ANATOMÍA E HISTOLOGIA DE LA CLOACA EN LAS HEMBRAS, Y LA GLÁNDULA MENTONIANA EN LOS MACHOS DE *Bolitoglossa nicefori* (CAUDATA: PLETHODONTIDAE)

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Trabajo de Investigación presentado como requisito parcial para optar al título de Biólogo

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INTRODUCCION GENERAL

La realización de estudios anatómicos e histológicos del sistema reproductivo y la glándula mentoniana en salamandras ha permitido conocer diferentes aspectos de su biología reproductiva. Sin embargo, con respecto a las salamandras suramericanas en general y en particular con la poco conocida salamandra *B. nicefori*, no se conocen ningún estudio. La realización de este trabajo de investigación permitirá obtener información relacionada con la biología reproductiva de esta especie, específicamente sobre dos estructuras relacionadas con la actividad reproductiva: la espermateca en las hembras y la glándula mentoniana en los machos.

Esta información permitirá comparar su morfología con la conocida para especies de la misma familia como de otras familias. Adicionalmente, puede aportar caracteres de uso filogenético, como por ejemplo la presencia (espermateca compleja) o ausencia (tubo cloacal, glándulas cloacales, receso cloacal) de determinadas estructuras cloacales y de la glándula mentoniana (tipo y orientación de las glándulas).

Para llevar a cabo esta investigación, se realizará un plan de trabajo basado en dos objetivos bien definidos, los cuales permitirán la interpretación de cortes seriados, para describir y comparar los resultados de estas dos estructuras en hembras y machos de *B. nicefori* en diferentes estados reproductivos, como con descripciones similares de otras especies pertenecientes a la misma familia y de otras familias.

Este trabajo se realizó en conjunto, pero fue necesario enviarlos por aparte a dos revistas especializadas, debido a la temática diferente para cada trabajo. Así, el trabajo titulado **FEMALE CLOACAL ANATOMY AND HISTOLOGY OF *Bolitoglossa nicefori* (CAUDATA: PLETHODONTIDAE)**, fue enviado a *Zoology*; y el trabajo titulado **THE MENTAL GLAND OF *Bolitoglossa nicefori* (CAUDATA: PLETHODONTIDAE)**, fue enviado a *Amphibia-Reptilia*.

JUSTIFICACION

La descripción y análisis de la cloaca y la actividad de sus glándulas asociadas, así como la actividad de la glándula mentoniana permite establecer una relación entre la actividad de cortejo y posterior apareamiento con el estado reproductivo de la hembra, debido a que las feromonas liberadas por estas glándula tienen un papel importante durante el comportamiento reproductivo de *B. nicefori*.

Así, la anatomía e histología del sistema reproductivo de las hembras (cloaca) y la glándula mentoniana en los machos son dos factores representativos en el estudio de la biología reproductiva de *B. nicefori*.

FEMALE CLOACAL ANATOMY AND HISTOLOGY OF *Bolitoglossa nicefori* (CAUDATA: PLETHODONTIDAE

RESUMEN

TITULO: ANATOMIA E HISTOLOGIA DE LA CLOACA DE LAS HEMBRAS DE *Bolitoglossa nicefori* (CAUDATA: PLETHODONTIDAE) [1]

AUTOR: Ezequiel González León[2]

PALABRAS CLAVES: *Bolitoglossa nicefori*, espermateca, anatomía cloacal, almacenamiento de esperma, Plethodontidae.

DESCRIPCION: La región cloacal de las hembras de *Bolitoglossa nicefori* en diferentes estados reproductivos de su ciclo reproductivo anual fueron estudiados por microscopia y comparados con descripciones de otros Plethodontidae. La anatomía e histología cloacal de *Bolitoglossa nicefori* es similar a otras salamandras Plethodontidae. La región cloacal consiste de un tubo cloacal anterior y una cámara cloacal posterior. El tamaño relativo del tubo cloacal corresponde al estado ancestral para Plethodontidae. En la región dorsal de la cámara cloacal se localiza la espermateca, cuyo tubo común diverge en dos cuellos de los túbulos que se ramifican en dos túbulos espermatecales dispuestos horizontalmente. Los cuellos de los túbulos y los túbulos espermatecales tienen un diámetro transversal similar. La glándula dorsal es rudimentaria y se relaciona con la condición reproductiva de la hembra; se observa solo en hembras sin esperma en su espermateca. El contenido secretor de la espermateca varía entre regiones y entre hembras adultas reproductivas y no reproductivas. La producción secretora, principalmente mucosubstancias acidas, esta relacionado al aumento del tamaño folicular durante el tiempo preovulatorio. El esperma se encontró solo en hembras periovulatorias; este mantenía un contacto mínimo con el epitelio espermatecal y según la reacción PAS/AB, las secreciones epiteliales no bañan el esperma almacenado. Futuros estudios son necesarios para saber si el esperma es embebido por el epitelio espermatecal o si es simplemente almacenado hasta la oviposición; también para determinar si la espermiofagia ocurre después de la oviposición.

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[1] Trabajo de Investigación.

[2] Facultad de Ciencias, Escuela de Biología, Director: Martha Patricia Ramírez Pinilla

ABSTRACT

TITLE: FEMALE CLOACAL ANATOMY AND HISTOLOGY OF *Bolitoglossa nicefori* (CAUDATA: PLETHODONTIDAE) [1]

AUTHOR: Ezequiel González León[2]

KEY WORDS: *Bolitoglossa nicefori*, spermatheca, cloacal anatomy, sperm storage, Plethodontidae.

DESCRIPTION: The cloacal region of *Bolitoglossa nicefori* females in different reproductive stages of their annual reproductive cycle was studied by light microscopy and compared with descriptions of other Plethodontidae. The spermathecal and cloacal anatomy of *B. nicefori* is similar to other plethodontid salamanders. The cloacal region consists of an anterior cloacal tube and a posterior cloacal chamber. The relative size of the cloacal tube corresponds to the ancestral state for Plethodontidae. In the dorsal region of the cloacal chamber is located the spermatheca, whose common tube diverges into two neck tubules that branch in several horizontally arranged spermathecal tubules. Neck tubules and spermathecal tubules have a similar transverse diameter. The dorsal gland is rudimentary and is related to female reproductive condition; it is observed only in females without sperm in their spermathecae. The secretory content of the spermatheca varied among its regions and between reproductive and non-reproductive adult females. Secretory production, principally acid mucosubstances, is related to the increase in ovarian follicular size during preovulatory time. Sperm was found only in periovulatory females; it maintains a minimum contact with spermathecal epithelium and according with PAS/AB reaction, epithelial secretions do not bathe the stored sperm. Future studies are needed to know if sperm is embedded by the spermathecal epithelium or if simply it is stored until oviposition; also to determine if spermiophagy occurs after oviposition.

[1] Investigation

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INTRODUCTION

The salamander females of the family Plethodontidae have a cloaca composed by a posterior cloacal chamber, an anterior cylindrical cloacal tube (Sever, 1978, 1994a), a single compound alveolar gland in the roof of the cloacal chamber, the spermatheca (Sever, 1991a, 1994a, 2003) that generally open between the union of the cloacal tube and the cloacal chamber on the cloacal papilla (Sever, 1978, 1994a, 2003), and associated glands. The cloacal tube begins anteriorly in the union of the oviducts, the Wolffian duct, and the bladder with the gut tube (Sever, 1978); it has different relative length among species and is absent in females of some Plethodontidae subfamilies (Sever, 1994a).

The spermathecae allow sperm storage for prolonged periods of time before fertilization/oviposition (Hardy and Dent, 1986; Sever, 2003), multiple matings, and provide the conditions for sperm competition (Sever and Brizzi, 1998). According to Sever (1991b, 1992d) and Brizzi et al. (1995) during the sperm storage a close contact between the sperm and the spermathecal epithelium occurs, it produces a secretory material which when released into the lumen bathes the stored sperm.

According to its structure, there are two types of spermathecal storage glands: (1) simple, constituted by numerous simple tubular glands (Ambystomatidae, Amphiumidae, Dicamptodontidae, Proteidae, Rhyacotritonidae and Salamandridae); and (2) compound, being a single compound alveolar gland present only in Plethodontidae (Sever, 1991a, 1994a, 2003; Sever and Brizzi, 1998). In oviparous species with internal fertilization sperm is released when the egg passes through the cloaca (Greven, 1998; Sever and Brizzi, 1998) during oviposition (Greven, 1998).

The complex spermathecae of Plethodontidae have three well-defined structures: (1) the common tube, open onto the roof of the cloacal chamber; (2) neck tubules, which radiate from the distal end of the common tube; and (3) spermathecal tubules, distal bulbs or enlarged acines where the sperm is effectively stored (Sever, 1992d, 1994a, 2003; Sever and Hamlett, 1998). Stored sperm does not last long after oviposition because in some species the epithelium of the distal bulbs is actively spermiophagic (Sever, 1992d, 2003; Sever and Brunette, 1993; Brizzi et al., 1995). After the reproductive stage, residual sperm enters in a degenerative process and, shortly afterwards, the spermathecae exhibit reduced lumina devoid of secretory products and stored sperm (Brizzi et al., 1995); but in some cases little sperm survives until the next breeding season (Sever, 1992d).

Bolitoglossa nicefori (San Gil Mushroomtongue Salamander) is an Andean Neotropical terrestrial salamander from the western slope of the Colombian Cordillera Oriental, at elevations 1400-1700 m. It inhabits rainforests and disturbed habitats like coffee plantations, among fallen leaves, herbaceous vegetation, and epiphytes (Ramírez-Pinilla et al., 2006). According to Ortega et al. (2009) females have seasonal breeding, since reproductive females only are found between November and February, so the cloacal morphology and its associated glands, and the presence of sperm in the spermatheca may vary in relation to the reproductive activity. This paper describes the anatomy and histology of the cloaca and its associated glands of *B. nicefori* females in different reproductive stages of their annual reproductive cycle; also compares its morphology with descriptions in other species of salamanders.

MATERIALS AND METHODS

Females in different reproductive stages and a juvenile female of *Bolitoglossa nicefori* were obtained from the Herpetological Collection of the Museo de Historia Natural, Universidad Industrial de Santander (UIS-A). They were collected in an organic coffee plantation at Hacienda El Roble (06°52´ N, 73°03' W, 1640 m altitude), and in a disturbed forest at Vereda Las Amarillas, Microcuenca La Venta (06°58´ N, 73°01´ W, 1400-2000 m altitude) Municipality of Piedecuesta, Santander, Colombia. Individuals were collected for past studies of reproduction, diet and microhabitat use (Ortega et al., 2009), cranial development (Dulcey et al., unpublished data), and appendicular development (Cabrera et al., unpublished data), under collecting and research permits granted by the local environmental authority (Corporación Ambiental para la Defensa de la Meseta de Bucaramanga).

The cloacal region of three adult reproductive, one adult post-reproductive, four adult previtellogenic, and one juvenile female of *B. nicefori* was studied. Reproductive females were considered those with yolked periovulatory follicles or with oviductal eggs. Post-reproductive females were those in which there was evidence of recent oviposition. Previtellogenic females those with adult body sizes but with previtellogenic follicles in their ovaries and without oviductal eggs; and juvenile females those with smaller body size than mature females and slightly differentiated ovaries and oviducts.

Snout-vent length (SVL) was measured from the tip of the snout to the posterior edge of the cloacal orifice. The cloacal region of each female was dissected and fixed in Bouin´s solution or neutral buffered 10 % formalin, and then stored in 70% ethanol. The entire cloacal region of each female was dehydrated in a series of ethanol and xylene, embedded in paraffin, serially sectioned at 7 µm with a rotary microtome. Some sections from each individual were stained with hematoxylin-eosin (general histology) and PAS-AB at pH 2.5 (neutral carbohydrates and acid mucopolysaccharides, respectively). Sections were viewed with an Olympus microscope and were photographed whit a digital camera. Anatomical and histological descriptions were done following the morphological nomenclature of Sever (1994a).

To express the relative size of the cloacal tube was used the proportion used by Sever (1986, 1991a, 1992a,b,c, 1994a) dividing the cloacal tube length by total cloacal length (CTL/TCL); the most anterior section of the cloacal tube was considered the one immediately posterior to the junction of Wolffian ducts and hindgut. The number of transverse sections of the cloacal tube and the cloacal chamber were counted to determine lengths. Finally, three-dimensional reconstructions were performed from the transverse serialized sections through the cloaca and spermatheca. This reconstruction was performed with Reconstruct software version 1.0.9.1 (Fiala, 2005). For this purpose, every certain number of sections was used (168 µm for cloaca and 28 µm for spermatheca).

RESULTS

Two sexually active females have the largest ovarian follicle diameters (2.35-3.2 mm), sperm in their spermathecae, and expanded highly convoluted oviducts (diameter 3.56 mm). The third female in reproductive condition have similar attributes, but without sperm in its spermatheca. The post-reproductive female has ovarian follicles of 1.01 mm, without sperm in the spermatheca, and convoluted and expanded oviducts (1.51 mm of diameter). Previtellogenic adult females have ovarian follicle diameters of 0.4-0.73 mm, without sperm in the spermathecae, and less convoluted oviducts (1 mm of diameter). The juvenile female had ovarian follicles of 0.26 mm and slightly differentiated oviducts (0.13 mm of diameter). This juvenile female did not exhibit a defined spermatheca.

Anatomy

The cloaca in *B*. *nicefori* female consists of an anterior cloacal tube and a posterior cloacal chamber. The cloacal chamber is elevated posteriorly to the spermatheca and dorsal gland and gradually decreases in height to the posterior end (Fig. 1). The cloacal tube presents a proportion CTL/TLC > 0.05. Both the cloacal tube and the posterior cloacal chamber have conspicuous wall folds (Figs. 2A-B); while at the anterior end of the cloacal chamber the walls are relatively smooth (Fig. 2C). Additionally, no females presented any type of cloacal recess.

The spermatheca is located in the dorsal region of the cloacal chamber, and is ventrally delimited by the cloacal papilla (Fig. 1). It consists of a common tube, whose distal end branches off into two neck tubules; at the end of these neck tubules are connected two spermathecal tubules that are extended caudally, bent ventrally and arranged horizontally under the neck tubules and the anterior segment of the common tube (Fig. 3). These spermathecal tubules are different from acinar bulbs; they are elongated and occasionally branched. Spermathecal tubules initially divide in two from the distal end of the neck tubules; then again each one diverges in two, not following a specific order. From a dorsal view, it is possible to observe variation in the symmetry of the spermathecal tubules; they can be symmetrical (Fig. 3B) or asymmetrical (Fig. 3D).

In vitellogenic females with sperm stored in their spermathecae the dorsal gland is not observed; whereas in previtellogenic and post-reproductive females it is possible to find a conspicuous dorsal gland. This gland presents 2-15 narrow tubules located on the roof of the cloacal chamber just below of spermathecal tubules at the beginning of the evagination of the cloacal papilla (Figs. 3C, 4 and 5B). Dorsal gland tubules secrete onto the cloacal lining in the dorsal medial region of the cloacal chamber.

The spermatheca and the dorsal gland were found poorly developed in the juvenile female; only the common tube, two neck tubules and a small dorsal gland were clearly defined. None of these structures has a defined lumen. In this female, there was no evidence of the formation of spermathecal tubules.

Histology

In *B*. *nicefori*, the posterior intestine has a simple columnar epithelium, whose apical cytoplasm reacts positively with PAS and AB (pH 2.5). The cloacal chamber is covered mostly by epidermis, except in its dorsal region where the cloacal tube presents an aglandular pseudostratified epithelium. The epidermal region of the cloacal chamber has a stratified epithelium with two differentiated layers: a basal cuboidal layer with nuclei at several levels and an apical layer of squamous cells. There are not glandular or ciliated cells associated with the epithelium of any region of the cloaca.

In previtellogenic, post-reproductive and sexually active (vitellogenic) females without sperm stored, the dorsal gland exhibits inactive condition: small lumen lined by a simple cuboidal epithelium with large nuclei occupying almost the whole of the epithelial cells, and without cilia (Fig. 4). The dorsal gland did not show secretory activity, and did not stain with PAS or AB (Table 1).

The spermatheca is surrounded by a thin myoepithelial layer and several melanocytes located in the underlying connective tissue making its location evident at macroscopic level. The three structures that comprise the spermatheca present transverse similar areas (Figs. 3B-D). The common tube and the neck tubules have a pseudostratified epithelium (Fig. 5), whereas the spermathecal tubules have a simple columnar epithelium with basal nuclei and evident eosinophilic secretory apex (Fig. 6).

The spermathecal epithelium has secretory granules ≤ 1 µm in diameter in post-reproductive, previtellogenic and sexually active (vitellogenic) females; however, the epithelium of the spermathecal tubules has a variable appearance (Fig. 2B and 5B). Seasonal variation occurs in the height of the spermathecal epithelium, it is higher in sexually active ($\bar{x}$ =

47.98 µm, range 20-76 µm) and post-reproductive ($\bar{x}$ = 58.10 µm, range 25-90 µm), that in pre-vitellogenic females ($\bar{x}$ = 36.08 µm, range 28-50 µm). In addition, sperm is present only in the spermathecae of periovulatory females at the end of the breeding season. Stored spermatozoa follow a parallel orientation to one another (Fig. 6C), and none was observed embedded in the cytoplasm of the spermathecal epithelium, only was found a minimal contact between the sperm and the spermathecal epithelium. Spermiophagy by the spermathecal epithelium cannot be readily demonstrated by light microscopy. Cilia were absent in any region of the spermatheca, both in females with or without sperm stored in their spermathecae.

Results of PAS/AB staining reaction for epidermal region of the cloacal chamber and spermathecal regions of *B. nicefori* are showed in Table 1. These PAS/AB staining reactions are different among the proximal and distal regions of the common tube, neck tubules, and spermathecal tubules. The connective tissue that surrounds all cloacal structures was eosinophilic and stained poorly with PAS.

DISCUSSION

The cloacal conformation and associated glands of *B*. *nicefori* is similar to those descriptions reported by Sever (1978, 1985, 1986, 1987, 1991a, 1992a,b,c,d, 1994a), Trauth (1984), Sever et al. (1990); Sever and Brunette (1993), Sever and Brizzi (1998) and Sever and Hamlett (1998) for plethodontid salamanders. The cloacal anatomy and histology of *B. nicefori* is similar to that is present in Salamandroidea, which exhibits a pseudostratified epithelium in the cloacal tube, replaced by epidermis in the cloacal chamber (Sever, 2003). Also, the cloaca has no ciliated cells in any of its regions, characteristic found in Plethodontidae (Sever, 1991a).

Among the species of *Bolitoglossa* examined by Sever et al. (1990) and Sever (1994a), *B. adspersa*, *B. platydactyla*, *B. subpalmata* (*Bolitoglossa-alpha*), *B. franklini* and *B. rufescens* (*Bolitoglossa- beta*), none exhibited a cloacal tube according to the relation CTL/TCL (CTL/TCL > 0.05 is the ancestral plethodontid state, according to Sever, 1994a). Therefore, Sever (1994a) stated that this was the only character in females Bolitoglossini that could characterize this clade. The relation found in *B. nicefori* CTL/TCL > 0.05 corresponds to the ancestral state suggested for Plethodontidae (Sever, 1994a); therefore the assumption that this proportion supports the clade Bolitoglossini should be re-evaluated. Sever (1994a) was based on a limited number of species of *Bolitoglossa* examined (5 of nearly 93 species). It is possible to expect intrageneric variation in this proportion when a major number of species of *Bolitoglossa* will be studied. The posterior elevation of the cloacal chamber respect to the spermatheca and dorsal gland is similar to the descriptions made by Sever et al. (1990) for some Bolitoglossini (*B. rufescens*, *B. adspersa*, *Batrachoseps wrighti*, *B. pacificus major*, *Hydromantes italicus*, *Dendrotriton bromeliacia*, *Oedipina poelzi* and *Thorius macdougalli*).

Plethodontidae females present at least one type of cloacal gland, the spermatheca (Sever, 1994a). The spermatheca of *B. nicefori* has the characteristics of the Plethodontidae spermathecae described by Sever (1978, 1985, 1986, 1987, 1991a, 1994a), Trauth (1984), Sever et al. (1990), Sever and Brunette (1993), Sever and Brizzi (1998), and Sever and Siegel (2006). The common tube also present in *B. nicefori* is considered an autapomorphy for the family (Sever, 1987). Contrary to *B. nicefori* which has two neck tubules and two spermathecal tubules invariably, in other species of Plethodontidae a variable number of narrow neck tubules can be found: 4 in *Plethodon dorsalis* (Sever, 1978), 15-20 in *Eurycea quadridigitata* (Trauth, 1983), 8-15 in *P. glutinosus* (Trauth, 1984), 6 in *P. cinereus* (Sever, 1978), 6-12 in *E. nana* and *E. neotenes*, 12-20 in *Typhlomolge rathbuni* (Sever, 1985), 16-26 in *Gyrinophilus porphyriticus,* 22-34 in *Pseudotriton montanus*, 18-32 in *P. ruber*, 24 in *Stereochilus marginatus* (Sever, 1986), 20-24 in *E. bislineata*, 9-13 in *Hemidactylium scutatum*, 16-22 in *Typhlotriton spelaeus* (Sever, 1987), and 20-24 in *E. cirrigera* (Sever and Brunette, 1993). On the other hand, in plethodontid salamanders the place of sperm storage is named the spermathecal bulb or distal bulb (Trauth, 1984; Sever, 1992d, 1994b, 1997, 2003; Sever and Brunette, 1993; Sever and Hamlett, 1998; Sever and Siegel, 2006) because generally, the common tube distally branches into narrow neck tubules that expand into acinar distal bulbs (Sever, 2003). However, in *B. nicefori* this region does not have an acinar bulbous shape. A similar conformation of the branched spermathecal tubules of female *B. nicefori* is found in other Bolitoglossini studied by Sever et al. (1990), including *Bolitoglossa-alpha* and *Bolitoglossa- beta*, and in the Hemidactyliini *E. quadridigitata* by Trauth (1983).

Sever and Brunette (1993) suggested that spermiophagy by the distal bulbs is energetically more advantageous that to expel the sperm across the narrow neck tubules and the common tube, since the sperm are quiescent in the spermathecae and energy is needed to expel the sperm through myoepithelial contractions. On the contrary, in *B. nicefori* the neck tubules and spermathecal tubules have a similar transverse diameter (Fig. 3) which would allow easily expel the sperm from the spermatheca, probably facilitated by the contractions of the myoepithelial cells. A similar function of the myoephitelium is described by Hardy and Dent (1986). This feature could be correlated with the possible non-spermiophagic function of the epithelium of the spermathecal tubules of *B. nicefori* since sperm would be expelled completely during the breeding season. Additionally, the spermathecal epithelium of *B. nicefori* presents a secretory granular apex whose secretions released into the lumen may have different functions (see below). Contrary to *B. nicefori*, *E. cirrigera* does no present secretory granules in the epithelium of the distal bulbs and the sperm are embedded in the apical cytoplasm and posteriorly degraded (Sever, 1991b, 1992d; Sever and Brunette, 1993). Thus, the spermiophagic function of the distal bulbs can be correlated with the complexity of the spermathecae (Sever and Brunette, 1993). In other species as *P. cinereus,* the secretory vacuoles are found in all regions of the spermatheca and do not present regionalization of secretory activity (Sever, 1997) as was observed in *B. nicefori*.

According to Sever (1994b), the symmetry of the spermatheca is based in the location of the distal ends of the tubules respect to the midline to the cloaca. In *P*. *cinereus*, symmetry is related to the number of the distal bulbs located on each side of the midline to the cloaca, in which nine of the fourteen examined specimens had spermathecal tubules to the left side, and five had tubules on the right side (Sever, 1978). Taking into account the description made in other Plethodontidae species by Sever (1994b), in *B. nicefori* a symmetrical spermatheca according to its location on the midline to the cloaca is observed (Figs. 3B and D).

However, as is observed in the Figure 3D, the asymmetry of the spermathecal tubules is evident and intraspecific variation according to the symmetric conformation of the spermathecal tubules occurs.

The epidermal region of the cloacal chamber and spermathecal epithelium of *B. nicefori* show variation to the PAS/AB reaction not only between breeding and non-breeding females, but also among the different regions of the spermatheca (Table 1). The absence of neutral mucopolysaccharides in previtellogenic females, and their presence in vitellogenic females with and without sperm in their spermathecae (Table 1), suggests that this secretion plays a role during the breeding season and can be hormonally regulated. In addition, in all regions of the spermatheca acid mucosubstances were found in different degrees (scant or abundant positive reaction); except in the distal portion of the spermathecal tubules in previtellogenic females. Additionally, the sperm stored in vitellogenic females does not react to any PAS-AB stain, suggesting that probably the sperm is not bathed by the secretions of the epithelial cells of the spermathecal tubules. The resolution of the light microscopy does not allow clearly observe whether the sperms are embedded into the epithelial cells or if they are bathed by secreting material; however, there is not indication that this is occurring in this species. In *E. cirrigera*, the spermathecal secretions bath the sperm stored after mating and prior to oviposition, and the spermathecal epithelium becomes spermiophagic after oviposition (Sever, 1992d). Probably, this same spermiophagic process occurs in *B*. *nicefori*, and the sperm is degraded shortly after oviposition, since no sperm was observed in the spermathecae of females collected one month after finished the breeding season.

In some Plethodontini (*P. ouachitae*) a narrow and tubular dorsal gland occurs and the epithelium does not react with diagnostic stain, therefore is considered a vestigial accessory gland. But contrary to this species, *Ensatina eschscholtzii* (Plethodontini) and Hemidactyliini females with dorsal gland present a short and tubular gland with an apical cytoplasm PAS + or AB +. In Desmognathinae females, the dorsal gland is rudimentary with narrow lumina, scant cytoplasm, and occasionally an AB + reaction (Sever, 1994a). The description realized for Bolitoglossini females (Sever, 1994a, 2003) is consistent with the description of *B. nicefori*, since it presents a narrow lumen, absence of a secretory granular apex (Fig. 4) and lacking of PAS/AB reaction. A notable feature is the presence of this dorsal gland in previtellogenic a post-reproductive female of *B. nicefori*; probably in vitellogenic females, the dorsal gland is not well-differentiated. Although inter and intraspecific variation in the presence of this dorsal gland occur in other plethodontid salamander as studied by Sever (1986, 1994a), there is no a correlation between the presence/absence of the dorsal gland and the reproductive status of females.

The regionalization of secretory activity (PAS/AB) of the spermatheca of salamanders has been studied in species with simple and complex spermathecae. In the simple spermathecae, it was not observed cytological variation among proximal and distal regions (Sever and Kloepfer, 1993) and no variation in the secretory material (Sever, 1994b). In complex spermathecae, Sever (1994b) found that there was a regionalization of secretory activity in 10 of 41 examined species. In six species, the PAS/AB reaction varied among regions of the spermatheca (the common tube and neck tubules are PAS + and AB +, and the distal bulbs are PAS + and AB – including to *B*. *adspersa*). In *E*.

cirrigera, Sever and Brunette (1993) found a PAS + reaction in neck tubules and common tube along the annual cycle, but the reaction was weak on the months following the breeding season. In other Hemidactyliini females, the spermathecal epithelium in specimens in breeding condition or with sperm in the spermathecae, the common tube, neck tubules and spermathecal acini were PAS +. The spermatheca in *E*. *eschscholtzii* and spermathecal acini in *Aneides* (both Plethodontini) are AB +. A similar AB reaction occurs in the common tube and the epithelium of the spermathecal acini of Desmognathinae females (Sever, 1994a).

While the PAS/AB reactions are differentially observed in the spermathecae of salamanders (Sever, 1994a,b, 1997; Sever and Siegel, 2006), the role of the secretory products is unknown (Sever, 1991b, 1994b). Although there are some associated features proposed as attraction, nutrition, degradation, training or management of a chemical/osmotic environment for sperm quiescence during storage (Hardy and Dent, 1986; Sever and Kloepfer, 1993; Sever and Brizzi, 1998; Sever, 2003), these secretions may have different roles in different species (Sever and Siegel, 2006). Additionally, these secretions may be released during storage or luminal degradation of sperm, mating or oviposition (Sever and Bart, 1996; Sever and Brizzi, 1998). However, the production of secretory products in the spermatheca coincides with an increase in ovarian follicular size and sexual activity (Sever, 1991b). In *B. nicefori*, this production of secretory products (principally neutral carbohydrates) occurs mainly when females are periovulatory.

In the juvenile female of *B. nicefori* the spermatheca is not clearly definite and the dorsal gland is poorly developed but conspicuous. This fact suggests that the development of the spermatheca is controlled by the hormones secreted by the mature ovary as other secondary sexual characters. Sever (1987) also found that in *Rhyacotriton olympicus*, the spermatheca and other cloacal glands are not well-developed related possibly to the sexually inactive condition and the presence of the small ovarian follicles.

In summary, the female cloacal region of *Bolitoglossa nicefori* is similar to the descriptions made for Plethodontidae and Bolitoglossini female salamanders without evident modifications in the general cloacal morphology; however, there are differences in the spermathecal tubules and neck tubules conformation. Future ultrastructural studies in *B. nicefori* are needed to clarify if the sperm is embedded by the spermathecal epithelium and its possible spermiophagic function, or simply as seems to be, the sperm is stored in the lumina of the spermathecal tubules of periovulatory females until oviposition.

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LITERATURE CITED

Brizzi R, Delfino G, Selmi MG, Sever DM. 1995. Spermathecae of *Salamandrina terdigitata* (Amphibia: Salamandridae): patterns of sperm storage and degradation. J Morphol 223: 21-33.

Fiala JC. 2005. *Reconstruct*: A free editor for serial section microscopy. J Microsc 218: 52-61.

Greven H. 1998. Survey of the oviduct of Salamandrids with special reference to the viviparous species. J Exp Zool 282: 507-525.

Hardy MP, Dent JN. 1986. Transport of sperm within the cloaca of the female red-spotted newt. J Morphol 190: 259-270.

Ortega JE, Monares JM, Ramírez-Pinilla MP. 2009. Reproductive activity, diet and microhabitat use in *Bolitoglossa nicefori* (Caudata: Plethodontidae). J Herpetol 43: in press.

Ramírez-Pinilla MP, Osorno-Muñoz M, Rueda JV, Amézquita A, Ardila Robayo MC. 2004. *Bolitoglossa nicefori*. 2006 IUCN Red List of Threatened Species. Downloaded on 21 April 2008.

Sever DM. 1978. Female cloacal anatomy of *Plethodon cinereus* and *Plethodon dorsalis* (Amphibia, Urodela, Plethodontidae). J Herpetol 12: 397-406.

Sever DM. 1985. Sexually dimorphic glands of *Eurycea nana*, *Eurycea neotenes* and *Typhlomolge rathbuni* (Amphibia: Plethodontidae). Herpetologica 41: 71-84.

Sever DM. 1986. Disparate sexual variation among *Gyrinophilus*, *Pseudotriton* and *Stereochilus* (Amphibia: Plethodontidae). Herpetologica 42: 301-323.

Sever DM. 1987. *Hemidactylium scutatum* and the phylogeny of cloacal anatomy in females salamanders. Herpetologica 43: 105-116.

Sever DM. 1991a. Comparative anatomy and phylogeny of the cloacae of salamanders (Amphibia: Caudata). I. Evolution at the family level. Herpetologica 47: 165-193.

Sever DM. 1991b. Sperm storage and degradation in the spermathecae of the salamander *Eurycea cirrigera*. J Morphol 210: 71-84.

Sever DM. 1992a. Comparative anatomy and phylogeny of the cloacae of salamanders (Amphibia: Caudata). III. Amphiumidae. J Morphol 211: 63-72.

Sever DM. 1992b. Comparative anatomy and phylogeny of the cloacae of salamanders (Amphibia: Caudata). IV. Salamandridae. Anat Rec 232: 229-244.

Sever DM. 1992c. Comparative anatomy and phylogeny of the cloacae of salamanders (Amphibia: Caudata). VI. Ambystomatidae and Dicamptodontidae. J Morphol 212: 305-322.

Sever DM. 1992d. Spermiophagy by the spermathecal epithelium of the salamander *Eurycea cirrigera*. J Morphol 212: 281-290.

Sever DM. 1994a. Comparative anatomy and phylogeny of the cloacae of salamanders (Amphibia: Caudata). VII. Plethodontidae. Herpetol Monogr 8: 276-337.

Sever DM. 1994b. Observations on regionalization of secretory activity in the spermathecae of salamanders and comments on phylogeny of sperm storage in female Amphibians. Herpetologica 50: 383-397.

Sever DM. 1997. Sperm storage in the spermatheca of the red-back salamander, *Plethodon cinereus* (Amphibia: Plethodontidae). J Morphol 234: 131-146.

Sever DM. 2003. Courtship and mating glands. In: Sever DM, editor. Reproductive biology and phylogeny of urodela. Science Publisher, Enfield, NH. pp 232-381.

Sever DM, Bart HL. 1996. Ultrastructure of the spermathecae of *Necturus beyeri* (Amphibia: Proteidae) in relation to its breeding season. Copeia 1996: 927-397.

Sever DM, Brizzi R. 1998. Comparative biology of sperm storage in female salamanders. J Exp Zool 282: 460-476.

Sever DM, Brunette NS. 1993. Regionalization of eccrine and spermiophagic activity in spermathecae of the salamander *Eurycea cirrigera* (Amphibia: Plethodontidae). J Morphol 217: 161-170.

Sever DM, Hamlett WC. 1998. Sperm aggregations in the spermatheca of female Desmognathine salamanders (Amphibia: Urodela: Plethodontidae). J Morphol 238: 143-155.

Sever DM, Kloepfer N. 1993. Spermathecal cytology of *Ambystoma opacum* (Amphibia: Ambystomatidae) and the phylogeny of sperm storage organs in female salamanders. J Morphol 217: 115-127.

Sever DM, Siegel DS. 2006. Sperm aggregations in the spermatheca of the red back salamander (*Plethodon cinereus*). Acta Zool 87: 331-340.

Sever DM, Heinz EA, Lempart PA, Taghon MS. 1990. Phylogenetic significance of the cloacal anatomy of female Bolitoglossine salamanders (Plethodontidae: Tribe Bolitoglossini). Herpetologica 46: 431-446.

Trauth SE. 1983. Reproductive biology and spermathecal anatomy of the dwarf salamander (*Eurycea quadridigitata*) in Alabama. Herpetologica 39: 9-15.

Trauth SE. 1984. Spermathecal anatomy and the onset of mating in the slimy salamander (*Plethodon glutinosus*) in Alabama. Herpetologica 40: 314-321.

FIGURE LEGENDS

Figure 1. Mid-sagittal section through the cloaca of a vitellogenic adult female of *Bolitoglossa nicefori*. Note the absence the sperm in its spermatheca. Section stained with hematoxylin-eosin (H-E). Cc, cloacal chamber; Co, cloacal orifice; Cp, cloacal papilla; Cs, common tube to the spermatheca; Ct, cloacal tube; Ep, epidermis; Li, large intestine; St, spermatheca; Ub, urinary bladder. Scale bar = 0,5 mm.

Figure 2. Transverse histological sections through the cloaca of a vitellogenic adult female *Bolitoglossa nicefori* without sperm in its spermatheca. Section stained with hematoxylin-eosin (H-E). **A:** Posterior end of the cloacal chamber, with conspicuous folds. **B:** Neck tubules and spermathecal tubules in the roof of the middle cloacal chamber. **C:** Anterior end of the cloacal chamber with relatively smooth walls, also it is observed the cloacal tube and its conspicuous folds. Cc, cloacal chamber; Ct, cloacal tube; Dg, dorsal gland; Nt, neck tubules; Stu, spermathecal tubules. Scale bar = 200 µm for A, and 300 µm for B and C.

Figure 3. Three-dimensional reconstruction of the spermathecae of female *Bolitoglossa nicefori*. **A and B:** Spermatheca with luminal sperm. **C and D:** Spermatheca without sperm. The distance between sections of cloacal walls is 168 µm and 28 µm for the spermathecae. Sections tilted 10° laterally (left) and anteriorly (right). Cc, cloacal chamber; Cp,

cloacal papilla; Cs, common tube to the spermatheca; Ct, cloacal tube; Dg, dorsal gland; Nt, neck tubules; Stu, spermathecal tubules.

Figure 4. Para-sagittal (A and B) and transverse (C) sections through the cloacae of vitellogenic adult females without sperm in their spermathecae of *Bolitoglossa nicefori*. **A:** Note the presence of the spermatheca and the dorsal gland. **B:** Detail of dorsal gland showed in A. **C:** Dorsal gland associated with the cloacal chamber. Sections stained with hematoxylin-eosin (H-E). Cc, cloacal chamber; Dg, dorsal gland; St, spermatheca. Scale bar = 200 µm for A, and 50 µm for B and C.

Figure 5. Transverse histological sections through the cloaca of a vitellogenic adult female *Bolitoglossa nicefori* without sperm in its spermatheca. Section stained with hematoxylin-eosin (H-E). **A:** Posterior end of the common tube. **B:** Anterior end of the neck and spermathecal tubules. Cc, cloacal chamber; Cs, common tube to the spermatheca; Dg, dorsal gland; Nt, neck tubules; Stu, spermathecal tubules. Scale bar = 200 µm for A and B.

Figure 6. Transverse histological sections through the spermathecal tubules of adult females of *Bolitoglossa nicefori*. Section stained with hematoxylin-eosin (H-E). **A:** Spermathecal tubule showing absence of sperm in the lumen. **B and C:** Spermathecal tubules showing sperm in the lumen with parallel orientation in C. Ac, apical cytoplasm; Lu, lumen

of the spermathecal tubule; Sp, sperm in the spermathecal tubules. Scale bar = 16 µm for A and B, and µm for 20 C.

TABLE 1. PAS/AB reactions (pH 2.5) of the epidermal region of the cloacal chamber and spermathecal epithelium in different regions of the cloaca of *Bolitoglossa nicefori* adult females in different reproductive stages. Note the regionalization of secretions in the different regions of the spermathecae and between the proximal and distal segments of each structure: spermathecal tubules, neck tubules and common tube. Abbreviations: A, absence; P, present; –, negative reaction; +, scant positive reaction; ++, abundant positive reaction.

Reproductive stage		**Sperm**	**Spermathecal tubules**		**Neck tubules**		**Common tube**		**Dorsal gland**	**Cloacal epithelium**	**Cloacal tube**
			Distal	Proximal	Distal	Proximal	Distal	Proximal			
Previtellogenic	PAS	A	−	−	−	−	−	−	P	−	−
	AB	A	−	+	+	+	+	+	P	+	+
Vitellogenic	PAS	A	+	+	+	+	+	+	P	−	−
	AB	A	+ +	+	+ +	+	+ +	+	P	+ +	+
Vitellogenic	PAS	P	+	+	+ +	+	+	+ +	A	−	−
	AB	P	+	+	+ +	+ +	+ +	+	A	+ +	+

FIGURES

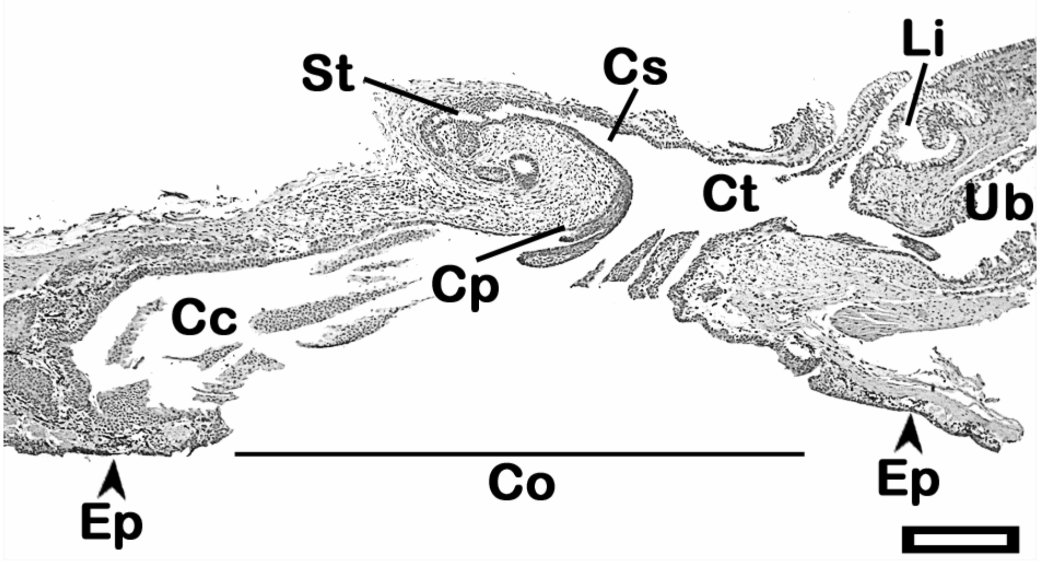
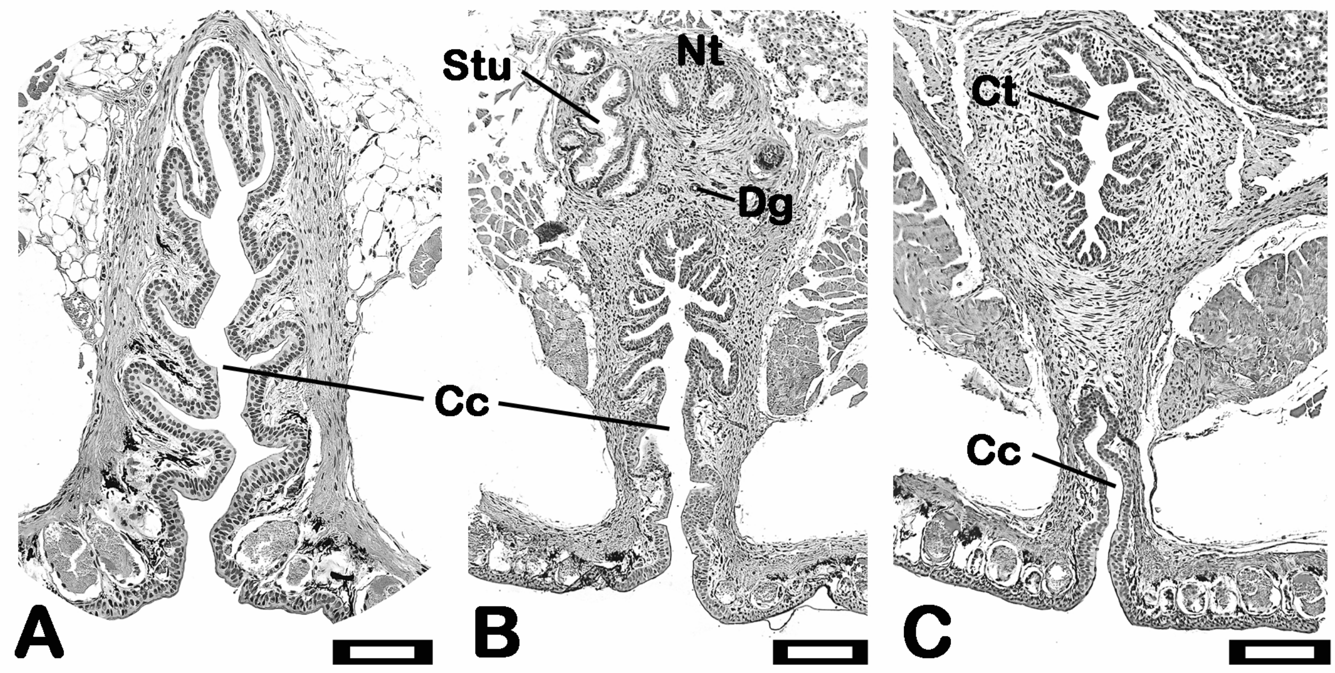


Figure 1.



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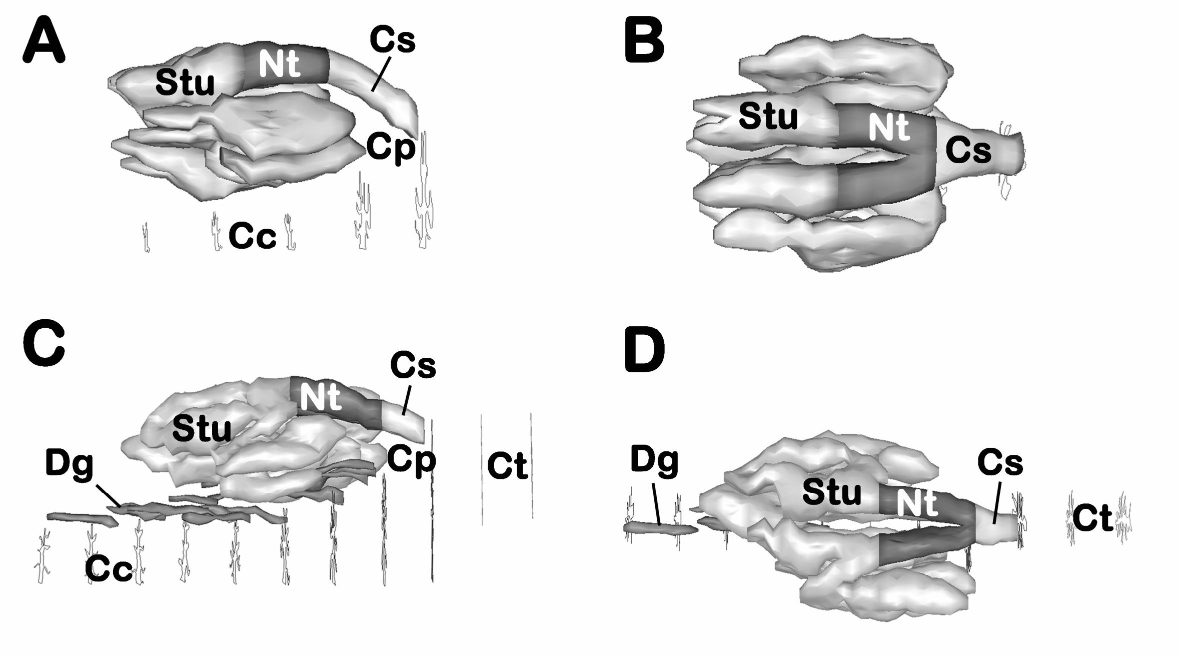

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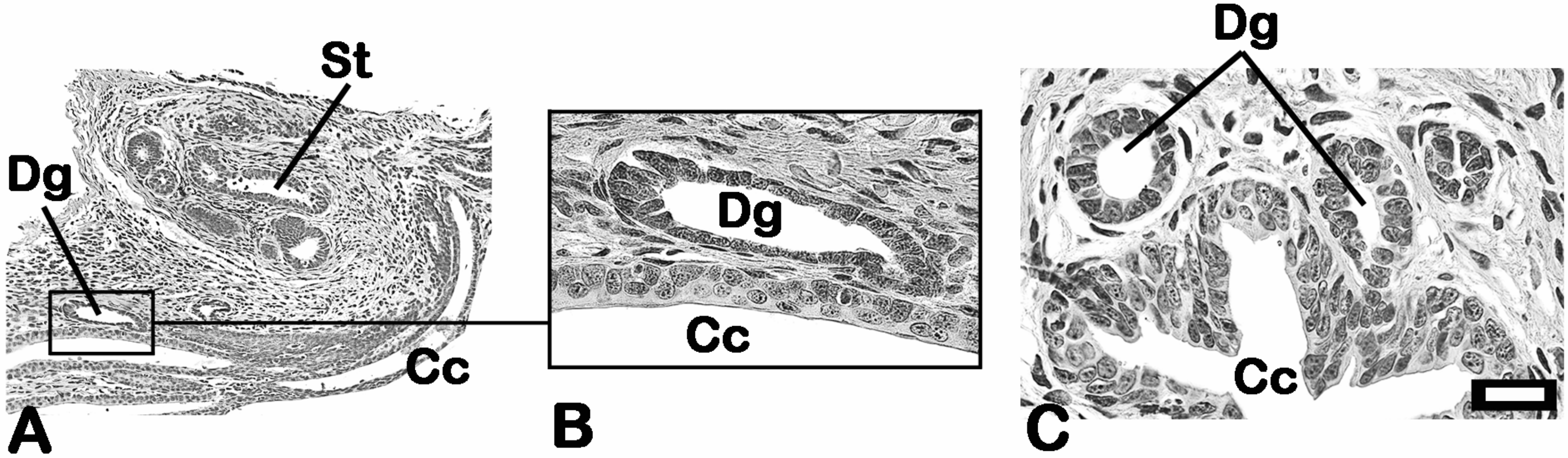

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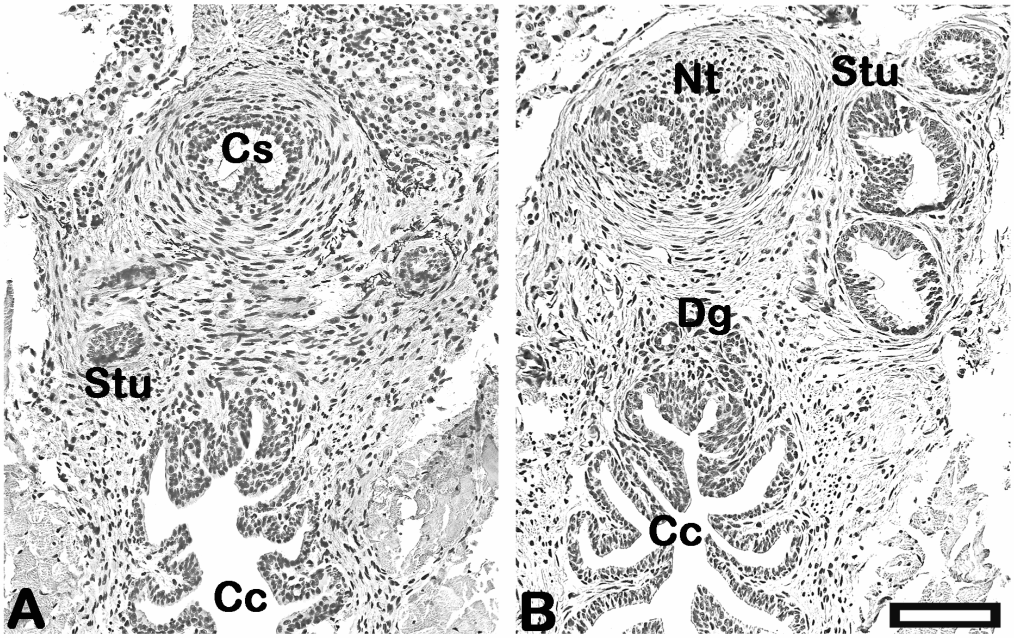

FIGURE 5.

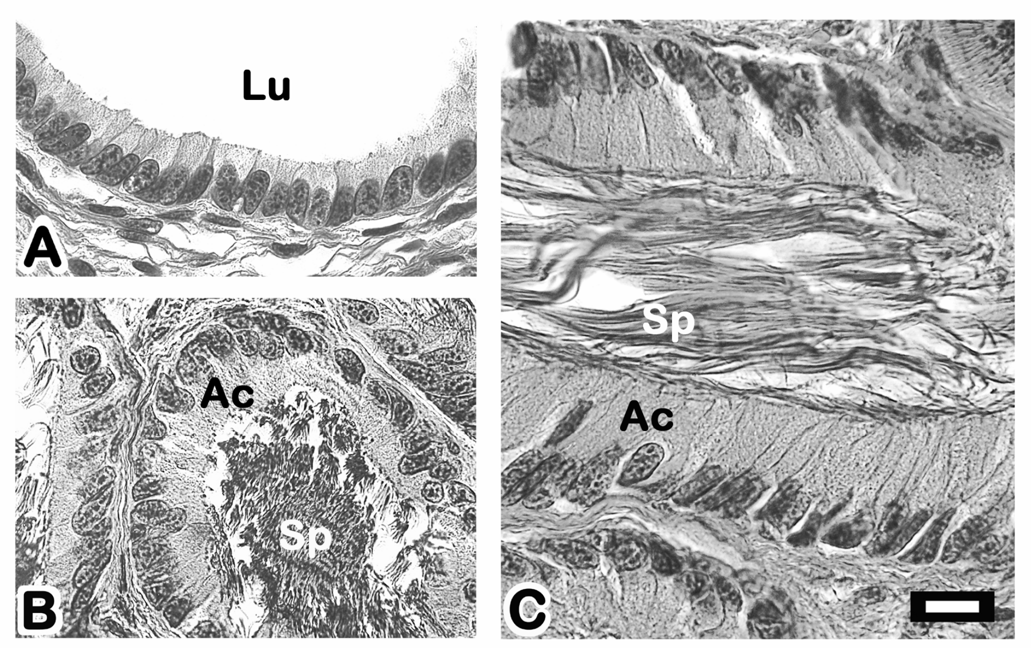

FIGURE 6.

THE MENTAL GLAND OF *Bolitoglossa nicefori* (CAUDATA: PLETHODONTIDAE)

RESUMEN

TITULO: La glándula mentoniana de *Bolitoglossa nicefori* (Caudata: Plethodontidae).[1]

AUTOR: Ezequiel González León[2]

PALABRAS CLAVES: *Bolitoglossa nicefori*, glándula mentoniana, Plethodontidae, cortejo, salamandra.

DESCRIPCION: La glándula mentoniana de la salamandra *Bolitoglossa nicefori* fue examinada por observación macroscópica y microscópica y comparada con descripciones morfológicas de otras especies del genero. La glándula mentoniana presenta forma ovalada que se desarrolla exclusivamente en machos reproductivos. Se compone de 225 a 710 glándulas tubulares simples de secreción merocrina en posición dorso-ventral. Los ductos de las glándulas tubulares tienen forma fusiforme. La reacción PAS/AB negativa es consistente con los resultados de los componentes glandulares de la glándula mentoniana de otros Plethodontidae. Los machos de esta especie producen esperma continuamente a lo largo del año, por lo tanto los machos adultos presentan una glándula mentoniana conspicua. Sin embargo, se observó una leve variación en la altura y diámetro de las glándulas tubulares y una variación significativa en el diámetro de los gránulos secretores. Esto puede estar relacionado con el hecho que los machos son potencialmente reproductivos todos los meses del año, pero las hembras se reproducen temporalmente definiendo la temporada reproductiva cuando la hipertrofia de la glándula mentoniana en los machos en mayor.

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[1] Trabajo de Investigación.

[2] Facultad de Ciencias, Escuela de Biología, Director: Martha Patricia Ramírez Pinilla

ABSTRACT

TITLE: The mental gland of *Bolitoglossa nicefori* (Caudata: Plethodontidae)[1]

AUTHOR: Ezequiel González León[2]

KEY WORDS: *Bolitoglossa nicefori*, mental gland, Plethodontidae, courtship, salamander.

DESCRIPTION: The mental gland of the plethodontid salamander *Bolitoglossa nicefori* was examined by macroscopic observation and light microscopy and compared with morphological descriptions of other species of the genus. The mental gland is an oval shaped pad which develops exclusively in reproductive males. It is composed by 255 to 710 simple tubular glands of merocrine secretion arranged in a dorso-ventral disposition. The outlets of the glands have a spindle-shaped or fusiform form. Negative stain reaction for PAS-AB is consistent with results of the glandular components of mental glands from other Plethodontidae. Males of this species produce sperm continuously throughout year and related to that all examined adult males showed conspicuous mental glands. However, it was observed a slight variation in the height and diameter of the tubular glands and a significant variation in the diameter of the secretory granules. It would be related to the fact that although males are potentially reproductive all months of the year, females are seasonal breeders and then there is a defined breeding season when the hypertrophy of the mental gland is the greatest.

[1] Investigation

[2] Faculty of Sciences, Program of Biology, Director: Martha Patricia Ramírez Pinilla

INTRODUCTION

In salamanders, chemical signals have a key importance in the courtship process. The male produces courtship pheromones from the specialized submandibular gland that hypertrophy only during the mating season (Sever 1975; Rollmann *et al.* 1999, 2000, 2003; Houck and Arnold 2003; Weichert 1945), increase female receptivity (Houck and Arnold 2003; Rajchard 2005; Rollmann *et al*. 1999, 2003), allowing the continuity of courtship (Sever 1976b; Rollmann *et al*. 2003), reducing the courtship length which in turn decrease the probability of disruption of mating by another male rivals or predators (Rollmann *et al*. 2003), and are probably involved in the mate choice (Rajchard 2005). These glands in the submandibular region of males were named mental gland by Noble (1927). They have been reported in males of one species in the Salamandridae (Sever 2003) and occur widely in Plethodontidae (Sever 1976b, 2003). According to Sever (1976b) many authors recognized that some intergeneric variation existed in the morphology of mental glands. The mental glands in Plethodontidae vary from round, oval, or broadened posteriorly, to elongate and bifurcate (Valentine 1963; Sever 1975; Dodd and Brodie 1976). Generally, the mental gland is constituted by groups of short, dorso-ventrally oriented tubules that secrete over a small to large area (Sever 2003), through the excretory ducts through the epidermis of the mental region (Testa *et al.* 1993); or form a fan-shaped cluster of short or long tubules, antero-posteriorly oriented that secrete at the apex of the lower jaw (Sever 2003). The mental gland is hypertrophied during the breeding season and then it is used as a sexually dimorphic character to distinguish between the sexes (Noble 1929; Weichert 1945).

Bolitoglossa nicefori (San Gil Mushroomtongue Salamander) is a Neotropical and endemic species from the Colombian Cordillera Oriental; it is found in disturbed humid forests and in coffee plantations, among fallen leaves, herbaceous vegetation and epiphytes (Ramírez-Pinilla *et al*. 2004). The analysis of the annual reproductive activity of a population of this species showed a breeding season during the driest months of the year and defined by the females (Ortega *et al*. 2009). The objective of this study was to describe and compare the anatomy and histology of the mental gland in juvenile and adult males of this species to allow intra and interspecific comparisons.

MATERIALS AND METHODS

Males in different reproductive stages of *Bolitoglossa nicefori* were obtained from the Herpetological Collection of the Museo de Historia Natural, Universidad Industrial de Santander. They were collected in an organic coffee plantation located in Hacienda El Roble (06º52´ N, 73º03´ W, 1640 m altitude) and in a disturbed forest at Vereda Las Amarillas, Microcuenca La Venta (06º58´ N, 73º01´ W, 1400-2000 m altitude) Municipality of Piedecuesta, Santander, Colombia. Individuals were collected for past studies of reproduction, diet and microhabitat use (Ortega *et al*. 2009), cranial development (Dulcey *et al*. unpublished data), and appendicular development (Cabrera *et al*. unpublished data), under collecting and research permits granted by the local environmental authority (Corporación Ambiental para la Defensa de la Meseta de Bucaramanga).

According to Ortega *et al*. (2009), adult males of this species present sperm in their testes and ducts through the year; therefore for this study were considered to be reproductive those males that presented the above characteristics. They were separated in two groups: active males when they were collected during the breeding season (November-February), and inactive males when they were collected outside of the breeding season (March-September). The mental glands of six active males, five inactive males, and one juvenile male of *B. nicefori* were studied.

Snout-vent length (SVL) was measured from the tip of the snout to the posterior edge of the cloacal orifice. Mental gland of each male was dissected and fixed in 10 % buffered formalin, dehydrated in a series of ethanol and xylene, embedded in paraffin, serially sectioned at 7 to 10 µm with a rotary microtome. Some sections from each individual were stained with hematoxylin-eosin (general histology) and with PAS-AB at pH 2.5 (neutral carbohydrates and acid mucopolysaccharides). For each mental gland we measured its length and width macroscopically; height and diameter of the tubular glands, diameter of the secretory granules, and length and width of the ducts of the tubular glands in their medial region, microscopically. We used a *t*-test to compare the difference between means with data obtained of diameter and height of the tubular gland and the secretory granules from active and inactive males. Additionally, testes were serially sectioned at 10 µm and stained with hematoxylin-eosin to precise the reproductive stage of each male.

RESULTS

Three of the active males had a conspicuous and hypertrophied mental gland; they have a SVL > 44.9 mm. The other three active males had an evident but less hypertrophied gland; it has a SVL > 42.3 mm. Three of the inactive males presented a slightly hypertrophied gland, they had a SVL > 48.56 mm; the other two inactive males showed a poor development of the gland, they had a SVL > 45.5 mm. The juvenile male (SVL 39.54 mm) did not present any evident indication of mental gland in the submandibular region.

Anatomy

Mental gland exhibits an oval form in cross-sections (Fig. 1) that changes in diameter from 1.82 mm of length and 3.58 mm of width (inactive males) to 2.82 of length and 3.94 mm of width (active males). It consists of approximately 255-386 (inactive males) to 318-710 (active males) simple tubular glands with dorso-ventral disposition (Fig. 2A, B) and non-uniform diameter.

Histology

In the submandibular region of the juvenile male were found common epidermal granular glands and there was no indication of any development of the mental gland (Fig. 3). In reproductive males, tubular glands presented a variable diameter and height (Table 1). The tubular glands of inactive and active males are composed by columnar glandular cells with basophilic basal nuclei and granular eosinophilic apex directed towards the lumen of the gland (Fig. 2, 4A). Surrounding the tubular glands there is a small layer of highly eosinophilic connective tissue. Several melanocytes surround the mental gland at different levels (Fig. 1B, 2B, C).

Tubular glands open individually at the bottom of the lower jaw, through small spindle-shaped or fusiform ducts (Fig. 2B, C). These ducts measure in their medial region 40 ± 7 µm length and 8.7 ± 3.7 µm width (Fig. 4B). They consist of two cells that form the duct itself. In turn, four epithelial cells surround these cells in a particular disposition: two cells located longitudinally to the major axis of the duct, and other two located perpendicular to this axis (Fig. 4B).

Each tubular gland has a merocrine secretion; the secretory apical granules are liberated into the lumen of the gland asynchronously (Fig. 4A). They are round or oval with variable diameters in males from different reproductive seasons (Table 1). One active male (SVL 44.52 mm) collected the last month of the reproductive season (mid-February), showed few secretory granules, whose mean size was the lowest among active males (diameter 2.3 mm). Staining reaction to PAS-AB was negative in all cases for the glandular content; however, the connective tissue that surrounds the mental gland was poorly stained for PAS. Only the diameter of the granules showed a statistically significant difference ($t = -1.86$, $df = 9$, $p < 0.05$) between active and inactive males. The other parameters, diameter and height of the tubular gland were not statistically different between active and inactive males ($t = -0.24$, $df = 9$, $p > 0.05$, and $t = -0.97$, $df = 9$, $p > 0.05$, respectively).

The testes of the juvenile male had several cists in different stages of the spermatogenesis; however, cists of early spermatids were the latest stage found and the excurrent ducts were empty. In the testes of adult males collected during breeding and non-breeding seasons were observed cists of the different stages of the spermatogenesis and spermiogenesis including mature sperm at the lumen of the seminiferous tubules (Fig. 5) and in the collecting ducts (Fig. 6).

DISCUSSION

The anatomy and histology of the mental gland of *Bolitoglossa nicefori* is similar to those described for most species of Plethodontidae. For this family and related to their shape Sever (2003) proposed five types of glands: (1) pad-shaped, relatively large and oval, in *Plethodon* and *Bolitoglossa*; (2) pad-shaped, relatively small, in *Hemidactylium* and *Eurycea*; (3) anterior protrusion, in *Desmognathus*; (4) elongate and bifurcated, in *Desmognathus wrighti*; (5) fan-shaped and elongated, in *Eurycea* and various Bolitoglossine genera. According to this classification, the mental gland of *B. nicefori* is within the first type which characterizes the genus.

In plethodontid salamanders with round or oval mental gland and dorso-ventrally oriented glands, there are a variable number of these glands (Table 2); in relation to the species in the Table 2, active males of *B. nicefori* present a mental gland of intermediate size, the number of the simple tubular glands is major possibly due to the fact that they present a non-uniform diameter and they are randomly arranged (Fig. 4A); additionally, the glands that compound the cluster of the mental gland have a different shape. *P. glutinosus* and *P. jordani* have cylindrical glands whereas in *Eurycea*, these glands are tubular or elliptical (Sever 1976b). The difference in size, number, and shape of simple glands in *B. nicefori* probably is due to their different diameters (Fig. 4A). These features were observed by Sever (1976b) in genera as *Thorius*, *Pseudoeurycea*, and some *Bolitoglossa* which present glands with a markedly non-uniform diameter.

The characteristic spindle-shaped or fusiform form of the ducts of tubular glands in the mental gland of *B. nicefori* is similar to that described by Testa *et al*. (1993) for *Hydromantes genei*, an European Plethodontidae (Sardinia, Italy), stating that presents an appearance of elliptical stomata which is lined by one or two cell. Other studies on the morphology of the mental gland only show longitudinal sections, so little is know regarding to the form and cell position of these ducts.

Although the mental gland has been described as a prominent feature in reproductive males (Sever 1976b) that hypertrophy only during breeding season (Rollmann *et al*. 2003), in *B. nicefori* it is evident in all adult males over the year without statistically different between diameter and height of the tubular gland in active and inactive males. Mental gland development would be a response to the secretion of testosterone in the active seminiferous tubules of *B. nicefori* as was suggested in *Eurycea quadridigitata* in which testosterone aids in the maintenance of sperm in vas deferens; and in turn, males show little seasonal variation in the presence and hypertrophy of the mental gland (Sever 1975). Sever (1976a) injected females of *E. quadridigitata* with testosterone solution and the chin area was stimulated to hypertrophy into a cluster equivalent to the mental glands, with abundant secretory product, similar in appearance and staining characteristic of males. The opposite case appears in *E. bislineata*, in this species the reproductive system of male is fully developed months before the breeding season; the mental gland begins to hypertrophy at the time that spermatids are being formed in the testes, but it begins to secrete actively only during the breeding season. It regresses almost completely outside the breeding season increasing the difficulty of distinguishing between sexes (Weichert 1945). According to Sever (1976b), there is a correlation between seasonal variation of the mental gland and release of hormones responsible for maintaining other secondary sexual characters. In *B. nicefori*, testes volume increase in the breeding season (Ortega *et al.* 2009), which allow to relate the hypertrophy of the mental gland and testicular activity during the breeding season.

There is insufficient information about the type of secretion of the glandular cells in the mental glands. *Taricha torosa* (Salamandridae) presents a holocrine secretory process as the glandular epithelium is destroyed during elaboration and expulsion of the small basophilic granules (Smith 1941). Different to the holocrine secretion described for *T. torosa*, the type of secretion observed in *B. nicefori* is merocrine and the eosinophilic secretory granules were observed filling the apex of all glandular cells in adult males from both breeding and non breeding seasons.

Negative staining reaction for PAS-AB, is consistent with Rollmann *et al*. (1999, 2000) results for the glandular components of the mental gland from *Plethodon* that includes protein components as PRF (plethodontid receptivity factor) and multiple isoforms of PRF. Additionally, in Plethodontidae there are other protein components as PMF (plethodontid modulating factor) (Houck *et al*. 2007) and SPF (sodefrin precursor-like factor) (Palmer *et al*. 2007). PRF and PMF stimulate the female olfactory system (Wirsig-Wiechmann *et al*. 2002, 2006), and produce differential effects on female behavior (Houck *et al.* 2007). In *Plethodon*, PRF increases female receptivity (Rollmann *et al*. 1999) and

PMF produces an opposite effect, decreasing female receptivity (Wirsig-Wiechmann *et al*. 2006; Houck *et al*, 2007). The combined effect between PRF and PMF interaction increase female receptivity, reducing mating time (Wirsig-Wiechmann *et al*. 2006). These two proteins may produce both sedative (PMF) and stimulatory (PRF) effects, facilitating courtship (Wirsig-Wiechmann *et al*. 2006). SPF is an ancestral pheromone in plethodontids, and its expression acts as courtship pheromones, increasing female receptivity (Palmer *et al*. 2007). Finally, PRF and SPF interaction in female mating behavior is unknown (Palmer *et al*. 2007). In males, variation among isoforms and chemical composition may vary the functional differences and their ability to stimulate females (Rollmann *et al*. 2000). It is possible that a seasonal variation exists in the chemical composition of the secreting products of the mental gland of *B. nicefori* allowing courtship of reproductive females during the breeding season.

Although mental gland development seems to be a secondary sexual feature related to hormonal control during the reproductive season, variations in the size of the gland (height, diameter of the simple tubular glands and diameter of secretory granules) between males from breeding and non-breeding seasons in *B. nicefori* indicate that although the males are reproductive the whole year and their mental glands are active continuously, their hypertrophy is maximal during the reproductive season when females are receptive to mating. Some unidentified factor additionally would stimulate the activity of this gland in order to synchronize its secretion with the time of female receptivity. Subsequent studies may determine the content of pheromones in the mental gland of *B. nicefori*, and their interactions related to the reproductive activity of the females.

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LITERATURE CITED

Dodd, C. K. and Brodie, E. D. 1976. Observations on the Mental Hedonic Gland-cluster of Eastern Salamanders of the Genus *Plethodon*. *Chesapeake Science* **17**: 129-131.

Houck, L. D. and Arnold, S. J. 2003. Courtship and Mating Behavior. In Sever, D. M. (Ed.): *Reproductive Biology and Phylogeny of Urodela* , pp. 383- 424, Science Publisher, Enfield, NH.

Houck, L. D., Palmer, C. A., Watts, R. A., Arnold, S. J., Feldhoff, P. W. and Feldhoff, R. C. 2007. A New Vertebrate Courtship Pheromone, PMF, Affects Female Receptivity in a Terrestrial Salamander. *Animal Behaviour* **73**: 315-320.

Noble, G. K. 1927. The Plethodontid Salamanders; Some Aspects of Their Evolution. *American Museum Novitates* **249**: 1-26.

Noble; G. K. 1929. The Relation of Courtship to the Secondary Sexual Characters of the Two-Lined Salamander, *Eurycea bislineata* (Green). *American Museum Novitates* **362**: 1-5.

Ortega, J. E., Monares, J. M., & Ramírez-Pinilla, M. P. 2009. Reproductive Activity, Diet and Microhabitat use in *Bolitoglossa nicefori* (Caudata: Plethodontidae). *Journal of Herpetology* **43**: in press.

Palmer, C. A., Watts, R. A., Houck, L. D., Picard, A. L. and Arnold, S. J. 2007. Evolutionary Replacement of Components in a Salamander Pheromone Signaling Complex: More Evidence for Phenotypic-Molecular Decoupling. *Evolution* **61**: 202-215.

Rajchard, J. 2005. Sex Pheromones in Amphibians: A Review. *Veterinary Medicine-Czech* **50**: 385-389.

Ramírez-Pinilla, M. P., Osorno-Muñoz, M., Rueda, J. V., Amézquita, A., Ardila-Robayo, M. C. 2004. *Bolitoglossa nicefori*. IUCN, Conservation International, and NatureServe. 2006. Global Amphibian Assessment. <www.globalamphibians.org>. Downloaded on 30 May 2008.

Rollmann, S. M., Houck, L. D. and Feldhoff, R. C. 1999. Proteinaceous Pheromone Affecting Female Receptivity in a Terrestrial Salamander. *Science* **285**: 1907-1909.

Rollmann, S. M., Houck, L. D. and Feldhoff, R. C. 2000. Population Variation in Salamander Courtship Pheromones. *Journal of Chemical Ecology* **26**: 2713-2724.

Rollmann, S. M., Houck, L. D. and Feldhoff, R. C. 2003. Conespecific and Heterospecific Pheromone Effects on Female Receptivity. *Animal Behaviour* **66**: 857-861.

Sever, D. M. 1975. Morphology and Seasonal Variation of the Mental Hedonic Glands of the Dwarf Salamander, *Eurycea quadridigitata* (Holbrook). *Herpetologica* **31**: 241-251.

Sever, D. M. 1976a. Induction of Secondary Sexual Characters in *Eurycea quadridigitata*. *Copeia* **1976**: 830-833.

Sever, D. M. 1976b. Morphology of the Mental Hedonic Gland Clusters of Plethodontid Salamanders (Amphibia, Urodela, Plethodontidae). *Journal of Herpetology* **10**: 227-239.

Sever, D. M. 2003. Courtship and Mating Glands. In Sever, D. M. (Ed.): *Reproductive Biology and Phylogeny of Urodela*, pp. 232-381, Science Publisher, Enfield, NH.

Smith, R. E. 1941. Mating Behavior in *Triturus torosus* an Related Newts. *Copeia* **1941**: 255-262.

Testa, R. F., Serra, G. P., Loffredo, F. and Riva, A. 1993. Ultrastructural Study of the Mental Body of *Hydromantes genei* (Amphibia: Plethodontidae). *Journal of Morphology* **217**: 75-86.

Valentine, B. D. 1963. The Mental Gland of the Salamander *Desmognathus wrighti* King. *The Ohio Journal of Science* **63**: 25-26.

Weichert, C. K. 1945. Seasonal Variation in the Mental Gland and Reproductive Organs of the Male *Eurycea bislineata*. *Copeia* **1945**: 78-84.

Wirsig-Wiechmann, C. R., Houck, L. D., Feldhoff, P. W. and Feldhoff, R. C. 2002. Pheromonal Activation of Vomeronasal Neurons in Plethodontid Salamanders. *Brain Research* **952**: 335-344.

Wirsig-Wiechmann, C. R., Houck, L. D., Wood, J. M., Feldhoff, P. W. and Feldhoff, R. C. 2006. Male Pheromone Protein Components Activate Female Vomeronasal Neurons in the Salamander *Plethodon shermani*. *BMC Neuroscience*. 2006; **7**: 26.

FIGURE LEGENDS

Figure 1 — Pad-shaped mental gland of adult male of *Bolitoglossa nicefori*. —**A–B.** Note the oval pad-shape in its outer and internal side, respectively. In B, it is possible to observe the presence of melanocytes on the top of the gland (arrowhead). Ep, epidermis; Mg, mental gland. Scale bar = 2 mm for A; 1 mm for B.

Figure 2 — Sagittal histological sections of the mental gland in active male of *Bolitoglossa nicefori*. Sections stained with hematoxylin-eosin (H-E). —**A**. Note the transition between the tubular glands of the mental gland and the typical skin glands surrounding the mental gland. —**B**. The tubular glands that constitute the mental gland open in ducts at the bottom of the lower jaw (arrowhead). —**C**. Detail of B in which it is observed the ducts that opens in the submandibular region (arrowhead) through the epidermis; further connective tissue that surrounds each gland (arrow) and melanocytes located between the tubular glands and the epidermis. Cts, connective tissue; Ep, epidermis; Tg, tubular gland. Scale bar = 100 µm for A and B; 50 µm for C.

Figure 3 — Sagittal histological section of the submandibular region of a juvenile male of *Bolitoglossa nicefori* in which only granular glands are observed. Sections stained with hematoxylin-eosin (H-E). Melanocytes (arrow). Cts, connective tissue; Ep, epidermis; Gg, granular gland. Scale bar = 40 µm.

Figure 4 — Transverse sections of the mental gland of an adult male of *Bolitoglossa nicefori* from non-breeding season. Sections stained with hematoxylin-eosin (H-E). —**A**. Tubular glands (Tg) that compose the mental gland are observed. It is possible to observe the merocrine asynchronous secretion of eosinophilic secretory granules. Some tubular glands present a lumen devoid of secretion and the glandular cells already have discharged their secretory granules (arrowhead), and in others, the lumen is still obliterated (Asterisk). —**B**. Detail of the region where are observed the ducts (arrowhead) of the simple tubular glands. Note the special form of the ducts (arrowhead) and the particular disposition of the cells that surround the duct. Tg, tubular gland. Scale bar = 100 µm for A; 20 µm for B.

Figure 5 — Spermiogenesis in adult male of *Bolitoglossa nicefori*. Sections stained with hematoxylin-eosin (H-E). —**A**. Testicular regions of spermatogenic activity in the cephalo-caudal axis. Testicular lobules with numerous cysts in different stages of spermiogenesis. —**B**. Detail of the testicular region in which are observed late spermatids (Et) forming thick bundles. Also spermatozoa (z) are observed. —**C**. Cyst with primary spermatocytes (Ps) in anaphase I of meiosis. Also are shown secondary spermatocytes (Ss). The lobules are separated by walls of thin connective tissue (arrowhead). Es, early spermatids. Scale bar = 100 µm for A; 20 µm for B and C.

Figure 6 — Male excurrent ducts in adult male of *Bolitoglossa nicefori*. Sections stained with hematoxylin-eosin (H-E). Transversal ducts show a cuboidal epithelium and the lumen (L) contains abundant spermatozoa

(z). The connective tissue contains melanocytes (arrowhead). Scale bar = 100 µm.

TABLE 1. Measurements of different parameters of the mental glands: tubular gland diameter, tubular gland height, and granules diameter in adult males of *Bolitoglossa nicefori* in different reproductive status. Males of *B. nicefori* are reproductive through the year and they were separated in two groups: active males when they were collected during the breeding season (November-February), and inactive males when they were collected outside of the breeding season (March-September).

	N	**Tubular gland diameter**			**Tubular gland height**			**Granules diameter**		
		Range	**Mean**	**SD**	**Range**	**Mean**	**SD**	**Range**	**Mean**	**SD**
Active males	6	121.2-152.4	137.5	12.48	225.6-497.5	353.7	96.12	2.3-5.3	4.1	0.94
Inactive males	5	123.3-147	135.6	9.43	213.8-375	295.3	56.98	2.2-3.9	3	0.72

TABLE 2. Comparative dimensions of the mental glands (length and width) and their individual glands (height and diameter) of Plethodontidae with dorso-ventrally oriented glands. Males of *Bolitoglossa nicefori* were separated in two groups since are reproductive through the year: active males (*) collected during the breeding season (November-February), and inactive males (**) collected outside of the breeding season (March-September).

Species	**Mental gland**		**Individual glands**		**Number of**	
	Length (mm)	**Width (mm)**	**Height (µm)**	**Diameter (µm)**	**Simple glands**	
Aneides aeneus	3.88	4.63		50-90		Sever 1976b
Plethodon jordani	3.73	4.29	566.4-613.6	106.2-200.6	143-251	Sever 1976b
*Bolitoglossa nicefori**	2.82	3.94	380-520	125-187	318-710	This study
Plethodon glutinosus	2.94	3.54	212.4-413.0	82.6-177.0	330-415	Sever 1976b
*Bolitoglossa nicefori***	1.82	3.58	250-400	130-160	255-386	This study
Typlotriton spelaeus	1.80	1.80		156.6-221.8	34	Sever 1976b
Hydromantes genei			~350	~120	~200	Testa *et al.* 1993
Hemidactylium scutatum				86.1-148.8		Sever 1976b

FIGURES

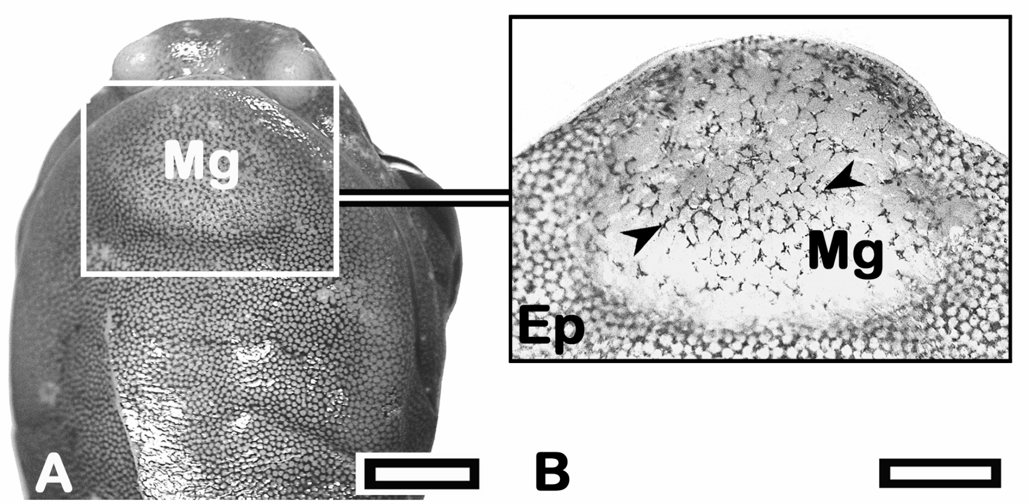
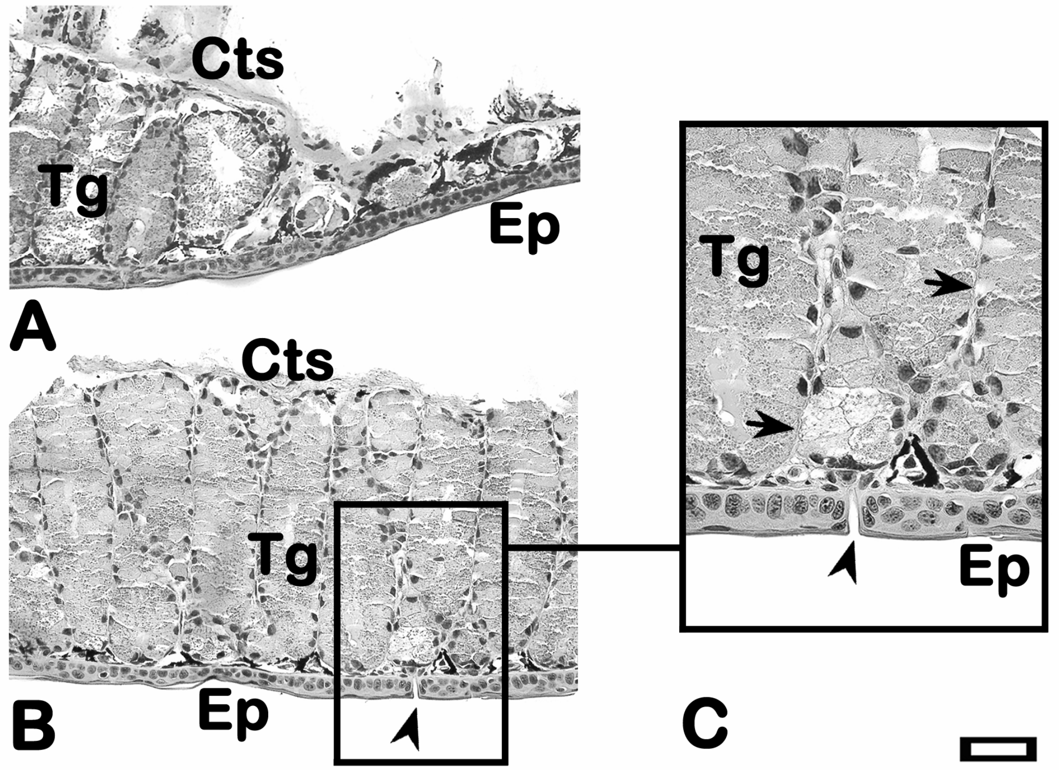


FIGURE 1.



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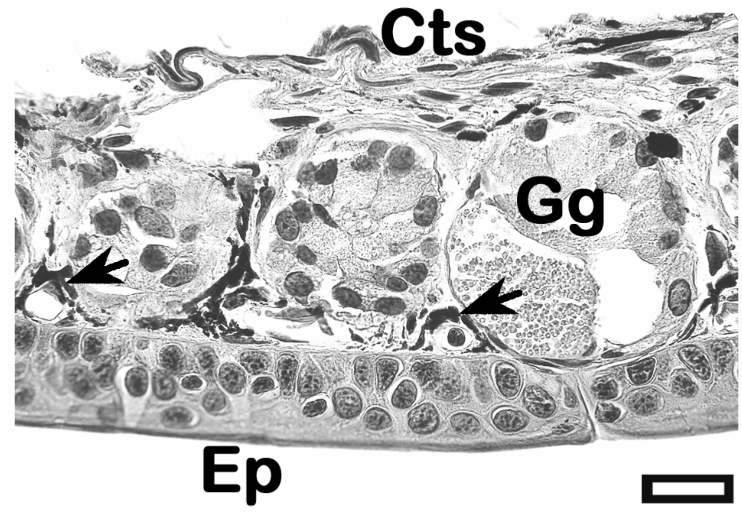

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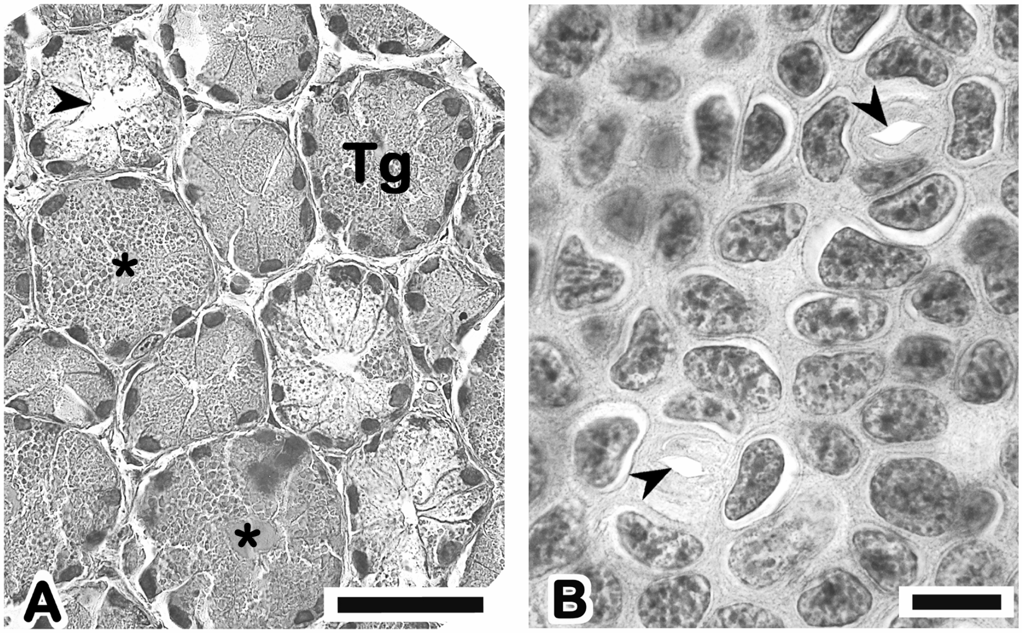

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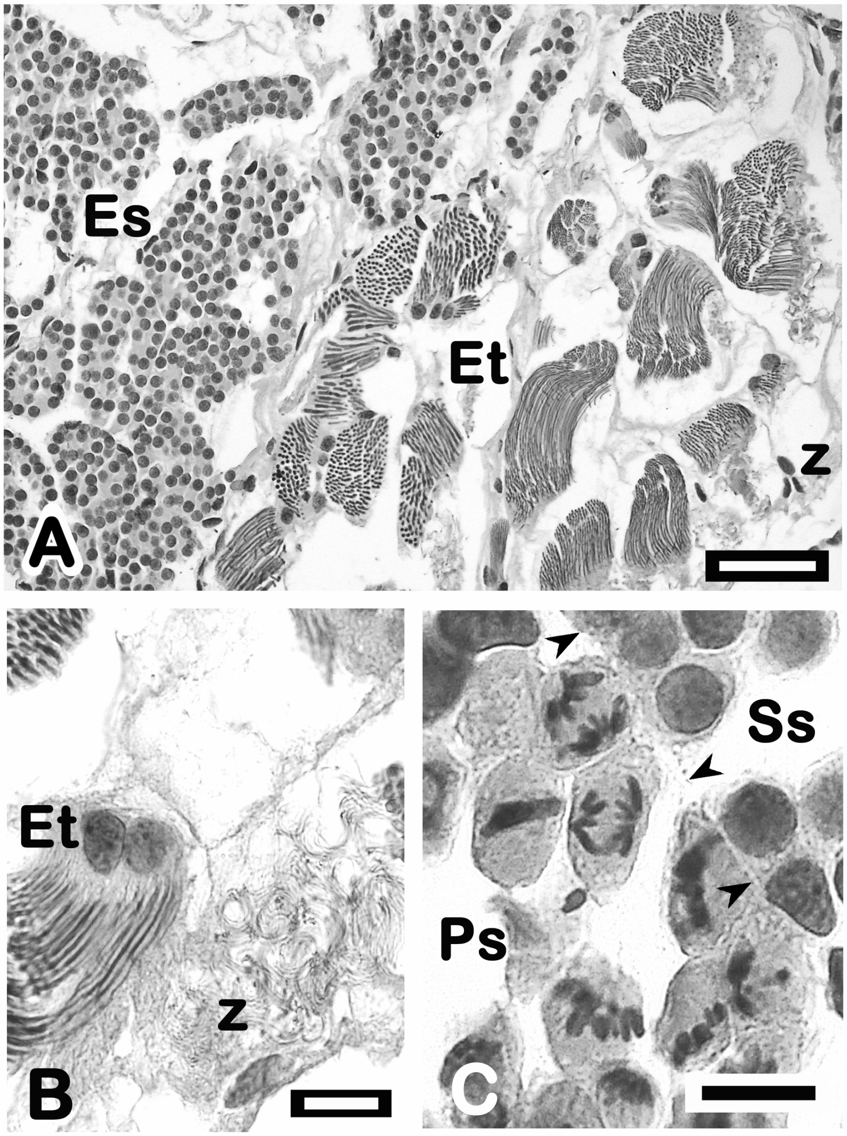

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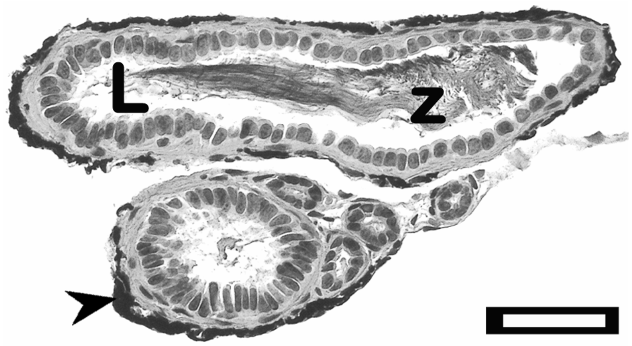

FIGURE 6.