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Anatomical, scanning electron microscopy, histological and histochemical studies of the orbital glands of the Egyptian agama *Trapelus mutabilis*

Fatma Abdel-Regal Mahmoud[1*], Samia Abdalla Gaber[2], Amany Salah Saad Mahmoud[2] and Ali Gamal Gadel-Rab[3]

Abstract

Background: The orbital gland of the group agama (Agamidae) is not well-studied. Here is a first description of anatomical and histological studies on the orbital gland of the Egyptian agama *Trapelus mutabilis*.

Results: The Harderian gland (HG) of the Egyptian agama is well-developed, with a tongue shape, appearing whitish in color, and reaching approximately 1.47 mm in length. The gland can be divided into head and tail regions and lies at the medial corner of the orbit ventral to the ventralis oblique muscle and dorsal to the medialis rectus muscle. Secretory product of the HG is released into the conjunctival fornix, which is deep into the nictitating membrane. The HG is a compound tubuloacinar type, consisting of columnar and pyramidal glandular cells with rounded nuclei and lumina that are variable in size and containing pigments in the connective tissue capsule that envelops the tail portion of this gland. The glandular cells of the HG react positively to periodic acid-Schiff (PAS) and alcian blue staining at pH 2.5. In contrast, lacrimal gland (LG) of Egyptian agama is a reduced mucous gland that appears whitish in color, opens on the surface by numerous orifices at the base of nictitating membrane, and reaches approximately 0.1 mm in length. The lacrimal gland is composed of a simple tubulo-acini of columnar cells with narrow lumen and basal oval-shaped nuclei and exhibits a weak response to PAS but a strong response to alcian blue at pH 2.5.

Conclusions: Morphology of LG and HG isn't impacted by features of the orbit, such as the existence or absence of nictitating membrane and/or fixed or movable eyelid. The small size of the LG of the Egyptian agama does not affect the performance of its main function in lubricating the surface of the cornea. The pigmentation envelope of the HG of the Egyptian agama allows heat to be absorbed in order to increase its secretion activity, thereby increasing the protection of the eyes against mechanical damage. Finally, both orbital glands may play a secondary function in digestion via indirect connection with the oral cavity.

Keywords: Eye, Harderian, Lacrimal, Gland, Agama

Background

The reptiles were the first tetrapods to develop both the Harderian gland (HG) and the lacrimal gland (LG) (Chieffi-Baccari et al., 1992). The HG was first discovered by Harder (1694) in the deer Dama dama. The HG is remembered to assume a significant part in earthly environments since it has not been recognized in fish,

*Correspondence: M_F11_7@yahoo.com

[1] Department of Zoology, Faculty of Science, Assiut University, Assiut, Egypt
Full list of author information is available at the end of the article

amphibian urodeles, and aquatic anurans. However, the gland is present in some secondary aquatic vertebrates, such as crocodilians and cetaceans (Dominguez-Perez et al., 2019).

Anatomical criteria based on its location in the anterior region of the orbit and its relationship with the nictitating membrane were used to characterize the HG. (Rehorek, 1997). The morphology of the HG isn't impacted by the existence or absence of eyelid (Rehorek et al., 1997). The HG is a huge exocrine secretory construction. When the nictitating membrane is present, the ducts of the HG open onto the corneal aspects of the nictitating membrane, or in the comparable location when the nictitating membrane is absent (Rehorek et al., 2006). In animals with the nictitating membrane, the HG is situated within the ocular orbit, medial and posterior to the eyeball (Boydak & Aydin, 2009). Santillo et al. (2006) studied in the HG the presence of d-aspartate of *Podarcis s. sicula* and its impact on gland secretion.

Most lizards have well-developed LGs that are located posterior, dorsal, and ventral to the eye. LGs are absent in chameleons, calotes, some geckos, Australian snake-lizards, and snakes (Wyneken, 2012) and in the small lizard *Podarcis s. sicula* (Chieffi-Baccari et al., 1990). The persistence of the LG has not been connected with the existence or absence of a brille. The LG is situated within the ocular orbit at either the posterior portion of the eyeball (Chieffi-Baccari et al., 1992) or the anterior portion of the eyeball (Chieffi-Baccari et al., 1990).

Glands structure is variable and, as a result, has many functions prescribed to them. It is considered that the main function of the LG and the HG is in orbital lubrication (Payne, 1994). However, recent studies have proposed additional extra-orbital functions for the HG and LG. It serves as a site for various immunological responses, a source of thermoregulatory lipids, a source of pheromones, a photoprotective organ, and part of the retinal-pineal axis (Dominguez-Perez et al., 2019; Payne, 1994). It has also been suggested that glands have a morphological link with the vomeronasal (Jacobson's) organ (VNO) (Silva & Antunes, 2017) and that it has a function in vomer-olfaction and chemosensory detection (Dominguez-Perez et al., 2019). Finally, the HG may have other activities in squamates, including serving as an accessory salivary gland and/or in the production and secretion of digestive enzymes (Dominguez-Perez et al., 2019).

Apparently, the orbital gland of the large group known as agama (Agamidae) has been studied in only one prior report. Bellairs and Boyd (1949) observed that, when present, the nictitating membrane physically divides the lacrimal duct and the HG and allows for secretions of the HG to enter the orbital environment. Therefore, the current study presents the first description of anatomical and histological investigations into the orbital gland of agama, *Trapelus mutabilis*. *Trapelus mutabilis* is an Egyptian agama endemic to Northern Africa that possesses relatively smooth scales (Wagner et al., 2011).

Methods

Five adult Egyptian agama lizards, *Trapelus mutabilis,* were collected from North Sinai, Egypt during the months of April and May. Live samples were brought to a comparative anatomy of vertebrate lab and dissected according to the guidelines of research ethics committees at Assiut University (www.enrec.org).

For our anatomy study, we fixed sample heads in 10% formalin for two weeks and then stored in 2% phenoxy ethanol for long-term preservation. Images were acquired using a Toup camp XCAM full HD camera connected to an Olympus microscope (model SZ61). The anatomical terminology of skull and orbital tissue was determined according to previously published guidelines (Nomina Anatomica Veterinaria, 2012).

For light microscopy experiments, the lower jaws were taken out from samples and heads were cut longitudinally into two halves. Eyes were then separated, along with their associated glands. Specimens were preserved in 10% neutral formalin for three days, after which they were decalcified in EDTA for two weeks. Then, A series of ethyl alcohols were used to dehydrate the samples, cleared in methyl benzoate for three days, which was embedded in paraffin wax and serially sectioned (7 m). Hematoxylin and Eosin and Masson's trichromic stains were used to stain the sections. (Drury & Wallington, 1980). Images were acquired on an Olympus camera (model DP74) connected to an Olympus microscope (model BX43).

Alcian blue at pH 2.5 and the periodic acid-Schiff tests were utilised in histochemical studies for the finding and characterization of acidic and neutral mucosubstances (PAS) (Ravetto, 1964).

For scanning electron microscopy (SEM) experiments, small pieces of eyes were cut and fixed directly in 5% glutaraldehyde in a cacodylate buffer for 48 h at 4 °C. Samples were washed in three changes of 0.1% cacodylate buffer, and the specimens were post-fixed in a cacodylate buffered solution of 1% osmium tetroxide for 2 h at 37 °C. Then washed three times in the same buffer, got dried out through an ascending series of ethyl alcohols, and infiltrated with amyl acetate for two days. The drying of specimens was accomplished by critical point drying using liquid carbon dioxide. Specimens were mounted and sputter-coated with gold. Finally, specimens were examined on a Jeol scanning electron microscope (J S M-5400I V) at 15 kV.

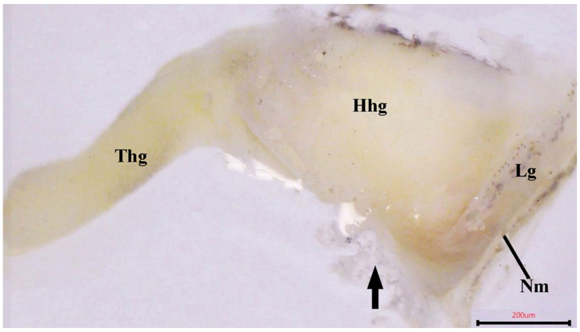

Fig. 1 A photomacrograph of the Harderian gland (Hg) and lacrimal gland (Lg) of Egyptian agama, *Trapelus mutabilis,* showing the shape of the Harderian gland with head (Hhg) implanted in the connective tissue capsule (arrow), granular tail (Thg), small lacrimal gland appearing nearly white in color, and the nictitating membrane (Nm)

Results

Orbital glands of Egyptian agama, *Trapelus mutabilis,* have two orbital glands, the HG and the LG.

Harderian gland

The HG of the Egyptian agama, *Trapelus mutabilis,* is well-developed and tongue-shaped. This gland appears close to white in color and can reach approximately 1.47 mm in length (Fig. 1). The HG lies at the medial corner of the orbit ventral to the ventralis oblique muscle and dorsal to medialis rectus muscle, which connects with the gland capsule by a connective tissue. The head (anterior region) is implanted in the connective tissue capsule, and tail (posterior region) passes postero-inferiorly (ventral) into the orbit without reaching the optic nerve (Fig. 2a–c).

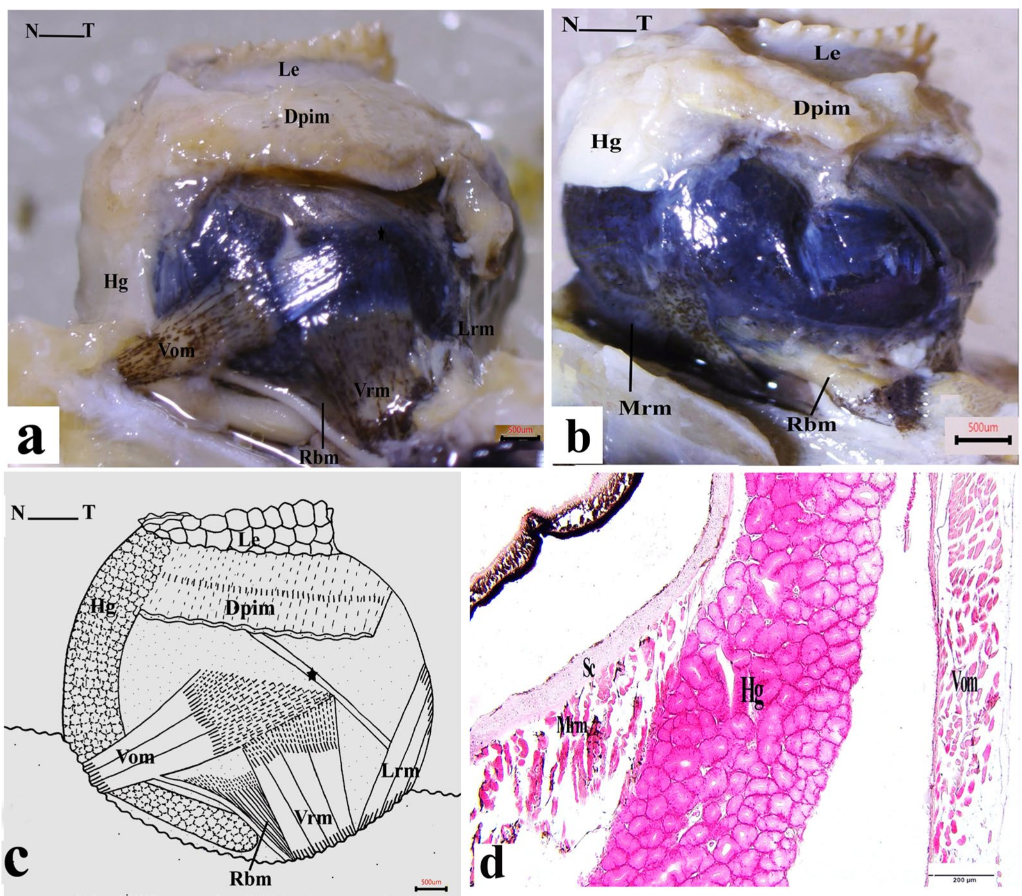

Fig. 2 A photomacrograph of the ventral view of the eye of Egyptian agama, *Trapelus mutabilis*, **a** after removing the partially depressor palpebral inferioris muscle (Dpim), **b** after the cutting ventralis oblique muscle (Vom), **c** drawn to clarify (**a**), **d** transverse section of the eye of Egyptian agama, *Trapelus mutabilis*, showing the localization of the Harderian gland (Hg) between the ventralis oblique muscle (Vom) and the medialis rectus muscle (Mrm) that attach on sclera cartilage (Sc) (H &E, × 100), lower eyelid (Le), ventralis rectus muscle (Vrm), and retractor bulbi muscle (Rbm)

The tail of HG of the Egyptian agama, *Trapelus mutabilis,* looks to be superficially granular and shows a considerable amount of pigments in the connective tissue capsule (Figs. 2d, 4a).

The duct of the HG of the Egyptian agama, *Trapelus mutabilis*, could be seen in microscopic detail. The gland's secretory product is released into the conjunctival fornix, which is deep into the nictitating membrane.

Histological analysis of the HG of the Egyptian agama, *Trapelus mutabilis,* showed that the gland is a compound tubuloacinar type. The acini of the gland consist of columnar and pyramidal glandular cells with rounded nuclei and lumina that are variable in size (Fig. 3a, b).

A thin connective tissue capsule surrounds the HG of the Egyptian agama that contains thin strands of collagenous fibers and a variable number of elastic fibers stained brown with the orcein stain (Fig. 4a, b). We observed blood vessels and nerve fibers penetrating into the glandular tissue (Fig. 3a).

Histochemical analysis of the HG of the Egyptian agama, *Trapelus mutabilis,* showed that the glandular cells are positive to the PAS reaction and with alcian blue at pH 2.5 give a bluish color (Fig. 5a, b).

Lacrimal gland

The LG of the Egyptian agama, *Trapelus mutabilis,* is a reduced mucous gland that appears nearly white in color and has a length of approximately 0.1 mm. The LG opens on the surface by numerous orifices on the base of nictitating membrane (Fig. 6b).

SEM analysis of the LG of the Egyptian agama, *Trapelus mutabilis,* showed many orifices of the glandular ductules that open on the surface of nictitating membrane base (Fig. 6b). Many orifices of the glandular ductules that open on the surface of nictitating membrane's base.

Histological analysis of the LG of the Egyptian agama, *Trapelus mutabilis,* showed that the LG is surrounded by

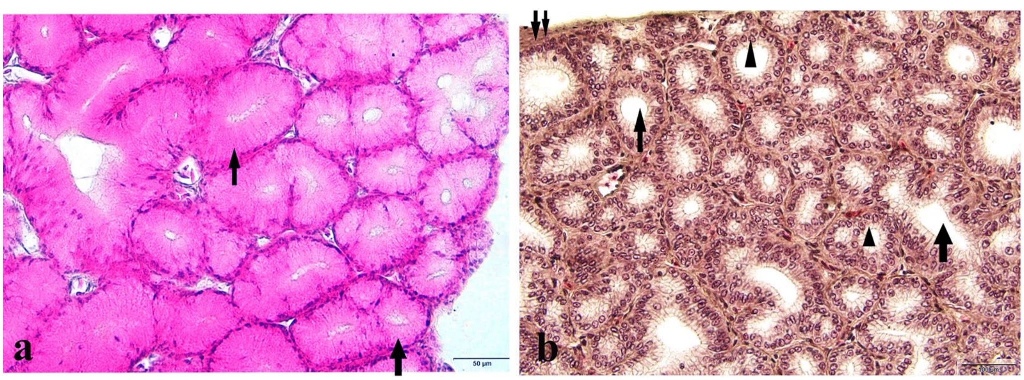

Fig. 3 A photomicrograph of a transverse section of the Harderian gland of Egyptian agama, *Trapelus mutabilis*, showing the Harderian gland is a compound tubuloacinar type (arrow) consisting of columnar and pyramidal glandular cells (arrowhead) with rounded nuclei and lumina that are variable in size (arrow); thin connective tissue capsule (double arrow) and blood vessels (arrowhead) penetrate into the glandular tissue (H &E, × 400, Masson's trichromic, × 200)

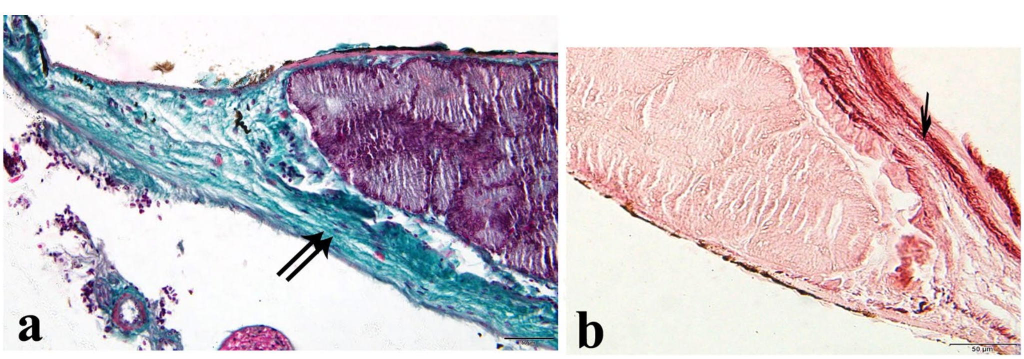

Fig. 4 A photomicrograph of a transverse section of the Harderian gland of Egyptian agama, *Trapelus mutabilis*, showing the connective tissue capsule of the Harderian gland contains collagen fibers (double arrows) and elastic fibers (arrow) (a, Masson's trichromic, b, Orcein, × 400)

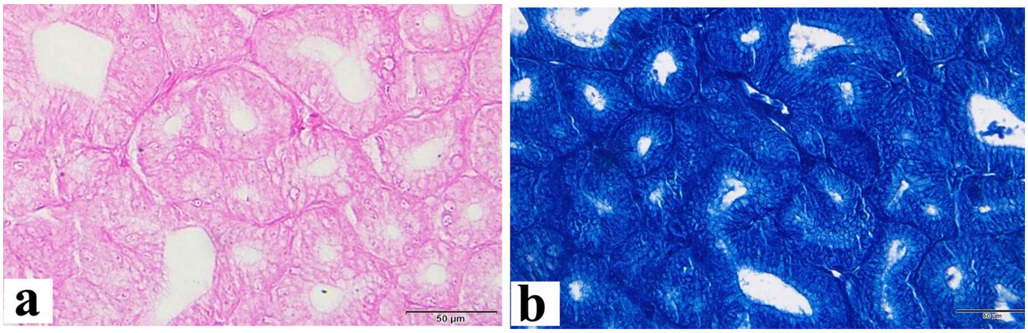

Fig. 5 A photomicrograph of a transverse section of the Harderian gland of Egyptian agama, *Trapelus mutabilis*, showing **a** glandular cells give a weakly positive reaction with PAS and **b** a bluish color with alcian blue at pH 2.5 (× 400)

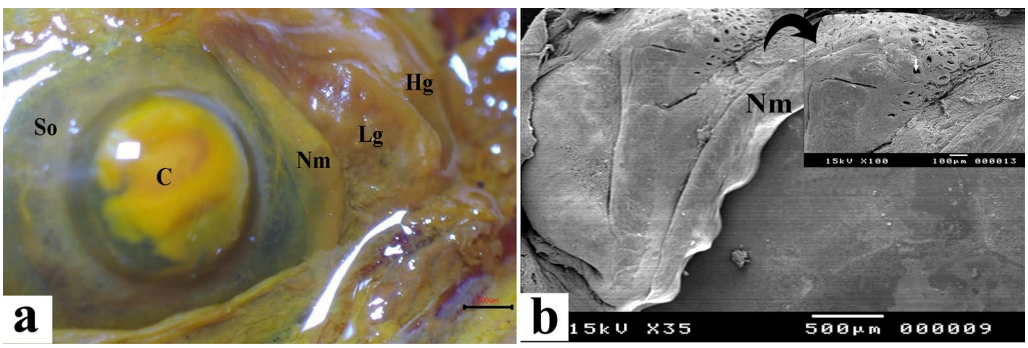

Fig. 6 A photomacrograph of the lacrimal gland of Egyptian agama, *Trapelus mutabilis*, showing **a** the position of the lacrimal gland (Lg) at nictitating membrane's base (Nm), Harderian gland (Hg), Cornea (C) and Sclera ossicle (So). **b** Scanning electron micrograph of the lacrimal gland of Egyptian agama, *Trapelus mutabilis*, showing numerous orifices of the lacrimal gland (arrow)

thick connective tissue, capsules of strands of collagenous fibers containing blood vessels, and scattered nerve fibers. This capsule separates the gland into acini. The LG consists of simple tubulo-acini of columnar cells with narrow lumen and oval-shaped nuclei located basally (Fig. 7a).

Histochemical analysis of the LG of Egyptian agama, *Trapelus mutabilis,* showed that the glandular cells are weakly positive to the PAS reaction but appear bluish in color with alcian blue at pH 2.5 (Fig. 7b, c).

Discussion

The main orbital glands of the lacrimal apparatus are the LG and HG (Jordan, 1990; Paynter, 1993). In the present study, both orbital glands in Egyptian agama, *Trapelus mutabilis,* were observed. We found that the HG was well-developed and large in size compared to the LG. Previous studies have suggested that these features of the glands are common across reptiles and birds, with the LG usually appearing less developed and smaller in size than the HG (Dimitrov, 2011; Dimitrov & Genchev, 2011; Henker et al., 2013; Payne, 1994).

The HG of Egyptian agama, *Trapelus mutabilis,* is located medioventrally at the corner of the orbit. This localization is similar in most terrestrial vertebrates, the anuran amphibian, reptiles, birds, and mammals, with the exceptions of higher primate, some types of mammals, and secondarily aquatic forms, including crocodiles and cetaceans (Boydak & Aydin, 2009; Burns, 1992; Dimitrov & Genchev, 2011; Kozlu & Altunay, 2011; Mobini, 2012; Payne, 1994). In Egyptian agama, the gland's secretory product is released into the conjunctival fornix, which is deep into the nictitating membrane. While the LG of Egyptian agama is reduced, it still functions and opens on the surface by numerous orifices.

Schwarz-Karsten (1933) provided details regarding the presence of the LG in reptiles, for example, Lacertcl uivipara, L. agilis, and L. muralis in Varanw, Anguis, Ablepharus, Nessia, Scimus, Chakides, and Mabuia. In Scimus, Chakides, and Mabuia, the gland was small. The LG is missing in calotes, Chameleon, Hemidactylus, Tarentob, Ophisops, Pygopus, and Sphenodon. Among the amphisbaenids described, the LG is absent. The absence of LG in calotes and Chhmeleo, as well as its

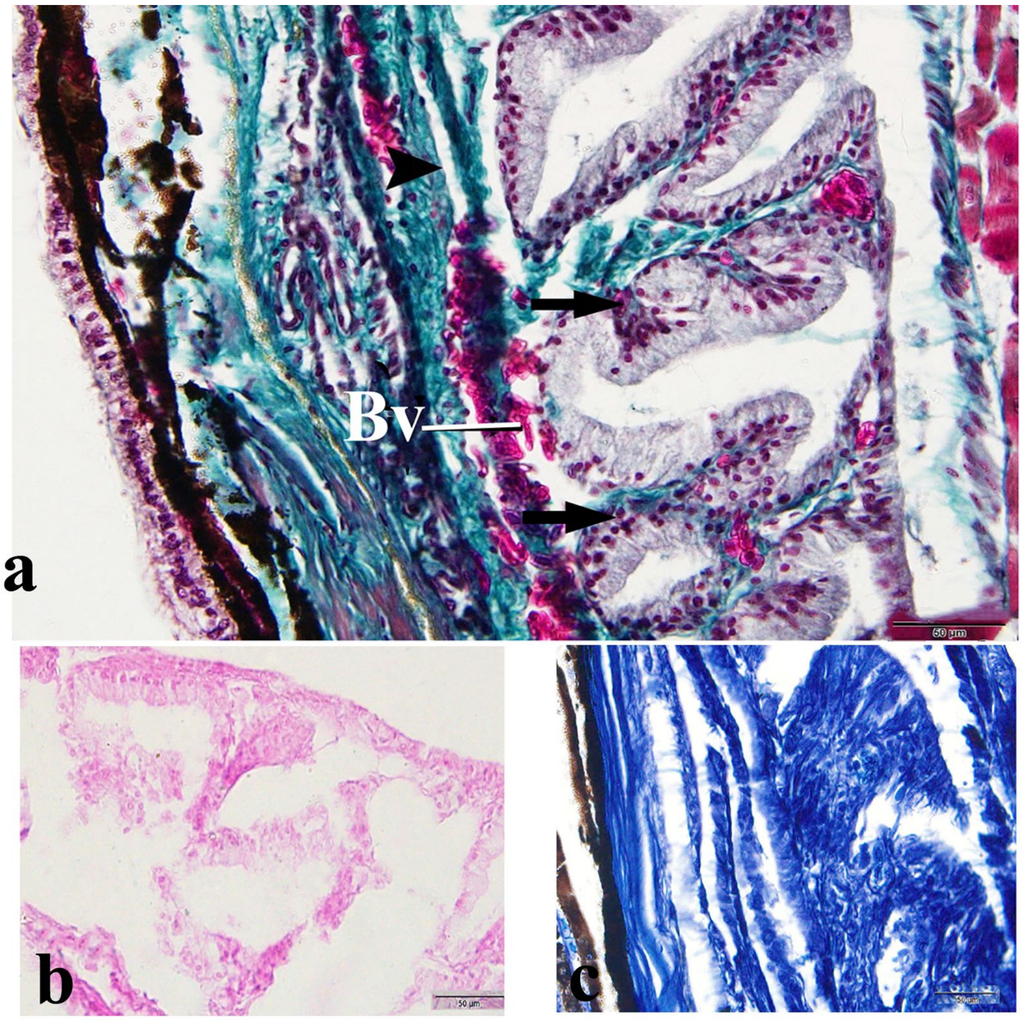

Fig. 7 A photomicrograph of a transverse section of the lacrimal gland of Egyptian agama, *Trapelus mutabilis*, showing **a** the lacrimal gland consists of simple tubulo-acini of columnar cells (arrow) with narrow lumen and is surrounded by thick connective tissue capsule of strands of collagenous fibers (arrowhead) containing blood vessels (Bv) (Masson's trichromic, × 400), **b** glandular cells are weakly positive to the PAS reaction and **c** bluish in color with alcian blue at pH 2.5 (× 400)

existence in Ablepharus and a few snakes, indicated that LG persistence was unrelated to the presence or absence of a brille. While Bellairs and Boyd (1947) determined that species that have a spectacle or brille have lost the nictitating membrane, there may not be a clear correlation between the presence of a nictitating membrane and LG. The present findings are consistent with this observation, as the nictitating membrane and LG were not developed in Egyptian agama.

Rehorek et al. (1993) suggested that the reduction in size of orbit or reduction in the corneal surface may influence structural changes in the HG, but later determined that there was no significant variation in relative sizes or structures of the HG.

Comparative morphological analyses have showed that the structure of the squamate is unaffected by orbital variations. Orbital reduction affected shape rather than size. This result implies that the shape of the HG varies within the squamata and occupies a large place in orbital space. In the species analyzed in this study, we found that the HG of Egyptian agama was tongue-shaped, similar to other reptiles as described previously by Rehorek et al. (1997, 2005, 2006). In chelonians, *Pseudemys scripta*, and *Testudo graeca*, the HG was oval-shaped (Chieffi-Baccari et al., 1992), and HG of pygopodid species, *Delma molleri*, and *Aprasia pseudopulchella* is bottle-shaped (Rehorek et al., 1999). Finally, the HG of *Pseudonaja textilis* is a multilobed structure that fills the orbital region (Rehorek et al., 1999, 2003).

Previous studies on several reptile species (Chieffi et al., 1992; Rehorek, 1997; Saint Girons, 1988) have described a species-specific volume of the HG but a general pattern that the gland is usually the largest orbital gland. In some anguimorph lizards and most snakes, the HG extends from the anterior to the posterior portion of the orbit, filling most of the orbital space. The largest squamate HG present in colubrid snakes, which have a large postorbital lobe (Minucci et al., 1992; Rehorek et al., 1999). In our study, we found that the HG of the Egyptian agama did not reach the optic nerve and postorbital lobe, suggesting it may be intermediate in size between reptile species.

Dullemeijer (1959) and Rehorek (1992) reported that, the size of the orbit seems to limit the relative development of the HG in both viperid snakes and skinks. The fully-developed HG has a large posterior expansion, and only in the sea snake has the poorly developed gland barely reach the posterior wall of the eyeball (Chieffi et al., 1992).

Moreover, the nearly white color of the HG and LG of Egyptian agama resembles those observed in several reptile species (Chieffi-Baccari et al., 1992; Rehorek et al., 1999; Sabry & Al-Ghaith, 2000). However, HG of *Alligator mississippiensis* appears reddish in color (Rehorek et al., 2005), the head of the HG in the lizard *Podarcis s. sicula* appears pinkish while the body appears gray (Chieffi-Baccari et al., 1990), and the LG of *Pseudemys scripta and Testudo graeca* was observed to be pinkish-gray (Chieffi-Baccari et al., 1992).

Most tetrapod vertebrates have a nasolacrimal duct connecting their orbital and nasal regions (Hillenius & Rehorek, 2005). However, the duct's terminal location in the nasal cavity differs among tetrapods. In both birds and crocodiles, the nasolacrimal duct connects the eye to the posterior portion of the nasal cavity (Bang & Wenzel, 1985; Rehorek et al., 2005). While the nasolacrimal duct of mammals opens into the anterior portion of the nose, and secretions, as well as those from the HG, drain through the nares (Hillenius & Rehorek, 2005; Rehorek et al., 2006). In many reptiles, the nasolacrimal duct opens into the duct of the vomeronasal organ (Rehorek, 1997), and then secretions may pass into the anterior oral cavity.

Despite these observations, variations exist in the vomeronasal organ, and the route of the nasolacrimal duct, particularly its precise connection with the HG and VNO (Bellairs & Boyd, 1947, 1949; Saint-Girons, 1982; Rehorek, 1997). Specific route of secretions of HG varies among reptiles in at least two examples. First, in snakes and some lizards, ducts of HG open into the nasolacrimal duct directly; previous research has proposed that secretions of HG may play role in the vomeronasal organ (Rehorek et al., 2000). The pathways of the nasolacrimal duct in snakes follow one of three general pathways with varied tortuosity and end in a single opening into the oral cavity, in close association with VNO duct (Souza et al., 2015). Secretions of HG drain into the conjunctival region then the nasolacrimal (Rehorek, 1997).

The discharge locations of secretory product of the LG and the HG of Egyptian agama showed that the orbital glands of agama have an indirect connection with the nasolacrimal duct and vomeronasal organ. This connection may be similar to that found in geckos and other lepidosaurs, in which HG ducts open onto the surface of cornea and are located only medial to the conjunctival puncta of the nasolacrimal duct without opening directly into the nasolacrimal duct (Bellairs & Boyd, 1947; Saint-Girons, 1982; Rehorek et al., 1997). One possibility is that the nasolacrimal duct of orbital glands of Egyptian agama passes through lacrimal foramen that is bordered medially by the prefrontolacrimal bones and inferiorly by the maxillary bones and palatines when secretions leave the orbit and travel laterally along the nasal capsule into the oral cavity. Secretions may therefore drain secondarily into the mouth via the nasolacrimal duct and may contribute to oral lubrication, or even digestion; this hypothesis agrees with previous work by Wyneken (2012).

In addition, the existence of pigments contained in the connective tissue capsule of the HG of the Egyptian agama is influenced by environmental conditions, such as background, light intensity and temperature. Temperature has a direct effect on pigment cell response (Frank et al., 2017). One of the most important factors influencing HG secretory activity is temperature. (Santillo et al., 2011). Minucci et al. (1990) reported that the activity of secretory products increases with the seasonal increase in temperature even when the light regime remained constant throughout the year. Changes in environmental temperature are coordinated with the annual variations in the secretory activity of the frog HG. As a result, perhaps pigment cells in the connective tissue capsule of HG of Egyptian agama assist in increasing the activity of secretory products that contain neutral and acidic mucopolysaccharides. In addition, the medialis rectus muscle that connects to the HG capsule by a connective tissue inferiorly may assist in emptying of the gland through voluntary muscular contraction (Tarpley & Ridgway, 1991).

Conclusions

The results of the present study suggest that morphology of LG and HG isn't impacted by features of the orbit, such as the existence or absence of the nictitating membrane or fixed eyelids. Egyptian agama has a small LG and a well-developed HG. The LG opens at the base of the nictitating membrane through numerous orifices, while the HG drains into the conjunctival fornix deep that leads into the nictitating membrane. Furthermore, the HG of the Egyptian agama is enveloped with pigmented capsules that allow heat to be absorbed in order to increase its secretion activity. The increased lacrimal secretion keeps the corneal surface moist, protecting the eyes from mechanical damage. Finally, the LG and HG of the Egyptian agama may share a role in digestion through an indirect connection with the oral cavity.

Abbreviations
HG: Harderian gland; LG: Lacrimal gland.

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FM, AG, SG, and AM designed and performed this work. All authors read and approved the final manuscript.

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Consent for publication
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Competing interests
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Author details
[1]Department of Zoology, Faculty of Science, Assiut University, Assiut, Egypt. [2]Department of Zoology, Faculty of Science, Aswan University, Aswân, Egypt. [3]Departmentof Zoology, Faculty of Science, Al-Azhar University, Assiut, Egypt.

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