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Can environmental DNA be used to detect first arrivals of the cane toad, *Rhinella marina*, into novel locations?

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Abstract

Eradicating invasive species is difficult, but success is more likely when populations are small after arrival. The cane toad, *Rhinella marina*, is an invasive pest species that threatens native fauna worldwide. Increasingly, environmental DNA (eDNA) is used as a technique to monitor the presence of invasive species given its power to detect low numbers of individuals. We aimed to investigate eDNA persistence in freshwater at three different temperatures (25, 30 and 35°C) and eDNA detection thresholds for *R. marina* using controlled experiments in aquaria. For the latter, two water volumes and two cane toad exposure times were used (800 or 200 L volume with 5 or 30 min exposure). A 15-ml water sample was collected from each replicated aquaria and preserved with 5 ml Longmire's buffer. Environmental DNA was extracted and four technical quantitative PCR replicates were analyzed targeting the cane toad *16S* rDNA mitochondrial gene. Environmental DNA decayed rapidly in water and was reliably detected for up to 3 days after cane toad removal, regardless of the temperature treatment. Also, cane toad eDNA was detected in the water after a 5-min initial exposure of a single individual in 800 L of water. Under the physical parameters tested here, a positive detection means that a cane toad has been in contact with the water body between 1 and 3 days prior to the sampling event. The results of the present study show the importance of eDNA for determining the presence of a species that occurs at low abundance in a small water body, such as at the onset of a cane toad invasion.

KEYWORDS

detectability, eDNA persistence, environmental DNA, first incursions, invasive species, monitoring

1 | INTRODUCTION

Invasive species constitute one of the major threats to native biodiversity worldwide. More specifically, Australia has a large number of introduced species that have successfully established and spread across the country, negatively impacting native biodiversity (Saunders, Cooke, McColl, Shine, & Peacock, 2010). The cane toad, *Rhinella marina* (Linnaeus), is a good example of an exotic species

that has quickly colonized new environments with an adverse effect on the native fauna (Saunders et al., 2010). Cane toads are native to South and Central America (Zug & Zug, 1979) and were deliberately introduced into Queensland, Australia, in 1935 to act as a biological control agent against cane beetles in sugar plantations (Sabath, Boughton, & Easteal, 1981). Their ability to adapt to new environments due to their wide temperature tolerance range, (Zug & Zug, 1979) and low predation on them due to their high toxicity, has enabled this species to steadily spread into most of northern Australia (Tingley et al., 2017).

Contemporary cane toad invasion fronts are advancing more rapidly than historical invasion fronts as new generations of toads have enhanced locomotor ability (Smith & Phillips, 2006). Additionally, human-induced spread through voluntary and involuntary toad transport to off-shore islands and distant locations (Tingley et al., 2017; White & Shine, 2009), dispersal corridors (Brown, Phillips, Webb, & Shine, 2006), and future climatic scenarios (Kearney et al., 2008) promote their widespread and rapid colonization. Despite this, there are some areas in mainland Australia, such as in the Kimberley and Pilbara (Western Australia), as well as off-shore islands, that remain free from cane toads (Tingley et al., 2017). Since eradication efforts are only successful when dealing with small groups of toads (Tingley et al., 2017), it is critical to continuously monitor those cane toad-free areas to detect early signals of their presence. This could trigger control efforts attempting to prevent cane toads from establishing new populations. The Australian government has an ongoing threat abatement plan in place that aims to maintain the cane toad-free status of off-shore islands and ecological communities at risk (Commonwealth of Australia, 2011). However, northern Australia faces some challenges that limit financial and human resources for regular cane toad monitoring using traditional methods: is remote, has poor accessibility, and has a sparse human population.

Traditional cane toad survey methods involve visual or auditory confirmation of an animal's presence. Night time spotlight surveys are common, but where cane toads are at very low densities provide false negative detections (Smart, Tingley, Weeks, Van Rooyen, & McCarthy, 2015). Sound detection requires toads to call, reducing the potential for detection (Tingley, Greenlees, Oertel, van Rooyen, & Weeks, 2019). Trapping using sentinel traps allows for longer periods of deployment, but even with attractive baits, individuals may still be missed (Tingley et al., 2019). Therefore, additional tools would be useful to detect new incursions of cane toads, especially at low numbers. Given that cane toads are associated with waterbodies (i.e., individuals must hydrate frequently, Schwarzkopf and Alford 2002), their presence could be accurately determined by detecting environmental DNA (eDNA) that they shed into the water when hydrating. In a recent review proposing innovative methods to control cane toads, Tingley et al. (2017) recognized the potential of eDNA as an early warning tool to reduce their spread.

One of the advantages of eDNA as an early detection tool over the other methods is that it can detect the presence of animals without requiring to hear or see them (Jerde, Mahon, Chadderton, & Lodge, 2011). Environmental eDNA is particularly useful for detecting low numbers of amphibian individuals in small water bodies (Dejean et al., 2012; Smart et al., 2015) and it therefore has the potential to detect the first invasion of cane toads. On the other hand, detection of rare species in large water bodies, such as lakes, could be more challenging and potentially require substantial sampling regimes that prevent operational use. For example, eDNA detection post-eradication of the invasive European carp in a temperate lake in Australia was only possible under an exponential increase in sampling effort, which was translated into high financial cost (Furlan, Gleeson, Wisniewski, Yick, & Duncan, 2019). Determining the assay sensitivity taking into account the survey method and the dispersion of eDNA at the target site is important in order to accurately interpret eDNA data (Furlan, Gleeson, Hardy, & Duncan, 2016). Nevertheless, including eDNA methods within current monitoring programs would allow having an extra tool in the early warning system toolbox. Another attractive feature of eDNA is that water samples can be collected by non-specialists, lending itself to citizen science participation in cane toad monitoring. This could be particularly useful to mitigate the spread of cane toads at off-shore islands (Tingley et al., 2019) and other remote mainland locations under risk of an invasion (Jerde et al., 2011).

Given the threat that cane toads pose to Australian biodiversity and the suitability of the eDNA technique to be used for continuous monitoring, two eDNA assays have been recently developed and validated (Edmunds & Burrows, 2019; Tingley et al., 2019). Tingley et al. (2019) developed an eDNA assay based on an 80 base pair (bp) fragment of the NADH dehydrogenase subunit 3 (ND3) mitochondrial gene. They tested it in locations of known cane toad occurrence as well as in an off-shore island without an established cane toad population. On the other hand, Edmunds and Burrows (2019) developed an assay based on a longer fragment of the *16S* gene (120 bp) and tested it in silico and in vitro against 42 Australian species. Both studies focused on determining the suitability of their assay to detect the target species. However, to utilize and interpret eDNA data into cane toad monitoring, it is important to determine whether the technique can detect low numbers of cane toads (i.e. an invasion front), and to understand eDNA persistence in water, as eDNA decay will limit its detectability over time (Barnes et al., 2014; Goldberg et al., 2016; Harrison, Sunday, & Rogers, 2019).

Environmental DNA persistence varies widely across taxa and is dependent on the environmental factors surrounding it (Barnes et al., 2014). Temperature and microbial activity have been widely discussed as important factors driving eDNA degradation (Goldberg, Strickler, & Fremier, 2018; Strickler, Fremier, & Goldberg, 2015). However, other physical factors such as pH and UV radiation are thought to interact and affect eDNA persistence, although there is no consensus as to their relative importance (Andruszkiewicz, Sassoubre, & Boehm, 2017; Mächler, Osathanunkul, & Altermatt, 2018; Strickler et al., 2015). In amphibians, eDNA persistence has been studied in both native and invasive species that occur at low densities. For example, a study testing salamander eDNA shedding rates and persistence found that individuals shed higher amounts of eDNA during the first 2 hr of exposure to aquaria and that the eDNA was undetectable after 3 days

of salamander removal (Pilliod, Goldberg, Arkle, & Waits, 2014). Longer eDNA persistence (1 week) was observed by Thomsen et al. (2012) who tested bullfrog and newt eDNA degradation in aquaria that housed individuals during 2 months. The authors hypothesized that eDNA detectability depends on both eDNA production and decay (Thomsen et al., 2012). Understanding eDNA decay under specific conditions is critical for robust data interpretation (Harrison et al., 2019). Here, we conducted two sets of controlled experiments in order to: (a) explore cane toad eDNA persistence in water at three different temperatures; and (b) determine the minimum time for which cane toad eDNA can be detected in water after a single exposure. The results from these experiments will help design eDNA field sampling programs and interpret eDNA field data for cane toad monitoring in areas that are at risk of invasion or at locations where cane toad eradication programs are underway.

2 | MATERIALS AND METHODS

2.1 | Study species

Adult cane toads were hand collected from Townsville, Australia, one week prior to the start of the experiments. They were kept in 50 L crates containing a layer of water on the bottom and a shade cloth preventing the toads from being permanently submerged in water. There were a total of 10 toads per crate and these were fed live crickets until 2 days before the beginning of the experiments to reduce the amount of organic material that they could add into the experimental aquaria and to minimize individual variation on eDNA concentration. Experiments were conducted at the TropWATER aquarium facility at James Cook University, Townsville (James Cook University ethics permit number A2577).

2.2 | Experiment 1—Persistence of cane toad eDNA in water

Environmental DNA is useful for surveillance, detection and monitoring as it can detect the prior presence of cane toads, even after they are no longer present at a waterbody. In order to determine the length of time that cane toad eDNA may remain at detectable levels, we tested eDNA persistence in water using three different temperature treatments: 25, 30, and 35°C. These temperatures were chosen because water bodies in northern Australia are likely to fluctuate within that range (Burrows & Butler, 2012). Moreover, those temperatures do not represent physiological stress to cane toads; the critical thermal maxima for cane toads is 40°C water temperature (Krakauer, 1970) and 30°C is considered a benign temperature (Overgaard et al., 2012). Additionally, cane toad eDNA has been previously captured and extracted from water bodies that exhibit warm temperatures (Edmunds & Burrows, 2019; Tingley et al., 2019).

Each temperature treatment consisted of six 20 L glass aquaria filled up to 15 L: five replicated experimental aquaria and one negative control aquarium (18 aquaria in total). Each aquarium had a polycarbonate lid to prevent water evaporation and cross-contamination from neighboring aquaria. Aquaria was kept in a water bath where temperature was regulated using a Thermoline™ programmable aquarium heater to ensure a constant temperature for each treatment. All aquaria and lids were decontaminated with 10% bleach and rinsed thoroughly with filtered freshwater prior to the commencement of the experiment. Free chlorine present in the aquaria before the start of the experiment was below the minimum detection threshold of 0.05 ppm.

A new DNA LoBind® 15 ml conical tube (Eppendorf) was placed inside each aquarium where it remained for the duration of the experiment. These tubes were used to collect a 15-ml water sample from the experimental aquaria, from where it was poured into a 50 ml DNA LoBind® conical tube (Eppendorf) pre-filled with 5 ml of Longmire's preservative solution (Renshaw, Olds, Jerde, Mcveigh, & Lodge, 2015). Prior to the commencement of the trials, a 15 ml water sample was taken from each aquarium to confirm that no existing cane toad eDNA was present in the water. It has been demonstrated that 15 ml of water can successfully capture eDNA in a water body when using a DNA precipitation method for eDNA extraction (Ficetola, Miaud, Pompanon, & Tab erlet, 2008). One single cane toad was placed in each experimental aquarium for a total period of one hour. Cane toads reliably urinate when they are handled; therefore, before they were placed in the treatment aquaria, we confirmed that they had emptied their bladders and were washed with clean water to ensure no extra eDNA was introduced in the aquaria. Due to the aquaria water depth and the fact that we did not provide cane toads with a resting structure, cane toads had to maintain their own buoyancy during the experiment. After the exposure time, cane toads were removed from all aquaria and a 15 ml water sample was taken from each aquaria. Water samples continued to be taken at 24 hr intervals over the following 7 days. New gloves were worn before handling of each aquarium to avoid cross-contamination within and between treatments.

Two types of negative controls were carried out at every sampling collection event. First, a “room control” was used to demonstrate that no eDNA was introduced in the samples due to air flow in the aquaria room. The room control consisted of opening a tube pre-filled with 5 ml of Longmire's preservative solution plus 15 ml of MilliQ water and leaving it open for the duration of the sample collection (approximately 45 min). Additionally, a “handling control” was used to control for any eDNA introduced in the samples from manual handling of the lids and tubes. This consisted of pouring 15 ml of MilliQ water into a tube containing 5 ml of Longmire's preservative solution after sampling the water from all experimental aquaria. Water samples and controls stored in the Longmire's solution were placed inside a plastic crate with a lid and kept at room temperature until eDNA extraction.

2.3 | Experiment 2—eDNA detection of single cane toads

The first arrival of cane toads to a naïve natural waterbody may be represented by very low numbers of individuals that make fleeting

visits. We tested the sensitivity of eDNA sampling in detecting even a fleeting visit of a single cane toad to a small water body, by placing single cane toads in plastic livestock troughs (51 × 117 × 228 cm), referred to from now on as "troughs," containing two different water volumes (200 and 800 L) over two different exposure times (5 and 30 min). The 5-min exposure time was chosen based on the minimum time that a cane toad comes in contact with a water body to hydrate (Schwarzkopf, pers. comm.). Each "water volume * exposure time" treatment consisted of three replicated troughs. Additionally, one negative control trough was introduced for each water volume (14 troughs in total). Trough order was randomized to account for possible effects of position. As the troughs were outdoors, shade cloth was placed over them to prevent non-experimental cane toads entering them. Mean water temperature at the 200 L treatment was 25.0 ± 0.02°C and 24.8 ± 0.06°C at the 800 L treatment. Prior to the commencement of the experiment, troughs and shade cloths were decontaminated with 10% bleach and rinsed thoroughly with tap water. Troughs were filled with tap water 2 days prior to the commencement of the experiment to allow chlorine evaporation. Free chlorine present in the troughs before the start of the experiment was below the minimum detection threshold of 0.05 ppm.

Before the start of the experiment, a 15 ml water sample was taken from each trough using a new DNA LoBind® 15 ml conical tube to confirm that no existing cane toad eDNA was present in the water. Randomly selected cane toads were individually placed at a random position in each treatment trough, taken out after the set time and a 15 ml water sample was taken from a different random position immediately after toad removal. Due to the troughs water depth and the fact that we did not provide cane toad with a resting structure, cane toads had to maintain their own buoyancy during the experiment. After the exposure of cane toads to their respective treatment, they were returned to the holding facility, where they were kept individually in separate crates until the next day, when they were exposed to the same treatment over the course of the experiment (7 days). Individual cane toad weight (g) was recorded at the beginning of the experiment in order to determine whether cane toad size was correlated with eDNA concentration in the water. Water samples were collected on days 1, 3, 5, and 7. Before cane toads were placed in the treatment troughs, they urinated when handled and were washed with clean water to ensure no extra eDNA was introduced in the troughs. Gloves were changed after handling each cane toad and each individual trough to avoid cross-contamination. One handling control was also performed at every sampling event. Following the previous experiment, water samples and controls were kept in plastic crates with lids at room temperature until eDNA extraction.

2.4 | Environmental DNA extractions

We used a DNA precipitation method protocol described in Edmunds and Burrows (2019). Briefly, we added 20 ml isopropanol, 5 ml sodium chloride 5 M, and 10 µl glycogen to the 20-ml aliquots of water and Longmire's solution and incubated samples at 4°C overnight. We then centrifuged this solution (3,270 g; 90 min; 22°C), discarded the supernatant, dissolved the pellet in 600 µl lysis buffer (guanidinium hydrochloride and TritonX), and froze the samples overnight. Subsequently samples were thawed, vortexed, and lysed for four hours at 50°C. After sample lysis, we added 1200 µl polyethylene glycol (PEG) precipitation buffer and 5 µl glycogen and incubated the samples overnight at 4°C. Finally, samples were centrifuged (20,000 g; 30 min; 22°C), the supernatant was discarded and the pellet was washed twice with 70% ethanol before resuspending it in 100 µl elution buffer. Given that Experiment 2 was conducted outdoors, we assumed that water samples contained environmental inhibitors, and therefore, eDNA extracted from those samples was purified using the DNeasy PowerClean Pro Cleanup Kit (Qiagen). A negative extraction control was added to each batch of eDNA extractions to ensure that no contamination was introduced during laboratory procedures (Goldberg et al., 2016).

In Experiment 1, we tested whether increasing glycogen concentration during extraction would allow recovering a higher eDNA yield. We compared the eDNA yield of extractions done using a commercial glycogen against those using an "in-house" glycogen of higher concentration (Appendix S1). No significant differences in eDNA yield between glycogen treatments were found (Appendix S1), and therefore, this factor was removed from downstream analyses.

2.5 | Quantitative PCR (qPCR)

DNA amplification of extracted eDNA was conducted using a species-specific primer pair targeting the *16S rDNA* mitochondrial gene of *R. marina* (Edmunds & Burrows, 2019). This primer pair had been previously tested in silico and in vitro against 31 Australian freshwater fish species, 5 Australian frogs, and 6 Australian freshwater turtle species to determine its specificity. It had also been validated using eDNA samples from Ross River, Townsville, Australia, where there is known cane toad presence (Edmunds & Burrows, 2019).

Quantitative PCR (qPCR) assays were performed on 96-well plates using a QuantStudio® 3 System thermocycler. Four qPCR technical replicates per biological sample were run on 10 µl assays containing 5 µl PowerUp SYBR® Green Master Mix, 0.5 µl forward *16S R. marina* primer (250 µM), 0.5 µl reverse *16S R. marina* primer (250 µM), 1 µl MilliQ water, and 3 µl DNA template. This number of technical replicates as well as DNA template used in the qPCR reaction is within the common practices in eDNA studies (Rees, Maddison, Middleditch, Patmore, & Gough, 2014). Each 96-well plate contained samples collected on one experimental day, including the handling, room, and extraction controls plus non-template controls (NTC). The qPCR was run as follows: a 2 min 50°C hold (uracil-DNA glycosylase, UDG, incubation) followed by an initial denaturation at 95°C for 2 min and subsequent 45 cycles of 95°C for 15 s and 65°C for 1 min at a ramp rate of 2.7°C per second. A melt curve analysis was then performed by transitioning from 65 to 95°C at a ramp rate of 0.15°C per second.

Environmental DNA extractions and qPCR set-up were performed in a dedicated low-DNA copy room at James Cook University, Townsville. The limit of detection (LOD) of the assay was determined through 11 serial dilutions of artificial DNA ranging from 6.5×10^{6} to 6.5×10^{-4} copies of DNA per µl, with four technical replicates of each template DNA concentration. The LOD was determined using a discrete threshold, whereby the lowest standard concentration of template DNA produced at least 95% positive replicates (Klymus et al., 2019). Therefore, we determined the assay's LOD to be 6.5×10^{-1} copies of DNA per µl.

2.6 | Data analyses

A common fluorescence threshold of 0.2 was used to determine threshold cycle (C_t) values for all amplifications. C_t values were then extrapolated to copies per assay using *R. marina* artificial DNA standard curve (Edmunds & Burrows, 2019). We reported the results of the present study as: (a) number of DNA copies per assay; and (b) percentage of positive detections (number of positive detections/total number of assays). These two measures vary slightly, since even trace amounts of DNA in a sample represent eDNA presence when calculating the percentage of positive detections.

Samples were considered putative positive detections when: (a) at least one qPCR technical replicate's amplification curve crossed the common florescence threshold within 40 cycles and were above the LOD (Goldberg et al., 2018) (b) melt curve analysis of an amplified replicate showed a peak at 80.938°C (0.554 and 0.513 confidence intervals) (Edmunds & Burrows, 2019); and (c) all negative controls in the qPCR plate did not amplify. Amplicons from a subset of putatively positive samples with representative melt peak values (T_m) were Sanger sequenced at the Australian Genome Research Facility (AGRF). A nucleotide BLAST was performed and amplicon sequences from the samples considered putative positive detections were considered as true detections if there was ≥99% pairwise identity with the *Rhinella marina 16S* gene.

We determined decay rate in Experiment 1 using the following monophasic exponential decay curve, which assumes that eDNA degrades at a constant rate through time:

$$N(t) = N_0 e^{-\lambda t}$$

where N_0 is the initial number of DNA copies per assay (day 0), N_t is the number of DNA copies per assay at a certain time (t), and λ is the eDNA decay rate. This model has been demonstrated to provide the best fit to fish eDNA decay rate (Lance et al., 2017). Two models were run, model 1 assumed that λ was different across temperature treatments while model 2 assumed that λ was the same across all treatments. Nonlinear fitting of the models was performed using GraphPad Prism version 7.00 for Windows, GraphPad Software, La Jolla California USA (www.graphpad.com).

For Experiment 2, differences between treatments were assessed using a template model builder (TMB) computer in the R package glmmTMB (Brooks et al., 2017). This R package uses maximum likelihood estimation, which allows for faster computing speed than Markov chain Monte Carlo sampling and is flexible for handling mixed effects and data over dispersion (Brooks et al., 2017). The dependent variable in our model was the number of DNA copies per assay at each experimental day, and the explanatory variables were water volume * exposure time (fixed factor), replicate trough (fixed factor), and experimental day (random factor). Two models were run: the first one testing replicate troughs as an additive fixed factor and the second one testing replicate troughs as a fixed interaction. The best performing model was chosen based on the corrected Akaike information criterion (AICc).

We further tested the likelihood of positive detections through time for both experiments using logistic regression with "glm" function in R (R Core Team, 2016), where the response variable was percentage of positive detections and the explanatory variable was experimental day.

Finally, the relationship between individual weight and number of DNA copies per assay during day 1 was analyzed using the Spearman's correlation test. We only considered the first experimental day, given that the eDNA accumulation through time could potentially have confounding effects on the relationship.

3 | RESULTS

3.1 | Experiment 1—Persistence of cane toad eDNA in water

Water temperature remained stable throughout the experiment for all treatments (24.91 ± 0.002°C *SE*; 30.04 ± 0.006°C *SE*; 34.93 ± 0.002°C *SE*). Additionally, no cane toad eDNA was detected in the aquaria prior to the start of the experiment nor in any of the negative controls. Amplicon sequences from samples considered putative positive detections matched the *Rhinella marina 16S ribosomal RNA* gene (accession number KF665157) between positions 89 and 310 bp with 99.5% pairwise identity.

Initial DNA concentration (day 0) varied across temperature treatments, with mean number of DNA copies per assay being the highest at the 30°C water temperature treatment (106.17 ± 41.38, *SE*) treatment, lowest at the 25°C treatment (0.51 copies per assay ± 0.25 *SE*), and intermediate at the 35°C treatment (45.35 ± 16.84, *SE*) (Figure 1).

eDNA degraded rapidly in all temperature treatments, with mean number of DNA copies per assay at the 25°C treatment reaching zero at day 1 of the experiment and onwards (Figure 1a). The 30°C treatment exhibited on average 1 copy of DNA per assay from day 3 onwards and reached zero at the end of the experiment (Figure 1b). Finally, the 35°C treatment exhibited on average 1 copy of DNA per assay from day 1 onwards, reached zero on day 4 (Figure 1c).

Based on the log likelihood ratio statistic, we concluded that the model assuming that eDNA decay rate (λ) was the same for all three water temperature treatments provided the best fit to the data (Table 1, Appendix S2). Therefore, regardless of water temperature, λ equaled 1.83 (0.89; 2.77, 95% CI) (Figure 1, Appendix S2).

The percentage of positive detections at the beginning of the experiment (day 0) were highest for the 30°C water temperature treatment (80%), followed by the 35°C (75%) and 25°C treatments (20%) (Figure 2). The percentage of positive detections decreased significantly through time in the two highest water temperature treatments (30°C: $F = -0.5355$, $P = <.0001$; 35°C: $F = -1.4615$, $p < .0001$). No significant differences in the percentage of positive detections through time were found for the 25°C water temperature treatment, where no positive detections were found from day 1 onwards. Percentage of positive detections for the 30°C temperature treatment reduced to more than half of the starting point from days 3 to 6 and only reached zero at the conclusion of the experiment on day 7(Figure 2b). Finally, positive detections for the 35°C temperature treatment decreased to 5% from day 3 and reached zero on day 4 onwards (Figure 2c).

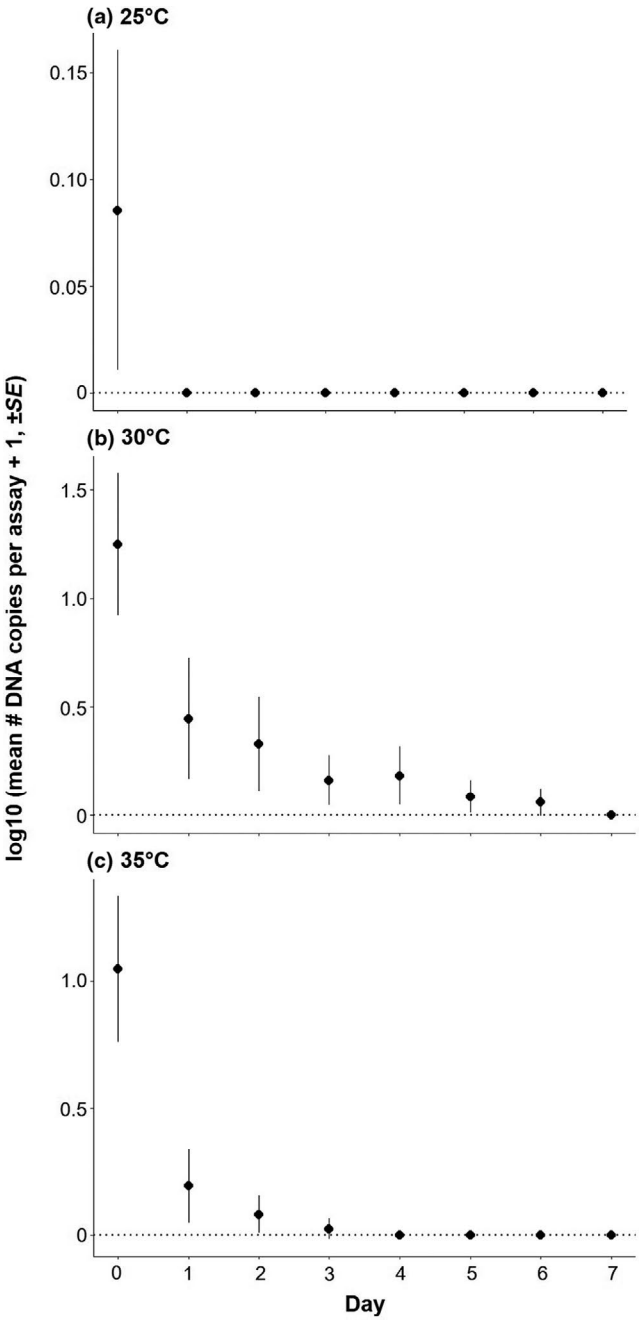

FIGURE 1 Mean number of DNA copies per 3 µl assay (log_{10} + 1, ±*SE*) through time for the: (a) 25°C; (b) 30°C; and (c) 35°C water temperature treatments. eDNA from samples taken on the even days were extracted using the commercial glycogen and the odd days were extracted using the in-house glycogen

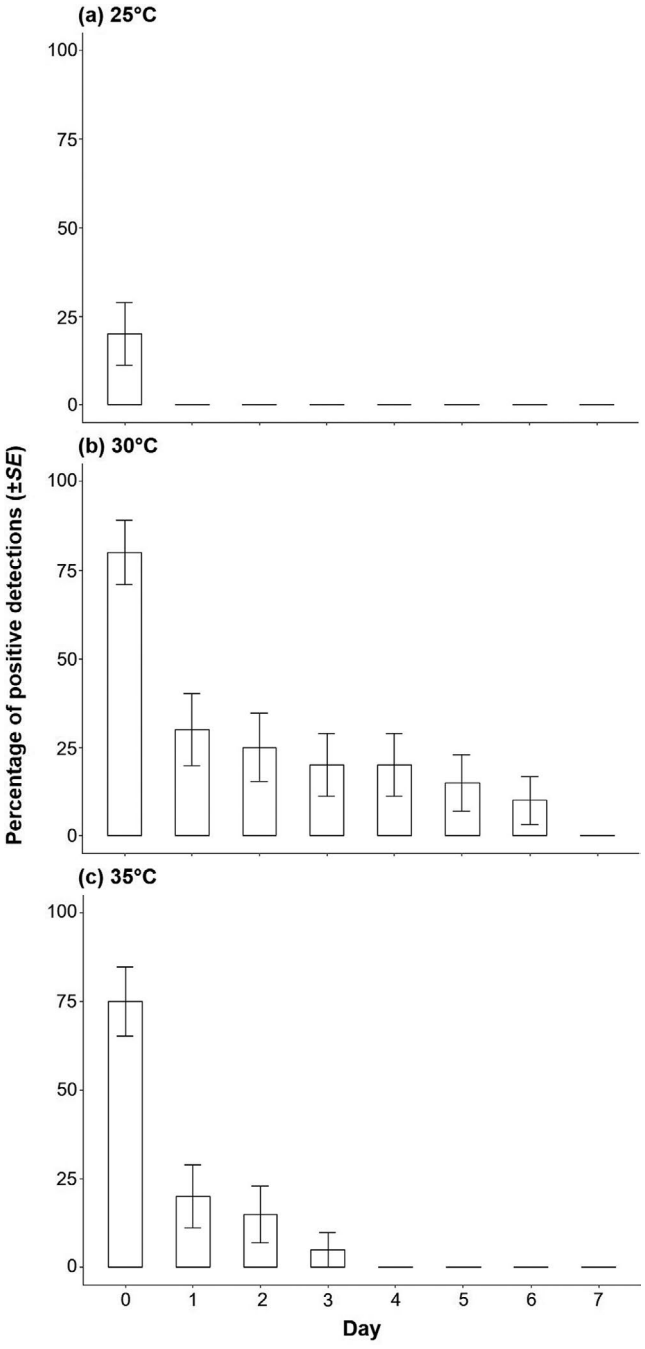

FIGURE 2 Percentage of positive cane toad detections (±*SE*) through time for the: (a) 25°C; (b) 30°C; and (c) 35°C water temperature treatments. eDNA from samples taken on the even days was extracted using the commercial glycogen, and the odd days were extracted using the in-house glycogen

The discrepancy between the number of DNA copies per assay and the percentage of positive detections at the 30 and 35°C treatments is due to the fact that even trace amounts of DNA in a sample (down to the LOD of 0.65 copies per µl) count as a positive detection and were indeed confirmed as cane toad DNA through Sanger sequencing. Therefore, although the 30°C treatment showed on average only one DNA copy per assay from day 3 onwards, there were still 2 (out of 20) qPCR technical replicates that showed cane toad DNA at day 6, which account for 10% positive detections. Additionally, the 5% positive detections detected on day 3 of the 35°C treatment were due to one qPCR technical replicate (out of 20) with a positive amplification.

3.2 | Experiment 2—eDNA detection of single cane toads

All experimental troughs were demonstrated to be free from cane toad eDNA at the start of the experiment, and none of the negative trough controls sampled during the experiment were found to contain cane toad eDNA. Amplicon sequences from the putatively positive detections exhibited 99.5% pairwise identity with the *Rhinella marina 16S ribosomal RNA* gene (accession number KF665157) between positions 82 and 310 bp. Cane toad DNA was detected in the water after the first day of exposing them to the experimental troughs in all treatments except 200 L × 30 min. Mean Ct values showed a slight decrease through time following the increase in eDNA concentration (Table 2, Appendix S2). Experimental troughs exhibited eDNA from the first day of cane toad exposure, with number of DNA copies per assay being the highest at the 800 L × 30 min treatment (4.04 ± 1.40), followed by the 800 L × 5 min treatment (1.00 ± 0.46), the 200 L × 5 min treatment (0.63 ± 0.33), and finally the 200 L × 30 min treatment with only one replicate with 1 DNA copy per assay. Environmental DNA concentration increased from day 1 to day 3 of the experiment and thereafter exhibited a slight decrease through time in all treatments, except the 800 L × 30 min treatment, where eDNA concentration showed a steadily increasing trend over time (Figure 3).

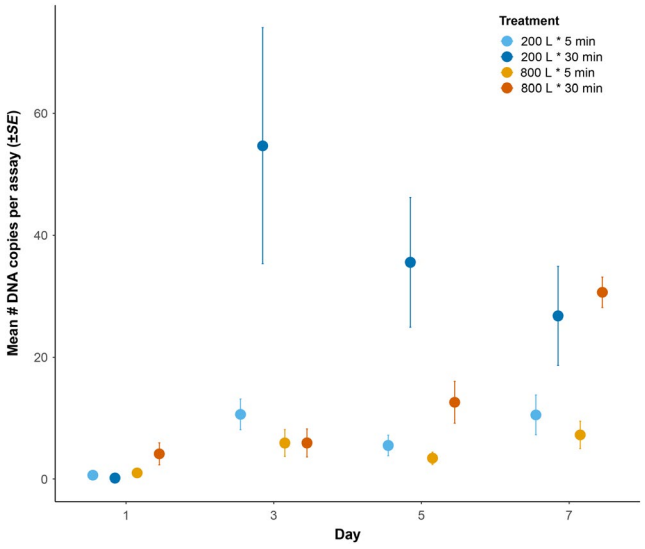

FIGURE 3 Mean number of DNA copies per 3 µl assay (±*SE*) through time for each water volume * exposure time treatment during 1 week of experimental period

The best performing model to assess differences among treatments through time was the one assuming that water volume * exposure time and replicate trough were interactions. The 800 L × 30 min treatment had significantly higher eDNA concentration than the 800 L × 5 min (F = −1.53747, p = .0065) and the 200 L × 30 min (F = −1.36383, p = .0033) treatments.

There was no significant correlation between cane toad size and eDNA concentration in the water at the beginning of the experiment (Spearman's rho = −0.35, p = .2713).

We detected a significant increase in percentage of positive detections through time in all treatments (800 L × 5 min: F = 0.3011, p = .0165; 800 L × 30 min: F = 0.6888, p = .0004; 200 L × 5 min: F = 0.2426, p = .0382; 200 L × 30 min: F = 0.7885, p = .0004). The 800 L × 30 min treatment exhibited 42% positive qPCR detections on the first day, and this value increased to 100% on day 7. The 800 L × 5 min treatment had 30% positive detections on the first day, increasing to 67% on days 5 and 7. The 200 L × 5 min treatment exhibited 17% positive detections on the first day of the experiment, increasing to 75% on day 3, and subsequently decreased to approximately 60% on days 5 and 7. The 200 L × 30 min treatment started with a very small amount of cane toad eDNA in the water (8% positive detections), rapidly increasing to 67% on day 3, 83% on day 5 and 92% on day 7 (Figure 4).

4 | DISCUSSION

The present study successfully detected cane toad eDNA in water samples after as little as 5 min exposure time of a single adult toad. We also demonstrated that although cane toad eDNA decays rapidly, it does so at the same rate across three different water temperatures (based on the best-fitted model) and can still be detected up to 7 days after cane toads were last present, although detections are more reliably obtained within 2–3 days of their most recent presence. This study suggests eDNA may provide a good short term (2–3 day) indication of the presence of cane toads in small waterbodies, even after quite short visits (5 min) from single toads.

4.1 | Experiment 1—Persistence of cane toad eDNA in water

The consistent decay rate of cane toad eDNA found in the present study (1.83 DNA copies per day) is in line with previous studies on amphibians. Environmental DNA of the Idaho giant salamander, *Dicamptodon aterrimus*, showed very similar decay rates after individuals were removed from their experimental tank held at 25°C (λ = 1.89, Pilliod et al., 2014). In our experiment, eDNA decay rate

was not dependent on water temperature, despite this factor frequently having a considerable effect on eDNA decay in other studies. A study analyzing eDNA decay in bighead (*Hypophthalmichtys nobilis*) and silver carp (*H. harmandi),* in the presence of multiple physical and chemical environmental factors, determined that increased water temperature promoted eDNA degradation, especially during the first three experimental days (Lance et al., 2017). However, Lance et al. (2017) tested a wider temperature range (4, 12, 20, and 30°C) than the present study and large differences in decay rates occurred between the minimum and maximum temperatures, whereas eDNA decayed at the same rate at both the 20 and 30°C treatments.

While we did not find differences in eDNA decay rate across the three temperature treatments, temperature had a clear effect on eDNA shedding rates. The initial eDNA concentrations in the aquaria kept at 25°C were, on average, one and two orders of magnitude lower than those in the aquaria kept at 35 and 30°C, respectively. We believe that differences in shedding rates could be due to a reduction of cane toad activity levels at the lower temperature. Locomotor performance of other *Rhinella* species has been shown to be significantly higher at 30 and 35°C than at 25°C in fully hydrated individuals (Titon & Gomes, 2017; Titon, Navas, Jim, & Gomes, 2010). More specifically, a study testing *Rhinella marina* metabolism at 20 and 30°C showed higher oxygen consumption with increasing test temperature (Seebacher & Franklin, 2011). The authors concluded that a cane toad's capacity for extended movement decreases at 20°C and cane toads have optimal performance at 30°C (Seebacher & Franklin, 2011). Accordingly, cane toads in the 25°C treatment were observed floating on the water surface, whereas toads in the 30 and 35°C treatments actively swam around the experimental aquaria for the whole duration of their exposure. We therefore conclude that higher cane toad activity and metabolism at warmer temperatures promoted eDNA shedding. Metabolic rate has a positive impact on eDNA shedding rates (Maruyama, Nakamura, Yamanaka, Kondoh, & Minamoto, 2014) and higher metabolism caused by warmer temperature has been linked to higher eDNA shedding rates in other ectotherms (Jo, Murakami, Yamamoto, Masuda, & Minamoto, 2019; Lacoursière-Roussel, Rosabal, & Bernatchez, 2016).

Percentage of positive detections across all temperature treatments declined 3 days after removing toads from the aquaria. Rapid eDNA degradation in water has also been observed in controlled mesocosm experiments testing eDNA persistence of the toad *Pelobates fuscus* (Thomsen et al., 2012). The authors found a reduction from 100% positive detections when one toad was present in the 80 L experimental aquaria, to 30% positive detections 2 days after removal, and no detections 9 days after removal (Thomsen et al., 2012). Another study testing persistence of amphibian eDNA, for the bullfrog *Lithobates catesbeianus*, showed that 80%–90% of the eDNA degraded only 3 days after tadpole removal from experimental microcosms (Strickler et al., 2015). The authors did not find significant differences in eDNA degradation rates between 20 and 35°C (Strickler et al., 2015). These studies corroborate our findings that amphibian eDNA degrades rapidly in water, and that temperature did not significantly affect eDNA persistence.

4.2 | Experiment 2—eDNA detection of single cane toads

The present study detected cane toad eDNA after only 5 min of exposure to water, in both experimental water volumes. Studies investigating DNA shedding rates of other amphibians also reported eDNA presence within one hour of first exposure (Piaggio et al., 2014; Pilliod et al., 2014). For example, Piaggio et al. (2014) detected presence of Burmese pythons after 15 min of immersion of the snakes into experimental pens. Our results add to the body of evidence that proposes eDNA as a sensitive technique to detect species even at low densities.

We observed statistically significant variability across replicate troughs in this experiment. Previous studies have hypothesized that the individual size of the study organism could be correlated to eDNA shedding rate (Pilliod et al., 2014; Thomsen et al., 2012). However, we failed to detect a significant correlation between cane toad body size and eDNA concentration in the water after the first day of exposure. This does not preclude an effect of individual physiological condition, age, and sex on shedding rates, given that body weight does not necessarily represent body condition accurately in amphibians (MacCracken & Stebbings, 2012). Significant differences in initial shedding rates have also been observed in bullfrog tadpoles of similar size and weight and that were kept under the same environmental conditions (Strickler et al., 2015). Additionally, physiological stress caused by osmotic imbalance or handling may influence eDNA shedding rates (Pilliod et al., 2014). In our study, each cane toad was washed with eDNA-free water before they were exposed to the treatment troughs to avoid introduction of eDNA that had not been produced during the experiment. This handling could have stressed the animals increasing the probability that eDNA would be shed quickly (i.e., after only five minutes of exposure).

While the effect of innate individual variability on shedding rates cannot be disregarded, it is also possible that the high variability among replicates found herein was due to patchy distribution of eDNA in the experimental troughs. We collected water samples from a random position in the troughs, which was different from the random position at which the toads were placed. Given that our experimental troughs were relatively large, low eDNA dispersion (Goldberg et al., 2018) or survey sensitivity (Furlan et al., 2016) could have impacted our ability to capture it. Environmental DNA detection relies on the probability of capturing the eDNA present in the water, and eDNA dispersion may be an important factor influencing detection rates (Goldberg et al., 2018). A process of dispersion and accumulation over multiple days may also explain why the detections in the troughs were variable at first, and then stabilized toward the last 2 days of the experiment. Therefore, it is important to maximize sampling effort in order to increase capture probability for eDNA (Furlan et al., 2016; Goldberg et al., 2018).

In the present study, the four treatments exhibited an initial increase of eDNA in the water (between days 1 and 3) and the 200 L × 5 min treatment showed slight decline between days 3 and 5. This may seem counterintuitive, since cane toad eDNA accumulation

in a closed system should have produced a continuous increase in eDNA concentration over time. Also, inherent variability in individual eDNA shedding rates (Pilliod et al., 2014), or insufficient replication (Furlan et al., 2016) could explain the observed pattern from days 1, 3, and 5. Another possible explanation of this slight drop in eDNA concentration could be that the microbial community had started to degrade the eDNA. It is well known that microbial enzymatic action can increase DNA degradation rates in water (Strickler et al., 2015). The experimental troughs were kept outdoors, and although they were covered with shade cloth, they were exposed to sunlight that promotes primary productivity and bacterial growth. Although we did not measure microbial activity in our experiments, we could hypothesize that after day 3, eDNA was being degraded at a rate higher than it was produced, and this rate stabilized toward the end of the experiment.

4.3 | Implications for using eDNA as a monitoring tool

This study demonstrates that eDNA analysis is sufficiently sensitive to detect the presence of just one adult cane toad that has spent as little as 5 min in a small waterbody (up to 800 L volume). Where a single cane toad is exposed to the same waterbody for just 5 min each day, the amount of eDNA present increases, indicating that the eDNA is accumulating faster than it is degrading. Our study indicates that in pristine experimental conditions, eDNA can be detected 2–3 days after its last presence. Freshwater bodies in tropical Australia are often turbid with high productivity, exposed to high UV radiation, and have diurnal fluctuations in pH and dissolved oxygen (Butler & Burrows, 2006). These factors promote microbial activity and negatively effect eDNA persistence in water (Lance et al., 2017; Strickler et al., 2015). Studies comparing eDNA decay under controlled conditions and in the field have found lower eDNA persistence in natural conditions after DNA source removal (Dejean et al., 2011; Pilliod et al., 2014). Under experimental conditions, salamander eDNA could be detected up to 3 days after exposure (Pilliod et al., 2014). However, when high density of salamanders was translocated into unoccupied streams, eDNA was only detectable up to 1 hr (Pilliod et al., 2014). Our results, therefore, should be taken conservatively, given that eDNA is likely to degrade at a faster rate in environments with high productivity and turbidity (Barnes et al., 2014).

Tingley et al. (2019) proved that eDNA can be used as an effective monitoring tool for invasive cane toads. However, knowledge of decay rates and minimum eDNA detection thresholds provides context for developing, and interpreting data from cane toad eDNA monitoring programs. Low densities of other amphibians have been successfully detected using eDNA (Olson, Briggler, & Williams, 2012) with even higher detection rates than traditional sampling methods (Tingley et al., 2019). Consequently, eDNA could constitute an effective tool for monitoring the first arrival of single or small numbers of cane toads into new locations, even when the invader is not physically sighted and may have visited a sampled waterbody some days prior. Additionally, eDNA monitoring can be used to inform eradication programs. The relative ease of eDNA sampling also enables participation in monitoring programs of non-specialist personnel, including residents living near monitoring locations, who can sample frequently.

There are three main considerations to be taken into account when using eDNA as a monitoring tool for new cane toad incursions. First, is the low abundance of the target species especially in large waterbodies (Furlan et al., 2016). Failure to detect a species that is present at a site could be due to imperfect field sampling or suboptimal laboratory processing (Schmidt, Kéry, Ursenbacher, Hyman, & Collins, 2013). Site occupancy models can help determine the sampling effort needed in order to obtain reliable occupancy estimation (Schmidt et al., 2013). If surveying a site of unknown presence or with very low abundance of the target species, increasing the number of sampling sites could help overcome bias in occupancy models (Schmidt et al., 2013). Additionally, suboptimal eDNA extraction techniques could also result in false negative detections. Using standardized laboratory procedures would allow results to be compared across studies/groups (Rees et al., 2014); however, the efficiency of eDNA extraction methods and post-extraction purification would depend on the study site. Setting gold standard tools for routine eDNA monitoring is needed in order to ensure that laboratories use widely accepted methods for eDNA extraction and screening. Second, given the high variability among replicates in our study, field sampling should be designed to maximize replication and number of sites surveyed (Goldberg et al., 2018). High variability among replicates is very common in eDNA studies, given that positive eDNA detections depend on the probability of capturing eDNA that is not evenly distributed in water (Goldberg et al., 2018). Therefore, increasing the number and spatial distribution of replicates is critical for improving precision and avoiding false negative detections (Pilliod, Goldberg, Arkle, & Waits, 2013; Turner et al., 2014). A careful sampling design taking into account the assay's sensitivity is needed in order to avoid false negative results when dealing with species that occur at low densities (see Furlan et al., 2019). Pilot studies, simulation tools, and knowledge of the ecology of the species and local environmental conditions can help determine the appropriate sampling design for each particular study (Goldberg et al., 2018; Roussel, Paillisson, Tréguier, & Petit, 2015). Replication is especially important when investigating first incursions of an exotic species or rare species (Turner et al., 2014), where false negative detections could be detrimental.

Finally, eDNA results should be quantified as percentage of positive detections given that it is a more informative measure of presence or absence than number of DNA copies per assay. If the aim of a surveillance program was to determine whether there are cane toads present in a given location, then even trace amounts of eDNA (1 DNA copy per assay) should be considered a positive detection. Also, care should be taken to avoid false positives arising from cross-contamination in the wild (e.g., mud, water, dirt with cane toad eDNA translocated by vehicles and people). By incorporating negative control samples at every field site and adopting "clean and

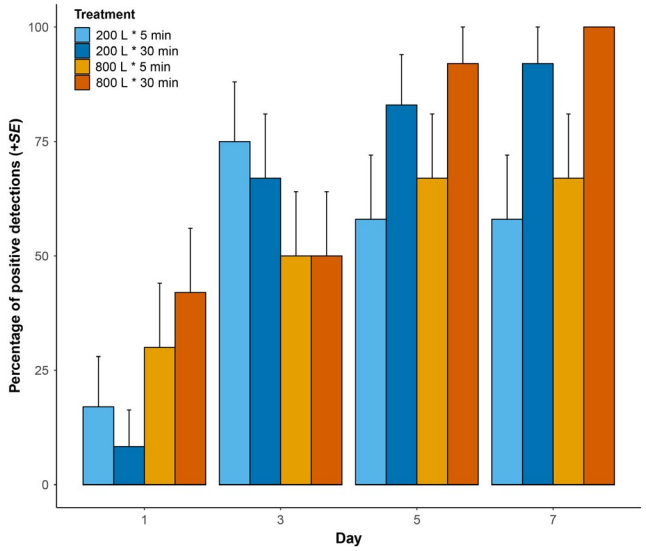

FIGURE 4 Percentage of positive cane toad detections (±*SE*) during one week of experimental period for all four water volume * exposure time treatments

consistent" sample collection protocols, contamination during sampling could be detected (Goldberg et al., 2016).

5 | CONCLUSIONS

Analysis of cane toad eDNA likely constitutes a sensitive approach for detecting the presence of low numbers of individuals (n = 1) in a small water body even after only five minutes of exposure. Based on our results, we can conclude that when a positive detection has been recorded from a field sample, a cane toad has been in contact with the water body between 1 and 3 days prior to the sampling event. We conclude that eDNA surveys can be effectively incorporated in surveillance and monitoring programs aimed at detecting new incursions or to inform eradication programs. Furthermore, eDNA surveys are easily conducted by non-specialists in daylight, enabling the participation of volunteer and citizen science programs.

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CONFLICT OF INTEREST

None declared.

AUTHOR CONTRIBUTIONS

C.V.R., D.B., and L.S. conceived and designed the study; C.V.R. carried out the experiments; C.V.R. and R.C.E. conducted laboratory analyses; C.V.R., A.I.A., J.M.S., and D.B. analyzed the data. All authors commented the final manuscript.

DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the supplementary material of this article.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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