Using environmental DNA to detect estuarine crocodiles, a cryptic-ambush predator of humans

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Abstract: Negative human–wildlife interactions can be better managed by early detection of the wildlife species involved. However, many animals that pose a threat to humans are highly cryptic, and detecting their presence before the interaction occurs can be challenging. We describe a method whereby the presence of the estuarine crocodile (*Crocodylus porosus*), a cryptic and potentially dangerous predator of humans, was detected using traces of DNA shed into the water, known as environmental DNA (eDNA). The estuarine crocodile is present in waterways throughout southeast Asia and Oceania and has been responsible for >1,000 attacks upon humans in the past decade. A critical factor in the crocodile's capability to attack humans is their ability to remain hidden in turbid waters for extended periods, ambushing humans that enter the water or undertake activities around the waterline. In northern Australia, we sampled water from aquariums where crocodiles were present or absent, and we were able to discriminate the presence of estuarine crocodile from the freshwater crocodile (*C. johnstoni*), a closely related sympatric species that does not pose a threat to humans. Further, we could detect the presence of estuarine crocodiles within an hour of its entry and up to 72 hours after the crocodiles were removed from aquariums. We conclude that eDNA could be a valuable tool for reducing human–wildlife conflict through early detection of the species.

Key words: Australia, *Crocodylus porosus*, cryptic ambush-predator, eDNA, estuarine crocodile, human predator, human–wildlife conflict, wildlife attacks

Large carnivores may pose a significant threat to humans and livestock (Messmer 2000, Treves and Karanth 2003, Mponzi et al. 2014). Consequently, human–wildlife conflicts (HWC) with large carnivores remains the greatest threat to their populations and is the primary reason they have been expatriated from much of their historic ranges (Woodroffe 2000, Kissui 2008). However, local communities may be amenable to coexisting alongside large carnivores if the conflicts can be resolved or mitigated (Proctor et al. 2018). The likelihood of HWC occurring can be reduced with the increased ability to detect an animal in time to initiate aversive actions (Campbell et al. 2015).

The estuarine crocodile (*Crocodylus porosus*) is the top predator within coastal, estuarine, freshwater, and marginal terrestrial ecosystems from East India throughout southeast Asia, to northern Australia and the Pacific Islands (Webb and Manolis 1989). The geographical range of the estuarine crocodile spans from East

India and Sri Lanka throughout southern China to Thailand, the Philippines, Sunda Islands (including Sumatra, Java, Borneo, Celebes, and Timor), northern Australia, Vanuatu, Fiji, and the Solomon Islands (Grigg and Kirshner 2015).

Estuarine crocodiles can grow up to 6 m in length (Britton et al. 2012). This species has been responsible for >1,000 human attacks in the past decade, of which 53% were fatal (CrocBite 2020). Estuarine crocodiles also prey upon livestock and domestic pets, making them a considerable threat to humans wherever these groups occur (Grigg and Kirshner 2015). The species typically inhabits turbid waters and is a highly cryptic ambush-predator, with the surviving victims typically reporting they were unaware of the crocodile's presence prior to the attack (Caldicott et al. 2005).

The large geographical range of the estuarine crocodile overlaps with 6 sympatric crocodilian species (i.e., fresh water crocodile [*C. johnstoni*], Philippine crocodile [*C. mindorensis*], New
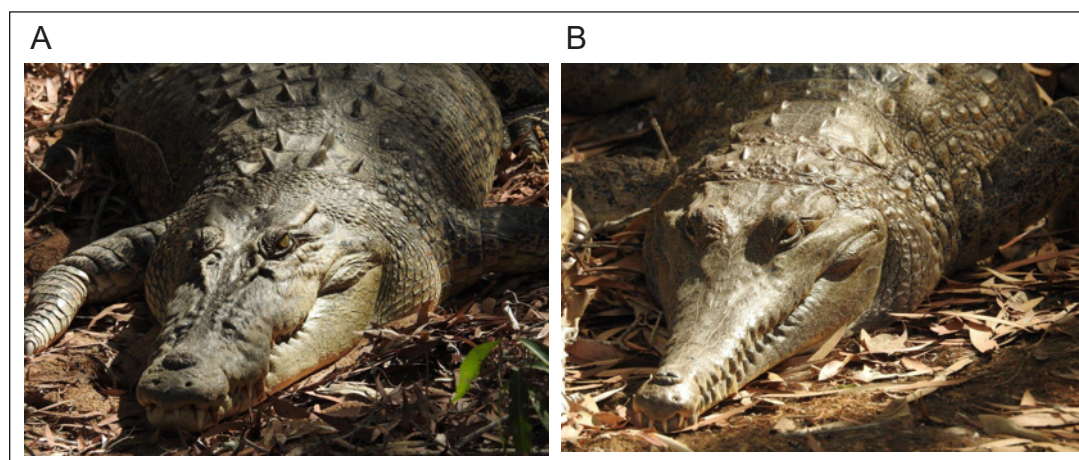
Figure 1. (A) Estuarine crocodile (*Crocodylus porosus*); (B) Freshwater crocodile (*C. johnstoni*).

Guinea crocodile [*C. novaeguineae*], false gharial [*Tomistoma schlegelii*], Siamese crocodile [*C. siamensis*], and mugger crocodile [*C. palustris*]), which do not pose as significant a threat to humans. In Australia, estuarine and freshwater crocodiles co-habit the same waterways, are often located in close proximity, and are frequently mistaken for each other (Figure 1; Fukuda et al. 2013).

The method for detecting crocodile presence has not changed since the 1970s (Messel et al. 1982). This method entails scanning the water surface, banks, and fringing vegetation with a spotlight, searching for the crocodiles' distinctive reflective eye shine. However, because crocodiles spend a significant pro-portion of their time underwater (Campbell et al. 2010*b*) and can remain submerged without surfacing for many hours (Campbell et al. 2010*a*), the spotlight method may not always detect a crocodile if present and/or differentiate between dangerous and non-dangerous co-habiting species.

The rapid technological development of genetic methods has greatly increased the specificity and sensitivity by which species can be detected in the environment from very low traces of their DNA shed into the environment (eDNA; Rees et al. 2014). The cost of these techniques has dramatically decreased in recent years, enabling broad scale sampling of the environment for the occurrence of the chosen species (Walker et al. 2017). This technique has been embraced by the conservation community and has become a relatively main-stream technique for detecting threatened and endangered species (Sigsgaard et al. 2015, Thomsen and Willerslev 2015, Simpfendorfer et al. 2016). The use of eDNA in HWC issues has similar potential but has yet to be explored.

We developed a technique whereby an estuarine crocodile could be positively detected in an experimental setting by sampling the water it had been inhabiting. The purpose of this study was to develop a DNA probe specific for estuarine crocodiles, so that it would be possible to discriminate its DNA from the closely related species that do not pose a threat to humans. We also assessed the sensitivity of the probe in a laboratory experimental setting.

Methods

To conduct our research, we sourced 4 captive estuarine crocodiles and 4 freshwater crocodiles from crocodile farms located in the Northern Territory of Australia (Figure 1). The crocodiles were manually restrained, and a small section of one of the tail scutes was removed with a scalpel. This is a common technique for marking crocodiles (Department of Biodiversity, Conservation and Attractions 2017) and is routinely carried out on crocodiles without an anaesthetic. The tissue samples used in this study were opportunistically collected from crocodile scutes marked as a component of routine management operations. An iodine veterinary spray was applied to the wound after excision. Clean instruments were used for each crocodile to avoid any DNA contamination. The samples were immediately

Table 1. Estuarine crocodile (*Crocodylus porosus*) eDNA detection assay DNA sequences. PCR product size = 105 bp.

DNA region	Sequence
"Croc_ND4 Gene_F" General crocodile forward primer	CCCTTCTAATCGCACTTCTATGG
"Croc_ND4 Gene_R" General crocodile reverse primer	GCGTGTTTGTTCAGAATGTTAGG
"Estuarine crocodile_ND4 gene probe" Estuarine crocodile - specific internal probe	TET-TGCGTCTATTACACTCTTACAGCTACTCCC-IDQ
Crocodylus porosus ND4 gene fragment (Identical to NCBI ACC#NC_008143.1)	CCCTTCTAATCGCACTTCTATGGGTGTATAAC ATAAAAGGAACTGCGTCTATTACACTCTTACA GCTACTCCCCCCAATAACCCTAACATTCTGAA CAAACACGC

placed in 99% ethanol before undergoing DNA extraction using the DNeasy blood and tissue kit (Qiagen 2006). These tissues were used to first develop and determine the sensitivity of the eDNA detection assay and also used as controls throughout the trials.

Designing the estuarine crocodile eDNA detection assay

The estuarine crocodile eDNA detection assay was developed using 29 freshwater and 969 estuarine crocodile mitochondrial DNA sequences retrieved from the National Centre for Biotechnology Information (NCBI) nucleotide database. The crocodile sequences were aligned using GENEIOUS® version 11.0.3 (Biomatters, Auckland, New Zealand) software. The crocodile-primer binding sites were chosen from the ND4 gene because it was specific for the *Crocodylus* family and had an internal species-specific sequence to estuarine crocodiles (Jarman et al. 2004, Deagle et al. 2006). Using the estuarine crocodile NCBI sequence ACC#NC_008143.1, the crocodile primers and the estuarine crocodile-specific probe with 9 sequence mismatches to freshwater crocodile were designed and synthesised with the IDT Oligotool (Table 1).

The probe was labelled with yellow TET™ fluorophore at the 5′ end and the "Iowa Dark" quencher at the 3′ end. The TET dye was only fluorescent when it bound to estuarine crocodile eDNA and was released from the quencher. The primers and probe were tested for specificity to the genera *Crocodylus*. The procedure was a "two-in-one" test, as detailed by Jarman et al. (2004) when the qPCR reaction was mixed with Quantitect SYBR® Green, because both crocodile species were detected at the green ~510 nm, but only the estuarine crocodile was detected at the yellow ~557 nm (Figure 2). The species was confirmed by searches of GenBank with the Nucleotide Basic Local Alignment Search Tool (BLASTn; Altschul et al. 1990).

The estuarine crocodile eDNA detection assay primers and probe were optimized by PCR and qPCR methods to ensure that the probe did not bind to the DNA of other animals. We optimized the binding specificity of the general crocodile primers in a PCR using different annealing temperatures from 53–65°C. The likelihood of undesirable primer fragments interfering with the reaction was assessed by mixing the primers with the Quantitect SYBR Green (Qiagen 2010) reaction mix and by performing a qPCR and melting curve analysis in a Rotor-Gene® Q (Qiagen 2010) using the crocodile-specific 60°C annealing temperature. We then optimized the test to only detect the estuarine crocodile using the PerfeCTa qPCR ToughMix and 0.1x BSA with the final qPCR conditions: 20 minutes at 94°C followed by 70 cycles of 94°C for 10 seconds, annealing at 60°C for 30 seconds, 72°C for 30 seconds, and 1 cycle of 7°C for 2 minutes to acquire a fluorescence TET™ signal. The least amount of eDNA that the estuarine crocodile-specific probe could detect was determined by diluting the pure estuarine crocodile DNA over 7 logs (from 75 ng/uL to 10 fg/uL) with 10 mM Tris (pH 8).

qPCR analysis of environmental DNA

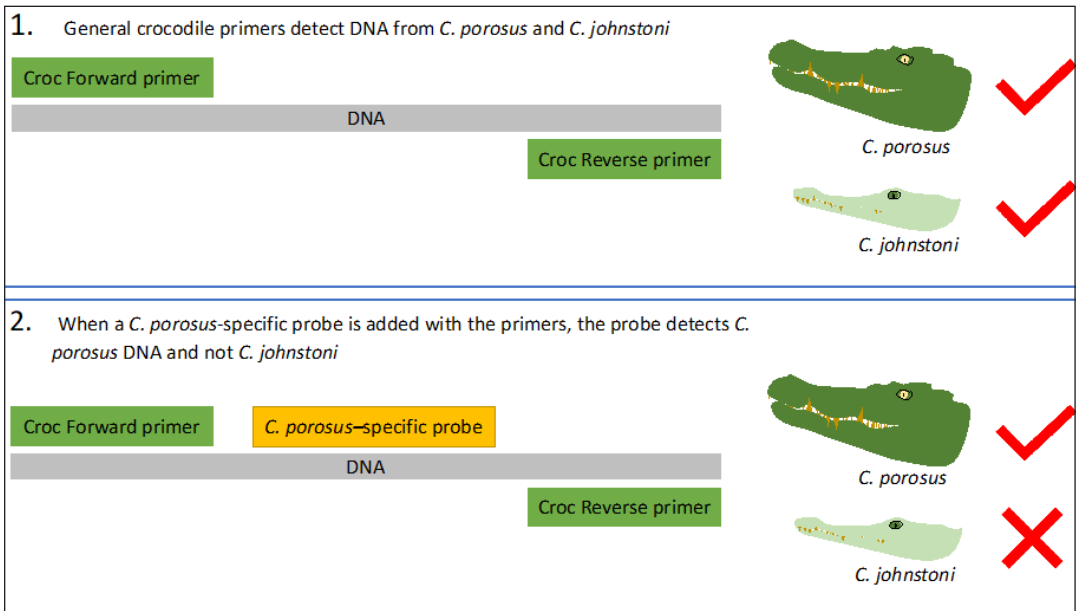Along with the experimental quality control samples to assess the likelihood of eDNA

Figure 2. Overview of the estuarine crocodile (*Crocodylus porosus*) eDNA detection assay. Test 1: both *Crocodylus* species are detected with the general forward and reverse crocodile primers at the green wavelength. Test 2: adding a TET™-dyed estuarine crocodile-specific probe to the reaction results in only estuarine crocodile eDNA being detected at the yellow wavelength.

contamination during the trial and extraction processes, each qPCR also included multiple positive and negative quality control samples to test for: eDNA contamination, non-specific probe binding, and reaction failure during a qPCR. To test if the reaction was successful, pure estuarine crocodile DNA was included as a positive control in each qPCR run. To ensure that the probe was only binding to saltwater crocodile eDNA, each run also included a pure freshwater crocodile DNA sample as a negative control. We also tested for the presence of eDNA contamination between samples and the reagents in a qPCR by including samples with no DNA added to the reaction mix. A qPCR reaction was only considered valid if the positive controls were detected and not the negative controls.

Aquarium eDNA trials

To determine the time taken for an estuarine crocodile to be detected within a body of water, and to determine the eDNA persistence under UV conditions, we undertook a series of aquarium trials. A 90-cm-long estuarine crocodile was placed into a 180-L tank of freshwater, and water samples (1 L) were collected in triplicates prior to the crocodile entering the water, and then at 0, 1, 3, 6, 12, 24, 48, 72, 96, 120, 144, and 168 hours from animal placement into the tank. For the eDNA persistence trial, 180 L of water was pumped from an enclosure containing 2 crocodiles (crocodiles housed for >2 weeks) into the trial tank placed outdoors in full sunlight. The water was sampled in triplicates at 0, 1, 3, 6, 12, 24, 48, 72, 96, 120, 144, and 168 hours. The positive control for each experiment was triplicate 1-L samples collected from water exposed to 2 ~90-cm estuarine crocodile for 420 hours.

The water samples were filtered through sterile 0.45-uM Mixed Cellulose Ester (MCE) filters (Merck Millipore). Any DNA present on these filters was isolated and purified using a DNeasy blood and tissue DNA extraction kit (Qiagen 2006). Samples were extracted according to the manufacturer's guidelines for tissue (step 1a) with minor modifications (see Day et al. 2019). To check for crocodile eDNA contamination during extraction, each trial included triplicate extractions of "blank filters" (filtered 1 L of high-pure water).

To test whether the reaction was successful, pure estuarine crocodile DNA was included as a positive control in each qPCR. To assess probe specificity for estuarine crocodile eDNA, each
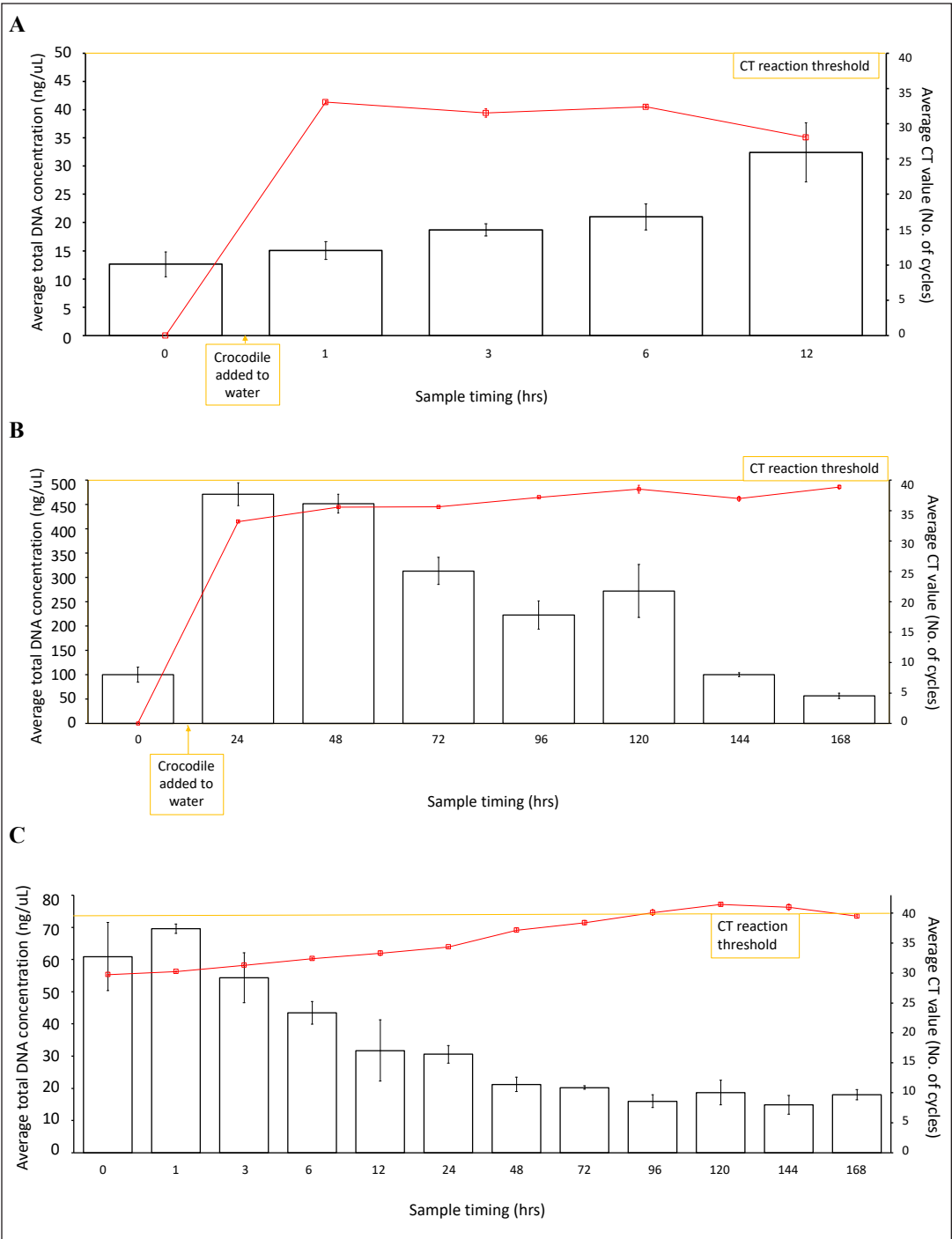
Figure 3. Estuarine crocodile (*Crocodylus porosus*) eDNA trials. (A) Estuarine crocodile eDNA detection in water and total DNA concentration over 12 hours. Samples were collected at intervals of 0, 1, 3, 6, and 12 hours. (B) Estuarine crocodile eDNA detection in water and total DNA concentration over 7 days. Samples were collected at intervals of 0, 24, 48, 72, 96, 120, 144, and 168 hours. (C) Estuarine crocodile eDNA persistence in water and total DNA concentration over 7 days. Samples were collected at intervals of 0, 1, 3, 6, 12, 24, 48, 72, 96, 120, 144, and 168 hours. Bars = average total DNA concentration. Red squares = average qPCR CT (cycle threshold) value for estuarine crocodile eDNA detection. Cycle threshold = 40. Crocodile added to trial water immediately after time 0 hours. All samples were 1 L in volume and collected in triplicate.

qPCR trial included a pure freshwater crocodile DNA sample as a negative control. We also tested for the presence of eDNA contamination between samples and the reagents in a qPCR by including samples with no DNA added to the reaction mix. A qPCR reaction was only considered valid if the positive controls were detected and not the negative controls. The sample was deemed negative if it took longer than 40 replication cycles to detect estuarine crocodile eDNA. This is because after 40 cycles, a positive detection was likely false and from non-specific replication of small DNA fragments present in the sample. For full test development details, results, and quality control results, see supplementary material (https://doi.org/10.5061/dryad.jwstqjq5p).

Results

The estuarine crocodile eDNA test detected crocodile eDNA after 1 hour of the crocodile's immersion in 180 L of water (Figure 3A). Measuring eDNA accumulation over 7 days showed that eDNA concentrations fluctuate but were detectable over the 7 days (Figure 3B). Positive controls also indicated that eDNA detections were possible for water exposed to 2 crocodiles for up to 3 weeks (supplementary figures). After the estuarine crocodile was removed from the water, its eDNA was detected for up to 7 days (Figure 3C). However, the detection was only reliable for the first 72 hours. Samples collected between 96 and 144 hours were considered unreliable because they required greater than the 40-cycle limit to produce enough estuarine crocodile eDNA for a positive result. Also, while eDNA was detected on the seventh day (168 hours), the average CT value (39.5 ± 0.3 CT) was close to the detection threshold (Figure 3C). As expected, the longer the crocodile was absent from the water, the longer it took to detect its eDNA, with the number of cycles needed for a signal increasing with time.

During the trial tests, the integrity and species discrimination of estuarine crocodile probe was not compromised because it amplified the pure estuarine crocodile DNA and not the negative control (pure freshwater crocodile DNA). It was unlikely that the eDNA results were compromised by estuarine crocodile eDNA contamination during the extraction process and at the start of the 12-hour and 7-day accumulation trials because no crocodile eDNA were detected for the tank water used for the trials, nor in the trial tank immediately after filling (0 hours). Also, crocodile eDNA contamination during the qPCR set-up and reaction were also unlikely for all trials because no signal was detected from the "no template control" (NTC) samples with no DNA added to the reaction mix. Further, pumping water from the crocodile enclosure to the trial tank outside for the eDNA persistence trial did not reduce the concentration of crocodile eDNA in the water (Figure 3 and supplementary figures).

Our estuarine crocodile eDNA assay was sensitive, and in a 20-uL qPCR reaction could reliably detect down to 8.7×10^6 copies of estuarine crocodile DNA per 1 uL. Estuarine crocodile DNA diluted beyond a concentration of 0.0001 ng/uL were not detected by the assay (supplementary figures). As well as sensitive, our eDNA test was 99% efficient at amplifying crocodile eDNA in a qPCR reaction.

Discussion

The technique developed in the present study shows promise as a survey tool to provide early detection of estuarine crocodiles, identifying estuarine crocodile presence against a backdrop of sympatric crocodilian species that do not pose a threat to humans. The technique is adaptable and could easily be applied to other HWC species. As far as we are aware, this is the first use of the eDNA technique for HWC resolution.

Our results suggest that estuarine crocodiles continually shed high volumes of DNA into water and this DNA remains stable for a number of days — even under full sunlight. The high amount of DNA shed by estuarine crocodiles into water, and its stability, means that this technique has the potential to monitor estuarine crocodile distribution over broad geographical ranges. This allows for samples to be collected by individuals without specialized knowledge or equipment and at a relatively low cost compared to traditional crocodile survey methods. The stability of the crocodile DNA would allow for samples to be collected from remote areas and transported some distance to a laboratory facility for analysis.

There are limitations of the eDNA technique

that need to be considered before we recommend its use as a management tool for early detection of cryptic ambush-predator crocodiles. First, there were a few days of lag time between taking the initial water sample to generating the detection results. However, the field of eDNA is rapidly evolving, assay times for detection are ever decreasing, costs are reducing, and instantaneous eDNA kits for detecting particular species will soon be available (Pomerantz et al. 2018). Second, the technique did not provide information about the animal's body size. This could lead to waterways being closed unnecessarily, and we recommend that the eDNA detection method be used as an initial broad scale determinant of crocodile presence, which is followed up by more targeted survey methods. Finally, as we only trialled this new methodology under laboratory conditions, we are uncertain of the probability of a false negative (not detecting a dangerous crocodile when it was present). This is a well-known limitation of the eDNA detection technique that can be resolved with rigorous field trails alongside more traditional detection methodologies (Simpfendorfer et al. 2016).

Management implications

The study showed that estuarine crocodiles continually shed high volumes of stable DNA into water and thus offers promise for using the technique to monitor estuarine crocodile distribution over broad geographical ranges. Samples can be collected without specialized knowledge or equipment and at a relatively low cost compared to traditional crocodile survey methods. The eDNA technique we developed has the potential for resolving other HWC through early detection of the problem species, but further research is required to realize its full potential.

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Supplementary material

Supplementary information and figures can be viewed through Dryad at https://doi.org/10.5061/dryad.jwstqjq5p.

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YUSUKE FUKUDA is a wildlife scientist for the Northern Territory Government in Darwin, Australia. He conducts population monitoring research for crocodilians in Australia and other countries, specializing in the population ecology and habitat analysis of crocodiles, especially *Crocodylus porosus* and *C. johnstoni*. He is interested in the process of the population decline and recovery in relation to human interventions. His recent focus has been to understand the movement and dispersal patterns of crocodiles among populations through genetic approaches. His research assists with the conservation of crocodiles, including the mitigation of human–crocodile conflicts and sustainable use of crocodiles as natural resources.

HAMISH A. CAMPBELL is an associate professor at Charles Darwin University in Darwin, Northern Territory, Australia. He integrates the disciplines of zoology and spatial ecology to create an innovative and novel research program in the emerging field of movement ecology. He is specifically interested in studying the spatiotemporal relationship between animals, conspecifics, predators, and their environment—which of these drives movement and how we can measure this quantitatively to assess individual responses to environmental heterogeneity. Understanding how animals move through space and time is fundamental to ecological processes, and his research develops and applies a range of novel technologies and analysis techniques. The findings for his research are used to guide management and recovery planning for threatened species, identify and alleviate key threatening processes, and resolve human–wildlife conflict issues.