Description of the tadpole of *Cruziohyla calcarifer* (Boulenger, 1902) (Amphibia, Anura, Phyllomedusidae)

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Specimens belonging to the genus *Cruziohyla* from Panama, Costa Rica and Honduras, collected by the scientific community as *Cruziohyla calcarifer* are now known to represent a different species, *Cruziohyla sylviae*. Similarly, the tadpole previously described for *C. calcarifer* also now represents that of *C. sylviae*. Here we describe the tadpole of the true *C. calcarifer* for the first time, including information on ontogenetic changes during larval development. The tadpole of *C. calcarifer* is characterised in having distinctive morphology, mouthpart features and markings.

Keywords: larvae, splendid leaf frog, reproduction, development, morphology

INTRODUCTION

The tadpole of *Cruziohyla calcarifer* (Boulenger, 1902), named *Agalychnis calcarifer* until 2005 (Faivovich et al., 2005), was previously described from specimens collected at La Selva Biological Research station, 2.6 km SE of Puerto Viejo de Sarapiqui, in the north of Costa Rica (Donnelly et al., 1987), which is approximately 1200 km from the type locality for that species. However, following a review of the genus in 2018, when the closely related species *Cruziohyla sylviae* Gray, 2018, was identified and described, it was confirmed that the only species of *Cruziohyla* known occurring at La Selva is *C. sylviae*. No specimens matching the description of the true *C. calcarifer* have ever been recorded at La Selva and the last specimens of *C. calcarifer* to be recorded in Costa Rica, and representing that species' northernmost point of distribution, are an adult male (UCR6480 [UCR = Universidad de Costa Rica]) and female (UCR6285) collected near the Panamanian border in 1996, and a juvenile collected just south of the port of Limon in 1997 (UCR7199).

By comparison, *C. sylviae* has a geographic range known to extend from Panama northwards through Costa Rica to Nicaragua and Honduras (Gray, 2018). As such, La Selva Biological Research Station is central to the known geographical range of *C. sylviae*, and this is the only species in the genus recorded there or any further north. La Selva is only 70 km from the Type locality of *C. sylviae*, being Guayacan, Costa Rica, and all specimens previously recorded as *C. calcarifer* at La Selva, and northward to Honduras, are confirmed as *C. sylviae*

(Gray, 2018). Subsequently, the only *Cruziohyla* tadpole found at La Selva and further north belongs to that of *C. sylviae*, and those found agree with the description provided by Donnelly et al. (1987) (Sub: *A. calcarifer*) (e.g. CRE 6697 [CRE = Natural History Museum of Los Angeles, USA] from Puerto Viejo de Sarapiqui, Costa Rica, and SMF 79425 [SMF = Forschungsinstitut und Natur-Museum Senckenberg, Germany] from Guasimo, Olancho, Honduras).

Cruziohyla calcarifer was described in 1902 (Boulenger, 1902), with the type specimen originating from Ecuador. To date, the species has remained extremely rare with almost nothing known of its breeding biology and the tadpole has not been described. Herein we describe the tadpole of *C. calcarifer*, a species confirmed as having a distribution from north-western Ecuador to only the very south-eastern part of Costa Rica. The description presented herein is based on wild-collected tadpoles and those produced by wild collected specimens from Ecuador, which have had their identity confirmed by 16s mitochondrial DNA (Gray, 2018).

METHODS

16 live juvenile specimens of *C. calcarifer* were obtained from Alto Tambo, Esmeraldas, Ecuador, a locality within the species' recognised range where specimens are considered representative of those from the type locality (Faivovich et al., 2010). The morphological characteristics of the specimens obtained fully match those for *C. calcarifer*, as defined by Boulenger (1902) and Gray (2018). Following exportation to Europe, a representative

specimen (e.g. MM1021 [MM = Manchester Museum, UK]) was genetically confirmed as a true *C. calcarifer* (Gray, 2018). The live specimens developed to adulthood and were subsequently housed at Manchester Museum, England, and also within the private herpetological collection of Konstantin Taupp in Germany.

Two pairs of adult *C. calcarifer* maintained in Germany produced fertile clutches of eggs on 25 April 2020 and 11 May 2020. The egg clutches consisted of 19 (15 fertile) and 11 (6 fertile) eggs that were laid on open leaves, from which five and four tadpoles hatched on 7 May 2020 and 23 May 2020, respectively. The tadpoles were maintained in an aquarium at 20.5 ˚C (+/- 0.5 ˚C), and grew slowly but consistently throughout their development. Tadpoles were staged according to Gosner (1960). Measurements of single representative tadpoles at given stages of development were taken directly with digital calipers to the nearest 0.1 mm, using a digital microscope, and also from accurately scaled digital images (Table 1). Of the nine tadpoles studied, two specimens were euthanised using MS222 at stages 26 and 37 and preserved in formalin so as to afford scientific description and the best direct morphological comparisons of congeneric species (Grosjean, 2005): *C. sylviae* (Donnelly et al., 1987: sub: *A. calcarifer*) and *C. craspedopus* (Hoogmoed & Cadle, 1991). The remaining seven tadpoles completed metamorphoses successfully, taking between 91–101 days from hatching to emergence.

The main description of the tadpole of *C. calcarifer*, its external morphology, is based on a well-developed specimen at Stage 37 (NHMUK/BMNH.2021.6359), and is further supported by a specimen at Stage 26 (NHMUK/BMNH.2021.6360). These specimens are housed with the type specimen of *C. calcarifer* (Boulenger, 1902) at the Natural History Museum, London (NHMUK). Other specimens examined or cited as part of the work are housed at: Manchester Museum (MMUK), Museo de Zoología, Pontificia Universidad Católica del Ecuador (QCAZ), Natural History Museum of Los Angeles, USA (CRE/LACM), Forschungsinstitut und Natur-Museum Senckenberg, Germany (SMF), Coleccion de Herpetología, Escuela de Biología, Universidad de Costa Rica (UCR). Institutional abbreviations used follow Frost (2020).

Terminology of the tadpole description is that of Altig and McDiarmid (1999) and Grosjean (2001). Acronyms used for tadpole measurements are as follows: TL (total length = direct line distance from tip of snout to posterior tip of tail), BL (body length = direct line distance from tip of snout to body terminus), TAL (tail length = direct line distance from body terminus to absolute tip of tail), BW (body width = greatest transversal distance of body), BH (body height = widest vertical point from ventral-dorsal surface), TMH (tail muscle height = vertical distance from the ventral margin of the tail muscle to dorsal margin of tail musculature at midpoint), UFH (upper fin height = maximum vertical distance from tail musculature to dorsal fin margin), LFH (lower fin height = maximum vertical distance from tail musculature to ventral fin margin), MTH (maximum tail height = vertical distance from dorsal to ventral fin margins at mid-point), ED (eye diameter = distance from anterior to posterior corner of eye), IOD (interorbital distance = shortest distance between the centre of the orbits), NW (nostril width), IND (internarial distance = shortest distance between the inner margins of the nostrils), RN (rostro-narial distance = straight line from anterior corner of nostril to tip of labium), RP (rostro-pupilar distance = straight line from anterior corner of eye to tip of snout), NP (nostril-pupilar distance = straight line from anterior corner of eye to posterior margin of nostril), ODW (oral disc width = greatest transversal distance from oral disc margins, LTRF (labial tooth row formula).

RESULTS

Body shape: Ovoid in lateral view, elliptical in dorsal view, depressed (body width = 9.5mm; body height = 8.2 mm), highest and widest at about midpoint of the body. Body length: Approximately 30 % of total length (BL = 16.2 mm; TAL = 53.4 mm). Snout shape: In dorsal and lateral profiles the overall snout shape is rounded. The rounded profile of the snout extends anteriorly, medial to distinct nares which are minimally raised and situated on a shallow fold either side of the mouth: The shallow fold slopes anteroventrally, from nostril to outer margin of oral disc. Nares: Small (0.4 mm) yet well-defined, positioned dorsolaterally, directed anteriorly, located on the same lateral plane as the centre of the pupil. Distance of nostrils from upper labia (RN = 1.5 mm), from the eye (NP = 4.2 mm), apart (IND = 3.6 mm).

Eyes: Dorsolateral, directed laterally, (ED = 2.0 mm), interorbital distance over twice that of internarial distance (IOD = 8.7 mm). Spiracle: Ventral, sinistral to the midline, short (1.0 mm), oblique spiracular opening (1.3 mm wide), situated approximately midway and at the posterior edge of the body, equal distance from eye and mouth (5.0 mm). Tail: Ventral and dorsal tail fins curve outward distally, narrowing toward the terminus; tail highest at the midpoint (MTH = 13.0 mm). Caudal musculature is moderately high and gradually tapers, not quite extending to the tip of the tail. Dorsal fin emerges at the junction of body and tail musculature, ventral fin does not extend onto the body. Height of dorsal fin and caudal musculature at the midpoint of the tail are approximately equal (4.2 mm), ventral fin slightly higher (4.6 mm). Anal tube: Short (1.5 mm), dextral to the caudal fin.

Mouth: Moderately small (ODW = 3.8 mm wide), anteroventral, directed anteriorly. Oral disc not emarginate, labia completely bordered by papillae. Anteriorly a single row of marginal papillae joined by very short length (n = 4 papillae) forming a second papillar row at the mid-dorsal margin. Additional papillae are present submarginally, diminishing to double row ventrally, having a short tertiary row and convex-shaped cluster of papillae mid-ventrally (Fig. 1c). Upper jaw sheath with fine serrations, medially convex, forming a smooth broad arch with long slender lateral processes extending distally. Lower jaw sheath V-shaped, with distinct sharply pointed serrations. Labial teeth present in two anterior (upper) and three posterior (lower) rows. LTRF: 2 (2) / 3 (1). Anterior (upper) rows are long,
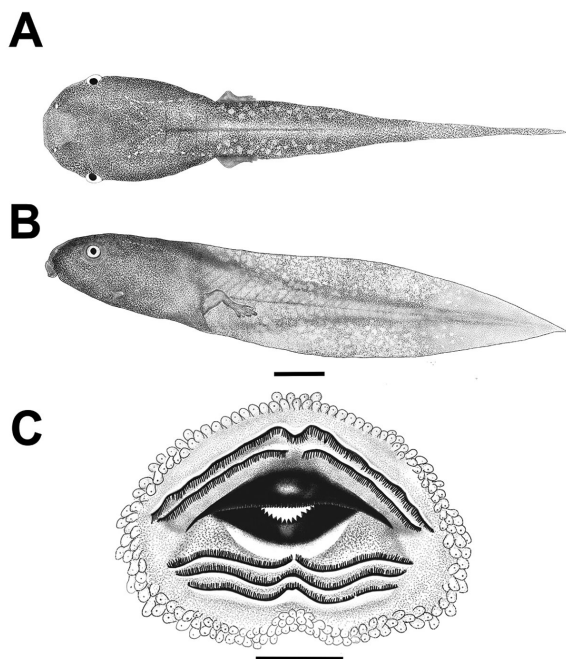
Figure 1. Tadpole of *Cruziohyla calcarifer* (NHMUK/BMNH 2021.6359) Stage 37. **(A)** Dorsal view; **(B)** Lateral view, scale bar = 5mm; **(C)** oral disc, scale bar = 1mm.

extending laterally nearly to the submarginal papillae, first row forms distinct dip medially, second upper row narrowly interrupted medially. Posterior rows becoming progressively shorter posteriorly, upper posterior labial teeth row narrowly interrupted medially, lower two rows are complete.

Coloration: In life, background colour of body at Stage 37 is tan brown, having fine pale yellow marbling on the dorsal and ventral surfaces of the body, the spiracle, and anterior part of tail. Tail musculature pale tan, pale grey reticulations to both dorsal and ventral fins extending three-fourths the length of the tail. A series of fine gold-coloured speckles on the body surface represent clearly visible lateral line configurations (Fig. 2). The Internal organs are not clearly visible through the ventral surface of the body. Iris is silver-grey, its periphery having gold reticulation. In preservative, coloration of body pale yellow having grey markings throughout, area anterior to the eyes pale yellow, dorsally a dark grey band extends interorbitally. Pancreas and duodenum seen through ventral surface of the body as light and dark areas respectively. Tail musculature predominantly pale yellow with fine grey mottling anteriorly. Dorsally the pale-yellow colour of the tail musculature extends onto body, symmetrically either side of a dark grey mediodorsal line. Spiracle, dorsal and ventral fins translucent. Iris is black. Oral disc and papillae remain translucent with fine dark speckling, oral surfaces of upper and lower jaw sheaths are black.

Ontogenetic changes:
Changes in colour
Stage 24: Body and tail very dark brown, tail musculature lighter brown. Iris brown with golden inner edge. Some internal organ coloration is visible through the ventral surface of the body, primarily the intestinal tube which is turquoise blue. **Stages 25–26:** The overall body coloration lightens considerably, the tail musculature is pink and redness of the heart muscle is visible through the ventral surface of the body. Externally the orientation of neuromasts forming the lateral line system are clearly visible as fine gold-coloured speckles on the body surface and thus able to be detailed (Fig. 2). **Stage 25–30:** Body tan-brown with fine yellow marbling to lateral body surfaces and anterior of tail. Pupil round, iris silver with reticulations, periphery has gold reticulation. Internally, the intestinal tube loses its blue coloration to become dark grey, externally a pink hind limb bud develops (Fig. 3). **Stages 30–39:** Spiracle is evident but transparent, brown/yellow marbling coloration on the body and at junction of caudal musculature becomes progressively more extensive and more contrasted in the later stages as the background body coloration becomes a darker brown. In later stages, undersides of toes develop dark brown pigmentation, tubercles and toe-pads pale yellow. **Stages 40–43:** Dorsal median, upper labium and tail is tan brown. Dorsal surfaces of arms, legs, flanks and eye-snout light grey-brown, concealed surfaces yellow. Distinct dark brown markings present on ventral thigh region. **Stage 43:** In contrast to the brown tail musculature the dorsal and lateral fins turn distinctly black as tadpole emerges and the tail atrophies.

Changes in morphology
Notable morphological changes include a distinct change in body shape: in the early stages (stages 25–26) the body is higher than wide, becoming dorsally depressed (wider than high) from Stage 26 (Table 1). This phenomenon is known in both other members of the genus (Donnelly et al., 1987; Hoogmoed & Cadle, 1991). At stages 25–26 the tail musculature and both dorsal and ventral fins at the midpoint are of equal width, whereas from Stage 26 the ventral fin is marginally higher than the caudal musculature and dorsal fin. At Stage 30 the bilateral myotonic muscle masses in the tail musculature are more clearly defined, the nostrils become more prominent either side of snout. At Stage 37 the pupil has developed an elliptical shape, the spiracle is still evident, and the dorsal lateral lines remain. Between stages 37–41 a notable increase in the growth of the tail length is seen compared with that of the body length, the hind limbs become well-developed, and at later stages further widening of the body is evident, attributable to the developing forearms that have not yet emerged. Between stages 39–40 the distance between the anterior labium and medial aspect of the snout reduces as the snout becomes truncate in lateral plane. From Stage 41, with the onset of metamorphosis, the tail begins to shrink, viewed from above the snout becomes more pointed, and the nostrils are further defined. A small notch also appears at the bottom of the pupil in respect of initiating the pupils' development to becoming vertical in shape. The outward facing calcar on the heel becomes apparent in some specimens.
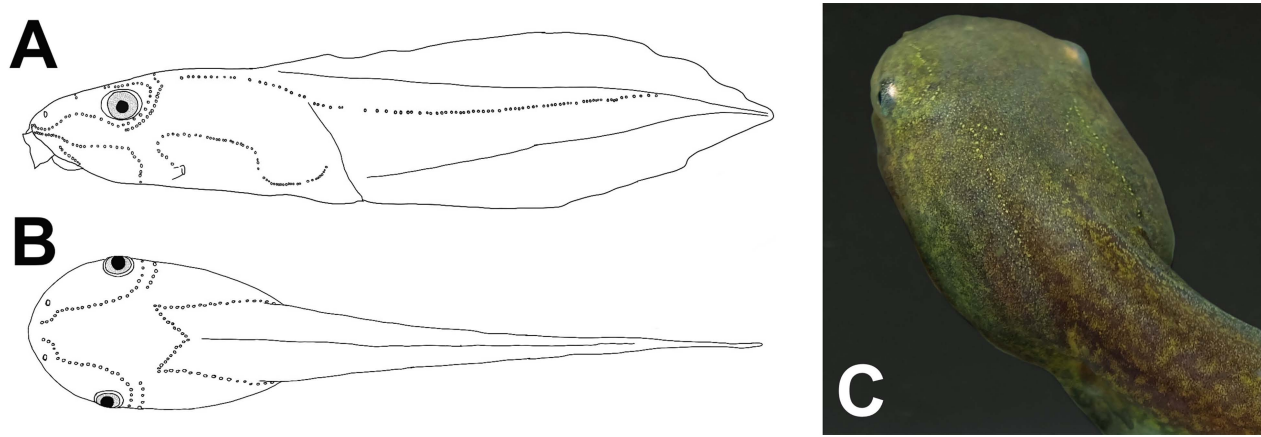
Figure 2. Tadpole of *Cruziohyla calcarifer* (NHMUK/BMNH 2021.6360) at Stage 25, showing lateral line organs. **(A)** Lateral view; **(B)** Dorsal view; **(C)** In life.

Table 1. Morphometric measurements (in mm) of single representative tadpoles of *Cruziohyla calcarifer* during ontogeny

Day from hatching	Day 1	Day 7	Day 14	Day 21	Day 28	Day 42	Day 54	Day 61	Day 70	Day 77	Day 82	Day 89
Stage	24	25	25	26	26	30	36	37	38	39	40	41
Total length	19.0	26.1	32.0	37.3	47.3	50.1	52.9	53.4	58.6	64.0	67.0	66.0
Body length	4.4	7.1	8.8	10.3	15.3	15.8	16.0	16.2	18.0	20.0	20.0	20.0
Tail length	14.6	19.0	23.2	27.0	32.0	34.3	36.9	37.2	40.6	44.0	47.0	46.0
Body Height	3.5	5.1	5.8	6.3	7.8	8.0	8.2	8.2	10.5	10.8	10.8	9.4
Body width	-	-	-	-	8.8	9.2	9.4	9.5	11.8	12.0	12.1	13.0
Tail muscle height	0.8	2.0	2.6	3.0	3.0	3.7	4.0	4.2	4.4	5.4	5.5	5.0
Dorsal fin height	1.2	2.0	2.6	2.9	3.0	3.2	4.0	4.2	4.4	4.4	4.8	4.0
Ventral fin height	1.7	2.0	2.6	3.0	3.2	3.6	4.2	4.6	4.8	5.5	5.5	4.5
Eye diameter	0.9	1.1	1.1	1.2	1.4	1.6	1.8	2.0	2.0	2.0	2.0	2.0
Eye to nostril	0.6	1.6	1.8	2.0	3.4	3.6	4.0	4.2	4.4	4.5	4.3	4.0

DISCUSSION

Reproductive behaviour, egg deposition and clutch size

Initial observations of the breeding behaviour of the true *C. calcarifer*, both in captivity and in the wild, indicate the species has reproductive traits which differ from both other members of the genus: *C. calcarifer* deposit their egg clutches on the lower central section of leaves overhanging small ponds or open water bodies, whereas *C. craspedopus* and *C. sylviae* lay egg clutches above flooded hollows or water cavities between buttresses of fallen trees (Marquis et al., 1986, sub: *A. calcarifer*; Donnelly et al., 1987: sub: *A. calcarifer*; Hoogmoed & Cadle, 1991; Caldwell, 1994: sub: *A. calcarifer*; Duellman, 2001, sub: *A. calcarifer*; Savage, 2002, sub: *A. calcarifer*; Kubicki, 2004, sub: *A. calcarifer*). The courtship and breeding behaviour witnessed for *C. calcarifer* appears most similar to that of *Agalychnis spurrelli* (Boulenger, 1913), where adult *C. calcarifer* congregate 'en masse' and lay their eggs on leaves in and around open ponds (personal communication with Miguel Solano in reference to observations in the mid-nineties in the Fila Carbon, Costa Rica). In the Changuinola drainage, Panama, groups of calling *C. calcarifer* males with females were also commonly found together in the understory above a headwater streamlet (Myers & Duellman, 1982),

and in Alto Tambo, Ecuador, 17 eggs of *C. calcarifer* were found deposited in the centre of an open leaf (Fig. 3a) with the species' tadpoles found in pools by the side of a road (e.g. QCAZ37723), together with those of *A. spurrelli*.

Egg clutch sizes of *C. calcarifer* produced in captivity (n = 11–19) are consistent with those found in the wild (n = 17) at Alto Tambo, Ecuador (Fig. 4a). These small clutch sizes are comparable with those produced by its sister species, *C. craspedopus*, which laid between 9–22 eggs (mean 14, n = 13) in captivity (personal observation of 3rd author). The numbers are consistent with clutch sizes for *C. craspedopus* in the wild: 14–21 eggs (mean 17, n = 10), Hoogmoed & Cadle, 1991 and 2–16 eggs (mean 10.8, n = 5), Block et al. (2003). However, in contrast to both *C. calcarifer* and *C. craspedopus*, egg clutch sizes for *C. sylviae* are repeatedly reported to be significantly larger: 30–40 eggs, Marquis et al., 1986 (sub: *A. calcarifer*); 13–27 eggs, Donnelly et al., 1987 (sub: *A. calcarifer*); 20–28 eggs, Caldwell, 1994 (sub: *A. calcarifer*); 10–54 eggs, Savage, 2002 (sub: *A. calcarifer*). More recent findings through committed long-term monitoring of wild *C. sylviae* in Costa Rica confirm that egg clutches of 12–38 eggs (mean 29, n = 22) are indeed representative for that species (personal communication, Pepo Marsant, 2020).
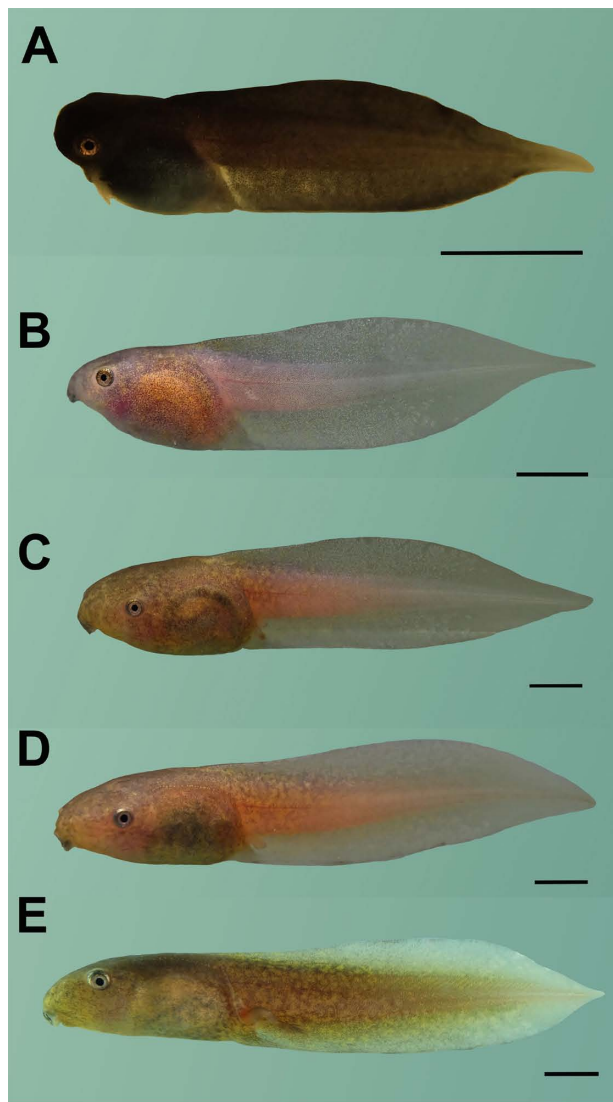
Figure 3. Tadpole of *Cruziohyla calcarifer* at various stages of development: **(A)** Stage 24; **(B)** Early Stage 25; **(C)** Stage 29; **(D)** Stage 34; **(E)** Stage 36. Scale bar = 5mm.

Tadpole development and characteristics

Development times in *Cruziohyla* tadpoles are highly variable between and within species, presumably due to the differing conditions, including water temperature, volume, tadpole density and food supply. The development of wild collected *C. calcarifer* tadpoles from Durango, Ecuador, took approximately 50 days from egg to metamorphosis (e.g. QCAZ 37745), whereas the development of captive *C. calcarifer* tadpoles raised during this work took approximately twice as long (Mean: 96 days). Similarly, *C. sylviae* tadpoles raised in captivity have taken between 66–78 days to metamorphose (mean 71 days; Gray, 2002, sub: *A. calcarifer*), compared with those raised by Donnelly at al., 1987 (sub: *A. calcarifer*) which took 6 months to complete metamorphosis. Allowing for varying developmental timescales, some growth differences between the species during ontogeny are reported. For example, in this study some increases in growth were seen in the tail length of *C. calcarifer* tadpoles between stages 37–41, similar to that reported for *C. craspedopus* tadpoles by Hoogmoed & Cadle, 1991, whereas in the tadpoles of *C. sylviae* reported on by Donnelly et al. (1987) (sub: *A. calcarifer*) tail lengths at the same stages showed little if any growth.

The mouthparts of tadpoles belonging to each *Cruziohyla* species share some common features as well as individual defining characteristics: Having a complete row of marginal papillae on the anterior labium is a characteristic seen in tadpoles of all members of the genus from Stage 27. The anterior (upper) top tooth row of *C. calcarifer* shows a distinct median dip, which it shares only with *C. craspedopus* (Hoogmoed & Cadle, 1991) and not *C. sylviae* (Donnelly et al., 1987: sub: *A. calcarifer*); Tadpoles of *C. calcarifer* also possess a short double row of papillae to the medial-upper labium which is a unique feature among the tadpoles in the genus. Apart from overall visual differentiation (Fig. 5), reproductive traits and larval characteristics can be combined when summarising the diagnostic comparisons one can make between the tadpoles of species in the genus *Cruziohyla* (Table 2).
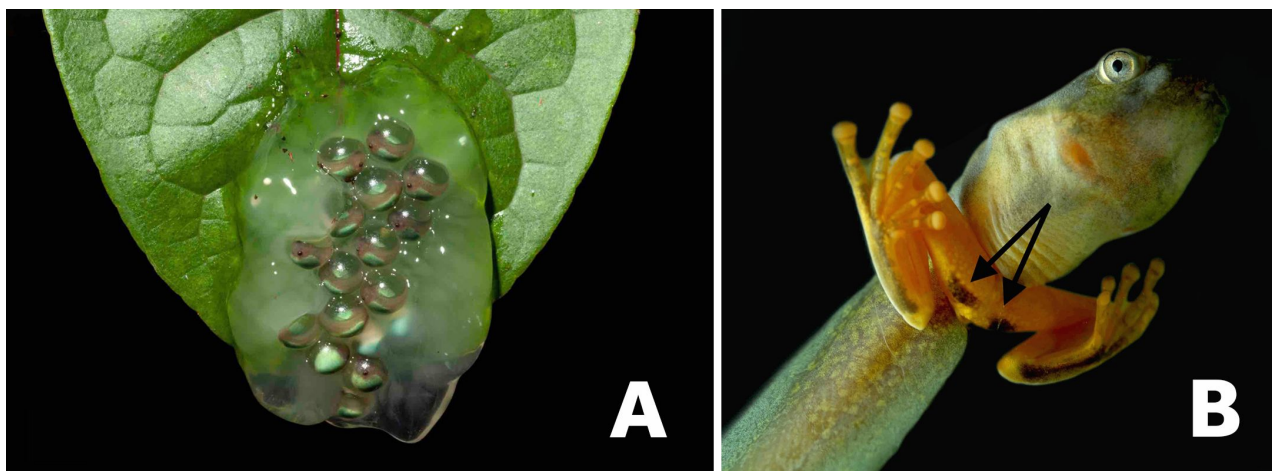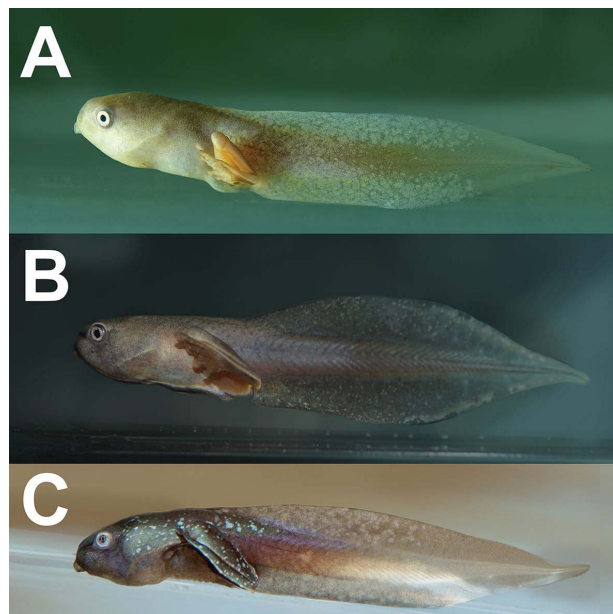

Figure 4. Tadpole of *Cruziohyla calcarifer*, in early and late stages of development: **(A)** Prior to hatching from small egg clutch laid on open leaf, Alto Tambo, Esmeraldas, Ecuador; **(B)** Showing dark markings on thighs, a unique characteristic of the tadpole.

Table 2. Diagnostic comparison of egg deposition and tadpoles in the genus *Cruziohyla*

	C. calcarifer	*C. sylviae*	*C. craspedopus*
Body: Widest point of body, flatness of dorsal surface, hind-limb shape	Body widest at midpoint. Back is not flattened. No indentation formed in tarsal fold	Widest point of body posteriorly. Back is not flattened. No indentation formed in tarsal fold	Body widest just behind eye. Back is distinctly flattened. Pronounced indentation in tarsal fold
Coloration: Overall body and tail colouration in later stages of development	Tan brown. No conspicuous spotting to body or tail	Dark olive-green to brown. No conspicuous spotting to body or tail	Dark olive-green to grey-black. Having conspicuous blue-grey spotting
Mouthparts: Papillae and tooth rows	Short double row of papillae to medial-anterior labium. Anterior top tooth row possesses distinct medial dip	Single row of papillae to medial-anterior labium. No top tooth row medial dip	Single row of papillae to medial-anterior labium. Anterior top tooth row possesses distinct medial dip
Markings: Dark ventral thigh markings present in later stages of development	Yes	No	No
Lateral lines: Neuromasts form defined 'M'- shape on dorsal body surface	Yes	No	No
Ecology: Site of egg deposition and water-body type	Deposits eggs on centre/tip of a leaf, over open water-bodies such as small roadside pools, ditches, ponds	Deposits eggs on roots, vines, leaf stems over flooded hollows or water cavities between buttresses of fallen trees	Deposits eggs on roots, vines, leaf stems over flooded hollows or water cavities between buttresses of fallen trees
Reproduction: Egg clutch size	< 25	10 – 55 >	< 25

Figure 5. Tadpoles of the genus *Cruziohyla* (Stage 41): **(A)** *Cruziohyla calcarifer*; **(B)** *Cruziohyla sylviae*; **(C)** *Cruziohyla craspedopus*.

The morphology and overall coloration of captive-bred *C. calcarifer* tadpoles originating from Ecuadorian *C. calcarifer* agree fully with wild specimens and those collected and raised in-country (e.g. QCAZ 37745). The dark brown markings on the ventral surfaces of the thigh region seen later in development (stages 40>) in the tadpole of *C. calcarifer* is a feature unique to this species (Fig. 4b).

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Author Contributions:

We can confirm that the above authors all played a high level of contribution to this research and that without each the work would not have been possible: The corresponding author instigated and led the project, conducted fieldwork in Ecuador, facilitated the acquisition of the key specimens, heavily researched comparative material, and wrote up the major part of the description. The second author was also involved in specimen acquisition, captive breeding and rearing the specimens concerned, measurements, and writing. The third author was likewise involved, breeding and rearing congener species to provide key data, specimen acquisition, and manuscript contributions. The fourth author contributed to writing and was also responsible for conducting the invaluable genetic work that clarified the identity of the specimens involved. The fifth author contributed greatly by illustrating the detailed morphology shown in the figures, and with manuscript contribution.

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