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## Short Communication

# Field surveys in Western Panama indicate populations of *Atelopus varius* frogs are persisting in regions where *Batrachochytrium dendrobatidis* is now enzootic

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The stunning Harlequin frogs of the genus *Atelopus*, once common, are now among the most imperiled of all amphibian species (La Marca et al. 2005; Zippel et al. 2006). Of 88 described *Atelopus* species in Central and South America, 65 (74%) are Critically Endangered (La Marca et al. 2005). The most pressing threat to these frogs is chytridiomycosis, a fungal disease caused by *Batrachochytrium dendrobatidis* (hereafter "*Bd*") and associated with die-offs of amphibians around the world (Berger et al. 1998; Longcore et al. 1999; Kilpatrick et al. 2010). In Western Panama, an epidemic wave spreading from west to east caused mass mortality events, resulting in catastrophic losses in amphibian diversity (Lips et al. 2006; Brem and Lips 2008; Woodhams et al. 2008; Kilburn et al. 2010), including declines in three *Atelopus* species: *A. varius*, *A. zeteki*, and *A. chiriquiensis*.

Relatively few studies have been conducted on the amphibian communities of Western Panama following chytridiomycosis outbreaks. Those few investigations that have focused on understanding community composition where *Bd* is now enzootic report differential survival among host species (e.g., Brem and Lips 2008), with some species putatively driven to local extinction (Gagliardo et al. 2008). In particular, *Atelopus* species were considered to be highly vulnerable to disease-induced extinction for multiple reasons. First, *A. varius* was used as an "indicator species" to monitor declines and thus helped to document *Bd* invasion and characterize *Bd*-related amphibian losses (Brem and Lips 2008). Second, *A. zeteki* has been repeatedly tested in controlled laboratory infection experiments and found to be highly susceptible to lethal chytridiomycosis (Bustamente et al. 2010; DiRenzo et al. 2014; Ellison et al. 2014). Third, recent immunogenetics research suggests that *A. zeteki* adaptive immune responses are suppressed by *Bd* (Ellison et al. 2014). Thus, *Atelopus* species have become important focal species in the study of chytridiomycosis dynamics and have also provided motivation for progressive conservation action (Gagliardo et al. 2008).

In October 2012, we revisited study sites that were surveyed for *Atelopus* from 2001 to 2004 (Richards and Knowles 2007) and we established new study sites in hopes of discovering extant populations of Critically Endangered *Atelopus* species. Using a measuring tape, a 200 m transect was developed and marked with flagging tape every 10 m. For our surveys, 2–3 observers walked these transects slowly, searching for amphibians according to established visual encounter survey protocols (e.g., Lips 1999). We captured all post-metamorphic amphibians we encountered using a fresh pair of gloves or inverted plastic bag to minimize transmission or infection and followed strict field hygiene protocols (Phillot et al. 2010). We noted the time and location of capture, identified the species, sex and age class, and measured

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snout-to-vent length and body mass. We also collected skin swab samples for all amphibians using standardized swabbing techniques (Hyatt et al. 2007). We preserved the skin swab samples (by freezing at -20 °C) to test for *Bd* infection using quantitative polymerase chain reaction (qPCR; Boyle et al. 2005; Hyatt et al. 2007). For the qPCR assay, we analyzed all samples in triplicate with an internal positive control (Hyatt et al. 2007) and used a dilution set of plasmid standards (obtained from Pisces Molecular, Boulder, Colorado) to quantify pathogen load. We converted plasmid copy numbers to zoospore copy numbers using the line of best fit ($r^2 > 0.999$) from a linear regression of log (plasmids) vs. log (zoospores) ($t4 = 210.6$, $P < 0.0001$) that we obtained by running the plasmid standard set alongside a series of standards containing known quantities of zoospores (obtained from Alex Hyatt, Australian Animal Health Laboratory). If one of three replicate wells turned up positive, we checked Cycle Threshold (Ct) value to determine whether non-amplification in two of three wells could have been caused by a low-level infection (near the detection threshold) and verified that the qPCR was not inhibited (IPC amplified normally). In cases of inhibition or Ct values far from the detection threshold, we re-ran and considered them positive if *Bd* was detected in any of the three re-run wells.

We surveyed 16 field sites from 2012 to 2013, 10 of which were sites where *Atelopus* species were found in 2004 (Richards and Knowles 2007), prior to the chytridiomycosis epidemic. The remaining six sites were chosen based on other biologists' sightings of *Atelopus*

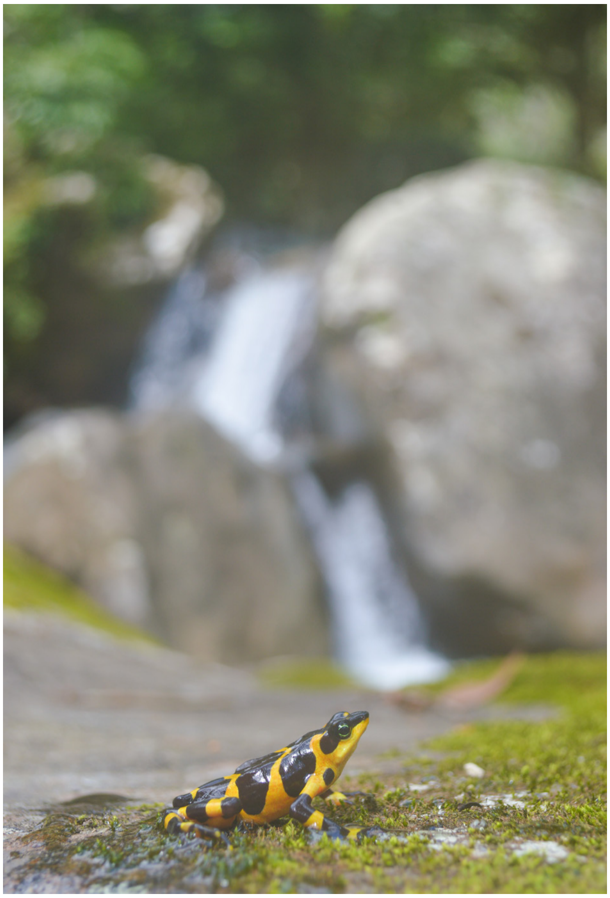

**Figure 1.** A female Harlequin frog, *Atelopus varius*. This species, classified as Critically Endangered by IUCN, has been found in small numbers in the mountains of Western Panama.

**Figure 2.** A pair of *Atelopus varius* in amplexus, found in the mountains of Western Panama.

**Table 1.** Summary of infection prevalence of *Batrachochytrium dendrobatidis* from amphibians found at three sites where *Atelopus varius* still persist.

| Site (Elevation) | Species | Prevalence | N | Lower 95% | Upper 95% |
|---|---|---|---|---|---|
| Highland 1 (735 m) | *Atelopus varius* | 0% | 0/1 | 0 | 0.975 |
| | *Colostethus flotator* | 0% | 0/1 | 0 | 0.975 |
| | *Craugastor bransfordii* | 0% | 0/1 | 0 | 0.975 |
| | *Craugastor crassidigitus* | 0% | 0/1 | 0 | 0.975 |
| | *Craugastor fitzingeri* | 0% | 0/1 | 0 | 0.975 |
| | *Lithobates warszewitschii* | 60% | 3/5 | 0.147 | 0.947 |
| | *Pristimantis cerasinus* | 100% | 1/1 | 0.025 | 1 |
| | *Pristimantis cruentus* | 0% | 0/2 | 0 | 0.842 |
| | *Pristimantis museosus* | 0% | 0/1 | 0 | 0.975 |
| | *Pristimantis ridens* | 0% | 0/1 | 0 | 0.975 |
| | *Sachatamia albomaculata* | 25% | 2/8 | 0.0715 | 0.591 |
| | *Terahyla spinosa* | 0% | 0/1 | 0 | 0.975 |
| | Glass frog metamorph | 0% | 0/1 | 0 | 0.975 |
| Highland 2 (521 m) | *Atelopus varius* | 0% | 0/6 | 0 | 0.459 |
| | *Colostethus flotator* | 0% | 0/7 | 0 | 0.41 |
| | *Colostethus panamensis* | 25% | 1/4 | 0.073 | 0.524 |
| | *Pristimantis ridens* | 0/1 | 0/1 | 0 | 0.975 |
| | *Rhaebo haemititicus* | 42% | 1/5 | 0.057 | 0.437 |
| | *Silverstoneia flotator* | 33% | 1/3 | 0.008 | 0.906 |
| | *Smilisca* spp. | 100% | 3/3 | 0.292 | 1 |
| Lowland 1 (0 m) | *Atelopus varius* | 0% | 0/1 | 0 | 0.975 |
| | *Craugastor bransfordii* | 0% | 0/2 | 0 | 0.842 |
| | *Craugastor crassidigitus* | 0% | 0/1 | 0 | 0.975 |
| | *Craugastor longirostris* | 0% | 0/1 | 0 | 0.975 |
| | *Craugastor fitzingeri* | 0% | 0/2 | 0 | 0.842 |
| | *Dendrobates auratus* | 0% | 0/3 | 0 | 0.708 |
| | *Dendrobates minnutus* | 0% | 0/1 | 0 | 0.975 |
| | *Diasperous* spp. | 0% | 0/3 | 0 | 0.708 |
| | *Pristimantis caryophyllaceus* | 0% | 0/1 | 0 | 0.975 |
| | *Rhinella alata* | 0% | 0/10 | 0 | 0.308 |
| | *Silverstoneia flotator* | 0% | 0/1 | 0 | 0.975 |

(e.g., Hertz et al. 2012) or predicted habitat suitability in species distribution models. We found persisting populations of *A. varius* at three of 16 (18.7%) field sites (Fig. 1, Table 1). At one site, we found one juvenile *A. varius*, five adult males, and two adult females, including one pair in amplexus (Fig. 2). We found individual adult males at each of the other two respective sites (Table 1). We have intentionally only provided general site information, rather than precise site coordinates, due to the risk of illegal animal collections.

We confirmed that *Bd* is present in two of these three populations based on detection of *Bd* on skin swabs from other species (e.g., *Lithobates warszewitschii* and *Sachatamia albomaculata*), but none of the *Atelopus* samples were *Bd* positive on these transects (Table 1). These sites vary in elevation from 45 to 750 m and all three are in areas where fungal epidemics were associated with massive amphibian declines from 2004 to 2006 (Lips et al. 2006; Brem and Lips 2008; Kilburn et al. 2010). We did not detect *A. zeteki* or *A. chiriquiensis* at any of our study sites. Although survey efforts for these species are still underway, the absence of these species is concerning because we know that they previously had restricted ranges (Zipple et al. 2006) and at least *A. zeteki* is known to be highly susceptible to chytridiomycosis in laboratory infection experiments (Bustamente et al. 2010; DiRenzo et al. 2014; Ellison et al. 2014).

We found that *A. varius* is persisting in multiple sites following a chytridiomycosis outbreak in western Panama. Furthermore, our positive qPCR results suggest that these populations have survived despite the presence of *Bd*. Prior to this study, Hertz et al. (2012) was the only study to document sightings of *A. varius* in the wild in Panama since 2006. Those observations were made in

2009 at a site in Santa Fe National Park. These populations may be persisting for a wide range of biotic (e.g., changes in host behavioral, innate or acquired immune responses, anti-*Bd* microbial communities) or abiotic (e.g., environmental/thermal conditions) reasons. However, because there have been few coordinated efforts to locate new populations, resurvey historical localities, or test for *Bd* infections, the question of how these populations have persisted—and whether any other *Atelopus* populations have survived—remains to be unraveled. We believe that the lack of post-decline survey effort has not been so much an oversight, but likely a consequence of the enormity of the challenge of monitoring these species while simultaneously establishing conservation programs to abate the threat of chytridiomycosis to entire amphibian communities.

Post-decline surveys are critical for conservation of *Atelopus* species, as well as for other neotropical amphibians. Documenting rediscovered species is critically important for informing conservation and management initiatives (Minteer et al. 2014) and, in this case, could be accomplished with photographs, rather than collecting the individuals. Moreover, understanding the variables that permit some populations to persist while others die out will be critical to conservation, especially since several species are being bred in captivity (e.g., *A. varius* and *A. zeteki*) with the expectation of one day returning them to the wild (Gagliardo et al. 2008; Zippel et al. 2011). Our discovery of extant populations of *A. varius* in *Bd*-enzootic areas underscores the importance of continued monitoring for species presumed to be "extinct in the wild," even after long periods without any sightings.

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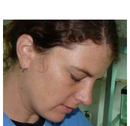
**Edgardo J. Griffith** is a world-renowned leader in amphibian conservation in Panama and one of the founders of the El Valle Amphibian Conservation Center (EVACC). Edgardo is one of the pioneers that has proven that multi-species *ex situ* amphibian conservation is the only option we have at the moment to fight the dramatic amphibian declines in Panama. His work has been featured in several conservation books, documentaries, and peer-reviewed scientific papers. Edgardo has 14 years working with the Panamanian amphibians, including the Panamanian golden frog *in situ* and breeding them *ex situ*.



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**Jamie Voyles** is currently an Assistant Professor at New Mexico Tech working on emerging infectious diseases in wildlife. She conducts chytridiomycosis research in Central America and in California. She is a member of multiple working groups investigating disease-related amphibian declines. She is actively involved in conservation initiatives, such as Amphibian Rescue and Conservation Project, and contributes to amphibianrescue.org and AmphibiaWeb.