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Low *Batrachochytrium dendrobatidis* prevalence in two persisting post-decline populations of endangered hylid frogs in western Panama

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When the amphibian chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*) emerged in western Panama in the early 1990s, it caused mass declines in many amphibian communities. In western Panama, amphibian mass mortality has been well documented (LIPS 1998, 1999, LIPS et al. 2006), thus it is known when and where amphibian populations have declined and which species were most severely affected. By comparing ecological traits of species that survive and species that decline, LIPS et al. (2003) noticed that montane, stream-associated amphibians are most likely to experience population declines. This pattern has most plausibly been explained by the physiology of the pathogen that grows at temperatures of 10–25°C with an optimum at 23°C, that stops growth and production of zoospores at 28°C, and dies off at above 30°C (PIOTROWSKI et al. 2004). Further, the zoospore as the infectious stage is motile in water with the aid of a terminal flagellum, so infection takes place in a moist environment (LONGCORE et al. 1999). These findings allowed predicting species susceptibility. Accordingly, montane species of the anuran families Bufonidae, Hylidae, and Ranidae that lay aquatic eggs, which hatch into tadpoles, as well as stream-dwelling craugastorids were most severely affected (LIPS 1999).

In recent years, several of these species have been reencountered after having experienced severe population declines (e.g. HERTZ et al. 2012). For some surviving populations of highly susceptible *Craugastor* species it has been hypothesized that habitats surrounded by dry and hot lowlands and with separated hydrographical systems may serve as natural refuges from the pathogen (PUSCHENDORF et al. 2005, ZUMBADO-ULATE 2011, KÖHLER et al. 2012). However, some species show signs of recovery that have experienced previously documented *Bd*-related declines. This seems to be in particular the case with stream-breeding hylid species (HERTZ et al. 2012). However, the reasons why some species are being rediscovered after severe declines are subject to ongoing investigation and might be related to increased disease resistance by the defensive mechanisms of the amphibian skin (WOODHAMS et al. 2007, 2014) or natural selection of genetic polymorphisms after pathogen exposure (SAVAGE & ZAMUDIO 2011), or by changes in *Bd* infection dynamics in the host-pathogen system (BRIGGS et al. 2010, SAPSFORD et al. 2015).

We registered the number of calling males within two persisting populations of highly *Bd*-susceptible hylids at two sites in the Panamanian central mountain range. We

Table 1. Results of amphibian specimens from La Fortuna Forest Reserve and Cerro Colorado, Serranía de Tabasará, Panama tested for the presence of *Bd* using real-time PCR. Abbreviations: Ct – qPCR threshold cycle, RFLF – Reserva Forestal La Fortuna, CS – Colorado sample, AH –Andreas Hertz field number.

Field number	Species	Life stage	Locality	Ct value	Result
RFLF 1	<i>Duellmanohyla uranochroa</i>	tadpole	La Fortuna	no Ct	negative
RFLF 2	<i>Duellmanohyla uranochroa</i>	tadpole	La Fortuna	41.4	questionable
RFLF 3	<i>Duellmanohyla uranochroa</i>	adult male	La Fortuna	no Ct	negative
RFLF 4	<i>Duellmanohyla uranochroa</i>	adult male	La Fortuna	no Ct	negative
RFLF 5	<i>Duellmanohyla uranochroa</i>	tadpole	La Fortuna	no Ct	negative
RFLF 6	<i>Duellmanohyla uranochroa</i>	tadpole	La Fortuna	no Ct	negative
RFLF 7	<i>Duellmanohyla uranochroa</i>	tadpole	La Fortuna	no Ct	negative
RFLF 8	<i>Duellmanohyla uranochroa</i>	adult male	La Fortuna	no Ct	negative
RFLF 9	<i>Duellmanohyla uranochroa</i>	adult male	La Fortuna	no Ct	negative
RFLF 10	<i>Duellmanohyla uranochroa</i>	adult male	La Fortuna	no Ct	negative
RFLF 11	<i>Duellmanohyla uranochroa</i>	tadpole	La Fortuna	no Ct	negative
RFLF 12	<i>Duellmanohyla uranochroa</i>	tadpole	La Fortuna	no Ct	negative
RFLF 13	<i>Duellmanohyla uranochroa</i>	adult male	La Fortuna	no Ct	negative
RFLF 14	<i>Duellmanohyla uranochroa</i>	adult male	La Fortuna	no Ct	negative
CS 1	<i>Isthmohyla graceae</i>	adult male	Ratón, Cerro Colorado	40.8	questionable
CS 2	<i>Isthmohyla graceae</i>	adult male	Ratón, Cerro Colorado	no Ct	negative
CS 3	<i>Isthmohyla graceae</i>	adult male	Ratón, Cerro Colorado	no Ct	negative
CS 4	<i>Isthmohyla graceae</i>	adult male	Ratón, Cerro Colorado	40.4	questionable
CS 5	<i>Isthmohyla graceae</i>	adult male	Ratón, Cerro Colorado	no Ct	negative
CS 6	<i>Smilisca sila</i>	adult	Ratón, Cerro Colorado	38.9	positive
AH 570	<i>Craugastor</i> sp.	adult	Ratón, Cerro Colorado	no Ct	negative
1	positive control			28.9	positive
2	positive control			27.3	positive

searched for *Duellmanohyla uranochroa* (COPE, 1875) tadpoles and adults in three tributary brooks of Río Hornito in the La Fortuna Forest Reserve (A: 8.6761°, -82.20621° at 1,410 m a.s.l.; B: 8.6750°, -82.20743° at 1,380 m a.s.l.; C: 8.67705°, -82.21003° at 1,290 m a.s.l.). We recorded one (male) voucher specimen's advertisement call at this site as a reference. The call recording and the specimen are deposited in the Senckenberg Museum Frankfurt herpetological collection (SMF 103275). All other specimens were released after processing. On July 25, 2013 between 19:00 and 22:00 h, we ran audiotranssects with two people walking 100 m upstream each brook. We counted calling males within each transect and took skin swab samples of all adults that were within reach, following the protocol of KRIGER et al. (2006). Additionally, we swabbed the mouthparts of all *D. uranochroa* tadpoles that were encountered following the protocol of RETALLICK et al. (2006).

At a second site in the Cerro Colorado region near Ratón (8.5422°, -81.8087° at 1,540 m a.s.l.), Comarca Ngöbe-Buglé, we monitored a persisting population of *Isthmohyla graceae* (MYERS & DUELLMAN, 1982) between 20:00 and 23:00 h on July 28, 2013. We counted the total number of adults at this site and took swab samples from every individual encountered. Additionally, we swabbed two individuals of other frog species, *Smilisca sila* (DUELLMAN & TRUEB, 1966), *Craugastor* sp., from this site, since

Bd had not been detected in the area before (HERTZ et al. 2012). Following return from fieldwork, swab samples were stored at -24°C until analysis.

Bd tests were performed at the Landesbetrieb Hessisches Landeslabor (LHL), Gießen, by Taqman real-time PCR assay following the protocol of BOYLE et al. (2004) as described in RASMUSSEN et al. (2012). Threshold cycles (Ct-values) below 39 cycles were considered positive, while in negative samples no target DNA was sequenced. Ct-values above 39 cycles are considered questionable, what can either mean a very low infection or a contamination of the swab.

At La Fortuna, we registered calling individuals of *D. uranochroa* as follows – A: three; B: three; C: five. We tested each seven adult males and tadpoles of *D. uranochroa* for *Bd*. Of all 14 samples, 13 were tested negative while one tadpole yielded a questionable result (Table 1). The seven sampled *D. uranochroa* tadpoles completely lacked keratinized mouthparts.

At Ratón, we counted five calling *I. graceae* males that were sitting in a partly flooded meadow. All were swab-sampled for the presence of *Bd*, three tested negative and two with questionable results (Table 1). The one *Craugastor* sp. specimen was tested negative for *Bd*, whereas the one *S. sila* specimen carried a low infection load (Table 1), representing the first positive *Bd* sample from the Cerro Colorado region.

Our results might allow to carefully hypothesize that numbers of adult *D. uranochroa* at La Fortuna continue to increase from only two adults found in the year 2009 during repeated fieldtrips (HERTZ et al. 2012) to eleven found in 2013 in a single night. This corresponds with reports on populations from Costa Rica (NatureServe & IUCN SSC ASG 2013). Although none of the tested animals were *Bd* clearly positive, our sample size is too small to conclude *Bd* disappearance from La Fortuna and thus more extensive swabbing would be needed. This might rather be negated as the complete loss of keratinized mouthparts in all examined tadpoles might likely be the result of *Bd* infection (KNAPP & MORGAN 2006). The fact that tadpole swabs yielded negative or questionable results is probably due to the complete loss of keratine in all tadpoles tested, what means a loss of substrate on the host to the parasitic pathogen and thus an associated reduction of *Bd* (MCMAHON & ROHR 2014). The potential high infection rate in *D. uranochroa* larval stages is a remarkable contrast to the observed *Bd* absence in adults that all appeared to be healthy and showed no signs of infection.

The first *Bd* positive report in *S. sila* from the Cerro Colorado region proves the presence of the pathogen in the area. However, the infection load in this specimen was very low. Of the five *I. gracieae* specimens we sampled, none was clearly infected (in terms of Ct-value, Table 1) nor did any specimen of this species display clinical symptoms of chytridiomycosis. However, the two specimens that yielded questionable results might have carried a very low *Bd*-load. Additional testing in the future would be necessary. The Ratón population of *I. gracieae* is the only persisting population of this species known and therefore of high conservation value. The Cerro Colorado region is entirely unprotected and the habitat is threatened by human activities (HERTZ et al. 2012).

BRIGGS et al. (2010) showed that in persistent frog populations, adults frequently clear and regain *Bd* infection, while long-lived tadpoles may act as a within species pathogen reservoir. Their model predicts that once a critical host density is reached populations might collapse again. To date, amphibian density at both sites studied by us is considerably low. It remains open to future studies to elucidate if the recovery of single Panamanian hylid populations is a result of enhanced immune defense following natural selection or just the current status of enzootic and epizootic dynamics in the host-pathogen system.

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