A Developmental Staging Table for *Physalaemus biligonigerus* (Cope, 1861) (Anura: Leptodactylidae)

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Abstract. *Physalaemus biligonigerus* is a leptodactylid frog that is widely distributed in southern South America and a common member of anuran assemblages that breed in temporary ponds near human settlements. Herein, we analyzed its embryonic and larval morphology through the completion of metamorphosis and constructed a normal table of development. In addition, the timing of development and growth data are provided. Development of *P. biligonigerus* from fertilization through metamorphosis takes 20–24 days. We recognized 46 discrete developmental stages on the basis of readily discerned, unambiguous, external traits that can be identified in preserved specimens. In contrast with the standard Gosner developmental table, we recognized several particularities in the development of this species. The staging criterion based on external gill morphology presented here is a novel approach to distinguish pre- and post-hatching stages, which could also be employed in other anuran species. We found that anal tube loss in *P. biligonigerus* occurs earlier than Gosner Stage 41; this trait is highly variable among species and should not be used as a standard criterion. Our analysis demonstrates the value of detailed specific staging tables to characterize anuran life cycles. Furthermore, we point out that the use of generalized standard tables might mask the richness of phenotypic variation during the ontogeny of anuran species.

Keywords. Development; Growth; Leptodactylid; Morphology; Ontogeny.

Resumen. *Physalaemus biligonigerus* es un leptodactílido ampliamente distribuido en el sur de Sudamérica y un miembro común de los ensambles de anuros que se reproducen en charcas temporales cerca de asentamientos humanos. En este estudio analizamos su morfología embrionaria y larval hasta el fin de la metamorfosis y construimos una tabla normal de desarrollo. Además, proporcionamos el tiempo de desarrollo y datos sobre crecimiento. El desarrollo de *P. biligonigerus,* desde la fertilización hasta el fin de la metamorfosis dura 20–24 días. Reconocimos 46 estadios discretos basados en rasgos fácilmente discernibles y no ambiguos de la morfología externa, los cuales pueden ser identificados en especímenes preservados. En contraste con la tabla de desarrollo estándar de Gosner, se reconocen varias particularidades en el desarrollo de esta especie. El criterio de estadificación basado en la morfología de las branquias externas aquí presentado representa una forma novedosa de distinguir las etapas pre- y post-eclosión, que podría emplearse en otras especies de anuros. Encontramos que la pérdida del tubo anal en *P. biligonigerus* ocurre antes del estadio 41 de Gosner; este carácter es altamente variable entre especies y no debería considerarse como un criterio estándar. Nuestro análisis demuestra el valor de tablas específicas y detalladas para caracterizar los ciclos de vida de anuros. Además, señalamos que el uso de tablas estándar generalizadas podría enmascarar la riqueza de la variación fenotípica durante la ontogenia de las especies de anuros.

INTRODUCTION

Most anurans are characterized by a biphasic life cycle, with two important processes of morphological changes. The first occurs between embryonic and larval stages, whereas the second occurs between the larval stage and adulthood (Duellman and Trueb, 1994). The change from egg to larva and from tadpole to adult needs to be well documented if one wants to compare ontogenesis between taxa. This requires an effective method to organize the progress of development (Dünker et al., 2000); therefore, the developmental tables are the most useful tool to order developmental events (e.g., Shumway, 1940; Taylor and Kollros, 1946; Limbaugh and Volpe, 1957; Schreckenberg and Jacobson, 1975; Sammouri et al., 1990; Iwasawa

and Yamashita, 1991; Nieuwkoop and Faber, 1994; Shi and Boucaut, 1995; Hall et al., 1997; Dünker et al., 2000; Fabrezi et al., 2009, Fabrezi et al., 2012). Accordingly, documenting variation in development and growth is important for understanding general developmental patterns and for detecting divergence processes in the ontogenesis that can resolve systematic relationships (Hall et al., 1997).

The external and generally rapid development of anurans facilitates the study of their life cycles. Consequently, data on developmental time in captivity or even in the field are relatively easy to obtain. Despite these advantages, developmental tables are available for fewer than 120 species, which represent less than 2% of the extant anuran diversity (Fabrezi et al., 2017). Moreover, knowledge about morphological variation during anuran de-

velopment is generally characterized on the basis of just a few standardized tables (i.e., Taylor and Kollros, 1946; Gosner, 1960; Nieuwkoop and Faber, 1994). Using these generalized tables neglects much interspecific variation in larval development and growth that may have had consequences in anuran diversification (Fabrezi, 2011).

We present here a detailed description of the embryonic and larval development of the leptodactylid frog *Physalaemus biligonigerus* (Cope, 1861), together with a staging table based on external morphological characters that synthesize the species development through the end of metamorphosis. We also provide species-specific features and data on developmental time and growth to facilitate comparison among populations and detect anatomical anomalies in the context of rapid environmental changes. Our results will also facilitate comparison of developmental patterns with other leptodactylid genera (e.g., *Pleurodema* Tschudi, 1838, *Leptodactylus* Fitzinger, 1826).

Physalaemus biligonigerus is distributed in northern and central Argentina, adjacent Bolivia, Paraguay, Uruguay, and southern Brazil (Fig. 1A; Frost, 2018). Adults are small to medium-sized, terrestrial, and sexually dimorphic. They have a conspicuous advertisement call and

reproduce during the warm, rainy season in temporary ponds near human settlements, even in highly disturbed environments like soybean fields. During amplexus the male uses his legs to beat the albuminous secretion with eggs released by the female, forming a mass of white foam that is placed near vegetation on the shore of the ponds (Cei, 1980). Foam nests contain approximately 1,050–1,690 eggs (Zaracho et al., 2005), and tadpoles are type IV according to Orton's (1953) classification.

Phylogenetic hypotheses for the species of *Physalaemus* Fitzinger, 1826 have been proposed in recent analyses of molecular data (Pyron and Wiens, 2011; Lourenço et al., 2015). Lourenço et al. (2015) recognized the *P. biligonigerus* species group, composed of *P. biligonigerus, P. marmoratus* (Reinhardt and Lütken, 1862), *P. santafecinus* Barrio, 1965, and *P. riograndensis* Milstead, 1960. Tadpoles of these four species share a similar oral disc morphology, which is characterized by the presence of two lower labial tooth rows. This might be a synapomorphy of these species (Lourenço et al., 2015) and defines an oral disc that truncates its development with regard to the plesiomorphic 2/3 larval labial tooth-row formula (Vera Candioti et al., 2011).
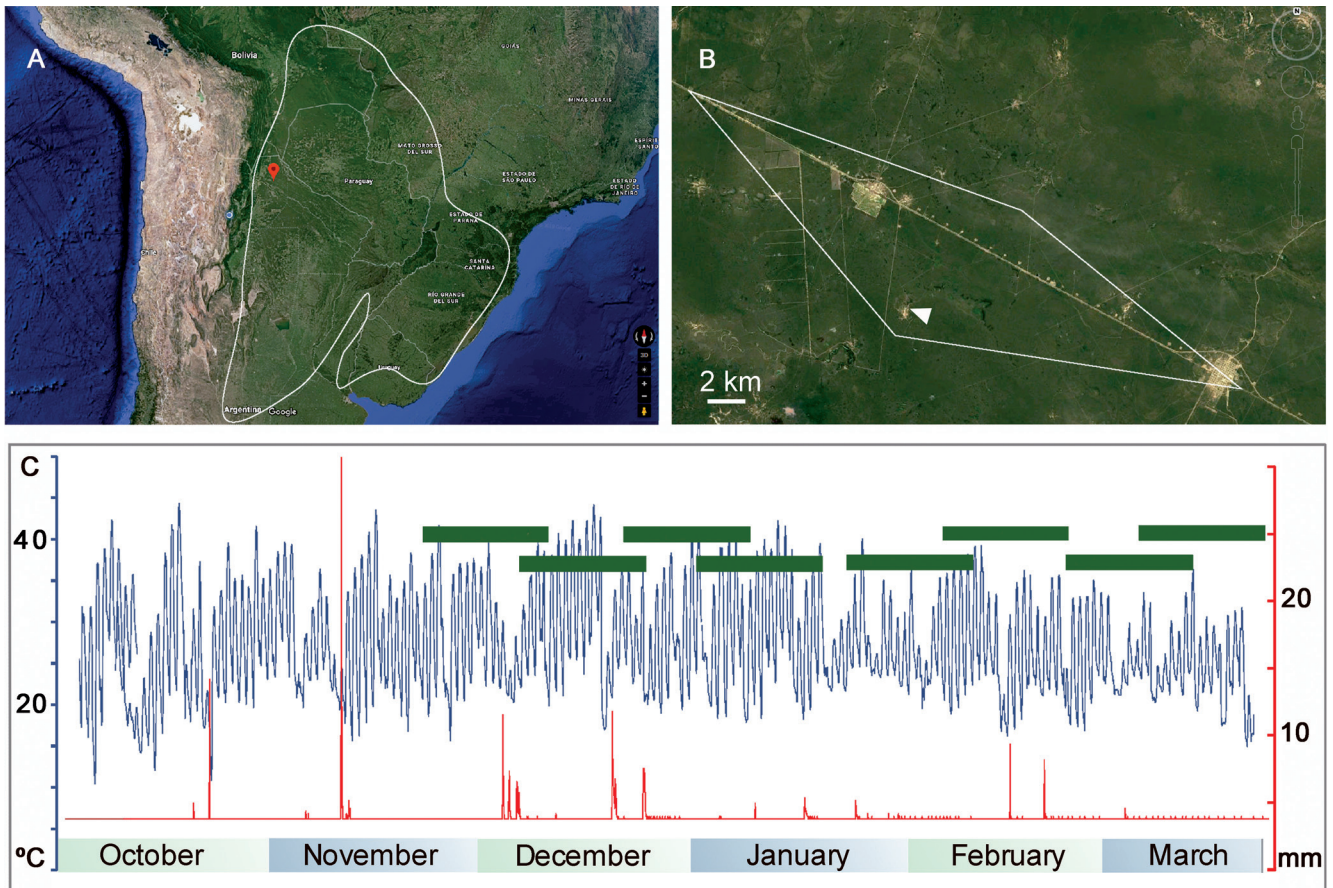

Figure 1. (A) Satellite image of southern South America showing *Physalaemus biligonigerus* distribution. The red mark indicates the locality of our study area. **(B)** Detail of the study area. The polygon delimits the area where field records of *P. biligonigerus* were taken. White arrow points the localization of a semi-automatic weather station. **(C)** Climatic diagram with temperature (°C; left axis) and rain (mm; right axis) records from October 2017 to March 2018. Green bars depict the presence of tadpoles of different age groups of *P. biligonigerus* over the summer.

A Developmental Staging Table for *Physalaemus biligonigerus* (Cope, 1861) (Anura: Leptodactylidae)
Mariana Chuliver, Marissa Fabrezi

151

Table 1. Synopsis of the diagnostic features defining stages in the embryonic and larval development of *Physalaemus biligonigerus.* Developmental time is given for Stages 1–26. Values are reported as $\bar{X} \pm$ SD.

Stage	Diagnostic features	Total length (mm)	Time (h)
1	1 cell, smooth eggs, pigmentation absent	1 ± 0.05	0
2	2 cells divided by complete cleavage furrow (meridional)	1 ± 0.05	1 ± 0.35
3	4 cells, embryo divided by second cleavage furrow (meridional)	1 ± 0.05	2 ± 0.35
4	8 cells, embryo divided by third furrow (supraequatorial), four micromeres in animal pole and four macromeres in vegetative pole	1 ± 0.05	2.5 ± 0.35
5 morula	16 cells, embryo divided by fourth and fifth cleavage furrows (meridional)	1 ± 0.05	3.33 ± 0.35
6 morula	32 cells, embryo divided by the sixth cleavage furrow (supra-equatorial)	1 ± 0.05	4 ± 0.35
7 blastula	Numerous, large, and well-defined cells, resulted from multiple and disorganized cleavage furrows.	1 ± 0.05	5 ± 0.35
8 blastula	Numerous tiny cells of subequal size	1 ± 0.05	7 ± 0.35
9	Dorsal lip of blastopore as a crescent-shaped slit in the vegetative pole	1 ± 0.05	10 ± 1.5
10 gastrulae	Lateral and ventral lips of the blastopore enclosing rounded yolk plug, its diameter approximately equal to 1/2 diameter of embryo (i.e., 0.5 mm)	1 ± 0.05	12 ± 1.5
11 gastrulae	Yolk plug diameter 1/4 of diameter of embryo (i.e., 0.25 mm)	1 ± 0.05	15 ± 1.5
12 gastrulae	Yolk plug diameter < 1/4 of diameter of embryo; embryo ovoid	1 ± 0.05	17 ± 1.5
13 neurula	anterior and dorsal regions of embryo denoted by broad anterior plate (neural plate) and pair of short neural folds	1.25 ± 0.05	20 ± 1.5
14 neurula	Neural folds elevated, defining deep groove (neural groove); embryo lengthens	1.25 ± 0.05	23 ± 1.5
15 neurula	Neural folds contact in the mid-dorsal surface; ventral yolk mass with uniform appearance	1.5 ± 0.05	26 ± 1.5
16	Anterior region with ventral depression (stomodeum) and short tail bud with rounded end defining cephalic and caudal regions, respectively.	1.5 ± 0.05	31 ± 2.5
17	Adhesive (= cement) glands on both sides of stomodeum, bulges of gill buds and eyes in cephalic region, pronephros protuberances in mediolateral position, anal tube in posteroventral region	1.5 ± 0.05	35 ± 2.5
18	Spherical and prominent gill buds, tail with dorsal and ventral fins outlined	1.75 ± 0.08	40 ± 3
19	Each gill bulge divided into 2 lobes by medial groove (first and second gill arches), anterior lobe divided in 2 filaments	2 ± 0.09	47 ± 3
20	First gill with 2–3 filaments, second gill divided into 2 lobes.	2.25 ± 0.12	54 ± 3
21	First gill with 4–5 filaments, second gill with 3 filaments	2.5 ± 0.13	59 ± 3
22	First gill with 5–6 filaments, second gill with 4 filaments	2.5 ± 0.18	65 ± 3
23	First gill with 6–7 filaments, second gill with 5 filaments	3.25 ± 0.17	75 ± 4
24	First gill with 7–8 filaments, second gill with 6 filaments, nostrils and mouth differentiated	4.25 ± 0.23	82 ± 4
25	Second gill with 7 filaments; oral disc with upper and lower lips	5.25 ± 0.23	120 ± 8
26	Oral disc with keratinized parts, operculum covering external gills partially or totally	6.25 ± 0.25	150 ± 8
27	Hind limb bud length ≥ 1/2 diameter	10.75 ± 1.5	
28	Hind limb bud length ≥ diameter	12.1 ± 0.96	
29	Hind limb bud length ≥ 1.5 × diameter	14.4 ± 1.1	
30	Hind limb bud length ≥ 2 × diameter	14.9 ± 1.65	
31	Hind limb bud paddle-shaped (autopodium with anterior and posterior indentations)	15.4 ± 1.4	
32	Autopodium asymmetrical with distal and postaxial elongation of primary axis (Toe IV)	16.3 ± 1.4	
33	Toes IV and V extended beyond autopodium boundaries, separated by indentations; estilopodium and zeugopodium differentiated	16.6 ± 2.9	
34	Indentation defining Toe III	17.8 ± 3.5	
35	Indentations defining Toes I and II	22.1 ± 1.6	
36	Toes IV and V separated and elongate, tarsus and pes differentiated	24.8 ± 2.1	
37	5 toes elongated and separated without interdigital tissue; ventrally, both hind limbs completely separated by anal tube	26.2 ± 2.1	
38	Anterior and posterior metatarsal tubercles	26.9 ± 2.5	
39	Digital subarticular tubercles, anal tube dorsal to hind limbs	28.9 ± 2.2	
40	Tarsal tubercle, fibers of *M. rectus abdominis* visible in the ventral body wall	28.6 ± 5.1	
41	Oral disc without keratinized teeth, upper eyelid.	28.8 ± 4.6	
42	Both forelimbs emerged fully formed, lower eyelid and nictitating membrane, oral disc without keratinized parts	25.6 ± 3.7	
43	Mouth commissure between the nares and anterior margin of eye	20.5 ± 4.5	
44	Mouth commissure prior to the anterior half of the eye, tail atrophied, with markedly different skin coloration, ventral fin lost, and dorsal fin reduced	11.9 ± 5.4	
45	Mouth commissure exceeding anterior half of eye, tail forming prominence with same color as skin of dorsal body	10.9 ± 1.8	
46	Complete tail loss, mouth commissure reaching posterior limit of eye	13.4 ± 1.1	

152

A Developmental Staging Table for *Physalaemus biligonigerus* (Cope, 1861) (Anura: Leptodactylidae)
Mariana Chuliver, Marissa Fabrezi

MATERIALS AND METHODS

To describe embryonic stages, eight amplectant pairs were collected around Salta city (24°45′28″S, 65°27′48″W), Argentina, in January and February 2014, 2015, and 2016. Each mating pair was kept in plastic containers with pond water until foam nests were obtained. The clutches were kept at ambient temperature and exposed to a natural light cycle. From two foam nests, we extracted samples every hour during the first 12 h and every 2 h until the larvae started feeding. From the remaining six foam nests, we took samples 36 and 48 h after fertilization (three foam nests each) every 2 h until the larvae started feeding. The number of individuals varied in each sample but included at least 20 per sample to establish developmental stages.

Descriptions of larval and metamorphic stages were based on individuals collected between 1998–2014 in Salta Province (Appendix). Additionally, we collected tadpoles from November 2017 to April 2018 in temporary ponds in a polygon comprising the coordinates 23°26′18.30″S, 63°4′44.46″W, where a semiautomatic weather station was installed (Fig. 1B).

Embryos and larvae were fixed in 10% formalin with 0.5% acetic acid. Fixed embryos were freed with forceps from the surrounding egg membranes and measured with the aid of a stereomicroscope. Body length and total length were measured with a caliper accurate to 0.25 mm. The total length of embryos was measured as the straight-line distance between the two furthest points in lateral view, while the total length of larvae was assessed as the straight-line distance between the tip of the snout and tip of the tail. Observations and photographs were made using a trinocular stereomicroscope Nikon with digital camera.

We defined the commencement of development by the appearance of egg clutches, thus indicating fertilization (t_0). The developmental time given in Table 1 is that required for at least 75% of the sample to reach a particular stage. We used at least 20 individuals per sample to establish their developmental timing. We used foam nests maintained in the laboratory to estimate developmental timing from fertilization to larval pre-feeding stages. To estimate developmental time for larval and metamorphic stages, we employed individuals collected from one small pond formed after a rainstorm, which was sampled every 5 d for three consecutive weeks.

All specimens are deposited in the Herpetological Collection of the Instituto de Bio y Geociencias del NOA (IBIGEO) and are listed in the Appendix. The terminology used to describe *Physalaemus biligonigerus* development was that used in developmental tables for *Incilius valliceps* (Wiegmann, 1833) (Gosner, 1960) and *Xenopus laevis* (Daudin, 1802) (Nieuwkoop and Faber, 1994) to simplify comparisons with those and other species.

RESULTS

We recognize 46 discrete developmental stages for *Physalaemus biligonigerus* from fertilization through the end of metamorphosis on the basis of readily discernible and unambiguous external morphological features. Fertilized eggs are embedded in a white foam nest (Fig. 2A). Each egg is uniformly white and surrounded by a transparent gelatinous capsule (Fig. 2B). When the eggs are free from their capsules, their average diameter is 1 mm (SD = 0.05 mm; n = 25). Fertilization is not evident until the first cleavage furrow appears. From fertilization to the end of gastrulation, embryos do not exhibit macroscopic changes in diameter or coloration (Fig. 2C–H). Cleavage is holoblastic and cell boundaries are well distinguished and rounded. We defined Stage 4 by the presence of four macromeres (vegetative pole) and micromeres (animal pole) with minor size differences between them. The 32 cell-stage (Stage 6) is reached 4 h after fertilization. Each new cleavage furrow appears on average every 40 min. After these stages, cleavage furrows become asymmetrical and differences between blastula stages (Stages 7–8) are evidenced by number, size, and organization of cells (Fig. 2D). At Stage 8, embryos acquire an externally compact appearance due to the homogenization and reduction of cell size. Cleavage is completed in 6–7 h. During gastrulation (Stages 9–12) the anteroposterior axis is defined; cells in the rounded yolk plug are polygonal and larger than those located outward of the plug (Fig. 2E–H).

The dorsoventral axis is defined during neurulation (Stages 13–15) by the formation of the neural plate in the anterior region and the neural folds in the dorsomedial region (Fig. 2I). At Stage 15 the embryo acquires a semicircular shape with ventral yolk mass. Stage 16 is defined by the appearance of the tail bud while in the subsequent stage (Stage 17) external gill bulges appear (Fig. 2J–K). For Stages 19–25, the number of branches in the second pair of gills is indicative of the stage. The gill developmental trajectory between Stages 19–24 is summarized in Figure 3. During these stages, segmentation of the axial musculature is observed, adhesive glands develop, the tail bud lengthens, and dorsal and ventral fins grow progressively in height (Fig. 2M–S).

The developmental stage in which hatching occurs in *Physalaemus biligonigerus* varies. Development in the foam nest lasts on average 62 ± 3 h. Some embryos hatch at Stage 21 after 59 h, while others hatch after 65 h when their external gill development is more advanced (i.e., at Stage 22). Upon hatching, embryos release their egg membranes and fall into the water where they are able to make moderate movements. However, at this stage tadpoles do not feed. At Stage 22, melanophores appear in the dorsal region of head and body, with pigmentation increasing through Stages 23–26 to cover the dorsal region of the head and body as well as the eye formation

A Developmental Staging Table for *Physalaemus biligonigerus* (Cope, 1861) (Anura: Leptodactylidae)
Mariana Chuliver, Marissa Fabrezi

153

Figure 2. External view of embryos and larvae of *Physalaemus biligonigerus,* from cleavage to first feeding stage. **(A)** Foam nest; **(B)** egg within the foam nest; **(C)** four-cell stage; **(D)** large-cell blastula; **(E)** early gastrula, dorsal lip of blastopore as crescent-shaped slit; **(F)** gastrula, lips of blastopore enclosing yolk plug; **(G)** late gastrula, yolk plug diameter 25% of diameter of embryo; **(H)** late gastrula with small yolk plug; **(I)** embryo showing formation of neural groove; **(J)** tail bud embryo; **(K)** stage with bulges outlined in different colors, eyes (blue), gills (red), pronephros (green), and future anal tube zone (violet); **(L)** stage with spherical and prominent gill buds; **(M–T)** Stages 19–25, during which segmentation of the axial musculature is observed, adhesive glands develop, tail lengthens, dorsal and ventral fins grow in height, and pigmentation progresses; **(U)** hind limb bud larvae. Stages indicated by codes S1–S27. Scale bars = 0.5 mm.

area (Fig. 2Q–T). The body shape becomes ovoid, the tail doubles its length, and dorsal and ventral fins increase in height during Stages 23–26 (Fig. 2R–T). The eyeball is defined at Stage 24 (Fig. 2R). At Stage 25 the mouth opening (stomodeum) is oval with sharp edges and upper and lower lips outline the oral disc, which does not exhibit keratinized structures (Fig. 4A). Adhesive discs are developed on both sides of the mouth. The maximum number of gill filaments is reached at Stage 25. The operculum starts developing at that stage and completely covers the gills in Stage 26 (Fig. 4B). Progress in the development of the operculum is asymmetrical, with the right external gills being covered first and the left gills still protruding in some individuals at this stage (Fig. 4B). The ventral body wall is translucent since no yolk remains, and the coiled intestine is visible.

Tadpoles of *Physalaemus biligonigerus* from Stage 27 to 40 have an ovoid body shape in dorsal and ventral views and their snouts rounded, with eyes and nostrils in a dorsal position (Fig. 5A). The oral disc is small and anteroventral with two anterior (A1–A2) and two posterior (P1–P2) tooth rows. The labial tooth row formula is 2(2)/2(1); the gap in A2 is wider than that of P1 (Fig. 5B). Marginal papillae are subconical with rounded tips and occur along the entire ventrolateral margin with a gap dorsally (Fig. 5B). The upper jaw sheath is arched and overlaps the lower jaw sheath, which is V-shaped. Both jaws are finely serrate and brownish. The oral disc shows all the diagnostic characteristics of the species at Stage 27, which are maintained until Stage 40. The spiracle is short, sinistral, and directed posterodorsally at mid-body; its opening is oval, with a diameter approximately equal to the length of the tube (Fig. 5C). The anal tube is short and continuous with the ventral tail fin and its opening is slightly dextral (Fig. 5D). Both the spiracle and anal tube preserve their morphology until Stage 40. The caudal musculature is

Figure 3. Representation of development of external gills of *Physalaemus biligonigerus* during Stages 19–24. The first pair of gills has two states (i.e., number of filaments) within the same stage. **(A)** Gill bulges divided into two lobes by a medial groove; at the end of the stage the groove between the lobes becomes deeper, with the anterior-most lobe divided into two filaments by a small distal commissure; **(B)** each lobe lengthens and subdivides forming filaments; **(C–F)** the number of gill filaments increases and lengthens in each stage. Stages indicated by codes S19–S24. Scale bars = 1 mm.

moderately robust (Fig. 5A). The dorsal and ventral fins are transparent with discrete groups of melanophores. The dorsal fin extends briefly over the body and is slightly higher than the ventral fin (Fig. 5A).

The hind limb buds appear at Stage 27 (Fig. 2U). Identification of Stages 27–30 is based on the limb bud length/diameter ratio, following the same criteria proposed by Gosner (1960; Fig. 6). At Stage 31 the hind limb bud is paddle-shaped and Stages 32–35 are identi-

fied by appearance of notches in the distal region of the hind limb, which indicates the successive delimitation of toes (Fig. 6B–D). Differentiation of segments in the hind limb occurs at Stages 31, 33, and 36 (Fig. 6B–C, E). Slight pigmentation distributed as dots on the dorsum of the limb appears at Stage 34 (Fig. 6D). Pigmentation then progresses towards the autopodial ventral surface (Stage 38). The anal tube is constricted as a result of the approximation of the hind limbs towards the body mid-
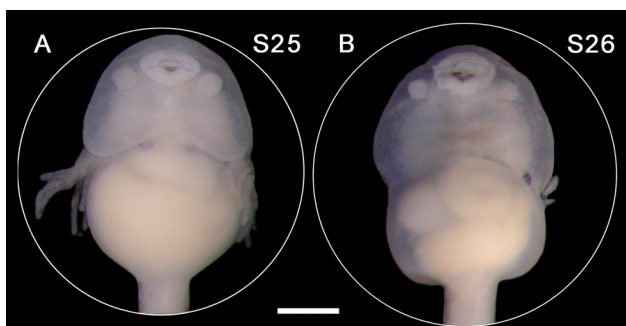

Figure 4. *Physalaemus biligonigerus* in Stages 25 and 26. **(A)** Ventral view of larval body at Stage 25; **(B)** ventral view of larval body at Stage 26, characterized by external gills covered by the operculum and keratinized structures in the oral disc. Stages indicated by codes S25 and S26. Scale bars = 0.5 mm.
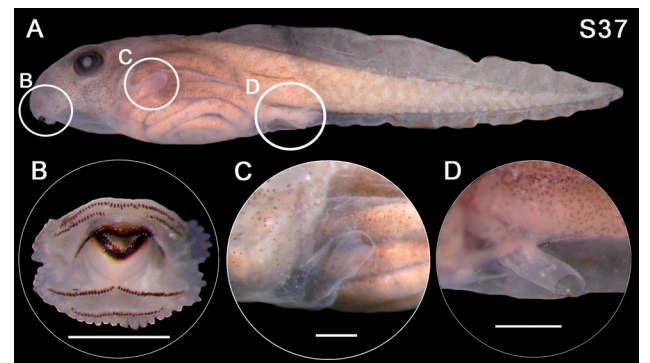

Figure 5. (A) Lateral view of general larval body aspect of *Physalaemus biligonigerus* at Stage 37 (S37) with oral disc, spiracle, and anal tube indicated by circles. **(B)** Oral disc; **(C)** Spiracle; **(D)** Anal tube (hind limb removed for better visualization). Scale bars = 0.5 mm.
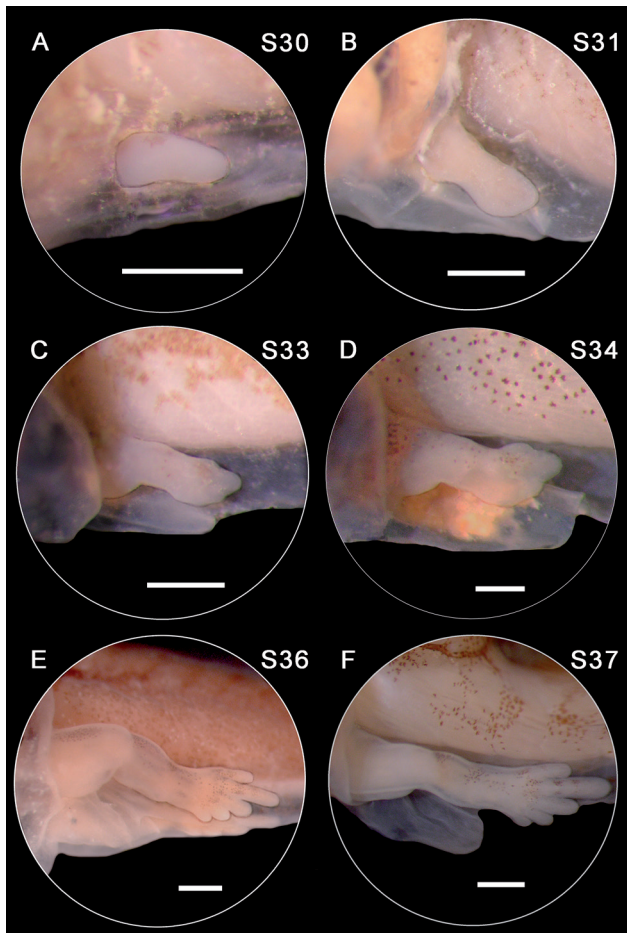
A Developmental Staging Table for *Physalaemus biligonigerus* (Cope, 1861) (Anura: Leptodactylidae)
Mariana Chuliver, Marissa Fabrezi

155

Figure 6. Development of the left hind limb of *Physalaemus biligonigerus* through Stages 30–37. Stages indicated by codes S30–S37. **(A)** Hind limb bud length more than twice its diameter. **(B)** Hind limb bud paddle-shaped. **(C)** Stylopodium and zeugopodium differentiated, Toes IV and V separated by indentations. **(D)** Toe III defined. **(E)** Tarsus and pes differentiated. **(F)** Five toes separated without interdigital tissue. Scale bars = 0.5 mm.

line at Stage 38. At Stage 39 the hind limbs are proximally united as a consequence of the fusion of the pelvic girdles at the body midline, so the anal tube is no longer connected with the ventral skin (Fig. 7A). Stage 41 is characterized by changes in the oral disc, the first of which is loss of keratodonts (Fig. 7C). The ventral body wall becomes opaque and thicker by the progress in development of the rectus abdominis muscle. The thighs become robust and the autopodial tubercles are prominent and pointed.

Forelimbs emerge at Stage 42 (Fig. 8A), the left one first, through the spiracle opening, and then the right one. The forelimb skin is rapidly incorporated into the body's integument. The anal opening is observed but the skin of the anal tube remains attached to the ventral fin. Stages 43–45 are primarily identified by the position of the mouth commissure relative to the eye and nostril in lateral view, as well as the degree of tail reduction (Fig. 8B–D). At Stage 43 the tail is at least 30% shorter than at Stage 42, but it retains its general shape; both the

dorsal and ventral fins decrease in height proximodistally, but the dorsal fin remains taller (Fig. 8B). Metamorphosis is completed at Stage 46 when the tail is fully resorbed (Fig. 8E). The transformed froglet has certain features of the adult, such as a tarsal tubercle and the pattern of bands on the limbs; however, it lacks other adult traits, such as the lumbar glands.

In ponds of the Chacoan environment within the polygon where we have made collections, we found adults of *Physalaemus biligonigerus* reproducing throughout the austral summer (Fig. 1C). Mating can occur many times in the same pond; hence, we found up to eight age groups that will complete their development over the course of the summer (Fig. 1C). Despite slight variations in color and size, we did not find significant morphological differences between larvae within or between ponds.

Total length and body length of embryos and larvae is plotted against their stages in Figure 9A. Early stages (cleavage, blastula, and gastrula; i.e., Stages 1–12) involve mostly morphological differentiation, but no growth. However, when hatching occurs, total and body length increase due to prevalent growth and further differentiation. Consequently, morphological variation increases during Stages 21–25. During feeding stages (Stages 27–40), size can vary considerably among individuals and size ranges for different stages often overlap. Tadpole variation in size at these stages might be related to spatial and temporal variables (i.e., precipitation, food availability,
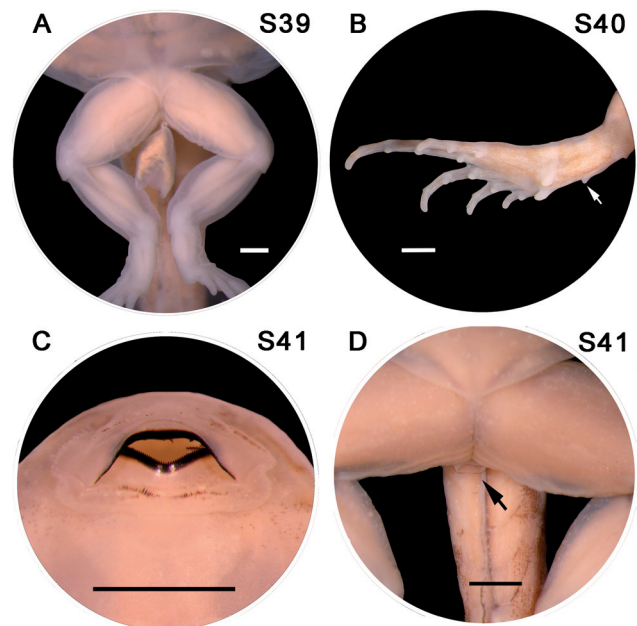

Figure 7. *Physalaemus biligonigerus* in Stages 39–41. **(A)** Hind limbs united proximally in the midventral line, constricting the anal tube; **(B)** hind limb at Stage 40 with tarsal (arrow) and prominent metatarsal and digital subarticular tubercles; **(C)** oral disc prior to metamorphosis showing absence of keratinized teeth; **(D)** anal opening (note that skin of the anal tube remains as a vestige attached to the ventral fin; arrow). Stages indicated by codes S39–S41. Scale bars = 1 mm.
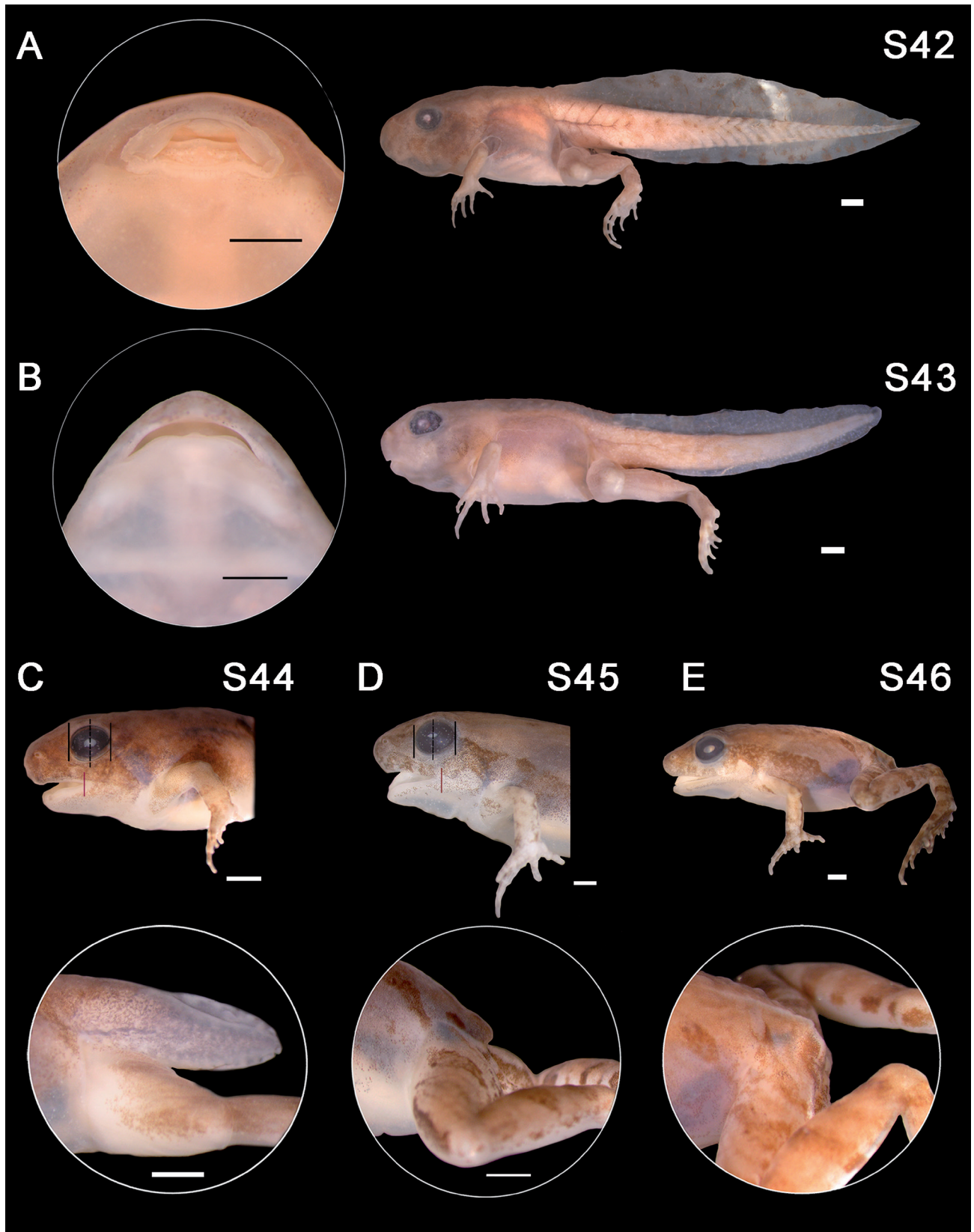
Figure 8. Metamorphosis of *Physalaemus biligonigerus.* **(A)** Oral disc and lateral view of whole organism in Stage 42; **(B)** Mouth and lateral view of metamorphic individual in Stage 43; **(C)** Lateral view of anterior part of body of metamorphic individual in Stage 44 with mouth commissure prior to anterior half of the eye and detail of tail resorption; **(D)** Lateral view of anterior part of the body of metamorphic individual in Stage 45 with mouth commissure exceeding anterior half of eye and tail bud; **(E)** Stage 46, representing froglet morphology. Stages indicated by codes S42–S46. Scale bars = 1 mm.
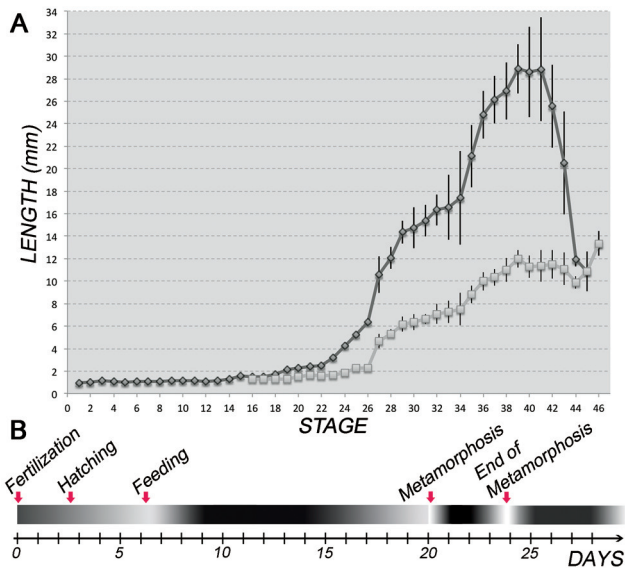
A Developmental Staging Table for *Physalaemus biligonigerus* (Cope, 1861) (Anura: Leptodactylidae)
Mariana Chuliver, Marissa Fabrezi

157

Figure 9. (A) Variation of total length and body length during the complete ontogenesis of *Physalaemus biligonigerus.* From Stage 27 up to Stage 40 there is approximately constant growth. During metamorphosis, total length decreases due to tail resorption; **(B)** schematic representation of embryonic and larval development of *P. biligonigerus* through time.

temperature, pond duration). Larval growth increases until Stage 39 when total length reaches its maximum (Fig. 9A). Despite the overall shortening during metamorphosis due to tail resorption, body length remains approximately constant. Complete development from fertilization to the end of metamorphosis occurs in 20–24 d (Fig. 9B). The first stages from fertilization to hatching occur rapidly in 2.5 d on average. The period from feeding stages to the onset of metamorphosis lasts about 16 d, while metamorphosis encompasses a brief period of 4 d (Fig. 9B).

DISCUSSION

In the present study, we divided embryonic and larval development of *Physalaemus biligonigerus* into 46 stages. External morphological changes used as criteria for staging fertilization, cleavage, gastrulation, and neurulation (Stages 1–15) were the same as those proposed by Shumway (1940) and Nieuwkoop and Faber (1994). In contrast, unlike the criteria based on characters of the living individuals proposed by Limbaugh and Volpe (1957) and synthesized by Gosner (1960), we proposed to distinguish Stages 18–25 on the basis of the number of filaments in external gills (Fig. 3). In the most commonly used tables, these stages are described through physiological characters (e.g., muscular movement, gill circulation, heartbeat), which are impracticable when studying preserved specimens.

Nokhbatolfoghahai and Downie (2008) organized ontogenetic sequences for 21 species belonging to six families based on Gosner (1960). These authors found that external gills varied in number, length, and shape of their secondary filaments (named filaments in this study) among species. Moreover, they showed that external gill development follows a logical sequence, adding filaments consecutively to each gill. In this way, the novel and purely morphological criterion proposed herein—based on external gill morphology— could be used to identify pre- and post-hatching stages in other anuran species,

In *Physalaemus biligonigerus,* we distinguished two different morphologies relative to the operculum development. At Stage 25 the operculum starts to develop and is barely visible at the base of the external gills, while at Stage 26 it partially or completely covers the external gills (Fig. 4). Conversely, in Gosner (1960) developmental table describes three states for operculum development; however, those states are short-lived and difficult to observe, so we reduced them to two discernable morphologies for *P. biligonigerus.*

The stages based on limb morphology (Stages 27–39; Fig. 6) in *Physalaemus biligonigerus* are in agreement with the criteria employed in previous published tables (e.g., Limbaugh and Volpe, 1957; Gosner, 1960). During these stages, we observed no major changes in the larval body morphology. For this reason, the hind limb ontogeny provides accurate and unequivocal features for staging this conservative period.

Stage 40 is defined by both the appearance of the tarsal tubercle and the visibility of fibers of the *rectus abdominis* muscle. The tarsal tubercle is a conspicuous morphological trait of several species of *Physalaemus,* but it is absent in others (Lynch, 1970; Nascimento et al., 2005; Fig. 7B). Consequently, for those species lacking the tarsal tubercle, the visibility of fibers of the *M. rectus abdominis* defines Stage 40, although interspecific variability in this trait should be tested. Additionally, in *P. biligonigerus* the anal tube has already been lost in Stage 39. Conversely, in the developmental table proposed by Gosner (1960) the anal tube disappears at Stage 41 just prior to metamorphosis. The timing of disappearance of the anal tube has been described for several anuran species and is highly variable (Fabrezi, 2011). As such, we advise caution when using this trait to stage development.

On the basis of scanning electron micrographs, Zaracho et al. (2003) described oral disc, external gill, adhesive organ, and operculum development for *Physalaemus biligonigerus* through Gosner Stages 19–25. We noted some differences in comparison with our results. For example, we have found that hatching occurs at Stages 21–22, whereas Zaracho et al. (2003) indicated hatching at Stage 19. This difference might be related to hatching plasticity, which is influenced by rearing conditions, oxygen concentration, temperature, and predator risk, among others. Furthermore, they neglected morphological changes of the gills as a result of the use of Gosner's table and made no ex-

Table 2. Developmental tables available for ~~leptodactylids~~ and their characteristics (modified from Fabrezi et al., 2017). ES: embryonic stage; LS: larval stage; DLC: development under laboratory conditions.

Species	Characteristic	Author
Engystomops spp.	23 ES, 3 d	Romero-Carvajal et al. (2009)
Engystomops guayaco (Ron et al., 2005)	23 ES, 77 h, 18–23°C	Andrade-Cahueñas (2015)
Physalaemus albonotatus (Steindachner, 1864)	46 ES and LS, DLC	Gómez et al. (2016)
Physalaemus nattereri (Steindachner, 1863)	46 ES and LS, 32 d	Vizzoto (1967)
Pleurodema bibroni Tschudi, 1838	12 ES, DLC, 195 h, 18°C	Izquierdo and Pereda (1964)
Pleurodema brachyops (Cope, 1869)	25 ES, DLC, 72 h	León-Ochoa and Donoso-Barros (1969)

plicit reference to developmental timing. As such, incorporating changes in the gill morphology provides a more reliable staging system, especially when working with preserved specimens.

The main differences between larvae of the species of the *Physalaemus biligonigerus* group (*P. biligonigerus, P. marmoratus, P. santafecinus, P. riograndensis*) are found in their oral disc morphologies. The presence of two lower labial tooth rows is known only in these four species, so it might be a synapomorphy of this species group (Lourenço et al., 2015). Additionally, there is a complete row of marginal papillae on the lower labium in *P. biligonigerus* and *P. santafecinus*, and there are two ventrolateral gaps in the lower labium in the remaining two species (Perotti and Céspedez, 1999; Nomura et al., 2003; Borteiro and Kolenc, 2007). Interestingly, variations within this configuration have been described previously among *P. biligonigerus* populations. Fernández and Fernández (1921) mentioned that tadpoles sampled in Santa Fe, Argentina, have a labial tooth row formula 2 (2)/2. However, we observed a narrow gap in P1 in the larvae examined in this study. This is in agreement with the description of Borteiro and Kolenc (2007) and Schulze et al. (2015) for populations in Uruguay and Bolivia, respectively. Another feature described by Schulze et al. (2015) and observed in their figure is the partly fragmented P2 keratodont row. Moreover, they described the presence of submarginal papillae laterally, absent in the descriptions of Fernandez and Fernández (1921), Borteiro and Kolenc (2007), as well as in the present work. The variation in the absence/presence of gap in P1 seems to be an alternative state of a polymorphic character. The fragmented P2 keratodont row and the presence of submarginal papillae can result from developmental alterations. Despite the variations detailed above and slight differences in color or body length, tadpole morphology of *P. biligonigerus* is in general highly conservative. This suggests that genetic variability within or even between populations that inhabit different ecoregions in South America is not necessarily translated into meaningful morphological diversity.

At the interspecific level, several aspects of *Physalaemus* tadpoles are well known (Miranda and Ferreira, 2009; Ruggeri and Weber, 2012; Schulze et al., 2015), but generally the morphology of larval stages and its spatiotemporal variations are interpreted using the standard table proposed by Gosner (1960). Studies about variation in the oral apparatus suggested important evolutionary changes in oral disc morphogenetic patterns (Vera Candioti et al., 2011). These findings could be enhanced with data on developmental timing supplied by specific developmental tables, which can explain heterochronic variations.

Remarkably, staging tables previously developed for other leiuperines focus on specific ontogenetic periods (particularly the initial phases; Table 2). Moreover, these tables do not provide developmental times and often lack adequate illustrations to distinguish key characters and intraspecific variation. Furthermore, the most widely used staging tables are built for species that are not necessarily widespread or have typical larvae (Shumway, 1940; Taylor and Kollros, 1946; Limbaugh and Volpe, 1957; Nieuwkoop and Faber, 1994). It is also noteworthy that the table proposed by Gosner (1960) is simplified from older ones for *Lithobates* (previously *Rana*) *pipiens* (Schreber, 1782) (Shumway, 1940; Taylor and Kollros, 1946) and for *Bufo* (now *Incilius*) *valliceps* (Limbaugh and Volpe, 1957). Gosner´s table is a helpful tool with well-defined characters per stage, but some of the characters are based only on physiological characters of living individuals. In contrast, the developmental table presented here for *Physalaemus biligonigerus* includes exhaustive descriptions and images for complete developmental phases, as well as data on time and size of each stage. This information can be useful for diverse biological studies (i.e., taxonomic, ecological, biogeographic) and mainly for studying variation among populations in different ecosystems. Moreover, the variation we found in the development of the external gills, operculum, and anal tube of *P. biligonigerus* indicates the relevance of having species-specific developmental tables to interpret the evolution of ontogenies.

Finally, some questions about the reproduction of *Physalaemus biligonigerus* remain, such as if adults (female, male, or both) are able to reproduce more than once per summer season. This and other questions remain unanswered for many Neotropical anuran species. Accordingly, staging of embryos and larvae represents the first step to understand evolutionary consequences of ephemeral water bodies on life cycles of the anuran species that inhabit the semiarid Chacoan environments.

A Developmental Staging Table for *Physalaemus biligonigerus* (Cope, 1861) (Anura: Leptodactylidae)
Mariana Chuliver, Marissa Fabrezi

159

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160

A Developmental Staging Table for *Physalaemus biligonigerus* (Cope, 1861) (Anura: Leptodactylidae)
Mariana Chuliver, Marissa Fabrezi

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APPENDIX

Physalaemus biligonigerus: ARGENTINA: **Salta:** Departamento Gral. José de San Martín: Hickman: RN 81, IBIGEO-A 679, IBIGEO-A 944, IBIGEO-A 958, IBIGEO-A 959, IBIGEO-A 984, IBIGEO-A 1011, IBIGEO-A 1079, IBIGEO-A 1158, IBIGEO-A 1793; Dragones: RN 81, IBIGEO-A 985; Misión Chaqueña: RP53, IBIGEO-A 957, IBIGEO-A 1795; Campichuelo: RN 34, IBIGEO-A 1043; Embarcación: RN34, IBIGEO-A 1157, IBIGEO-A 1813; Departamento General Güemes: RN 34, IBIGEO-A 1797; Departamento Capital: Lomas de Medeiros, IBIGEO-A 1808–1816.

A Developmental Staging Table for *Physalaemus biligonigerus* (Cope, 1861) (Anura: Leptodactylidae)
Mariana Chuliver, Marissa Fabrezi

161