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How many Trinidad stream frogs (*Mannophryne trinitatis*) are there, and should they be regarded as vulnerable to extinction?

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The Trinidad stream frog *Mannophryne trinitatis* is a Trinidad endemic inhabiting small seasonal forest streams throughout the northern and central range hills. IUCN has assessed the species as Vulnerable, but the evidence for this remains anecdotal. We surveyed the northern range population at five sites over three consecutive years using visual encounter and audio surveys, also using removal sampling at two of the sites. We further tested for the presence of chytrid infection at six sites in one year. Removal sampling revealed densities of about 100 and 600 frogs per 100 m of stream, resulting in a conservative estimate of 3.5 million frogs in the northern Range when taking the total length of suitable streams into account. None of the 116 frogs were positive for chytrid, and no frog showed skin lesions or clinical signs of disease. Along with a lack of evidence for decline in the extent and quality of Trinidad stream frog habitat, we conclude that this species should no longer be regarded as under threat. Our results combined with previous work should provide a basis for future assessments of this species.

Key words: chytrid infection, IUCN Red List, *Mannophryne trinitatis*, removal sampling, Trinidad stream frog

INTRODUCTION

It is well known that there is a world-wide biodiversity crisis, with large numbers of species experiencing population declines and many threatened with extinction. Among the tetrapod vertebrates, amphibians appear to be the most seriously affected group, with an initial assessment showing 32.5% of species as globally threatened, compared to 12% of birds and 23% of mammals (Stuart et al., 2004). The IUCN Amphibian Specialist Group maintains the amphibian Red List (IUCN, 2014), updating information on the conservation status of each species as it becomes available. However such information is not easy to obtain, particularly given that the world list of described amphibians is expanding at over 100 new species each year (Frost, 2015; AmphibiaWeb, 2015).

In this paper, we focus on a single tropical species, the Trinidadian stream frog *Mannophryne trinitatis* (Garman 1887) and attempt to assess its population size and the potential threats to its future. Previously, *M. trinitatis* was assumed to occur in both Trinidad and the nearby Paria Peninsula of Venezuela. However, Manzanilla et al. (2007, 2009) established that the Venezuelan and Tobago populations are distinct species (*M. venezuelensis*, and *M. olmonae*, respectively). Jowers and Downie (2004) showed that *M. trinitatis* in Trinidad occurs in two geographically distinct regions in the Northern Range mountains (88 km long and up to 16 km wide) and the Central Range hills (60 km long and up to 8 km wide), separated by a plain about 20 km wide. *Mannophryne trinitatis* was later also confirmed along the southern coast in the Trinity Hills (Mohammed et al., 2014). Jowers et al. (2011) analysed mtDNA from ten Northern Range and four Central Range localities and recovered three geographically unstructured distinct clades.

IUCN's original assessment of the status of *M. trinitatis* (2004) listed it as Vulnerable and declining, which was not altered in the revised assessment (Angulo, 2010). An additional factor is the possible impact of chytrid fungus, established as a key factor underlying the declines of many amphibian populations around the world (e.g., Kilpatrick et al., 2010). Alemu et al. (2013) expected that *M. trinitatis* might be at risk from chytrid because of the species' close association with water, its stream-dwelling tadpoles and the occurrence of close intra-specific interactions capable of leading to high levels of transmission. However, they detected chytrid in only two out of 12 populations with low prevalences (3 and 23%). In addition, infected frogs showed no evidence of clinical disease, as was also the case in an earlier study on *M. olmonae* in Tobago (Alemu et al., 2008). Our aim was to assess the populations of *M. trinitatis* at a range of sites that could be used in future years to

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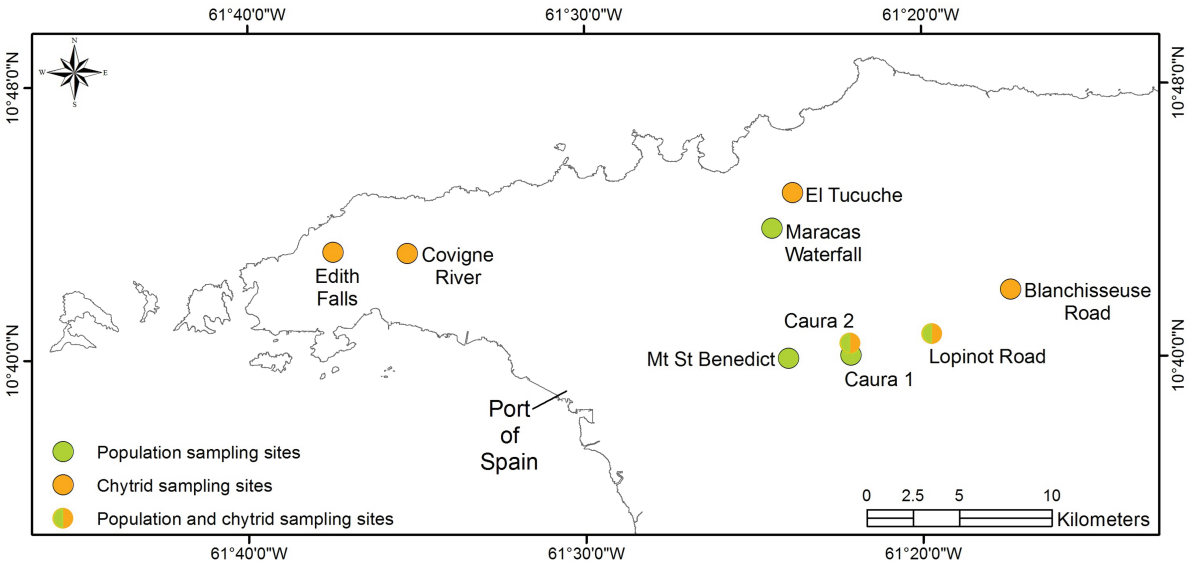

Fig. 1. *M. trinitatis* sampling sites for both population estimates and chytrid sampling, as detailed in Table 1.

determine population trends. In addition, we carried out new sampling for chytrid at sites previously assessed by Alemu et al. (2013). We use the obtained information to discuss the conservation status of *M. trinitatis*.

MATERIALS AND METHODS

Study species

Mannophryne trinitatis is a member of the family Aromobatidae (Frost, 2015; AmphibiaWeb, 2015), and inhabits steep stream sides in hilly forested regions. Females have bright yellow throats and defend small territories close to streams, whereas males lack the yellow throat pigmentation but turn jet black when calling (Wells, 1980). Eggs are deposited on damp ground in crevices or under leaves and rocks and are guarded by males; hatched tadpoles are transported by males to predator-free sections of streams or pools up to 20 m away from a stream (Downie et al., 2001; Jowers & Downie, 2005). *Mannophryne trinitatis* is regarded as largely day-active, calling throughout daylight hours depending on the season and weather (Murphy, 1997).

Survey sites

Surveys were conducted between June and August 2012–14 by a changing team of four trained surveyors with one of us (MSG) always present to ensure consistency. The Northern Range sites were Caura 1 and 2, Mount St Benedict, Maracas Waterfall and Lopinot (Fig. 1). Caura 1 and 2 are intermittent first-order stream sites in closed canopy secondary forest flanked by steep rocky banks. The Mount St Benedict site is a second- order stream bordered by sloping rocky banks in closed canopy secondary forest. Maracas Waterfall is at the base of a cascade of water with adjacent rivulets and pools where the frogs occur; the site is more open than the other sites and can be described as disturbed, since the waterfall is a popular visitor destination. The Lopinot site is again an intermittent first- order stream descending between steep rocky banks in closed canopy secondary forest (Fig. 2). Co-ordinates and dimensions of the sites obtained through a combination of GPS recorders, maps, and using a 50 metre tape measure are shown in Table 1.

Daytime (1000–1200 or 1500–1700 hours) and dusk (1815–1915 hours) surveys were conducted five times each year at each site, with at least one day between successive surveys. Torches were used both day and night. For visual encounter surveys (VES), three observers spread across the width of the site, walking slowly up the stream valley searching for frogs; both daytime and night surveys took 20–60 minutes depending on the site. The fourth member of the team recorded each frog found as male (not calling), male (calling) or male (transporting, including the number of tadpoles), female, unidentified adult, or juvenile (defined as distinctly smaller than adults). Audio surveys we conducted on the same day on two or three survey points per site, determining the number of calling frogs over a period of two minutes by averaging the results of the four observers. We also made recordings using a Roland R-05 recorder.

In order to better relate the survey data with an estimate of the total population size, we also carried out removal sampling at two sites (Lopinot: 2012–14; Caura: 2013–14). Previous work has shown that *M. trinitatis* cope

Fig. 2. The stream site sampled at Lopinot Road with measuring tape in place.

Table 1. Co-ordinates and dimensions of study sites.

Site	Co-ordinates	VES Length (m)	Audio survey length (m)	Mean Width(+/- S.D.)(m)
Caura 1	10°40'03.36''N 61°22'08.04''W	33.3	50.9	4.3+/-4.3(n=12)
Caura 2	10°40'24.96'' N 61°22'09.12''W	56.5	83.7	5.6+/-1.4(n=19)
Lopinot Road	10°40'40.44'' N 61°19'42.96''W	97.5	122.5	3.9+/-0.8(n=21)
Mt St Benedict	10°39'58.32'' N 61°23'58.56''W	76.1	111.2	8.8+/-2.8(n=21)
Maracas Waterfall	10°43'47.28'' N 61°24'27.36''W	105	127.4	9.5+/-6.0(n=17)

well with removal to a laboratory setting for several days (Downie et al., 2001), and its social structure (territorial females) and habitat preference (staying close to linear rocky streams) renders removal sampling suitable to estimate population sizes (Wells, 1980). Sampling was performed by teams with four members over three or four successive days (morning and late afternoon, 2 hours each), using hand-held nets or manual capture. All frogs were taken to the University of the West Indies, St Augustine, to be measured (weight to the nearest 0.001g; snout-vent length (SVL) to the nearest 0.1 mm), sexed and photographed (dorsally and ventrally). Frogs were transferred to 90 x 30 x 30 cm glass aquaria containing a layer of damp leaf litter at a temperature of about 28°C. Rotting mangoes were placed in the tanks as a source of fruitflies for the frogs. Tanks were misted with dechlorinated tap water mornings and evenings. After sampling, frogs were returned to their sites of capture. The casualty rate was about 1% (escapes or deaths).

Chytrid sampling

Chytrid sampling was conducted from June to August 2014 at six sites (Fig. 1). Frogs were captured during the day with nets prior to transfer to individual plastic bags. Each frog was examined for any signs of damage or skin lesions. After each use, nets were sprayed with a 0.5% sodium hypochlorite solution and rinsed in distilled water. SVL was measured using callipers (to the nearest 0.1 mm) while the frogs were in the bags. Using disposable nitrile gloves changed after each frog, each frog was then transferred to a clean plastic petri dish and weighed using a Pesola PPS200 digital pocket scale to the nearest 0.01 g. Frog were sampled for chytrid by lightly spraying with dechlorinated tap water, then swabbing 25 times using sterile cotton-tipped swabs (Fisher) according to the standard chytrid protocol (Boyle et al., 2004; Brem et al., 2007) using dry storage rather than ethanol preservation (Hyatt et al., 2007). Using equal pressure and rotation for each stroke, each frog was swabbed five times on the abdomen, each thigh and each foot. Each swab was placed in a sterile labelled vial. After swabbing, each frog was returned to its bag before all frogs were released at their site of capture. All equipment was sanitised using bleach following each site visit.

Samples were immediately placed in a cool box containing ice for transport before storing at -20°C at the University of the West Indies. Samples were later transported to the UK on ice and transferred to -80°C. Polymerase chain reaction (PCR) analysis of the samples was conducted following washing of each swab (to release frog tissues and cells) in 50mM TrisHCl (pH 8.0), 10 mM EDTA proteinase K (100 mg/ml) buffer. Total DNA extraction was performed using phenol-chloroform protocols (Sambrook & Russell, 2001). Control PCR analysis to demonstrate PCR quality DNA extraction was performed using 16S rDNA primers (16Sar 5'-CGC CTG TTT ATC AAA AAC AT-3' and 16Sbr 5'-CCG GTC TGA ACT CAG ATC ACG T-3'; Palumbi, 1996). Extracted DNA samples were tested for the presence of chytrid using exisiting ITS/5.8S primers (Boyle et al., 2004; ITS Chytr 5' CCTTGATATAATACAGTGTGCCATATGTC-3' and 5.8S Chytr 5'-AGCCAAGAGATCCGTTGTCAAA-3'). Positive control DNA for *Batrachochytrium dendrobatidis* was supplied by Prof. Andrew Cunningham, Institute of Zoology, London.

Table 2. Maximum number of adult *M. trinitatis* encountered on daytime visual surveys per year per 100m (NS = not surveyed).

Site	2012	2013	2014
Caura 1	90	171	93
Caura 2	76	257	235
Mt St Benedict	99	204	160
Maracas Waterfall	25	27	33
Lopinot	NS	46	34

Table 3. Maximum number of adult *M. trinitatis* encountered on dusk visual surveys per year per 100m.

Site	2012	2013	2014
Caura 1	27	108	60
Caura 2	30	195	143
Mt St Benedict	63	114	156

Extent of habitat and population estimate

To determine the *M. trinitatis* population size, an estimate of the total length of suitable stream sites was needed. Individuals are mainly found along gently sloping stream reaches rather than steep rocky falls (Jowers & Downie, 2004), and we used 45 degree stream slopes as a cut-off. The length of such streams in the Northern Range was determined using digitised streams and contour data from the 1976 topographic map series for Trinidad. A digital elevation model (DEM) with a resolution of 30 m was created for the Northern Range using the Topo to Raster algorithm with contour data as input in ArcGIS v.10.0. The DEM was used to generate the gradient of each cell using the Slope algorithm. Using a 2007 landcover classification (Department of Geomatics, University of the West Indies), all forested areas were extracted for use. The spatial extent of slopes below 45 degrees and forested areas was developed by intersecting the two datasets to derive a total stream length constituting suitable *M. trinitatis* habitat.

RESULTS

Visual encounter (VES) and audio survey results are shown in Tables 2–4, normalising the data to the number of frogs per 100 m. The different sites surveyed had 'natural' lengths determined by features such as cliffs, and because the population below such a feature was continuous, we surveyed the entire stretch of stream valley rather than designate an arbitrary transect length. In all cases, daytime VES and audio surveys generated the highest and lowest numbers, respectively. Contrary to our expectations we also recorded *M. trinitatis* at night. Overall, the data for 2012 gave the lowest numbers. VES results tended to increase as the survey season progressed while audio results decreased (detailed data not shown), suggesting that survey teams may have over-estimated numbers of calling males at the onset of a season. The maximum number of frogs per 100 m varied considerably between sites and years for all sites except Maracas Waterfall.

Results for removal sampling are shown in Table 5. There is no evidence for removal sampling having any effect on population sizes, and numbers based on removal sampling are more than twice as high as numbers derived from VES (normalised to 100 m). Frogs captured at Lopinot and Caura were 56% and 64% females, respectively. We stopped removal sampling at Lopinot in 2014 after a single visit because of the low numbers found, which was likely linked to a prolonged dry season and lack of water.

No frogs from a total of 116 sampled tested positive for chytrid (Table 6), and we did not observe any signs of skin lesions or clinical disease (data not shown). Figure 3 shows that our extraction protocols yielded DNA suitable for PCR analysis, and the chytrid control PCRs were able to amplify control DNA at 10 and 100 zoospore genome equivalents of *B. dendrobatidis*.

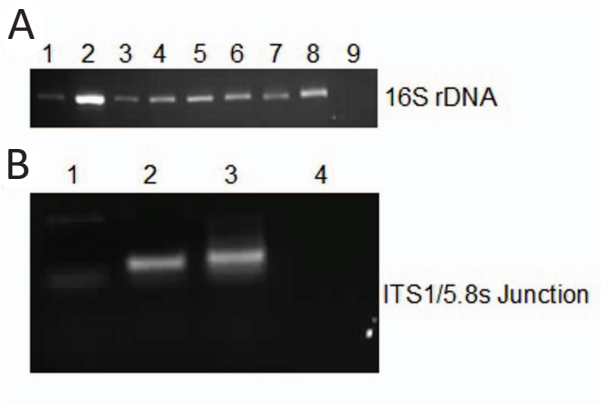

Fig. 3. Electrophoretic analysis of chytrid swab samples. A) Lanes 1-8 are rRNA control PCR analyses of eight randomly chosen DNA extractions from the swabs to ensure that extracted DNA was of sufficient amount and quality for PCR analysis. Lane 9 is a negative control. B) Control PCR analysis using chytrid specific primers (Boyle et al., 2004) ; Lane 1 – Control using 20 ng *M. trinitatis* DNA; Lane 2 -10 zoospore genome equivalents (zsp GE); Lane 3 - 100 zsp GE; Lane 4 – negative control. Gels not shown for negative chytrid gels from swabs.

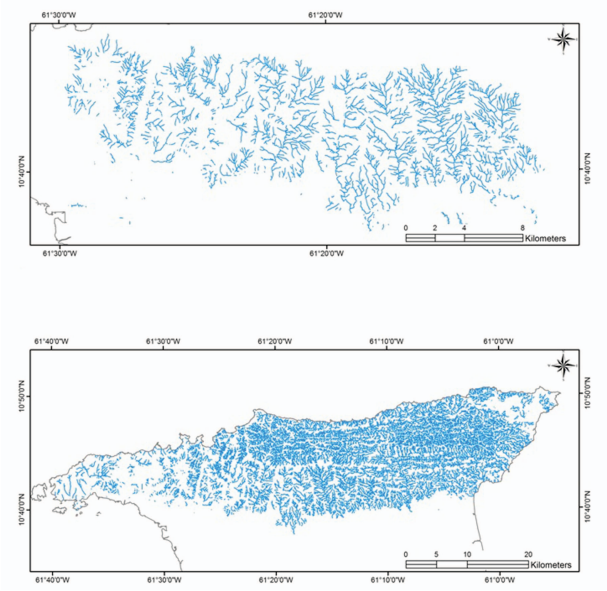

Fig. 4. Streams considered as suitable *M. trinitatis* habitat: A – Caroni River basin region of the Northern Range; B – the entire Northern Range.

Table 4. Maximum number of calling male *M. trinitatis* encountered on day time audio surveys per year per 100m (NS = not surveyed).

Site	2012	2013	2014
Caura 1	24	14	25
Caura 2	29	33	33
Mt St Benedict	19	13	20
Maracas Waterfall	12	13	9
Lopinot	NS	4	12

Figure 4 shows the estimated extent of suitable *M. trinitatis* habitat in the Northern Range. The total length of suitable streams is 722.09 km in the Caroni catchment and 3,460.96 km in the Northern Range.

DISCUSSION

According to the best of our knowledge, the present study is the first attempt to quantify the size of the entire population of *M. trinitatis*. Schmidt & Pellet (2010) have discussed the problems of relating counts from different amphibian sampling methods to actual population size, emphasising variation in detection probability. Our data from audio and visual sampling confirm this finding, as deriving a scaling factor to relate maximum counts to the population estimates based on removal sampling was impossible (data not shown). The assumptions behind effective removal sampling are closed populations, equal and constant catchability of individuals, and constant catching efforts (Hayek, 1994). Working on salamanders, Petranka & Murray (2001) concluded that their population estimates were conservative due to a lack of decline in catch size for one species and a fluctuating catchability for another species. In the case of *M. trinitatis*, the population is essentially closed by the need for individuals to stay at the vicinity of streams and by the territorial behaviour of females. Egg guarding and larval transportation by males may alter their catchability, possibly contributing to the female-biased sex ratio revealed by removal sampling. Individual *M. trinitatis* are difficult to catch in the field (termed 'rocket frogs', Royan et al., 2010), and our removal sampling catches declined at Lopinot but not at Caura; uncaught frogs were detectable hiding in crevices, and we added them to the total count at the end of the sampling period. As in Petranka & Murray (2001), our removal sampling population size estimates should therefore be regarded as conservative.

Alemu et al. (2013) swabbed 123 frogs from 12 sites in July-August 2007 for the presence of chytrid, recording two sites with positive individuals (Blanchisseuse Road and Aripo River). Our results from six sites revealed no positives for chytrid, despite a large sample size across a range of elevations (the two positive sites had the highest altitude of all sites studied by Alemu et al. 2013). We however tested a substantial proportion of juveniles at Covigne River only, despite the previous finding that juveniles are more affected by chytrid (Alemu et al. 2013). Taken together, our evidence suggests that there is no evidence for chytrid as a cause for population declines in *M. trinitatis*. Chytrid also appears absent in 15 other species from Trinidad (Shepherd et al., unpublished data), suggesting that it has not become a major threat.

Since 2004, IUCN lists *M. trinitatis* as Vulnerable following standard IUCN criteria and protocols (extent of occurrence < 20,000 km^2, decline in the extent and quality of habitat, decreasing population size). Hailey & Cazabon-Mannette (2011) further suggested that the split of *M. trinitatis* from *M. venezuelensis* along with the discovery of chytrid infection could lead to an upgrade of its conservation status to Endangered. While the distribution area is less than 3,000 km^2, we do not consider the distribution as severely fragmented within the main areas where *M. trinitatis* occurs. It is found around first- and second-order streams draining the steeper slopes of the hills, only becoming absent where those streams become larger rivers on the valley floors. Jowers et al. (2011) found three well-resolved clades among the 14 *M. trinitatis* sites they sampled from, but there was a lack of geographic structuring, an indication that these populations are not sufficiently isolated to allow genetic divergence.

Table 5. Removal sampling results for *M. trinitatis* per site per year. In the total column, the number in brackets is the number of frogs visible but not catchable at the final catch: the Total/100m column includes these uncaught frogs.

	Catch number								
Site year	1	2	3	4	5	6	7	Total	Total/ 100m
Lopinot 2012	18	24	3	10	9	12	15	91 (+6)	99
Lopinot 2013	33	18	12	10	13	16	9	111(+8)	122
Lopinot 2014	20	3	-	-	-	-	-	23	24
Caura 2 2013	50	58	57	53	46	53	-	317(+53)	655
Caura 2 2014	70	77	53	52	40	39	-	331(+61)	694

Table 6. *M. trinitatis* sites and frogs sampled for chytrid.

Site	Co-ordinates	Frog numbers			
		Adult males (transporting)	Adult females	Juveniles	Positive for chytrid
Edith Falls	10°43'08.5" N 61°37'29.0" W	5	13	2	0
Covigne River	10°43'06.0" N 61°35'16.9" W	4	8	9	0
El Tucuche	10°44'50'' N 61°23'50" W	4	4	0	0
Caura 2	10°40'24.96" N 61°22'09.12" W	6(1)	14	0	0
Lopinot Road	10°40'40.44'' N 61°19'42.96'' W	9	18	0	0
Blanchisseuse Road	10°41'57.7" N 61°17'23.2" W	7(2)	13	0	0

What is the extent and quality of *M. trinitatis* habitat? The Northern Range valleys contain abundant cocoa and coffee plants, in addition to mango trees and other introduced plants as indicators of previous more intense cultivation; primary forests are rare (Oatham, pers. comm.). Abandoned cacao plantations have previously been described as suitable for local amphibians (Hailey & Cazabon-Mannette, 2011), and there is no evidence that vegetation changes have had a negative effect on *M. trinitatis*. Hailey & Cazabon-Mannette (2011) note that the closure of sugar production has released abundant land for development in Trinidad, taking the pressure off the Northern Range. As evidenced by its presence at the Maracas Waterfall, *M. trinitatis* generally appears tolerant of human presence, and localised quarrying in the Northern Range affects only small areas of the extent of *M. trinitatis* sites. A national government report gives an estimate of deforestation in Trinidad as 0.8% per year over the period 1990–2000, but also notes the occurrence of forest regeneration following a decline in agriculture (Environmental Management Authority, 2011). In terms of population trends, Alemu et al. (2013) suggested declines for one site (Tamana Cave), where the population size however appears to largely fluctuate according to annual rainfall regimes since 1982 (JRD, personal observation). In the Northern Range, an extrapolation from our finding of about 100 *M. trinitatis* per 100 metres of stream at Lopinot on to our estimate of 3,500 km of suitable habitat would result in 3.5 million frogs. Given that Caura held a higher density, we consider this a conservative number.

Overall, our results suggest that *M. trinitatis* should be listed as Least Concern or Near Threatened rather than Vulnerable. Although *M. trinitatis* only occurs on Trinidad, it is widespread and abundant which is in line with the IUCN descriptors for the latter (IUCN, 2014). We hope that our survey results combined with those of Jowers & Downie (2004) and Alemu et al. (2013) can provide a baseline for *M. trinitatis* distribution and abundance on which future assessments can be grounded.

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