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ORIGINAL ARTICLE

Practical eDNA sampling methods inferred from particle size distribution and comparison of capture techniques for a Critically Endangered elasmobranch

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Abstract

Environmental DNA (eDNA) methods are increasingly applied in the marine environment to identify species and community structure. To establish widely applicable eDNA techniques for elasmobranchs, we used the Critically Endangered largetooth sawfish (*Pristis pristis* Linnaeus, 1758) as a model species for: (1) assessing eDNA particle size distribution; (2) assessing the efficiency of long-term preservation of water samples; and (3) comparing the efficiency and detection sensitivity of filtration and precipitation methods. Water samples (1 L) collected from a tank containing one largetooth sawfish specimen were sequentially filtered through five filter membranes of decreasing pore size (20, 10, 5, 1.2, and 0.45 μm). The proportion of sawfish eDNA retained within each size class was determined through quantitative real-time PCR (qPCR) using a species-specific TaqMan probe assay. A linear mixed-effects model (lme) showed that the 1.2 and 20 μm filters captured most of the eDNA particles present in the sampled water. Additionally, whole water samples (0.375 L) were preserved in Longmire's buffer, stored at tropical ambient temperatures (26.3°C ± 3.0 SD) and extracted at five time points: immediately, one, two, and three months after collection, as well as frozen and extracted three months later, to assess the preservation efficiency of Longmire's buffer via qPCR analysis. A linear mixed-effects model showed that samples maintained maximal eDNA yield for at least three months after collection at ambient storage. Lastly, when comparing the filtration and precipitation methods, filtration using 0.45 μm pore size was more sensitive to capture of largetooth sawfish eDNA than filtration with 20 μm filter or water precipitation. However, water precipitation was more efficient when accounting for volume of water processed. These results provide options for best capture and preservation of elasmobranch eDNA.

Madalyn K. Cooper and Cecilia Villacorta-Rath should be considered joint first author.

KEYWORDS
ecology of eDNA, eDNA sampling methods, elasmobranch eDNA, marine species monitoring, non-invasive surveys, threatened species

1 | INTRODUCTION

Anthropogenic impacts are placing growing pressure on aquatic ecosystems, as evidenced by widespread biodiversity declines and an increased number of threatened species (Halpern et al., 2008; He & Silliman, 2019). This warrants immediate attention if we are to mitigate the risk of extinction to species and the detrimental societal and economic consequences (Halpern et al., 2008; He & Silliman, 2019; Hoffmann et al., 2010; Pereira et al., 2012; Worm et al., 2006). However, studying threatened species is often even more challenging in the marine environment than on land due to vastness of the ocean, making much of it inaccessible (McCauley et al., 2015; Webb & Mindel, 2015) and the inefficiency of many traditional survey methods (e.g., Shaw et al., 2016; Sigsgaard et al., 2015; Simpfendorfer et al., 2016; Smart et al., 2015). Non-invasive survey methods are favorable for the detection of threatened species, given the threat that invasive sampling methods pose on individual survivorship (e.g., Hermosilla et al., 2015). Environmental DNA methods have been applied to detect a variety of threatened species, and have demonstrated success where traditional, invasive sampling methods are otherwise ineffective (Shaw et al., 2016; Sigsgaard et al., 2015; Simpfendorfer et al., 2016; Smart et al., 2015). All organisms naturally release DNA into their local environment through excretion, epidermal shedding, reproduction, or post-mortem decay, which can be isolated from filtered or whole water samples (Taberlet et al., 2012). This eDNA can be isolated and subsequently screened for the target species DNA using real-time quantitative PCR (qPCR; e.g., Bylemans et al., 2017; Erickson et al., 2016; Levi et al., 2019; Lugg et al., 2018). Although eDNA methods are increasingly being used in the marine environment, the interpretation of eDNA data can be imperfect due to the myriad of factors that influence detectability (Furlan et al., 2015; Harrison et al., 2019).

Factors that influence the detectability of aquatic eDNA particles include its state and fate. Aquatic environmental DNA is presumed to be made up of a complex mixture of intact cells, organelles, and DNA fragments dissolved in water or bound to particles (Sassoubre et al., 2016; Turner et al., 2014). In the environment, eDNA is degraded through the synergistic effect of biotic and abiotic factors (Harrison et al., 2019; Huerlimann et al., 2020; Jo & Minamoto, 2021) and can continue to degrade the following collection if preservation and storage conditions are not optimal (Spens et al., 2016). An additional aspect driving differences in eDNA detectability is that eDNA sampling approaches differ in the size of particles that they target (i.e., whole water precipitation vs. filtration, or filtration with different filter pore sizes). As a result, improving our knowledge of eDNA in its natural state and how best to capture and preserve it in samples before arrival at the laboratory will improve the detection of target species. This is particularly relevant when dealing with rare species, when eDNA concentration in the natural environment might be lower than that of more abundant species.

Thus far, much of the understanding on the state and fate of eDNA has been focused on teleosts (i.e., bony fishes; Barnes et al., 2020; Jo et al., 2019; Sassoubre et al., 2016; Turner et al., 2014; Wilcox et al., 2015). These studies target organellar (mitochondrial) genome regions, because these occur at high copy numbers and organelles are abundantly distributed within a single cell (Martellini et al., 2005). The size of organelles, specifically mitochondria, is therefore useful information for the design of eDNA surveys that aim to detect mitochondrial gene fragments, especially when adopting a filtration eDNA capture method. The size of mitochondrial-derived particles that are captured is likely to vary, depending on the tissue source, development stage, or water temperature-dependent degradation (Jo et al., 2019; Takeuchi et al., 2019). Current literature suggests that mitochondrial-derived eDNA particles from bony fish are most abundant between the 1.2 and 10 μm size class (Jo et al., 2019; Sassoubre et al., 2016; Turner et al., 2014; Wilcox et al., 2015), and that this eDNA is therefore most likely still contained within its organelle, cell, or is clustered together in clumps (Furlan et al., 2015).

As with capture of the eDNA itself, its effective preservation is equally important to avoid degradation and subsequent false negative detection. The recommended approach is to extract the eDNA as soon as possible following capture to minimize further risk of degradation (Hinlo et al., 2017; Yamanaka et al., 2016). However, a primary advantage of eDNA sampling is the ability to conduct minimally invasive, rapid, and low-cost field work in remote regions, which often precludes rapid laboratory processing. Environmental DNA is, therefore, captured using filter membranes, or in whole water samples, and then immediately preserved at ambient temperature using a preservation solution. For example, for filtered samples; Longmire's buffer (Renshaw et al., 2014), SPYGEN CL1 buffer (Cantera et al., 2019), Qiagen AL1 buffer (Majaneva et al., 2018), ethanol (Hinlo et al., 2017), or silica gel or beads (Bakker et al., 2017; Majaneva et al., 2018), and for whole water samples; Longmire's buffer (Williams et al., 2016), alcohol and sodium-acetate (Doi et al., 2017), or benzalkonium chloride (Takahara et al., 2020). Ambient storage in a preservation solution also removes the necessity for cold storage, which is an advantage in remote or distant sampling sites away from the laboratory (Huerlimann et al., 2020; Ladell et al., 2019; Williams et al., 2017). In particular, Longmire's buffer can be made inexpensively and conveniently in-house and has demonstrated efficiency at preserving eDNA at ambient temperature (Edmunds & Burrows, 2020; Renshaw et al., 2014; Williams et al., 2017), and up to 8 months after sample collection (Mauvisseau et al., 2021).

The use of eDNA to survey elasmobranchs (sharks and rays) is a growing field given the advantages that this method possesses

over traditional ones for sampling species of high conservation concern (i.e., one-third of sharks, skates, and rays are threatened with extinction; Dulvy et al., 2021). For example, qPCR-based eDNA detection is highly sensitive and specific to the target species, particularly those of low abundance, and is non-invasive circumventing risks to species susceptible of capture-induced stress (e.g., Budd et al., 2021; Lehman et al., 2020). Despite this, there are still fewer studies on eDNA of this taxon (reviewed by Le Port et al., 2018) compared to bony fishes. Elucidating the efficacy of eDNA field surveys, including an increased understanding of the physical state of eDNA in water, will lead to more robust and reliable detection of elasmobranch eDNA. It is anticipated that eDNA survey data on the contemporary occurrence and distribution of threatened shark and ray species will become an asset to practitioners designing tailored management and conservation interventions for shark and ray species.

Here, we describe the particle size distribution of aqueous mitochondrial-derived eDNA from an elasmobranch species of high conservation concern, the Critically Endangered largetooth sawfish (*Pristis pristis* Linnaeus, 1758). We also assess differences in sensitivity and efficiency of three eDNA capture methods with respect to capture of intra- and extra-cellular DNA. Lastly, we determined the long-term storage efficiency of eDNA in Longmire's buffer. Studies of largetooth sawfish eDNA characteristics and dynamics may be directly transferrable to eDNA studies of other elasmobranch species and especially sawfish relatives of the group Rhinopristiformes, which are of high conservation concern (Dulvy et al., 2016; Kyne et al., 2020).

2 | MATERIALS AND METHODS

2.1 | Experimental set up

Environmental DNA was sampled from an outdoor saltwater tank at James Cook University, Cairns, Australia (−16.816658°, 145.687867°) on 5 September 2019 using individual sterile polyethylene bottles (described below). The tank contained one largetooth sawfish (*Pristis pristis*) and several small reef fish (green chromis *Chromis viridis*, longfin bannerfish *Heniochus acuminatus*, and blue tang *Paracanthurus hepatus*). The individual largetooth sawfish (male, approx. 1 m total length) had been housed in the tank for 5 months prior to sample collection (April 2019) and was fed a diet comprised exclusively of mullet (family Mugilidae). Capture, handling, and husbandry of the sawfish in this study were according to James Cook University animal ethics A2584.

The tank water volume was 33,048 L and the total amount of water for the whole tank system was 75,000 L, which was filtered using biological and mechanical filtration, including sand, wool, live rock, protein skimmers, ozone, algae scrubbers, and mangroves. The water was collected from Trinity Inlet, Cairns, and was pre-filtered prior to use in the tank. Trinity Inlet is not expected to be inhabited by largetooth sawfish as indicated by discontinuity of records for the species along the Queensland coast, excluding Princess Charlotte Bay, in the past two decades (Wueringer, 2017). On the day of sampling, the tank water pH was 8.2, salinity was 35 ppt, and water temperature was 24.5–26.5°C.

2.2 | Particle size fractionation

A size fractionation experiment was conducted to understand the particle size distribution of naturally occurring aquatic particles. Evidence suggests that the majority of macro-organismal eDNA is efficiently captured by filter pore sizes between 1 and 10 μm (Turner et al., 2014). Additionally, studies in turbid waters have proven that target species eDNA can be effectively captured using 20 μm filter pore sizes (Cooper et al., 2021; Robson et al., 2016). Therefore, we tested filter pore sizes ranging from 0.45 to 20 μm. The filter series included three types of nylon net filters (20, 10, and 5 μm nominal pore sizes, 47 mm diameter; Merck). Nylon net was the only filter material type available for these larger pore size filters at the time of this study (see also Turner et al., 2014). As a result, we selected nylon membrane filters for the two smaller pore sizes (1.2 and 0.45 μm nominal pore sizes, 47 mm diameter; Merck).

To physically separate the aquatic particle size classes, triplicate 500 ml of water samples was collected directly from the tank containing the largetooth sawfish using individual sterile polyethylene bottles and were sequentially filtered using filter pore sizes from largest to smallest. Specifically, the triplicate water samples were each filtered through 20 μm filters held in individual sterile filter housing units (Thomas et al., 2018; Smith-Root, Washington, United States) that were attached to a diaphragm pump (Grover Scientific, Townsville, Australia) using clear 10 mm nylon tubing. Following filtration, each water sample was collected in a new, sterile polyethylene bottle and then filtered through 10 μm filters that were housed in new filter housing units. This method was repeated for each step of the sequential filtration experiment. Diaphragm pumps and nylon tubing were reused throughout the experiment because they are downstream of the filter and so were not considered a contamination risk. The external surfaces of the pumps and tubing were cleaned by wiping with 10% bleach and reverse osmosis-purified (RO) water and gloves were changed at each step to minimize the risk of contamination.

Immediately after filtration at each point of the experiment, filters were cut in half using sterile forceps and scissors. Each filter half was then placed in 1.5 ml of Longmire's buffer (Longmire et al., 1997) contained in a 2 ml LoBind® microtube (Eppendorf South Pacific Pty Ltd; Lecerf & Le Goff, 2010) to minimize potential loss of low-copy DNA due to biochemical or electrostatic retention and stored at ambient room temperature until extraction. One half was archived for future use. In addition, triplicate 500 ml water samples were filtered once through 0.45 μm nominal pore size nylon membrane filters to estimate target capture efficiency when target particles are ultimately captured by a single filtration, hereafter termed the "single filtration samples." A filtration negative control consisting of passing 500 ml MilliQ water through a clean 0.45 μm filter was taken before tank water sample filtration.

2.3 | Preservation efficiency of Longmire's buffer at ambient temperature

Whole water samples were collected from the same tank containing the juvenile largetooth sawfish for eDNA extraction via precipitation. Five replicate 375 ml water samples were collected and decanted into a new, clean HDPE plastic bottle (700 ml capacity) containing 125 ml Longmire's preservative buffer. The final volume of each tank replicate was therefore 500 ml. Additionally, a filed control consisting of decanting 375 ml laboratory-grade water into a container filled with 125 ml Longmire's buffer was included. To test the integrity of eDNA preserved in Longmire's buffer over time, each tank replicate was split into five different treatments (100 ml per treatment) upon arrival to the laboratory: (1) time 0; (2) time 1; (3) time 2; (4) time 3; and (5) frozen (see below for description of treatments). For ease of storage at their treatment conditions, each 100 ml subsample in each treatment was thoroughly mixed by inversion and then decanted into five 20 ml aliquots in 50 ml LoBind® falcon tubes (Eppendorf South Pacific Pty Ltd, New South Wales, Australia; Figure S1).

Environmental DNA from the time 0 treatment was extracted immediately upon arrival to the laboratory. Samples subjected to time 1–3 treatments were kept in a dark box in an outdoor area at James Cook University, Townsville, in order to subject them to the true ambient temperature experienced throughout the study period (Table 1). DNA was then extracted 1 month (time 1), 2 months (time 2), and 3 months (time 3) after collection. During the 3 months, ambient storage temperature ranged from 16.1 to 35.3°C and averaged 26.3°C (±3.0 SD). More specifically, accumulated mean ambient temperatures (± SD) were 24.4°C (±3.0), 25.5°C (±2.9), and 26.3°C (±3.0) for time 1, 2, and 3 samples, respectively. Samples from the frozen treatment were stored at constant −20°C for 3 months prior to eDNA being extracted (Table 1).

2.4 | Comparison of capture efficiency between filtration and whole water precipitation

The eDNA capture efficiency of filtration through 0.45 μm filters, 20 μm filters, and water precipitation was evaluated. Here, copy number estimated from qPCR Ct values were used for the 0.45 μm treatment from the single filtration samples; for the 20 μm treatment from the 20 μm filters used in the particle size fractionation experiment; and for the precipitation treatment from the time 0 treatment used in the preservation experiment.

2.5 | Environmental DNA extraction and quantification

Environmental DNA of all samples was extracted using a glycogen-aided precipitation extraction method described in Edmunds and Burrows (2020; Data S1). Extractions of the Longmire's buffer-preserved filter paper samples followed additional modifications for filter papers stored in 2 ml microtubes detailed in Cooper et al. (2021). An extraction control was included for each batch of extracted samples. Extracted eDNA from each sample was eluted in 100 μl UltraPure distilled water (ThermoFisher Scientific Pty Ltd) in a 2 ml LoBind® microtube (Eppendorf South Pacific Pty Ltd; Lecerf & Le Goff, 2010). Following extraction, each sample was briefly vortexed before 5 μl subsamples were taken for quantification of total recovered eDNA using a Quantus™ Fluorometer dsDNA System (Promega Pty Ltd). Extracted eDNA samples were stored at 4°C until qPCR screening.

2.6 | Quantitative PCR analysis

A partial fragment of the largetooth sawfish *12S* ribosomal RNA was amplified using a QuantStudio 5 quantitative real-time PCR machine (Life Technologies, ThermoFisher Scientific Pty Ltd) with a previously optimized primer and TaqMan probe assay (Cooper et al., 2021; Table 2). To estimate copy number, triplicate 12-point standard curves were run in adjacent wells on the same qPCR plate. For generation of standard curves, double-stranded synthetic DNA fragments (gBlocks™; Integrated DNA Technologies Pty Ltd) were synthesized to match the largetooth sawfish target fragment (Table S1) and serially diluted by 8-point $\log_{10}$ (1E+08–10 copies per assay) and 4-point $\log_2$ (5–0.65 copies per assay).

qPCR analysis was performed in six replicate 10 μl reactions run in adjacent wells on a MircoAmp™ Optical 384-well plate (Applied Biosystems, ThermoFisher Scientific). Additionally, each qPCR plate included a triplicate no-template control (NTC) sample. Each reaction contained 3 μl template eDNA, 5 μl 2× TaqPath ProAmp Multiplex Master Mix (Applied Biosystems), 0.3 μM forward and reverse primer, 0.25 μM probe, and adjusted to 10 μl with MilliQ

TABLE 1 Temperature probe data for storage of samples used in the preservation time series experiment

Treatment name	Treatment period (day/month)	Average (°C)	Standard deviation	Minimum	Maximum
Time 0	6/9	–	–	–	–
Time 1	6/9–10/10	24.4	3.0	16.1	31.6
Time 2	6/9–11/11	25.5	2.9	16.1	34.8
Time 3	6/9–09/12	26.3	3.0	16.1	35.3
Frozen	6/9–09/12	−20	0.0	–	–

TABLE 2 Primer information for largetooth sawfish *12S* ribosomal DNA eDNA assay (from Cooper et al., 2021). MGB-NFQ; Minor groove binding and non-fluorescent quencher, VIC® fluorescent dye, Applied Biosystems

Oligo name	Oligonucleotide (5′–3′)	Concentration (nM)	GC content (%)	T_m (°C)	Amplicon size (bp)
*P. pristis*_12_F	GTGCCTCAGACCCAC CTAGA	300	60	60.6	179
*P. pristis*_12_R	CATCATACTGTTCGT TTTTTCTTAGGAG	300	59.1	59.1	
*P. pristis*_12_P	VIC-AAATGAACTAA CCTTCAATACG-MGB-NFQ	250	31.8		

water. Cycling conditions consisted of initial denaturation at 95°C for 10 min, followed by 50 cycles of 95°C for 15 s and 60°C for 1 min. QuantStudio Analysis Software version 1.4.2 was used to analyze threshold cycle value (Ct) based on automatic baseline and manually determined threshold fluorescence values (0.7 ΔRn). All amplicons were sent to the Australian Genome Research Facility (AGRF Pty Ltd) for clean-up and bidirectional Sanger sequencing for verification that the product amplified was from the target species. Species-specificity of each sequence was confirmed using BLASTn searches against the entire NCBI nucleotide database. Detections were considered true positives and were used in subsequent analyses if amplification curves crossed the fluorescence threshold within 50 cycles, BLASTn search of sequence matched target species with ≥98% pairwise identity, and corresponding negative controls exhibited no amplification.

To test for qPCR inhibition, we used a TaqMan™ Exogenous Internal Positive Control (IPC) qPCR assay (Applied Biosystems; Hartman et al., 2005) with a custom internal probe modification (i.e., TAMRA-VIC changed to ABY-QSY) so as to not compromise amplification efficiency of the target, which uses a VIC-labeled reporter dye, in the same qPCR reaction. We applied this assay in duplexed reactions, as per the manufacturers' optimized conditions, with the 3 µl of eDNA in three technical replicates. Three reactions containing only IPC DNA were included as "inhibitor-free" positive controls. To distinguish types of inhibition, we used an IPC ΔCt of three cycles as the threshold (Hartman et al., 2005). Specifically, IPC ΔCt of three or more cycles was considered partial inhibition and no amplification for the IPC was considered complete inhibition.

2.7 | Data analyses

Differences in largetooth sawfish *12S* rDNA copy were assessed using linear mixed effects (lme) and generalized least squares (gls) models with a Gaussian distribution in the "nlme" R package (Pinheiro et al., 2013), using copy number (inferred from 12-point standard curves; $1 \times 10^{08-0.25}$ copies per reaction) as response variable. Prior to model testing, copy number data from the sequential filtration experiment were log transformed to reduce skewness and conform to normality (Figure S4). Given that the data were auto-correlated, we accounted for non-independence of the response variable (Figure S3), by including the "AR-1" auto-correlation structure (corAR1; Zuur et al., 2009). The explanatory variables tested in the full model for the sequential filtration experiment were filter pore size (fixed effect) and tank replicate and technical replicate (nested random effects). For the degradation experiment, the explanatory variables were treatment (fixed effect), tank replicate, and technical replicate (nested random effects). Models were fitted using the restricted maximum likelihood (REML) function. The best performing model was chosen based on the Akaike Information Criterion (AIC). Post hoc multiple comparisons of means were conducted using the general linear hypotheses function (glht) Tukey contrasts and included a Bonferroni correction in the "multcomp" R package (Hothorn et al., 2020).

Differences in sawfish eDNA capture sensitivity (total number of DNA copies captured) and capture efficiency (relative sawfish eDNA capture per 100 ml of processed water) across different methods were assessed using a linear model (lm). This analysis used the single filtration, 20 µm filter, and water precipitation samples as model methods for eDNA capture. In both cases, the response variable was *12S* rDNA copy number and the explanatory variable was eDNA capture method. Three different models were run to test; (i) the effect of technical replicate as an additive factor, (ii) the interaction between technical replicate and eDNA capture method, and (iii) eDNA capture method as the sole explanatory variable. The unbalanced sampling design (three tank replicates used for water filtration and five tank replicates used for water precipitation) was corrected using a type II analysis of variance (ANOVA) in the "car" R package (Fox & Weisberg, 2018). Post hoc paired comparisons of means were performed using Tukey's HSD. Based on data normality testing, copy number data were log transformed and relative copy number data were square root transformed (Figure S4).

Finally, the coefficients of variation (CV; standard deviation divided by the mean) for all experimental data were assessed using a one-way ANOVA to evaluate the relative stability of sawfish *12S* rDNA copy numbers across filter pore size, preservation treatment, and method.

All statistical analyses were completed in R v 3.5.0 (R Core Team, 2013) and figures were generated in GraphPad Prism 8.1.0. Results of model testing can be found in Tables S3–S6.

3 | RESULTS

Largetooth sawfish eDNA was detected in all qPCR reactions (excluding negative and no-template controls) in the particle size

distribution (n = 90) and Longmire's buffer preservation (n = 150) experiments and in the single filtration sample (n = 18). The standard curve used to estimate *12S* rDNA copy number had a y-intercept of 38.97 cycles, slope of −3.11, efficiency of 109.29%, and R^2 of 0.98. All tested filter samples and whole water samples showed no evidence of inhibition (IPC ΔCt range: 0.18–1.09 cycles). Finally, field and instrument controls, extraction negative controls, and qPCR no-template controls tested negative for largetooth sawfish eDNA and all sequenced amplicons matched to reference *P. pristis* sequence, confirming true positive detection (Data S2).

3.1 | Estimation of particle size distribution

The best performing model was the one assessing DNA copy number as a function of pore size and field and technical replicates (Table S2). There were significant differences in the estimate of sawfish *12S* rDNA copy numbers across different pore sizes (Figure 1a; Table S3). The mean total number of copies (±SE) captured in the experiment was 17,748.4 (±6037.5). Overall, the 1.2 μm filter retained the highest number of copies (10,413.7 ± 3263.7 copies). Copy number estimates for the 1.2 μm filter were 3.1 times higher than those for the 20 μm filter (3356.1 ± 348.6 copies; $\beta = 0.83$, SE = 0.16, $z(90) = 5.1$, $p < 0.001$). Copy number estimates for the 20 μm filter were on average 3.9 times higher than the subsequent 10 μm filter (851.5 ± 94.5 copies; $\beta = 1.37$, SE = 0.18, $z(7.57)$, $p < 0.001$) and 1.7 times higher than the 5 μm filter (2029.5 ± 324.5 copies; $\beta = 0.58$, SE = 0.18, $z(90) = 3.21$, $p = 0.01$). Capture on the 5 μm filter was on average 2.4 times higher than the 10 μm filter ($\beta = 0.79$, SE = 0.16, $z(90) = 4.87$, $p < 0.001$). Copy number estimates for the 10 and 0.45 μm filters, which retained the least number of copies, were not significantly different (851.5 ± 94.5 vs. 1097.6 ± 71.5 copies, respectively), which retained the least number of copies. Total dsDNA (ng/μl) values followed the same trend as sawfish copy number. Relative variability, measured through the CV, was not significantly different and constant across filter pore sizes (Figure S2A).

3.2 | Preservation efficiency of Longmire's buffer at ambient temperature

For this experiment, the best performing model was the one including treatment time, as well as filed and technical replicates as explanatory variables (Table S4). There were significant differences in estimated sawfish *12S* rDNA eDNA copy numbers across different treatments (time period; Figure 1b; Table S5). Mean estimate copy numbers per treatment ranged from 1102.8 to 1509.6. The highest number of copies were observed in treatment time 2 (2 months; 1509.6 ± 71.1 copies) and time 3 (3 months; 1342.5 ± 46.4 copies). Mean estimate copy number from time 2 and 3 were on average 1.2 times higher than time 0 (processed immediately after collection; 1215.3 ± 58.7 copies) and time 1 (1 month; 1207.1 ± 75.9 copies), and 1.3 times higher than frozen (extracted after 3 months of storage at −20°C; 1102.8 ± 658 copies). Though this difference was only significant for comparisons of time 2 with time 0 ($\beta = 294.32$, SE = 77.96, $z(150) = 3.78$, $p < 0.01$) and time 1 ($\beta = 302.55$, SE = 79.33, $z(150) = 3.81$, $p < 0.01$). Differences in mean estimated copy number were also significant for comparisons between time 2 and frozen ($\beta = 406.81$, SE = 78.01, $z(5.22)$, $p < 0.001$) and time 3 and frozen ($\beta = 239.65$, SE = 78.01, $z(150) = 3.07$, $p < 0.05$). Relative variability (CV) values were consistent across all treatments but were lowest in time 3 samples (Figure S2B), though this was not significantly different. Total dsDNA (ng/μl) values followed the same trend as sawfish copy number (Figure 1b).

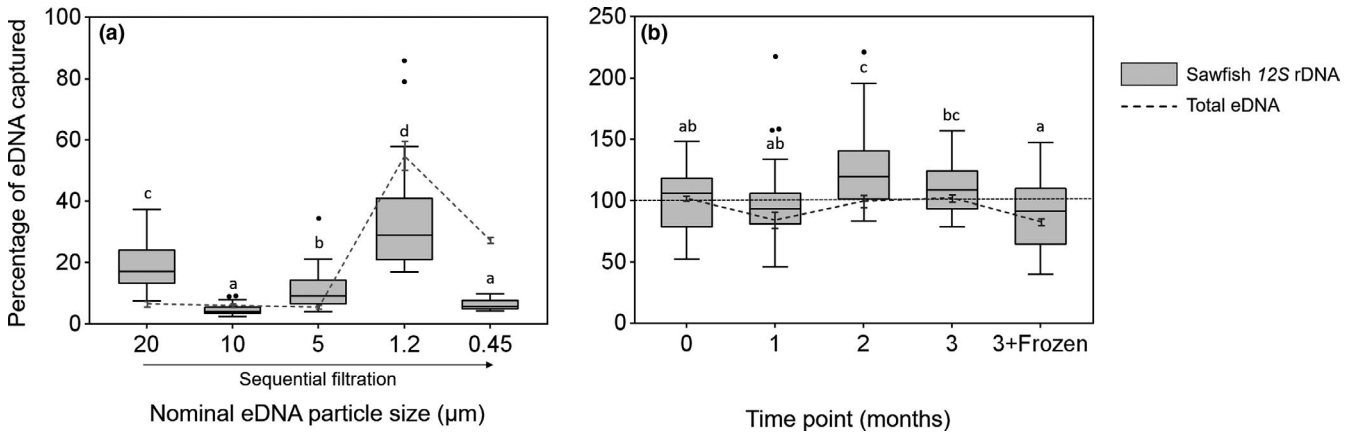

FIGURE 1 Percentage of largetooth sawfish (*Pristis pristis*) *12S* rDNA copies captured as eDNA (Tukey boxplots, black points are outliers) and total eDNA (line graph, bars are SE) (a) captured in each particle size class (μm) following sequential filtration of 1 L water samples and (b) preserved from whole water samples in Longmire's buffer solution stored at ambient temperature for 0, 1, 2, and 3 months and frozen for 3 months. In B a horizontal dotted line was plotted at 100%, the theoretical maximum preservation rate, which was represented by the average *12S* rDNA copy number estimate for time 0. Percentage of eDNA captured in each filter pore size class was determined by dividing DNA copy number for each filter pore size by the total sum of DNA copies in the experiment. For the whole water samples, copy number estimates for all qPCR technical replicates in each treatment were averaged and divided by the average copy number estimate for time 0, which was used to represent maximal yield (100%)

3.3 | Comparison of capture sensitivity and efficiency across methods

The best performing models to assess differences in capture sensitivity and efficiency of sawfish *12S* rDNA eDNA estimate copy number considered capture method as the sole explanatory variable (Table S6). Mean estimate copy numbers differed significantly across all three capture methods ($F_{(2,63)} = 113.24$, $p < 0.001$). The single filtration method (filtering 1 L of water through a 0.45 μm filter) yielded the highest mean number of copies (23,152.2 ± 1788.6 copies), performing better than the 20 μm filtration method (3356.1 ± 348.6 copies; $\beta = -2.64$, SE = 0.18, $t(63) = -14.96$, $p < 0.001$) and the precipitation method (time 0, processed immediately after collection; 1215.3 ± 58.7 copies; $\beta = -1.54$, SE = 0.16, $t(63) = -9.74$, $p < 0.001$; Figure 2a). The precipitation method also performed better than the 20 μm filtration method ($\beta = 1.1$, SE = 0.16, $t(63) = 6.98$, $p < 0.001$). Although relative variability (CV) values were not statistically different across capture methods, they showed a decreasing trend from single filtration to precipitation samples (Figure S2C). Total dsDNA (ng/μl) yield followed a similar pattern to mean sawfish copy number (Figure 2a).

Relative estimate copy numbers also differed significantly across all three capture methods ($F_{(2,63)} = 184$, $p < 0.001$). The precipitation method, which captured 7390.19 ± 519.19 per 100 ml, outperformed both the single filtration (2315.22 ± 229.38 copies per 100 ml; $\beta = 1.14$, SE = 0.16, $t(63) = 9.74$, $p < 0.001$) and 20 μm filtration methods (335.61 ± 34.86 copies per 100 ml; $\beta = -0.82$, SE = 0.12, $t(63) = -6.98$, $p < 0.001$). Total dsDNA (ng/μl) yield followed a similar pattern to mean sawfish copy number (Figure 2b).

4 | DISCUSSION

Environmental DNA detectability of rare or threatened species relies on the successful capture and preservation of eDNA particles. Therefore, the study of target species particle size distribution is important to help the user select the most effective sampling method to capture existing eDNA. Additionally, the preservation of captured eDNA particles prior to processing and analysis can influence the risk of false-negative detection due to eDNA degradation. Based on our models, the particle size distribution of largetooth sawfish eDNA in marine tank water was non-linear and most abundant at 1.2–5 and ≥20 μm size classes. Additionally, Longmire's buffer preserved the integrity of largetooth sawfish eDNA in whole water samples for at least 3 months at tropical ambient temperature. Finally, when the volume of water was standardized across capture methods, precipitating largetooth sawfish eDNA from whole water samples exhibited higher capture efficiency than filtering water through either a 0.45 or 20 μm filter. Conversely, filtration was more sensitive to capture of largetooth sawfish eDNA than precipitation due to the higher volume of water that was able to be processed. Given these findings, and as to our knowledge this is the first study to investigate eDNA particle size distribution and preservation efficiency of any elasmobranch species, we discuss methodological points relevant for the capture of elasmobranch aqueous mitochondrial eDNA.

4.1 | Particle size fractionation

Our findings show that the mitochondrial-derived eDNA particle size from largetooth sawfish was most predominantly in the 1.2–5 and ≥20 μm size classes and that the distribution was nonlinear. The former result is generally consistent with comparable studies on bony fishes by Turner et al. (2014), Wilcox et al. (2015), and Sassoubre et al. (2016), where mitochondrial eDNA particles were predominantly in the 1 μm size class. More recently, Barnes et al. (2020) multi-species analyses demonstrated that 1 μm filters captured most of the eDNA available in experimental ponds, supporting a growing body of evidence in eDNA particle size across fish species. A 20 μm pore size filter is not often used in particle size distribution studies, which precludes a detailed comparison to the results presented here. Yet, Turner et al. (2014) found that common carp (*Cyprinus carpio*) mitochondrial eDNA copies in the 20 μm size class were twice as abundant as in the 10 μm size class and whole water precipitation sample. Jo et al. (2019) quantified Japanese jack mackerel (*Trachurus japonicus*) mitochondrial eDNA particle size with a greater resolution by using an additional size fraction (i.e., 3 μm) and showed that particles were most abundant in the 3–10 μm size fraction.

In this study, the 1.2 μm filter captured 58.7% of target eDNA and this was almost one order of magnitude greater than the preceding 0.45 μm filter. This suggests that the eDNA in the study system was likely derived from within intact mitochondrial organelles, or mitochondria within cells, and not extra-cellular/organellar DNA. Eukaryote mitochondria range between 0.5 and 10 μm in size (mean 0.75–3 μm), but this can vary considerably depending on the cell type, physiological state, organ, and species (Miyazono et al., 2018). For aquatic organisms, the regular apoptotic shedding of epithelial cells releases apoptotic cellular bodies with intact mitochondria, which supports the hypothesis that intact mitochondria can exist and persist in the water column and are available for capture. However, we cannot rule out the possibility of capture of some extra-cellular/organellar DNA, given that the breakdown of large eDNA particles into smaller particles is positively correlated with higher water temperature and time since deposition (Jo & Minamoto, 2021; Jo et al., 2019). This is partially owing to increased microbial growth at elevated temperature and an associated microbial-utilization of DNA for phosphorus (Strickler et al., 2015), which is likely to be a feature for warm tropical waters (Huerlimann et al., 2020). Therefore, the filtration fractionation method used here is likely to oversimplify the reality of particle size distribution, given the above factors and the expected, but unmeasured, variation induced by the tropical tank system and the behavior of the inhabiting sawfish. In addition, differences in filter membrane material type can also affect eDNA yield (Hinlo et al., 2017; Turner et al., 2014). In this study, differences in the

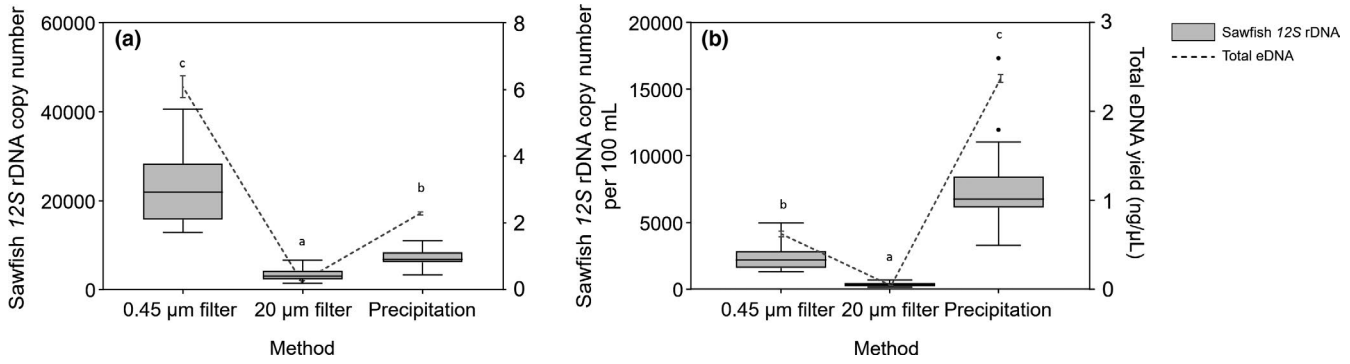

FIGURE 2 Plots showing the difference in largetooth sawfish (*Pristis pristis*) *12S* rDNA copy (a) capture sensitivity and (b) capture efficiency across different methods (filtration of 1 L through a 0.45 µm filter or 20 µm filter and precipitation of 100 ml of water). The total number of *12S* rDNA copies captured was used as an index of overall method sensitivity. Additionally, the relative number of *12S* rDNA copies captured per 100 ml of processed water was used as a measure of method efficiency

physical texture and uniformity of the pores across the five nylon membrane and nylon net filter types (Data S3) is likely to have produced differences in total eDNA yield.

The >20 µm filter size class comprised the second greatest proportion of largetooth sawfish mitochondrial eDNA, wherein capture was 3.9 times greater than the preceding 10 µm filter. The latter trend was also observed for common carp eDNA from lake and pond environments (Turner et al., 2014). This suggests that eDNA may exist in greater abundance at larger size classes than is often referred to by eDNA survey users, who intuitively use the smallest possible filter pore size suitable to the conditions. Mitochondria that comprise aqueous eDNA from macrofauna originate predominantly from waste products, shed epidermal tissues and secretions, and on the occasion of birth or death, reproductive material or post-mortem debris. Depending on this biological source, mitochondria can be arranged within the shed/released cells or in large aggregates of biological material. These particles may remain suspended in stratified seawater, or transported horizontally by currents (Wotton & Malmqvist, 2001). Given that we sampled the tank water during the period that a single sawfish was present, it is possible that we directly captured clumps of biological material. However, large particles, for example, fecal pellets, may rapidly settle out of shallow still water (Wotton & Malmqvist, 2001). In addition, eDNA may be associated with other large particles such as algal cells and sediments (Barnes et al., 2020). Overall, we suggest that larger pore sizes (1.2–20 µm) are effective for capture of mitochondrial-derived eDNA using filtration. To our knowledge, this is the first study to provide evidence of eDNA particle size distribution of elasmobranchs and can be applied to eDNA studies targeting other sharks and ray species. This is particularly informative for users conducting field sampling in challenging environments (e.g., turbid or highly productive environments) where the use of larger filter pore sizes will commensurately increase the viable filtrate volume and ameliorate challenges with rapid filter clogging (Egeter et al., 2018; Goldberg et al., 2018; Hinlo et al., 2017; Kumar et al., 2021; Sanches & Schreier, 2020; Wittwer et al., 2018). Notably, the advent of high-volume filtration methods, such as tow nets (Sepulveda et al., 2019), that use >60 µm mesh to process 1000's of liters of water relies on the capture of eDNA in large particle sizes. These methods have already demonstrated increased detection sensitivity for rare taxa in large study areas. For example, Villacorta-Rath et al. (2021) reported a high eDNA detection frequency of a Critically Endangered rainforest frog 22.8 km downstream from the population when using a large-volume filter unit that could process >1000 L of stream water.

4.2 | Preservation efficiency of Longmire's buffer

Our findings demonstrate that Longmire's buffer is a viable method for preserving eDNA (3:1 of water and buffer) in water samples at ambient tropical temperatures (mean 26.3°C ± 3.0). We not only demonstrated eDNA integrity in whole water samples over a longer study period (i.e., 3 months compared to 56 days; Williams et al. (2016); and 8 weeks; Edmunds and Burrows (2020)), but also showed that samples in Longmire's buffer can be stored frozen upon arrival to the laboratory and extracted three months later without a significant compromise of eDNA yield. Specifically, when samples extracted on arrival at the laboratory (time 0) were considered as the control (i.e., assumed 100% recovery of sawfish eDNA), all other Longmire's-preserved samples stored for 1–3 months at ambient tropical temperature and 3 months at −20°C performed equally or better. A similar result was reported recently by Mauvisseau et al. (2021), who showed that filter preservation in Longmire's buffer for eight months before eDNA extraction yielded significantly more eDNA than extracting eDNA from filters immediately after collection. This builds on a growing body of evidence that endorses long-term, ambient storage (reviewed by Kumar et al., 2020) and thus supports the application of eDNA methods in underrepresented, challenging, or remote environments that require storage versatility, which is arguably the most appealing and valuable use of eDNA.

A possible explanation for the greater yield over time is an increase in cell lysis efficiency of Longmire's buffer over an extended period prior to eDNA extraction. Longmire's buffer relies

on sodium dodecyl sulfate and Ethylenediaminetetraacetic acid to inhibit enzyme activity and sodium chloride and Tris preserve integrity of DNA while lysing cellular components (Longmire et al., 1997). Longmire et al. (1997) originally suggested the use of Longmire's buffer for storing nucleated red blood cells for up to 1 year prior to DNA extraction. Longmire's buffer may even protect eDNA from the destructive effects of freeze-thawing (Takahara et al., 2015), as demonstrated here. As with Renshaw et al. (2014), storage at elevated temperatures may also have facilitated cell lysis.

While this Longmire's buffer preservation experiment was not replicated for eDNA on filter papers, we acknowledge the growing body of literature that demonstrates the utility of Longmire's buffer for long-term, ambient temperature storage of filter papers (Mauvisseau et al., 2021; Renshaw et al., 2014; Spens et al., 2016; Wegleitner et al., 2015). Longmire's buffer was used as the storage solution for filter papers in this study, as it has been previously validated for dwarf sawfish (*Paraponera clavata*) eDNA detection on filter samples collected and stored at ambient field temperatures above 30°C in northern Australia (Cooper et al., 2021).

4.3 | Method sensitivity and efficiency

The performance of eDNA surveys relies on optimal methods for the capture and preservation of target particles. It is evident that capture of eDNA from target species that are rare or in low abundance is especially difficult, so choice of method is important. The results of this study suggest that there are trade-offs between sensitivity and efficiency for different eDNA capture methods. Overall, our model suggests that filtering 1 L of water through a single 0.45 μm filter is more sensitive to detection of target eDNA than filtering 1 L through a 20 μm filter, or precipitating 100 ml of water. This was shown as largetooth sawfish eDNA yields that were on average 6.9 and 3.1 times greater, respectively. Yet, when the volume of water sampled was standardized across methods, which was used as a proxy measure of method efficiency, the precipitation method outperformed both filtration methods. In this case, yields per 100 ml were on average 3.2 and 22 times greater than 0.45 and 20 μm filters, respectively. Existing literature on methods comparison also provide evidence of differences in efficiency and sensitivity, supporting either precipitation (Muha et al., 2019; Piaggio et al., 2013; Raemy & Ursenbacher, 2018), or filtration (Eichmiller et al., 2016; Hinlo et al., 2017; Peixoto et al., 2020), but in context of the study system and species. We suggest that the choice of method is dependent on the characteristics of the water body, namely turbidity or suspended organic material. For example, high turbidity paired with the associated problems introduced by filter clogging may make the precipitation method more attractive.

We qualify the results presented in this study by suggesting that it is generally unlikely that users of the filtration method would filter only 100 ml using a 0.45 μm filter, and even less likely with a 20 μm filter. The volume of water that can be processed in a single filtration event can be several orders of magnitude larger than precipitation (e.g., ≥2000 L; Sepulveda et al. (2019), but typically 0.5–5 L vs. 15–100 ml), making it a more sensitive method and therefore the more popular choice for capture of eDNA. In addition, the ease at which hundreds of filters can be stored and extracted post-collection compared to whole water samples is a major advantage (e.g., for 100 samples; 47 mm disc filters folded in 2 ml microtubes stored in a single 100-well storage box vs. 3 L of water in 100 50 ml falcon tubes as a minimum). In contrast, it is evident that the precipitation method captures both intra- and extra-cellular/organellar eDNA and, because of this, the method can be more efficient than filtration (Minamoto et al., 2015; Muha et al., 2019; Piaggio et al., 2013). Its other major advantages are the portability and low cost of field equipment, simplicity of handling, and reduced chances of contamination in the field, which allows for engaging with non-specialists for sample collection (Villacorta-Rath et al., 2020).

Yet the advantages of both methods are offset by their limitations, which are especially problematic for rare species detection. In the case of filtration, where it is recommended to maximize collection of trace eDNA through use of a small pore size (Minamoto et al., 2015; Turner et al., 2014) or increase filtrate volume (Sepulveda et al., 2019), highly turbid or productive environments will cause rapid filter clogging (Robson et al., 2016). Filtering water in turbid environments could be considered one of the most widespread, yet undesirable methodological challenge (Cooper et al., 2021; Ip et al., 2021; Robson et al., 2016; Sanches & Schreier, 2020), but may be compensated with the use of larger pore size filters (e.g., 1.2–20 μm, as in this study; Barnes et al., 2020), prefiltration (Takahara et al., 2013), or multiple filter replicates (Hunter et al., 2019). The downside of these options would be the increase in cost and time for field and laboratory processing of additional replicates (Sepulveda et al., 2019). In addition, filtration can also concentrate a higher amount of qPCR inhibitors in the samples (Raemy & Ursenbacher, 2018; Sepulveda et al., 2019), therefore, inhibition testing should be routinely applied to confirm that any negative result is not due to qPCR inhibition. Conversely, Williams et al. (2017) suggest that whole water sample collection is the most optimal method for eDNA capture in turbid waters to avoid problematic filter clogging. Yet, whole water precipitation is limited by the collection of smaller water volumes, as the DNA extraction step is limited by centrifuge size, which may be especially undesirable in large rivers and lakes, or the open ocean where eDNA is highly dispersed or diluted. The volume and weight of water samples may be expensive to ship via air freight regionally or internationally and whole water samples must be subsampled for extraction, which increases the cost and time of DNA extraction proportionally. Other options that were not tested here include filtration with a syringe and enclosed filter (e.g., Sterivex filters; Buxton et al., 2018; Spens et al., 2016); however, we recommend pilot studies are carried out to test the suitability of the method for the study system.

It is important to note that the results of the present study are based on eDNA collected from a tank housing the target species

and, therefore, we would expect a much lower concentration of target eDNA in the wild. Given the threatened status of many elasmobranch species, we consider that this study will improve the implementation and interpretation of eDNA surveys and thereby strengthening its usefulness in providing crucial baseline information for management practitioners and researchers.

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CONFLICT OF INTEREST

No conflicts of interest have been declared by the authors.

AUTHOR CONTRIBUTIONS

M.K.C., C.V.R. and D.B. conceived and planned the experiments. M.K.C., L.C. and C.V.R. planned and carried out sample collection with supervision from A.B. and C.H. who collected the animal and managed its husbandry. M.K.C., C.V.R. and L.C. conducted the experiments and laboratory analyses. M.K.C. and C.V.R. conducted the statistical analyses and interpretation of the data. C.A.S., D.R.J. and D.B. contributed to interpretation of the results. M.K.C. and C.V.R. took the lead in writing the manuscript. All authors provided critical feedback and contributed to the final manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are provided in the main text and Supporting Information of this article. The consensus sequence data from Sanger sequenced qPCR amplicons are available in the Supporting Information.

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