# Release trial of captive-bred variable harlequin frogs *Atelopus varius* shows that frogs disperse rapidly, are difficult to recapture and do not readily regain skin toxicity

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**Abstract** Variable harlequin frogs *Atelopus varius* have declined significantly throughout their range as a result of infection with the fungal pathogen *Batrachochytrium dendrobatidis* (Bd). The Panama Amphibian Rescue and Conservation Project maintains an ex situ population of this Critically Endangered species. We conducted a release trial with surplus captive-bred *A. varius* individuals to improve our ability to monitor frog populations post-release, observe dispersal patterns after freeing them into the wild and learn about threats to released frogs, as well as to determine whether natural skin toxin defences of frogs could be restored inside mesocosms in the wild and to compare Bd dynamics in natural amphibian communities at the release site vs a non-release site. The 458 released frogs dispersed rapidly and were difficult to re-encounter unless they carried a radio transmitter. No frog was seen after 36 days following release. Thirty frogs were fitted with radio transmitters and only half were trackable by day 10. Tetrodotoxin was not detected in the skins of the frogs inside mesocosms for up to 79 days. Bd loads in other species present at sites were high prior to release and decreased over time in a pattern probably driven by weather. No differences were observed in Bd prevalence between the release and non-release sites. This trial showed that refinements of our methods and approaches are required to study captive *Atelopus* frogs released into wild conditions. We recommend continuing release trials of captive-bred frogs with post-release monitoring methods, using an adaptive management framework to advance the field of amphibian reintroduction ecology.

**Keywords** Amphibia, *Atelopus varius*, *Batrachochytrium dendrobatidis*, Bd, chytridiomycosis, ex situ conservation, radiotracking, reintroduction, skin toxins

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## Introduction

Variable harlequin frogs *Atelopus varius* are brightly coloured Critically Endangered frogs occurring in Costa Rica and Panama that have declined significantly as a result of chytridiomycosis, a disease caused by the fungal pathogen *Batrachochytrium dendrobatidis* (Bd; Escobedo-Galván et al., 2013; McCaffery et al., 2015; Lewis et al., 2019). Most species in the genus *Atelopus* are now threatened with extinction (La Marca et al., 2005; Scheele et al., 2019), leading the Panama Amphibian Rescue and Conservation Project to develop captive management and ex situ reproduction programmes of extant populations of *Atelopus* in Panama (Gratwicke et al., 2016; Lewis et al., 2019). Persisting wild populations have been observed in some warmer climates that are less suitable for the fungus, leading to the formulation of a 'climate refuge' hypothesis (Puschendorf et al., 2011; McCaffery et al., 2015), in which it is proposed that reduced Bd transmission could allow *Atelopus* populations to persist in spite of significant Bd-related mortality (Lampo et al., 2017). In addition, some persisting populations have evolved anti-Bd skin secretions that could explain their persistence (Voyles et al., 2018), and recent migration and gene flow are evident in these populations, raising the potential of genetic rescue through outbreeding (Byrne et al., 2021). However, it was recently demonstrated that skin secretions of the Panamanian golden frog *Atelopus zeteki* could increase susceptibility to chytridiomycosis (Gass & Voyles, 2022), and thus understanding specific

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components of skin defence is required (reviewed in Woodhams et al., 2023).

The threat of Bd in Panama has not abated, but captive-bred animals have been a valuable tool in efforts to understand the disease and test potential solutions in ex situ contexts (Lewis et al., 2019; Becker et al., 2021). Rapid declines of animals following Bd outbreaks have led to the disappearance of highly susceptible species before in situ disease dynamics could be adequately studied (McCaffery et al., 2015), and studies of small persisting populations are challenging because of sample size limitations (Perez et al., 2014). A few studies of Bd in wild *Atelopus* populations suggest that thermal context is important in persistence and disease dynamics (Richards-Zawacki, 2010; Lampo et al., 2017; Sauer et al., 2018). Carefully designed in situ studies could shed valuable light on the potential for interventions that could favour those frogs facing disease threats (Scheele et al., 2014). However, conducting in situ studies of released animals is not straightforward, and captive-bred animals face many threats in addition to disease as they transition back into the wild. Furthermore, concern has been expressed that susceptible species that can develop high Bd loads could act as supershedders of Bd, and so their release could have negative impacts on other species living at the release site (DiRenzo et al., 2014).

There are many unique, taxa-specific challenges when performing a reintroduction of *A. varius* frogs. For example, *A. varius* is aposematically coloured and diurnal and probably relies on its toxins for predator defence. *Atelopus varius* is known to possess tetrodotoxin (TTX), a powerful water-soluble toxin thought to be of bacterial origin, and when reared in captivity the production of these chemical defences is reduced or eliminated (Daly et al., 1997). This raises questions about both the mechanism of production and how long it will take a reintroduced frog to regain its chemical defences. The recovery time of natural skin defences is an important question for future reintroductions, as without these defences, predators could potentially pose a greater threat to reintroduced animals than the chytrid fungus. We hypothesize that skin toxicity will recover upon exposure to natural diets and/or symbiotic bacteria.

Another issue to consider is that wild *A. varius* are known to be territorial, and both sexes exhibit homing behaviour to within 1 m of their original territory if displaced (Crump, 1986). When conducting surveys, males are more frequently encountered on stream banks than females (Crump, 1988), and it is unclear whether these differences are because of habitat preferences, skewed sex ratios or differences in detectability. After the emergence of Bd at a site, the chances of re-encountering females increased, suggesting that this water-borne disease could have more rapidly reduced stream-dwelling males than females that are less closely linked to streamside habitats (McCaffery et al., 2015). When placing captive-bred animals into the wild, understanding sexually dimorphic dispersal behaviour could be critical to disease ecology, site fidelity and sex-based recapture probabilities.

Intensively monitored pilot releases have been recommended to facilitate understanding of the threats faced by animals after reintroduction, but poor recapture rates are a recurring problem in many amphibian reintroduction projects, limiting the potential for effective research or adaptive management (Linhoff et al., 2021). Post-release monitoring of reintroduced populations is challenging because of low or variable detection probabilities and high numbers of unobservable individuals. Developing methods to monitor released animals effectively is therefore an essential requirement to advance in situ conservation of this threatened species.

The primary objective of this study was to conduct the first release trial of *A. varius*. Our goals were to (1) improve our ability to monitor populations post-release, (2) observe dispersal patterns as individuals transition from captivity to the wild, (3) increase our understanding of immediate threats to released animals and (4) observe whether natural skin toxin defences can be restored after a transition to the wild through implementing outdoor mesocosm trials. A synchronous objective to the release trial was to observe Bd dynamics in wild amphibian communities at the release site compared to a similar site where no release was conducted.

## Study area

We conducted the study in the Donoso area, Colón Province, Republic of Panama, within the Río Caimito basin at 50–140 m elevation. The natural vegetation of this area comprises tropical lowland evergreen rainforest, subjected to c. 4,500 mm mean annual precipitation (IGNTG, 2016). Estimated precipitation is 2,500–3,000 mm during the rainy season (mid May to mid December) and 1,000–1,200 mm during the dry season (mid December to mid May; ETESA Hidrometerología, 2020). A large open-pit mining operation is being developed in the Donoso area. We selected two accessible first-order perennial streams with protected, forested catchments within the known range of *A. varius* that appeared to be suitable habitat even though they are not known historical localities for the species. The nearest known historical locality for *A. varius* was c. 10 km to the south. We simultaneously monitored the amphibian community at the release stream (8.91582°N, 80.66270°W) and a control stream c. 650 m away (8.91186°N, 80.65733°W).

## Methods

### Provenance of animals

We selected 470 captive-bred *A. varius* individuals for the release trial. The individuals were 1–2 years old and were

derived from seven clutches, representing 14 lowland founder animals from the Donoso area that had been collected as part of the Panama Amphibian Rescue and Conservation Project. These comprised 279 females (mean weight 4.5 ± SD 1.7 g, mean snout to vent length 39 ± SD 5 mm) and 191 males (mean weight 3.0 ± SD 0.9 g, mean snout to vent length 33 ± SD 4 mm). Pre-release, we swabbed and confirmed as negative for Bd one frog from each holding tank housing up to 10 same-sex individuals, and a veterinarian (EK) examined all individual animals.

## Tracking protocol

We fitted 12 males and 18 females with radio transmitters to observe the dispersal patterns of the frogs and compare the movements of males and females. We used 0.31 g LB-2X transmitters (Holohil Systems Ltd, Carp, Canada; 6–12% of the body mass of the animals) with a 21 day battery life and a mean detection range of 80–120 m depending on habitat and topography. We attached the transmitter to the waist of the frog using cotton threaded through 1 mm (inner diameter)–2 mm (outer diameter) surgical silicone tubing (Plate 1; Bartelt et al., 2000; Rowley & Alford, 2007; Pašukonis et al., 2014). The transmitter of larger frogs had an additional tubing attachment around the chest. A test on a captive frog showed that the cotton thread degraded in c. 30 days, serving as an auto-release mechanism (Rowley & Alford, 2007). We released animals at 5–10 m intervals along the 150 m stream transect on 17 January 2018. Weather permitting, we attempted to locate animals once daily using a Biotracker radio receiver (Lotek, Ontario, Canada), and we documented all recapture locations using a GPS with a mean error of 7 ± SD 3 m at the release site. We searched with the radio telemetry equipment up to c. 30 m upstream and c. 30 m downstream of the release site. After 11 days, we removed radiotracking devices at the first recapture and released the frogs. We mapped dispersal paths using *QGIS 3.10.2* (QGIS Development Team, 2021). We were able to calculate the 11- or 12-day dispersal distance for seven males and eight females using the Haversine formula (Robusto, 1957), to determine the distance moved between the release location and the last post-release encounter 11 or 12 days later. The dispersal distances between male and female frogs did not meet assumptions of normality or homogeneity of variance for parametric tests, so we compared them using a non-parametric Wilcoxon rank-sum test in *R 4.0* (R Core Team, 2021).

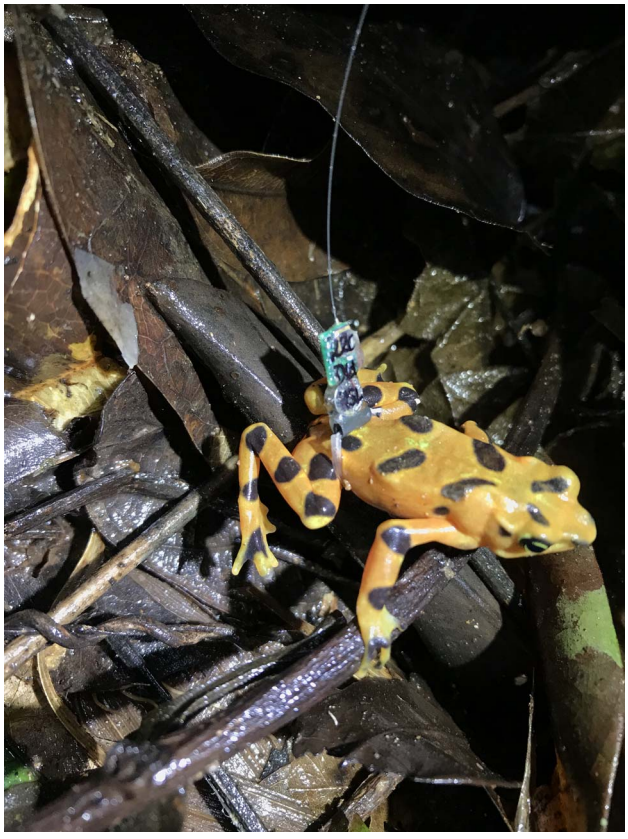

PLATE 1 Variable harlequin frog *Atelopus varius* fitted with a 0.3 g radio transmitter.

## Mark–recapture

We adopted a robust recapture design for this study as it assumes open populations, allowing for immigration, mortality and recruitment between intensive concurrent resampling periods that assume closed populations (Pollock, 1982). This method has been successfully used to monitor wild *Atelopus* populations of 50–100 animals (Tarvin et al., 2014). We uniquely marked frogs for recapture analysis through a combination of a single toe-clip and coloured visual implant elastomer (NW Marine Technology, Anacortes, USA), injected into the palm of a hand or foot (Dodd, 2010). We took individual dorsal and ventral photos of each frog as a back-up to validate individual IDs if necessary using pattern-mapping (Dodd, 2010). We released 428 frogs for the mark–recapture study. We divided these into 15 groups (28–30 frogs in each group) that we freed along the margins of the release stream split into 15 pre-marked transect segments every 10 m along a 150 m transect on 17 January 2018 (Supplementary Material 1). We selected six gravid females visually determined to be at grade 5 on the *Atelopus* gravidity scale of Bronson et al. (2021). We housed each gravid female in its own small enclosure and paired her with an unrelated male the night before release, and we released the four males that amplexed the gravid females as amplectant pairs; we released all others individually. We established three survey transects at the release site: transect 1 was directly on the stream and where the animals were released; transects 2 and 3 were 10 m either side of transect 1, in parallel. One trained surveyor walked

transects 2 and 3 in a single direction, searching c. 2 m either side in a standard visual encounter survey, with effort standardized by transect length (Heyer et al., 1994). Two trained surveyors walked the stream transect, with each focusing on their side of the stream and bank. We resampled the transects by visually resurveying for *Atelopus* only on seven occasions, each consisting of 3 consecutive sampling days on which we walked each transect once per day. We conducted these diurnal visual encounter surveys immediately following release, at 10 days after release and, subsequently, at c. monthly intervals for 5 months. We analysed recapture data using *Rcapture 1.4.3* in *R*, following a robust design (Pollock, 1982; Rivest & Baillargeon, 2019). We swabbed all recaptured frogs from day 10 onwards to check for Bd.

## Skin toxicity

We evaluated recovery of TTX skin toxicity using three groups of frogs. We euthanized immediately the first group of five frogs (pre-release) to examine TTX quantities in captivity, and we housed individually the second group of 12 frogs in 12 outdoor terrestrial mesocosms filled to depths of 10–15 cm with leaflitter collected from the field environment. Each mesocosm was an 80 × 76 × 45 cm cage constructed from 0.7 cm gauge firm plastic mesh. We euthanized five frogs at day 27, and we euthanized the remaining seven frogs at day 79. We euthanized frogs using a topical overdose of 20% benzocaine anaesthetic Orajel (AVMA, 2007; Cecala et al., 2007). We preserved dissected skins from euthanized frogs in 50 ml Falcon tubes filled with methanol and stored at −80 °C prior to extraction and analysis.

We used an analytical method adapted from Bane et al. (2016), as follows: we filtered crude extracts from all samples through a 1 ml Norm-Ject disposable syringe attached to an Acrodisc CR 13 mm, 0.2 µm polytetrafluoroethylene membrane filter. We evaporated the methanol from the crude extracts in vacuo in a DR 120 SpeedVac with the drying rate on low. We stored the dried crude extracts at −80 °C until ready for liquid chromatography–mass spectrometry analysis, at which point we reconstituted them in 100 µl of high-performance liquid chromatography-grade methanol and transferred them via micropipette into 0.25 ml polypropylene autosampler vials for liquid chromatography–mass spectrometry analysis. We analysed the crude extracts via high-performance liquid chromatography using a Shimadzu LC-20 liquid chromatograph equipped with a Phenomenex Luna HILIC column (3 µm, 150 × 4.6 mm) and an Applied Biosystems SCIEX API 2000 triple quadrupole mass spectrometer. We separated the samples with a binary mobile phase flowing at 0.4 ml/min consisting of water (1 l) with formic acid (75 µl) and ammonium hydroxide (300 µl; solvent A) and 70:30 water:acetonitrile (1 l) with formic acid (100 µl; solvent B). The gradient was as follows: 70% B (2 min hold) ramped to a mobile phase concentration of 30% B over 3 min (5 min hold) before being ramped back to 70% B for the remainder of the run. Each run lasted 15 min. We determined the behaviour of TTX using a commercially available TTX standard (Toronto Research Chemicals, North York, Canada); under these conditions, the retention time was c. 6.7 min. We performed mass spectrometry analyses using positive electrospray ionization in single quadrupole scan mode or multiple reaction monitoring mode scanning for transitions of 320→ 302 and 320→ 162 (Tsujimura & Yamanouchi, 2015). For the multiple reaction monitoring mode scan, we optimized the declustering potentials and collision energies for each transition at 30 and 50 V, respectively. We obtained all spectra under *Analyst 1.6.2* (SCIEX, Framingham, USA) control. This method successfully detected small TTX quantities from other amphibian species (K. Minbiole & J.A. Tasca, unpubl. data, 2019), and it would have isolated TTX previously reported from two wild *A. varius* populations (Kim et al., 2003) or 4-epi-TTX, which is usually found in nature to be present in smaller quantities than TTX. Other TTX or saxitoxin analogues (e.g. zetekitoxin AB), often found in *Atelopus* species and reviewed previously (Pearson & Tarvin, 2022), would not have been detected using this method.

We used a complementary targeted amplicon approach to determine the proportion of skin bacteria that could produce TTX on frogs in the mesocosms on pre-release and days 28 and 79. Skin bacteria have been collected and analysed previously (Kueneman et al., 2022). We assembled a sequence file of 16S rRNA gene sequences of known TTX-producing bacteria from Vaelli et al. (2020), where the bacteria were cultured from the toxic rough-skinned newt *Taricha granulosa*. The *T. granulosa* and matching *A. varius* bacterial sequences are provided in Supplementary Materials 2 & 3.

## Amphibian community dynamics

We conducted one diurnal and one nocturnal visual encounter survey along a 150 m stream transect in the release (i.e. transect 1) and control streams (Heyer et al., 1994), 6–7 days prior to the release and, subsequently, at c. monthly intervals for 5 months. These surveys were additional to the ones focused only on *Atelopus*. Two trained personnel visually searched the stream and c. 2 m on either side for amphibians, one working on either stream bank. We captured all amphibians using a freshly gloved hand (or using a fresh plastic bag) and then swabbed them to detect Bd. We calculated the community species richness at each site over the six repeated diurnal and nocturnal samples during the 155-day monitoring period using the *vegan* package in *R* (Oksanen et al., 2019).

### Detection of *B. dendrobatidis*

We swabbed the skin of individual frogs using a sterile MW113 rayon swab, with 60 passes total (i.e. 10 midventral, 10 on each flank, 10 on each thigh, 5 on the ventral side of each foot). We stored the swabs dry in cryovials that were frozen at −20 °C prior to DNA extraction and quantitative PCR analysis. The extraction of DNA from swabs followed Hyatt et al. (2007). We performed the quantitative PCR analysis according to the protocol of Boyle et al. (2004) with some modifications, as indicated herein. We used 20 µl reactions (Kriger et al., 2006b) in a Roche LightCycler 96 system. Each reaction contained 5 µl of sample and 15 µl of a master mix. The master mix contained 10 µl of Roche FastStart Essential DNA Probes Master, 0.25 µl of Roche LightCycler uracil-DNA glycosylase to avoid PCR carryover contamination, 0.8 µl of 18 µM ITS1-3 Chytr and 0.8 µl of 18 µM 5.8S Chytr primer solutions, 1 µl of 5 µM ChytrMGB2 probe solution, 2 µl of 10X Exo IPC mix and 0.4 µl of 50X Exo IPC DNA TaqMan Exogenous Internal Positive Control Reagents (Applied Biosystems No. 4308323) for uncovering false negatives from reaction inhibition (Hyatt et al., 2007). We tested the samples in triplicate, along with five Bd-negative and one or two Bd-positive controls on every plate (Boyle et al., 2004; Kriger et al., 2006a). We considered samples with two or three positive reactions to be Bd positive. We repeated a few samples to confirm these results. We quantified the infection load of Bd-positive samples based on the number of zoospore equivalents (Boyle et al., 2004). We used tenfold serial dilutions of the standards that ranged from 0.1 to 10,000 zoospore equivalents of the JEL 423 Bd strain (originally isolated in Panama). We averaged the quantified values and multiplied them by 100 to compensate for the extraction and dilution of the samples.

## Results

### Tracking data

After release, we obtained 151 relocations of radiotracked animals, with a mean of 5 relocations and 8.3 days of tracking time per animal (Supplementary Material 4). Radiotracked frogs dispersed rapidly beyond the resurvey area, and by day 9 or 10 only c. 50% of radiotracked frogs were found inside the area associated with transects within the general release area (Table 1). Data were not recorded on days 4 and 5 because of heavy rainfall. All radiotracked frogs tested negative for Bd on the last day of sampling prior to removal of the transmitter. Females dispersed further than males (Wilcoxon rank sum test $W = 49$, $P = 0.013$), 62% of females (5/8) dispersed > 50 m in the first 11 or 12 days following release and two of these females dispersed > 100 m. Only 14% of males (1/7) dispersed > 50 m in that period (Table 2, Fig. 1). The mean dispersal distances of females (n = 8) and males (n = 7) were 65.0 and 22.7 m, respectively (Table 2). Of 30 tracked frogs, we recovered only half at the end of the first 10 days. We recovered one detached transmitter on day 1. We could not detect five frogs after day 2, and we last saw seven frogs on days 6–8. We traced one of the frogs that disappeared on day 8 to a Savage's thin-toed frog *Leptodactylus savagei* hole and so it could have been predated. One frog had a malfunctioning transmitter but was incidentally encountered on days 3 and 7.

Table 1 Number and per cent of radiotracked variable harlequin frogs *Atelopus varius* found inside the mark–recapture survey area in Panama (Fig. 1) after release day.

| Day | Radiotracked frogs found (n) | Recaptures associated with resurvey transects (%) |
|---|---|---|
| 1 | 11 | 100 |
| 2 | 23 | 73 |
| 3 | 9 | 66 |
| 6 & 7 | 21 | 80 |
| 8 & 9 | 19 | 60 |
| 10 | 11 | 54 |
| 11 & 12 | 16 | 56 |

### Mark–recapture

We recaptured 78 unique individuals of the 428 non-radiotracked frogs that we released (Table 3), the last of which we saw 36 days following release. We had 35 recaptures on day 1, which decreased to 23 recaptures on day 2 and 25 recaptures on day 3. Taken together with the rapid

Table 2 Dispersal distance of 15 variable harlequin frogs calculated between the site of release and the last recapture on day 11 or 12 (Fig. 2). The median dispersal distances of females (n = 8) and males (n = 7) were 55.5 and 14.0 m, respectively. Females dispersed further than males (Wilcoxon rank-sum test $W = 49$, $P = 0.013$). All frogs tested negative for *Batrachochytrium dendrobatidis* on the last recapture.

| Sex | ID | Dispersal distance (m) | Day |
|---|---|---|---|
| Female | VA081 | 16 | 11 |
| Female | VA176 | 27 | 11 |
| Female | VA114 | 42 | 11 |
| Female | VA185 | 54 | 11 |
| Female | VA038 | 57 | 11 |
| Female | VA178 | 89 | 12 |
| Female | VA179 | 117 | 12 |
| Female | VA173 | 118 | 12 |
| Male | VA134 | 8 | 11 |
| Male | VA153 | 11 | 11 |
| Male | VA162 | 13 | 11 |
| Male | VA158 | 14 | 11 |
| Male | VA149 | 16 | 11 |
| Male | VA155 | 24 | 11 |
| Male | VA143 | 73 | 11 |

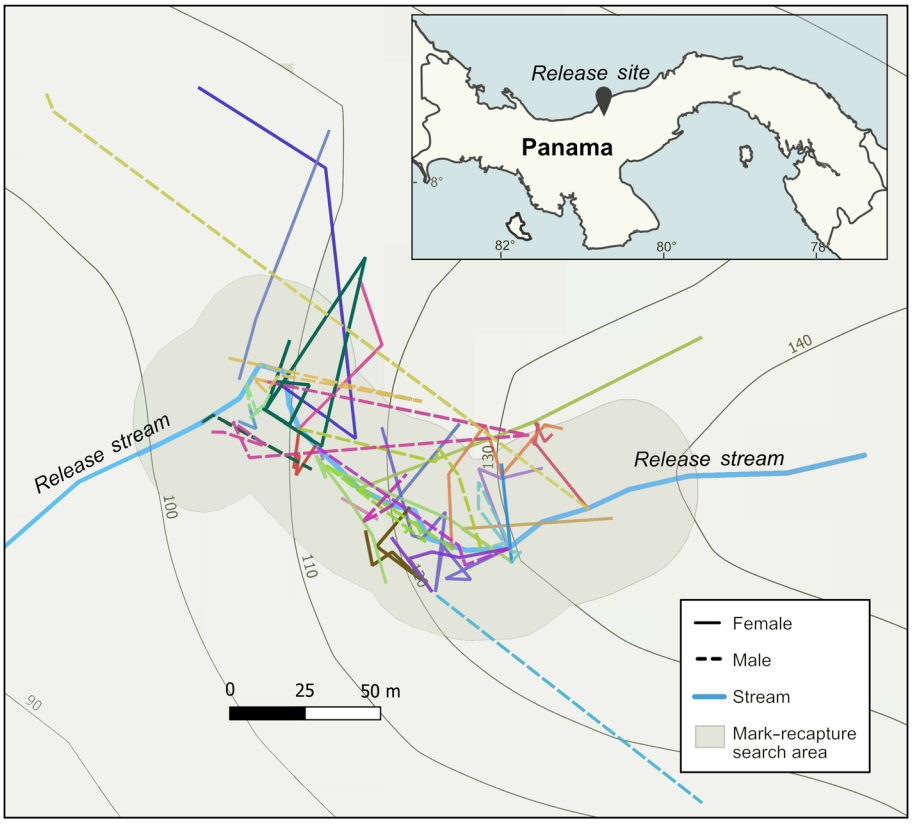


FIG. 1 The release site in Panama, showing the paths of 15 radiotracked variable harlequin frogs *Atelopus varius* (7 males, 8 females) within the first 11 or 12 days of release (Table 2). Frogs dispersed rapidly beyond the mark–recapture area (colours indicate individuals). Females dispersed a mean of 65 m, and males a mean of 23 m.

dispersal of frogs out of the radiotracking study area (Table 1), it appears that the assumption of a closed population for the first recapture period was not satisfied. The closed population estimate for that period was 133 animals ($M_o = 133.6 \pm SE$ 24.5, Akaike information criterion = 36.50) or 30% of the known number of released animals (Table 3). We did not record sufficient numbers of recaptures in subsequent recapture periods to implement the planned robust recapture design (Table 3). Two of 13 of the frogs recaptured on day 10 tested positive for Bd, and we detected no frogs on days 11 or 12 (Supplementary Material 5), possibly because of heavy rain and reduced detectability. We are uncertain as to the fate of these individuals as we were unable to observe them. Anecdotally, we observed courtship behaviour amongst untracked animals, with male frogs calling and fighting from rocks in the stream, and on day 7 we observed one male amplexing a female (Plate 2). We also observed that following release some individuals became a darker green colour, probably reducing their detectability.

## Skin toxicity

We could not detect TTX on any of the captive-bred *A. varius* prior to release (n = 5). Frogs did not appear to regain toxicity in mesocosms, as all animals euthanized at day 27 (n = 5) and at day 79 (n = 7) also tested negative for TTX. A targeted amplicon sequencing approach detected bacteria on the skin matching known TTX producers described previously (Vaelli et al., 2020). The proportion of reads was < 10% of the bacterial community and increased substantially with time from < 1% in captivity to c. 8% in the outdoor mesocosms. The sequences from *A. varius* that matched with TTX producers are described in Supplementary Material 3 and included four genera: *Pseudomonas*, *Aeromonas*, *Shewanella* and *Sphingopyxis*. By comparison, the proportion of skin bacteria predicted to contribute to anti-Bd defence function increased from c. 40–60% over the same period in the mesocosms (Fig. 2; based on microbiome sequencing reported in Kueneman et al., 2022).

## Amphibian community dynamics

During the amphibian community surveys, 83.7 person-hours resulted in 28 species and 230 individuals being found at the two sites. The Chao 1 species richness estimates were similar for both sites: 24.6 ± SE 2.2 for the release site and 23.6 ± SE 2.2 for the control site. The number of frogs and number of species encountered varied over the survey period. The pattern of change in both community metrics was similar at both the release and control sites (Fig. 3), indicating that the cause of the observed changes occurred at both sites and was unlikely to be a result of the release of

TABLE 3 Variable harlequin frog recaptures following the release day of 17 January 2018 (i.e. day 0) when we released 428 individuals for the mark–recapture study. We resurveyed all transects on three concurrent days in each recapture period. In recapture period 2 it is probable that heavy rains on days 11 and 12 affected the detectability of the frogs and we did not attempt a population estimate.

| | | Experimental day | Recaptures (n) | Population estimate |
|---|---|---|---|---|
| Recapture period 1 | Visit 1 | 1 | 35 | |
| | Visit 2 | 2 | 23 | |
| | Visit 3 | 3 | 25 | 133.6 ± SE 24.5 |
| Recapture period 2 | Visit 1 | 10 | 13 | |
| | Visit 2 | 11 | 0 | |
| | Visit 3 | 12 | 0 | |
| Recapture period 3 | Visit 1 | 34 | 0 | |
| | Visit 2 | 35 | 0 | |
| | Visit 3 | 36 | 1 | |
| Recapture period 4 | Visit 1 | 76 | 0 | |
| | Visit 2 | 77 | 0 | |
| | Visit 3 | 78 | 0 | |
| Recapture period 6 | Visit 1 | 97 | 0 | |
| | Visit 2 | 98 | 0 | |
| | Visit 3 | 99 | 0 | |
| Recapture period 7 | Visit 1 | 118 | 0 | |
| | Visit 2 | 119 | 0 | |
| | Visit 3 | 120 | 0 | |
| Recapture period 8 | Visit 1 | 145 | 0 | |
| | Visit 2 | 146 | 0 | |
| | Visit 3 | 147 | 0 | |
| *Total* | | | 97 recaptures (78 individuals) | |

*Atelopus*. Bd prevalence was variable, probably because of the low total numbers of frogs swabbed on each sampling occasion, but Bd prevalence was highest at both sites immediately preceding release, exceeding 40% at both sites then declining at both sites, which had a similar mean Bd prevalence of c. 23% (Table 4, Fig. 3). The observed high Bd prevalence at the time of release was probably associated with weather conditions. On the first sampling occasion immediately preceding release, we observed two dead frogs (*Craugastor talamancae* and *Pristimantis* sp.) with high Bd loads exceeding 1 million zoospore equivalents (Supplementary Material 6). Approximately 3–18% of the frogs we encountered in some months (January, February and May) had high Bd loads exceeding 100,000 zoospore equivalents (Supplementary Material 6), including a released individual of *A. varius*.

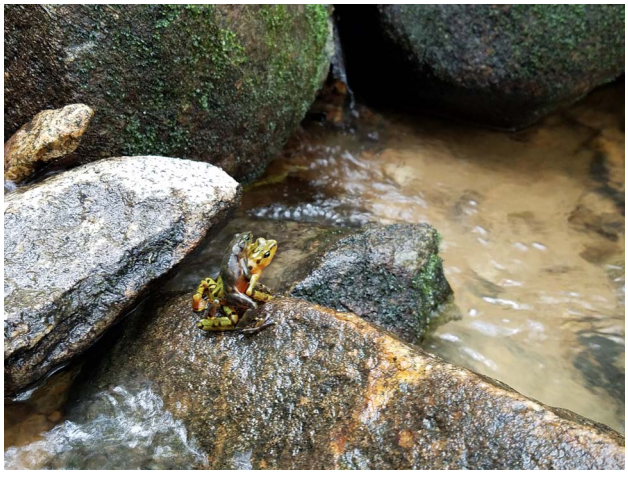

PLATE 2 Male (top) and female (bottom) amplectant pair of *A. varius* observed following male–male aggressive interactions with an undepicted male on day 7 following release.

## Discussion

The goal of this study was to understand how captive-bred *A. varius* transition into a wild situation. Frogs did not regain any detectable skin toxicity in the first 2.5 months post-release. We found that frogs dispersed rapidly and were difficult to re-encounter unless they had a working radio transmitter or were housed in a mesocosm. The low re-encounter rates prevented us from gaining insight into the fate of these animals. Some released animals rapidly contracted Bd, but within the wild frog community Bd prevalence rates at the release site and a control site were similar during the post-release observation period. We planned our release trial around the early dry season in Panama, but unseasonable continuous heavy rain negatively affected our work. Heavy rain on some census days prevented us from conducting radiotracking and probably reduced the detectability of frogs during visual encounter

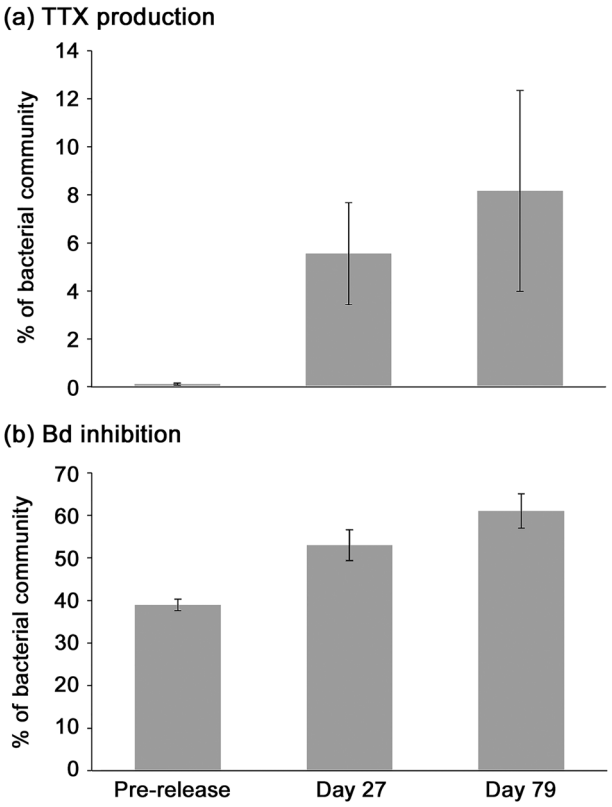


FIG. 2 Per cent of the bacterial community (mean reads ± SE) thought to (a) produce tetrodotoxin (TTX) or (b) inhibit growth of *Batrachochytrium dendrobatidis* (Bd) on the skin of *A. varius* held in outdoor mesocosms based on targeted amplicon sequencing described in Kueneman et al. (2022).

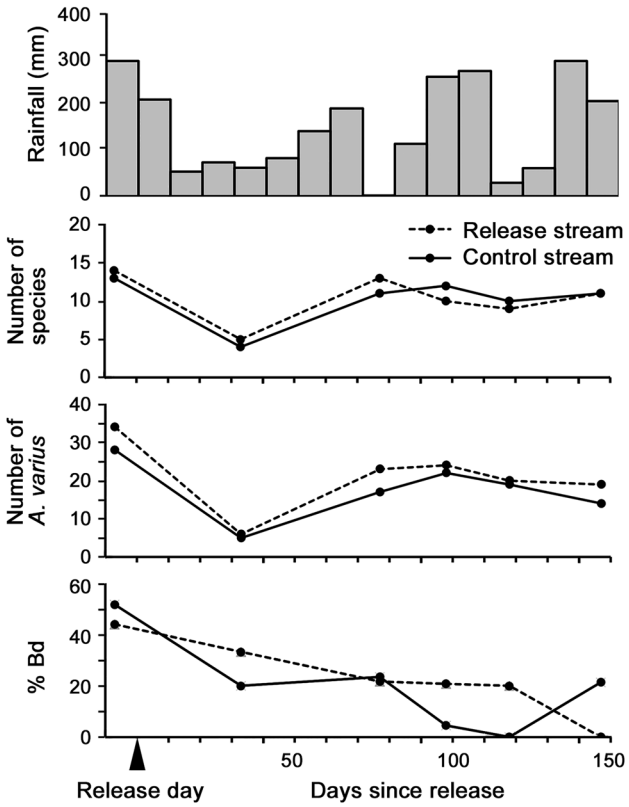


FIG. 3 Ten-day rainfall totals, number of species observed, total number of all *A. varius* counted and *Batrachochytrium dendrobatidis* (Bd) prevalence in frog communities (Table 4) on 150-m long stream transects along the release and control streams that were surveyed both diurnally and nocturnally at regular intervals before and after the release day of 17 January 2018.

surveys. During a subsequent visit to the release site c. 1 year later (3 July 2019), we recorded the presence of three frog species but did not observe individuals of *A. varius* along the three surveyed transects or in surrounding areas.

The radiotracking method offered a labour-intensive way to track a limited number of frogs (≤ 30) for a short duration. Nonetheless, 50% of the radiotracked animals disappeared in just 10 days, possibly due to transmitter failure, predation or dispersal beyond the area of detectability. This method was suitable for mapping dispersal over longer distances. We were able to discern sex-based dispersal differences, but the GPS location, with an error of 7 m ± SD 3 m, was not sufficiently precise to measure finer-scale movements and territory establishment within 10 m, which would have required an intensive manual quadrat survey grid (Crump & Pounds, 1989) or a high-resolution forest mapping approach (Ringler et al., 2016). Male *A. varius* and *A. zeteki* are usually encountered in higher densities along streams (Crump, 1986; McCaffery et al., 2015). Males are more territorial and defend their territories through calls, semaphoring behaviour and aggressive interactions (Lindquist & Hetherington, 1996). It is probable that males are simply more detectable than females, but it is also possible there is a sex-ratio skew in the population (Crump, 1988; Crump & Pounds, 1989); however, our observations suggest that the females are less tied to stream banks and could disperse further.

We released many animals for the mark–recapture study: the equivalent of 285 frogs per 100 m stream segment. This number is large even for pre-Bd *Atelopus* populations. One *A. varius* site using mark–recapture documented 5–16 individuals/100 m, and another *A. zeteki* site found 34–75 individuals/100 m (McCaffery et al., 2015). We released frogs at much higher densities, hoping that even with significant dispersal outside the core release area, a sufficiently high density would remain in the core recapture transects to conduct effective mark–recapture evaluations of the population. However, our transect search-based mark–recapture population estimate methods were not sufficient to recapture frogs even with the large number of animals released. Both the dispersal area and density of frogs within the release area changed rapidly (Fig. 1). It is possible that the detectability of the frogs changed as some brightly coloured animals assumed an olive colouration post-release. As frogs settled into their new environment, they probably found

TABLE 4 Summary of community survey data at the release and control sites ranked by total number of variable harlequin frogs sampled. Binomial 95% CIs are shown for species for which we recorded > 10 individuals. Chao 1 species richness estimates for the release site and control site were similar: 24.6 ± SE 2.2 and 23.6 ± SE 2.2, respectively. Bold indicates species where at least one individual had a *Batrachochytrium dendrobatidis* (Bd) load > 100,000 zoospore equivalents (Fig. 3). Full data are in Supplementary Material 6.

| | Release stream | | Control stream | | Total | | |
|---|---|---|---|---|---|---|---|
| Taxon | n | Bd+ | n | Bd+ | n | Bd+ (%) | Bd 95% CI |
| ***Craugastor talamancae*** | **30** | **10** | **10** | **4** | **40** | **35** | **21–52** |
| *Rhinella alata* | 25 | 5 | 10 | 4 | 35 | 26 | 12–43 |
| *Lithobates vaillanti* | 6 | 1 | 12 | 3 | 18 | 22 | 6–48 |
| *Craugastor crassidigitus* | 8 | 0 | 8 | 2 | 16 | 13 | 2–38 |
| *Teratohyla spinosa* | 5 | 1 | 11 | 0 | 16 | 6 | 0–30 |
| *Smilisca phaeota* | 8 | 2 | 6 | 0 | 14 | 14 | 2–43 |
| ***Lithobates warszewitschii*** | **3** | **2** | **8** | **2** | **11** | **36** | **11–69** |
| *Pristimantis cruentus* | 2 | 1 | 8 | 2 | 10 | 30 | 3–60 |
| ***Pristimantis* sp.** | **4** | **1** | **6** | **1** | **10** | **20** | **3–56** |
| *Craugastor bransfordii* | 8 | 2 | | | 8 | 25 | 3–65 |
| ***Pristimantis cerasinus*** | **4** | **1** | **2** | **1** | **6** | **33** | **4–78** |
| *Silverstoneia flotator* | 3 | 0 | 3 | 0 | 6 | 0 | |
| *Espadarana prosoblepon* | | | 4 | 0 | 4 | 0 | |
| *Incilius coniferus* | 4 | 0 | | | 4 | 0 | |
| *Leptodactylus savagei* | 3 | 0 | 1 | 0 | 4 | 0 | |
| *Agalychnis callidryas* | 1 | 0 | 2 | 0 | 3 | 0 | |
| *Andinobates minutus* | 2 | 1 | 1 | 0 | 3 | 33 | |
| *Boana rufitela* | | | 3 | 0 | 3 | 0 | |
| *Craugastor fitzingeri* | 1 | 0 | 2 | 0 | 3 | 0 | |
| ***Craugastor* sp.** | **1** | **1** | **2** | **2** | **3** | **100** | |
| *Phyllobates lugubris* | 1 | 0 | 2 | 1 | 3 | 33 | |
| ***Atelopus varius*** | **2** | **1** | | | **2** | **50** | |
| *Diasporus citrinobapheus* | 2 | 1 | | | 2 | 50 | |
| *Diasporus* sp. | 2 | 0 | | | 2 | 0 | |
| *Colostethus* sp. | | | 1 | 0 | 1 | 0 | |
| *Oophaga vicentei* | | | 1 | 1 | 1 | 100 | |
| *Pristimantis ridens* | 1 | 0 | | | 1 | 0 | |
| *Sachatamia ilex* | | | 1 | 0 | 1 | 0 | |
| *Total number* | **126** | **30** | **104** | **23** | **230** | **23** | **18–29** |
| *Number of species* | **23** | **14** | **22** | **11** | **28** | | |
| *Bd prevalence (% of individuals)* | **23.8** | | **22.1** | | | | |

hiding spots that further reduced their detectability, which could only be overcome with the aid of a radio transmitter (O. Garcés & E. Lassiter, pers. obs., 2018). Even when contained in mesocosms, these animals were not conspicuous and required considerable effort to find (O. Garcés & E. Lassiter, pers. obs., 2018). A more appropriate recapture

methodology would have involved a visual encounter survey design that accommodated a more intensive search method (such as zigzag searches between numbered stakes or intensive quadrat sampling; Heyer et al., 1994), encompassing a much larger projected dispersal area. Another alternative would have been developing a recapture survey method that quantifies search effort and accommodates a changing dispersal area determined by radiotracking a subset of frogs. Regardless, recapturing individuals is likely to remain the greatest limiting factor for monitoring future release trials of *Atelopus*.

One of the problems with releasing an aposematically coloured animal without chemical defences back into the wild is likely to be predation. We did not observe direct predation on the frogs, but it can be inferred from the anecdotal observation of a frog disappearing into a burrow containing a large *L. savagei* and from the rapid disappearance of 50% of the radio transmitters that frogs could have been captured by predators. Captive-raised *Atelopus* lack TTX (Daly et al., 1997; present study), and it is currently unclear how TTX is acquired, although it is postulated to be derived from the frogs' diet or from symbiotic bacteria (Yotsu-Yamashita & Tateki, 2010). The absence of previous information on the skin toxins of wild *A. varius* from the study area precludes comparisons of the natural levels of TTX present in their skin. Studies of Californian newts have shown they can demonstrate TTX increases in captivity (Hanifin et al., 2002) and experience rapidly inducible changes in TTX defences over short periods (Bucciarelli et al., 2017), and that TTX could be bacterially derived (Vaelli et al., 2020). Similarly, it was shown previously that association with toxic parents was necessary to induce detectable TTX in Chinese red-bellied newts *Cynops orientalis* (Mebs & Yotsu-Yamashita, 2021). Captive-bred *Atelopus* appear to rapidly regain their wild microbiome when held in wild environments (Estrada et al., 2022; Kueneman et al., 2022), but after 79 days there was no detectable TTX in the skin of our study animals, thus indicating the value of research into artificial restoration of skin defences prior to release, with dietary provisioning (Kudo et al., 2017) or probiotics. Although putative TTX-producing bacteria were detected at high abundances (up to 8% of reads at day 79) in the mesocosms compared to the 1–2% of reads from the same bacteria detected in toxic rough-skinned newts *T. granulosa* (Vaelli et al., 2020), our results indicate that different strains of bacteria or additional factors might be needed to induce toxicity.

The threat of Bd in Panama has not been mitigated, but there is ongoing research into the evolution of anti-Bd skin secretions and exploration of genetic rescue possibilities (Voyles et al., 2018; Byrne et al., 2021). Rather than setting explicit recovery goals, the aims of this release trial were focused on comprehending the other threats faced by *Atelopus* frogs as they transition back into the wild and on understanding Bd dynamics in the released frogs and the community. Our expectation, based on laboratory studies of *A. zeteki*, was that Bd-related mortality would not begin until c. 2–4 months after exposure (Becker et al., 2011, 2015, 2021; Langhammer et al., 2013), but this is highly variable depending on Bd load, temperature and hydric environment (Bustamante et al., 2010). The released frogs mostly disappeared before we could make any observations, but Bd prevalence in the surrounding frog community was high, probably because of the weather conditions (wet and cool), with two dead Bd-positive non-*Atelopus* frogs observed, and 2/13 of the released *A. varius* were Bd positive by day 10. This was an unseasonably rainy period despite us planning for the release to occur in the dry season. The month of January 2018 received 1,167 mm of rain in this area (Fig. 3), whereas rainfall amounts were more characteristic in the years before and after the trial, with 89 mm in January 2017 and 54 mm in January 2019 (Empresa de Transmisión Eléctrica, S.A. rainfall data, Station 105003, Coclé del Norte, Panama).

One of the concerns about releasing highly susceptible frogs into the environment is that they could develop high Bd loads and become supershedders, which could be detrimental to the persisting frog community as a result of them increasing the environmental disease load (DiRenzo et al., 2014). However, a follow-up experimental study did not validate the supershedder hypothesis (DiRenzo et al., 2018). When comparing our control stream and release stream, we observed that disease loads prior to release were high and declined over time in a pattern probably driven by climatic effects. However, we did not observe any differences in Bd prevalence at the two streams. Any statistical comparisons will require replication, and we present our data (Supplementary Materials 6) for future meta-analysis.

It is evident that surplus individuals from captive collections of threatened amphibians afford numerous research opportunities to enable us to understand the threats they face and to study these animals ex situ and in situ (Lewis et al., 2019). This study revealed that we will need to refine our methods and approaches to study captive amphibians released into the wild. Although the use of mesocosms has proven to be a useful and efficient method for conducting experiments and monitoring *Atelopus* individuals within their natural habitats (Estrada et al., 2022; Klocke et al., 2023; this study), our study also showed that simply increasing the numbers of frogs released into the wild is not sufficient to ensure repeated observations of individuals using visual encounter survey methods. This release trial demonstrated that even if we had a species of frog that shows resilience to the amphibian chytrid fungus in captive settings, we do not have satisfactory tools to perform adequate post-release monitoring in the wild. Radiotracking *Atelopus* individuals is an expensive and labour-intensive method that

has potential for use (Klocke et al., 2023; this study), but it requires further testing and improvement. Moreover, we lack a good understanding of the other potential threats facing amphibians previously acclimated to captive conditions. We recommend continued release trials of captive-bred frogs with intensive post-release monitoring methods using an adaptive management framework to advance the nascent field of amphibian reintroduction ecology. Given that 62 of 94 *Atelopus* species are considered to require ex situ conservation measures for species management (IUCN, 2022) and that the action plans of regional conservation initiatives place captive survival insurance colonies for future reintroduction amongst their conservation priorities (Valencia & Fonte, 2021, 2022), we anticipate that our findings will be useful for other ex situ conservation efforts for *Atelopus* species.

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**Author contributions** Study design, grant writing: BK, BG, RI; captive animal care, breeding: JG, HR; fieldwork: BK, OG, EL, JG, AH, EK, HR, BG, RI; laboratory analysis: EI, KM, JAT, DGW; data analysis: BK, DGW, BG, RI; writing: BK, LL, DGW, BG, RI; supervision: BG, RI; revision: all authors.

**Conflicts of interest** The funders acknowledged above had no role in the study design, data collection and analysis, decision to publish or preparation of the manuscript.

**Ethical standards** This study abided by the *Oryx* guidelines on ethical standards. We obtained the approval 2016-0311-2019-A10 from the Smithsonian Tropical Research Institute Animal Care and Use Committee and the scientific permits SE/APB-1-17 and SE/APH-1-18 from the Panamanian Ministerio de Ambiente to conduct this study.

**Data availability** The data supporting the findings of this study are available within the supplementary materials.

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