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Environmental DNA survey of *Rhinella marina* (cane toad) on Mulgumpin (Moreton Island) – June 2022

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TropWATER Centre for Tropical Water and Aquatic Ecosystem Research

JAMES COOK UNIVERSITY AUSTRALIA

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INTRODUCTION

Cane toad (*Rinella marina*) invasions have had a substantial effect on Australian biodiversity (Shine 2010). They currently occupy coastal areas of Queensland, New South Wales, the Northern Territory, and northern Western Australia. Some offshore islands remain free from cane toad due to their distance from established populations on the mainland. Some islands, however, are at higher risk of cane toad incursions due to their proximity to the runoff plume of a river and could receive cane toads during wet season (Tingley *et al.* 2017). Also, accidental or intentional cane toad displacement into offshore islands is common in Queensland (Tingley *et al.* 2017). However, toads that are transported into offshore islands would only have the ability to establish a population if there are multiple individuals, both male and female (Tingley *et al.* 2017). Eradication once cane toad populations are established is almost impossible (Smart *et al.* 2018), with very few successful examples (White and Shine 2009). Therefore, detecting new incursions is critical for successful eradication (Tingley *et al.* 2017; Smart *et al.* 2018).

Traditional survey methods can have limited resolution to detect new cane toad incursions. The most commonly used method involves counting toads at night time, when animals are active, although visual detection may fail at very low toad densities (Smart *et al.* 2018). Sentinel traps are also widely used, but even with attractive baits they may still miss individuals (Tingley *et al.* 2019). Since cane toads are associated with water bodies, their presence or absence could be accurately determined by capturing the environmental DNA (eDNA) that they shed into the water when hydrating. Environmental DNA detection of an invasive aquatic amphibian, the smooth newt (*Lissotriton vulgaris vulgaris*) showed to be more reliable than trapping (Smart *et al.* 2015). The high sensitivity of eDNA detection to low number of individuals suggests that this technique could be better at accounting for false negative errors than traditional methods (Smart *et al.* 2015). However, strict field and laboratory methods need to be put in place in order to avoid contamination leading to false positive detections (Smart et al. 2015).

Mulgumpin (Moreton Island) is located near Brisbane, and it is a very popular tourist destination. A small number of live and dead adult cane toads have been observed around the island and cane toad detection dogs have indicated cane toad presence (Tingley et al. 2019). Recently, an eDNA survey carried out on Mulgumpin showed that cane toad populations are not established on the island (Tingley et al. 2019). As cane toads are established on mainland Australia, adjacent to Mulgumpin, there is a high risk of introduction of this species to Mulgumpin due to large amounts of vehicular and

pedestrian traffic between the mainland and the island. Further, recent flood and heavy rain events have given cause to dead cane toads observed washed ashore on Mulgumpin.

The aim of the present work was to determine presence/absence of cane toad eDNA using a *16S Rhinella marina* primer pair developed at TropWATER, James Cook University, across four sites of Moreton Island during the June 2022 sampling campaign.

METHODS

Sample collection

Environmental DNA sampling kits were supplied by TropWATER and sent to the Brisbane City Council prior to their field collection. Field collection was carried out by the Brisbane City Council employees, following TropWATER's eDNA collection protocol. In 2022, water samples were collected from five different sites (Table 1). Three 30 mL field water sample replicates were collected at each site along with one field blank, to ensure that no contaminating target eDNA was introduced to a site during sample collection.

Table 1 *Sampling sites for the collection of water on Mulgumpin for cane toad eDNA detection conducted in June 2022.*

	Location	Site Number	Sample Number	Latitude (°S)	Longitude (°E)	Collection date
Mulgumpin (Moreton Island)	Creek Near Tangalooma Ranger Station	1	1 2 3	-27.150914 -27.15144 -27.15191	153.367071 153.367* 153.36787	21/04/2022
	Bulwer North Beach Creek	2	1 2 3	-27.031421	123.366075	19/06/2022
	Creek Near Ranger Station	3	1 2 3	-27.151459	153.367315	20/06/2022
	North Creek Cowan Township	4	1 2 3	-27.122284	153.366677	20/06/2022
	N/A	5	1 2 3	-27.736851	153.175236	23/04/2022

* Approximate geographic coordinates

eDNA extraction

Environmental DNA extractions were carried out in a dedicated eDNA laboratory at TropWATER, James Cook University, Townsville. Prior to laboratory extraction of eDNA, bench top surfaces and floors in a dedicated eDNA laboratory were decontaminated, and field sample tubes were wiped

down, with 10% v/v bleach, as per standard operating procedure. We followed a DNA precipitation method protocol described in (Villacorta-Rath *et al.* 2020). Briefly, 20 mL isopropanol, 5 mL sodium chloride 5M and 10 µL glycogen was added to the collected samples and incubated at 4°C overnight. Samples were then centrifuged at 3,270 g for 90 min at 22°C, supernatant discarded, and remaining pellet dissolved in 600 µL lysis buffer and stored overnight at -22°C. Samples were then thawed, vortexed and lysed for four hours at 50°C, and precipitated with polyethylene glycol (PEG) buffer with glycogen, by overnight incubation at 4°C. Samples were then centrifuged at 20,000 g for 30 min at 22°C, the supernatant discarded and the pellet washed twice with 70% ethanol and resuspending in 100 µL elution buffer. DNA was then purified using the DNeasy PowerClean Pro Cleanup Kit (Qiagen) as per the manufacturer's instructions. An extraction control consisting of was added to each batch of eDNA extractions to ensure that no contamination was introduced during laboratory procedures (Goldberg *et al.* 2016).

Inhibition testing

Water samples were tested for inhibition by spiking 18 x 10^3 copies of double-stranded synthetic DNA fragments (gBlocks™ Integrated DNA Technologies Pty Ltd, New South Wales, Australia) of a species that does not occur on Mulgumpin (*Channa striata*). Each field sample was tested in duplicate qPCR replicates. A baseline was created by spiking the same amount of spike DNA into laboratory-grade water. A sample was considered inhibited if it exhibited a shift in Ct value of three or more cycles ($\Delta Ct \geq 3$) when compared to spiked laboratory-grade water (Cao *et al.* 2012).

Detection of species-specific eDNA by quantitative PCR (qPCR)

Detection of cane toad eDNA was performed using a species-specific primer pair developed at TropWATER, targeting the *16S Rhinella marina* mitochondrial gene (Edmunds and Burrows 2019).

qPCR assays were run on the QuantStudio™ 3 Real-Time PCR System (Thermo Fisher Scientific Australia Pty Ltd) in a white 384-well plate sealed with optical films (Thermo Fisher Scientific Australia Pty Ltd). Eight technical replicates of each sample were used, representing 48% of the total available DNA elution volume (Fig. 1). Additionally, three no template control (NTC) samples and three positive reference samples were used. The NTC samples did not contain the target species DNA and their lack of amplification indicated that no contamination was introduced during plate handling. The positive reference samples, containing cane toad eDNA and therefore their positive amplification proved that our primer pair was able to amplify the target species eDNA.

Each qPCR assay consisted of 6 µL of template DNA and 14 µL of master mix (10 µL PowerUp SYBR Green Master Mix; 1 µL forward primer at 5 µM; 1 µL reverse primer at 5 µM; 2 µL laboratory-grade water). Thermal cycling conditions were as follows: initial denaturation and activation at 95°C for 2 min then 55 cycles of 95°C for 15 secs and 65°C for 1 min. A subsequent melt curve analysis was performed to generate dissociation curves by transitioning from 65°C to 95°C at 0.15°C sec^{-1}.

The qPCR plate was analysed with a common fluorescence threshold (0.2) using QuantStudio™ Design and Analysis Software (version 1.4.2; Thermo Fisher Scientific Australia Pty Ltd). All samples that expressed positive amplification within the assay criteria: amplification curve crossed the common fluorescence threshold within 45 cycles and the melt curve analysis showed a dissociation temperature peak at 80.938°C (-0.554 and 0.513 confidence intervals), were sequenced at the Australian Genome Research Facility (AGRF) to confirm positive detection. Samples that expressed positive replication that were close to, but outside, the assay criteria were also sent for sequencing as a measure of certainty.

A field site was considered positive for cane toad detection if at least one of the total technical qPCR replicates for that site met the following criteria: 1) qPCR amplification falls within the assay criteria, 2) Subsequent Sanger sequence data showed >98% pairwise identity with the *16S Rhinella marina* gene, and 3) corresponding control field blanks and extraction blanks were not contaminated with target species.

RESULTS

Inhibition tests

No shift in Mean Ct value ≥ 3 was observed in any spiked field samples, nor spiked control field blank, indicating that no inhibition was present in the qPCR assays.

Screening of field samples for *Rhinella marina* (cane toad) eDNA

Based on the assessment criteria for positive detection of cane toad for this assay, Site 5 had positive detection for the target species in one of the field replicates (Table 2). The location of this site was not indicated on the data sheets provide. No sample site on Mulgumpin detected cane toad eDNA within the assay criteria. One field sample from Site 4 on Mulgumpin, North Creek Cowan Township, returned a positive sequence for *Rhinella marina*, however, the characteristics of this qPCR replicate were outside of the qPCR assay limits and not considered a true positive for cane toad presence. Samples that presented as positive in qCPR were confirmed by sangar sequencing. All control field blanks and extraction controls were verified to be absent of the target species eDNA by qPCR.

Table 2 *Summary of cane toad eDNA detections using the 16S* Rhinella marina *mitochondrial gene from five sites on Mulgumpin (Moreton Island), Queensland.*

Location	Site	Target Species	# Field samples	Field Samples # Positive detections	Field Samples % Positive detections	qPCR analysis # Technical replicates	qPCR analysis # Positive detections	qPCR analysis % Positive detections
Creek Near Tangalooma Ranger Station	1	*Rhinella marina*	3	0	0	0	0	0
Bulwer North Beach Creek	2	*Rhinella marina*	3	0	0	0	0	0
Creek Near Ranger Station	3	*Rhinella marina*	3	0	0	0	0	0
North Creek Cowan Township	4	*Rhinella marina*	3	0	0	0	0	0
N/A	**5**	***Rhinella marina***	**3**	**1**	**33.33**	**24**	**5**	**20.83**

DISCUSSION

As part of an on-going cane toad monitoring campaign on Mulgumpin (Moreton Island), Queensland, Brisbane City Council employees collected water samples for eDNA analysis from five sites considered to be at high risk of human-assisted cane toad incursion. Cane toad eDNA presence was detected at sampling site 5, in one of the three field replicates, constituting positive detection in 33.33% of samples taken at this site. Five of the 24 technical (qPCR) replicates were positive for the target species, indicating a positive detection rate of 20.83% across all technical replicates analysed at this site.

Although no location information was specified in the data sheets returned for Site 5, the GPS co-ordinates supplied on the data sheet indicate that this sampling site was on the mainland, and not on Mulgumpin. As such, our analysis indicates that no cane toad eDNA was present in water sampled from the corresponding sites on Mulgumpin. One qPCR technical replicate at Site 4 (North Creek Cowan Township) on Mulgumpin retuned a positive match for *Rhinella marina* after Sangar sequencing. However, the qPCR result for this sample was outside the assay criteria and returned a pairwise identity match for sequences below 98%, and as such is not considered positive. This putative detection may be a similar circumstance to that seen in the 2020 cane toad survey, where eDNA was recorded in a single detection at two sites on Mulgumpin, Tangalooma Resort and Cowan Bypass. It was concluded that the most likely source of target species eDNA detected at these sites was via residual mud from the tyres of vehicles coming from the mainland. As with one of the samples from the 2020 survey, this current sample was taken at a water body near the Cowan township. It is likely the same circumstance, where cane toad DNA has been brought to this collection site via vehicular traffic and very small amounts of target DNA have been picked up in our eDNA assay.

Of interest, there was no eDNA detected in samples taken from Site 1 (Creek Near Tangalooma Ranger Station), where sampling took place in April 2022. This timeframe was shortly after heavy rain events that occurred in southeast Queensland and dead cane toads were noted washed up on beaches at Mulgumpin. The implication being that the eDNA assay used by TropWATER accurately assesses presence of live cane toads in a water body, and that presence of dead cane toads likely does not confound the assay.

RECOMMENDATIONS

As per previous sampling years (Villacorta-Rath and Burrows 2021), the putative presence of one positive sample on Mulgumpin, which was outside the parameters of the assay criteria, it may be concluded the most likely source of target species eDNA detected at these sites was introduced via residual mud from the tyres of vehicles coming from the mainland. It would be useful to continue to collect samples for eDNA analysis after heavy rain events, when live or dead toads may be washed onto the island from flood plumes. Successive sampling in areas where dead cane toads have washed ashore after flood events would confirm whether dead cane toads deposit detectable amounts of eDNA, that may interfere with the detection of presence/absence of live cane toads.

To be confident that no populations of cane toad have established on Mulgumpin, it is recommended that surveillance of cane toad eDNA in water bodies continue. Subsequent surveys should be focussed at sites of higher risk from cane toad incursions.

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