Apparent lack of chytrid infection in northeast Tobago's frogs

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ABSTRACT

Six species of Tobago frogs (including the stream frog *Mannophryne olmonae*) from 11 sites across the northeast of the island were tested in 2016 for the presence of the pathogenic chytrid fungus *Batrachochytrium dendrobatidis*. No chytrid was detected in any of the 176 samples (20-39 frogs per species). These results contrast with a 2006 survey which found chytrid in 25% of the *M. olmonae* individuals sampled, though none in smaller samples of four other species. Together with similar results from Trinidad, our findings indicate that the infection may have died out or be at a very low level in Trinidad and Tobago. However, there is a need to sample from the south of Tobago and from two threatened species not yet assessed.

Key words: amphibians, Caribbean, chytridiomycosis, Tobago

INTRODUCTION

The infectious disease chytridiomycosis whose aetiological agent is the fungus *Batrachochytrium dendrobatidis* (Bd) has been identified as one cause of the widespread population declines afflicting the World's amphibians (Stuart *et al*. 2004; Kilpatrick *et al*. 2010). In recent years it has become apparent that amphibian species vary widely in their susceptibility to the disease, with some acting as carriers while themselves remaining asymptomatic, whilst others suffer high mortality rates (Venesky *et al*. 2014).

In Trinidad and Tobago, Alemu *et al*. (2008,2013) reported the occurrence of Bd infection in both of the endemic aromobatid species, *Mannophryne olmonae* (Tobago) and *M. trinitatis* (Trinidad) although they saw no signs of clinical disease in either species. Greener *et al*. (2017) and Shepherd *et al*. (2016) carried out new surveys for chytrid in Trinidad, re-sampling *M. trinitatis* but also testing other species, both from single-species sites and from multi-species assemblages. Greener *et al.* found no chytrid infected individuals in a sample of 116 *M. trinitatis* from six sites, including some sites where Alemu *et al.* (2013) collected chytrid-positive samples. Shepherd *et al*. found no chytrid in 245 frogs from a further 15 species sampled across nine widely separated sites, both single and multi-species assemblages. In the light of these findings, it seemed worthwhile to re-test Tobago's frogs for chytrid, ten years after the previous assessment. We report on the results of these tests here.

METHODS

Site and species selection

Chytrid swabbing was conducted in Tobago between June and August 2016. A total of eleven sites were visited and six species sampled (Table 1; Figure 1), all in the north-east of the island. At two sites, only *Mannophryne olmonae* was sampled. At the other eight sites 1-4 species were sampled. Table 1 lists only the species that were sampled at each site, not the complete list of species detected at each site. *M. olmonae* is a day active frog, so sampling occurred during daylight hours. The other five species are active and were sampled at night. Scientific names follow Frost (2017).

Sampling methods

Frogs were caught by hand or with the aid of a small hand-net, and were transferred to individual polythene bags. The collection team were all trained in frog identification by JRD and had Murphy (1997) available to check any doubtful identifications. Where possible, chytrid sampling took place at the capture site and frogs were released once all had been swabbed. On occasion, when the number of frogs captured was very large, or the weather too wet for reliable swabbing, the frogs in their bags were transferred to our base in Charlotteville, swabbed there and returned to their capture site next morning. Swabbing was all carried out by RT to ensure uniform technique, and followed the standard protocol (Brem *et al*. 2007), as used also in Trinidad by Greener *et al*. (2017) and Shepherd *et al*. (2016). Gloves were routinely used for frog handling and discarded after each use, as were the polythene bags, to avoid any cross contamination. In addition, nets were disinfected with bleach after use. Clinical grade sterile Deltalab swabs were used to sample the skin of each frog. They were stored in sterile 1ml vials with 0.5 ml ethanol added to preserve the collected DNA, and sealed with a screw cap. Vials were stored in our base freezer and later transferred to the UK by air in a cool bag.

Each site was sampled only once per species, to avoid

Fig. 1. Map of northeast Tobago showing the sites sampled for this study.

any risk of sampling the same individuals more than once. The exception to this was Dead Bay pond where ten *Leptodactylus validus* were sampled one night early during the study, and then a further 25 were sampled six weeks later; the intention was to test whether there was any evidence of a change in chytrid occurrence over time, given the multi-species breeding assemblages at this site.

Sample analysis

DNA was extracted using Bioline genomic extraction kit and standard PCR carried out as described by Shepherd *et al*. (2016). The positive control DNA for Bd was supplied by Professor Andrew Cunningham, Institute of Zoology, London.

RESULTS

A total of 188 frogs were sampled, with the smallest species sample size being 27 (Table 2). DNA could not be extracted from 12 samples, so the total number of analysed samples was 176. No sample tested positive for chytrid. As insurance that we had performed the assay correctly, our negative control showed no DNA and our positive controls with low and higher amounts of chytrid DNA both showed positive. Since no Tobago sample gave positive results, there was no basis for a comparison between the two *Leptodactylus validus* samples collected from Dead Bay pond at different times, so they are all presented together in Table 2. Of the 12 samples which lacked extractable DNA, ten were from the Tobago glass frog, *Hyalinobatrachium orientale* (reducing the number of that species that could be tested to 20). During the fieldwork, no frogs were observed with clinical symptoms of chytrid infection. A few frogs did look unhealthy e.g. a few *Boana xerophylla* (previously *Hypsiboas crepitans*) had green growths on their hands and feet, but this is not a symptom normally associated with chytridiomycosis.

DISCUSSION

Alemu *et al*. (2008) carried out their survey during June-September 2006. They sampled 84 *Mannophryne olmonae* from five northeast rivers (Argyle, Bloody Bay, Doctor's, King's Bay and Louis d'Or) and three Northside Road streams, but caught most of these frogs (64) at Doctor's. At Doctor's, they found 29.7% positive for chytrid; chytrid was also detected at two other sites (Argyle and one of the Northside Road streams), giving 25% positives for the whole sample. They also tested four other species: two each of *Leptodactylus validus*, *Pristimantis charlottevillensis* and *Hyalinobatrachium orientale*, and 34 *Rhinella marina,* all of which were negative for chytrid. None of the chytrid-positive frogs showed clinical signs of disease.

Our survey, ten years after that of Alemu *et al*., covered several of the same sites (Argyle, Louis d'Or, Doctor's) and therefore provides a direct comparison. The difference in results could relate to sample size (Alemu

Table 1. Descriptions of the eleven sites, with the frog species sampled from each. Site names are as on local maps, except where such names are lacking and have been given by University of Glasgow expeditions e.g 'Mystery' river. Abbreviations for frog names: OL, *Mannophryne olmonae*; VA, *Leptodactylus validus*; PU, *Engystomops pustulosus*; BX, *Boana xerophylla*; CV, *Pristimantis charlottevillensis*; HO, *Hyalinobatrachium orientale*. GPS co-ordinates differed slightly at different locations within each site; for simplicity, we give only one set of co-ordinates for each site.

Site name and GPS co-ordinates	Species collected	Site characteristics
Hermitage N11.31435, W060.57454	HO,CV,BX, VA	Medium to high vegetation with *Heliconia* and ferns.Canopy closed where glass frogs were located, and stream running constantly. Elsewhere, canopy open, stream intermittent. Substrate rocks and pebbles.
Cambleton N11.31667, W060.55733	CV,VA	Medium to high vegetation with *Heliconia* and some bamboo. Canopy closed, stream intermittent. Substrate: leaf litter.
Merchiston N11.28638, W060.54179	OL, BX, CV	Medium height vegetation, mostly small plants. Canopy open, stream intermittent. Substrate: leaf litter.
Louis d'Or 'original' N11.27180, W060.56355	CV	High vegetation with *Heliconia* and ferns at some points, elsewhere, bamboo, ferns and open canopy. Stream constant. Substrate: leaf litter, rocks, pebbles.
Louis d'Or 'new' N11.27049, W060.56311	OL, CV	Medium height vegetation with *Heliconia*, ferns and small plants. Closed canopy, stream intermittent. Substrate: leaf litter.
Main Ridge N11.28667, W060.59545	HO,CV	High palms and ferns. Canopy closed, stream constant. Substrate: rocks, silt, mud.
'Mystery' N11.31566, W060.62614	OL	High ferns and small plants with canopy partly closed. Stream constant. Substrate: leaf litter, rocks, pebbles.
Argyle Waterfall N11.25953, W060.58602	OL	Low ferns, canopy open. Major river. Substrate: rocks and pebbles.
Doctor's river N11.31104, W060.53991	OL, HO, CV	High *Heliconia* and ferns where glass frogs were found. Elsewhere, smaller plants including low ferns, canopy open, stream constant. Substrate: leaf litter, rocks, pebbles.
Dead Bay river N11.29070, W060.63354	HO, CV, VA	High *Heliconia* and ferns where glass frogs were found, canopy open, stream constant. Substrate: leaf litter, rocks, pebbles.
Dead Bay pond N11.29152, W060.63214	BX,VA,PU	Pond surrounded by low vegetation; open canopy. Edges part concrete, part grass. Bottom of pond muddy with rotting timber.

Table 2. The number of individual frogs of each species sampled at each site. *= no DNA extracted. Species name abbreviations as in Table 1.~=additional sample 6 weeks later than the first.

Site	Numbers of each species					
	HO	OL	VA	PU	BX	CV
Hermitage	6(*1)	0	2	0	1	6(*1)
Cambleton	0	0	1	0	0	2
Merchiston	0	18	0	0	5	2
Louis d'Or original	0	0	0	0	0	2
Louis d'Or new	0	2	0	0	0	2
Main Ridge	11(*5)	0	0	0	0	5
Mystery	0	4	0	0	0	0
Argyle Waterfall	0	4	0	0	0	0
Doctor's	4	3	0	0	0	3
Dead Bay river	9(*4)	0	1	0	0	5
Dead Bay pond	0	0	10(~25)	30(*1)	25	0
TOTALS	30	31	39	30	31	27

et al. caught 64 at Doctor's alone, while our *M. olmonae* sample was 31 in total from five sites). However, if chytrid was going to take hold in Tobago, it would be expected to spread in the ten years since the first survey and possibly to affect other species. Instead, we found no chytrid in *M. olmonae* or in substantial samples of five other species. Of these, three (*P. charlottevillensis*, *B. xerophylla* and *H. orientale*) were found at the same sites as *M. olmonae* and therefore might have been expected to have become infected. Research elsewhere (Scheele *et al.* 2017) shows that asymptomatic infections in one species can amplify the harmful effects on a susceptible species where the two co-occur. The Dead Bay pond we surveyed is used as a breeding site by several species in very large numbers (*Trachycephalus typhonius* and *Dendropsophus minutus* in addition to those we surveyed: JRD, personal observations) and would therefore be expected to act as a place where chytrid, if present, could easily spread. Our conclusion therefore is that chytrid, while present in at least one species ten years ago, is now absent or at very low levels or in very restricted locations.

One deficiency in our results was the lack of DNA in 12 of our samples. Ten of these cases were from *H.orientale* (Table 2), suggesting that the failures may relate more to the species than to our technique overall. These are very small delicate frogs and it may be that in some individuals, our swabbing was too gentle to extract an adequate sample.

One of Alemu *et al.*'s concerns was that they had found chytrid in one of Tobago's endemic species. At that time, IUCN rated *M. olmonae* as Critically Endangered, so the discovery of chytrid in the population was particularly worrying. Since then, following work by Lehtinen *et al.* (2016), the conservation status of *M. olmonae* has been softened to Vulnerable. When added to our findings on chytrid, Lehtinen *et al*.'s results provide a more hopeful view of the future of this species.

Both of the investigations into chytrid's status in Tobago's frogs have been conducted in the northeast of the island. This is justifiable since the northeast is the principal location of Tobago's endemic and threatened species (Murphy 1997). However, not all of the threatened species (IUCN 2016) have been assessed for chytrid: *Pristimantis turpinorum* is a Tobago endemic with a very restricted range, an IUCN rating of Vulnerable, and should be assessed; *Flectonotus fitzgeraldi* occurs in Tobago, Trinidad and Venezuela, but has an IUCN rating of Endangered- the problem is that its habits make it extremely difficult to capture in adequate numbers to assess properly.

The occurrence and impact of chytrid in the Caribbean has been patchily reported. Olson *et al.* (2013) provided maps of chytrid's distribution up to 2011. Caribbean islands positive for chytrid were Cuba, Hispaniola (Dominican Republic), Puerto Rico, Dominica and the British Virgin Islands, whilst those showing no chytrid were Jamaica, Montserrat, Barbados and Grenada. Other islands appeared not to have been assessed, although Olson *et al.*'s dataset omitted Alemu *et al*.'s (2008) Tobago study. Since then, Greenhawk *et al*. (2017) and Sabino-Pinto *et al*. (2017) have confirmed the presence of chytrid at low levels of prevalence in Puerto Rico and Cuba respectively. In contrast, Hudson *et al.* (2016) reported the devastating effects on the Critically Endangered endemic mountain chicken (*Leptodactylus fallax*) of the arrival of chytrid first in Dominica and later in Montserrat. Rodriguez-Brenes *et al.* (2016) discussed the importance of studying chytrid prevalence in low altitude tropical locations where the infection can be asymptomatic but act as a reservoir for spread to cooler, wetter, often montane habitats where mortality occurs. Our findings give some grounds for optimism concerning Tobago's frogs, but regular monitoring is advisable.

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REFERENCES

Alemu,J.B., Cazabon, M.N.E, Dempewolf, L., Hailey, A., Lehtinen, R.M., Mannette, R.P., Naranjit, K.T. and **Roach, A.C.J.** 2008. Presence of the chytrid fungus *Batrachochytrium dendrobatidis* in populations of the Critically Endangered frog *Mannophryne olmonae* in Tobago, West Indies. *Ecohealth*, 5:34-39.

Alemu, J.B., Cazabon-Manette, M.N.E., Cunningham, A.A., Dempewolf, L., Hailey, A., Mannette, R.P., Naranjit, K.T., Perkins, M.W. and **Schmidt-Roach, A.C.J.** 2013. Presence of the chytrid fungus *Batrachochytrium dendrobatidis* in a Vulnerable frog in Trinidad, West Indies. *Endangered Species* R*esearch,* 20: 131-136.

Brem, F., Mendelson III, J.R. and **Lips, K.R.** 2007. Field-sampling protocol for *Batrachochytrium dendrobatidis* from living amphibians using alcohol preserved swabs (version 1.0). Conservation International, Arlington, Virginia, USA, p.1-10.[On-line] Available at http://www.amphibians.org (Accessed May, 2017).

Frost, D.R. 2017. Amphibian Species of the World:

an online reference, version 6.0 American Museum of Natural History, New York, USA. [On-line] Available at http://research.amnh.org/herpetology/amphibia/index.html. (Accessed 4 October, 2017)

Greener, M.S., Shepherd, R., Hoskisson, P.A., Asmath, H. and **Downie, J.R.** 2017. How many Trinidad stream frogs (*Mannophryne trinitatis*) are there, and should they be regarded as Vulnerable to extinction? *Herpetological Journal,* 27:5-11.

Greenhawk, N., Zlotnik, S., Billy, L.M., Boas, S. and **Gabel,S.** 2017. Baseline amphibian survey and sampling of Bd in the Icaco and Hormiga valleys, Puerto Rico. *Phyllomedusa*, 16: 63-69.

Hudson, M.A., Young, R.P., D'Urban Jackson, J., Orozco ter Wegel, P., Martin, L., James, A., Sulton, M., Garcia, G., Griffiths, R.A., Thomas, R., Magin, C., Bruford, M.W. and **Cunningham, A.A.** 2016. Dynamics and genetics of a disease driven species decline to near extinction: lessons for conservation. *Scientific Reports*, 6:e30772.

IUCN. 2016. The IUCN red list of threatened species. International Union for the Conservation of Nature and natural Resources. [On-line] Available at www.iucnredlist.com (Accessed 4 October, 2017)

Kilpatrick, A.M., Briggs, C.J., and **Daszak, P.** 2010. The ecology and impact of chytridiomycosis: an emerging disease of amphibians. *Trends in Ecology and Evolution*, 25: 109-118.

Lehtinen,R.M., Calkins, T.L., Novick,A.M. and **McQuigg, J.L.** 2016. Reassessing the conservation status of an island endemic frog. *Journal of Herpetology*, 50:249-255.

Murphy, J.C. 1997. Amphibians and Reptiles of Trinidad and Tobago. Krieger Publishing Company, Malabar, Florida.

Olson, D.H., Aanensen, D.M., Walker, S.F., Bielby, J., Garner, T.W.J., Weaver, G., Group T.B.M. and **Fisher, M.C.** 2013. Mapping the global emergence of the amphibian chytrid fungus. *PLoS ONE,* 8: e56802.

Rodriguez-Brenes, S., Rodriguez, D., Ibanez, R. and **Ryan, M.J.** 2016. Spread of amphibian chytrid fungus across lowland populations of tungara frogs in Panama. *PLoS ONE*, 11: e0155745.

Sabino-Pinto,J., Bletz, M.C., Iturriaga, M., Vences, M. and **Rodriguez, A.** 2017. Low infection prevalence of the amphibian chytrid fungus *Batrachochytrium dendrobatidis* (Chytridiomycetes: Rhizophydiales) in Cuba. *Amphibia-Reptilia*, 38:243-249.

Scheele, B.C., Hunter, D.A, Brannelly, L.A., Skerratt, L.F. and **Driscoll, D.A.** 2017. Reservoir-host amplification of disease impact on an endangered amphibian. *Conservation Biology*, 31: 592-600.

Shepherd, R., Hoskisson, P.A., and **Downie, J.R.** 2016. Apparent absence of chytrid infection in Trinidad's frogs. *Living World: Journal of the Trinidad and Tobago Field Naturalists' Club*, 2016: 19-22.

Stuart, S., Chanson, J.S., Cox, N.A., Young, B.E., Rodrigues, A.S.L., Fishman, D.L. and **Waller, R.W.** 2004. Status and trends of amphibian declines and extinctions worldwide. *Science*, 306: 1783-1786.

Venesky, M.D., Raffel, T.R., McMahon, T.A. and **Rohr, J.R.** 2014. Confronting inconsistencies in the amphibian-chytridiomycosis system; implications for disease management. *Biological Reviews*, 89: 477-483.