

Breed fast, die young: demography of a poorly known fossorial frog from the xeric Neotropics

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Abstract. We successfully used skeletochronology to provide the first detailed demographic data regarding *Ceratophrys stolzmanni*, a cryptic, fossorial amphibian inhabiting the xeric Neotropics. We observed a female-biased sexual size dimorphism, but no differences in age parameters between the two sexes. Growth rate is accelerated during the first year of life, both before and after metamorphosis, followed by a rapid sexual maturation and a short lifespan. Both males and females reached sexual maturity before they were one year old, the mean age was two years, and longevity was low for both sexes, with only 2% of the tested individuals, all females, reaching the age of four years. We discuss the implications of the observed aging and growth patterns for the long-term survival and conservation of the species, comparing our results to other tropical species.

Key words. Amphibia, Anura, Ceratophryidae, age, size, longevity, sexual dimorphism, Pacific dry forest.

Introduction

Neotropical seasonally dry forests are currently recognized as one of the most threatened ecosystems worldwide (ESPINOSA et al. 2012). In Ecuador, this type of habitat has been strongly impacted by human activity with more than 87% of the original area being transformed, mostly for agriculture and cattle farming (RON et al. 2011). Considering its low representation in the National Protected Area System (0.3%), this ecosystem should be a priority for conservation (BANDA et al. 2016). Neotropical dry forests are endemism hotspots (LINARES-PALOMINO et al. 2009), and although plant and bird diversity are relatively well studied, hardly any information is available for other groups (ESCRIBANO-ÁVILA 2016).

Effective conservation requires understanding species' natural history and identifying factors that influence extinction risks and the ability to recover after perturbations (SELWOOD et al. 2015). Life history traits, like longevity, age

at first reproduction, and body size are amongst the most important parameters for the study of populations and a prerequisite for designing effective conservation strategies (BIEK et al. 2002). Age- and size-related parameters determine the fitness of individuals and as such are under strong selective pressure (STEARNS 1992). While measuring size is a straightforward affair in most amphibian species, reliably estimating age by means of capture-mark-recapture techniques will require time and financial investment, which are not always available. As an alternative, skeletochronology is currently being used for robust estimation of age in amphibians and reptiles (SINSCH 2015), with a large body of data accumulating. However, this knowledge is biased towards temperate species. Data concerning tropical amphibians is still scarce (SINSCH 2015), with even baseline information lacking for whole families (BIEK et al. 2002) and even though the diversity of adaptation and degree of threat are much higher in tropical environments. Skeletochronology uses cross sections of long bones to reveal in-

dividual growth patterns in organisms that have their growth cycles synchronized with environmental seasonality, such as temperature or rainfall regimes (SMIRINA 1994; SINSCH et al. 2007). Therefore, periods of arrested growth are marked in the hard tissue of these organisms, which through staining appear as intensely coloured, dense lines (i.e., lines of arrested growth, LAGs) (CASTANET & SMIRINA 1990). The formation of LAGs is considered to be genetically controlled and correspond to seasonal periods of inactivity (hibernation or aestivation) (SINSCH 2015). Besides being a reliable tool, skeletochronology has also the advantage of not requiring the sacrifice of animals, since bones from clipped phalanges can be used to obtain accurate age estimates.

The frogs belonging to the South American family Ceratophryidae have attracted a lot of interest, especially in the pet trade, due to their peculiar shapes and voracious appetite, but most scientific studies have focused on phylogeny and morphologic adaptations (WILD 1997, FABREZI 2011, FAIVOVICH et al. 2014), whereas little information is available regarding their ecology and life-history (DUELLMAN & LIZANA 1994, SCHALK et al. 2014, FABREZI et al. 2016). One of the least known species of this family is the Pacific horned toad – *Ceratophrys stolzmanni*, a fossorial frog inhabiting tropical xeric lowland forests in coastal Ecuador and Peru. There is a general lack of information about its life history, with observations being limited to a small number of individuals in captivity (ORTIZ et al. 2013) and mostly dealing with reproductive aspects. Because of its restricted distribution and high pressures on its native forest habitat through logging and expansion of agriculture, *C. stolzmanni* is currently assessed as a vulnerable species by the IUCN (ANGULO et al. 2004). It is active only during the rainy season when heavy rains will trigger explosive breeding. Larval development is fast, with metamorphosis occurring as quickly as two weeks after oviposition (SZÉKELY et al. 2017). Over the course of the dry season, individuals remain buried in the ground, forming a protective cocoon that reduces water loss and so protects them from dehydration (FAIVOVICH et al. 2014).

To understand the natural history of this species and support the conservation of its habitat, we here present the first data regarding age and growth parameters for a population inhabiting the dry forests of southern Ecuador. Because the extreme seasonal variation in the precipitation regime imposes a cessation of activity on this species, we believe that *C. stolzmanni* is an adequate species for studying the expression of growth marks by means of skeletochronology.

Materials and methods

Study area

Our study took place in Arenillas Ecological Reserve, located in El Oro Province, southwestern Ecuador (03°34' S, 80°08' E, 40 m above sea level). The area has been under military protection for the last 60 years, after which,

in 2001, it was incorporated into the Ecuadorian National Protected Area System, and in 2005 was declared an Important Bird and Biodiversity Area (code ECo35, BirdLife International 2016). The total area occupied by Arenillas Ecological Reserve has been decreasing continuously, from 17.083 ha in 1994 to 14.282 ha in 2001, and 10.277 ha in 2012 (CAMACHO & ULLAURI 2013). This loss was caused by illegal logging and the ongoing expansion of shrimp farms and cultivated land within the officially protected area. The reserve protects one of the last remnants of original Pacific dry forests, an ecosystem with low deciduous trees and a dense herbaceous understorey (ESPINOSA et al. 2016). The climate is characterized by a dry season from June to December (less than 10 mm of monthly precipitation for at least four months) and a rainy season, from January to May. Total annual precipitation averages 667 mm, with large fluctuations between years. The mean annual temperature is 25°C, with a 3.4°C variation between the coldest and warmest months (ESPINOSA et al. 2016). Nine species of amphibians, representing five families, have been reported from the reserve (SZÉKELY et al. 2016).

Data collection

A total of 153 adult individuals (79 males and 74 females) and 92 freshly metamorphosed individuals were sampled in 2015 (January through April). They were caught along transects in the forest; for each individual we recorded the following measurements: body mass using a My Weigh 300Z portable scale (0.1 g precision), snout–vent length (SVL) and head width using a Dial-Max calliper (0.1 mm precision). The males were recognized by the presence of secondary sexual characters such as dark coloration on throat and nuptial pads on forelimbs. We collected the third digit of the right forelimb from all adults and from five freshly metamorphosed froglets. All individuals were released at their capture sites thereafter, and the bone samples were preserved in 96% ethanol; tissues that were not used for skeletochronology (skin and muscle) were deposited at the Museo de Zoología de la Pontificia Universidad Católica del Ecuador, Quito, Ecuador (QCAZ 66291–62491).

Skeletochronology

Our tissue preparation protocol followed the method established by CASTANET & SMIRINA (1990) with minor modifications. We removed skin and soft tissues, decalcified the bones in 5% nitric acid for 2 to 18 min, depending on sizes, and afterwards left them to soak in tap water overnight. We embedded the bones in TissueTek embedding medium for cryotomy, cut 12–16 µm cross sections with a Tehsys CR 3000 cryotome, stained these with Ehrlich's hematoxylin for four hours, and finally washed them in distilled water for three minutes. Sections with the smallest marrow cavity and the thickest cortical bone were mounted on slides using Aquatex (aqueous mounting agent for microscopy,

Merck Millipore) and photographed them using an Olympus E-620 microscope-mounted camera (Olympus CX 31 microscope) with Quick Photo Micro 2.3 software. Three independent observers (US, DC and FS) counted the lines of arrested growth (LAGs) in 3–5 sections per individual. Faintly stained bone marks, situated in close proximity of the annual LAG were considered double/multiple lines and counted as one year (LECLAIR et al. 2005, SINSCH et al. 2007).

The parameters analysed follow the definition by LESKOVAR et al. (2006): (1) age at sexual maturity as the minimum estimated age of breeding individuals; (2) size at sexual maturity as the average SVL of individuals with the minimum reproductive age; (3) longevity as the maximum age; (4) potential reproductive lifespan as the difference between longevity and age at sexual maturity; (5) median lifespan as the mean of age distribution. Since the distance between consecutive LAGs is a good indicator of growth, the abrupt rapprochement of LAGs was regarded as the attainment of sexual maturity (SMIRINA 1994).

Data analysis

Analyses were carried out in R environment (R Core Team 2016), with the significance level set at 0.05. Size-related parameters were normally distributed (body mass after ln-transformation; Shapiro-Wilk test, $P > 0.05$) and conformed to the assumption of homoscedasticity (Levene's test, $P > 0.05$). To detect differences between sexes, we used the Wilcoxon-Mann-Whitney test for age distribution, and Welch's t-test for morphometric variables. To test the effect of age on size (SVL, body mass), we used a General Linear Model (GLM, Gaussian distribution) with age and sex as predictors.

We used the packages FSA (OGLE 2014) and nlstools (BATY et al. 2015) to compute von Bertalanffy's growth model (VON BERTALANFFY 1938) following the formula modified by BEVERTON & HOLT (2012):

$$SVL_t = SVL_{max} \times (1 - e^{-k \times (t - t_0)}),$$

where SVL_t is the expected or average SVL at the time (or age) 't', SVL_{max} is the asymptotic average SVL, 'k' is the growth rate coefficient, and ' t_0 ' is the time or age when the average SVL was zero. We fitted von Bertalanffy's growth model and estimated growth parameters (VBGPs) by non-linear least squares regression. Two estimated VBGPs were considered significantly different at the 0.95 level when their confidence intervals (CI 95%) did not overlap.

We propose an alternative method to estimate juvenile post-metamorphic growth by analysing the bone growth pattern observed in cross sections. The attainment of sexual maturity is associated with reduced growth rates, corresponding to LAG spacing being reduced in the periosteal bone. In *C. stolzmanni*, juvenile post-metamorphic growth is most evident in the periosteal bone, bounded between the line of metamorphosis and the first LAG (which, in

this case, corresponds to the time when sexual maturity is reached). With ImageJ software v. 1.50i (RASBAND 1997–2016), we measured two parameters in those cross-sections of the diaphysis where the line of metamorphosis was evident: (1) the distance between the line of metamorphosis and the first LAG, at the widest growth area in the periosteal bone, as a proxy of terrestrial growth during the first year of life (TG), and (2) the longest diameter of the first LAG (D). Using these two parameters, we computed a relative growth index: $RGI = (2 \times TG)/D$, which allowed us to test if growth before sexual maturity was a source for the observed sexual size dimorphism in *C. stolzmanni*.

Results

Body size and sexual size dimorphism

At metamorphosis, juveniles had on average 34.4 mm (SVL), and 4.14 g (body mass), but with large individual variation (Table 1). For adults, significant differences were found between the two sexes (Table 1) in that females were overall larger and heavier than males (Welch's t-test, SVL: $t_{142,18} = -8.84$, $P < 0.001$; mass: $t_{145,6} = -10.63$, $P < 0.001$). In the same age group, females were on average between 6 and 11% longer and between 32 and 57% heavier than the males, with differences increasing with age. For both sexes, body mass and SVL were significantly correlated (Pearson correlation – male: $r^2 = 0.67$, d.f. = 76, $P < 0.001$; female $r^2 = 0.63$, d.f. = 72, $P < 0.001$).

Age structure and age at first reproduction

We found cross-sections in which LAGs were clearly marked in 152 (99%) adult individuals (Fig. 1). The only individual that showed poorly expressed LAGs was excluded from the analysis. The metamorphosis line was evident in 73% of adults and in two of the five juveniles. Also, sections of 59.4% individuals showed double or/and multiple LAGs, of which 91% showed a multiple first LAG, while only 26% showed a multiple LAG in their second year of life, and 2% had a multiple LAG in the third year. *Ceratophrys stolzmanni* usually breeds in February–March, and metamorphosis occurs in March–April, depending on climatic conditions. Because adult samples were collected before and during the reproduction period (January–March), the precise age of the individuals is slightly (1–3 months) lower than the number of LAGs. For example, individuals in the 1-year old age group (showing one LAG), are 9–11 months old.

The age structure of the studied *C. stolzmanni* population is illustrated in Fig. 2. The youngest breeding individuals were 1 year old (both males and females). Average SVL and body mass at first reproduction were 60.7 mm and 21.6 g for males and 64.4 mm and 28.4 g for females (Table 1). There were no significant differences in the mean age between the two sexes (Wilcoxon-Mann-Whitney test, $n_{males} = 78$, $n_{females} = 74$, $U = 2609$, $P = 0.46$). The median

Table 1. Morphometric variables of *Ceratophrys stolzmanni* according to sex and age. Parameters for which males are significantly different from females of same age (Welch's t test, corrected with the Bonferroni method for multiple comparisons) are marked with asterisks: ** - $P < 0.01$, *** - $P < 0.001$.

Number of LAGs		n	SVL mean \pm SD (range) (mm)	Head width mean \pm SD (range) (mm)	Body mass mean \pm SD (range) (g)
0	at metamorphosis	92	34.45 \pm 4.85 (23.8–47.9)	18.17 \pm 2.71 (11.7–24.3)	4.14 \pm 1.89 (1.2–11.9)
1	male	14	60.67 \pm 4.69 (53.9–70.4)	30.08 \pm 1.84 (27.7–33.6)	21.58 \pm 5.14 (16–32.1)
	female	7	64.41 \pm 7.0 (55–75.9)	31.42 \pm 3.4 (28.3–36.8)	28.38 \pm 8.55 (18.7–40.8)
2	male	49	59.22 \pm 3.65 *** (52.6–69.1)	29.22 \pm 1.67 *** (24.9–33.3)	21.87 \pm 4.71 *** (14.1–36.7)
	female	51	65.44 \pm 4.33 (56.8–77.5)	31.66 \pm 2.36 (27.7–37)	31.05 \pm 6.55 (17.9–43.8)
3	male	15	59.5 \pm 2.67*** (53–63.1)	29.06 \pm 1.9 ** (25.5–31.6)	21.06 \pm 3.07 *** (14.6–27)
	female	13	66.32 \pm 3.51 (61.9–72.2)	31.16 \pm 1.43 (28.1–34)	33.16 \pm 5.48 (24.8–40.4)
4	female	3	63.33 \pm 3.85 (59–66.4)	30.53 \pm 1.85 (28.4–31.7)	30.26 \pm 5.56 (24.1–34.9)

value was 2 years for both sexes. Longevity was 4 years in 3 females (2% of tested individuals). Potential reproductive lifespan was 2 years for males, and 3 years for females.

On average, after metamorphosis, during their first year of life, males were able to increase their mass 5.21 times and SVL 1.76 times, while females increased their mass 6.85 times and SVL 1.87 times. Size could not be significantly predicted by age (GLM–SVL: $F_{(1,150)} = 3798.7$, $P = 0.748$; mass: $F_{(1,150)} = 11.142$, $P = 0.373$); age classes overlapped in size and variation amongst the one year old cohort was the largest.

Table 2. Statistics for von Bertalanffy growth parameters in *Ceratophrys stolzmanni* males and females. SE = standard error, CI = confidence interval, SVL_{max} = asymptotic body size, k = growth coefficient.

Sex	Parameter	Estimate	SE	CI 95%	P
Males	SVL_{max}	59.32	0.401	58.52–60.12	<0.001
	k	5.79	13.12	-20.37–31.97	0.66
Females	SVL_{max}	65.67	0.668	64.34–67.00	<0.001
	k	2.17	0.548	1.08–3.27	<0.001

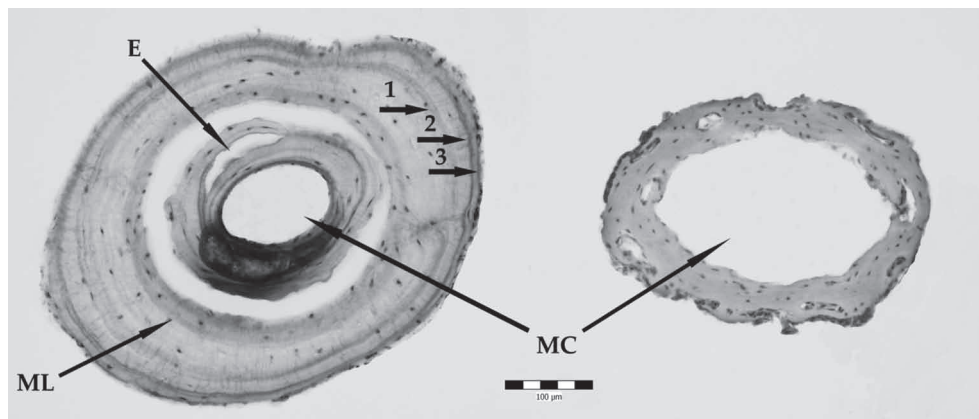


Figure 1. Cross-sections through *Ceratophrys stolzmanni* phalanges: left. three-year old male; right. freshly metamorphosed juvenile. LAGs are indicated by black arrows and numbers. ML – metamorphosis line; MC – marrow cavity; E – endosteum. LAG 1 is triplicated, LAG 2 is duplicated. LAG rapprochement is visible after LAG 1, indicating the attainment of sexual maturity.

Table 3. Life-expectancy of tropical amphibians (studies carried out at latitudes between 23°27' N and 23°27' S) living in xeric or arid environments that impose the cessation of activity. Min. age - minimum age reported for sexually mature individuals and longevity, in years.

Family	Species	Sex	Min. age	Longevity	Location	Source
Bufonidae	<i>Bufo pentoni</i>	M	2	4	Senegal	(BARBAULT et al. 1979)
		F	2	4		
Bufonidae	<i>Nimbaphrynoides occidentalis</i>	M	0.3	2	Liberia	(CASTANET et al. 2000)
		F	0.3	5		
Microhylidae	<i>Scaphiophryne gottlebei</i>	M	<1	2	Madagascar	(GUARINO et al. 2010)
		F	1	2		
Mantellidae	<i>Boophis occidentalis</i>	M	4	11	Madagascar	(ANDREONE et al. 2002)
Mantellidae	<i>Mantella expectata</i>	M	1	3	Madagascar	(GUARINO et al. 2010)
		F	1	3		

Growth

von Bertalanffy models fitted growth in *C. stolzmanni* in both sexes (residual standard error, males: 3.118 on 71 degrees of freedom, females: 3.912 on 68 degrees of freedom), but the growth rate coefficient could not be estimated accurately in males (Table 2). The asymptotic average SVL was significantly higher in females, while the growth pattern followed a more abrupt decreasing trajectory in males compared to females (Fig. 3). We computed the RGI in 33 males and 32 females, and found no significant differences between the two sexes (Welch's t-test, $t_{60,23} = -1.882$, $P = 0.065$).

Discussion

We provide the first detailed morphometric and demographic data for a population of the Pacific horned frog, showing that the skeletochronological method can successfully be used to determine the age in this species. Like other tropical species living in xeric environments (Table 3), Pacific horned frogs grow fast during their first year until

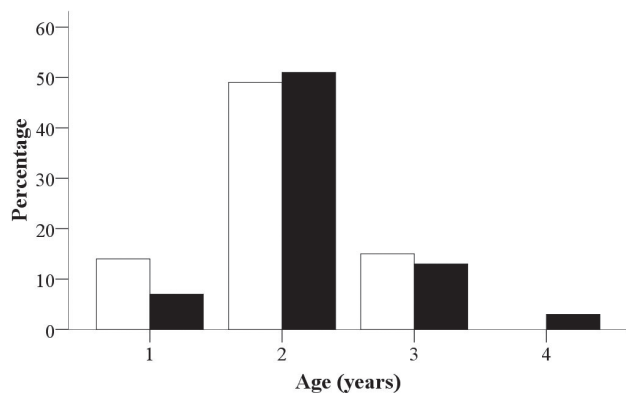


Figure 2. Age structure of *Ceratophrys stolzmanni* in Arenillas Ecological Reserve, Ecuador, determined by skeletochronology. Open bars - males, full bars - females.

reaching sexual maturity at a young age. The growth rate of adults is marked by a sharp decrease once sexual maturity has been reached, and lifespan is short. Tropical amphibians tend to have shorter lifespans than those inhabiting colder habitats, higher altitudes or latitudes (SMIRINA 1994, MORRISON & HERO 2003); also, longevity is generally correlated with age at maturity (GUARINO et al. 2003). In other species of the family Ceratophryidae from semi-arid subtropical South American dry Chaco, reported values of longevity (based only on a small number of individuals per species) are: *Lepidobatrachus llanensis*, six years; *Lepidobatrachus laevis*, seven years; *Chacophrys pierotti*, six years; and *Ceratophrys cranwelli*, 14 years (FABREZI & QUINZIO 2008). However, no data regarding the age structure of populations are available for comparison.

The sampled *C. stolzmanni* population consisted predominantly of young individuals, with the majority being two years old, and only three females having lived to an age of four years. Since our observations were not restricted to the breeding season, the fact that we sampled fewer 1-year

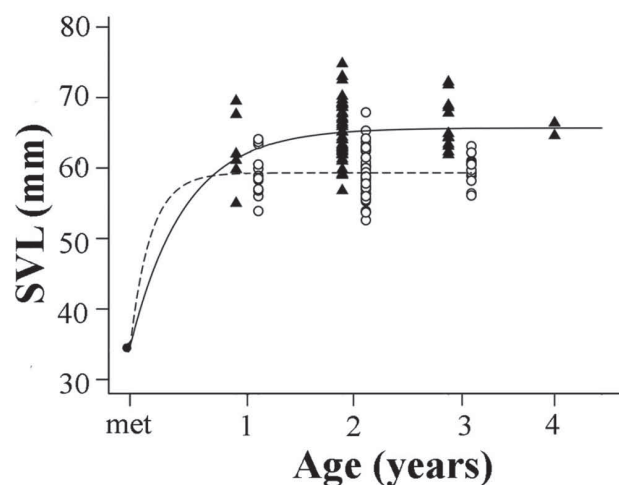


Figure 3. Growth described by von Bertalanffy's model in *Ceratophrys stolzmanni* males (open circles, dotted line) and females (full triangles, continuous line). met - metamorphosis, full circle - average size at metamorphosis.

olds than 2-year olds might reflect either a lower detectability of this age group, or a fluctuation in survival rates between the years.

Growth is accelerated during the first year, both before (SZÉKELY et al. 2017) and after metamorphosis. In the 2–3 weeks of larval development, the tadpoles of *C. stolzmanni* show one of the fastest growth rates recorded for any anuran species (for a comparison, see RICHTER-BOIX et al. 2011). Our data suggest that they may have reached 57 and 53% of their adult SVL (males and females, respectively) at metamorphosis. The rapid development and accelerated growth rate in larval stages seem to be a characteristic of Ceratophryidae, and are considered a response to life in unpredictable environments with long periods of inactivity due to extreme dryness (FABREZI 2011). Both males and females reach sexual maturity in their first year of life, after which growth is greatly decelerated, probably because of a large investment into reproduction. The rapid onset of sexual maturity is regarded as an adaptive response to high annual mortality rates (METCALFE & MONAGHAN 2003) since it is more advantageous to breed early and at a small size than to delay maturity and run an increased risk of not breeding at all.

Variation in size per age class was large, and different age classes overlapped for all tested morphometric values, rendering size measurements unreliable for estimating age in this species. Large variations in size at the same age can be explained by different individual growth rates before maturity – both before and after metamorphosis. Data obtained from skeletochronological and mark-recapture studies showed that size is a poor predictor for age in amphibians (HALLIDAY & VERRELL 1988) because the growth rate before maturity has a much larger impact on size than age. Female Pacific horned frogs are larger than males, which is the case in the vast majority of amphibians (SHINE 1979). In species where fecundity is correlated with size, this is considered to be the result of different pressures on the two sexes, with the selection for larger size being stronger on females (SHINE 1989). In some cases, sexual size dimorphism is caused by differences in age at first reproduction (MONNET & CHERRY 2002). Since sexual maturity was reached by both male and female *C. stolzmanni* before the age of one year, we infer that their dimorphism is determined by different growth rates. This assumption is also supported by von Bertalanffy's model estimates of asymptotic average snout–vent length, which was significantly higher in females. Our study suggests that von Bertalanffy models using skeletochronological data are of limited use in species that grow fast and are short-lived, i.e., the age class width of one year is too wide to adequately represent growth rates. RGI indicated that both males and females experienced the same growth rates from metamorphosis to sexual maturity (i.e., at one year old), which is corroborated by the lack of a significant sexual size dimorphism in the one-year age class. Our results indicate that growth rates after reaching sexual maturity shape the growth models and sexual size differences in *C. stolzmanni*.

As in other species (SINSCH et al. 2007), a relatively large proportion of individuals formed double and/or multiple LAGs. The presence of these lines is usually attributed to a decrease in growth rate, which can be caused by a variety of environmental and internal factors (CASTANET & SMIRINA 1990). In the case of *C. stolzmanni*, we suspect that the presence of double/multiple LAGs may be the reflection of unfavourable periods during the active season that restricted activity and thus growth (e.g., intervals with low precipitation during the rainy season, or excessive growth of grass vegetation that can impede movement and foraging).

The short reproductive lifespan of the species has important conservation implications, because a persistent drought of 2–3 consecutive years with low recruitment would endanger the survival of the whole population (MARSH & TRENHAM 2001). A recent study emphasizes the vulnerability of the Southern Region of Ecuador, and especially of the dry-forests in this area, to the predicted future climate scenarios (EGUIGUREN-VELEPUCHA et al. 2016). Prolonged droughts associated with El Niño/La Niña oscillations have been shown to affect population densities especially in short-lived tropical amphibians (e.g., *Eleutherodactylus coqui*; STEWART 1995), and have been proposed as the main cause for the disappearance of other species (POUNDS & CRUMP 1994); their influence is far from being understood, however.

The endangered Tumbes-Piura dry forest ecoregion was chosen as one of the 66 priority ecoregions for the conservation of Neotropical amphibians (LOYOLA et al. 2008). Conversion of tropical dry forest into agricultural land was demonstrated to negatively impact on amphibian assemblages (SUAZO-ORTUÑO et al. 2008), and the consequences can be exacerbated by climate change. We highlight the urgent need for effective conservation of Arenillas Ecological Reserve, as increased anthropogenic pressures and land-use modification will probably lead to further fragmentation of the Pacific horned toad habitat and reduce opportunities for successful reproduction and recolonization, so that the risk of catastrophic declines would be augmented in species with short lifespans and few reproductive opportunities, such as *C. stolzmanni*.

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