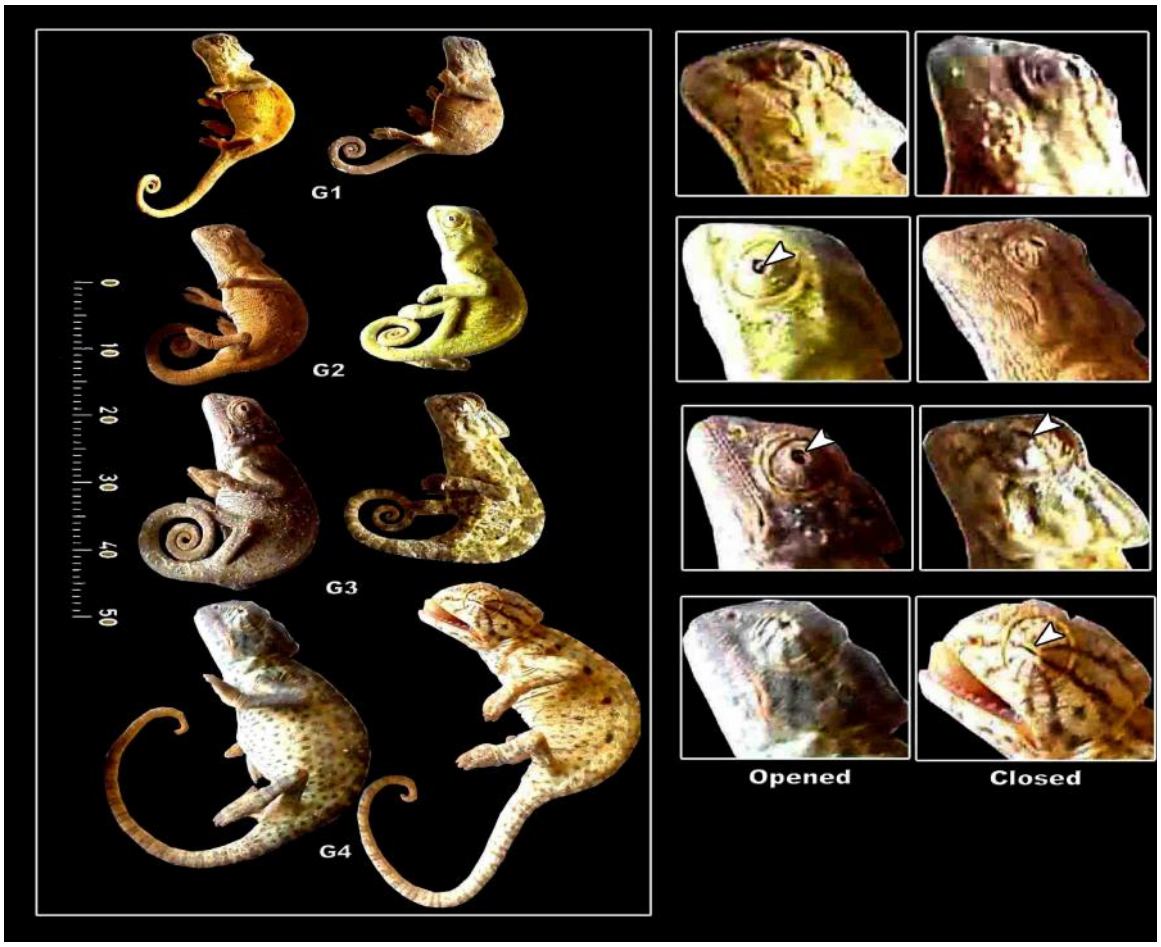

Fig.1. Lateral view photomacrographs of different stages of developing *Chameleo chameleon* (G1, G2, G3 & G4). Arrowed head indicated the conical-shaped structure of eye ensheathed with eyelids and appeared either in open and closed conditions.

1. Cornea:

The cornea is composed of multilayered epithelium, Bowman's layer, compacted stroma with regularly arranged collagenous layer infiltrated by numerous keratocytes and bordered by the endothelium. In advanced stages of development, vacuolated epithelium firstly appeared in G2 and widespread in G3& G4. (Fig. 2 A-A3).

Corneal immunostaining with GFAP, revealed a dark-brown reaction in peripheral epithelium and deep stromal and endothelial layers of G1& G2 as well as deep stromal layer of G4 (Tab. 2, Fig. 2 B-B3). Also, immunostaining of the iNOS was detected in the epithelium, Bowman's membrane and deep stromal layer and endothelium of G3& G4 (Tab .2, Fig. 2 C-C3).

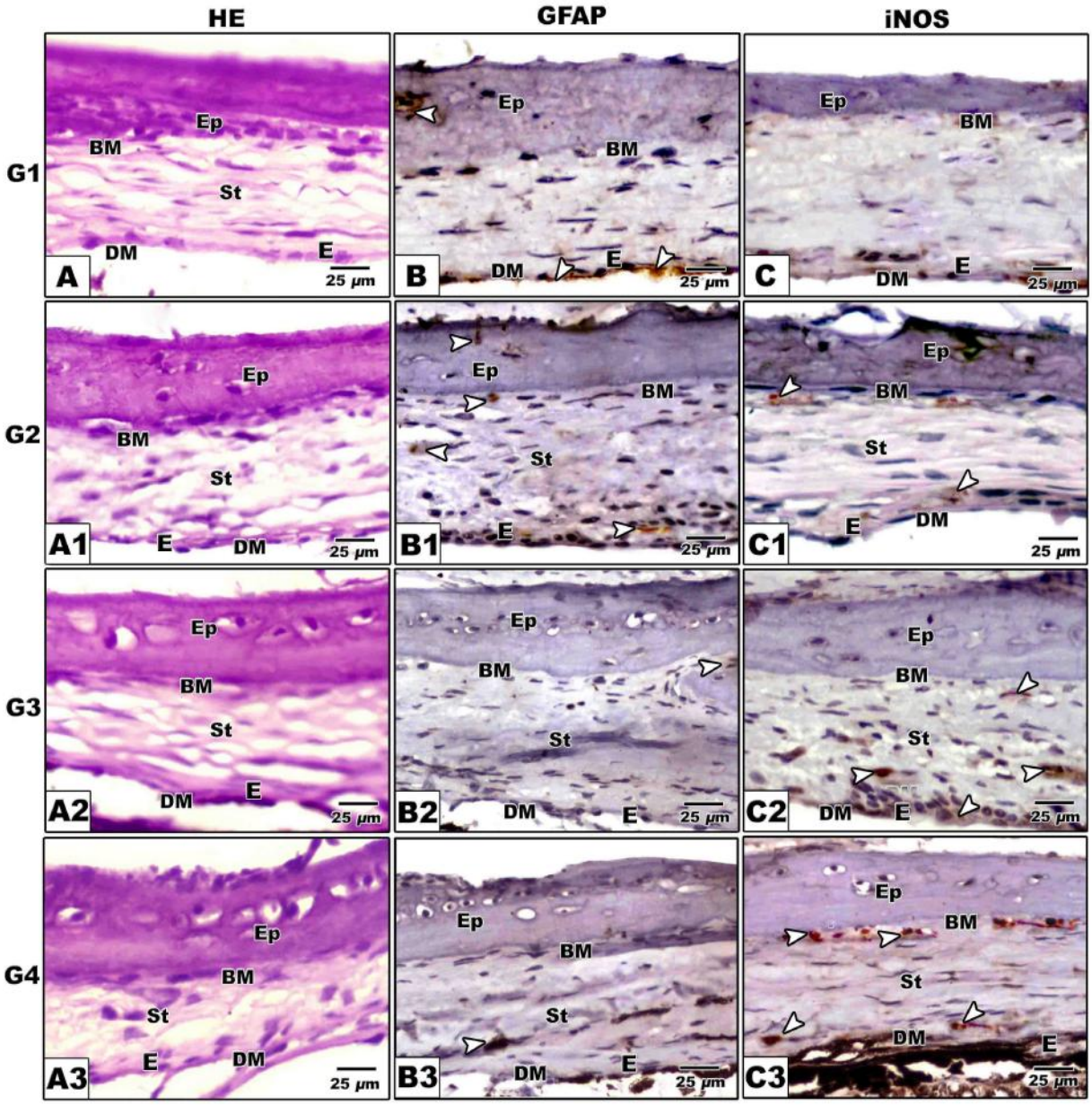

Fig.2. Photomicrograph of histological sections of cornea of different developing stages of *Chameleo chameleon*. A-A3. Showing multilayered epithelium, Bowman's layer, heavily nucleated stroma and endothelium. B-B. Showing intense immunostaining of GFAP in epithelium, stroma and endothelium of G1, G2& G4. C-C3. Showing immune staining of iNOS more intense in G3& G4.
Abbreviations; **BM**, Bowman's membrane; **DM**, Descemet's membrane; **E**, Endothelium; **Ep**, Epithelium; **St**, Stroma.

Tab (2): Immunoreactivity of GFAP and iNO of cornea, retina and Harderian gland of G1, G2, G3 and G4 of *Chameleo chameleon.*

		G1		G2		G3		G4	
		GFAP	iNO	GFAP	iNO	GFAP	iNO	GFAP	iNO
Corneal layers	Ep	+	-	+	-	-	-	-	-
	BM	-	-	++	+	+	+	-	+++
	St	-	-	+	+	-	++	++	++
	DM	+++	-	++	-	-	+++	-	+++
Retinal cell layers	PhR	+	-	++	-	+++	+	-	++
	ONL	+	-	++	-	++	++	+	+++
	OPL	+	+	++	-	+++	++	+	+++
	INL	+	-	+	+	+++	+	+	+++
	IPL	++	-	++	-	+++	-	++	+++
	GCL	+	-	+++	-	++	+	+	+
Harderian gland	Ac	++	+	++	-	++	++	-	+++
	SD	+	-	+	-	++	+	+	++

Abbreviations; **Ac**, Acini; **BM**, Bowman's membrane; **DM**, Descemet's membrane; **Ep**, Epithelial layer; **GCL**, Ganglion cell layer; **GFAP**, Glial fibrillary acidic protein; **iNO**, Inducible nitric oxide; **INL**, Inner nuclear cell layer; **IPL**, Inner plexiform layer; **ONL**, Outer nuclear cell layer; **OPL**, Outer plexiform layer; **PhR**, Photoreceptor cell layer; **SD**, Secretory ducts; **St**, Stroma; -, Negative; +, Weak ; ++, Moderate ; +++, Intense immunoreaction

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2. Retina:

Table (3) illustrates the thickness of retinal layers in the developing individuals. The whole retinal thickness was apparently decreased with the growth of the young chameleon.

Tab (3): Mean thickness of retinal cell layers in (μm) of G1, G2, G3 and G4 of *Chameleo chameleon.*

	R	PhR	ONL	OPL	INL	IPL	GCL	NFL
G1	116.75±2.78	11.35±1.14	5.21±1.12	17.25±1.71	30.91±1.98	24.31±1.54	4.38±0.87	2.08±0.32
G2	109.09±3.10	11.38±1.31	5.78±1.32	18.41±1.91	30.30±2.21	25.08±2.01	4.04±0.65	2.14±0.26
G3	108.27±2.22	13.53±1.29	6.98±1.25	24.24±2.1	28.34±1.67	26.23±1.78	4.92±0.48	3.22±0.18
G4	102.14±1.98	12.98±1.40	7.77±0.87	19.74±1.51	30.41±1.87	28.91±1.56	3.1±0.37	2.88±0.12
F-test	1.354	1.286	8.34	12.580	7.154	4.231	4.523	2.145
P < 0.05	S	S	IS	S	S	S	S	IS

Each result represents the mean±SE (n=5). Significant at P <0.05; Abbreviations; **GCL**, Ganglion cell layer; **INL**, Inner nuclear cell layer; **IPL**, Inner plexiform layer; **NFL**, Nerve fiber layer; **ONL**, Outer nuclear cell layer; **OPL**, Outer plexiform layer; **PhR**, Photoreceptor cell layer; **R**, Retina.

The retinae of examined developing *Chameleo chameleon* are composed of two plexiform layers, two nuclear layers, a ganglion cell layer, a nerve fiber layer and the photoreceptor layer. The outer nuclear layer composed of single or double cell layers composed to more thickened inner nuclear cells. Also, outer plexiform layer thickness is decreased comparing to the inner plexiform. The ganglion cells are abundant and distributed in contact with nerve fibers and being less numerous compared to the young ones. The retinae outlined in its outer margin, in contact with photoreceptors, by pigmented epithelium which contained dark-brown pigmentation. This heavy melanosomal distribution protects the retina from

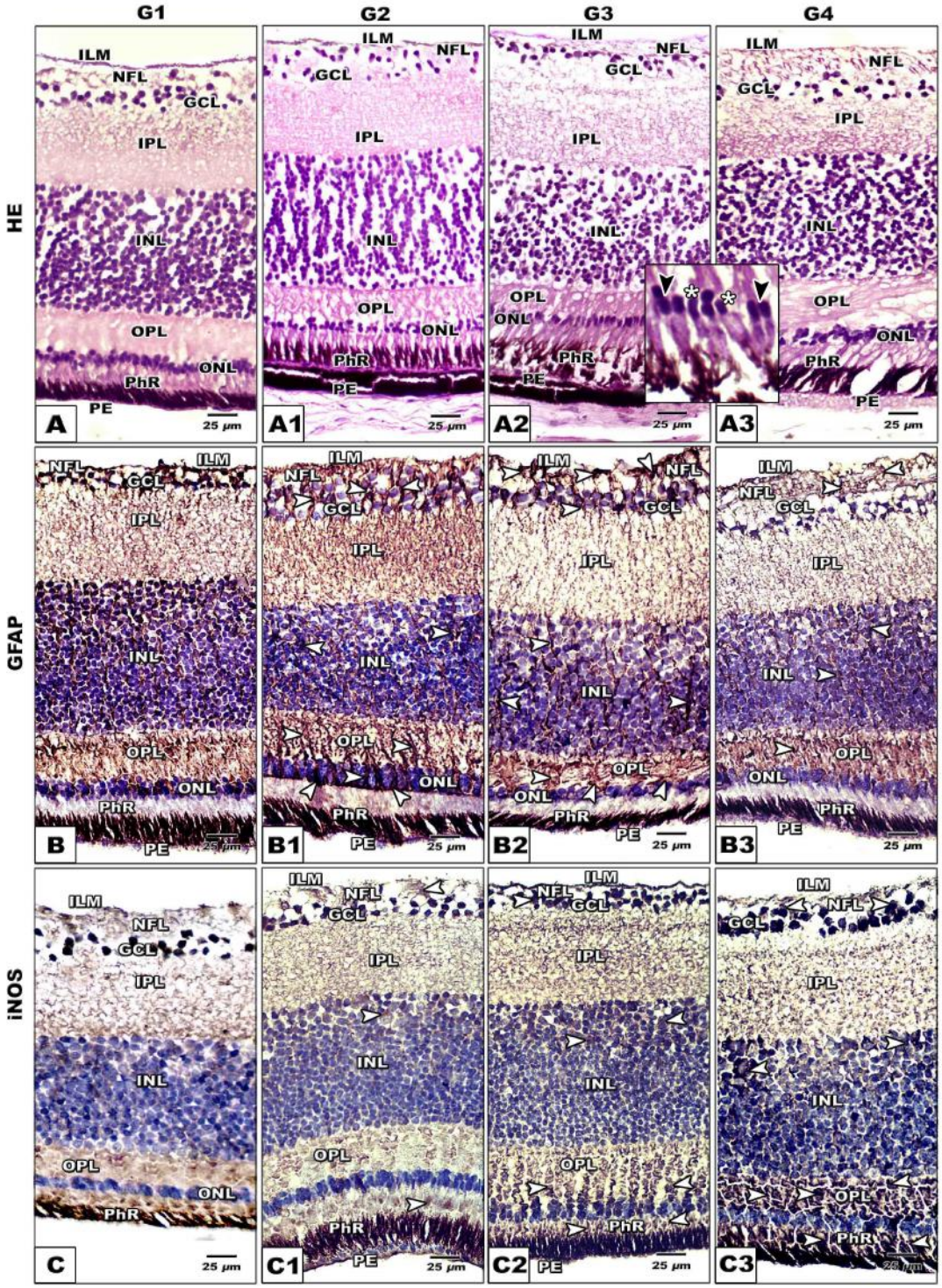

Fig.3. Photomicrograph of histological sections of retina of different stages of *Chameleo chameleon*. A-A3.Showing developed pigmented epithelium, photoreceptor, outer plexiform layer, inner & outer nuclear layer inner plexiform layer, ganglion and nerve fiber layer. (Stars refer to the single cone and black arrows for double cone). B-B3.Showing GFAP immunostaining dark-brown reactions in nerve fiber and ganglion layer, nuclear, outer plexiform and outer nuclear layer more intense in G1 and G2. C-C3. Showing more immunostaining of induced nitrous oxide synthase in ganglion, inner& outer nuclear layer and photoreceptors especially in G4.

Abbreviations; **GCL**, Ganglion cell layer; **ILM**, Inner limiting membrane; **INL**, Inner nuclear layer; **IPL**, Inner plexiform layer; **ONL**, Outer nuclear layer; **NFL**; Nerve fiber layer; **OPL**, Outer plexiform layer; **PE**, Pigmented epithelium; **PhR**, Photoreceptors).

sudden bright light exposure (Fig. 3 A-A3). The photoreceptors of *Chameleo chameleon* are mainly cones to be adapted for diurnal life and as in figure 3 (A2& A3) it is obviously double cones (black arrows) and single cones (stars).

GFAP immunostaining revealed dark-brown reactions in nerve fiber and ganglion layer, inner nuclear, outer plexiform and outer nuclear layer especially in G2 and G3 compared to the other developing stages (Tab. 2, Fig. 3 B-B3). Immunostaining with iNOS was markedly detected in ganglion, inner & outer nuclear layer and photoreceptors especially in G3&G4 compared to the other developing stages (Tab. 2, Fig. 3 C-C3).

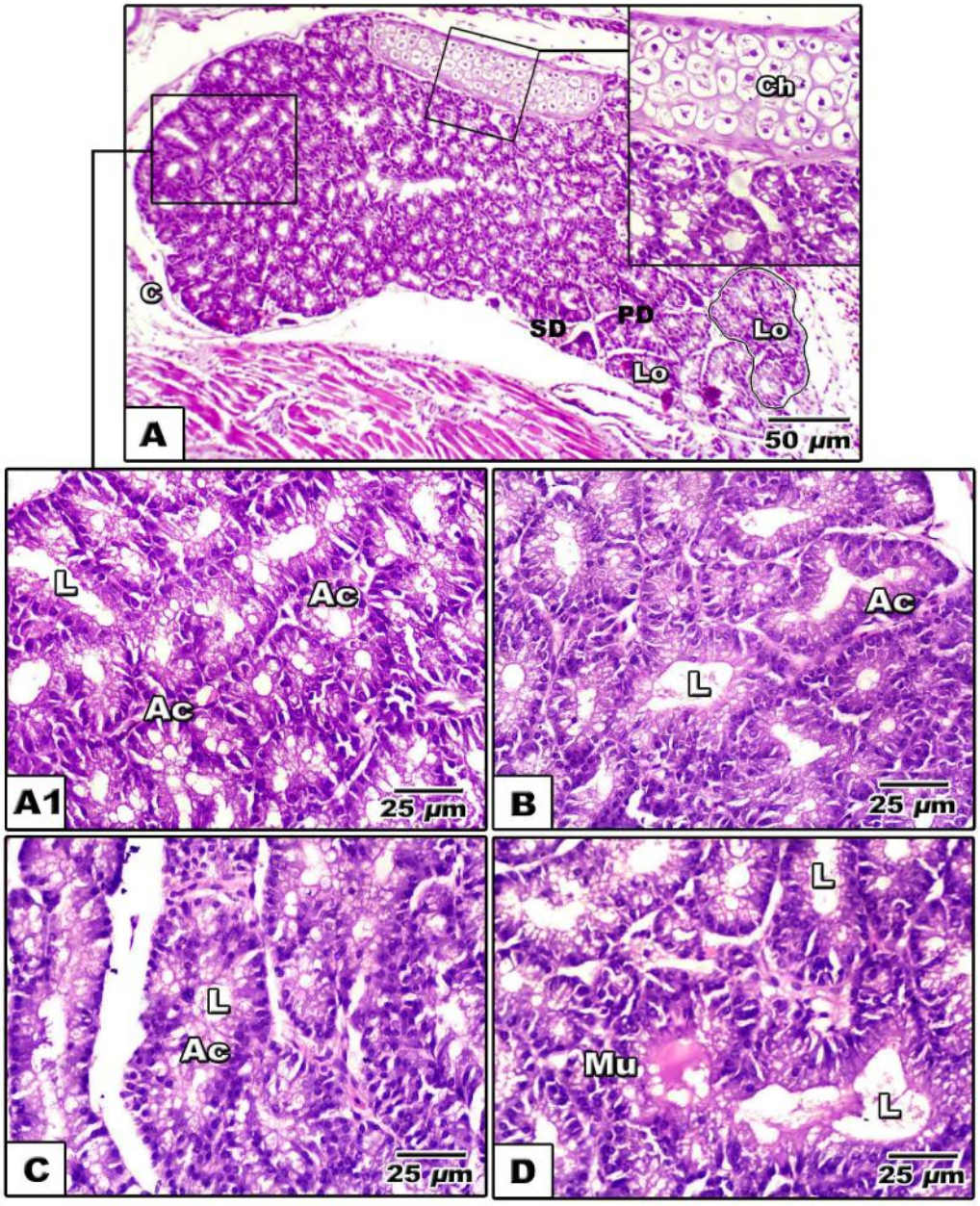

Fig.4. Photomicrograph of histological sections of Harderian gland of different developing stages of *Chameleo chameleon*. A. Showing kidney-shaped harderian gland of G1 surrounded by a capsule of connective tissue. The gland composed of lobules, each lobule has acini opens into primary and secondary secretory ducts. A1-C. Showing alveoli lined by cuboidal cells and central lumen. D. Showing follicles possess hyaline mucinous secretion inside its lumen.

Abbreviations; **Ac**, Acinus; **C**; Cortex; **Ch**, Chondrocytes; **L**, Lumen; **Lo**, Lobule; **Mu**, Mucinous secretion; **PD**, Primary duct; **SD**, Secondary duct.

3. Harderian gland:

Histologically, the Harderian a gland is mainly composed of two lobes, each lobe of all developing stages, is kidney-shaped with small (upper or dorsomedial) and large (lower or medial) lobes with the optic nerve in between. The gland is encircled with thin capsule of connective tissue. Septa from this capsule penetrate into the gland dividing it to many lobules. Each lobule is separated by fine interlobular spaces. The overall shape of the tubuloalveoli is not rounded, but being elongated and tortuous and some of them become bifurcated. Each alveolus has a lumen ensheathed by columnar or conical cells. The lobules are separated from each other by fine connective tissue. These alveolar cells and their lumina attained a considerable hypertrophy with the presence of mucinous secretion in G4 (Fig. 4 A-D).

Following immunostaining with GFAP, the follicle epithelial lining cells showing positive GFAP intense reaction in G4 (Tab. 2, Fig. 5 A-A3). On the other hand, iNOS was immunostained in cytoplasm of follicle cells and their tubules of G1& G3 and G4 (Tab. 2, Fig. 5 B-B3).

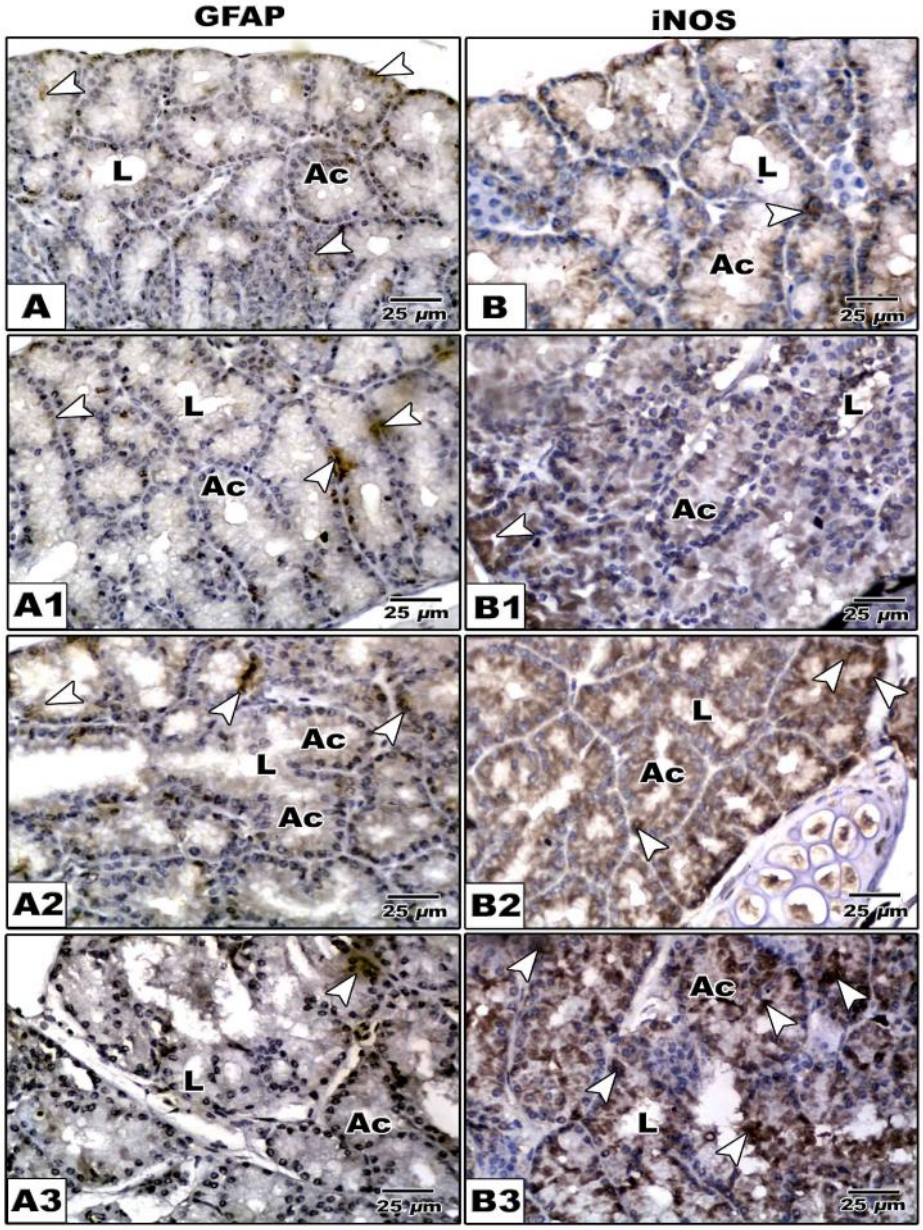

Fig. 5. Photomicrograph of immunostaining GFAP and induced nitrous oxide synthase of Harderian gland of developing stages of *Chameleo chameleon*. A-A3. Showing intense immunostaining of GFAP localized in epithelial lining cells. B-B3. Showing intense immunostaining of iNOS in follicle cells.
Abbreviations: **Ac**, Acinus; **L**, Lumen.

Discussion

Macroscopically, the eyes protrude laterally from the head taking a cone-shaped structure. The eyelid fused to the pupil to protect the eyes, leaving only a small part to expose. During opening, a circular to oblique ocular opening appeared meanwhile during closure a longitudinal slit of ocular region appeared. The structural organization of the cornea allows it to move in a wide range of its surrounding environments.

Flanders [1] and Kirmse et al. [2] reported that the chameleons have a characteristic high mobility of eyes. Both eyes move independently [3, 4].

Concerning the cornea, it is composed of several cell layers as a multilayered epithelium, Bowman's membrane, stroma, Descemet's membrane, and endothelium. The corneal endothelium (CE) is a single hexagonal cell that separates the corneal stroma from the aqueous humor of the anterior chamber. Transparency of the cornea is carried out by regulation of stromal hydration through the barrier and pump functions of the corneal epithelium [23].

Following immunostaining, both GFAP and iNOS immunoreactivity were intense in the epithelium and deep stromal layers including the endothelium. This increased immunoreaction reflects the state of cornea innervations.

Following the investigation of chick cornea [24], mice [25] and human [26], there is a close similarities of corneal innervations. Nerves were found to be infiltrated throughout the cornea especially with apparent increase around the corneal limbus and continuous toward the central cornea. In mice and human, dense nerve network present at the mid-peripheral zone, and anterior stromal nerves. Nerves from all directions converged towards the infero-central cornea to form a characteristic whorl pattern. On the other hand, the nerves appear to be directly entering the cornea, and extend both dorsally and ventrally, forming a pericorneal nerve ring around its entire circumference. The pattern of innervation and lining endothelium reflects the apparent distribution of iNOS and GFAP.

On the other hand, the histological structure of retina characterized by increased inner plexiform and inner nuclear layer compared to apparent decrease of outer nuclear and outer plexiform layer. There was a detected increase of ganglion cells and presence of single and double cone. In addition to heavy pigmentation and melanosomal activity in the pigmented epithelium which plays an important role in the protection of retina from sudden light exposure. All these retinal structural accommodations reflect the diurnal living of the studied *Chameleo chameleon.* The photoreceptors composed mainly of single and double cones which become more visualized in G3 and G4 compared to poorly differentiate one in G1.

Similar findings were reported in iguanids, agamids, scincids, lacertids, anguids, pygopodids and varanids [27-30]. Taking in consideration that the retina is a specialized tissue responsible for the reception and transduction of light stimuli derived from the outside environment. Retinal cells including the photoreceptors (rods and cones), horizontal, bipolar and ganglion cells are coordinated in preserving retinal function. During retinal neurogenesis, structural organization of retinal cells rely its functional activities. Following immunostaining, there was an intense immunoreaction of GFAP in nerve fiber and ganglion layer, inner nuclear, outer plexiform and outer nuclear especially in the early young age G1 and G2 compared to the other developing stages.

Glial fibrillary acidic protein (GFAP) was found to be localized mainly in astrocytes [31,32] and Müller cells [33]. The Müller cells penetrate the entire thickness of the retina and their basal processes align in the nerve fibre layer to form septa that fasciculate the axons of the ganglion cells. The processes of the astrocytes are confined to the ganglion cell layer and to the nerve fibre layer. In the latter, the astrocytic processes run parallel to and between the axons of a given nerve fibre bundle [34].

Also, iNOS was markedly detected in ganglion, inner& outer nuclear layer and photoreceptors especially in the advanced developing chameleon (G4) compared to the other developing stages. This would reflect the maturation phase of the retinal cells.

It is known that the nitric oxide is a gaseous free radical produced by the enzyme nitric oxide synthase (NOS) which participated in the intracellular signaling processes in the nervous system [35,36]. Nitric oxide synthase (NOS), expressed in three types, neuronal, endothelial, and immunologic. Neuronal and immunologic NOS have been detected in the retina. It plays a great role for managing the developmental aspects of embryonic retinal neuronal cells, such as neurotransmitter release, proliferation and cell death. Neuronal NOS is important for producing nitric oxide in photoreceptors and bipolar cells [37]. The apparent increased NOS reactivity indicates the maturity of

chameleon retina in advanced stages to be highly adapted for predation.

Our study demonstrated that Harderian gland has follicular structure with epithelial lining cells and interfollicular tissues contain thin collagenous fibrous tissue. By advancement of growth, the Harderian gland produces mucinous secretion.

These finding agree to the work of Rehorek et al.[18,19] who observed similar mucins and serous secretion in reptilian Harderian gland. Similar results were reported in the study in the Ostrich [38], adult domestic geese [22] and rodents [39,40] whose indicate the presence of neutral and acid mucins as well as glycoprotein secretion.

GFAP and iNOS immunoreactivity were detected in the follicle epithelial lining cells and interfollicular tissue. The increased affinity to immune reaction at the periphery and inbetween of the follicles reflects the innervation of the Harderian gland. This involved in the stimulation of the acini to produce its mucous secretion for cleaning and sterilizing the eye.

Similar results were reported by Huhtala et al. [41] who identified the presence of acetylcholinesterase-positive nerves infiltrated through the bundles in the intertubular connective tissue. These bundles sent finer branches around the acini. The blood vessels distributed in between the Harderian follicles and surrounded by a dense plexus of acetylcholinesterase-containing fibres.

Finally, the authors concluded that there is a co-ordination between, cornea, retina and Harderian glands for preserving their function.

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How to cite this article:
Ahmed A. El Mansi, Dalia A. Sabry, Yosra A. Fouda. (2016). Development of eye regions and Harderian glands in young *Chameleo chameleon*. Int. J. Adv. Res. Biol. Sci. 3(3): 57-67.