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Ultrastructure and Histochemistry of the Adhesive Breeding Glands in Male *Gastrophryne carolinensis* (Amphibia: Anura: Microhylidae)

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The histology, histochemistry, and ultrastructure of the adhesive breeding glands of male *Gastrophryne carolinensis* are described. Adhesive glands are mutlicellular exocrine glands in the dermis of the sternum and forearm that cause the male to adhere to the female during amplexus. The epithelial cells have distinct plasma membranes, and the product consists of electron-dense secretory granules that fill the cytoplasm and are released intact by an apocrine process. We support one previous study and contradict another report by finding that adhesive glands react positively for neutral carbohydrates and negatively for glycosaminoglycans and proteins. The ultrastructural results, the first on these organs, confirm that adhesive glands are derived from mucous glands and not serous glands.

THE presence of multicellular mucous and serous (granular) exocrine glands in the dermis of metamorphosed skin is a synapomorphy for extant Amphibia (Houck and Sever, 1994). In addition, many amphibians have lipid glands and mixed mucous–serous glands in the dermis, plus specialized glands representing modified mucous or serous glands (Brizzi et al., 2003). These specialized glands include poison glands used in defense and breeding glands used in social communication and reproduction (Thomas et al., 1993; Brizzi et al., 2003; Sever, 2003).

Among the more unique types of breeding glands are adhesive glands in the pectoral region of certain male frogs that cause the venter of the male to adhere to the skin of the dorsum of the female during amplexus (Fig. 1A). Such glands were first reported in the Microhylidae for *Kaloula conjuncta* from the Phillipines by Taylor (1920), who stated that males adhere to females by virture of a slimy secretion from the belly. Inger (1954) later reported ''belly glands'' from *K. picta* and *K. rigida*, and noted that they were absent in other members of the genus. Fitch (1956) described adhesion during amplexus of the North American microhylid *Gastrophryne olivacea*, and subsequently, Conaway and Metter (1967) described adhesive glands in *G. carolinensis*. Adhesive glands are also known from male *Breviceps* in the South African family Brevicipitidae (Poynton, 1964; Visser et al., 1982). The adhesion of bisexual pairs during amplexus in these species has been proposed to aid in reproduction by one of the following mechanisms: 1) protecting a female's backside from a rival male (Fitch, 1956), 2) keeping pairs together in case of a mating disturbance (Fitch, 1956), 3) helping a male with short arms stay amplexed to his potential mate (Wager, 1965; Conaway and Metter, 1967), or 4) causing strong adherence for burrowing into a nesting chamber (Visser et al., 1982).

Detailed descriptions of adhesive glands are limited to light microscopy and histochemical analysis in only three species, *Gastrophryne carolinensis*, *G. olivacea* (Conaway and Metter, 1967; Metter and Conaway, 1969; Holloway and Dapson, 1971), and *Breviceps gibbosus* (Visser et al., 1982), with conflicting histochemical results reported by Conaway and Metter (1967; describe a protein secretion) and Holloway and Dapson (1971; describe a mucous secretion) for *Gastrophryne*. Conaway and Metter (1967) provide evidence for an apocrine mode of secretions in adhesive glands and along with Holloway and Dapson (1971), hypothesize that these glands derive from mucous glands. In this study, we confirm and extend previous observations on the light microscopy and histochemistry of the adhesive glands of *G. carolinensis*. We also present the first ultrastructural description of adhesive glands and compare the fine structure of these glands to mucous and serous glands.

MATERIALS AND METHODS

Specimens.—A pair of *Gastrophryne carolinensis* in amplexus was captured on 22 August 2006 in Ponchatoula, Tangipahoa Parish, Louisiana and sacrificed by means of placement in MS-222 solution (protocol approved by the Institutional Animal Care and Use Committee of Southeastern Louisiana University). The amplexed pair was subsequently submerged in Trump's fixative (1:1 2.5% glutaraldehyde:3.7% formaldehyde; in cacodylate buffer at pH 7.2) for 48 hrs. Skin from the venter of the amplexed male was removed and divided into two halves at the midline. The left section was used for light microscopy, whereas the right section was used for electron microscopy. Adobe Photoshop 7.0 (Adobe Systems, Inc., San Jose, CA) was used for editing and printing of micrographs.

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Light microscopy.—Tissues from the left half of the venter were rinsed for 1 hr in tap water and dehydrated in a graded series of ethanol (70%, 95%, for 1 hr each, 100%, two cycles for 30 min each). Tissues were then placed in toluene (two cycles for 30 min each) and subsequently embedded in paraffin blocks for sectioning with a MR3 (Research and Manufacturing Co., Tucson, AZ) microtome. Sections 10 micrometers (μm) thick were cut and affixed to albumenized slides. Alternate slides were stained with hematoxylin-eosin (H&E) for general histological examination, alcian blue 8GX (AB) at pH 2.5 for carboxylated glycosaminoglycans and periodic acid-Schiff's (PAS) for neutral carbohydrates or bromophenol blue (BB) for proteins. All histological techniques followed Kiernan (1990). Slides were viewed under a Leica DM2000 (Leica Microsystems, Wetzlar, Germany) microscope, and images were obtained via a Leica DFC420 (Leica Microsystems, Wetzlar, Germany) digital camera.

Electron microscopy.—Tissues from the right half of the venter were rinsed in deionized water and then post-fixed for 90 min in 2% osmium tetroxide. Tissues were subsequently rinsed in deionized water, dehydrated with a graded series of ethanol (70%, 95%, 100%, for 1 hr each), soaked 30 min each in 1:1 100% ethanol:propylene oxide followed by pure propylene oxide, and embedded in an epoxy resin (EmBed 812, Electron Microscopy Sciences, Fort Washington, PA). Ultra-thin sections, at 70 nm, were achieved by use of DiATOME diamond knives (DiATOME, Biel, Switzerland) on a RMC MT7 (Research and Manufacturing Co., Tucson, AZ) ultramicrotome. Ultra-thin sections were placed on copper grids and stained with uranyl acetate and lead citrate. Grids were viewed and photographed with a JEOL JEM 100S (JEOL USA, Peabody, MA) transmission electron microscope.

RESULTS

Histology and histochemistry.—Using Conaway and Metter (1967) as a guide, adhesive glands were located in the sternal region of *Gastrophryne carolinensis* and at the base of the inner arm (Fig. 1B). In these regions, adhesive glands are more numerous and histologically distinct from typical serous and mucous glands. Adhesive glands are composed of a simple cuboidal epithelium surrounding a large luminal area that is connected to the surface of the epidermis by a small duct (Fig. 1C). The epithelium of the breeding glands is strongly eosinophilic, intensely PAS+, AB−, and BB− (Table 1). Eosinophilic and PAS+ secretory material can be observed in the lumen of these glands and on the surface of the epidermis during amplexus (Fig. 1C). Mucous glands appear much smaller than adhesive glands and exhibit pyrimoidal secretory cells surrounding an empty lumen (Fig. 1C). The epithelium of the mucous glands is basophilic, PAS+, AB+, and BB− (Table 1). The serous glands lack an obvious lumen and contain their products in a syncytium (Fig. 1C). This syncytium is eosinophilic, intensely BB+, PAS+, and AB−.

Ultrastructure.—The three distinct gland types were identified for ultrastructural analysis from comparison with the histological results above. The substance produced in the adhesive glands is electron dense and contained in tightly packed secretory granules with small mitochondria interspersed (Fig. 1D). These granules dominate the cytoplasm of the cuboidal epithelial cells during amplexus and because of their high concentration, exhibit the polyhedral-like shape common in tightly packed clusters (Brizzi et al., 2003; Fig. 1D,F). Myoepithelial cells surround the periphery of these glands (Fig. 1D). Rough endoplasmic reticulum is abundant in the basal portion of the epithelial cells surrounding irregularly shaped, heterochromatic nuclei (Fig. 1E). Microvilli are abundant on the luminal border of the epithelium (Fig. 1E). Intercellular canaliculi are narrow. Desmosomes can be observed basally connecting epithelia (Fig. 1E), but apical junctions appear to be lost during amplexus.

Our ultrastructural analysis demonstrates an apocrine mode of secretion in adhesive breeding glands. The secretory granules dissociate from the epithelium intact, surrounded by cytoplasmic material, including microvilli (Fig. 1F). Individual granules then combine to form a uniform secretory material that dominates the lumen during amplexus (Fig. 1F). Secretory material is highly concentrated on the surface of the epidermis upon which the ducts leading from the adhesive glands open (Fig. 1F insert).

Serous glands differ greatly in ultrastructure from adhesive breeding glands. These glands contain secretory granules of varying electron densities in a syncytium (e.g., no discernable membranes are observed between cells that make up the serous glands; Fig. 2A). Heterochromatic nuclei can be observed around the periphery of the syncytium with myoepithelial cells surrounding the serous glands (Fig. 2A). Serous syncitia possess dense cytoplasms that make it difficult to observe the cytoplasmic contents of the syncytium (Fig. 2A).

In contrast to serous glands, mucous glands are ultrastructurally similar to adhesive glands. Electron-dense secretory granules fill the pyramidal epithelium (Fig. 2B). Heterochromatic nuclei are basally to centrally located, depending on secretory phase, and myoepithelial cells envelop the mucous glands (Fig. 2B). Intercellular space is slightly increased, while microvilli decrease in number compared to that of adhesive breeding glands (Fig. 2C). Rough endoplasmic reticulum (Fig. 2D) and mitochondria (Fig. 2C) are abundant in the epithelium, while desmosomes (Fig. 2C) can be observed joining the epithelial cells together apically. The major differences between the mucous glands and the adhesive breeding glands are the smaller size of the mucous glands compared to that of the adhesive breeding glands and a merocrine mode of secretion in the mucous glands in contrast to an apocrine mode in the adhesive breeding glands.

DISCUSSION

Metter and Conaway (1969) studied the development of adhesive glands in *Gastrophryne carolinensis* following treatment of juvenile males and females with testosterone and regression of adhesive glands in castrated males. Due to the similarities in an undeveloped or regressed condition, they proposed that adhesive breeding glands are derived mucous glands, a hypothesis supported by the histochemical results of Holloway and Dapson (1971). The histochemical and ultrastructural results presented here further support this hypothesis. These distinctive glands produce a neutral carbohydrate material and contain all the cellular machinery of a derived mucocyte, including polyhedral secretory

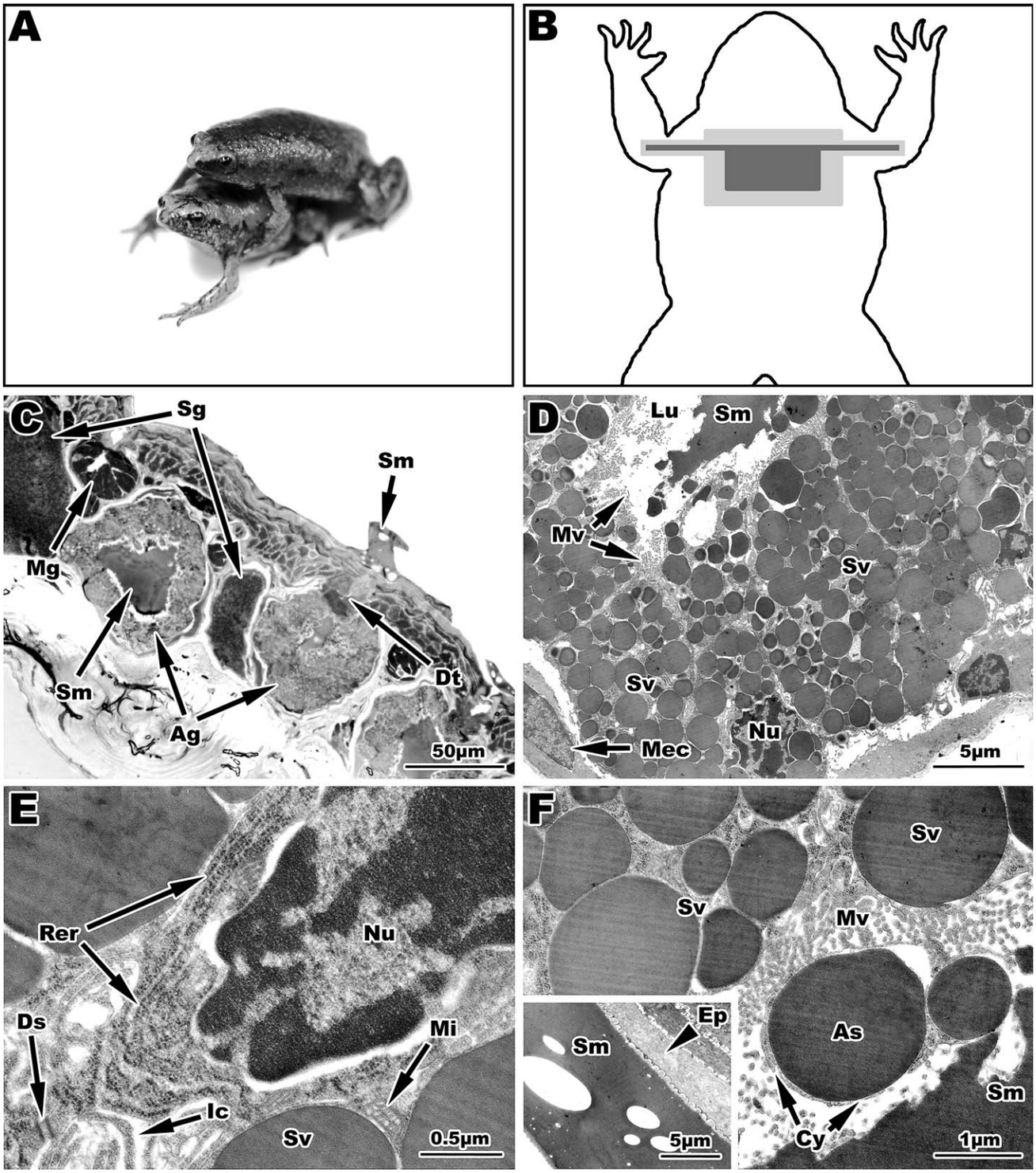

Fig. 1. (A) *Gastrophryne carolinensis* exhibiting axillary type amplexus. (B) Cartoon of the venter of *G. carolinensis* (dark gray is an area including almost entirely adhesive glands and mucous glands; light gray area is mixed with adhesive glands, mucous glands, and serous glands; unshaded areas contain mainly serous and mucous glands). (C) Light micrograph of light gray area in (B) showing serous and adhesive glands; scale bar = 50 μm. (D) Electron micrograph of (C) demonstrating ultrastructure of the adhesive breeding glands; scale bar = 5 μm. (E) Higher magnification of (D) exhibiting cytoplasmic components of the epithelial cells; scale bar = 0.5 μm. (F) Higher magnification of (D) showing the apocrine type secretion utilized by the adhesive breeding glands; scale bar = 5 μm; Insert, accumulation of secretory material on the epidermis; scale bar = 1 μm. Ag, adhesive gland; As, granule released through an apocrine mechanism; Cy, cytoplasm; Ds, desmosome; Dt, duct; Ep, epidermis; Ic, intercellular canaliculi; Lu, lumen; Mec, myoepithelial cell; Mg, mucous gland; Mi, mitochondria; Mv, microvilli; Nu, nucleus; Rer, rough endoplasmic reticulum; Sg, serous gland; Sm, secretory material; Sv, secretory granules.

Table 1. Histochemistry of the Epithelium in *G. carolinensis* Skin Glands. (−)–no reactivity, (+)–reactivity, (++)–intense reactivity; see materials and methods for abbreviations.

	Stain			
Gland type	H&E	BB	PAS	AB
Serous	Eosinophilic	++	+	−
Mucous	Basophilic	−	++	+
Adhesive	Eosinophilic	−	++	−

granules, abundant rough endoplasmic reticulum, and stacked Golgi complexes (Brizzi et al., 2003). However, Conaway and Metter (1967) reported that the adhesive glands secrete a protein rather than a mucoid substance. Holloway and Dapson (1971) questioned the histochemical analysis of Conaway and Metter (1967) and recorded results similar to ours. The release of intact secretory granules with cytoplasm, and the absence of apical junctions, supports the hypothesis of Conaway and Metter (1967) that adhesive glands utilize an apocrine mode of secretion for product release.

The adhesive glands in the microhylid genus *Kaloula* may differ significantly from those in *Gastrophryne*. In *Kaloula*, Inger (1954) describes adhesive glands as single celled epidermal aggregations on the ventors that vary in distribution depending on the species. Inger (1954) notes that females of *K. conjuncta* may also be involved in adherence, as they have well-developed mucous glands dorsally. No histological or ultrastructural work has been done on the adhesive glands of *Kaloula*.

Because Brevicipitidae and Microhylidae do not form a monophyletic clade (Frost et al., 2006), along with the fact that adhesive glands in other anurans have not been reported (including all other members of the Brevicipitidae and Microhylidae not discussed here), adhesive glands in these two taxa might have evolved independently. Although Visser et al. (1982) believed the adhesive glands in *Breviceps gibbosus* resembled those of *Gastrophryne*, the mechanism by which adhesion occurs is quite different and involves secretions from both sexes, as suggested by

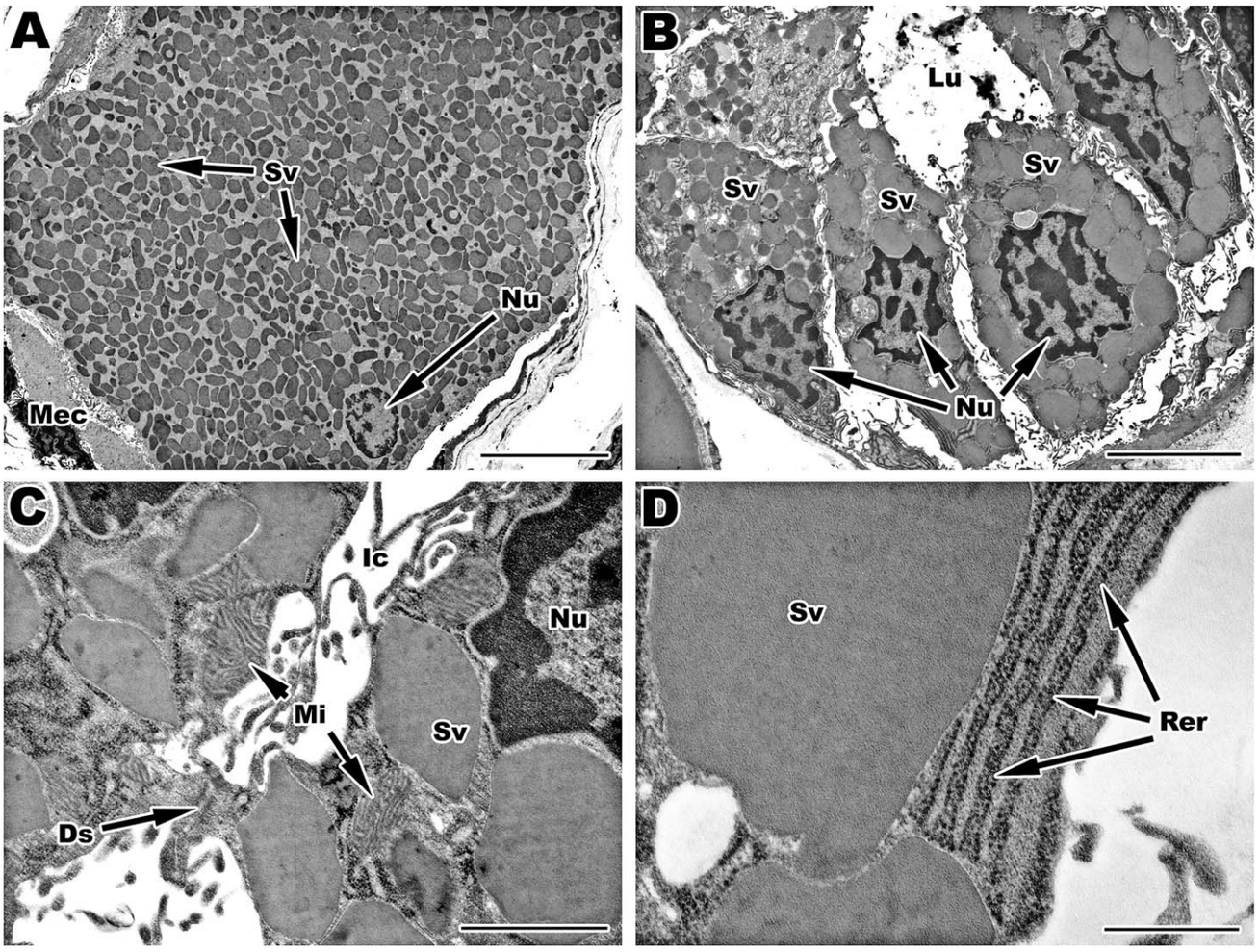

Fig. 2. (A) Overview of the ultrastructure of a serous gland in the sternal region of *Gastrophryne carolinensis*; scale bar = 10 μm. (B) Overview of the ultrastructure of a typical mucous gland in the skin of *G. carolinensis*; scale bar = 5 μm. (C) Higher magnification of (B) showing the cytoplasmic contents in the epithelium of a mucous gland and lateral surface relationships between mucocytes; scale bar = 1 μm. (D) Higher magnification of (B) focusing in on abundant rough endoplasmic reticulum in mucous glands; scale bar = 0.5 μm. Ds, desmosome; Ic, intercellular canaliculi; Lu, lumen; Mec, myoepithelial cell; Mi, mitochondria; Nu, nucleus; Rer, rough endoplasmic reticulum; Sv, secretory granules.

Inger (1954) for *Kaloula conjuncta*. Adhesive glands seem less abundant on the sternum of the male than on the dorsum of the female of *B. gibbosus*, and Visser et al. (1982) proposed that adhesion is caused by the mixing of adhesive substances from the female with serous secretions from the male. Thus, the female has evolved the role as the ''sticky'' partner. However, in another species, a *B. adspersus* male was discovered stuck to the back of a non-conspecific, *Tomopterna delalandei*, which is not known to glue during amplexus (Jurgens, 1978). Thus, the sex responsible for adhesive secretions could be variable within a genus. Histological and ultrastructural observations on *Kaloula* and ultrastructural work on *Breviceps* is desired. We also encourage the search for adhesive glands in other species within the Microhylidae and Brevicipitidae, and indeed, among other groups of anurans whose reproductive cycles are unknown.

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