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## Correspondence

# The clutch structure of *Pleurodema tucumanum* (Anura: Leptodactylidae)

Daiana P. Ferraro[1,2], María E. Pereyra[2], Diego Baldo[3] & Julián Faivovich[2,4]

[1)] Instituto Nacional de Limnología (INALI-CONICET), Paraje el Pozo S/Nº, S3000ACT, Santa Fe, Argentina

[2)] División Herpetología, Museo Argentino de Ciencias Naturales "Bernardino Rivadavia"-CONICET, Ángel Gallardo 470, C1405DJR, Buenos Aires, Argentina

[3)] Laboratorio de Genética Evolutiva, Instituto de Biología Subtropical (CONICET-UNaM), Facultad de Ciencias Exactas Químicas y Naturales, Universidad Nacional de Misiones, Félix de Azara 1552, N3300LQF, Posadas, Argentina

[4)] Departamento de Biodiversidad y Biología Experimental, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Buenos Aires, Argentina

Corresponding author: Daiana ferraro, e-mail: dferraro@macn.gov.ar

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Anurans have a vast diversity of reproductive modes (Salthe & Duellman 1973). Foam nests are a particularly peculiar mode of oviposition and have evolved independently in several anuran lineages (see Faivovich et al. 2012 for summary and discussion). During amplexus, females release ova and a proteinaceous fluid, while males release sperm, and either one (female or male) or both parents employ a beating behaviour to trap air bubbles in the fluid (Heyer & Rand 1977). The foam is a mixture of proteins produced in the oviduct, and when coupled with beating action to incorporate air it will result in a more or less stable foam nest structure. This structure is difficult to maintain indefinitely and has a tendency to collapse over time unless stabilized mechanically or kinetically by additional processes. As proteins are potentially susceptible to surface effects, those within the foam may often become denatured (Cooper & Kennedy 2010). The stability of foam nests has been correlated to surfactant proteins that were discovered within the foam (Cooper et al. 2005, Hissa et al. 2008, Fleming et al. 2009, Cooper & Kennedy 2010).

Anatomically, the anuran oviduct is composed of four sections (ostium, pars recta, pars convoluta, and ovisac), and in females of foam-nesting species, the terminal portion of the pars convoluta is enlarged into what has been termed pars convoluta dilata by Furness et al. (2010). This portion has been associated with the secretion of foam precursors (Bhaduri 1953). In the Neotropical anuran clade composed by the genera *Edalorhina*, *Engystomops*, *Physalaemus*, and *Pleurodema* (Leiuperinae, Leptodactylidae), the ancestral oviposition mode is a foam nest floating on water (Grant et al. 2006, Pyron & Wiens 2011, Faivovich et al. 2012). Interestingly, while the clade *Physalaemus* + *Engystomops* + *Edalorhina* includes more than 50 foam-nesting species, its sister taxon, the genus *Pleurodema*, includes only 15 species with four known oviposition modes (Faivovich et al. 2012).

*Pleurodema tucumanum* Parker, 1927 is a medium-sized frog that inhabits mainly the Chacoan region of Argentina, and marginally the Monte region (Ferraro & Casagranda 2009). While *P. tucumanum* was historically considered a foam-nesting species (Cei 1956, 1980, di Tada et al. 1976, Duellman & Veloso 1977, Perotti 1994, 1997), Martori et al. (1994) described its clutch as gelatinous, ovoid plate-like masses, in which eggs are arranged along a single plane. In the phylogenetic analysis of *Pleurodema* conducted by Faivovich et al. (2012), *P. tucumanum* is nested in the *Pleurodema brachyops* clade, a group of foam-nesting species. Consequently, Faivovich et al. (2012) hypothesized that the egg-clutch structure in *P. tucumanum* could be explained by the evolutionary loss of beating behaviour in the male.

In this note, we describe the clutch structure of *P. tucumanum* based on lab and field observations, and provide notes on the oviducts based on histological sections. We then discuss the hypothesis proposed by Faivovich et al. (2012) on the basis of our results.

Field observations and specimens were collected from three temporary ponds in Argentina: Provincial Road Nº 5, Junín Department, San Luis Province (32°18'34.6" S, 65°16'32.0" W, 623 m a.s.l., on 13–14 January 2013); Totora

Pampa, National Road Nº 9 and access to Villa Atamisqui, Atamisqui Departament, Santiago del Estero Province (28º27'48" S, 64º08'44" W, 131 m a.s.l., on 22–23 February 2009); and Río San Ignacio, La Cocha Departament, Tucumán Province (27º44'34" S, 65º40'34" W, 592 m a.s.l., 20 November 2011). Voucher specimens are deposited in the following Argentinean collections: Museo Argentino de Ciencias Naturales "Bernardino Rivadavia"-CONICET (MACN; Buenos Aires), Museo de La Plata (MLP; La Plata, Buenos Aires), and Laboratorio de Genética Evolutiva, Instituto de Biología Subtropical CONICET-UNaM (LGE; Posadas, Misiones).

Overall anatomy (MACN 46224) and histological sections (MLP A. 1960) of oviducts were studied. Oviduct samples were obtained from a specimen fixed in 10% formalin for 24 hours and subsequently stored in 70% ethanol. The oviduct was cut into 5-µm sections and the samples were dehydrated, post-fixed in Bouin, and paraffin-embedded. Serial sections were stained with haematoxylin and eosin (Martoja & Martoja-Pierson 1970) and Masson's Trichrome (Bradbury & Gordon 1990) stain for general cytology and histology. In addition, histochemical tests were performed in order to characterize the secretory products of glands, including Periodic acid-Schiff (PAS; Cook 1990) for both neutral mucopolysaccharides and mucoproteins, and Alcian blue (AB; Cook 1990) 8GX at pH 2.5 for primarily carboxylated acidic glycosaminoglycans. Stained sections were examined with a Nikon Eclypse 200 microscope and photographically captured with a Nikon DS-U2 digital camera.

Calling activity of *Pleurodema tucumanum* was observed in three temporary ponds.

In San Luis, *P. tucumanum* was reproducing alongside *Leptodactylus bufonius*, *L. mystacinus*, *Physalaemus biligonigerus* (Leptodactylidae), and *Rhinella arenarum* (Bufonidae). In Villa Atamisqui, *P. tucumanum* was reproducing syntopically with *Pleurodema guayapae*, and with *Pleurodema borellii*, *Leptodactylus chaquensis*, and *L. latinasus* (Leptodactylidae) in Rio San Ignacio. *Pleurodema tucumanum* started to vocalize in the evening and continued during the night, calling from the edges of temporary ponds or at some distance on the slopes leading to the ponds or in the water (Fig. 1). In San Luis, eight specimens of *P. tucumanum* were collected during its re-

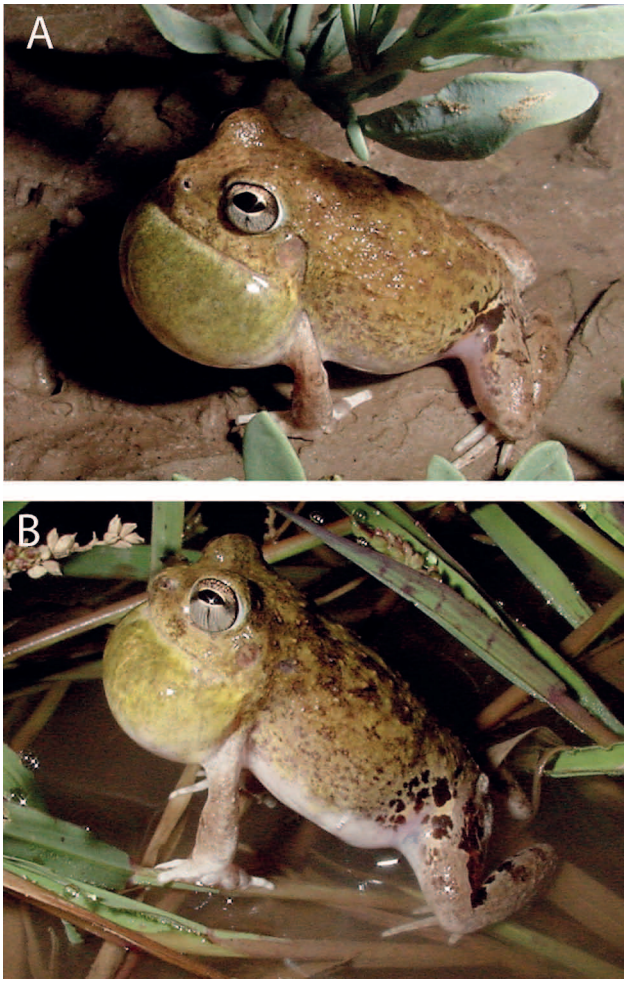


Figure 1. Males of *Pleurodema tucumanum* calling in Totora Pampa (Atamisqui Departament, Santiago del Estero province), A) on land; B) in the water.

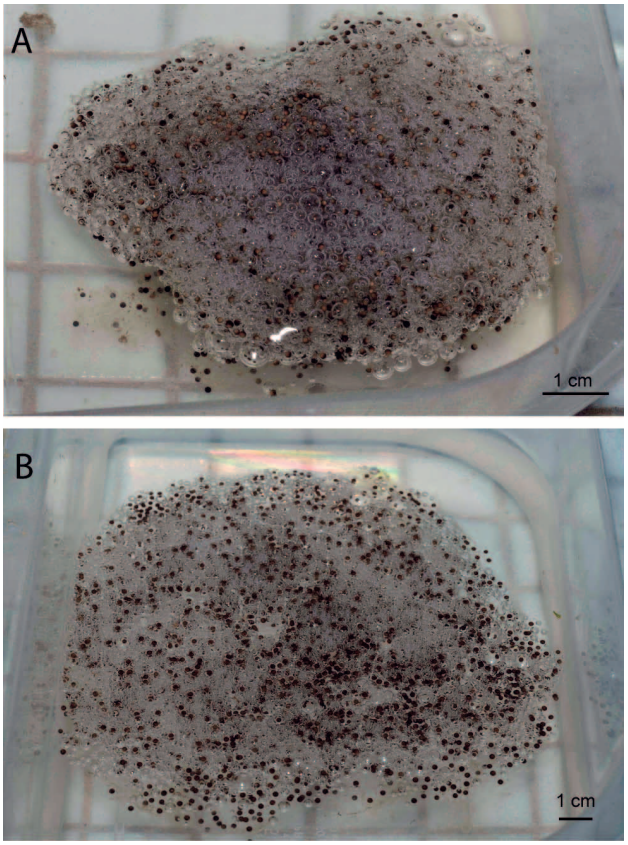


Figure 2. Foam nest built by an amplectant pair of *Pleurodema tucumanum* (MACN 46223–46224) inside a plastic vessel. A) Foam nest at the end of construction; B) foam nest 7.30 hrs after the pair finished its construction.

productive activity (MACN 46223–46230). Amplectant pairs of *P. tucumanum* observed in the field and laboratory were in axillary amplexus. An amplectant pair (MACN 46223–46224) contained in a plastic vessel filled with water (about 100 ml) started producing eggs at 01:00 h of 15 January 2013. Once the female released a transparent and gelatinous substance with the ova, the male started beating it rapidly with his hind legs. This behaviour was repeated many times (not counted) until the foam nest was completed. The foam mass contained air bubbles of different sizes and pigmented eggs at different planes (Fig. 2A). By the morning of 15 January 2013, the nest had doubled its diameter and flattened out, as though the air bubbles had coalesced (Fig. 2B). Subsequently, the clutch took on the shape that is usually found in the field, i.e., a floating and ovoid plate-like mass in which the eggs are arranged along a single plane (see Faivovich et al. 2012, Fig. 6C).

An amplectant pair collected at Río San Ignacio (LGE 2311 and 2316) also deposited eggs in a foam nest within the plastic bag, but, while foam nest construction was not observed, the nest found the morning following their capture had similar characteristics to those reported for the amplectant pair from San Luis. Egg deposition and larval development were observed in Río San Ignacio and Totora Pampa, and both took place in aquatic environments. Clutches observed in the field were similar in appearance to those observed in the laboratory (Fig. 3).

The oviduct of *Pleurodema tucumanum* has a short pars recta, a tightly coiled pars convoluta, and the ovisac. The terminal portion of the pars convoluta is expanded (Fig. 4A). The dissected female of *P. tucumanum* was gravid and had oocytes along the oviduct and the ovisac. The oviductal lumen is narrow when oocytes are absent, but when oocytes are present, the wide lumen is almost filled by them. Histologically, the oviduct consists of a thin external wall serosa, a thin lamina propria, and an internal mucosa. The mucosal layer is formed by an epithelium and a glandular stratum. In the anterior and medial regions of the pars convoluta, this layer projects into the lumen of the oviduct, developing elliptical lobes formed by glands. The epithelium consists of cuboidal ciliated cells and goblet cells (Fig. 4B), while in the glandular stratum, the glands are circular in cross-section, with a narrow lumen, and enlarged longitudinally. The glands are formed by columns of about 10 to 15 closely packed secretory cells with a central duct that drains into the oviductal lumen (Figs 4B, C). The mucosa of the dilated section of the pars convoluta has an epithelium similar to those of the first sections of the pars convoluta. The glands are circular in cross-section, have a lumen larger than those of the other regions of the oviduct, and are full of content (Fig. 4D). In the final portion of the oviduct, the lumen is collapsed and the facing epithelia are in contact. Histochemically, both glands and epithelia were PAS-positive along the whole oviduct. The AB analysis turned out positive in the glands and epithelia only in the first two sections, while the last section showed only AB-

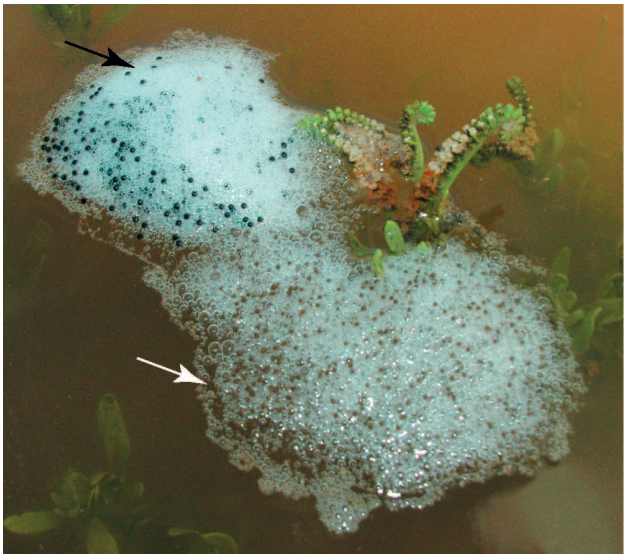

Figure 3. Aspects of completed foam nests of *Pleurodema guayapae* (black arrow) and *Pleurodema tucumanum* (white arrow) in Villa Atamisqui, Santiago del Estero Province. Note the dark brown eggs of *P. guayapae* compared to the pale brown ones of *P. tucumanum*.

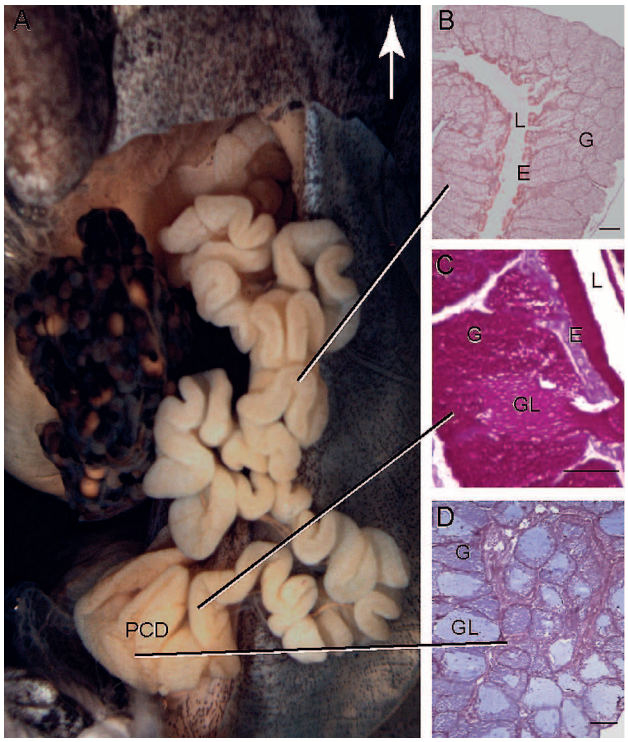


Figure 4. Oviduct of *Pleurodema tucumanum*. A) In situ-view of the left oviduct; note the pars convoluta dilata (PCD). White arrow indicates cranial direction. B) Histological section of the proximal zone of the pars convoluta. Masson's trichrome. Scale bar 100 µm. C) Histological section of the distal section of the pars convoluta. Periodic acid-Schiff. Scale bar 50 µm. D) Histological section of the pars convoluta dilata. Masson's trichrome. Scale bar 100 µm. E – epithelium; G – gland; GL – glandular lumen; L – oviductal lumen; S – secretory cells.

positive epithelia. These results together indicate that both proteins and glycoconjugates are secreted into the oviductal lumen of *P. tucumanum*.

Our observations indicate that the male of *P. tucumanum* builds a nest by beating female oviductal secretions during amplexus, in the same manner as reported for foam-nesting leiuperines (Martin 1970, Heyer & Rand 1977, Hödl 1986, 1990, 1992, Halloy & Fiaño 2000, Valetti et al. 2014). Unlike other leiuperines, however, the foam nest of *P. tucumanum* will soon coalesce into a superficial plate-like mass. Interestingly, an apparently similar collapsing foam nest has also been described in the limnodynastid clade (Frost et al. 2006, Pyron & Wiens 2011) composed of the genera *Lechriodus* and *Platyplectum* by Anstis (2013).

Martori et al. (1994) described the clutch of *P. tucumanum* as a gelatinous ovoid plate-like mass. However, they noticed that they were observing clutches that were already two or three days old, as was inferred by the larval stages present. Their description corresponds to our observations, but missed the fact that the plate-like mass was actually the result of the foam nest having coalesced. Their description has therefore caused some confusion, as it has been supposed to imply that no actual foam nest was involved. Based on that report, Faivovich et al. (2012) suggested that the egg-clutch structure of *P. tucumanum* could simply constitute the unbeaten oviductal secretion. This hypothesis must be rejected simply because it was based on the false premise that *P. tucumanum* does not build foam nests. Besides, we observed the male of *P. tucumanum* beating the female secretion during amplexus. Thus, while gametes are released in a foam nest, embryos and early larvae develop in a gelatinous ovoid plate-like mass. In this sense, the clutch structure of *P. tucumanum* is unique amongst those *Pleurodema* species whose oviposition modes are known.

The pars convoluta dilata, the section of the oviduct in which the foaming secretion is produced, was observed in *P. tucumanum* (present study) as well as in the other foam-nesting *Pleurodema* species, i.e., *P. borellii* (Alcaide et al. 2009), *P. brachyops* (Furnes et al. 2010), and *P. cinereum* (Bhaduri 1953), all of which are included in the *P. brachyops* clade (Faivovich et al. 2012). An enlarged posterior region of the pars convoluta has also been observed in other foam-nesting anurans (see Furnes et al. 2010: Table 1, and reference therein). Histological and histochemical properties of the oviductal glands of *P. tucumanum* agree with those described for other foam-nesting species (Alcaide et al. 2009, Furnes et al. 2010).

While foam nests are usually stable for many days (e.g., in *Engystomops pustulosus*, Cooper et al. 2005, Fleming et al. 2009), those of *P. tucumanum* will collapse soon after spawning. Long-term stability of foam nest has been related to a mixture of proteins that occur in foams. It has been suggested that initial foam formation is facilitated by specific surfactant proteins and further stabilized by subsequent molecular re-engagements (Cooper et al. 2005). Surfactant activity so far has been associated mainly with the proteins ranaspumin (isolated from the foam nests of the leptodactylids *Engystomops pustulosus* and *Leptodactylus vastus*; Cooper et al. 2005, Hissa et al. 2008) and ranasmurfin (isolated from the foam nests of the rhacophorid *Polypedates leucomystax*; McMahon et al. 2006). Considering our observations (i.e., oviduct of *P. tucumanum* with pars convoluta dilata and brief stability of the foam nest produced), we suspect that foam composition is modified here, especially with regard to the proteins that provide stability to the foam nest structure. Foam composition and stability result from the combination and interaction of many molecules and possibly even the mechanics of foam nest construction, and their complexity should be explained considering these factors. Unfortunately, no biochemical characterization of foam components is available for species of *Pleurodema*, nor is there an exhaustive survey of nest construction and structure in leptodactylids in general, comparable to Dalgetty & Kennedy's (2010) detailed study on *Engystomops pustulosus*.

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