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# Reproductive biology and geographic variation of *Zachaenus carvalhoi* (Anura: Cycloramphidae), a Brazilian Atlantic Forest frog

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**Abstract**

**Reproductive biology and geographic variation of *Zachaenus carvalhoi* (Anura: Cycloramphidae), a Brazilian Atlantic Forest frog.** Information is presented on the reproductive biology, habitat, behavior, morphology, and variation in advertisement call in *Zachaenus carvalhoi,* a rare frog for which basic natural history data are lacking. The species inhabits forest leaf litter in the states of Minas Gerais and Espírito Santo in southeastern Brazil. Reproductive individuals were found from October to December; females are slightly larger than males. The International Union for Conservation of Nature currently lists *Zachaenus carvalhoi* as Data Deficient. We suggest transferring the species to the Near Threatened category based on the IUCN criteria A2 because of the decline of the size and quality of its severely fragmented habitat.

**Keywords:** conservation, morphology, sexual dimorphism.

**Resumo**

**Biologia reprodutiva e variação geográfica de *Zachaenus carvalhoi* (Anura: Cycloramphidae), um anuro da Mata Atlântica.** Apresentamos aqui informações sobre biologia reprodutiva, habitat, comportamento, morfologia e variação no canto de anúncio de *Zachaenus carvalhoi*, um anuro raro para o qual dados básicos de história natural são escassos. A espécie habita o folhiço de florestas nos estados de Minas Gerais e Espírito Santo, sudeste do Brasil. Indivíduos em estado reprodutivo são encontrados de outubro a dezembro, e as fêmeas são pouco maiores que os machos. A União

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Internacional para a Conservação da Natureza atualmente inclui *Zachaenus carvalhoi* na categoria Dados Deficientes. Sugerimos transferir a espécie para a categoria Quase Ameaçada, baseados no Critério A2, que inclui diminuição contínua da extensão e qualidade do seu habitat, já severamente fragmentado.

**Palavras-chave:** conservação, dimorfismo sexual, morfologia.

## Introduction

*Zachaenus carvalhoi* Izecksohn, 1983 is a small, stout brownish frog that inhabits the forest floor (Figure 1). It was described from Santa Teresa, state of Espírito Santo, southeastern Brazil (Izecksohn 1983). In the last decade, additional specimens have been reported from the states of Minas Gerais and Espírito Santo (Dayrell *et al.* 2006, Verdade *et al.* 2009, Motta *et al*. 2010, Salles and Maciel 2010) (Figure 2). These new records, although mainly anecdotal, provide information on diet, activity period, habitat, defensive and calling behavior, and the description of the advertisement call (Verdade *et al.* 2009, Moura *et al.* 2012, Guimarães *et al.* 2013, Zocca *et al.* 2014, Mollo-Neto *et al*. 2015). Herein, we present data from field and laboratory work that complement the information on the reproductive biology of *Z. carvalhoi*. These data, together with information from the literature, allow us to reassess the listing of the species position in the IUCN Red List, where it currently is classified as Data Deficient (DD) (IUCN 2016).

**Figure 1.** A male *Zachaenus carvalhoi* from Parque Estadual da Serra do Brigadeiro, Minas Gerais state, southeastern Brazil. Photo: Diego J. Santana.

## Materials and Methods

We conducted the field work at Juiz de Fora municipality (JF; 21°41'20" S, 43°20'40" W; 700–900 m a.s.l.; datum WGS 84), state of Minas Gerais, in the Atlantic Forest Domain (Ab'Saber 1970). The frogs were collected from three forest fragments: Fazenda Floresta (370 ha; 21°44'33" S, 43°17'32" W; 810 m a.s.l.; datum WGS 84); Reserva Biológica Municipal Poço D'Anta (277 ha; 21°45'15" S, 43°18'39" W; 850 m a.s.l.; datum WGS 84); and Parque Municipal da Lajinha (88 ha; 21°47'32" S, 43°22'51" W; 900 m a.s.l.; datum WGS 84). The average temperature in the municipality of Juiz de Fora varies around 19°C and the yearly precipitation is about 1500 mm. The rainy season extends from October to April (January the rainier month with an average precipitation of 298 mm). From May to September the temperatures are lower and the rains are less frequent (Cesama 2016). We installed three 100 m, straight-line transects of pitfall and funnel traps (Heyer *et al.* 1994) in each forest fragment. Each transect contained five 20 L buckets intercalated with five pairs of funnel traps; these were linked by one meter high plastic fences. There was a total of 45 buckets and 90 funnel traps in Juiz de Fora municipality. The traps

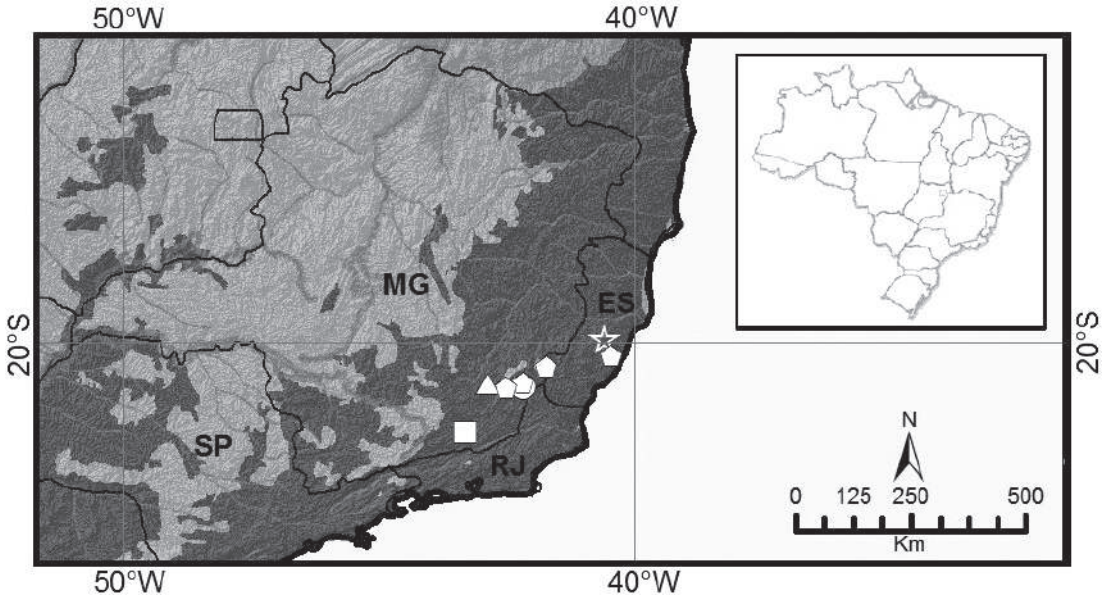


**Figure 2.** Map showing the geographic distribution of *Zachaenus carvalhoi*. The type locality, Santa Teresa (Izecksohn 1983) municipality (star); the municipality of Juiz de Fora (square); Vi osa (triangle); Eugen polis (circle); and from left to right, the municipalities of Muria (Motta *et al.* 2010), Pedra Dourada (Dayrell *et al.* 2006), Ibitirama (Verdade *et al.* 2009), and Cariacica (Tonini *et al.* 2010) (pentagons). Abbreviations indicate the Brazilian states of Minas Gerais (MG), Esp rito Santo (ES), Rio de Janeiro (RJ), and S o Paulo (SP).

were open continuously from October 2008 to April 2009, and were inspected twice a week (total sampling effort = 9,180 buckets/day and 18,360 funnels/day). Two fragments (Reserva Biológica Municipal Poço D'Anta and Parque Municipal da Lajinha) were also surveyed in an active four-day search in November 2009. Three persons searched the leaf litter in 40 plots (1 $m^2$) both in the afternoon and at night; thus, there was 24 h of active search of 40 $m^2$ of litter. We marked the plots in areas where calling males of *Zachaenus carvalhoi* were observed, in an attempt to locate egg clutches and tadpoles. After recording data, we released most frogs at the same place we captured them. We euthanized a voucher series of individuals ($N$ = 27) using a lethal dose of anesthetic, preserved in 10% formalin, and stored in 70% alcohol at the herpetological collections of the Universidade Federal de Juiz de Fora (UFJF) and Museu de Zoologia da Universidade de São Paulo (MZUSP).

To document call variation, we studied calls from Parque Estadual da Serra do Brigadeiro [the locality from which Guimarães *et al.* (2013) described the call], as well as (1) Parque Municipal da Lajinha fragment, Juiz de Fora, where one call was recorded in November 2009 with a digital recorder Panasonic RR-US470®, and (2) Viçosa municipality (20°47'46" S, 42°51'29" W; datum WGS 84), where 16 calls emitted by a single male were recorded in October 2011 with a digital recorder Olympus LS-10 linear PCM® and an ATR55 Telemike TM® directional microphone. The digital recordings were analyzed using Raven Pro 1.3 for Macintosh (Cornell Laboratory of Ornithology). The acoustic variables measured were call

dominant frequency, call length, number of notes per call, and number of pulses per note. Acoustic terminology follows that of Duellman and Trueb (1994). The recordings are deposited at the sound collections of "Laboratório de Evolução e Diversidade", Universidade Federal do ABC (ZUFABC 19), and "Laboratório de Anfíbios e Répteis", Universidade Federal do Rio Grande do Norte (ASUFRN 202–203).

We examined and measured preserved material (Appendix I) to assess morphological variation between *Zachaenus carvalhoi* from the municipalities of Ibitirama (Espírito Santo state) and Juiz de Fora (Minas Gerais state), and to determine whether the species is sexually dimorphic. We compared different samples with Student's *t*-test, and used only adults in the comparisons (SVL > 25 mm, according to our data on testicular development). Measurements were taken with Mitutoyo calipers, as follow: snout-vent length (SVL) measured from the edge of the snout to the vent opening; head length (HL) perpendicular to the imaginary line linking the angle of jaws to the tip of the snout; head width (HW) between the angle of jaws; horizontal eye diameter (ED) from posterior to anterior corners of the eye; eye-nostril distance (END) from the anterior corner of the eye to the outer edge of the nostril; interorbital distance (IOD) between the inner edges of the eyelids; hand length (HaL) ventrally, from the junction of radio-ulna and carpal to the tip of Finger III; femur length (FeL) ventrally, from the cloacal opening to the knee joint; tibia length (TL) ventrally, from the knee to the tarsal-metatarsal joint; foot length (FoL) ventrally, from the heel to the tip of the Toe IV; free part of humerus (FPH) from the elbow to the edge of the axilla; forearm length (FL) from the elbow to the wrist joint; carpal tubercle length (CTL) at greatest length, toward Finger I; metatarsal tubercle length (MTL) ventrally, at greatest length, toward Toe I; Finger I length (F1L) on the right hand, from the base to the tip of the Finger I; Finger II length (F2L) ventrally, on the right hand, from the base to the tip of the Finger II.

We dissected 38 specimens from the UFJF ($N$ = 35) and MZUSP ($N$ = 3) collections, and used them to study sexual dimorphism and reproductive biology in *Zachaenus carvalhoi*. We sexed the individuals by gonadal examination. We checked females for the presence of oocytes in the ovary, and inferred testicular development in males by measuring the length and width of the right and left testicles in ventral view. We used an ellipse formula to estimate the surface area of each testicle. Data on testicles development were analyzed in a regression plot using the SVL as dependent variable. We used a Student's *t*-test to compare male and female body proportions.

## Results

### *Temporal Abundance Variation*

A total of 49 individuals of *Zachaenus carvalhoi* was captured during field work in Juiz de Fora from October 2008–April 2009; all but one (collected actively in the trail), was captured using passive search (or 0.002 individual per trap day summing buckets and funnels). The active search for frog on plots was conducted in November 2009, and added a single frog to this number (or 0.025 frog/m$^2$). All frogs were captured during the rainy season: October ($N$ = 7), November ($N$ = 13), December ($N$ = 14), January ($N$ = 5), February ($N$ = 6), March ($N$ = 3), and April ($N$ = 2). We captured the frogs in all fragments studied, with the Parque Municipal da Lajinha having the largest sample ($N$ = 20).

Figure 3 shows the SVL range of specimens captured by month in Juiz de Fora (data from specimens deposited at UFJF herpetological collection, $N$ = 11, plus 27 measured before setting free specimens during field work). The smallest juvenile was captured in December. There are individuals from two SVL classes in October, November, and December that juveniles (SVL < 22mm) and fully grown up adults (SVL > 25mm).

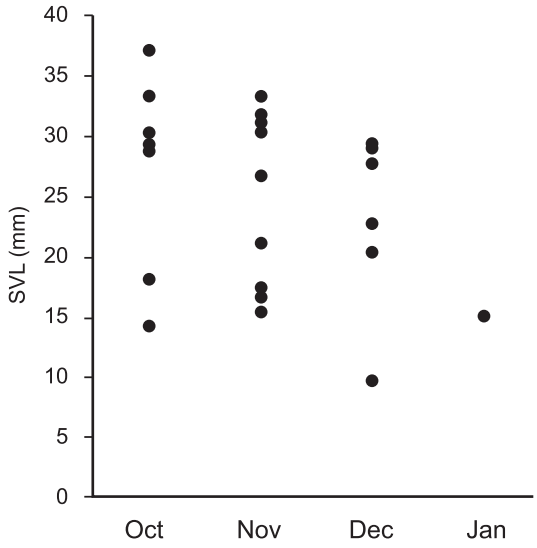


**Figure 3.** SVL variation (in mm) in *Zachaenus carvalhoi* captured by month during fieldwork in Juiz de Fora, Minas Gerais state, southeastern Brazil.

Capture did not elicit defensive behavior from the *Zachaenus carvalhoi*. However, we did observe the frogs biting each other, as well as other species (e.g., *Rhinella ornata*) when two or three individuals were put together in a plastic bag.

*Advertisement Call*

The frogs were heard on only two occasions in Juiz de Fora on 04 and 05 November 2009. They were calling infrequently (with intervals of 20 min or more) hidden under the leaf litter and only at night, beginning around 20:00 h when it was raining hard. The male recorded was captured buried approximately 12 cm under leaf litter. At Viçosa, several males were heard inside the forest fragment calling actively from at least 19:00 h to 23:00 h amid the leaf litter, and under fallen trunks, where they were buried under 5–20 cm of substrate.

The call of *Zachaenus carvalhoi* from Juiz de Fora is composed of four notes, with 9 pulses/note. The call length is 0.20 s, and the dominant frequency is 2584.0 Hz. The calls from Viçosa have 1–5 notes, with 2–6 pulses/note. When the frog emitted sequences of calls, the call length varied from 0.17–0.32 s (0.24 ± 0.50) and the dominant frequency from 2067.2–2756.2 Hz (2519.38 ± 250.50). The recordings from Juiz de Fora and Viçosa are similar to those from Parque Estadual da Serra do Brigadeiro (Table 1). The data available on temperature and vocalizing male size are similar among localities. The most significant difference between the calls we recorded in Juiz de Fora and Viçosa is the number of males calling, but we did not test for call parameters variation related to density of vocalizing males.

*Reproduction and Sexual Dimorphism*

We examined gonads of 38 individuals—15 males, 15 females, and eight juveniles that were not sexed. We did not find sexual dimorphism in external morphology. Male *Zachaenus carvalhoi* lack nuptial pads and spines, and the arms are not hypertrophied. The vocal sac is single, subgular, and internal (Liu 1935); thus, it is not conspicuous and may be mistaken for a preservation artifact. The dorsal and ventral color patterns are polymorphic, and sex-independent. Females are slightly larger than males (females SVL = 30.90 ± 2.70; males SVL = 28.90 ± 1.90; $p = 0.04$); nevertheless, there are no clear differences between male and female body proportions (Table 2).

Figure 4 shows the relation between SVL and testicles development (inferred by the sum of the area of the ellipsoid surface of left and right testicles). Testicular development in males of about 30 mm SVL is highly variable; this may relate to reproductive period. It also seems that the curve representing the relation between testicular area and SVL steepens in males having a SVL equal to or greater than 25 mm, which may be related to sexual maturity. Ten females collected in October, November, and December had developing oocytes; their number varies

**Table 1.** Summary of call parameters for *Zachaenus carvalhoi* reported in the literature: ST, Santa Teresa municipality in Esp rito Santo state from the original description of the species (Izecksohn 1983); and PESB (*N* = 3 male; *N* = 18 calls) from the original description of advertisement call (Guimar es *et al*. 2013). Additionally, there are data from calls recorded in the field at Juiz de Fora (JF; *N* = 1 male; *N* = 1 call), and Vi osa (VI; *N* = 1 male; *N* = 16 calls), all at the Minas Gerais state, southeastern Brazil. Localities reflect a northern to southern gradient. *Guimar es *et al*. (2013) "group of pulses" correspond to which we name notes.

| Call parameters | ST | PESB | VI | JF |
|---|---|---|---|---|
| Temperature (°C) | - | 21.5 | 20 | 20 |
| Male SVL (mm) | - | 28.6 | - | 29.0 |
| Dominant frequency (Hz) | - | 2067.2–2584.0 | 2067.2–2756.2 | 2584.0 |
| Call length (s) | 3 | 0.20–0.41 | 0.17–0.32 | 0.20 |
| Notes per call | 7–8 | 3–6* | 1–5 | 4 |
| Pulses per note | - | 2–12* | 2–6 | 9 |

**Table 2.** Measurements of male and female *Zachaenus carvalhoi* at Juiz de Fora and Ibitirama municipalities, southeastern Brazil. Only individuals with SVLs greater than 25 mm were measured. For abbreviations, see Material and Methods. Data are presented as (mean ± standard deviation) and range.

| | Males from Juiz de Fora (*N* = 10) | Males from Ibitirama (*N* = 2) | Females from Juiz de Fora (*N* = 13) |
|---|---|---|---|
| SVL | (28.8 ± 2) 25.59–31.72 | (29 ± 0.8) 28.42–29.61 | (30.9 ± 2.7) 26.26–37.03 |
| HL | (11.6 ± 0.7) 10.53–12.56 | (12.3 ± 0) 12.35–12.44 | (12.6 ± 1.1) 10.7–14.44 |
| HW | (13.4 ± 0.8) 11.87–14.47 | (14.1 ± 0.3) 13.96–14.4 | (14.3 ± 1.3) 12.62–16.88 |
| ED | (2.4 ± 0.2) 2.1–2.92 | (2.4 ± 0.1) 2.32–2.55 | (2.6 ± 0.5) 1.94–3.38 |
| END | (2.1 ± 0.2) 1.77–2.53 | (2 ± 0.5) 1.64–2.42 | (2.5 ± 0.4) 2.05–3.32 |
| IOD | (3.7 ± 0.4) 3.24–4.27 | (3 ± 0.2) 2.95–3.24 | (4.0 ± 0.6) 3.14–5.24 |
| FPH | (2.8 ± 0.5) 2.18–3.99 | (2.2 ± 0.3) 1.96–2.46 | (3.3 ± 2.0) 1.59–9.36 |
| FL | (6 ± 0.5) 5.08–6.83 | (6.8 ± 0.4) 6.54–7.14 | (6.6 ± 0.9) 5.4–8.45 |
| HaL | (6.9 ± 0.5) 6.26–7.7 | (7.5 ± 0) 7.57–7.62 | (7.5 ± 0.5) 6.84–8.14 |
| FeL | (12.9 ± 1.2) 10.4–10.41 | (14.1 ± 0.4) 13.88–14.51 | (14.0 ± 1.4) 11.6–16.71 |
| TL | (11.9 ± 0.6) 10.89–13.09 | (12.6 ± 0.2) 12.4–12.8 | (12.7 ± 1.0) 10.78–14.65 |
| Fol | (18.2 ± 1.2) 16.19–19.82 | (19.6 ± 1.1) 18.85–20.43 | (19.3 ± 4.0) 13.28–29.04 |
| CTL | (1.6 ± 0.1) 1.29–2.01 | (1.6 ± 0.1) 1.48–1.74 | (1.7 ± 0.2) 1.43–1.98 |
| MTL | (1.3 ± 0.2) 1.04–1.69 | (1.7 ± 0.2) 1.53–1.95 | (1.4 ± 0.1) 1.24–1.64 |
| F1L | (2.1 ± 0.2) 1.76–2.48 | (2.4 ± 0.1) 2.3–2.52 | (2.3 ± 0.2) 1.97–2.61 |
| F2L | (1.8 ± 0.2) 1.52–2.21 | (2 ± 0.2) 1.9–2.27 | (2.0 ± 0.3) 1.46–2.36 |

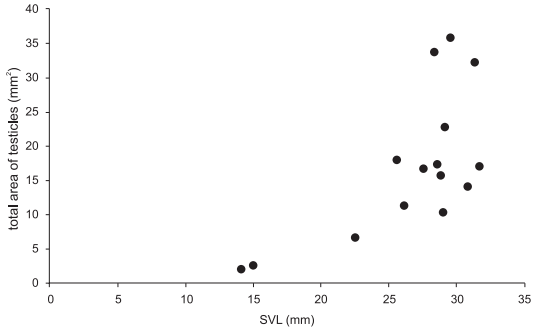


**Figure 4.** Relation between SVL (in mm) and testicular development (in mm²) in *Zachaenus carvalhoi*.

from 41–65 (55.90 ± 8.20). Only three females have mature oocytes. They were collected in October ($N$ = 2) and December ($N$ = 1), and the total number of mature oocytes varies from 18–21, with an average diameter of 3.30 ± 0.50 mm. Mature oocytes are uniformly yellowish and lack dark pigmentation at the animal pole. There was no correlation between female SVL and number of oocytes. Females with a SVL of 30 mm have from 20 to more than 60 oocytes in development.

*Morphometric Variation*

We found no differences in external morphology among the populations. There is overlap in SVL among Juiz de Fora, Ibitirama, and Santa Teresa municipalities; the smallest frog was from Juiz de Fora (25.6 mm) and the largest from Santa Teresa and Juiz de Fora (37.0 mm). Leg proportions also overlap (femur length/SVL: Santa Teresa 45–53%, Ibitirama 48–49%, and Juiz de Fora 41–50%; tibia length/SVL: 43–50%, 45–65%, and 37–46%; and foot length/SVL: 67–75%, 63–71%, and 59–88%).

## Discussion

The compilation of data on the natural history and distribution of *Zachaenus carvalhoi* from the literature and our study indicates that the species is a diet generalist (Mollo-Neto *et al*. 2015), forest-dependent, and has a uniform morphology and advertisement call (Guimarães *et al*. 2013, this work). The distribution is associated with the mountain complexes (800–1200 m a.s.l.) within the Atlantic Forest domain between the Rio Doce and Rio Paraiba do Sul (Verdade *et al*. 2009, this work). As originally described (Izecksohn 1983), the call at the type locality is "rough" with seven to eight notes lasting about three seconds; thus, it was composed of a few more notes and lasted longer than the records from Parque Estadual da Serra do Brigadeiro (Guimarães *et al.* 2013), Juiz de Fora, and Viçosa (Table 1). Reproduction is restricted to the rainy season, and may include two spawns per period. The adult females unexpectedly had twice as many immature oocytes than the number we found in females with mature oocytes. Although Coelho-Augusto *et al.* (2013) reported a clutch size of 37 eggs in *Z. carvalhoi*, they, in fact, examined the oocytes of a collected female. The clutch size of *Z. carvalhoi* is 20 eggs (Zocca *et al.* 2014), similar to the number of mature oocytes we found (18–21), and concordant with the clutch size (10–31) known in the sister species *Zachaenus parvulus* (Lutz 1944, Heyer and Crombie 1979, Van Sluys *et al.* 2001). *Cycloramphus faustoi* (Brasileiro *et al.* 2007) and *C. stejnegeri* (Noble 1924) are the other cycloramphid frogs that have terrestrial spawns for which we know the clutch sizes—31 and 41 eggs, respectively (Heyer and Crombie 1979, Brasileiro *et al.* 2007). The froglets of *Z. carvalhoi* produced during a reproductive season take 6 months to mature and reaching a SVL of 25 mm or more (Figures 2–3). Despite the geographical distance (~ 350 km between Juiz de Fora and the type locality of *Zachaenus carvalhoi* in Santa Teresa in the state of Espírito Santo), we found no differences in external morphology between the populations. There is overlap in the measurements among populations from Juiz de Fora, Ibitirama, and Santa Teresa municipalities. The similarity in external morphology may indicate that gene flow is

maintained among the populations despite the fragmented habitat, or, more probably, that morphological evolution is conservative. To test either hypothesis, we would need phylogeographic studies using fast-evolving molecular markers.

We think that the data described are sufficient to remove *Zachaenus carvalhoi* from the Data Deficient category of International Union for Conservation of Nature (IUCN) Red List. The distributional range of *Z. carvalhoi* includes an area larger than 20,000 km$^2$, mostly inside protected areas. However, these fragments are threatened by their surroundings (e.g., coffee and *Eucalyptus* plantations in Zocca *et al*. 2014, pastures in Viçosa, and urban areas in Juiz de Fora). The frogs occur in low densities (2.5 individuals per 100 m$^2$ in Juiz de Fora; 0.07 per 100 m$^2$ in Santa Teresa, Zocca *et al.* 2014; nine *Z. parvulus* per 100 m$^2$ in Ilha Grande, for comparison, Van Sluys *et al.* 2001). The subpopulations are becoming more isolated as fragmentation continues and the overall quality of the habitat in the Atlantic Forest declines, especially at the highly populated states of Minas Gerais and Espírito Santo in southeastern Brazil. Based on the above data, we infer reduction of the population sizes in *Z. carvalhoi* (A2), decline in the area that the species inhabits, as well as in its range and the quality of suitable habitat. Accordingly, we propose that *Z. carvalhoi* be classified under Near Threatened (NT) category of IUCN Red List.

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**Appendix I.** *Specimens examined.*

*Zachaenus carvalhoi:* BRAZIL. Espírito Santo: Ibitirama (Parque Nacional do Caparaó): MZUSP 139102, 140431, 140432. Minas Gerais: Juiz de Fora: MZUSP 142413, UFJF 663, 674–678, 692, 708, 753, 760, 773, 781, 792–830, 862–865.

*Zachaenus parvulus:* BRAZIL. Rio de Janeiro: Rio de Janeiro: MZUSP 297, 102160; Rio de Janeiro (Tijuca): MZUSP 102083, 102085, 102087, 102088, 102091, 102093–102097, 102099–102101, 102104, 102106, 102115–102120, 102122, 102124, 102125, 102127–102135, 102137, 102159, 126127.