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# Environmental DNA (eDNA) survey of largetooth sawfish in south-central Arnhem Land

Report

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Cover photographs

Front cover: Sample collection at Wilton River (photo: Mimal Land Management).

Back cover: Wilton River Crossing sampling site (photo: Mimal Land Management).

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# Acronyms and abbreviations

**eDNA**..... environmental DNA

**LOD**..... limit of detection association

**NESP**..... National Environmental Science Program

**qPCR**..... quantitative real-time polymerase chain reaction

**TropWATER**... Centre for Tropical Water and Aquatic Ecosystem Research

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# Executive summary

Sawfish populations worldwide have suffered major declines and there is an urgent need to determine the extent of their geographical distributions. While fisheries-dependent data and citizen science reports have provided some data on contemporary occurrence, this is not enough to accurately confirm distributions of these species. Targetted surveys using traditional methods have provided valuable information, however the ability to conduct this research is challenged by the highly turbid and often remote habitats that sawfishes inhabit. In addition, these sampling methods are often time intensive and require highly trained staff and equipment that are not always readily available. Environmental DNA (eDNA), or the DNA shed by organisms into their environment, constitutes a non-invasive, time- and cost-effective tool to assess species detection without having to trap or sight individuals. Sample collection requires only simple equipment, which allows researchers to enagage with non-specialists and conservation stakeholders for sample collection. This also increases the monitoring capacity by orders of magnitude and builds on the capacity for widespread, comprehensive assessment. In the present study we engaged with the Mimal Land Management for eDNA sample collection at four river systems in south-central Arnhem Land using a simplified field protocol developed at TropWATER. The Mimal Rangers collected samples from eight sites along four different river systems in the Northern Territory: Goyder, Barrapunta, Wilton and Mainoru Rivers. We extracted eDNA from water samples and screened it for presence of largetooth sawfish. Although no positive detections were obtained, the field protocol was user-friendly and easy to follow by non-specialists. We propose the method as a useful tool for future surveys and provide recommendations on strategies to maximise detection probability.

# 1. Introduction

Sawfishes (family Pristidae), a family of shark-like rays, are considered among the most threatened marine fishes in the world (Harrison & Dulvy, 2014). Three major factors account to their threatened status: low population growth rates, high catchability in fisheries and high commercial value of their fins (Dulvy et al., 2016). The Global Sawfish Conservation Strategy states the need for capacity building towards effective sawfish conservation and management (Harrison & Dulvy, 2014), however, information of the contemporary distribution of sawfish is limited, hindering conservation initiatives (Dulvy et al., 2016). Traditional sawfish survey methods include visual observations, fishing using gillnets or lines, traditional ecological knowledge, and public encounter data (Simpfendorfer et al., 2016). Of these, the most useful method for compiling important biological data has been capture with gillnets and line fishing, however, fishing becomes logistically difficult in remote and sparsely populated regions where sawfish are known to occur (Simpfendorfer et al., 2016). More specifically, in the Northern Territory, sawfish species distribution data is scarce, despite the fact that they constitute bycatch of recreational and commercial fisheries (Field, Charters, Buckworth, Meekan, & Bradshaw, 2008). Largetooth sawfish (*Pristis pristis*) occur in freshwater systems in the Northern Territory. This species has a large historical distribution, including coastal waters of 75 countries, however, it has experienced a 61% decline in geographic size range (Dulvy et al., 2016). There is an urgent need to apply less laborious field methods to detect presence of these threatened species in order to determine the current extent of their distribution.

Environmental DNA (eDNA) is the DNA released into the environment via mucous, faeces, skin cells, etc., and that can be isolated from water samples and screened for species of interest (Jerde, Mahon, Chadderton, & Lodge, 2011). Environmental DNA is gaining momentum as a monitoring tool given its sensitivity to detect the presence of species that occur at low abundance (Sigsgaard, Carl, Møller, & Thomsen, 2015). It is a time and cost-efficient method of assessing species distribution and in some cases, it has shown to have higher sensitivity to rare species that net surveys (Thomsen et al., 2012). Environmental DNA analysis has successfully been applied to detection of largetooth sawfish in Australia (Simpfendorfer et al., 2016) as well as smalltooth sawfish, *Pristis pectinata*, in the western Atlantic (Lehman et al., 2020). Environmental DNA analysis has the potential to build the capacity of citizen scientists and traditional owners to participate in sawfish regional conservation action through sample collection. However, existing sawfish eDNA studies involved collecting eDNA present in the water through water filtration. The downside of this eDNA capture technique is that it involves many procedurial steps, imposing higher risk of sample contamination (Huerlimann, Cooper, Edmunds, Villacorta-Rath, Le Port, et al., 2020), especially when dealing with eDNA of species of conservation concern where obtaining genuine positive detections is critical.

Despite major advances in filtering technology that make sampling easier (e.g., portable pumps) and less prone to contamination (e.g., Sterivex™ filter unit), filtering water is a laborious technique and is often not attractive to non-specialists who could otherwise be usefully involved in eDNA-based monitoring programs. In addition, when filtration is done off-site, water samples need to be kept on ice and filtered within 24 hours of collection in order to prevent further eDNA degradation and maximize eDNA yield (Hinlo, Gleeson, Lintermans, & Furlan, 2017). Remote sampling locations in northern Australia can be several hours away

from laboratories where filtration is often performed and keeping samples on ice is difficult due to high ambient temperature (Huerlimann, Cooper, Edmunds, Villacorta-Rath, LePort, et al., 2020). An alternative to filtration is to mix whole water samples with buffer or preservation solutions, however, preservation of eDNA samples at ambient temperatures above 30°C in the tropics where sawfish occur requires a temperature-stable preservative solution (Huerlimann, Cooper, Edmunds, Villacorta-Rath, LePort, et al., 2020).

TropWATER has developed a simplified eDNA field method that is routinely used for collection of whole water samples and preservation at ambient tropical temperatures for extended periods (Edmunds & Burrows, 2020). The method uses a non-toxic, non-alcohol preservation solution called Longmire's buffer to preserve eDNA in whole water samples (Villacorta-Rath & Burrows, 2019, 2020; Villacorta-Rath, Edmunds, & Burrows, 2019; Villacorta-Rath & Burrows, 2020). This method has also been successful in preserving sawfish eDNA on filters (Cooper et al., *in review*) and in unfiltered water samples for up to three months (unpublished data), allowing for samples to be shipped from remote areas to the laboratory facility in Townsville. In addition, the TropWATER field method, which involves collecting whole water samples, can be carried out with ease by non-specialists without formal training by just following an instruction manual. The protocol has been tested at different locations and for detection of other target species, including fish and amphibians (Villacorta-Rath & Burrows, 2019, 2020; Villacorta-Rath et al., 2019; Villacorta-Rath & Burrows, 2020). Whole water collection and immediate preservation represents a lower risk of contamination than conventional water filtration methods. The development of robust water sampling methods that can be used by citizen scientists and/or traditional owners will allow eDNA samples to be collected by the wider community. Community participation will not only raise awareness but will assist in comprehensive sawfish distribution data to be collected. This crucial baseline information can advise regional conservation actions for species protection and habitat conservation.

In the present study, we engaged with Mimal Land Management Rangers, south central Arnhem Land, Northern Territory, to conduct sawfish eDNA sampling during July-August 2020. Environmental DNA sampling was carried out by Mimal following TropWATER's field protocol across eight sites in four river systems: Goyder, Barrapunta, Wilton and Mainoru Rivers, in some of which there is anecdotal evidence of sawfish presence. The aims of the present study were to: (1) trial our eDNA field sampling methods without any formal training and just following a field manual; (2) screen field samples for presence of largetooth sawfish eDNA.

This study and report complements similar work undertaken by the researchers on Groote Eylandt in the Northern Territory, which can also be found on the hub website.

# 2. Methodology

## 2.1 eDNA sampling

Water samples for eDNA analysis were directly collected and preserved from eight sites in four river systems during July-August 2020 (Table 2.1, Figure 2.1). At each site, five replicate 500 mL samples were collected using a new, clean bottle and decanting into a new, clean jar containing 125 mL of Longmire's preservative solution. At every site, a field blank was also taken to ensure that the process of sample collection did not introduce contamination. The field blank consisted of decanting 500 mL of laboratory-grade water into a jar containing 125 mL of preservative solution. The final volume of all field samples, including controls, and preservative solution was therefore 625 mL.

*Table 2.1. Field sites in south-central Arnhem Land, Northern Territory, sampled for largetooth sawfish eDNA detection.*

| River system | Site name | Latitude | Longitude | Collection date |
|---|---|---|---|---|
| Goyder | Old Crossing | 13°01'44.832'' | 134°58'33'' | 11/08/2020 |
| | New Crossing | 12°57'56.741'' | 135°00'48.096'' | 11/08/2020 |
| Barrapunta | Barrapunta River | 13°09'24.918'' | 134°51'50.098'' | 11/08/2020 |
| Wilton | Wilton River Crossing | 13°39'7.345'' | 134°22'7.416'' | 06/08/2020 |
| | Moon Dreaming | 13°43'49.856'' | 134°26'36.192'' | 29/07/2020 |
| | Fish Hole | 13°55'20.693'' | 134°25'54.480'' | 04/08/2020 |
| Mainoru | Mainoru Crossing | 13°57'4.368'' | 133°57'54.225'' | 20/08/2020 |
| | Mainoru Station | 14°02'23.794'' | 134°05'49.534'' | 20/08/2020 |

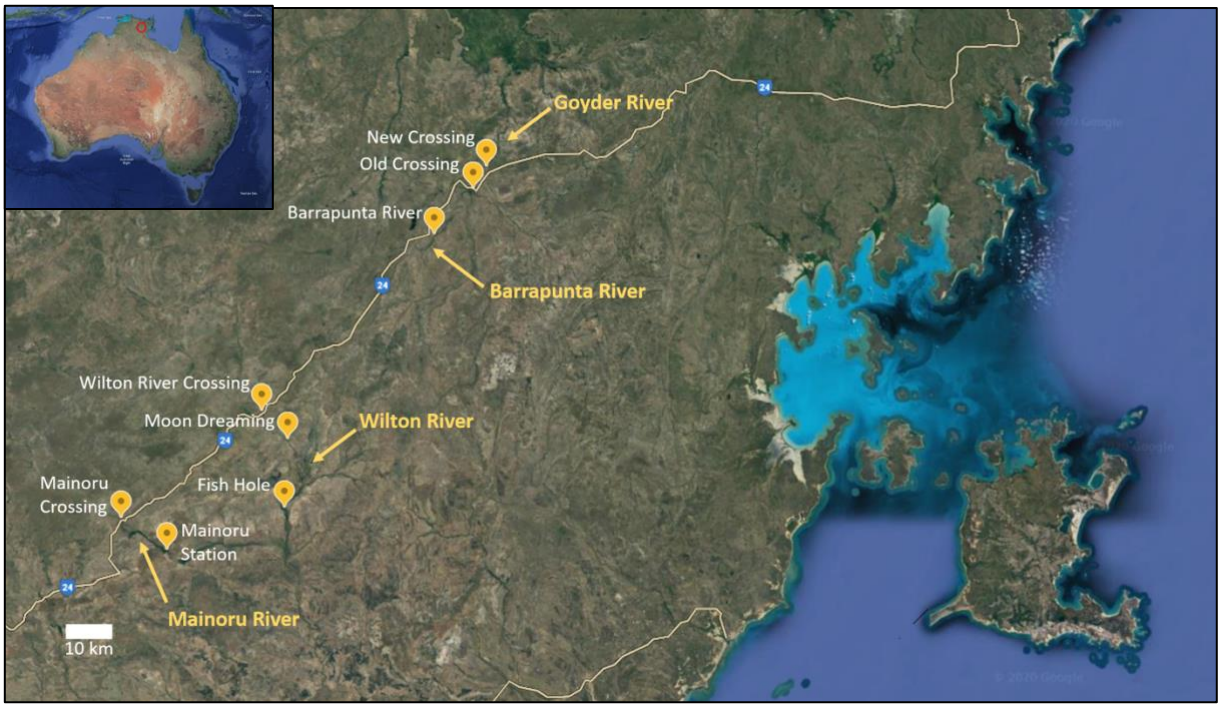


*Figure 2.1. Field sites in south-central Arnhem Land, Northern Territory, sampled for largetooth sawfish eDNA detection. Sampling site names are indicated in white font, while River systems are indicated in yellow font and yellow arrows.*

## 2.2 Environmental DNA extractions

Environmental DNA extractions were carried out at the dedicated TropWATER eDNA laboratory, James Cook University, Townsville. Prior to eDNA extraction, bench top surfaces and floor were decontaminated with 10% bleach and subsequently wiped with water and ethanol. Jars and lids containing the field samples were wiped using the same procedure to avoid cross contamination during tube handling. A volume of 300 mL of water sample plus Longmire's preservative solution of each field sample was aliquoted into 15 Falcon tubes of 50 mL capacity, each containing 20 mL of water sample and preservative solution for eDNA extraction (Figure 2.2).

We followed a glycogen-aided isopropanol precipitation protocol developed at TropWATER, as described by Edmunds and Burrows (2020). For all extractions, 20 mL sample aliquots were mixed with 5 µL glycogen (200 mg/mL), 20 mL isopropanol and 5 mL NaCL (5 M). Samples were then incubated overnight at 4°C and subsequently centrifuged at 6,750 g for 10 min to form a pellet. The supernatant was then discarded, and pellets were dissolved in 600 µL of lysis buffer (guanidinium hydrochloride and TritonX; pH 10), transferred into a 2 mL tube, and frozen overnight. Environmental DNA present in the samples was lysed at 50°C for five hours and a subsequent precipitation step was carried out by adding 1 µL glycogen and 1,800 µL polyethylene glycol (PEG) buffer to the samples. Samples were centrifuged at 20,000 g for 30 min to form a pellet that was then washed twice using 70% ethanol. After the ethanol washes, the pellet was dried and eDNA was resuspended in 100 µL MilliQ water. Finally, eDNA was purified using the Qiagen DNeasy® PowerClean® Pro Cleanup kit and eluted in 100 µL elution buffer (Figure 2.2). A negative extraction control (EC) was added to

each batch of eDNA extractions to ensure that no contamination was introduced during laboratory procedures (Goldberg et al., 2016).

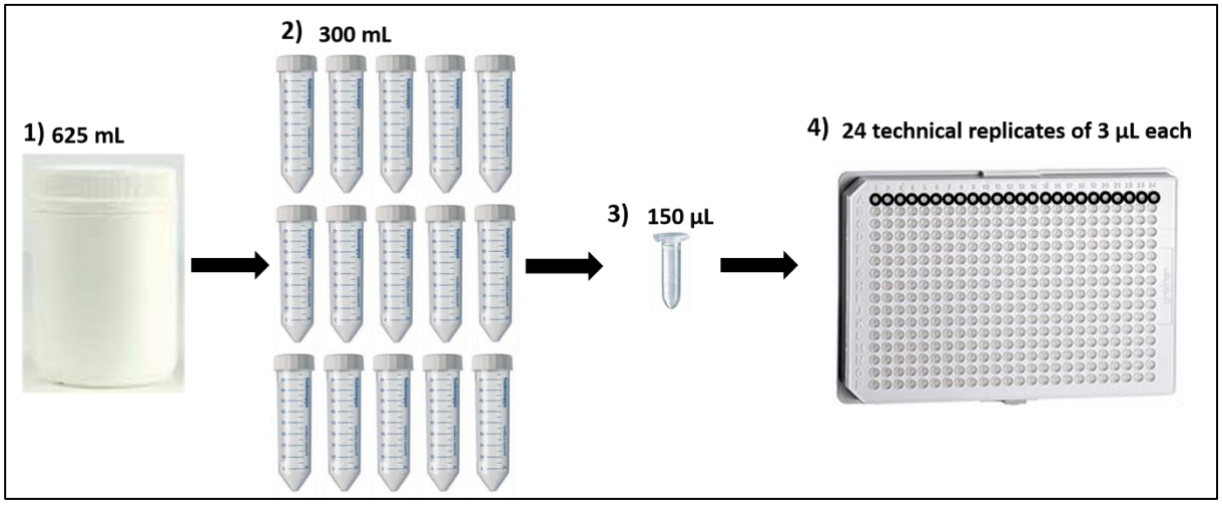


*Figure 2.2. Field samples consisting of 625 mL of water plus preservative buffer, concentrated into 100 µL of DNA eluted in laboratory-grade water, out of which 72 µL were loaded into a qPCR plate*

## 2.3 Detection of species-specific DNA by quantitative polymerase chain reaction (qPCR)

Detection of largetooth dwarf sawfish was performed using species-specific qPCR assays. The assays include unique primer pairs and TaqMan MGB probes that were previously developed and optimised at James Cook University (Cooper et al., *in review*). The assays were designed to detect a short segment of the *12S* rRNA mitochondrial gene of largetooth sawfish (179 bp) and dwarf sawfish (111 bp) with an LOD at 1.25 and 5 copies per reaction, respectively. The *12S* segments were chosen based on adequate interspecific sequence divergences and intraspecific sequence similarity using reference sequences in Geneious 10.2.6 software. The largetooth sawfish 12S assays uses forward primer 5'-GTGCCTCAGACCCACCTAGA-3', reverse primer 5'-CATCATACTGTTCGTTTTTTCTTAGGAG-3', and probe 5'-VIC-AAATGAACTAACCTTCAATACG-MGBNFQ-3'. The dwarf sawfish 12S assays uses forward primer 5'-GGTGCCTTAGATCCACCTAGAG-3', reverse primer 5'-CTGACGTATTGAAGGTGGGTTCT-3', and probe 5'-FAM-CATTTCTTGCTATCAACC-MGBNFQ-3'.

qPCR assays were run on a QuantStudio™ 5 Real-Time PCR System (Thermo Fisher Scientific Australia Pty Ltd) in a total of seven MicroAmp™ Optical 384-well plates sealed with optical films (Applied Biosystems, ThermoFisher Scientific, Victoria, Australia). Twenty-four technical replicates of each field sample were run on qPCR (Figure 2.2).

Each technical replicate consisted of 10 µL reactions containing 3 µL of template DNA and 7 µL of master mix (5 µL TaqPath™ ProAmp™ Multiplex Master Mix; 0.3 µL *P. pristis* forward primer at 100 µM; 0.3 µL *P. pristis* reverse primer at 100 µM; 0.25 µL of *P. pristis* TaqMan™

MGB probe; 1 µL Bovine Serum Albuminum, BSA; 1 µL MilliQ® water). Thermal cycling profile was as follows: initial denaturation at 95°C for 10 min, followed by 55 cycles of 95°C for 15 sec and 60°C for 1 min. In each plate, six positive reference samples containting largetooth sawfish 12S synthetic DNA (gBlocks® Gene Fragments; Integrated DNA Technologies Pty Ltd, New South Wales, Australia) were added, as well as three non-template controls (NTC) and EC from all eDNA extractions. The NTC and EC samples did not contain the target species DNA and their lack of amplification indicated that no contamination was introduced during plate handling or extraction, respectively.

Inhibition was tested for using a custom QSY-ABY labelled TaqMan™ Exogenous Internal Positive Control (IPC) qPCR assay (Applied Biosystems; Hartman, Coyne, & Norwood, 2005). A volume of 0.7 µL of IPC DNA was added to three technical replicates of one field sample at each site, selected randomly. Additionally, three reactions containing the same volume of IPC DNA was added to three NTCs to be used as positive controls. Any amplification with a Ct delay of > 3 cycles was considered to be due to qPCR inhibition (Hartman et al., 2005).

## 2.4 Inhibition tests

Inhibition was tested for using a custom QSY-ABY labelled TaqMan™ Exogenous Internal Positive Control (IPC) qPCR assay (Applied Biosystems; Hartman, Coyne, & Norwood, 2005). A volume of 0.7 µL of IPC DNA was added to three technical replicates of one field sample at each site, selected randomly. Additionally, three reactions containing the same volume of IPC DNA was added to three NTCs to be used as positive controls. Any amplification with a Ct delay of > 3 cycles was considered to be due to qPCR inhibition (Hartman et al., 2005).

## 2.5 Data analysis

All plates were analysed with a common fluorescence threshold (0.4) using QuantStudio™ Design and Analysis Software (version 1.4.2; Thermo Fisher Scientific Australia Pty Ltd) before export and subsequent analyses in Microsoft Excel. Samples were considered positive for largetooth or dwarf sawfish detection ifthere was a fluorescence signal and the association amplification curve crossed the common fluorescence threshold within 50 cycles.

# 3. Results

## 3.1 Inhibition tests

Extracted eDNA from water samples showed a Ct difference of less than 3 cycles, indicating that the eDNA was not inhibited.

## 3.2 Detection of sawfish eDNA via qPCR

Based on the assessment criteria to consider a positive detection of the target species, no presence of largetooth or dwarf sawfish at any sampling site was detected. All field and extraction control samples were verified to be devoid of the target species eDNA by qPCR.

# 4. Discussion

In the present study, we enagaged with Mimal Land Management Rangers, south central Arnhem Land, Northern Territory, to collect eDNA samples from four river systems where there is anecdotal evidence of sawfish occurrence. Although we did not find sawfish eDNA in the samples, our field method proved to be user-friendly and was successfully followed by Mimal Rangers.

## 4.1 Field collection method

Environmental DNA analysis relies on collecting trace DNA suspended in water and therefore is very sensitive to contamination. Contamination can occur and multiple stages of the analysis, in both the field and the laboratory and blank controls need to be implemented in order to monitor potential contamination (Goldberg et al., 2016). These include: field controls, eDNA extraction negative controls and qPCR negative controls (Goldberg et al., 2016).

Laboratory contamination is easier to eliminate by conducting all eDNA extractions and qPCR in a dedicated “low DNA copy” space (Taberlet, Waits, & Luikart, 1999). However, field contamination could be harder to avoid, especially when involving non-specialists in sample collection, who might not be aware of the special care that carrying out this work requires. Since avoiding contamination can be particularly hard to avoid when conducting protocols that involve many procedurial steps, such as on-site filtration, TropWATER’s simplified method involves collecting water samples and preserving them directly in Longmire’s buffer. This buffer can preserve eDNA integrity in the samples for up to three months (unpublished data) and therefore can allow for enough time to ship samples back to the laboratory. In the laboratory, samples can be aliquoted into smaller tubes that would fit a bench centrifuge and eDNA is extracted via precipitation (Villacorta-Rath & Burrows, 2019, 2020; Villacorta-Rath et al., 2019; Villacorta-Rath & Burrows, 2020).

Our simplified field collection method was sucesfully applied by Mimal Rangers. Importantly, the fact that we did not observe positive amplification in any of the field control samples collected in the present study demonstrated that the field method was stringent enough to avoid contamination during field sampling.

## 4.2 Sawfish eDNA detection

The application of eDNA as a detection tool for elasmobranchs (sharks and rays) in dynamic coastal environments is still in its infancy but presents an unparalleled opportunity to survey rare shark and ray species rapidly and effectively. We did not detect presence of largetooth sawfish eDNA at eight sampling sites in south central Arhnem land. There are many factors that could explain the lack of positive detections and lack of sawfish eDNA detections in the present study is not always indicative of the species absence at the sampling location. The capture and detectability of eDNA is contingent on not only the release of eDNA from the species when it is present in the habitat, but also the persistence and degradation of eDNA in the water (Thomsen et al., 2012).

Very little is known about the source of eDNA from elasmobranchs or the rate it is shed into the environment, which can depend on a number of factors including the species, size,

abundance, life stage, stress, and water conditions (Harrison, Sunday, & Rogers, 2019). Lack of such information makes inference of non-detections difficult. Moreover, eDNA transport and degradation in the water column can also affect the ability to detect eDNA (Harrison et al., 2019). On the other hand, given that eDNA degradation generally occurs within the first two weeks after it has been deposited (reviewed by Harrison, Sunday, & Rogers, 2019), it is plausible that false negative detections may be a result of rapid degradation. Therefore, eDNA detection relies on the target species having been present in the area within the approximate time of sampling. Aside from biological factors, there are technical factors that may also influence detection sensitivity. This includes failure to capture target eDNA in small water volumes or in only few field replicates when sampling in dynamic coastal habitat. Accordingly, survey replication and sampling across multiple time points are important steps to improve the sensitivity of the survey method.

Sawfishes in northern Australia are rare and patchily distributed and capture and sighting records are lower than they were historically (Peverell, 2005; Stevens et al., 2008). In the case of largetooth sawfish, low catch rates have been documented throughout the Northern Territory, even in areas of known sawfish occurrence (Kyne, Pillans, 2017). Additionally, very low capture records of juveniles may indicate the low survival rates of this species during early stages (Kyne et al., 2017). Independent surveys of dwarf sawfish in the Gulf of Carperntaria have also reported low capture rates (Field et al., 2008). Similarly, sawfish eDNA surveys in northern Australia indicated that detections were generally sparse and uneven. For example, approximately 15% of surveyed sites in the Kakadu region were positive for dwarf sawfish eDNA, and positive replicates contained only small amounts of detectable DNA (Cooper et al., *in review*). The survey in this current study was undertaken in December, which precedes the local pupping season of all four Indo-West Pacific sawfishes. Neonate and juvenile largetooth sawfish occupy freshwater sections of rivers and move into estuarine systems when they mature (Thorburn et al., 2007; Thorson, 1982b, 1982a), however, there is no evidence to suggest that the saltwater lake is a nursery habitat for largetooth sawfish. Especially considering there is not a substantial freshwater flow. In addition, seasonality, habitat fidelity, and spatial movements (Kyne et al., 2017) may also affect detection rates. The fact that we did not detect largetooth sawfish eDNA could indicate that the species were not present at the sampling sites during field work. It is suspected that sawfish exhibit seasonal movements (Kyne et al., 2017) and that females typically return to the same pupping ground and that pups will stay in nursery grounds for up to a year. However, there is no published evidence of where these pupping grounds exist in the Northern Territory and eastern Gulf of Carpentaria.

# 5. Recommendations

Conducting eDNA surveys during pupping seasons, when gravid females are expected to return to riverine environments could provide a higher chance of detecting sawfish eDNA. Alternatively, it is also likely that sawfishes are rare in south central Arnhem Land, and given that catch rates and sightings are variable, eDNA surveys should ideally be repeated seasonally in order to account for the natural variability of sawfish occurrence. Finally, conducting eDNA sampling shortly after an unconfirmed sawfish sighting could substantiate the utility of the technique for Ranger groups who wish to continue employing eDNA detection methods for species of local importance or conservation concern. These findings are relevant and important for research efforts that aim to reveal crucial baseline information for sawfishes, where conservation efforts are impeded by lack of technologies to reliably monitor them.

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