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A new scent organ for *Gymnodactylus* lizards (Squamata: Phyllodactylidae) and an updated evolutionary scenario for the origin of squamate epidermal glands

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Semiochemical dispersion-based communication is a determinant in squamate behaviour and reproduction. This communication mode is driven by different scent glands and is distributed widely among squamate lineages. However, most studies of such communication have focused on femoral glands, and there is little literature about semiochemical-based communication among clades that lack this structure. In this study, we describe a cloacal scent gland in *Gymnodactylus* lizards and discuss its evolutionary significance. This sexually dimorphic holocrine gland is located in the posterior cloacal lip, promoting the dispersion of its chemical compounds. Morphological analysis shows that this gland is derived from the inner epidermal generation and shares numerous characteristics with the femoral and pre-cloacal glands. Histochemical analysis confirms a lipid composition. Our study is the first report of this organ and provides the first autapomorphy for *Gymnodactylus*. Our histological and comparative approach provides a model for the evolution of this organ together with a reinterpretation of the evolutionary hypothesis proposed for the origin of generation glands and follicular glands. Finally, we also propose a terminology scheme to standardize the nomenclature of these different structures based on their origin and morphology.

ADDITIONAL KEYWORDS: cloaca – holocrine gland – integument evolution – pheromones.

INTRODUCTION

Chemical signals are the most basal mode of communication occurring in all forms of life (Wyatt, 2003; Steiger *et al.*, 2011). In animals, physiologically active substances known as pheromones are important in different biological activities (Karlson & Luscher, 1959). Pheromones are used to trigger many behavioural and physiological phenomena, especially the recognition and mate choice of conspecifics (Quay, 1972; Steiger *et al.*, 2011; Martín & Lopez, 2014). A wide range of chemical compounds are recognized as pheromones, which are usually secreted by specialized glandular structures that in vertebrates are known as scent glands (Brennan & Zufall, 2006). These glands are generally derived from epidermal specializations and have been studied both morphologically and biochemically in squamates (Martín & Lopez, 2014) among many other groups (Muller-Schwarze & Mozell, 1977).

Squamates are the most diverse group of reptiles, with approximately 10 000 species (Uetz *et al.*, 2018) widely distributed throughout the globe (Vitt & Caldwell, 2013). Among the various forms of communication used by squamates for territorial defence (Kratochvíl & Frynta, 2002; Brandão & Motta, 2005), predator evasion (Dial *et al.*, 1989; Bealor & O'Neil Krekorian, 2006) and reproduction (Nicholson *et al.*, 2007), semiochemical dispersion by pheromones, which are recognized

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by the vomeronasal organ, is widely used across many groups (Filoramo & Schwenk, 2009; Martín & Lopez, 2014). These pheromones are produced by scent glands or by scale-specific specializations generally associated with the cloacal region that independently evolved in various squamate groups (Sánchez-Martínez *et al.*, 2007; Mouton *et al.*, 2010).

In squamates, the most well-studied epidermal specialization responsible for the production of pheromones is the femoral glands (Cole, 1966; García-Roa *et al.*, 2017). These follicular holocrine glands (also referred to as tubule-follicular, tubule-alveolar and tubule-acinar glands) secrete their contents through pore-modified scales present on the ventral portion of the femur, by laying them directly onto the substrate (Imparato *et al.*, 2007; Khannoon *et al.*, 2013). These organs are present in a broad range of lizard families and are widely used as taxonomic and sexually dimorphic characters (Cole, 1966). Although relatively scarce, morphological and histochemical characterizations of the femoral glands have contributed to knowledge of semiochemical dispersion, and its role in lizard biology (Iraeta *et al.*, 2011; Martín & Lopez, 2014). Recently, gas chromatography and mass spectrometry of femoral gland secretions has revealed a great diversity of volatile and semi-volatile compounds such as esters, steroids, ketones, carboxylic acids, alcohols and proteins, which are expected to act semiochemically (Khannoon *et al.*, 2011; Khannoon, 2012). These compounds play important roles in the interactions between individuals of many squamate species, predominantly through vomeronasal stimuli by volatile compounds or ultraviolet visual stimuli mediated by proteins (Alberts, 1989; Khannoon *et al.*, 2010). Although femoral glands are the most well studied epidermal specialization involved in semiochemical dispersion, relatively little is known about chemical communication between squamate taxa that lack this structure (García-Roa *et al.*, 2017).

Few squamate clades show epidermal specializations in the form of pre-cloacal glands that are also involved in semiochemical dispersion (Antoniazzi *et al.*, 1993; Valdecantos *et al.*, 2014). However, little is known about such organs because they are usually described for single species or for a restricted group of species. Antoniazzi *et al.* (1993; 1994) and Jared *et al.* (1999) studied the precloacal organ of the amphisbaenid *Amphisbaena alba*. They showed that this organ, situated on the external surface of the anterior cloacal lip, has a significant number of similarities to lizard femoral pores in external morphology, cytology and dispersion mechanisms (Antoniazzi *et al.*, 1993, 1994; Jared *et al.*, 1999). Valdecantos *et al.* (2014) recently described and characterized a precloacal organ in *Liolaemus* lizards. These structures are follicular glands that are similar to the femoral glands of lizards and precloacal glands of amphisbaenians in terms of their morphology and secretory mechanism (Jared *et al.*, 1999; Imparato *et al.*, 2007). Based on morphology, femoral and pre-cloacal organs are both classified as epidermal follicular glands (Mayerl *et al.*, 2015).

Other types of less complex epidermal glandular specializations named 'generation glands', 'escutcheon glands' or 'callose structure', among other less common terms, are described for some genera of gekkotan (Maderson, 1967, 1968, 1972), iguanid (Dujsebayeva *et al.*, 2007, 2009) and cordylid lizards (Mouton *et al.*, 2010, 2014). Nevertheless, the aforementioned differ from femoral and precloacal tubule-follicular organs in numerous morphological aspects, such as body position, morphology and tissue homology (Maderson, 1972; Dujsebayeva *et al.*, 2009; Louw *et al.*, 2011). While femoral and precloacal glands are well-developed structures with clearly defined lobes and secretory portions, generation glands are usually composed of a thin layer of holocrine secretory cells. This group of cells, located on the scale surface of different body regions, does not have lobes and, in contrast to femoral and precloacal glands, has a less complex secretory mechanism without forming a secretory plug (Maderson, 1972; Van-Wyk & Mouton, 1992; Dujsebayeva *et al.*, 2009; Louw *et al.*, 2011). Moreover, while femoral and precloacal glands are derived from the inner epidermal generation (more specifically from the stratum germinativum and α-layer), generation glands are derived from the outer epidermal generation layer that dedifferentiated and lost during the sloughing cycle (Van-Wyk & Mouton, 1992; Mouton *et al.*, 1998).

Many squamate clades reportedly lack specialized glandular structures, and the mechanism by which they perform chemical communication remains unknown (Mayerl *et al.*, 2015). With the exception of snakes, which possess a pair of cloacal scent glands (Oldak, 1976), only a few putative scent organs have been described for other squamate species (Burkholder & Tanner, 1974; Saint-Girons & Newman, 1987; Trauth *et al.*, 1987; Cooper & Trauth, 1992; Valdecantos *et al.*, 2015). Here we describe a previously unreported glandular cloacal organ from lizards of the genus *Gymnodactylus* Spix, 1825. *Gymnodactylus* are endemic to Brazil and are widely distributed in the Cerrado, Caatinga and Atlantic Forest biomes. Some *Gymnodactylus* species have been previously studied in numerous ecological, phylogeographical and evolutionary aspects (Colli *et al.*, 2003; Pellegrino *et al.*, 2010; Domingos *et al.*, 2014; Amorim *et al.*, 2017). However, with the exception of some morphological studies focused on folidosis, little is known about the anatomy of this group (Kluge, 1964; Abdala, 1996; Abdala & Moro, 1996; Vanzolini, 2004, 2005; Cassimiro & Rodrigues, 2009). Our aim here was to provide a detailed morphological description of this new organ focused on its anatomical position, structural and ultrastructural characteristics, secretory mechanism and histochemical composition.

Furthermore, we discuss the importance of these findings from an anatomical, evolutionary and phylogenetic perspective and place this organ as a putative synapomorphy for *Gymnodactylus* species.

MATERIAL AND METHODS

Biological material

Eleven specimens of *Gymnodactylus amarali* (eight males and three females) were collected in the municipality of Nova Xavantina, Mato Grosso State, Brazil (September 2017) under permit number 56601-1 (ICMBio/IBAMA). The animals were killed via an anaesthetic overdose of thionembutal 100 mg/mL and lidocaine 20 mg/mL (1:1). The posterior cloacal lip was removed from each specimen and fixed as required by each analytical method (see descriptions below). Specimens were fixed in 3.7% formaldehyde and deposited in the collection of the Laboratory of Vertebrate Comparative Anatomy (LACV). This project was approved by the ethics committee of the CEUA (–UnB 150406/2015).

In an attempt to describe and compare the histological anatomy of the new gland, four preserved specimens of *Gymnodactylus darwinii*, one of *G. guttulatus*, and three of *Phyllopezus pollicaris*, the sister-group of *Gymnodactylus* (Gamble *et al.*, 2015), were analysed using histological methods and scanning electron microscopy (SEM; see details below).

To establish the phylogenetic occurrence of the organ, we analysed the cloacal region of 13 other gekkotan species, all South American genera of Phyllodactylidae, including all valid *Gymnodactylus* species, four species of Gekkonidae, and one species of Sphaerodactylidae, totalling 330 analysed specimens, as follows. Phyllodactylidae: *G. amarali* (N = 117), *G. geckoides* (N = 27), *G. darwini*, (N = 23), *G. guttulatus* (N = 17), *G. vanzolinii* (N = 17), *Phyllopezus pollicaris* (N = 38), *Thecadactylus rapicauda* (N = 4), *Thecadactylus solimoensis* (N = 10) and *Homonota uruguayensis* (N = 10); Gekkonidae: *Lygodactylus klugei* (N = 13), *Hemidactylus brasilianus* (N = 20), *Hemidactylus mabouia* (N = 22) and *Hemidactylus palaichtus* (N = 2); Sphaerodactylidae: *Coleodactylus brachystoma* (N = 12). The aforementioned specimens were obtained from Brazilian Herpetological Collections (CHUNB-UnB, LAFUC-UnB, UFMG, MCP-PUCRS, UFMT and LACV-UnB). A complete specimen list is provided in Supporting Information, List S1.

Histology

The posterior cloacal lips of ten specimens were removed: *Gymnodactylus amarali* (female = 1; male = 2; fresh samples), *G. darwinii* (female = 1; male = 2; ethanol-preserved samples), *G. guttulatus* (male = 1; ethanol-preserved sample), and *Phyllopezus pollicaris* (female = 1; male = 2; ethanol-preserved samples). Fresh samples were cold fixed in phosphate-buffered saline solution with 3.7% formaldehyde. All samples were dehydrated in a graded ethanol series (70%, 90% and 100%), cleared in xylene, embedded and mounted in paraffin. For male samples, semi-serial sections of 4–6 µm thickness in the sagittal and transverse planes were obtained with a Leica rotary microtome, whereas female samples were serially sectioned. After adhesion in glass slides (12–24 h at 60 °C), sections were stained using the haematoxylin-eosin routine stain method (Bancroft & Gamble, 2002). Slides were analysed and scanned at high resolution in an Evos FL Auto microscope.

Scanning electron microscopy

We analysed the posterior cloacal lip of three *G. amarali* (female = 2; male = 1; fresh samples) and two *G. darwinii* (female = 1; male = 1; ethanol-preserved samples) under SEM. Fresh samples were cold fixed in Karnovsky fixative solution for 24 h. Fresh and ethanol-preserved samples were post-fixed in 1% osmium tetroxide and dehydrated in increasing grades of acetone (30%, 50%, 70%, 90% and 100%). The samples were critical point dried with CO_2 at 37 °C in a Balzaers CPD030 device, mounted in metallic stubs and coated with gold in a Leica EM SCD005 sputter coater. Analyses were performed using a Jeol JSM-7000F scanning electron microscope.

Histochemistry

To identify lipids, samples from adult male specimens of *G. amarali* were cold fixed in 0.1% osmium tetroxide (N = 1), and in a modified Zenker's fixative solution (addition of 50 mg chromic acid in 50 mL Zenker's solution) (N = 2), both for 24 h at 4 °C. On combining potassium dichromate and mercury chloride (from Zenker's solution) with chromic acid, most lipid compounds become insoluble, enabling their recognition after routine xylene clearing and paraffin-embedding (Casselman, 1955; Heslinga & Deierkauf, 1961). After fixation and standard ethanol dehydration, xylene clearing and paraffin embedding, sections of 5–10 µm thickness were stained in Oil Red stain for lipids, and haematoxylin-only for nuclei differentiation. Osmium tetroxide-fixed samples were stained with haematoxylin only. After staining, sections were trickled with glycerin jelly at 60 °C and covered with laminulae.

For whole gland three-dimensional evaluation, one sample was cold fixed in phosphate-buffered saline solution containing 3.7% formaldehyde for 24 h, stained in Sudan Black stain for 24 h, cleared in 1.5% potassium hydroxide for 48 h and preserved in glycerin.

Transmission electron microscopy

One sample from a *G. amarali* adult male was cold fixed in Karnovsky fixative solution for 24 h, post-fixed in 1% osmium tetroxide, contrasted in 2% uranyl acetate, dehydrated in increasing grades of acetone and embedded in epoxy resin. Ultrathin serial sections were obtained using a Leica EM-UC7 ultramicrotome and analysed under a Jeol JEM-1011 transmission electron microscope.

RESULTS

Gross morphology, sexual dimorphism and phylogenetic occurrence

We observed a gland on the cloacal region of *Gymnodactylus* lizards, previously unreported in the literature. This gland is located transversally on the cloacal posterior lip. Its location causes a caudal protrusion of the posterior cloacal lip, overlapping the first and second roll of tail scales (Fig. 1A–D). Based on this anatomical position, the gland was named the 'postero-proctodeal gland' (PPG).

The *Gymnodactylus* PPG was visible macroscopically by eye in males, despite being relatively small. In living specimens, the gland is visible after slight traction of the tail, while in preserved specimens it is necessary to use forceps to visualize the posterior lip under a stereomicroscope. Its opaque coloration and contrast with adjacent tissues makes it easily distinguishable both *in vivo* and in preserved specimens (Fig. 1A–D). The gland occurs in both adults and juveniles, and the smallest specimen in which it was possible to identify the PPG using a stereomicroscope was a juvenile male of *G. darwini*, with a snout–vent length of 33.8 mm (UFMG1338). In females, the PPG is reduced and not distinguishable under the stereomicroscope (Fig. 1E). However, it may be visualized in histological (Fig. 2C) or SEM preparations (Fig. 3G). Therefore, this structure can also be used as a sexually dimorphic character in adult specimens.

The PPG was distinguishable in males of all *Gymnodactylus* species (*G. amarali*, *G. geckoides*, *G. darwini*, *G. guttulatus* and *G. vanzolini*), but absent in its sister group (*Phyllopezus pollicaris*) together with all of the other gekkotan species analysed: *Thecadactylus rapicauda*, *T. solimoensis*, *Homonota uruguayensis*, *Lygodactylus klugei*, *Hemidactylus brasilianus*, *Hemidactylus mabouia*, *Hemidactylus palaicthus* and *Coleodactylus brachystoma*. Thus, the available evidence suggests the presence of this gland is a morphological synapomorphy for *Gymnodactylus*.

Histological description

The PPG is a holocrine acinous gland composed of between two and seven acini, or lobes, in males. In the sagittal plane, the acinus displays sub-lobulations in the cortical region, and an active secretory portion in the central region of each acinus (Fig 2A, C). In the transverse plane, it is possible to identify the limits of each acinus and the secretory portion directly associated with the central region (Fig 2B). These inner secretory portions are in contact with the terminal (outer) secretory portion located in the final segment of the cloacal lumen (Fig 2A). Both inner and terminal secretory portions are composed of the remains of extracellular non-secreted material lacking cellular compounds (Fig 2A, B). In the terminal secretory portion, the remains of the extracellular matrix are often degraded and expelled (Fig. 2; A-E; Supporting Information, Fig. S2).

In females, the PPG is not distinguishable by eye or under the stereomicroscope. A single and very small lobe is found in histological sections of *G. amarali*, displaying a single outer secretory portion. The basal glandular epithelium shows three projections towards the centre of the gland, possibly corresponding to lobule precursors (Fig. 2D). In the female *G. darwinii* sample, a smaller single lobe was found. In contrast to the *G. amarali* sample, no signs of constrictions possibly relating to lobe formation were observed in *G. darwinii* (Fig. 2E). Based on these findings and the SEM results shown below, we regard the PPG as a vestigial structure in female *Gymnodactylus* lizards.

In both males and females of the *Gymnodactylus* species, the PPG is derived from the inner epidermal generation (stratum germinativum and α-layer) and is composed of a layer of squamous cells. The stratum germinativum is composed of a single layer of cells in the outermost cortical region with a greatly reduced cytoplasmic volume. From the second outermost layer, the cells are broadly larger, with a large eosinophilic cytoplasm. General modifications of the posterior cloacal lip epidermis are present: (1) in the base of the posterior lip, with enlargement of the basal stratum germinativum and keratinizing α-cell layer; (2) at the cloacal-scale transition with the end of the outer epidermal generation of the scales; and (3) at the beginning of the specialized glandular tissue from the stratum germinativum (Fig. 2E, F). The cloaca to tail scales transition is marked by the morphology of the adjacent connective tissue which changes from loose to dense, confirming the presence of the PPG in the proctodeal region of loose connective tissue.

The protuberant posterior cloacal lip of *P. pollicaris* is mainly composed of muscular and conjunctive tissue comparable to that of the *Gymnodactylus* samples (Fig. 2F). Nevertheless, histological analysis of the basal keratinizing layer in the proctodeal region of male *P. pollicaris* samples revealed remarkable thickening of the stratum germinativum and α-keratinizing layer in the same position as the *Gymnodactylus* posterior cloacal lip. Throughout this keratinizing layer enlargement in *P. pollicaris* males, the cells have a

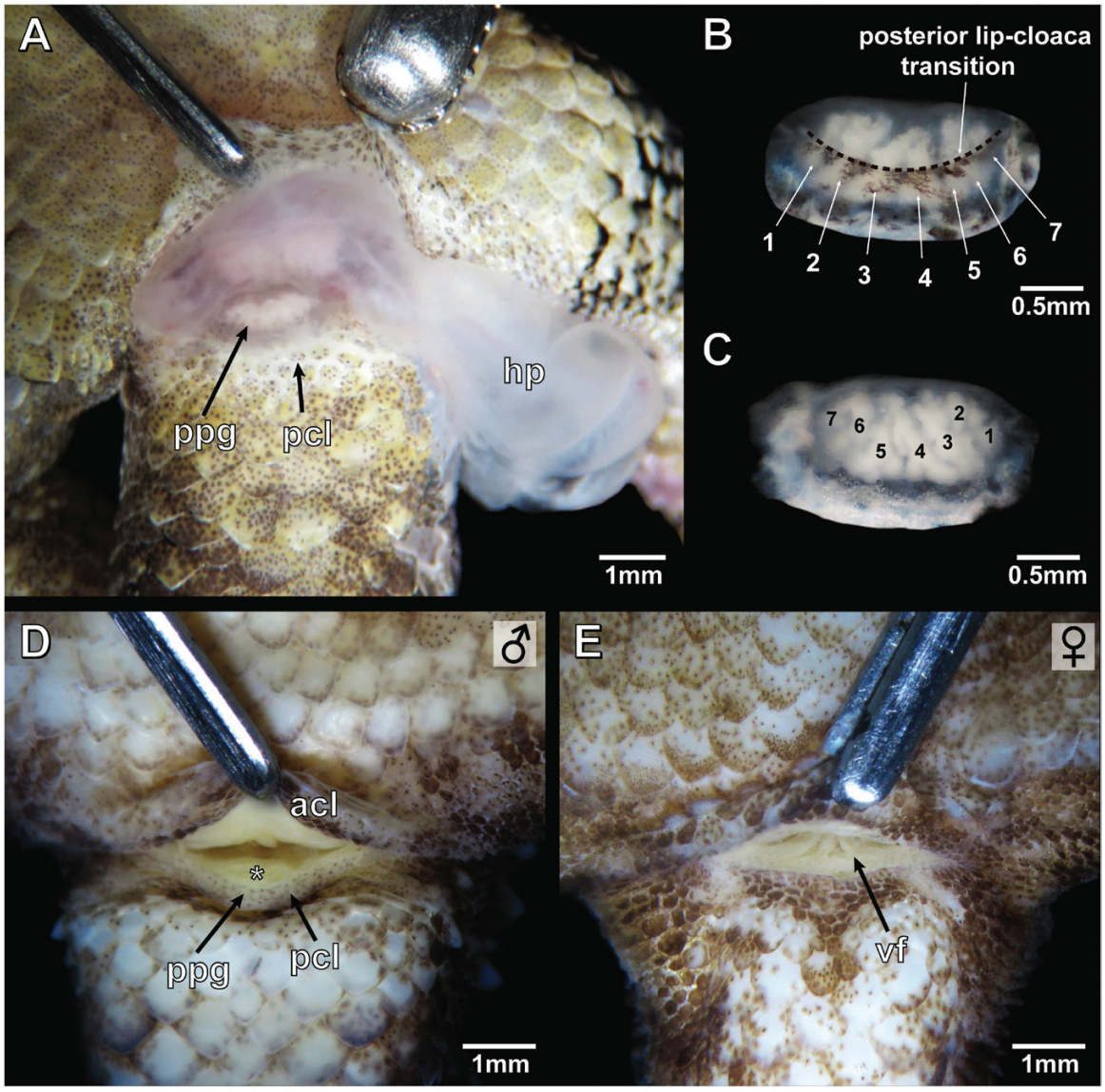

Figure 1. Stereomicrographs of the gross anatomy of the *Gymnodactylus* postero-proctodeal gland. The gland is visible in fresh (A–C) and fixed (D) males but not distinguishable in females (E). A dissected gland from a male *G. amarali* specimen is shown in outer (B) and inner (C) views, showing seven lobes. Asterisk in D shows the secretory portion of the posterior-proctodeal gland. Key: acl, anterior cloacal lip; hp, hemipenis; pcl, posterior cloacal lip; ppg, posterior-proctodeal gland; vf, vaginal folds.

hypertrophied appearance in comparison with the epidermis itself. However, no constrictions indicating the occurrence of lobes or any other morphological evidence for holocrine (or other type of) secretion were observed (Fig. 2F).

Scanning electron microscopy analysis

SEM revealed a caudal protuberance in the posterior cloacal lip in males, with a row of pits corresponding to the outer secretory portion of the gland. The secretory portion protrudes from the gland surface and shows a considerable degree of desquamation and hypertrophy in fresh *G. amarali* tissue (Fig. 3A, E). In the alcohol-preserved *G. darwinii* sample, less protrusion and hypertrophy were observed, clearly distinguishing the inner secretory portion associated with each lobe (Fig. 3B, F). In contrast to males, in females no protuberance was observed in the posterior cloacal lip, being continuous with the vaginal folds (Fig. 3C, D). Nevertheless, the vestigial gland of females was distinguishable under SEM by the presence of a single

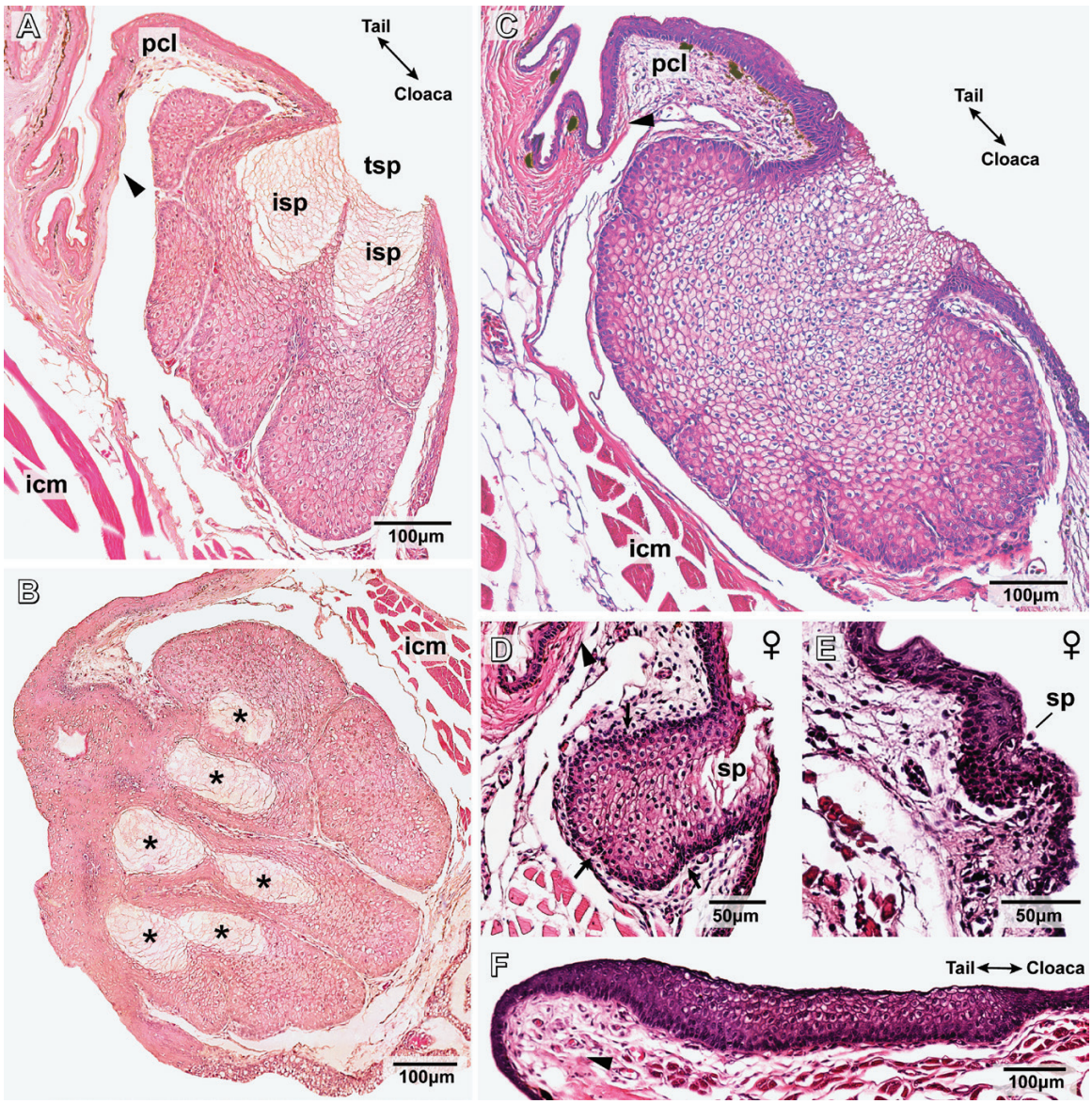

Figure 2. Histological sections of the *Gymnodactylus* posterior-proctodeal gland stained with haematoxylin and eosin. Samples from a male *G. amarali* in sagittal (A) and transverse (B) view. Sample from a male *G. darwinii* (C) in sagittal view. Sample from a *G. amarali* female (D) and *G. darwinii* female (E) in sagittal view, and the cloacal posterior lip of a *Phyllopezus pollicaris* male (F). Asterisks in B show inner secretory portions of each lobe. Arrows in D indicate constrictions in the cortical region. Arrowheads in A and C–F indicate the connective tissue transition from loose to dense, indicating the cloacal proctodeal region. Key: icm, isquio-caudalis muscle; isp, inner secretory portion; pcl, posterior cloacal lip; sp, secretory portion; tr, cloacal lip–gland transition; tsp, terminal secretory portion.

small outer secretory portion, which is located medially on the posterior cloacal lip (Fig. 3G).

Histochemical description

Sections stained using the oil red method exhibited strong dye retention, suggesting a lipid constituent of the gland. Each lobe showed a gradient of dye retention, with the cortical region being more strongly coloured than the central portion. The secretory cells displayed a large number of granules in the cytoplasm. In the secretory portion, lysed cells showed weaker dye retention. Specimen LACV3237 had a larger gland and showed stronger retention than specimen LACV3236, although both had similar snout–vent lengths (48.5 and 47.5 mm, respectively) and were captured in the same field expedition (Fig. 4A, B). Samples stained with osmium tetroxide also

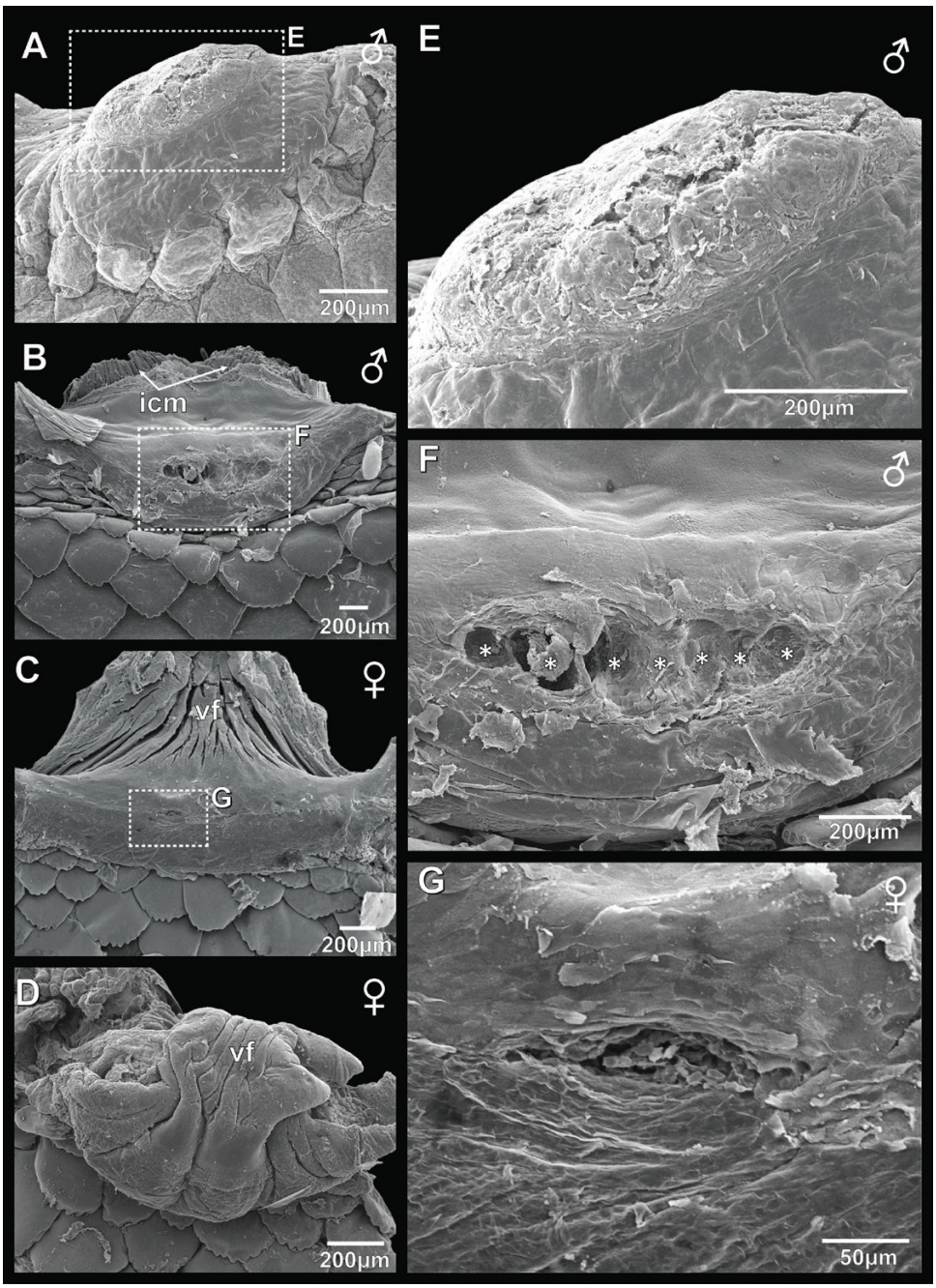

Figure 3. Scanning electron micrographs of the *Gymnodactylus* posterior-proctodeal gland. Micrographs from male *G. amarali* (A), male *G. darwinii* (B), female *G. darwini* (C) and female *G. amarali* (D). Secretory portions are shown in detail in males (E, F) and a female (G). Asterisk shows the secretory portion of each lobe (F). Key: icm, isquio-caudalis muscle, vf, vaginal folds.

showed strong dye retention, especially in the cytoplasmic granules (Fig. 4D).

The whole sample cleared and stained with Sudan Black B also showed a strong lipid signal. Using this method, it was possible to see the presence of seven lobes forming the PPG under stereomicroscopic analysis (Fig. 4C).

Ultrastructural considerations

Transmission electron microscopy showed cells ranging from cuboidal to polyhedral in form with a large number of electron-lucent vesicles throughout the glandular epithelium. In the secretory portion of each lobe, in addition to the terminal secretory portion, cells were lysed exposing their cytoplasmic

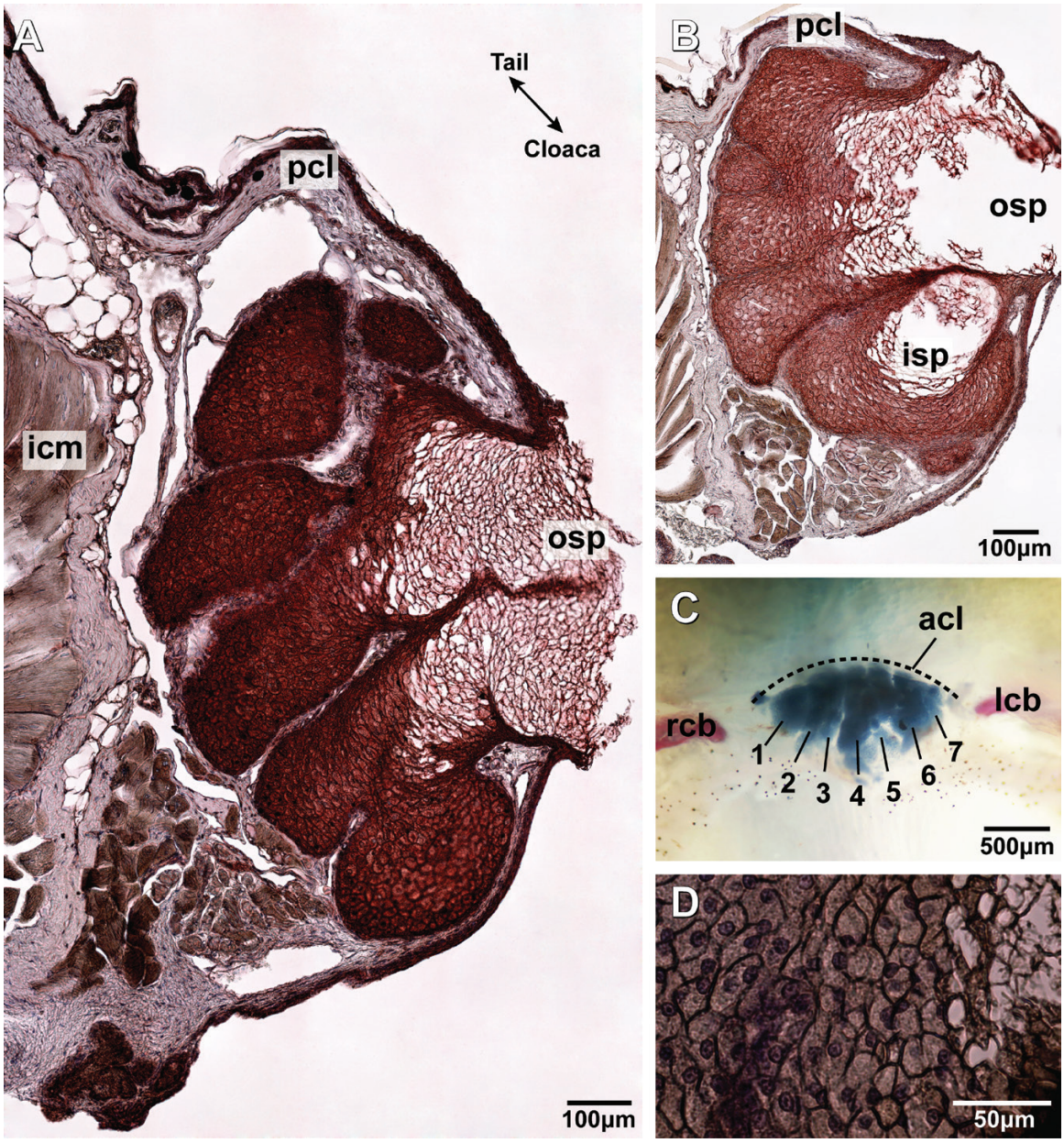

Figure 4. Histochemical preparations of the *Gymnodactylus* posterior-proctodeal gland. A and B, sections from *G. amarali* in sagittal view stained with Oil Red stain. C, whole gland of *G. amarali* stained with Sudan Black B, with adjacent tissue cleared with potassium hydroxide and cloacal bones stained with Red Alizarin S. D, section from *G. amarali* in sagittal view stained with osmium tetroxide. Key: acl, anterior cloacal lip indicated by trace; icm, isquio-caudalis muslce; isp, inner secretory portion; lcb, left cloacal bone; osp, outer secretory portion; pcl, posterior cloacal lip; rcb, right cloacal bone.

content as expected for holocrine secretions (Fig. S2A–D).

DISCUSSION

Squamates are recognized for their ability to produce chemically rich secretions that are involved in many biological and behavioural aspects such as defence, predator evasion and reproductive dynamics (Bealor & O'Neil Krekorian, 2006; Khannoon *et al.*, 2010; Martín & Lopez, 2014). In recent decades, an increasing number of studies have targeted Squamata chemical communication from morpho-physiological, biochemical and behavioural points of view, and a considerable range of morphological structures and pheromone

compounds have been described (Khannoon *et al.*, 2011; Martín & Lopez, 2014). However, the majority of studies have focused on femoral glands, and little is known about lizards that lack these glands, such as *Gymnodactylus* and numerous other taxa (e.g. other phyllodactylid and iguanid lizards) (Frost & Etheridge, 1989; Cajade *et al.*, 2013). Furthermore, there is limited evidence of chemical communication between clades in such taxa and few studies focus on discussing the evolutionary adaptations that compensate for the absence of femoral and pre-cloacal glands in lizards. Our study describes a novel gland with new biological characteristics among lizards of the genus *Gymnodactylus*.

Pheromone-producing structures have been reported as being related to epidermal specializations mainly present in the femoral region (Cole, 1966), but occasionally reported for the inner cloacal region (Burkholder & Tanner, 1974; Flachsbarth *et al.*, 2009; Valdecantos *et al.*, 2015) and also in abdominal and tail scales (Maderson, 1967, 1968, 1972). Besides morphological aspects and lipid chemical nature, the secretory mechanism may also be considered evidence of the pheromone dispersion of the *Gymnodactylus* PPG. A strongly supported hypothesis for the dissemination of pheromones to the environment by lizards is that they use excrement as a delivery mechanism for cloacal secretions by impregnating this with glandular semiochemical compounds (Alberts, 1993). In *Gymnodactylus*, the position of the terminal secretory portion of the PPG, located at the posterior cloacal lip, would allow the compounds to be deposited both in the excrement and in the substrate.

The cloacal gland present in *Gymnodactylus* lizards can be considered as an epidermal specialization, similar in morphology and secretory mechanism to those glands of the femoral and precloacal regions as these are follicular holocrine glands (Antoniazzi *et al.*, 1993; Imparato *et al.*, 2007). On the other hand, the *Gymnodactylus* PPG differs from generation glands of the outer epidermal generation layer found in some *Gonatodes*, *Lygodactylus*, *Oplurus* and cordylid lizards, in that these two types of epidermal specialization are derived from distinct epidermal layers differing in position, morphology and secretory mechanism (Maderson, 1967, 1968; Dujsebayeva *et al.*, 2009; Louw *et al.*, 2011; Mouton *et al.*, 2014).

The squamate epidermis has been the focus of many studies since the description of the unique mechanism of sloughing and its relationship to the evolution of epidermal glandular specializations in different body regions in many lineages (Maderson & Chiu, 1970). Following the evolutionary model of Maderson (1967, 1972), the organ present in the *Gymnodactylus* cloacal lip shows the same tissue developmental pattern described for the femoral and pre-cloacal organs, deriving from the presumptive inner epidermal generation skin layer, and more specifically from the inner stratum germinativum and α-keratinizing cells histological layer. However, although tissue homologies are well known among lizard pheromone organs, the signals that lead to the differentiation of glandular structures in specific regions of the body remain unknown. Furthermore, the evolutionary novelties that lead to the existence of other chemical communication mechanisms remain poorly understood (Shine & Mason, 2012) together with the degree of natural (Bealor & O'Neil Krekorian, 2006) and sexual selection (Trauth *et al.*, 1987; MacGregor *et al.*, 2017). As we found for *Gymnodactylus* species and considering the unique epidermal dedifferentiation capacity among squamates, we expect that other taxa should have unknown epidermal specializations in addition to femoral, precloacal and generation glands for chemical communication.

Evolution of scent glands in lizards

The possible evolutionary relationship between generation and follicular glands remains a matter of great debate for epidermal glandular specialization in lizards. Maderson & Chiu (1970) were the first to hypothesize on the origin and evolution of generation and follicular glands based on previous studies of a variety of Gekkotan taxa. The authors argued for a non-homologous origin of generation and follicular glands. Yet, it was postulated in their evolutionary model that follicular glands could have evolved from ancestral generation glands that had become enlarged in a specific femoral row of scales, thus giving rise to femoral organs (Fig. 5A).

The morphological aspects found in the PPG of *Gymnodactylus* species and the enlarged condition found in the posterior cloacal lip of *P. pollicaris* (the sister group of *Gymnodactylus*) suggest a different interpretation for the origin and evolution of generation glands and follicular glands. As the *Gymnodactylus* gland is located after the transition between the proctodeal epidermis and the first row of tail scales, it is unlikely that the PPG represents a derived condition of ancestral generation glands present in tail scales. An evolutionary scenario based on Maderson & Chiu (1970) for the *Gymnodactylus* PPG would argue that ancestral generation glands surrounding the posterior cloacal lip have undergone changes since the origin, and during the evolution of the PPG. However, in its posterior cloacal lip, *P. pollicaris* has a protuberance composed primarily of muscular tissue, and shows a remarkable thickening of the α-keratin layer and the stratum germinativum located in the same position as the PPG in *Gymnodactylus* lizards (Fig. 2F). This leads us to hypothesize that the thickening found

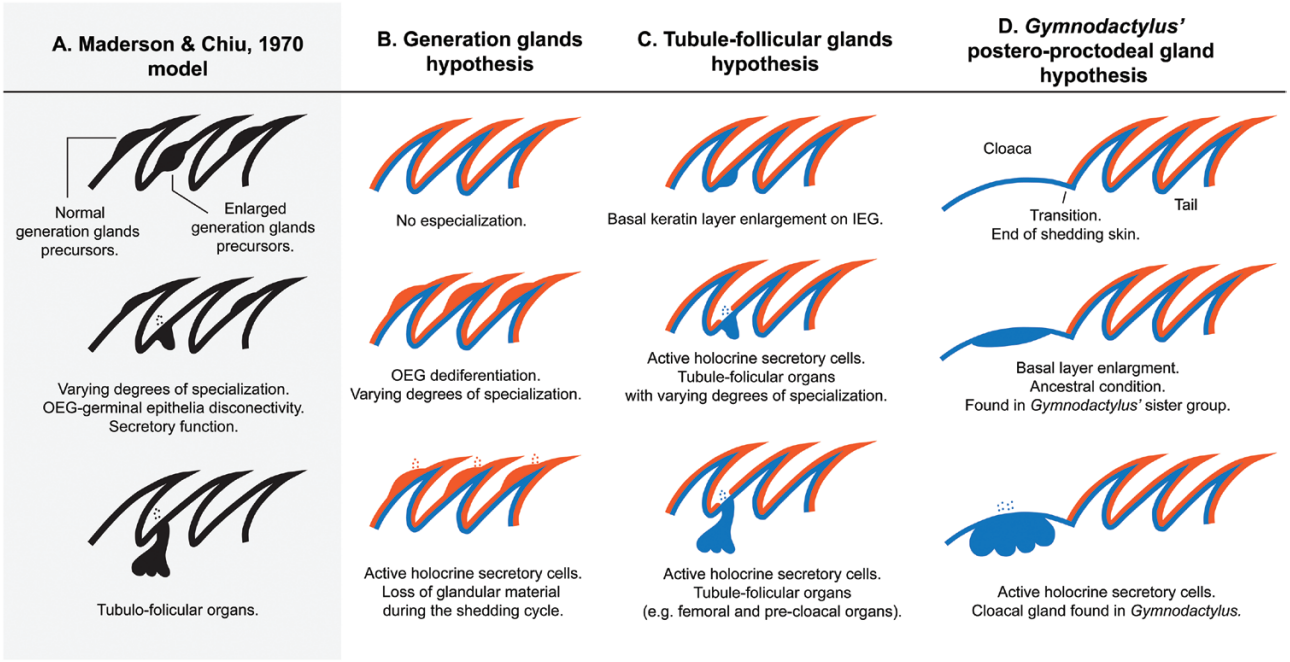

Figure 5. An evolutionary scenario for the *Gymnodactylus* posterior-proctodeal gland, and an updated evolutionary hypothesis for generation glands and tubule-follicular glands. A, a simplified representation of the Maderson & Chiu (1970) evolutionary model. B and C, hypothesis for the independent evolution of generation glands and tubule-follicular glands from the outer epidermal generation and basal keratin layer, respectively. D, an evolutionary scenario for evolution of the *Gymnodactylus* posterior-proctodeal gland. The outer epidermal generation layer is depicted in orange, and the stratum germinativum and α-keratin layer in blue. Key: IEG, inner epidermal generation skin layer; OEG, outer epidermal generation skin layer.

in *P. pollicaris* represents the ancestral state of the *Gymnodactylus* PPG and that this gland evolved through differentiation of the stratum germinativum and α-keratin layer (Fig. 5D). Further studies should investigate the ultrastructural and histochemical characteristics of the thickening found in *P. pollicaris* and determine which molecular signals trigger the epidermis to form this enlarged morphology. Studies should be carried out in other phyllodactylid genera to determine if there is and how the chemical communication occurs in such groups.

Generation glands are formed by secretory material outlying the epidermal generation layer, whereas follicular glands (sometimes confused with generation glands; see nomenclature section below) are derived from the stratum germinativum and the basal α-keratin layer. Given the non-homologous origin of these two epidermal specializations, we present a new hypothesis for the independent origin of both generation and follicular glands (Fig.5B, C). We hypothesize that generation glands originated from an ancestral dedifferentiation of the outer epidermal generation that later developed holocrine secretory function (Fig. 5B). On the other hand, it is possible that follicular glands evolved independently from hypertrophied regions of the stratum germinativum and α-keratin layer (as found in *P. pollicaris*) with posterior dedifferentiation in active holocrine secretion cells (Fig. 5C). As such, based on the morphological and histological characteristics of modern squamate scent glands, generation and follicular glands probably have independent evolutionary origins, driven by specific signalling of the epidermis on the outer and inner epidermal generation layers.

Nomenclature and homology of lizard follicular glands and scale-specific generation glands

We review the nomenclature of lizard pheromone glands and suggest improved practices and standardization in the use of all terminology relating to generation and follicular glands. Among lizards, epidermal glandular specializations can be found in the form of cloacal, precloacal, femoral and scale-specific glandular structures (Cole, 1966; Maderson & Chiu, 1970; Mayerl *et al.*, 2015). The terms 'preanal organ', 'preanal gland' and 'preanal pores' have been used with reference to femoral glands and femoral pores found in a wide range of lizard species (e.g. Kluge, 1967; Maderson & Chiu, 1970; Chiu & Maderson, 1975; Frost

& Etheridge, 1989). These are conceptually incorrect terms because lizards do not have a distinct anal region. Moreover, because they may lead to misinterpretations in relation to femoral structures (those present on the femoral surface), and proper precloacal structures (those present on the anterior lip of cloaca), we suggest that authors refrain from using the aforementioned terms, limiting instead to the terms 'femoral glands' for those on the femoral surface and 'pre-cloacal glands' for those on the antero-cloacal region.

According to Valdecantos *et al.* (2014), another misinterpretation regarding precloacal glands relates to the body position and tissue homology of generation glands referred to as follicular precloacal glands, and has etymological and other problems. As a general example, Dujsebayeva (2007) histologically characterized the epidermal scale-specific glandular structures present in agamids and referred to 'preanal' or 'precloacal callose scale' as terms for scale-specific glandular specializations present in agamids. These structures, similar to those described for a number of gekkotans (Maderson, 1968, 1972), do not have the same tissue origin as the follicular scent glands known as femoral and precloacal organs. Dujsebayeva *et al.* (2009) subsequently interpreted the scale-specific glandular structures present in agamids as generation glands, a term coined by Maderson & Chiu (1970), and discussed its tissue homology in corroborating the general model proposed by Maderson. As such, we also regard follicular glands present in the cloacal (such as the organ here described for *Gymnodactylus*), femoral and precloacal regions as individual organs with the same tissue origin, but not homologous structures. Conversely, we consider all glandular scale-specific structures relating to different body regions derived from the outer epidermal generation layer as generation glands. In addition, we suggest that future morphological studies consider investigating such structures under a histological approach because clear distinction is needed between follicular and generation glands. In fact, these two types of epidermal specialization do not have the same tissue origin (Fig. 5) (Maderson, 1972; Dial *et al.*, 1989; Dujsebayeva *et al.*, 2009) and a clear distinction between them is needed to provide a better understanding of integument evolutionary modifications and its use for the taxonomy of squamates.

Sexual dimorphism

The PPG of *Gymnodactylus* is an epidermal glandular specialization present in both males and females, although it shows dimorphism in terms of size. In males, this gland is multi-lobed and approximately ten times larger than in females with only one microscopically distinguishable lobe found in the latter. Similar cases relate to other pheromone glands in different vertebrate groups, thus suggesting male-specific ontogenetic growth control of scent glands and other secondary sexual characteristics mediated by sexual hormones (Chiu & Maderson, 1975; Izzo *et al.*, 1982; Krohmer *et al.*, 2004; Rollins & Staub, 2017).

In lizards, sexual dimorphism in epidermal glandular specializations is well documented. Many species show a male-only condition for the presence of pheromone glands, while there are fewer cases of the female-only condition (Cole, 1966). For those species in which pheromone glands are present in both sexes, differences in gland size are common, with females showing less-developed glands and secreting less content than males (Chamut *et al.*, 2009; Valdecantos *et al.*, 2014), as we found for *Gymnodactylus* lizards. Nevertheless, cases in which there are no morphological differences between males and females have also been reported (Antoniazzi *et al.*, 1993; Imparato *et al.*, 2007).

The PPG as a morphological synapomorphy for *Gymnodactylus*

Despite being a well-established taxonomic group, the genus *Gymnodactylus* has a problematic morphologically based taxonomic history, as discussed by Vanzolini (1953, 2004, 2005). Initially, *Gymnodactylus* included a wide range of gekkonid lizards from the Old and New Worlds that share the absence of digit dilations as a synapomorphy. After taxonomic the genus now consists of five formally described South American species, lacking any morphological autapomorphic character (Vanzolini, 2004, 2005; Cassimiro & Rodrigues, 2009). Therefore, the current diagnosis of *Gymnodactylus* is determined based on the combination of six morphological characters that, separately, are also found in other related genera (Kluge, 1964; Vanzolini, 1968). Few studies have investigated the morphology of South America gekkotans from a phylogenetic perspective, thus making it difficult to distinguish between some genera using morphological characters alone (Kluge, 1964; Abdala & Moro, 1996). With the description of this organ, found only in *Gymnodactylus*, we hereby define the first morphological autapomorphic character for the genus, supporting the recent morphological and molecular phylogenetic hypothesis that suggests the monophyletism of *Gymnodactylus* (Silva-Jr, 2010; Domingos *et al.*, 2014; Gamble *et al.*, 2015).

CONCLUSION AND FURTHER HYPOTHESES

Among squamates, scent organs are present in the form of follicular glands derived from the inner epidermal generation layer and other epidermal specializations present on the scale surface (generation glands)

derived from the outer epidermal generation layer (Maderson, 1967, 1972). Here, we describe and characterize a cloacal scent organ present in *Gymondactylus* lizards. The available evidence suggests that this previously unknown organ is the first putative morphological autapomorphic character for the genus. Moreover, we discuss its role in chemical signalling and the influence of this for our understanding of the evolution of squamate scent glands.

Morphological descriptions have shown structural similarities and tissue homology of scent glands present in the femoral and precloacal regions of lizards and amphisbaenians. As such, the characteristics of the cloacal organ we describe here are congruent with the femoral and precloacal organs found in other squamate groups, and on this basis classified as a follicular gland (Mayerl *et al.*, 2015). Moreover, our histochemical analysis indicates a lipid composition of the *Gymnodactylus* PPG, consistent with scent glands among different lizard lineages that also show a prevalence of lipid compounds.

Morphological comparisons of scent organs among lizards and amphisbaenians, together with their phylogenetic distribution, suggest three principal hypotheses concerning the evolution of scent organs among squamates: (1) it is probable that all squamates share the genetic basis responsible for the evolutionary emergence of holocrine epidermal structures; (2) the squamates unique capacity for dedifferentiation of epidermal layers during the sloughing cycle may have led to the parallel emergence of morphological novelties in the form of holocrine structures in different lineages, and (3) generation and follicular glands have independently evolved from specific signalling restricted to generation of the inner and outer epidermis. Further evolutionary and developmental studies may clarify the mechanisms and epidermal-specific signals responsible for the emergence of these different structures among different lineages.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

Figure S1. Histological sections of the *Gymnodactylus dawinii* postero-proctodeal gland stained with haematoxylin and eosin. A, whole gland in sagittal view. B, magnification of cortical region. C, magnification of the cortical-secretory region. Key: icm, isquio-caudalis muscle; pcl, posterior cloacal lip; sp, secretory portion; tr, cloacal lip–gland transition.

Figure S2. Postero-proctodeal gland of a male *Gymnodactylus amarali* under histology (A) and transmission electron microscopy (B–D) analysis showing electron-lucent granules in the cortical region (B, C), with asterisks indicating cellular lysis in the secretory portion (B). Arrowheads indicate cytoplasmic granules in E and F. Key: nuc, nuclei; pcl, posterior cloacal lip; vs, lepidic vesicles. Arrowheads in A indicate the magnified regions shown in B and C. Arrowhead in C indicates the magnified region shown in D.

List S1. Voucher numbers of the specimens analysed.