METHODS AND SOFTWARE

Development of highly sensitive environmental DNA methods for the detection of Bull Sharks, *Carcharhinus leucas* (Müller and Henle, 1839), using Droplet Digital™ PCR

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Abstract

**Background:** As apex and mesopredators, elasmobranchs play a crucial role in maintaining ecosystem function and balance in marine systems. Elasmobranch populations worldwide are in decline as a result of exploitation via direct and indirect fisheries mortalities and habitat degradation; however, a lack of information on distribution, abundance, and population biology for most species hinders their effective management. Environmental DNA analysis has emerged as a cost-effective and non-invasive technique to fill some of these data gaps, but often requires the development of species-specific methodologies.

**Aims:** Here, we established eDNA methodology appropriate for targeted species detections of Bull Sharks, *Carcharhinus leucas*, in estuarine waters in the northern Gulf of Mexico.

**Materials and Methods:** We compared different QIAGEN® DNeasy® extraction kit protocols and developed a species-specific Droplet Digital™ PCR (ddPCR) assay by designing primers and an internal probe to amplify a 237 base pair portion of the ND2 gene in the mitochondrial genome of *C. leucas*. To validate the developed methods, water samples were collected from known *C. leucas* habitat and from an ex situ closed environment containing a single *C. leucas* individual. The effectiveness of the assay in an open environment was then assessed by placing one *C. leucas* into a flow-through mesocosm system and water samples were collected every 30 min for 3 hr.

**Results:** The developed *C. leucas*-specific assay has the ability to detect target DNA concentrations in a reaction as low as 0.6 copies/µl. DdPCR reactions performed on water samples from known habitat and 30 min after a shark was added to the closed environment contained 1.62 copies/µl and 166.6 copies/µl of target *C. leucas* eDNA, respectively. *Carcharhinus leucas* eDNA was detected in the flow-through system within 30 min, but concentrations remained low and variable throughout the duration of the experiment.

**KEYWORDS**
elasmobranch, Gulf of Mexico, habitat use, mitochondrial genome, threatened species, water sample
1 | INTRODUCTION

Elasmobranchs (sharks, skates, and rays) play a crucial role in marine ecosystems as apex and mesopredators, influencing prey abundance, behavior, and trophic interactions across multiple trophic levels in marine food webs (Ferretti, Worm, Britten, Heithaus, and Lotze 2010; Ritchie et al. 2012). Healthy elasmobranch populations help to maintain ecosystem function, increase biodiversity, and buffer against invasive species and transmission of diseases (Heithaus, Frid, Wirsing, and Worm 2008; Ritchie et al. 2012). However, many elasmobranch populations are in decline as a result of exploitation via direct and indirect fisheries mortalities and habitat degradation (Dulvy et al. 2014). The life history strategies of many elasmobranchs are characterized by late maturity, longevity, and low fecundity, making the recovery of exploited populations a biologically slow process (Garcia et al., 2008; Hoenig and Gruber 1990). According to the International Union for Conservation of Nature (IUCN) Red List of Threatened Species, one-quarter of elasmobranch species are estimated to be threatened with extinction and almost one-half are categorized as Data Deficient, meaning there are insufficient data to properly assess their conservation status (Dulvy et al. 2014). Robust data on species distribution, abundance, biology, and population biology are necessary to enact appropriate conservation strategies for the maintenance of healthy elasmobranch populations; unfortunately, such data are often incomplete or lacking for many species (Dulvy et al. 2014).

Analysis of environmental DNA (eDNA) has recently emerged as an alternative, powerful approach to fill data gaps on the distribution, habitat use, abundance, and population biology of aquatic species (Ficetola, Miaud, Pompanon, and Taberlet 2008), including elasmobranchs (Sigsgaard et al. 2016). All organisms leave traces of DNA in the environment through shedding of cellular debris, skin cells, blood, and biological waste, all of which can be collected in water samples (Rees, Maddison, Middleditch, Patmore, and Gough 2014); however, differences in how organisms shed DNA (i.e., mucus, scales, feces) suggest that eDNA accumulation may differ across species (Le Port, Bakker, Cooper, Huerlimann, and Mariani 2018), requiring taxon-specific research. In targeted species detections, water samples are typically filtered. DNA extractions are performed on the resulting particulate material, and extracted DNA samples are analyzed using a quantitative real-time polymerase chain reaction (qRT-PCR) platform with species-specific primers, developed to amplify a small DNA fragment in the target species (Foote et al. 2012; Taberlet, Coissac, Hajibabaie, and Rieseberg 2012). The collection of water samples is a cost-effective and efficient method of surveying elasmobranch populations when compared to traditional survey methods involving setting nets or lines, which can have high incidence of bycatch and inflict varying degrees of stress to both target and nontarget species (Larson et al. 2017; Lewison, Crowder, Read, and Freeman 2004). Post-release recovery and survival tends to vary widely across species, with some species being particularly sensitive to net capture and handling (Stobutzki, Miller, Heales, and Brewer 2002). With a well-designed sampling scheme, eDNA methodologies offer increased sensitivity for detecting the presence of rare species while negating the need to capture, handle, or even observe the target species (Port et al. 2016; Rees et al. 2014). In elasmobranchs, eDNA methods have been used in targeted species detections for the Critically Endangered Largetooth Sawfish, Pristis pristis (Simpfendorfer et al. 2016), the Endangered Maugean Skate, Zearaja maugean (Weltz et al. 2017), the Vulnerable Chilean Devil Ray, Mobula tarapacana (Gargan et al. 2017), and the Vulnerable Great White Shark, Carcharodon carcharias (Lafferty, Benesh, Mahon, Jerde, and Lowe 2018). Furthermore, eDNA has been used to assess population characteristics in the Endangered Whale shark, Rhincodon typus (Sigsgaard et al. 2016) and to estimate shark diversity in tropical habitats using metabarcoding (Bakker et al. 2017; Boussarie et al. 2018).

Bull Sharks, Carcharhinus leucas (Müller and Henle, 1839), are found in temperate, subtropical, and tropical latitudes globally and are distinctive as one of only a few sharks that can use freshwater for extended periods of time (Thorson 1962; Thorson 1971; Thorson, Cowan, and Watson 1973). As upper trophic level predators, they play a crucial role in maintaining ecosystem health across both marine and freshwater habitats (Every, Pethybridge, Fulton, Kyne, and Crook 2017; Polovina, Abecassis, Howell, and Woodworth 2009; Ritchie et al. 2012). Using acoustic telemetry data to examine the habitat use of C. leucas in northern Gulf of Mexico waters, Drymon et al. (2014) found C. leucas may preferentially select higher-quality, less-urbanized rivers, although a spatially limited acoustic array hindered a full evaluation of this pattern. Targeted eDNA surveys of C. leucas could provide a cost-effective, sensitive method to examine this pattern more widely, as there could be substantial ecological implications of such habitat preference. Here, we establish an eDNA methodology appropriate for targeted species detections of C. leucas in estuarine waters in the northern Gulf of Mexico. Specifically, we compare total eDNA yields for different QIAGEN® DNeasy® DNA extraction kit protocols and develop a species-specific C. leucas eDNA assay using a relatively novel, Bio-Rad® Droplet Digital™ PCR (ddPCR), platform to detect low quantities of target DNA. Finally, we apply these methods to investigate the detectability of C. leucas eDNA in known habitat in the northern Gulf of Mexico and in ex situ closed and flow-through environments containing a single C. leucas individual.

2 | MATERIALS AND METHODS

2.1 | Laboratory controls

Strict laboratory controls were implemented throughout this study to reduce the risk of cross-contamination and contamination by exogenous DNA (see Deiner, Walser, Mächler, and Altermatt 2015; Goldberg et al. 2016). Water processing, DNA extractions, and PCR amplifications were conducted in physically separated laboratory spaces to prevent cross-contamination between stages. Negative controls were incorporated into every stage of sample processing, and PCR was performed on them to check for potential
TABLE 1 Eighteen genetically similar exclusion elasmobranch species found in the Gulf of Mexico

| Common name              | Species name                                      |
|--------------------------|--------------------------------------------------|
| Nurse Shark              | Ginglymostoma cirratum                           |
| Shortfin Mako            | Isurus oxyrinchus                                |
| Dusky Smoothhound        | Mustelus canis                                   |
| Tiger Shark              | Galeocerdo cuvier                                |
| Great Hammerhead         | Sphyrra mokarran                                 |
| Scalloped Hammerhead     | Sphyrra lewini                                   |
| Bonnethead               | Sphyrra tiburo                                   |
| Atlantic Sharppose Shark | Rhizoprionodon terraenovae                       |
| Lemon Shark              | Negaprion brevirostris                           |
| Finetooth Shark          | Carcharhinus isodon                              |
| Blacknose Shark          | Carcharhinus acronotus                           |
| Sandbar Shark            | Carcharhinus plumbeus                            |
| Spinner Shark            | Carcharhinus brevipina                           |
| Dusky Shark              | Carcharhinus obscurus                            |
| Silky Shark              | Carcharhinus falciformis                         |
| Blacktip Shark           | Carcharhinus limbatus                            |
| Cownose Ray              | Rhinoptera bonasus                               |
| Atlantic Stingray        | Hypanus sabina                                   |

Note: These 18 genetically similar exclusion species, and Carcharhinus leucas, were tested for species-specificity of the developed primers and internal probe on the Bio-Rad® QX200™ Droplet Digital™ PCR platform. All tissue samples were collected from the Gulf of Mexico.

2.2 | Water sample collection and filtration

Water samples throughout this study were collected just below the surface of the water in 1 L high-density polyethylene Nalgene® bottles precleaned in a 10% bleach solution and sanitized under ultraviolet (UV) light for 20 min. New gloves were used to collect each water sample and samples were stored on ice in a cooler until filtration using a vacuum pump could take place, which occurred within 24 hr of collection (see Pilliod et al. 2013), except where otherwise noted. Water samples were filtered in a dedicated, precleaned laboratory space that had never had C. leucas tissue or total genomic DNA (gDNA) present. Each 1 L water sample was inverted at least three times to ensure homogenization of particulate matter and was then vacuum-filtered using 47-mm-diameter, 0.8-μm nylon filters, which were replaced when clogging occurred every ~350 ml (e.g., three filters per 1 L) and preserved in 95% ethanol at room temperature, unless noted otherwise (see Appendix S1). During all water filtration, filters were handled with designated sterile forceps for each sample and gloves were changed in between samples to avoid cross-contamination.

2.3 | DNA extraction methods

Due to the wide variety of DNA extraction methods used in eDNA literature (Renshaw, Olds, Jerde, McVeigh, and Lodge 2015), we compared eDNA extraction kits to establish an appropriate method for the nylon filters used to filter water samples in this study. The QIAGEN® DNeasy® Blood & Tissue Kit is a frequent choice for DNA extractions from filters in eDNA studies, but with numerous variations (see Rees et al. 2014). The performance of this kit, using the Goldberg et al. (2011) variation incorporating QIAshredder™ spin columns was compared to that of an extraction kit designed specifically for water samples, the QIAGEN® DNeasy® PowerWater® Kit. The Goldberg et al. (2011) protocol incorporating QIAshredder™ spin columns was selected because in preliminary trials, it yielded higher relative quantities of DNA compared to some other variations (Appendix S2). Additionally, four variations of physical disruption methods to dislodge the particulate matter from the filters prior to digestion were tested with each extraction method: (a) no physical disruption, (b) bead beating, (c) filter scraping, and (d) freezing filters with liquid nitrogen and crushing them using an autoclaved mortar and pestle. The QIAGEN® DNeasy® PowerWater® Kit contained bead beating as part of the standard manufacturer’s protocol, so this step was eliminated for the no physical disruption variation to determine if this step was a critical factor in DNA yields. Three × 1 L water sample replicates were used in each extraction/physical disruption treatment, collected from Mobile Bay, Alabama using the water collection and filtration protocols described. To eliminate the filter preservation step, the filters for each 1 L sample were immediately placed into the appropriate lysis buffers (see Hinlo et al. 2017). The DNA extracts for each 1 L water sample were combined and the DNA qualities were assessed using 2% agarose gel and the relative quantities were measured using Thermo Fisher Scientific NanoDrop™ spectrophotometer technology, with each extract measured four times.

2.4 | Development of a species-specific assay

To develop a species-specific assay, primers and an internal probe were manually designed in conserved regions of the mitochondrial (mtDNA) NADH dehydrogenase 2 (ND2) gene within C. leucas, but variable regions across 23 genetically similar, exclusion elasmobranch species, using sequences available from GenBank and aligned via CodonCode Aligner v. 7.0 (see Appendix S3). Forward (BULLND2F6: 5′-TCCGGGTTTATACCCAAATG-3′) and reverse (BULLND2R5: 5′-GAAGGAGGATGGATAAGATTG-3′) primers were designed first to PCR-amplify a 237 base pair portion of the mtDNA ND2 gene in C. leucas. The primers were first tested using gDNA extracted from five C. leucas individuals from northern Gulf of Mexico waters using conventional PCR. Each PCR consisted of 10 mM Taq buffer, 1.5 mM MgCl2, 0.3 μM of each primer, 0.1 mM dNTPs, 1 U of Taq polymerase, ~25 ng/μl of each DNA extract, and PCR-grade water for a final reaction volume of 25 μl. PCR cycling conditions began with...
initial denaturation at 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 59°C for 30 s, and 72°C for 30 s, final extension at 72°C for 7 min, and a final hold at 4°C. Primers were also tested against one individual of each of 18 other genetically similar, local exclusion species, collected from the Gulf of Mexico (Table 1) to assess specificity. The primers amplified DNA in the target species, C. leucas, but also amplified DNA from some of the nontarget species tested. To increase the species specificity of the assay, an internal PrimeTime® double-quenched ZEN™/IOWA Black™ FQ probe labeled with 6-FAM (BULL_IBFQ: 5'-CAACACTAACTATAAGTCTCAACCCAAATC-3') was designed to amplify the target gene in only C. leucas.

DdPCR reaction mixtures and cycling conditions were optimized for C. leucas by systematically adjusting the concentrations of primers (300–1,000 nM) and internal probe (100–250 nM), cycle number (30–40 cycles), ramp rate (0.5–2.0°C/s), annealing temperature (54–66°C), elongation time (1–2 min), and the amount of gDNA (0.2–25.0 ng/µl). The optimized ddPCR mixture contained 1X Bio-Rad® ddPCR supermix for probes (no deoxyuridine triphosphate (dUTP)), 750 nM of each primer, and 250 nM of probe, and 1.1 µl of extracted DNA, adjusted to a final volume of 22 µl with PCR-grade water. DdPCR droplets were generated for each 22 µl reaction using the Bio-Rad® QX200™ AutoDG™ Droplet Digital™ PCR System (Instrument no. 773BR1456) and thermal cycling conditions were as follows, using a ramp rate of 1°C/s: initial denaturation at 95°C for 10 min, followed by 35 cycles of 94°C for 30 s and 56°C for 2 min, followed by enzyme deactivation at 98°C for 10 min, and a final hold at 4°C. To ensure the optimized assay was species-specific for C. leucas using the ddPCR platform, the primers and probe were tested using these ddPCR reaction and cycling conditions, in replicates of three, with 0.2 ng/µl of gDNA extracted from five C. leucas individuals and one individual of each of 18 other genetically similar, local exclusion species, collected from the Gulf of Mexico (Table 1).

All ddPCR data were analyzed with the Bio-Rad® QX200™ Droplet Reader and QuantaSoft™ software using the Rare Event Detection (RED) analysis, a manual detection threshold set to 3,000 amplitude (the horizontal pink line) using the QuantaSoft™ Rare Event Detection analysis. Event number refers to the number of droplet events generated for a given well or sample; Ch 1 amplitude measurement refers to the level of fluorescence emitted by a droplet event; and each column is a single well.

2.5 | Collection of positive water samples

Carcharhinus leucas eDNA samples were obtained via the collection of water samples from known C. leucas habitat and ex situ experiments. These experiments were conducted in accordance with the laws of the state of Alabama and under the IACUC protocols (IACUC Protocol Number 974304). All measures were taken to reduce the pain or stress the animal underwent during testing; therefore, the water used in the ex situ experiments were from natural shark habitat. Water was collected from the coastal waters of Mobile Bay, Alabama, known C. leucas habitat, in April 2017 and placed into a precleaned, circular fiberglass, closed-system tank (~120 cm wide and held a volume of ~711 L), and six × 1 L water samples were immediately collected from this tank to determine whether target eDNA was present in the ambient water. A bubbler was added to the tank to keep the system oxygenated and one wild-caught juvenile male C. leucas, ~930 mm total length, was added to the tank. To acquire a confirmed positive C. leucas eDNA sample, after 30 min, six × 1 L water samples were again collected from the tank. These water samples were used in aspects of method development (see Appendix S1) and to validate the developed genetic assay.

To test the effectiveness of the developed C. leucas assay in an open system with a single target species present, a flow-through mesocosm (~365 cm wide containing a volume of ~14,500 L) at Dauphin Island Sea Lab, Alabama was maintained in April 2017. The flow rate of the mesocosm was designed to mimic flow in a coastal system at ~30 cm³/hr, with complete system turnover at approximately 2 hr. One wild-caught juvenile male C. leucas, ~930 mm total length, was introduced to this system and five ×
1 L water samples were collected immediately (time 0.0), spanning the diameter of the mesocosm; this sampling regime was repeated every 0.5 hr for 3 hr, allowing for complete turnover of the system. Water samples were stored in a −20°C freezer for 1 month, due to laboratory equipment constraints, similar to Bakker et al. (2017) and Gargan et al. (2017), and were thawed at room temperature prior to filtration.

Water samples from these experiments were vacuum-filtered using 47-mm-diameter nylon 0.8-μm filters (three per 1 L), which were preserved in 95% ethanol at room temperature (Appendix S1) and DNA extractions followed the Goldberg et al. (2011) protocol incorporating the QIAshredder™ spin columns (Appendix S2). DdPCR amplifications were carried out in replicates of five, using the optimized C. leucas assay previously described in this study. All ddPCR reactions were set up using aerosol barrier filter pipette tips and designated pipettes, separate from those used in setting up PCR reactions, were used to add eDNA extracts to the reactions. DdPCR results were analyzed using the Bio-Rad QX200™ Droplet Reader and QuantaSoft™ RED analysis, a manual detection threshold of 3,000 amplitude, and the LoD.

3 | RESULTS

3.1 | Optimal eDNA methods

The Goldberg et al. (2011) protocol using the QIAGEN® DNeasy® Blood & Tissue Kit and QIAshredder™ spin columns yielded higher relative quantities of total eDNA from filters compared to the QIAGEN® DNeasy® PowerWater® Kit protocol, across all variations in physical disruption methods (Figure 2). The DNA yields from the four physical disruption methods used with the Goldberg et al. (2011) protocol were similar: No physical disruption yielded a total DNA average of 61.19 ng/μl (SE = 1.65), bead beating the filters yielded 56.83 ng/μl (SE = 6.75), filter scraping yielded 56.78 ng/μl (SE = 1.77), and freezing filters with liquid nitrogen and crushing yielded 64.93 ng/μl (SE = 2.36) (Figure 2). Since the total DNA yields were similar across these methods and because the addition of a physical disruption step is time-consuming and allows for an additional opportunity for contamination by exogenous DNA, we determined the optimal DNA extraction method for our purposes to be the Goldberg et al. (2011) protocol with no physical disruption method.

The combination of primers and probe designed in this study were demonstrated to be species-specific for C. leucas in our study area by successfully amplifying target DNA in all ddPCR replicates for the five C. leucas individuals and not amplifying DNA in any of the ddPCR replicates of the 18 local exclusion species or PCR negative controls. The LoD, as determined using the Bio-Rad QX200™ Droplet Reader and QuantaSoft™, was the 1:10,000 dilution, corresponding to 2.5 pg of target DNA in the reaction (Figure 3). There were several positive droplets present above the manual threshold in the 1:10,000 dilutions, and the standard errors did not include zero or overlap with those of the 1:100,000 dilutions. In contrast, there were no positive droplets in the 1:100,000 dilutions and the standard errors overlapped with zero, suggesting C. leucas DNA could not be reliably detected at this dilution (Figure 3). Using the number of copies of target DNA/μl in the 1:10,000 dilutions and applying the lower standard error as the relaxed detection threshold for each of the two individuals (see Baker et al. 2018), the average LoD threshold was determined to be 0.6 copies/μl in a reaction.

3.2 | Analysis of water samples

Using the developed ddPCR assay and the QuantaSoft™ RED analysis with a manual detection threshold of 3,000 amplitude, an average of 1.62 copies/μl (SE = 0.12) of C. leucas DNA was detectable in the ddPCR reactions from water samples collected from known habitat, Mobile Bay, without visually confirming the presence of C. leucas (Figure 4). In the ex situ positive eDNA experiment, 30 min after a C. leucas was added to the closed tank containing this water, large amounts of target eDNA were present, with an average concentration of 166.6 copies/μl (SE = 3.01) in the ddPCR reactions (Figure 4). In the flow-through mesocosm experiment, when applying a lower LoD of 0.6 copies/μl to the data analysis, target C. leucas DNA was not detectable in any of the ddPCR replicates at time 0.0 but was detectable in all ddPCR replicates 0.5 hr after the shark was added (Figure 5). Average target eDNA concentration peaked by 1.0 hr, with an average of 5.8 copies/μl (SE = 0.27) across all ddPCR replicates, and then declined over the next hour (Figure 5). By 2.0 hr, the average concentration of C. leucas eDNA dipped below the LoD, with positive detections in only two of the five ddPCR replicates for this sample (Figure 5). There was a second, smaller spike in C. leucas eDNA by 2.5 hr, that again decreased, but the average concentration...
The use of eDNA as a tool to study the distribution and ecology of marine species has increased substantially in recent years (Bakker et al. 2017; Foote et al. 2012; Lafferty et al. 2018; Port et al. 2016). However, careful consideration and optimization of the methods employed in such studies are necessary, ultimately allowing for an appropriate interpretation of the results. Here, we found filtering water with nylon 0.8-μm filters, preserving the filters in 95% ethanol (Appendix S1), and then performing DNA extractions using the Goldberg et al. (2011) protocol with the QIAGEN® DNeasy® Blood & Tissue Kit and QIAshredder™ spin columns to be an appropriate method of isolating total eDNA from water collected from the northern Gulf of Mexico. Although the number of replicates in the experiment was small, the Goldberg et al. (2011) protocol was found to outperform the PowerWater® kit across all four physical disruption methods, despite the latter being specifically designed and marketed for eDNA extractions from water samples, and at a higher cost. The total DNA yields used to evaluate the performances of these extraction methods are unlikely to be accurate in an absolute sense due to the inability of NanoDrop™ spectrophotometer technology to decipher DNA from other possible biological macromolecules, but the relative differences between DNA yields were substantial. The combination of primers and internal probe for the mtDNA ND2 gene designed in this study are optimized for *C. leucas* in the estuaries in the northern Gulf of Mexico; however, whether they are appropriate (e.g., species-specific) for use in other geographic regions, such as northern Australia, or in fully marine waters, where there may be additional species of closely related carcharhinids present, requires further testing. The LoD determined in this study shows the sensitivity and detection capability of the developed assay and was demonstrated to be sufficient for *C. leucas* eDNA detection in Mobile Bay and in ex situ positive samples. However, the LoD may require further refinement through additional dilution series between the 1:10,000 and 1:100,000 dilutions before being used in data analysis for large numbers of field samples. Furthermore, due to potential differences across ddPCR machines, we recommend the LoD to be refined independently for each machine, using the LoD here as a starting reference point for this assay.

The ability of ddPCR to detect low concentrations of target DNA, for example, 2.5 pg of *C. leucas* DNA in this study, means this platform may be less likely to produce false negatives when used alongside an appropriate sampling regime and water processing methods (e.g., spatial and depth coverage, volume collected, filter pore size). False negatives can occur when target DNA is captured in water samples but is not detected due to limitations of the genetic assays employed (Darling and Mahon 2011; Ficetola et al. 2015; Goldberg et al. 2016; Lahoz-Monfort, Guillaumet-Arroita, and Tingley 2016). To date, the majority of studies that use eDNA in targeted species detections have used qRT-PCR, but the detection capabilities of this platform may be limited, when compared to those of ddPCR (Doi, Takahara, et al. 2015; Doi, Uchii, et al., 2015).
difference in detection abilities between the two PCR platforms is likely due to fundamental differences in how they quantify target DNA. DdPCR quantifies the starting DNA copy number present in a sample using end-point PCR without reference to a standard (absolute quantification) (Whale et al. 2012), making it more sensitive and precise assay, ideal for eDNA applications targeting a single target species. Additionally, the RED analysis setting using the Bio-Rad QuantaSoft™ software is designed to identify low copy numbers of target DNA in a background largely composed of nontarget DNA copies (Bio-Rad Droplet Digital™ PCR Applications Guide). Given the ability of ddPCR to detect such low quantities of DNA, it may replace qRT-PCR in eDNA research (Doi, Uchii, et al., 2015; Nathan, Simmons, Wegleitner, Jerde, and Mahon 2014) assessing the distribution, habitat use, and abundance of species found in low abundance and/or are of conservation concern (Baker et al. 2018; Hunter et al. 2018; Tréguier et al. 2014), including elasmobranchs (Bohmann et al. 2014; Lafferty et al. 2018). However, we caution that the ability to detect such low quantities of DNA also increases the potential for false positives (Goldberg et al. 2016; Huggett, Cowen, and Foy 2015). All eDNA studies, but especially those using ddPCR, require strict field and laboratory controls and procedures be in place to reduce the potential for false positives, typically the result of contamination by exogenous DNA or cross-contamination of samples (see Ficetola, Taberlet, and Coissac 2016). In addition to the contamination controls described by Goldberg et al. (2016), Deiner et al. (2015), and Port et al. (2016), when using ddPCR, we also suggest: (a) using two cleaning methods for decontamination of all field and water filtration equipment (e.g., a bleach wash, plus autoclaving, and/or UV light exposure), (b) that water filtration is conducted in a laboratory space that has never had tissue or gDNA from the target species present, (c) that gloves and any tools are changed in between samples during water filtration (see Pilliod, Goldberg, Arkle, and Waits 2013), (d) that negatives be incorporated into field collection, water filtration, DNA extraction, and PCR, with each negative run through to PCR (see Bakker et al. 2017; Jerde, Mahon, Chadderton, and Lodge 2011), (e) that a designated pipette, separate from that used to set up reactions, be used to add DNA extracts to ddPCR reactions, and (f) that multiple replicates for each sample are run during ddPCR (see Rees et al. 2014). Strict field and laboratory controls will ensure the authenticity and reliability of eDNA results, which is increasingly critical in eDNA research using highly sensitive technologies, such as ddPCR, especially when the results of such studies will be used to inform conservation and management initiatives (Hunter et al. 2017; Hunter et al. 2018).
Fundamental research on the accumulation, persistence, and degradation of elasmobranch eDNA is necessary to improve the interpretation of results in eDNA field research. Here, we have shown that after adding a shark into closed and flow-through systems, target eDNA was detectable within 30 min. In the flow-through system, the initial spike in target eDNA that occurred between 0.5 and 1.0 hr could be due to initial stress experienced by the shark after being added to the mesocosm, causing it to expel more DNA (e.g., Barnes et al. 2014). The overall decrease in target eDNA between 1.0 and 2.0 hr may be the result of the shark acclimating to the environment and releasing less DNA or turnover of water in the mesocosm if the shark is releasing DNA into the system in pulses rather than continuously; however, this has not been explicitly explored in elasmobranches. The inability to detect C. leucas DNA in some of the ddPCR replicates at 2.0 and 3.0 hr, despite the confirmed presence of a shark and the use of a highly sensitive ddPCR assay, suggests there may have been very little C. leucas DNA present at those times, which could occur if DNA was shed in pulses, and then flowed out of the mesocosm. However, this pattern could also be indicative of sampling error, where C. leucas DNA was present, but not captured, highlighting the need for careful consideration of sampling regime as well as the interpretation of the results of eDNA studies. Because mesocosm water samples were frozen after collection, it cannot be completely ruled out that the eDNA degraded prior to filtration (Hinlo et al. 2017; Takahara, Minamoto, and Doi 2015); however, the concentrations of the total eDNA extracts from these samples were not unusually low compared to the other eDNA extracts analyzed for this study. Furthermore, other eDNA studies have frozen water samples prior to filtration without apparent negative effects (Bakker et al. 2017; Gargan et al. 2017) making it unlikely to be the sole explanation for the observed patterns of C. leucas DNA detected in this experiment. Ideally, these experiments should have been replicated and included a second tank without a shark as a negative control, with water samples immediately after collection; however, due to limited facilities and the constraints of using live animals, these improvements to the study design were not feasible. Regardless, this is the first elasmobranch eDNA study that has placed a single target animal into closed and then open, flow-through systems to quantify target eDNA from a single animal over time, creating a baseline for future ex situ research. In comparison, other eDNA studies of elasmobranches have acquired positive eDNA samples by collecting water samples from aquaria with the target species present (e.g., Simpfendorfer et al. 2016) or collecting water samples from known habitats, but without visually confirming the presence of the target species (e.g., Weltz et al. 2017). Future studies should assess DNA accumulation over different timescales than presented here, as well as how altered flow rates, water conditions (pH, temperature), weather conditions (photoperiod, cloud cover), and number and size of target species impact the accumulation and persistence of elasmobranch eDNA in marine systems.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

AUTHOR CONTRIBUTIONS

All authors contributed to the conception and design of the study, the acquisition and the interpretation of the data, and writing the manuscript.

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DATA AVAILABILITY STATEMENT

The datasets generated during and/or analyzed during the current study are available from N.M. Phillips on reasonable request.

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**SUPPORTING INFORMATION**

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

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