



Breeding site fidelity of the critically endangered toad *Atelopus cruciger* (Anura: Bufonidae): implications for its conservation

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Abstract. Harlequin Toads (Bufonidae: *Atelopus*) are among the most diverse Neotropical amphibians, but 84% of the known species are threatened with extinction mainly due to the fungal disease chytridiomycosis. With no effective tools to mitigate chytridiomycosis in the wild being available, captive breeding is currently the only option to ensure the long-term survival of many Harlequin Toad species. However, the life history and ecology of most species is still largely unknown. Using spatially explicit capture–recapture data from 2005–2013, we studied breeding habitat use and reproductive phenology of the critically endangered Rancho Grande Harlequin Toad, *Atelopus cruciger*. This species is a seasonal breeder that reproduces exclusively during the dry season. Adult males and females, but also juveniles, migrate from the forest to the river at the beginning of the dry season and return to the forest as soon as the rainy season begins, although a few adult males may remain on the riverbank throughout the year. Despite these seasonal displacements, the toads return to the same locations on the riverbank every year. Both female and male adult toads exhibit a strong breeding site fidelity, occupying small areas ($\leq 5 \text{ m}^2$) along the river margins. Adults were mainly active on rocky substrate and vegetation during the day, with males occupying significantly more elevated spots closer to the stream than females. Male territories tend to overlap, but we observed no aggressive or territorial defence behaviour. We discuss the implications of habitat use and high site fidelity for in situ and ex situ conservation measures of this critically endangered toad.

Key words. Amphibia, Harlequin Toads, reproductive phenology, seasonal migration, habitat use, home range, captive breeding, Venezuela.

Introduction

The Neotropical genus *Atelopus* DUMÉRIL & BIBRON, 1841 is the largest in the family Bufonidae, with 99 species described to date (FROST 2021) and several undescribed forms (LÖTTERS et al. 2011). *Atelopus* species, commonly known as Harlequin Toads, are colourful, diurnal, small-sized anurans that live near fast-flowing streams in most American tropical wet ecosystems. Their geographic distribution ranges from Costa Rica to Bolivia, and east to the lowlands of the Guiana Shield (LÖTTERS 1996, RUEDA-ALMONACID et al. 2005, LÖTTERS et al. 2011), with their greatest diversity occurring in the northern Andes, above 1,500 m a.s.l.

Over the last 40 years, *Atelopus* experienced the most drastic population declines and extinctions ever record-

ed in a species-rich amphibian genus (LA MARCA et al. 2005, LÖTTERS 2007, SCHEELE et al. 2019). According to the IUCN Red List of Threatened Species, 87.2% of the assessed *Atelopus* species are Extinct (3 spp.), Critically Endangered (62 spp., of which more than half are considered Possibly Extinct), Endangered (14 spp.), or Vulnerable (3 spp.) (IUCN 2022). In Venezuela, eight of the nine known Harlequin Toad species are listed as Critically Endangered (four as Possibly Extinct), and the other, *A. vogli*, is considered Extinct (IUCN 2022). Chytridiomycosis, a fungal disease caused by the chytrid fungus *Batrachochytrium dendrobatidis* (Bd), has presumably driven most population declines and extinctions of Harlequin Toads, although some have also been linked to climate change or habitat loss (BONACCORSO et al. 2003,

POUNDS et al. 2006, LIPS et al. 2008, TARVIN et al. 2014, SCHEELE et al. 2019).

In recent years, some Harlequin Toad species have been rediscovered after they had seemingly disappeared for decades (JAYNES et al. 2022). However, only a few lowland species (e.g. *A. cruciger*, *A. elegans*, *A. flavescens*, *A. hoogmoedi*, *A. pulcher*) and some highland species in the Sierra Nevada de Santa Marta (*A. arsyecue*, *A. carrikeri*, *A. laetissimus*, *A. nahumae*) appear to still maintain reasonably large populations (e.g., VON MAY et al. 2008, LUGER et al. 2009, LAMPO et al. 2012, 2017, FLECHAS et al. 2012, RUEDA-SOLANO et al. 2016, ROCHA-USUGA et al. 2017, Global Wildlife Conservation 2019, GRANDA-RODRÍGUEZ et al. 2020, PÉREZ-GONZÁLEZ et al. 2020). For most of the other species, only few individuals have been detected (YÁÑEZ-MUÑOZ et al. 2010, ENCISO-CALLE et al. 2017, TAPIA et al. 2017, GONZÁLEZ-MAYA et al. 2018, BARRIO-AMORÓS et al. 2020, JAYNES et al. 2022, BARRIO-AMORÓS & TORRES 2023). Because no effective long-term in situ treatments for this disease are as yet available (KNAPP et al. 2022) and we do not fully understand how some populations are able to cope with the fungus, maintaining captive colonies of priority species for possible future reintroduction programs are one of the main strategies recommended for saving Harlequin Toads from extinction (VALENCIA & FONTE 2021). Ex situ breeding programs for about two dozen amphibian species have thus been established, and some reintroduction trials are currently in progress (e.g., COLOMA & ALMEIDA-REINOSO 2012, GAWOR et al. 2012, GRATWICKE & MURPHY 2016, LEWIS et al. 2019, ESTRADA et al. 2022, Amphibian Ark 2022, KUENEMAN et al. 2022).

Currently, the Rancho Grande Harlequin Toad, *Atelopus cruciger* (LICHTENSTEIN & VON MARTENS, 1856), is the only Venezuelan Harlequin Toad with two known large and stable populations. Despite continuing efforts to find this species in its former habitats, only two subpopulations, both with endemic infections, have been discovered in lowland habitats (RODRÍGUEZ-CONTRERAS et al. 2008, LAMPO et al. 2012, 2017). Although adults are highly vulnerable to chytridiomycosis, transmission rates apparently are low and juvenile recruitment rates sufficiently high to compensate for disease-induced mortality. However, scenarios that increase *Bd* transmission could rapidly push these populations to collapse and the species possibly to extinction. The Rancho Grande Harlequin Toad is currently classified as Critically Endangered and recommended for ex situ rescue measures (LÖTTTERS 2007, VALENCIA & FONTE 2021, LAMPO et al. 2022), but its natural history and ecology are still largely unknown despite it having been a very abundant and common species in the past. Although other species (e.g., *A. varius/zeteki*) have provided valuable insights into the how-to of conserving target species for which life history information is scant, some temporal spatial aspects of reproduction vary between *Atelopus* species (LÖTTTERS 1996). Species-specific natural history data are critical for the success of captive breeding (MICHAELS et al. 2014) and reintroduction programs, however.

Here, we studied patterns of habitat use and breeding activity of *A. cruciger* in a remnant population between 2005 and 2013 to aid measures to ensure the long-term survival of this Venezuelan Harlequin Toad.

Materials and methods

Study site

Our study was conducted on the Cata River, on the lower boundary of the Henri Pittier National Park on the northern slope of the Cordillera de la Costa (Aragua State), Venezuela (Fig. 1a). The climate here is tropical (mean annual temperature 29°C, mean annual precipitation 795.6 mm), with a dry season between December and May (mean monthly precipitation < 10 mm), and a wet season between June and November (mean monthly precipitation 60 to 190 mm, peaking in July–August). At the study site, the river is approximately 4 m wide and has clear neutral water (pH 6.52). Its banks, of sand and gravel, feature large boulders on which *Cyclanthus bipartitus* plants (Cyclanthaceae) grow (Fig. 1b). During the dry season, some river sections may dry up, while the water level can rise to up to 2 m during the rainy months. Two to 4 m from the shore along the banks, the terrain rises abruptly to form a steep slope covered by riparian deciduous primary forest (HUBER 1986).

Field data collection

Fifty mark-recapture sessions were carried out between October 2005 and April 2013 in a 250 × 3 m transect along the western margin of the Cata River (transect origin 10°26'19.3" N, 67°43'28.3" W; altitudinal range between 120 and 220 m a.s.l.). Due to logistical constraints, these surveys were not distributed equally over the years, and time intervals between sessions varied between 30 and 96 days. Surveys were conducted during daylight hours, matching the activity period of the studied species, usually between 09:00 to 17:00 h, but varied in duration (number of sampling hours/session) depending on the abundance of toads. All individuals sighted were photographed in dorsal view to allow individual identification based on the unique and permanent pattern of adults (Fig. 2). A numeric code was assigned to each adult toad, and a digital photographic catalogue was assembled and updated after each sampling session. The classification of individuals as new or recapture was determined by visually comparing them with the photos in the catalogue. Once caught, individuals were sexed and their snout–vent length (SVL) measured to the nearest 0.1 mm with a calliper. All individuals smaller than 20 mm SVL were classified as juveniles or subadults (LÖTTTERS et al. 2004, LAMPO et al. 2023). Their sexual identity was judged by the shape of the thumb and forearms (long and slender in females vs. short and robust in males), the presence/absence of a brownish keratinized pad at the base of the thumb (present only in males), the presence/absence of externally visible eggs within the body cavity (females),

and/or their behaviour (calling males, pairs in amplexus). Once processed thus, the toads were released at their capture sites.

During the 2005–2007 sampling sessions, the exact point of detection for each toad was marked with a coloured and labelled flag, and its position was registered in a x/y grid, by measuring the distances to the beginning of the transect and to the water's edge perpendicularly. We

categorized the encounter substrate as sand, gravel, rocks, leaf litter, roots, trunks, or vegetation (specifically *Cyclanthus bipartitus*), and recorded the height above the ground at which each individual was discovered. Additionally, we estimated the canopy cover straight above its position (as percentage of shadow in an area of 1 m²). After 2007, we continued to record only the distance to the start-off point of the transect, and the substrate type.

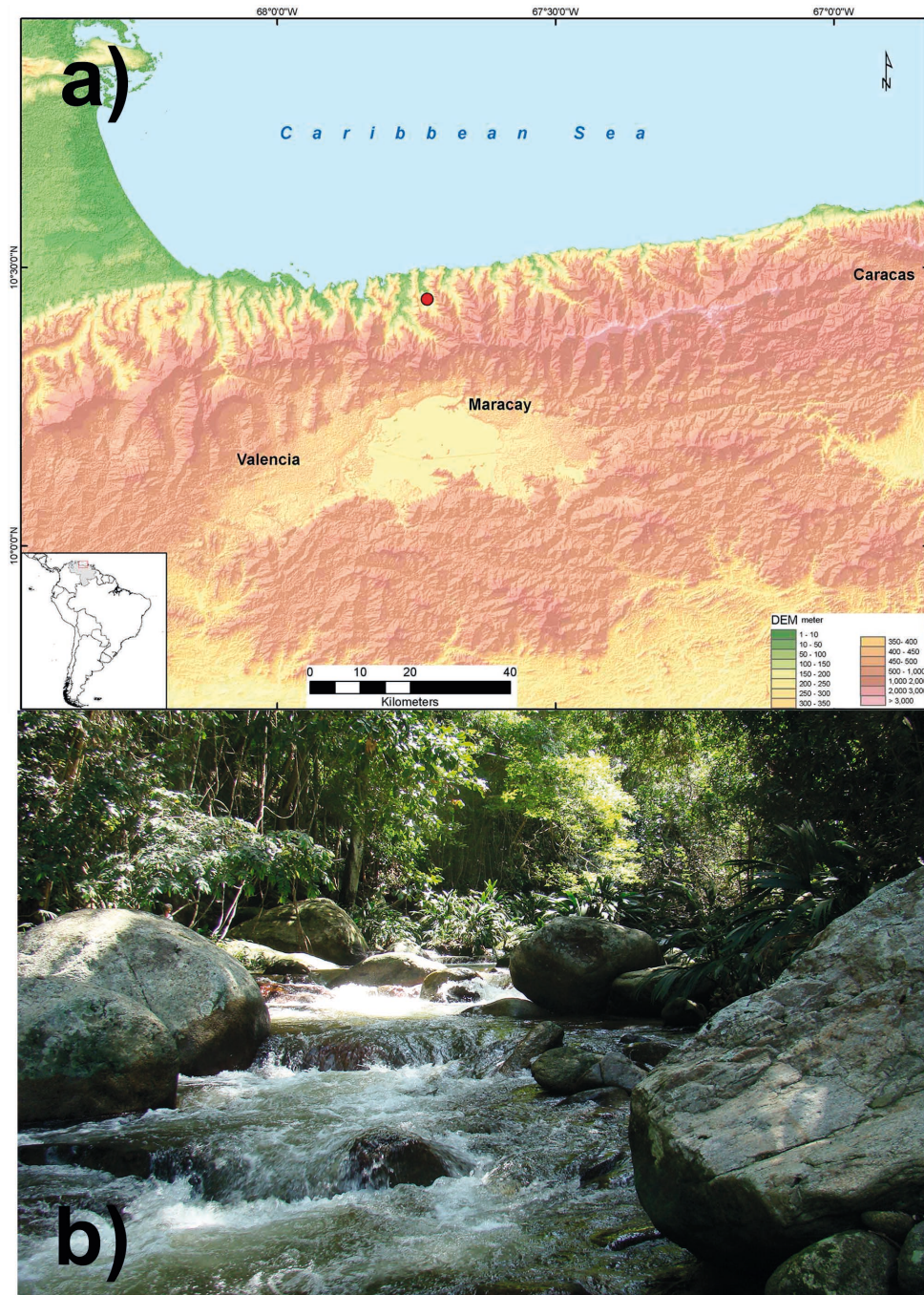


Figure 1. Schematic map showing (a) the location of the study area on the northern slope of the Cordillera de la Costa, Venezuela, and (b) a general view of our study site on the Cata River. Photo: CELSA SEÑARIS.

Temperature and precipitation data between 2005 and 2011 were obtained from a weather station 2.8 km from the study site, in Ocumare de La Costa (INIA 2014). For 2012–2013, precipitation data was available only from a more distant station in Maracay (INAMEH 2014).

Data analysis

We explored differences in the spatial distribution of males, females and juveniles by comparing their distances from the shoreline and the height above ground using the 2005–2007 dataset. Because neither of these variables was normally distributed (distance from shoreline, Shapiro-Wilk normality test $W = 0.814$; $p < 0.00001$; height: $W = 0.827$, $p < 0.00001$), we used the Kruskal-Wallis Rank Sum Test incorporated in R software to determine if positions differed in at least one group. To identify which groups differed, pairwise comparisons were made with a Pairwise Wilcoxon Rank Sum test with a Bonferroni correction for multiple testing.

We generated minimum convex polygons (MCPs) (MOHR 1947) for adult toads with three or more captures within the same year based on their x/y coordinates in the transect (2005–2007 dataset) using the software compo-

nent Animal Movement Analysis 2.0 (HOOGE & EICHENLAUB 1997) in ArcGis 9.1 (ESRI, Redlands, CA, USA). A detailed history of recapture for those adults with ≥ 3 sightings are provided in Supplementary Table S1. The estimated MCP areas approximate the area used for breeding activity of individuals, but not their entire home ranges, because these toads will migrate into the forest, where they become practically undetectable, after the breeding season has ended. The MCPs could therefore estimate the entire home ranges only for the few males that remained on the riverbank throughout the year. To infer potential encounters between adults, we estimated the degree overlap of MCPs after slicing the 2005–2007 dataset by year. That is, overlap was estimated between MCPs generated by captures that occurred within the same breeding season (for most animals) or within the same year (for the few males that were present all-year round). The history of recapture for those adults with ≥ 3 sightings and their exact positions can be found in the Supplementary Table S1.

To test whether the toads had some degree of fidelity to specific areas along the river margin or, alternatively, tended to move randomly along the shoreline, we explored the relationship between the distance between successive sightings of the same individual and the time between these sightings. The spatial distance between successive sightings

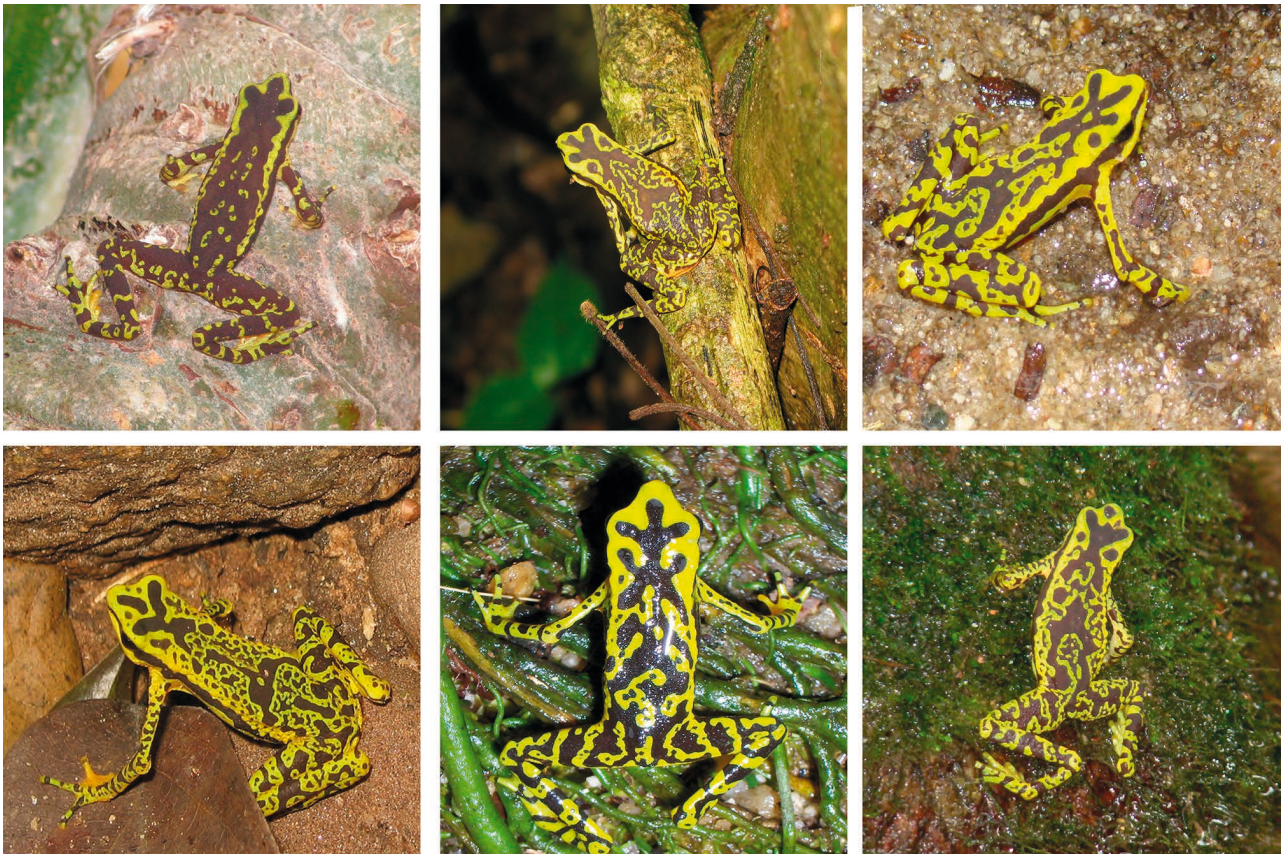


Figure 2. The unique dorsal pattern on each adult Rancho Grande Harlequin Toad, *Atelopus cruciger*, allows unequivocal individual recognition. All images depict males. Photos: CELSA SEÑARIS, FERNANDO J. M. ROJAS-RUNJAIC, MARGARITA LAMPO.

was approximated by the linear distance between their x-positions (one-dimension along the river) using the complete 2005–2013 dataset. The data was fitted to a linear-increase function. The theory of diffusion predicts that if organisms disperse randomly, we can expect them to spread from their initial sighting points with time (OKUBO & LEVIN 2001). Therefore, distance between sightings is expected to increase with time. The absence of such increasing relationship (null hypothesis negated) indicates that other non-random mechanisms retain individuals within certain areas.

Results

We encountered individuals of *A. cruciger* in 96% of the 50 sampling sessions conducted between October 2005 and April 2013. Only in two sessions, during which there were signs of recent flooding (e.g., fallen tree trunks and branches, detached vegetation, and rich accumulations of leaf litter), no toads were observed. The numbers of individuals sighted, and consequently the density of detected individuals in the transect, varied considerably between sampling sessions, with maximum values during the dry season (December–May), and minima during the rainy season (June–November) (Fig. 3). The maximum recorded density was 0.136 individuals detected/m² (102 individuals) in April 2013, after an exceptionally prolonged and severe drought. In sampling sessions carried out during the rainy season, only 2–4 toads were observed in the transect (0.003–0.005 individuals detected/m²).

We identified 399 distinct adult males, 212 adult females, and 82 juveniles or subadults. Adult SVLs ranged between 22.9 and 33.6 mm (mean 26.3 mm) for males, and from 29–43.2 mm (mean 31.8 mm) for females. Twenty-nine toads (SVL < 22 mm) could not be unequivocally sexed, and were classified as subadults. Between 2006 and 2013, 14 couples in amplexus were encountered, all of them during sampling sessions in dry periods. In December 2007, a string of desiccated eggs was found on a rock in the middle of the riverbed. Metamorphs (SVL 10–12 mm) leaving the water and moving into the forest were observed in abundance in April–June 2006, May 2009, and May 2012, that is at times corresponding to the beginning of the rainy season (Fig. 3). No egg masses or tadpoles were observed despite aquatic surveys (from the surface and by snorkelling underwater) carried out occasionally during this study or in additional field explorations until 2020.

The studied Harlequin Toads were active only in the day, and easily detected along the river margins, especially when perched on rocks (≥ 25 cm diameter) or on leaves and roots of *Cyclanthus bipartitus* plants. On a few occasions (9% of the sightings), the individuals were partially hidden in the leaf litter, under branches or trunks. Individuals were usually observed in places totally or partially shaded by canopy cover; in half of the sightings the canopy cover was 76–100%, in 26.4% it was 51–75%, in 15.5% 26–50%, and only 7.1% of toads were seen in areas with less than 25% of canopy cover. Seventy-eight percent of all individuals were found within 1.50 m from the shoreline, and 12% on top of rocks emerging from the riverbed. Only 10%

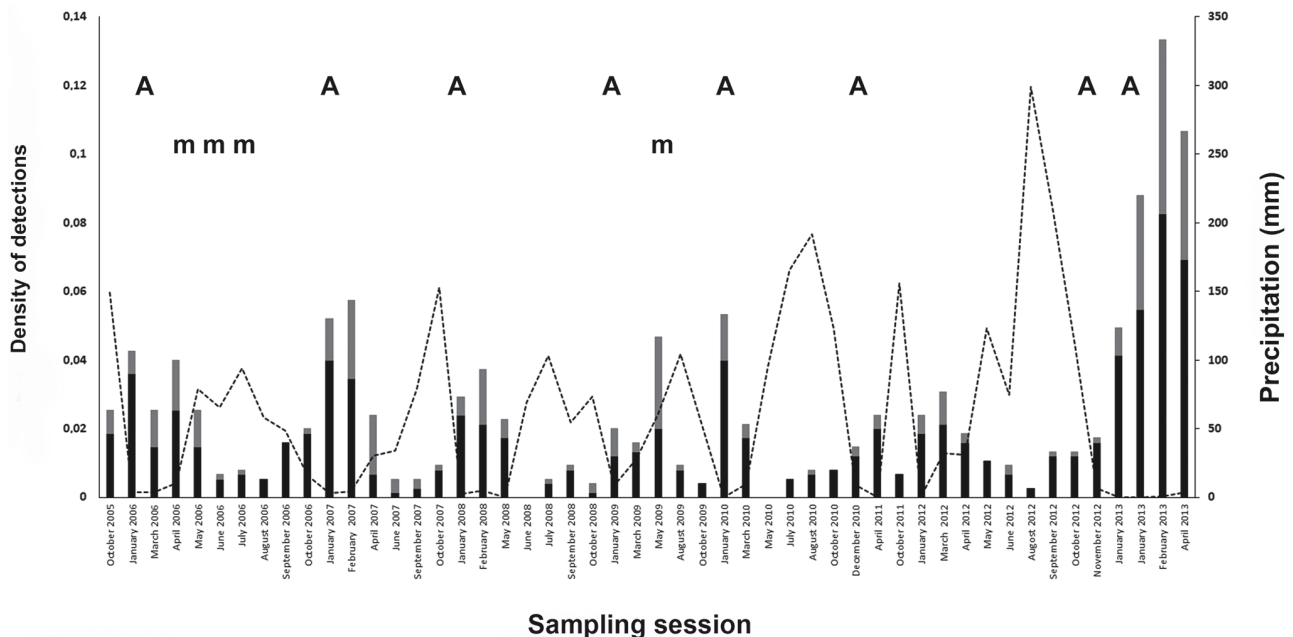


Figure 3. Density of detections (number of individuals detected/m²) of adult males (black) and adult females (grey) of *Atelopus cruciger* observed during sampling sessions between October 2005 and April 2013 on the Cata River, Venezuela. The dotted line corresponds to monthly rainfall as per the weather stations at Ocumare de la Costa (2007–2011) and Maracay (2012–2013). A = pairs in amplexus, m = metamorphs (SVL 10–12 mm).

of the toads were sighted more than 2 m from the edge of the river (Fig. 4). Significant differences were found between males and females in their distance from the shoreline ($\chi^2 = 7.63$, $df = 2$, $p = 0.022$): males were closer to the water as compared to females ($p = 0.043$). Juveniles exhibited no significant differences in their horizontal or vertical positioning as compared to males or females. Most toads perched on, or close to, the ground (< 1 m), but adults were occasionally also observed climbing on rocks or trunks up to 2 m above ground. We detected differences in perching height between groups ($\chi^2 = 6.23$, $df = 2$, $p = 0.044$): males tended to perch in higher spots than females ($p = 0.036$), and juveniles showed no difference to either adult group (Fig. 5).

Of the 399 adult males encountered during this study 64.4% were observed only once, 21.8% were captured twice, and the remaining 13.8% were logged with three or more sightings. Remarkably, one male was recorded with 12 sightings, between January 2010 and April 2013. Adult females produced a similar capture pattern, with most of them (81%) observed only once, 12.7% twice, and 6% were recorded with three to six sightings.

The maximum time between the first and the last observation of an individual was 1,157 days (3.17 years) for a male, and 683 days (1.87 years) for a female. Fifty-eight percent of the recaptured males and 51% of the females were sighted in successive months during the same breeding season (maximum 120 days); 21% of males, and 42% of the females were recaptured in the dry season of the following year (360–390 days) (Fig. 6). One fifth of adult males (~20%) were logged as recaptures in both dry and wet season sampling sessions, indicating that they stayed along the river all year long. Those females that were seen for longer than

a year were recaptured only in the next sampling sessions corresponding to the dry period.

Adult toads obviously tend to stay in small areas along the banks of the river. The mean linear distance between two successive recapture positions was 2.21 m (95% CI: 0–4.76) for males, and 2.08 m (95% CI: 0.99–5.15) for females. The frequency distributions of distance and time between captures indicate that most recaptures occurred within the first 100 days and 25 m of the initial encounter. There is no evidence of an increase in distance with the number of days elapsed between captures (Fig. 6). Exceptionally, individuals were found far away from their initial capture point, though: one male was recaptured 160 m and one female 56 m from their previous positions (time between recaptures: 38 and 31 days, respectively).

For those individuals with three or more recaptures the average MCP size was 4.57 m² (95% CI: 0.81–8.32) for males ($N = 22$), and 0.23 m² (95% CI: 0.04–0.43) for four females. Seventy-three percent of the adult male MCPs had overlaps ranging from 0.13 to 100%, with a pairwise average of 23.04% (95% CI: 13.8–32.28) (Supplementary Table S2). MCPs of six males (27%) did not exhibit any overlap with other males. Female MCPs were completely separated spatially from each other, but some had overlaps with male MCPs. Of the four estimated female MCPs, one overlapped 95.6% with three male MCPs (pairwise overlap ranged from 6.9 to 63%), and another shared 21.7% of its territory with a male MCP. Despite the expansive spatiotemporal overlaps between adult males, no physical aggressive or territorial defence behaviour between adult males was observed during our study. Only on one occasion, a brief interaction between an adult male and a juvenile/subadult was observed: the adult male walked towards the juvenile,

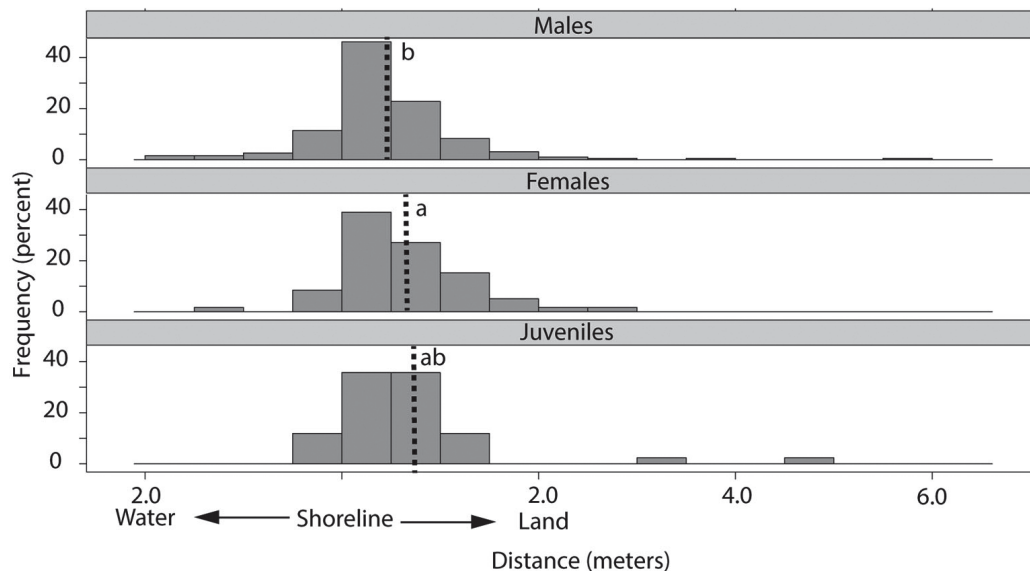


Figure 4. Percent of Rancho Grande Harlequin Toads observed at different distances from the shoreline of the Cata River (October 2005 – February 2007). The dotted line indicates the mean distance for each group. The rankings of distances differed significantly between groups (Kruskal-Wallis test). Letters indicate pairwise differences (Wilcoxon test).

emitted successive low-intensity vocalizations, made with a circular waving movement with one hand, and chased the juvenile to the edge of the rock (F. J. M. ROJAS-RUNJAIC 2006, unpubl. field notes and video).

Discussion

Our data indicate that the Rancho Grande Harlequin Toad is a strictly seasonal breeder with strong breeding site fidelity. This non-random pattern of habitat and season utilization suggests that reproductive activity is not homogeneous in time or space. Understanding the factors driving breeding choices are valuable for designing strategies for the captive propagation and future reintroduction of captive-bred individuals.

As with most tropical anurans and particularly other Harlequin Toad species (DUELLMAN & TRUEB 1994, LÖTTERS 1996, ROCHA-USUGA et al. 2017), the breeding phenology of *A. cruciger* is highly dependent on rainfall-drought patterns. Its reproductive activity starts at the beginning of the dry season, usually in December, when adult males and females (but also some juveniles) migrate from the adjacent forest to the river margin. Their abundance on the riverbanks peaks during the driest months (rainfall < 10 mm), typically between January and February. With the first rains of the wet season, all toads return to the forest, except for a few males that will stay near to the river all year round. In years of long and severe droughts, as we observed in late 2012 and early 2013, breeding activities may start earlier, during October and November, as is evident when the abundance of these toads at the river margins increases notably. In contrast, reproductive activities can be partially or totally disrupted by atypical or anticipated rains and flash floods, even during the dry season. A drop in local rainfall appears to play a fundamental role in triggering breeding in wild *A. cruciger* populations, with

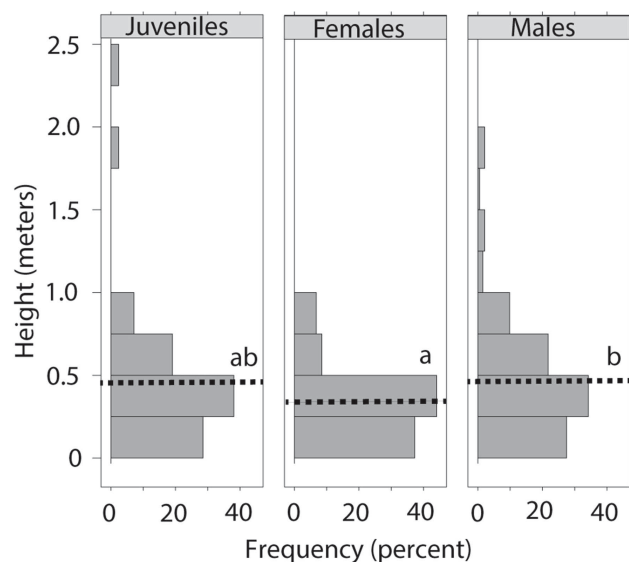


Figure 5. Percent of Rancho Grande Harlequin Toads observed at different perching heights above the ground on the Cata River (October 2005 – February 2007). The dotted line indicates the mean height for each group. The rankings of heights differed significantly between groups (Kruskal-Wallis test).

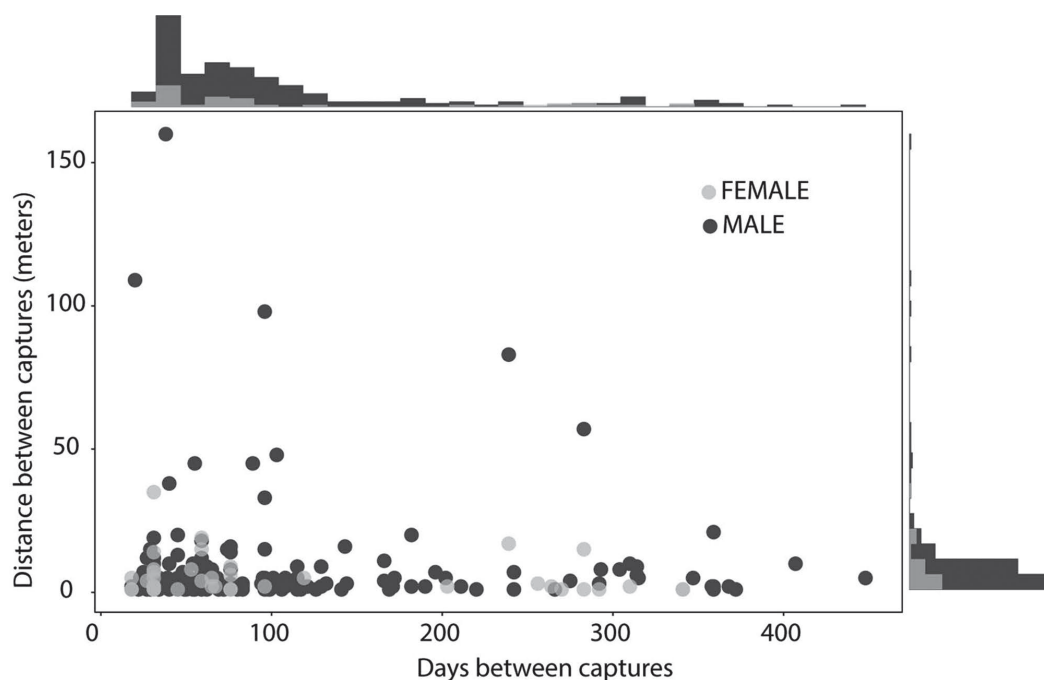


Figure 6. Relationship between distance and time elapsed between successive captures of Rancho Grande Harlequin Toads on the Cata River, Venezuela.

the duration of the breeding period depending on year-to-year rainfall variations, as also has been noted in *A. laetissimus* (ROCHA-USUGA et al. 2017). This high dependency of *A. cruciger* breeding phenology on climatic variables suggests that precipitation and humidity need to be managed to trigger amplexus in captivity, a strategy that has also proven effective for the ex situ breeding programs for other Harlequin Toads (LUGER et al. 2009, GAWOR et al. 2012).

Seasonal migrations for reproductive purposes between the forest and river edge are common in Harlequin Toads (e.g., DOLE & DURANT 1974, LÖTTTERS 1996, KARRAKER et al. 2006, LUGER et al. 2009, NICOLAÏ et al. 2017). The frequent presence of juveniles of *A. cruciger* at the river edge during the breeding period though is puzzling. Higher food availability near the water during the dry season could explain this phenomenon, but *A. cruciger* feeds predominantly on ants (GONZÁLEZ et al. 2012), which are typically more abundant in forested habitats than at riverbanks (COSTA et al. 2010). Furthermore, humidity levels remain relatively high in the forests adjacent to the rivers during the dry months (HUBER 1986, ZINCK 1986, PRADA & TORRES 1996), thus the attraction of juveniles to the riverbank does not seem to be associated with desiccation avoidance. Alternatively, juveniles may be accompanying adults to become familiar with the reproductive habitat and migration routes (e.g., social learning; KELLEHER et al. 2018) or simply adhere to inherited directional migration programming (LANDLER 2022). Females of *A. cruciger* were often seen migrating in groups from the forest to the river's edge along moist paths. It is possible that they use cues for selecting migration routes that minimize risks (e.g., desiccation, predators), or reduce costs associated with new habitat prospecting. Also, in some anuran species, conspecific attraction plays an important role in juvenile habitat selection (PIZZATTO et al. 2016, FOLT et al. 2018) and might be a relevant mechanism of population dynamics here as well. If conspecific attraction is a key factor for habitat selection in juveniles of *A. cruciger*, soft release strategies will be beneficial not only for restoring skin microbiomes that are key for the animals' health (KUENEMAN et al. 2022), but also to allow individuals to develop familiarity and affinity with the habitat while still enjoying protective measures. In this sense, the preservation of the integrity of the riparian forest must be a priority as it forms the habitat of the Rancho Grande Harlequin Toad during the non-breeding season, but also to maintain migratory routes towards the breeding habitat, and the possible connections between different sub-populations along the river basins.

Although *A. cruciger* may travel some distance between the forest and the riverbanks, each breeding season sees adults return to the same locations rather than to new sites. Site fidelity has been documented within a season (< 50 days) in *Atelopus* cf. *senex* (under the name *A. varius*), *A. cruciger*, *A. hoogmoedi*, *A. pulcher* and *A. zeteki* (SEXTON 1958, CRUMP 1986, 1988, POUNDS & CRUMP 1989, LUGER et al. 2009), in a year of sampling *A. carbonerensis* (DOLE & DURANT 1974, under the name *A. oxyrhynchus*), and in two successive reproductive seasons in *A. laetissi-*

mus (GRANDA-RODRÍGUEZ et al. 2020) and in males of the *A. flavescens* complex (RINGLER et al. 2022). Our observations indicate that *A. cruciger* males can maintain the same, small breeding territory (median: 4.57 m²) for at least three consecutive years. In the case of the males that stay on the riverbanks all year round, territories (i.e., total home range) did not exceed 16 m². This behaviour has been suggested to enhance mate attraction, improve knowledge of resources (e.g., spawning sites, shelters, food) or possible escape routes (WELLS 2007). For example, females could use their experience from previous breeding seasons to select and reuse optimal spawning sites, as was suggested to be the case in *A. zeteki* and *A. flavescens* (KARRAKER et al. 2006, GAWOR et al. 2012). For males, knowledge of advertising sites to effectively display their visual signalling, – i.e., bright forefoot waving, and hindfoot raising, improve call transmission, and/or to detect females arriving from the forest could increase their chances of pairing. Although couples arriving already paired at the riverbanks have been documented in some Harlequin Toad species (SEXTON 1958, DOLE & DURANT 1974, TARVIN et al. 2014), we never saw an amplexant couple of *A. cruciger* in the forest or away from the shoreline in our opportunistic surveys. We therefore presume that Rancho Grande Harlequin Toad females are clasped only when they arrive at the river. We consider that the maintenance of small territories in elevated places allows males to spot females earlier as they approach the riverbank.

We found no evidence of physical aggressive behaviours, territorial defence, males chasing females inside their territories, forming mating balls around a female, or amplexus displacement attempts of the kinds documented in other *Atelopus* species (CRUMP 1988, ROCHA-USUGA et al. 2017, RUEDA-SOLANO et al. 2022). Prolonged amplexus appears to be a common pattern in *Atelopus* (LÖTTTERS 1996, RUEDA-SOLANO et al. 2022), and has been interpreted as an effort to monopolize females when the male:female ratio is skewed towards males (DUELLMAN & TRUEB 1994). This does not seem to be the case in the Cata River population of *A. cruciger* with its almost balanced reproductive toad sex ratio of 1.06:1 (LAMPO et al. 2017) and the absence of obvious male–male competition for females. However, male aggressive behaviours involving physical contact have been documented in several Harlequin Toad species under captive conditions, and this behaviour may obviously cause distress and compromise the welfare of males when housed in colonies (CIKANEK et al. 2014). The lack of observations of aggressive behaviours or physical contact in *A. cruciger*, despite the substantial overlap between male territories, suggests that fights between males are uncommon and possibly not a concern for housing several males together in captive breeding settings. However, high densities of males have the potential of triggering behaviours that are not commonly seen in the wild, and therefore should be avoided in captivity.

Despite hundreds of hours spent locating oviposition sites or tadpole microhabitats in the river where post-metamorphic toads are abundant, no tadpoles and only

one desiccated eggs mass have been observed during this study. The lack of knowledge about the microhabitat requirements for females to spawn or tadpoles to develop can hinder captive breeding programs for *A. cruciger*. However, assisted reproduction techniques developed for other Harlequin Toad species, including artificially inducing gamete release and in vitro fertilization, have opened up new avenues if spontaneous spawning will prove difficult to achieve (NARANJO et al. 2022). On the other hand, *Atelopus* tadpoles are in general poorly understood, and less than 30% of the species' larvae have been described (MARCILLO-LARA et al. 2020, PÉREZ-GONZÁLEZ et al. 2020). According to HAAS (1995), juveniles (SVL 7.0 mm) of *A. pulcher* (under the name *A. spumarius*) left the water about two months after spawning, and the time for larval development to stage 41 of the *A. flavescens* complex in ex situ conditions is 100–130 days (GAWOR et al. 2012). In captivity, Rancho Grande Harlequin Toad tadpoles hatched two days after oviposition, and small hind limbs were visible 47 days later (MEBS 1980). The times observed between the peak amplexing and the find of ~10-mm froglets suggests 90–120 days for the aquatic phase (e.g., eggs and tadpole) of *A. cruciger* in our study population. A priority focus of new research in wild populations should be to fill the existing information gap on the development and the microhabitats of pre-metamorphic stages. It is necessary to describe the spawning sites, habitats of tadpoles, and pre-adult phases of the life cycle of the Rancho Grande Harlequin Toad. Additionally, and despite our opportunistic observations made during this study, it is necessary to extend population monitoring to include the adjacent forest, probably the main habitat of juveniles and adults *A. cruciger* during the non-breeding season.

The high site fidelity and small home ranges of Harlequin Toads (< 100 m²) (DOLE & DURANT 1974, LUGER et al. 2009, GRANDA-RODRIGUEZ et al. 2020, RINGLER et al. 2022, this work) suggest limited dispersal of, and reduced gene flow between, subpopulations, especially in fragmented landscapes. The only two currently known subpopulations of the Rancho Grande Harlequin Toad are more than 10 km apart, and separated by rugged terrain with steep slopes and discontinuous forest vegetation cover. The degree of genetic divergence between these two subpopulations is unknown. For *A. varius*, a significant decrease in genetic diversity between persisting subpopulations following *Bd*-related declines was found by BYRNE et al. (2020), and certain landscape features (e.g., abrupt steep slopes) can act as barriers to gene flow (RICHARDS-ZAWACKI 2009). For critically endangered species with small population sizes, the use of genetic data for making informed decisions can make the difference between the success and failure of conservation programs. The two isolated populations of the Rancho Grande Harlequin Toad warrant quick actions to assess the inter- and intra-subpopulation genetic diversity and delineate evolutionary significant units for in situ and ex situ management. For now, we recommended that each known subpopulation of this Critically Endangered species be treated as an independent management unit.

Our data provide relevant information on the breeding habitat use and reproductive phenology of *A. cruciger*, the first goal of the “Harlequin Toad (*Atelopus*) Conservation Action Plan 2021–2041” (VALENCIA & FONTE 2021). These results are especially important not only in a descriptive context of its natural history, but also for establishing the most suitable combination between in situ conservation strategies and captive breeding programs for future reintroductions. For example, captive-bred toads can be gradually exposed to natural environmental conditions, including diet and substrates that are characteristic of their breeding natural habitat, by housing them temporarily in about 5-m² field enclosures (mesocosms) along the stream. Such a soft-release approach will facilitate effective monitoring of the individuals that are to be reintroduced and so assist with finding and refining adaptive management strategies for making possible the transition from captivity back into the wild.

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Supplementary data

The following data are available online:

Supplementary Table S1. Detailed history of recapture and the position of each adult individuals of *Atelopus cruciger* with ≥ 3 sightings (October 2005–February 2007) in Cata river transect.

Supplementary Table S2. Breeding activity area (MCPs) and pairwise overlap (in meters and %) between males and females of *Atelopus cruciger* with ≥ 3 recaptures during the period October 2005–February 2007.

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