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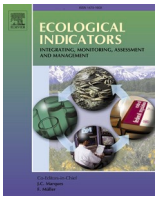

First detection of critically endangered scalloped hammerhead sharks (*Sphyrna lewini*) in Guam, Micronesia, in five decades using environmental DNA

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ABSTRACT

Among the hammerhead sharks, scalloped hammerheads (*Sphyrna lewini*) have undergone the steepest population declines worldwide. Due to their high susceptibility to exploitation, the species is now classified as 'critically endangered', the most threatened category listed by the International Union for Conservation of Nature. There is an urgent need for data on the distribution of *S. lewini* to inform the design and implementation of effective conservation management strategies, and mitigate the risk of global extinction. Environmental DNA (eDNA) is emerging as a powerful method to monitor the geographic distribution, population trends, and habitat usage of rare and endangered species. In comparison to traditional survey methods, eDNA methods offer lower cost, higher detection rates, and are non-invasive. At present, there is no targeted eDNA assay for the detection of *S. lewini* and existing methods to assess their distribution are either fisheries-dependent, leading to bias, or costly and laborious, leading to impracticality in regions of low or unknown abundance. Here we present an optimised workflow for the detection of *S. lewini* presence using eDNA methods, and apply these to successfully detect scalloped hammerhead sharks in Guam, of the western Pacific Ocean, where their presence has not been scientifically reported since the 1970s. The detection of *S. lewini* by eDNA survey methods was achieved from a single-day sampling effort, demonstrating the efficacy of the technique and workflow. If implemented, the eDNA survey methods developed here will enable the rapid generation of information on the distribution of scalloped hammerhead sharks in the western Pacific, and likely globally, and assist in the accurate placement of no-take reserves to best enable the species' recovery.

1. Introduction

Collectively, sharks, rays, and chimeras (class Chondrichthyes) are at substantially greater extinction risk than almost all other vertebrate lineages (Dulvy et al., 2014). According to indicators established by the United Nations and the Convention on Biological Diversity, oceanic sharks have undergone the most rapid rate of decline, leading to three-quarters of species in this far-ranging group now threatened with extinction (Pacoureau et al., 2021). Although habitat loss and degradation are prominent threats, overfishing (both targeted and bycatch) is the primary driver of chondrichthyan population declines, with many larger species targeted specifically for the shark fin trade (Dulvy et al., 2014; Pacoureau et al., 2021). Healthy chondrichthyan populations help to maintain ecosystem function, increase biodiversity, as well as buffer against invasive species and transmission of diseases (Heithaus et al., 2008; Ritchie et al., 2012). As such, there is an urgent call for changes in the implementation and enforcement of conservation and management initiatives in order to better protect and restore

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chondrichthyan populations and, in turn, strengthen the ecosystems they support (Dulvy et al., 2014; Pacoureau et al., 2021).

Effective marine protected areas can result in fourteen times more shark biomass in comparison to fished areas, and allow for exponential increases in shark biomass in isolated regions (Edgar et al., 2014). This spatial approach to conservation requires an understanding of the distribution, habitat and population trends of species, yet approximately half of all chondrichthyans are classified as 'data deficient' according to International Union for Conservation of Nature (IUCN) criteria (Dulvy et al., 2014). This substantial gap in knowledge may be a direct result of the rarity of sharks in many areas (MacNeil et al., 2020; Roff et al., 2018). Low levels of abundance lead to inefficacy of standard sampling techniques, such as visual observations, and can render labour intensive methods, such as tagging, logistically and economically ineffective (Postaire et al., 2020; Robbins et al., 2012). Furthermore, methods requiring manual handling are associated with increased stress and incidental mortalities, which can exacerbate issues of low abundance and therefore need to be applied cautiously (Drymon and Wells, 2017; Heupel et al., 2020; Marshall et al., 2015). To overcome these obstacles, there is a need to develop highly sensitive and non-invasive methods that can assess species distribution, best inform the placement and management of marine protected areas and, ultimately conserve these ecologically important species

Environmental DNA (eDNA) monitoring is an emerging and powerful method to assess the geographic distribution, population trends, and habitat use of many aquatic species (e.g., Lacoursière-Roussel et al., 2016; Sigsgaard et al., 2017; Stewart et al., 2017; Thalinger et al., 2019). Environmental DNA methods rely on the principle that all animals deposit DNA into the environment they inhabit via excretion and shedding (reviewed by Thomsen and Willerslev, 2015). Species-specific genetic information can be recovered from environmental samples such as seawater and, importantly, sample collection for use with eDNA methods is non-invasive and does not harm the target species or its environment (see below for examples in chondrichthyans). For this reason, the method has garnered specific interest in the field of conservation biology (Barnes and Turner, 2016; Cristescu and Hebert, 2018; Thomsen and Willerslev, 2015). In chondrichthyans, targeted eDNA assays have been developed for eight species: largetooth sawfish (*Pristis pristis*; Simpfendorfer et al., 2016), smalltooth sawfish (*Pristis pectinata*; Lehman et al., 2020), Chilean devil rays (*Mobula tarapacana*; Gargan et al., 2017), maugean skates (Zearaja maugeana; Weltz et al., 2017), whale sharks (*Rhincodon typus*; Sigsgaard et al., 2017), white sharks (*Carcharodon carcharias*; Lafferty et al., 2018), bull sharks (*Carcharhinus leucas;* Schweiss et al., 2020), and blacktip sharks (*Carcharhinus limbatus*; Postaire et al., 2020). In maugean skates, *Z. maugeana*, eDNA in seawater samples collected from field sites was shown to decay beyond the limit of detection within as little as four hours, indicating that eDNA detections provide a temporally relevant indicator of species presence (Weltz et al., 2017). Furthermore, in *C. limbatus*, targeted eDNA survey methods yield results equivalent to extensive fishing surveys and acoustic telemetry, with markedly lower sampling effort and reduced cost (Postaire et al., 2020). These examples demonstrate that eDNA surveys offer a highly suitable and effective method to assess the distribution of rare and threatened chondrichthyan species.

Creation and validation of an effective eDNA monitoring survey involves the careful design of a highly sensitive and specific assay, followed by three validation stages including in silico, in vitro, and in situ testing (Goldberg et al., 2016). Quantitative PCR (qPCR) using species-specific primers offers a highly sensitive method of eDNA detection, and with the addition of a TaqMan (Applied Biosystems, California, United States) Minor Groove Binding (MGB) probe, has been demonstrated as effective in detecting a single copy of target eDNA isolated from water samples (Wilcox et al., 2013). The design of primer and probe sequences for these assays requires adequate assessment of sequence variability in order to define a region conserved within the target species, but variable between the target and co-occurring species. Once the assay is designed, it can be tested in silico using a curated database generated from publicly available sequences, but in many cases will require the generation of additional sequences due to lack of, or incomplete, data for many species (Kwong et al., 2012). Once the sequence database is created, alignments of target, closely related and co-occurring species should be carried out (e.g., Takahara et al., 2012). In vitro tests may then be performed using tissue-derived and/or synthetic DNA to demonstrate both specificity and sensitivity of the assay to the target species alone, likely requiring testing of multiple primer-probe combinations (e.g., Wilcox et al., 2013). Finally, once the assay is designed it can then be tested in situ (field) and/or ex situ (tank experiments or other aquaria) to demonstrate the assay's efficacy in detecting target species' eDNA (e.g., Schweiss et al., 2020). Successful amplification of the target fragment is confirmed through sequencing of the amplified qPCR products (Goldberg et al., 2016). Execution of each of these design and validation steps is essential to the generation of a highly sensitive and species-specific eDNA assay.

In addition to the development of a highly sensitive and specific assay, sample collection and DNA capture methods must be optimized to obtain high quantity and purity eDNA. Field collection methods for the concentration of eDNA from water most commonly involve precipitation or filtration, with the latter being the method of choice for the identification of chondrichthyans thus far (Le Port et al., 2018 and references therein). In comparison to precipitation methods, filtration methods offer the ability to process large volumes of water (e.g., 20 L; Simpfendorfer et al., 2016), can be carried out on-site thus reducing potential eDNA degradation (e.g., Weltz et al., 2017) and can result in higher detection rates (Deiner et al., 2015). While filter pore size may be dictated by environmental variables (e.g., turbidity; Robson et al., 2016), eDNA can be successfully recovered at pore sizes from 0.2 to 20, and up to 180 μm, depending on the aquatic environment (Turner et al., 2014). The recovery of eDNA captured on the filter is also an important consideration, and will vary depending on the method of eDNA extraction. While QIAGEN DNeasy Blood and Tissue Kit (QIAGEN, Hilden, Germany) has been utilised by many studies, and has been shown to outperform other column-based methods (e.g., Goldberg et al., 2011), precipitation methods of DNA extraction yield greater DNA overall (Edmunds and Burrows, 2020; Deiner et al., 2015). DNA isolated from aquatic environments commonly contains substances that inhibit qPCR reactions, such as humic, phytic and tannic acids or, in high concentrations, non-target eDNA may become inhibitory (Lance and Guan, 2020). Inhibition can be measured by shifts in cycle thresholds or controlled for with the use of commercially available internal positive controls or secondary primers targeting endogenous eDNA (Cao et al., 2012; Furlan and Gleeson, 2017). Inhibitors, if detected, can be overcome by purification with silica or gel extraction columns (Lloyd et al., 2010), the dilution of the eDNA extracts (McKee et al., 2015) and/or the use of effective inhibitor resistant qPCR master mixes (Cao et al., 2012; Lance and Guan, 2020). Optimisation of these methods enables the attainment of high quantity, high purity eDNA, thereby increasing the probability of target eDNA capture, reducing potential qPCR inhibition and, ultimately, allows for increased accuracy in the detection of the target species.

Hammerhead sharks (family Sphyrnidae) are among the most vulnerable of all chondrichthyan groups to extinction, particularly the larger bodied scalloped, smooth (*Sphyrna zygaena*) and great (*Sphyrna mokarran*) hammerheads (Gallagher and Klimley, 2018). Not only are their fins highly valued in the global shark fin trade (Abercrombie et al., 2005; Clarke et al., 2006), their characteristic cephalofoil and unique feeding behaviour dictates that individuals are especially vulnerable to longline bycatch (Camhi et al., 2009; Gulak et al., 2015) and their sensitivity to capture stress leads to high susceptibility to at-vessel and post-release mortality (Morgan and Carlson, 2010). Scalloped hammerheads have suffered especially steep population declines over at least the last half-century; the third steepest of all oceanic sharks assessed (Pacoureau et al., 2021). Accordingly, the species was the first

shark to be protected by the U.S. Endangered Species Act in 2014 and has recently been reassessed by the IUCN as 'critically endangered' (Rigby et al., 2019). Evidence of population structure (Pinhal et al., 2020 and references therein) and limited *trans*-oceanic movements indicates that, despite their global distribution (Compagno, 1988), management decisions for *S. lewini* should be population-specific (Duncan et al., 2006). The most recent global phylogeographic genetic analysis for *S. lewini* proposes a centre of evolutionary origin in the Indo-Pacific with some population differentiation evident, particularly at the ends of the species range in the Gulf of Mexico and the Tropical East Pacific, which are separated by the Isthmus of Panama (Duncan et al., 2006; Daly-Engel et al., 2012). Management decisions are further complicated by the complex spatial organisation of *S. lewini* within populations (Coiraton et al., 2020). While adult females exhibit strong philopatry, males demonstrate the ability to migrate across oceanic expanses, increasing population connectivity and gene flow (Daly-Engel et al., 2012). Young scalloped hammerheads are known to aggregate in shallow coastal waters, utilising them as important nursery habitats for extended periods (Duncan and Holland, 2006). Nursery habitats for *S. lewini* these have been identified in Mexico (Bejarano-Álvarez et al., 2011; Rosende Pereiro and Corgos, 2018), Fiji (Brown et al., 2016), Colombia (Quintanilla et al., 2015), Hawaii (Duncan and Holland, 2006), southern Africa (Diemer et al., 2011), and Australia (Simpfendorfer and Milward, 1993; Tobin et al., 2014). Substantial anecdotal evidence suggests that there may be an additional nursery area for *S. lewini* in Guam, Micronesia (Department of the Navy, 2005; NMFS, 2015; Smith et al., 2010). The identification of nursery areas is becoming an essential component of conservation and management plans for shark species as these areas provide valuable refuges from predation (Heupel et al., 2007; Kinney and Simpfendorfer, 2009).

Despite their critically endangered status, complex spatial organisation and resultant difficulty detecting through traditional survey methods, no targeted eDNA assay exists to assess the distribution of *S. lewini*. In this study, we present the development, optimisation, and validation of a non-invasive assay to detect the presence of scalloped hammerhead sharks from filtered seawater. The techniques are applied in Guam to provide scientific evidence in support of anecdotal evidence of their presence in the area, where extensive alternative survey methods have been unable to record their presence for five decades. These results, combined with further implementation of the assay, will assist in the rapid generation of data urgently required to inform management decisions to assist the species' recovery.

2. Materials and methods

2.1. Assay design

To enable in silico assay design and specificity testing, a reference sequence database was built from publicly available (National Centre for Biotechnology Information; NCBI; Sayers et al., 2019) mitochondrial DNA (mtDNA) sequences from the target species, *S. lewini,* as well as 27 co-occurring shark and ray species (based on Myers and Donaldson, 2003), eight additional sphyrnid species and two later-determined high sequence similarity species (collectively referred to as exclusion species herein; listed in Table A1). To supplement available mtDNA sequence data, tissue samples from the target species and two additional sphyrnid species known to occur in the Pacific Ocean were obtained from multiple sources and locations across the western Pacific. These included 21 scalloped hammerheads, nine great hammerheads (*S. mokarran*), and seven winghead sharks. Genomic DNA (gDNA) was extracted from tissue samples using the CTAB method (Doyle and Doyle, 1990) including overnight digestion with proteinase K. Partial 12S ribosomal RNA (12S), 16S ribosomal RNA (16S), cytochrome *c* oxidase subunit I (COI), and NADH dehydrogenase 2 (ND2) sequence fragments were amplified by end-point PCR using MyTaq Red Mix (Bioline, London, UK) following the manufacturer's instructions. Primers and annealing temperatures are listed in Table A2. Amplified products were sent to the Australian Genome Research Facility (AGRF Pty Ltd, Brisbane, Australia) for purification and bidirectional Sanger sequencing. Forward and reverse sequence reads were imported into Geneious v10.2.6 (http://www.geneious.com), trimmed to remove low quality bases, pairwise aligned and consensus sequences were submitted to NCBI GenBank (http://www.ncbi.nlm.nih.gov/genbank/) under accession numbers MT881496 – 881543; MT883243 – 883276; MT883461 – 883,491 and MT883961 – 883995.

To determine the most suitable region for primer probe placement, all publicly available sphyrnid sequences and representative carcharhinid, the oceanic whitetip shark (*Carcharhinus longimanus;* KM434158), were aligned in Geneious using MUSCLE with 10 iterations (Edgar, 2004) and trees assembled using IQ-TREE v1.6.12 (Nguyen et al., 2015) using the default settings including ultrafast bootstrap analysis (Hoang et al., 2018) and, for COI, an increased number of iterations (n = 5000). Based on adequate interspecific sequence divergence and intraspecific sequence similarity (Fig. A1), the 12S and 16S rRNA sequences were selected for primer and probe design. To determine the best placement for primer and probe sequences within 12S and 16S rRNA fragments, exclusion species sequences were mapped to the *S. lewini* 12S reference sequence JX827259 using Geneious in-built read mapper with the default settings. The resulting alignments were visually inspected to identify regions with the greatest number of mismatches between *S. lewini* and exclusion species sequences for 3′ primer regions, and throughout the length of the probe. Multiple iterations of primer and probe design were performed with assistance from a Technical Application Specialist (Thermo Fisher Scientific Australia, Victoria, Australia) which were later subject to laboratory sensitivity and specificity testing (see below). The primer-probe combination that resulted is situated at the 3′ end of the *S. lewini* 12S rRNA gene, from base pair (bp) position 781 to 897 (117 bp total fragment length).

2.2. Assay specificity testing

To allow for specificity testing, gDNA was extracted as described above from twelve exclusion species for which tissue samples were available (Table A1) and highly degraded fragments were removed using Sera-Mag SpeedBeads (GE Healthcare; Chicago, Illinois, United States) prepared following the method developed in Rohland and Reich (2012). Total dsDNA concentration was quantified using QuantiFluor (Promega; Madison, Wisconsin, United States) fluorometric nucleic acid quantitation and measured on a Quantus Fluorometer (Promega; Madison, Wisconsin, United States). For use as qPCR controls, double-stranded gBlocks™ Gene Fragments (Integrated DNA Technologies; Coralville, Iowa, United States) were designed and synthesised for scalloped hammerhead, great hammerhead, smooth hammerhead (also occurs in the Pacific, no tissue samples available) and oceanic whitetip shark (co-occurring in Guam, high sequence similarity but no tissue available). Each of the gBlocks fragments were designed to be approximately 200 bp in length and include a 7 bp reverse complemented region, allowing for the preclusion of within-plate qPCR contamination that would not be possible with tissue-derived gDNA (Table A3).

The calculated nearest neighbour melting temperatures were 65 °C and 61 °C for the forward and reverse primer, respectively (Table A4). To determine the maximum annealing temperature that reliably produced a PCR product and allowed for the highest specificity, a gradient primer-only endpoint PCR from 55 to 70 °C was carried out, followed by a primer-probe qPCR using 62 °C, 63 °C and 64 °C. Specificity testing for annealing temperature was tested against *S. lewini*, *S. mokarran*, *S. zygaena*, and *C. longimanus* gBlocks fragments at an estimated 10 000 copies per reaction. Apart from the annealing temperature, thermal cycling parameters followed those recommended for TaqMan Environmental Master Mix 2.0 (EMM; Life Technologies; Carlsbad, California, US); 95 °C for 10 min followed by 60 cycles of 95 °C for 15 sec, 62 °C for 1 min. To determine the minimum primer concentration that reliably

produced a product and allowed for the highest specificity, concentrations between 300 and 800 nM at 100 nM increments were tested. Specificity testing for primer concentration was performed against gDNA from the 12 exclusion species previously mentioned at 1 and/or 0.1 ng/µl, as well as gBlocks fragments for *C. longimanus* and *S. zygaena* at an estimated similar concentration of 10 000 and 1000 copies per µl. Standard curves using 10-fold serial dilutions of the *S. lewini* gBlocks fragment (from 10 000 to 1 copy/reaction) and *S. lewini* gDNA (from 10 to 1×10^{-4} ng/ul) were analysed using the *S. lewini* 12S TaqMan assay and Ct values from each dilution were compared to ensure similar properties.

2.3. Assay sensitivity testing

To determine the ability of the assay to detect *S. lewini* eDNA at low copy numbers, as would be expected in the field, limit of detection (LOD) was measured by creating a 12-point standard curve from the *S. lewini* 12S gBlocks fragments, using a two-fold serial dilution starting at a theoretical 125 copies per reaction (25 copies per µl) and ending at 0.06 copies per reaction (0.01 copies per µl). Eight qPCR replicates were run for each standard. Data from the replicate standard curves were analysed in RStudio (v1.2.5042) running R (v4.0.0) using the Generic qPCR Limit of Detection (LOD) / Limit of Quantification (LOQ) calculator R Script (Klymus et al., 2020; Merkes et al., 2019). A three-parameter logistic function with an upper limit of one was selected as the best fitting model [lack of fit test: $F(93, 9) = 0.21$, $p = 0.99$)] and was used to determine the effective LOD for each quantity of PCR replicate.

2.4. Field sample collection methods

Field eDNA samples were collected from fives sites within the Apra Harbor and Orote Peninsula nearshore waters of Guam, Mariana Archipelago, including 1) Inner Harbor, 2) Sasa Bay (a marine preserve), 3) Middle Shoals, 4) Orote Point, and 5) Blue Hole (Fig. 1). These sites were selected based on anecdotal observations of *S. lewini*, and were accessed by boat during three sampling efforts occurring throughout March-April 2018, November 2018, and February-March 2019. Each sampling effort consisted of a single visit of approximately one hour or less to each of the five sites, throughout which at least ten replicate samples were collected as described below.

Prior to each field trip departure, field equipment and storage bins were decontaminated with 10% *v/v* bleach (active ingredient: Sodium Dichloroisocyanurate Dihydrate) for at least 20 min before thoroughly rinsing with reverse osmosis (RO) water. Filter cartridges, containers used to hold Ultrapure water for field blanks, forceps, and scissors were bleach decontaminated for a further 40 min then ultraviolet (UV) sterilised in a DNA-free PCR cabinet. Screw-top microcentrifuge tubes (2 mL; Sarstedt, Germany) were UV sterilised and filled with 1.5 mL DNA-free Longmire's solution (100 mM Tris pH 8, 100 mM EDTA pH 8, 10 mM NaCl, 0.5% SDS; Longmire et al., 1997) or non-denatured 96% ethanol solution. Reusable items (e.g., filter cartridges, forceps, and scissors) were used only once per field trip. Between sampling sites, the pump and tubing were wiped with 10% *v/v* bleach and RO water and

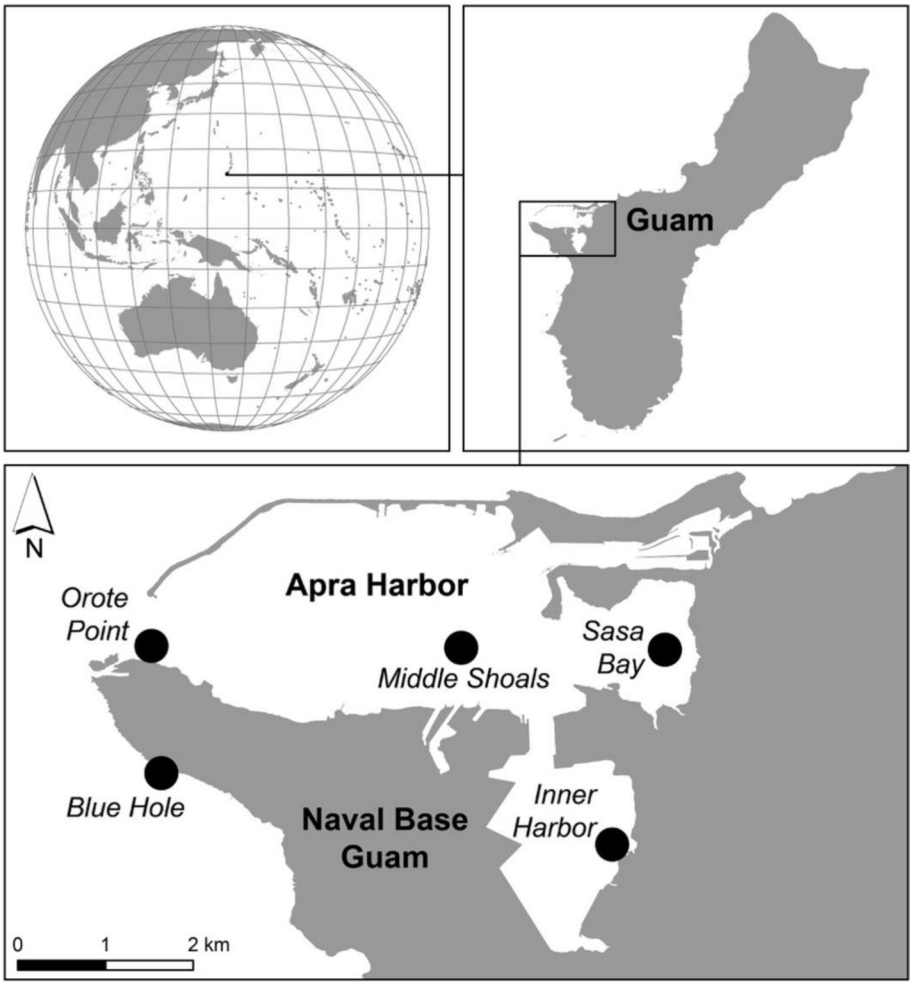

Fig. 1. Map of Apra Harbor, Guam. Black dots indicate eDNA sampling sites.

gloves changed.

At each site, at least ten replicate surface water samples were filtered through a nylon net filter (47 mm diameter; Merck, Darmstedt, Germany) housed in a filter cartridge (either 3D printed or Smith-Root; Washington, United States) and connected to a battery-powered diaphragm pump (Grover® Scientific, Queensland, Australia; www.groverscientific.com) using 10 mm vinyl tubing. Water samples were filtered directly from the ocean by submerging the filter cartridge approximately 1 ft below the water surface. Prior to sample collection, a field blank was collected by filtering 500 mL of Ultrapure water. Following filtration, each filter was folded and cut in half using sterilised equipment and each half was transferred into a 2 mL microtube containing either 1.5 mL of non-denatured 96% ethanol (March-April 2018 field collection) or 1.5 mL of Longmire's buffer (November 2018 field collection and thereafter).

To optimize filter pore size for capture of *S. lewini* eDNA, three different combinations of seawater volume and filter pore size were tested. These combinations were designed such that similar sampling effort was required for each combination of seawater volume and filter pore size, including 1 L and 1.2 μm, 5 L and 5 μm and 10.0 L and 10 μm, with approximately 5 min or less for each of ten to twelve filtrations per site (Table A6). The optimised method uses ten replicates of 10.0 L of seawater filtered through a pore size of 10 μm. All samples were stored at ambient temperature during the sampling period and transported to the University of Guam, Marine Laboratory, where Longmire's preserved samples were stored at ambient temperature and ethanol preserved samples were stored at 4 °C until shipment to James Cook University (JCU), Bebegu Yumba campus, for subsequent eDNA extraction and qPCR analysis. Upon arrival at JCU, Longmire's preserved samples were stored at ambient temperature and ethanol preserved samples were stored at −20 °C.

2.5. eDNA extraction and purification methods

To optimise preservation, extraction and purification methods, all ethanol-preserved filter halves (March 2018 field collection) and one of each of the Longmire's preserved filter halves from the November 2018 collection were extracted using the QIAGEN DNeasy® blood and tissue kit (DNeasy, herein) according to the manufacturer's protocol and the modifications in Table A7. The remaining Longmire's preserved halves from the November 2018 collection, and any samples collected thereafter, were subject to a precipitation method adapted from Edmunds and Burrows (2020) using either 0.6, 2 or 3 μg of glycogen in total. A brief description of the protocol and modifications for filter papers are provided in Table A7 and in Cooper et al. (in press). Extracted eDNA was purified using either the OneStep™ PCR Inhibitor Removal Kit (Zymo IR; Zymo Research; Irvine, California, United States) or DNeasy PowerClean Pro Clean up Kit (QIAGEN PCP; QIAGEN Hilden, Germany) following the manufacturer's instructions and stored at −20 °C until subsequent analysis. A flow diagram containing extraction and purification information for samples used in methods optimisation as described above can be found in Figures A2.1 and A2.2. The final extraction and purification workflow uses the precipitation method with the largest quantity of glycogen (total 3 μg), followed by Zymo IR purification.

2.6. eDNA quantification and inhibition testing

To optimise filtration methods, samples collected from Sasa bay during the March 2018 field collection were subject to quantification of total eDNA yield (n = 10 per seawater volume and filter pore size combination) using SYBR chemistry and generic fish primers Fish_16S_R (5′ - CTCAGATCACGTAGGACTTTA − 3′; Furlan and Gleeson, 2017) and 16SallR (5′ - CGCTGTTATCCCTAGGGTAACT − 3′; Tollit et al., 2009). For all other quantification purposes, eDNA yield was measured as total dsDNA concentration using the Quantifluor dsDNA system and fluorescence (excitation/emission: 504/531 nm) on an Enspire multimode plate reader (Perkin Elmer; Waltham, Massachusetts, United States) or Quantus Fluorometer (Promega; Madison, Wisconsin, United States). Inhibition was quantified using the spiking-followed-by-dilution approach developed by Cao et al. (2012) where inhibition is assessed based on the average Ct difference between spiked-undiluted (SU) and 5-fold spiked-diluted (SD) samples ($\Delta Ct_{SD\text{-}SU}$) and compared to the expected ΔCt of 1.32, which assumes an amplification efficiency of 2 and a natural variability between replicates of 0.5 cycles ($\Delta Ct_{expected} = \log_2 5 - 2 \times 0.5 = 1.32$). A detailed description of the method can be found in the supporting information of Cao et al. (2012). Inhibition was also assessed using TaqMan Exogenous Internal Positive Control reagents (IPC; Applied Biosystems; California, United States) according to the manufacturer's protocol.

2.7. Criteria for positive detection

For a detection to be considered positive, requirements are as follows: 1) a minimum of one positive detection in one technical (qPCR) replicate in one biological (field sample) replicate; 2) no amplification in extraction blanks (see Table A7 for description of blanks) or non-template qPCR controls and; 3) 100% sequence identity of the amplified fragment to *S. lewini* 12S gDNA based on Sanger sequencing.

2.8. Aquarium validation

To validate the efficacy of the assay, the *S. lewini* 12S primers and probe were tested on eDNA collected from an aquarium housing a single juvenile female scalloped hammerhead, amongst multiple other shark and ray individuals and species, at Cairns Aquarium in November 2017 (a full list of these species can be found in Table A9). Five 1 L water samples were collected as per the field sample collection methods described above using a filter pore size of 10 μm. The samples were stored in non-denatured 96% ethanol at ambient temperature during transport and then stored at −20 °C until extraction using the DNeasy method.

2.9. Field application

To test the ability of the optimised field sampling, laboratory eDNA extraction, purification and qPCR assay for the presence/absence detection of *S. lewini* in the field, samples from a single sampling effort during February-March 2019 were analysed. The assay included 5 μl of eDNA template in nine technical replicates for each of 12 biological replicates taken from each of the five field sites. Quantification and inhibition tests were also carried out for six biological replicates per site using the dsDNA quantification and spiking-followed-by-dilution methods described above. The TaqMan Exogenous IPC was added to at least three technical replicates of two biological replicates for each site.

3. Results

3.1. Assay specificity and sensitivity

Here a highly sensitive and specific assay to target scalloped hammerhead sharks was successfully designed, optimised, and validated. Multiple iterations of primer testing led to a final primer-probe combination with at least eight bp mismatches to closely related sphyrnid species *Sphyrna tiburo* (11 bp), *E. blochii* (11 bp), *S. mokarran* (8 bp), *Sphyrna tudes* (8 bp) and *S. zygaena* (8 bp), respectively (Fig. 2; Table A1). The optimised assay uses 0.3 μM of each primer and 0.25 μM of TaqMan probe and an annealing temperature of 62 °C. Specificity testing resulted in no amplification of gDNA from the twelve exclusion species tested and no amplification from gBlocks fragments from *S. zygaena* or *C. longimanus*. The final assay exhibited no primer-probe

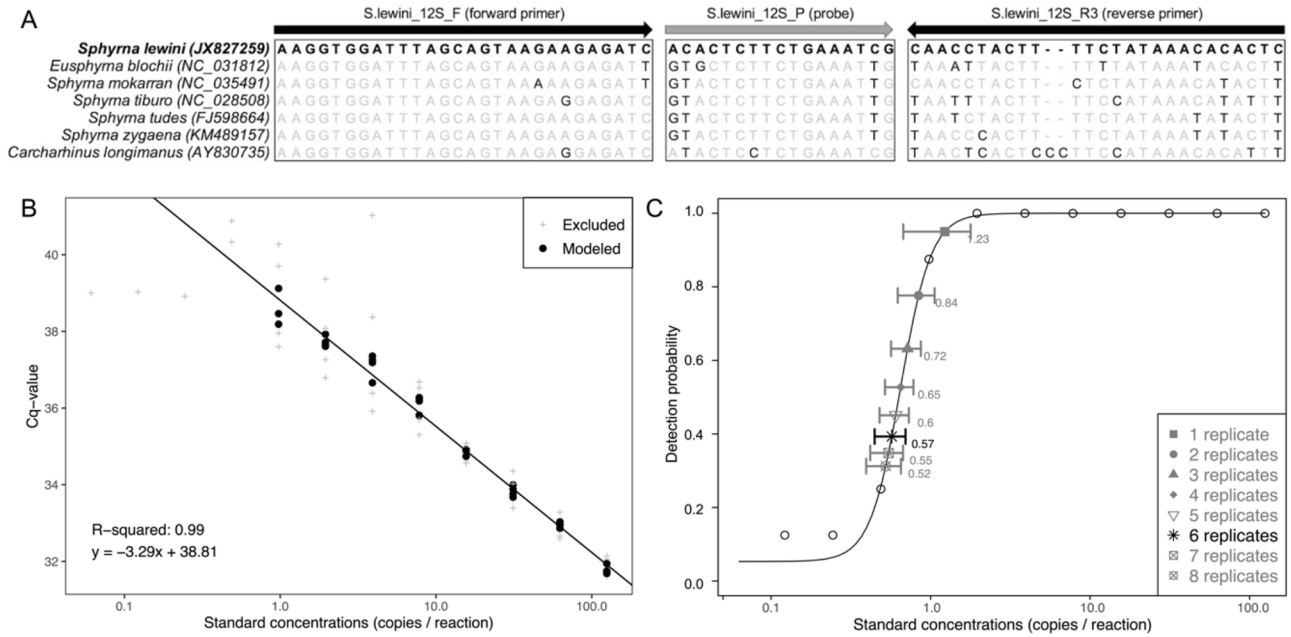

Fig. 2. Specifications for *Sphyrna lewini* 12S eDNA assay. **A.:** Alignment of *S. lewini* 12S primers and probe (bold nucleotides) with the target regions of publicly available Sphyrnidae species sequences and representative Carcharhinidae species *Carcharhinus longimanus*. The reverse primer (S.lewini_12S_R3) is depicted as the reverse and complement of the primer sequence. Grey scale nucleotides match corresponding bases in the primer or probe sequence. Black nucleotides are mismatches between primer and target sites. An alignment including all exclusion species can be found in Fig. A3. **B.:** Standard curve results from a 12 point two-fold serial dilution of *S. lewini* 12S gBlocks fragments, from a theoretical 125 copies per reaction to 0.06 copies per reaction. Standards with concentrations at and below 0.49 copies (serial dilutions number 9 to 12) were not included in copy estimate calculations because they detected below 50%. **C.:** Results from a three-parameter logistic function used to determine the effective LOD for each quantity of qPCR replicates. The effective LOD for six technical replicates (black text) is 0.57 copies per reaction.

amplification of any of the exclusion species tested, where the target species amplified in all reactions at 100-fold lower concentrations, and 50% of all reactions at 1000-fold lower concentration. In addition to high specificity, the assay demonstrates sensitivity to *S. lewini* found across the western Pacific as primer-probe sequences were designed using sequences from China, Papua New Guinea and Australia, validated using *S. lewini* gDNA and eDNA from Australia (tissue samples and aquarium samples, respectively) and eDNA from field sites in Guam, Micronesia. The assay also demonstrates sensitivity to low copy *S. lewini* DNA, with a modelled LOQ of 16 copies/reaction and the effective LOD using six technical replicates for each biological replicate determined as 0.52 copies/reaction (0.1 copies/µl; Fig. 2).

3.2. eDNA yield and purity optimisation

3.2.1. Filter pore size, preservation medium and eDNA extraction comparison

Filtration times between different seawater volume and pore size combinations were comparable, with a mean ± SD time of 3.68 ± 3.25 min overall, including 4.03 ± 5.03 min for 1 L of seawater through 1.2 µm filter pores size, 2.54 ± 1.07 min for 5 L seawater through 5 µm filters and 4.46 ± 1.89 for 10.0 L seawater through 10 µm filters for samples collected during March and April 2018 (Table A6). Comparison of total eDNA yield using SYBR and generic marine fish primers indicated that 5 and 10 L of seawater filtered through 5 and 10 µm filters, respectively, yielded greater total eDNA (Ct mean ± SD 30.7 ± 0.3) compared to 1 L of seawater through 1.2 µm filters (Ct mean ± SD 35.1 ± 0.4). Detection comparisons revealed zero detections in samples filtered using a pore size of 1.2 µm (n = 26 biological replicates, n = 182 technical replicates) and 23 detections each using filter pore sizes of 5 and 10 µm (n = 104 biological replicates and n = 806 technical replicates each). As such, only 5 and 10 µm filters were used in further testing.

Multiple hypothesis testing for each combination of filter size (5 and 10 µm), preservation medium (ethanol and Longmire's), and extraction method (DNeasy and precipitation) revealed that the combination of 10 L of seawater, 10 µm filters, Longmire's preservation buffer and precipitation extraction resulted in the highest dsDNA yield compared to all other directly comparable treatments ($p < 0.05$; Fig. 3). More specifically, regression analysis highlighted a positive association between the use of Longmire's preservation buffer and precipitation extraction on total dsDNA yield [$b = 5.82$, $t(44) = 4.25$, $p < 0.001$; Table A8], with an additional increase in yield where a combination of 10 µm filters and precipitation extraction is used [$b = 6.13$, $t(44) = 3.16$, $p < 0.01$; Table A8]. DNA integrity assessed by gel electrophoresis revealed no visible differences between treatment groups (data not shown).

Inhibition testing of all 10 µm-filtered Longmire's-preserved samples using the spiking-followed-by-dilution approach revealed evidence for the presence of qPCR inhibitors in precipitation-extracted samples, but not DNeasy-extracted samples. However, the difference in mean ΔCt_{SD-SU} between the two groups was only 1.37 cycles, which is unlikely to equate to a loss in detection ability (Fig. 3). As precipitation extracted samples produced significantly higher eDNA yield and demonstrated only partial and slight qPCR inhibition, the precipitation extraction method was selected for usage and further testing.

3.2.2. Glycogen quantity and purification method comparison

Multiple hypothesis testing for each combination of glycogen quantity and purification method highlighted that samples extracted with a total of 3 µg of glycogen and purified with the Zymo IR kit produced significantly higher dsDNA yield compared to all QIAGEN PCP purified samples ($p < 0.05$; Fig. 3). Linear modelling highlighted a significant positive effect of increasing total glycogen quantity [$b = 0.41$, $t(68) = 4.327$, $p < 0.05$; Table A8] on total dsDNA yield but a negative association between the use of QIAGEN PCP purification and total dsDNA yield compared to no purification [$b = -7.13$, $t(68) = -6.09$, $p < 0.001$;

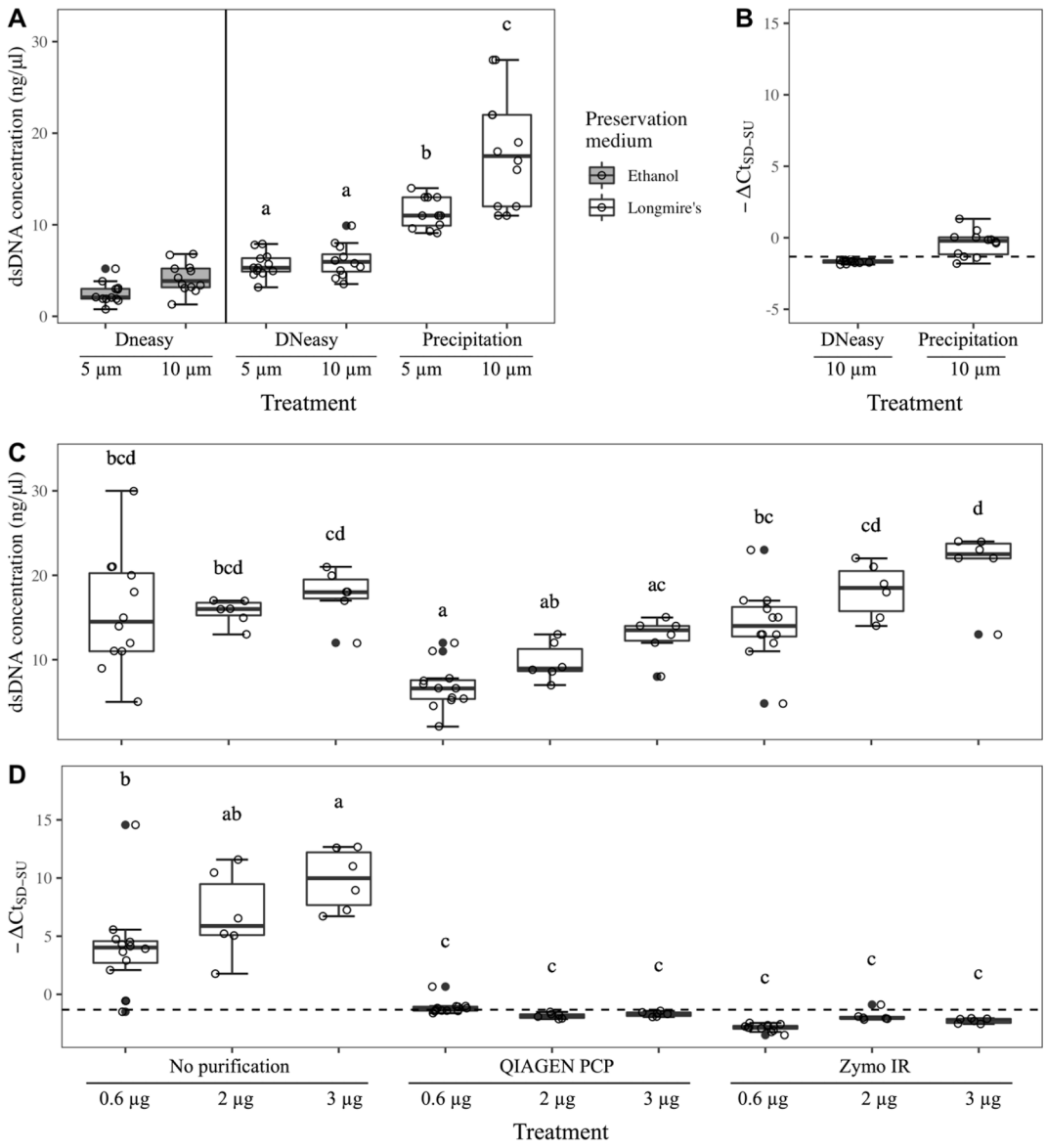

Fig. 3. Environmental DNA collection and capture optimisation results presented as double stranded DNA (dsDNA) quantity in ng/µl and qPCR inhibition as $-\Delta Ct_{SD-SU}$, measured by fluorescent dsDNA binding dye and spiking-dilution assay, respectively. **A.:** Reveals that eDNA filtered using 10 L of seawater and 10 µm filters, preserved in Longmire's buffer and extracted using the precipitation method offer the highest dsDNA concentrations and **B.:** suggests comparable purity. **C.:** Reveals that eDNA extracted using the highest quantity of glycogen and purified using Zymo IR yields the highest dsDNA concentrations and in **D.:** with sufficient purity. The dashed line represents the expected $-\Delta Ct_{SD-SU}$ value for an uninhibited sample, indicating that samples falling above this line show signs of qPCR inhibition. The x axis indicates treatment, including eDNA extraction method (DNeasy or precipitation method), filter pore size and filtrate amount (either 5 or 10 µm filters using 5 and 10 L of water respectively), purification method (QIAGEN PCP or Zymo IR) and total glycogen content used in extractions. In A and B, all samples were purified using Zymo IR and in C and D all samples are collected using 10 µm filters and extracted using the precipitation method. Letters denote pairwise comparisons using Tukey's Honest Significant Difference and Bonferroni correction following linear regression. March-collected samples, separated by the vertical line in A, were not included in statistical analysis.

Table A8].

Inhibition testing by spiking-followed-by-dilution approach revealed evidence for qPCR inhibition in all unpurified samples, and some evidence for incomplete inhibition in samples that were purified using QIAGEN PCP and extracted using 0.6 µg glycogen (Fig. 3). Multiple hypothesis testing for each combination of glycogen quantity and purification method revealed no significant difference in inhibition (as ΔCt_{SD-SU}) for any of the purified samples, regardless of purification method, but all unpurified samples had significantly lower ΔCt_{SD-SU} values than purified samples (Fig. 3). Linear modelling highlighted a significant negative effect of glycogen quantity on qPCR inhibition [b = -0.47, t (66) = -5.66, $p < 0.001$]. Conversely, there was a significant positive effect of both the QIAGEN PCP [b = 3.53, t (66) = 3.22, $p <$ 0.01; Table A8] and Zymo IR [b = 5.4726, t (66) = 5.01, $p < 0.001$; Table A8] on qPCR inhibition compared to unpurified samples. Significant interactions between glycogen quantity and both purification methods with positive estimates highlights that both the QIAGEN PCP [b = 0.53, t(66) = 4.47, $p < 0.001$; Table A8] and Zymo IR [b = 0.41, t (66) = 3.46, $p < 0.001$; Table A8] kits largely resolve the inhibition effect of increased glycogen addition as indicated by $-\Delta Ct_{SD-SU}$ values close to or below the inverse inhibition threshold (−1.32) in the majority of purified samples (Fig. 3). As Zymo IR-purified samples produced significantly higher yield and performed comparably in qPCR inhibition testing, the precipitation extraction method with 3 µg glycogen and Zymo IR purification was chosen for usage and subsequent testing.

3.3. Aquarium validation results

A total of 29 aquarium eDNA technical replicates of five biological replicates produced a mean ± SD Ct of 37.67 ± 1.29. All re-amplified products sent for sequencing (n = 14) were confirmed as having 100% *S. lewini* sequence identity. At the time of sampling, the tanks contained five additional shark species (n = 11 individuals) and five ray species (n = 10 individuals; Table A9) and just one *S. lewini* individual, demonstrating the specificity of the assay within a sample containing a mix of eDNA from close relatives.

3.4. Field application results

Both total dsDNA concentration and inhibition as $\Delta Ct_{SD\text{-}SU}$ were highest and most variable in samples obtained from Inner Harbor (Fig. A4). No inhibition was detected by the TaqMan Exogenous IPC reagents. Two positive detections, one from Inner Harbor (three technical replicates, two biological replicates; Ct 41.73, 46.42 and 48.80) and one from Orote Point (one technical replicate; Ct 40.44) were obtained. All Sanger sequences of amplified products returned 100% identity to *S. lewini* 12S reference sequence.

3.5. Optimised workflow

The optimized workflow to detect scalloped hammerhead eDNA from Apra Harbor, Guam is summarised in Fig. 4. Specifically, for each of 10 replicates per field site, 10 L of seawater is filtered through 47 mm diameter nylon net filters with 10 µm pore size using a Grover Scientific battery-powered diaphragm pump. To introduce redundancy, immediately after filtration filter papers are cut in half and each half is preserved in a separate 2.0 mL screw cap tube with 1.5 mL of Longmire's buffer and stored at ambient temperature. The eDNA collected and captured onto the filter is extracted using the precipitation method of Edmunds and Burrows (2020) with the modifications for filters specified in Table A7. The resulting 100 µl of eDNA is purified using the Zymo OneStep PCR Inhibitor Removal Kit. The final *S. lewini* 12S assay developed here uses a forward (5′-AAGGTGGATTTAGCAGTAAGAAGAGATC-3′; S. lewini_12S_F) and reverse (5′-GAGTGTGTTTATAGAAAAGTAGGTTG-3′, S. lewini_12S_R3) primer pair combined with a TaqMan MGB probe (6FAM-ACACTCTTCTGAAATCG-MGB, S. lewini_12S_P; Applied Biosystems). The eDNA is subject to qPCR analysis using this *S. lewini* 12S primer probe combination at concentrations of 0.3 µm and 0.25 µm, respectively, along with 10 µl of TaqMan Environmental Master Mix and 5 µl of eDNA template in each of six 20 µl reactions per field sample. Thermocycling parameters follow the manufacturer's instructions with the exception of the annealing temperature, which is set to 62 °C. For each plate, at least three non-template qPCR controls were run, each containing 5 µl of Ultrapure water in place of template eDNA. All technical replicates that result in qPCR amplification are subject to bi-directional Sanger sequencing and confirmation of *S. lewini* eDNA detection. A Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) checklist is included in Table A5).

4. Discussion

Here an optimised eDNA workflow (Fig. 4) for the assessment of critically endangered scalloped hammerhead shark presence was developed and applied. The efficacy of the method was demonstrated through the detection of *S. lewini* in Guam, confirming previous and substantial anecdotal reports of their presence in the area (NMFS, 2015; Smith et al., 2010; Department of the Navy, 2005). To our best knowledge, this is the first published evidence of the presence of *S. lewini* in the Mariana Islands since 1971 (Kami, 1971). The field collection method enables the efficient processing of large volumes of water and is adaptable to suit diverse aquatic environments. Following sufficient site-specific optimisation steps, the sampling method requires only a pump, tubing and filter with housing. Samples are preserved in Longmire's buffer, which is non-flammable, non-toxic, and able to be stored and shipped at ambient temperatures above 40℃ for up to six weeks (Edmunds and Burrows, 2020). The laboratory protocols presented here involve routine molecular procedures (DNA extraction and qPCR) carried out with careful consideration of contamination, and are optimised to minimise qPCR inhibition while retaining high eDNA yield; an important consideration for rare species. The primer-probe combination was developed using *S. lewini* sequences from Australia, Papua New Guinea and China and validated on eDNA collected from an aquarium in Cairns, Australia as well as field sites in Guam, Micronesia. This eDNA

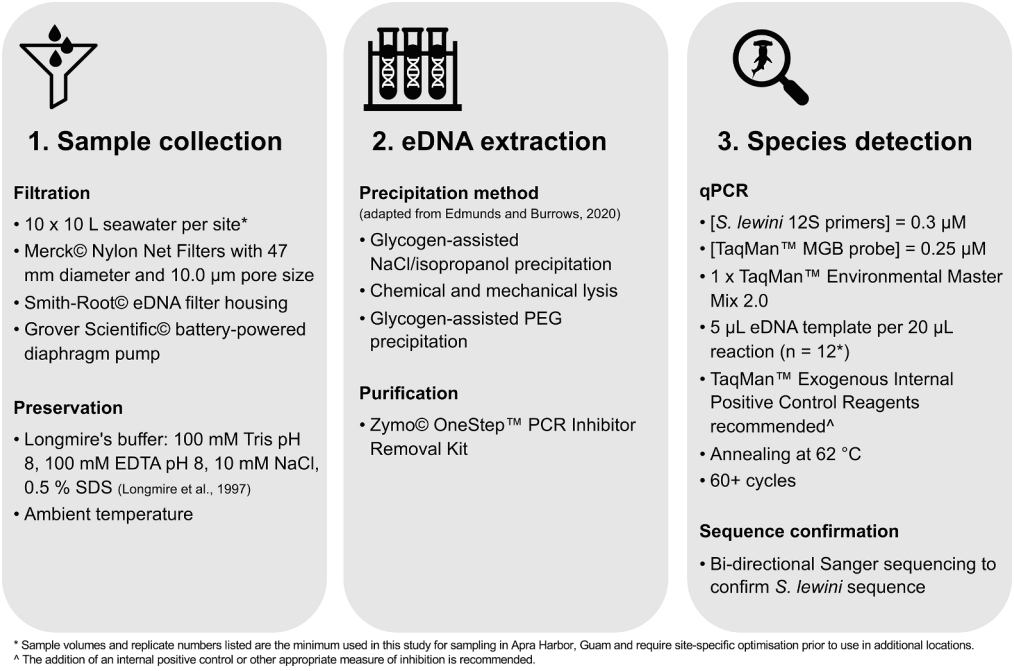

Fig. 4. Summary of the optimised eDNA workflow for the detection of scalloped hammerhead sharks (*Sphyrna lewini*).

survey method has been developed to offer a highly efficient, non-invasive, cost-effective, user-friendly survey technique to facilitate existing and ongoing research on the global distribution of *S. lewini* and assist in efforts to enable the species' recovery.

4.1. Application and implications for conservation

Environmental DNA survey methods, including the workflow presented here, offer non-invasiveness, increased accuracy, low cost and high detection probability in comparison to alternative survey methods (Sigsgaard et al., 2017; Valentini et al., 2016). For example, while various tagging methods have greatly advanced understanding of *S. lewini* movements, tagging requires intensive research effort and tag retention and/or recapture rates are generally low. Mark-recapture methods executed by Duncan and Holland (2006) reported a recapture rate of just 3.7%, with a total fishing effort of 3,562 hook hours. Acoustic tagging performed by Wells et al. (2018) and Nalesso et al. (2019) reported that less than half of the tags deployed (16 of 33 and 27 of 84 tags, respectively) resulted in a monitoring duration of more than four months. Spaet et al. (2017) and Jorgensen et al. (2009) each report satellite tagging information for a single individual, likely due to the associated costs. Most recently, Heupel et al. (2020) satellite tagged eight *S. lewini* but only three remained attached for the intended tracking duration of 180 days. The authors reported that failure to reliably capture large adults limited the research conclusions, noted that tag shedding is a common issue which may be exacerbated by hammerhead shark behaviour, and suggested additional sampling methods be implemented in future (Heupel et al., 2020). While these tagging studies significantly advance understanding of many biological and ecological variables that, at present, eDNA methods are unable to reveal, information on distribution and habitat usage can be more rapidly and cost-effectively obtained using the environmental DNA method presented here. The detection of *S. lewini* within a single visit of less than one hour to each of Inner Harbor and Orote Point sampling sites in Apra Harbor, Guam demonstrates the efficacy of eDNA methods to rapidly generate such information, and indicates that the technique can be appropriately applied where the objectives are primarily focused on assessing species presence.

The *S. lewini* assay also offers increased accuracy and detection rates when compared to non-tagging methods, such as those that rely on visual identification and/or sightings. The three species comprising the large hammerhead shark complex (*S. lewini, S. zygaena,* and *S. mokarran*) are morphologically similar and therefore difficult to visually differentiate (Gallagher and Klimley, 2018). These morphological similarities can lead to misidentification of hammerhead species whether live (e.g., Baited Remote Underwater Video Systems, BRUVs; S. Mukherji, *pers. comm.* 2020) or dead (fish markets; Clarke et al., 2006). Visual observations have proven an unsuccessful method of detection of *S. lewini* in the Mariana Islands, with nine years of biennial surveys conducted, including 84 towed-diver surveys and 39 belt transect surveys in Guam specifically, being unable to detect the presence of *S. lewini* (Zgliczynski et al., 2013). Similarly, 32 years of aerial surveys over five decades did not result in any reported sightings of *S. lewini* in Guam (Martin et al., 2016). Notably, both in-water and aerial surveys were able to detect co-occurring shark and ray species (including *Carcharhinus amblyrhynchos, Triaenodon obesus, Carcharhinus melanopterus and Mobula alfredi*) with varying success (Martin et al., 2016; Zgliczynski et al., 2013). Most recently, a global survey of sharks and rays using BRUVs also did not observe the presence of *S. lewini* in Guam (C. Simpfendorfer, *pers. comm.* 2020; MacNeil et al., 2020). While visual methods enabled the detection of alternative elasmobranch species in these studies, in Guam they have been unsuccessful for the detection of *S. lewini*.

An inability to detect scalloped hammerheads through visual methods is likely due to low abundance, behavioural factors and may be further exacerbated by complex spatial organisation of differing size and age classes (Coiraton et al., 2020). For example, while adult *S. lewini* exhibit differing migration patterns between pelagic and coastal environments both within and between the sexes (Daly-Engel et al., 2012; Harry et al., 2011), juveniles are likely to aggregate in deep, turbid areas to reduce risk of predation (Duncan and Holland, 2006). Furthermore, an absence of visual detections for *S. lewini* likely indicates very low levels of abundance, with Guam ranked the worst of 58 examined nations for relative abundance of reef sharks (MacNeil et al., 2020) and the third lowest of 46 nations evaluated for average reef biomass (Cinner et al., 2016). Although Guam is situated within the Micronesia Regional Shark Sanctuary, completed in 2015, MacNeil et al. (2020) suggest that poor governance and overfishing have played a major role in the exceptionally low probability of reef shark presence in Guam. In addition to low abundance, behavioural factors may further exacerbate difficulties in recording species presence as *S. lewini* is reported to be shy and highly susceptible to stress (Balakrishnan et al., 2014; Gulak et al., 2015; Tristram et al., 2014). While closely related *S. mokarran*, are reported to be commonly observed on BRUVs, *S. lewini* are not (MacNeil et al., 2020). Previous reports of *S. lewini* in Guam are solely based on personal observations and anecdotal reports of rare sightings of solitary individuals (Department of the Navy, 2005; NMFS, 2015; Smith et al., 2010) with photographic evidence of a recent sighting in Blue Hole in November 2018 (Fig. A4). The successful detection of *S. lewini* in Guam demonstrated here in comparison to previous and extensive visual surveys in Guam indicates that the eDNA methods developed here are highly efficient, sensitive and thus appropriate for locations in which the *S. lewini* abundance is low or not yet known.

4.2. Assay specificity and sensitivity

Reliable detection of rare species using environmental DNA can be achieved through the development of highly-specific qPCR primers (Wilcox et al., 2013). In this study, primer development was facilitated by the curation of an exclusion species mtDNA sequence database using published sequences, in addition to those generated specifically for assay design. Alignment of these sequences revealed *S. lewini* mtDNA was highly variable for COI, leading to difficulties in identifying regions conserved within-species. Conversely, *S. lewini* mtDNA sequences were highly conserved for ND2 fragments, leading to difficulties identifying regions that were variable between-species. Sequences for 12S and 16S demonstrated adequate sequence variability for primer-probe design, offering adequate within-species sequence conservation and between-species variability. This allowed for the design of primer probe combinations that were specific to *S. lewini* and non-specific to all closely related and co-occuring species. The alignment of sequences for species closely related to *S. lewini* also enabled the identification and removal of sequences from samples that have likely been misidentified or are otherwise not representative. For example, *S. lewini* 12S accession AF448021 exhibited 99.69% similarity with *S. mokarran* accession AF448022 but only 94.7% similarity to *S. lewini* accession JX827259. Similarly, one tissue sample donated by fishers included in this study was identified as *S. lewini*, but all mtDNA amplicons exhibited higher similarity to *S. mokarran* sequences, and were therefore omitted. As with all barcoding approaches to species identification, the use of mitochondrial sequences can result in the incorrect assignment of hybrid individuals to the maternal species (Ward et al., 2008). While there are no reported hybrids between *S. lewini* and *S. mokarran*, hybridisation has been reported between *S. lewini* and their closest relative, the recently described Carolina hammerhead (*Sphyrna gilberti;* Barker et al., 2019). While *S. gilberti* does not occur in the Pacific, this is a necessary consideration for use of the assay in the US Atlantic, and may be crucial in South Carolina. Generally, however, unless there is evidence for repeated back-crossing and introgression, the presence of target species mtDNA in eDNA samples can be considered as indicative of species presence (Wilcox et al., 2013). These examples highlight the importance of a robust sequence database and thorough understanding of sequence specificity for eDNA assay design, and awareness of assay limitations.

Multiple iterations of primer and probe design were carried out based on the curated sequence database, with not all resulting assays leading to species-specific amplification of *S. lewini*. This was despite the presence of primer-probe mismatches against all exclusion species tested. Environmental DNA qPCR assay specificity is not only influenced by base pair mismatches, but by their location in the primers and probe (Wilcox et al., 2013). Minor groove binding (MGB) probes such as those used in TaqMan assays enhance specificity by forming highly stable duplexes with DNA targets, exhibiting far higher melting temperatures enabling shorter probe lengths and lower background fluorescence (Kumar et al., 1998; Kutyavin et al., 2000). In particular, a mismatch in or near the MGB binding region (i.e., in the terminal 2–5 residues, but not the final 3′ base pair, which has little effect; Kumar et al., 1998) leads to increased specificity compared to mismatches in the remainder of the MGB-probe and within non-MGB probes (Kutyavin et al., 2000). The *S. lewini* 12S probe presented here includes at least a single bp mismatch in the terminal second and third base pair for the vast majority of exclusion species, as well as throughout the remainder of the probe (Fig. A3). Testing of the probe with primers that included fewer mismatches than those in the optimised assay revealed non-specific amplification in slit eye shark (*Loxodon macrorhinus*) and oceanic whitetip shark both of which do not contain a mismatch in the terminal base pair (Fig. A3). Thus, the positioning of mismatches in the second and third terminal base pair for the *S. lewini* 12S probe likely enabled the high specificity of the assay against exclusion species.

TaqMan MGB assay specificity is also heavily influenced by the position of base pair mismatches in the primers. Previous work on rare bull trout (*Salvelinus confluentus*) demonstrated that primer mismatches were particularly influential when situated at the 3′ end, but much less so at the 5′ end (Whiley and Sloots, 2005; Wilcox et al., 2013). The *S. lewini* 12S primer pair presented here was designed such that mismatches occurred at least in the 3′ of the forward primer of all co-occurring species sequences and throughout the entire reverse primer for all exclusion species' sequences (Fig. A3). Recent work in critically endangered sawfish species identified that at least five mismatches in the total TaqMan assay were sufficient to eliminate false positives, specifically with at least three mismatches in the probe (Cooper et al., in press). The TaqMan assay designed here contains a minimum of five mismatches in the primers alone, a minimum of two mismatches in the probe and a minimum of eight mismatches in total to all exclusion species for which sequence data were available. While two exclusion species that do not co-occur in Guam had no mismatches in the forward primer, including pig eye shark (*Carcharhinus amboinensis*) and sliteye shark gDNA from both species were successfully excluded by the assay. Furthermore, while the closely related great hammerhead exhibited the lowest number of mismatches, eight in total, gDNA from this species was also successfully excluded by the assay. The final *S. lewini* 12S assay exhibited no amplification of any of the exclusion species for which gDNA was available when tested at concentrations 1000-fold higher than those that allowed for detection of the target species. The demonstrated specificity of the optimised primer combination may therefore be attributed to both the 3′ positioning and high number of mismatches present.

Designing highly sensitive assays is also particularly important for the detection of rare species because target copy number within the eDNA sample is likely to be very low (Wilcox et al., 2013). Here, modelling LOD using gBlocks fragments revealed the assay is highly sensitive, resulting in modelled LOD values for the *S. lewini* 12S assay that were similar to the most sensitive of all 36 environmental DNA assays subject to the same statistical analysis in Klymus et al. (2020) and those presented in Wilcox et al. (2013). It is worth noting that sensitivity to synthetic DNA and eDNA is likely to differ substantially, largely due to the presence of environmental contaminants that act as qPCR inhibitors, such as humic acids (Green and Field, 2012). Extensive optimisation was carried out in order to reduce qPCR inhibition, discussed in further detail below. In summary, the assay developed here is both highly sensitive and specific, with a very low LOD for *S. lewini* and an inability to amplify all exclusion species tested. These attributes best increase the chances of detecting rare, endangered scalloped hammerhead sharks in an eDNA mix of co-occurring and highly abundant and/or closely related species.

4.3. Sample collection and eDNA preservation considerations

For eDNA to be an effective and practical tool for the detection of rare species, sampling protocols must enable the processing of large volumes of water and rapid preservation of genetic material. Filtration using portable, commercially available pumps (Grover® Scientific), 10 μm nylon filters and Longmire's storage buffer allowed for sampling of 100 L per site in under an hour with no requirement to ship large volumes of water, or to store samples on ice. Both the volume filtered and pore size used are large in comparison to previously reported species-specific elasmobranch eDNA sampling methods which typically collect 1–4 L but up to 20 L per site using 0.22 to 0.8 μm filter pore sizes (Gargan et al., 2017; Lehman et al., 2020; Schweiss et al., 2020; Sigsgaard et al., 2017; Weltz et al., 2017). Preliminary analysis revealed that total eDNA yields and *S. lewini* detections were increased using a larger volume of water and pore size where sampling time is approximately equivalent, although site-specific biotic and abiotic factors are likely to dictate which filter pore size is best suited to detecting *S. lewini* in regions beyond those sampled here. For example, high turbidity may necessitate the use of 20 μm filter pore sizes, which have been shown to be effective for the detection of rare sawfish species (Simpfendorfer et al., 2016). Immediate filtration also allows for immediate preservation and therefore a reduction in the risk of degradation, which occurs rapidly in aquatic eDNA samples. For example, in the endangered maugean skate eDNA in seawater samples collected from field sites decays beyond the limit of detection within as little as four hours (Weltz et al., 2017). Furthermore, sample storage at both 4 and −20 °C for greater than 24 h has been shown to lead to a reduction in copy number, and as such, immediate filtration or precipitation is ideal for rare species (Hinlo et al., 2017). While the use of ethanol for sample preservation may be precluded by area-specific attributes (e.g., climate, equipment availability and legality), Longmire's solution can be transported without limitation and can effectively preserve eDNA at ambient temperatures, including those above 40 °C for up to 6 weeks (Edmunds and Burrows, 2020; Renshaw et al., 2015; Spens et al., 2017; Williams et al., 2016). Preservation of the filter directly into a lysis buffer such as Longmire's also eliminates the need for removal of the buffer prior to extraction, which likely improves eDNA retention and reduces contamination risks compared to protocols requiring ethanol removal and dry steps (Hinlo et al., 2017). In summary, the use of portable eDNA pumps and Longmire's solution enables immediate filtration and preservation of samples suitable for locations with varying accessibility and climatic conditions.

4.4. Recommendations for eDNA extraction and minimising inhibition

The optimisation of eDNA extraction methods are important to maximise eDNA yield, particularly in low abundance species, while reducing the concentration of environmental contaminants that act as qPCR inhibitors. While extraction kits are a convenient and common method of eDNA extraction, they typically lead to low total yield compared to precipitation methods (Deiner et al., 2015; Renshaw et al., 2015; Turner et al., 2015). PEG-based precipitation has the additional advantage of omitting many of the toxic chemicals associated with Phenol-Chloroform-Isoamyl alcohol extraction, but retains high eDNA yield (Edmunds and Burrows, 2020). The addition of high concentrations of glycogen also leads to significant increases in eDNA yield, as was the case in this study. During precipitation, glycogen serves as an inert carrier molecule for DNA, thus aiding DNA recovery (Russell and Sambrook, 2001). The results presented here demonstrated significantly higher total eDNA yield in precipitation-extracted samples compared to

DNeasy-extracted samples, however, high yield does not necessarily indicate higher detections. This is largely due to the co-purification of substances that act as qPCR inhibitors (Eichmiller et al., 2016; Green and Field, 2012). While it is difficult to compare detection rates in samples from field sites where species are in low abundance, because detections do not occur at high enough frequencies to allow for robust statistical analysis, inhibition was instead tested using a spiking-dilution method developed by Cao et al. (2012). These comparisons revealed that the combination of the precipitation method with Zymo IR was optimal to relieve inhibition and retain sufficient yield. Recent work has shown that the combination of Longmire's preservation and precipitation extraction improves detections in sawfish compared to ethanol preservation and QIAGEN extraction (Cooper et al., in press). Furthermore, the TaqMan EMM used here has been specifically developed for environmental samples, and shown to resolve inhibition and offer higher detection rates compared to other master mixes (Cao et al., 2012; Doi et al., 2015). As such, the use of Longmire's buffer combined with precipitation extraction, followed by OneStep™ PCR Inhibitor Removal Kit (Zymo Research) and TaqMan EMM is recommended to sufficiently relieve inhibition and retain high yield for eDNA detection, but that inhibition should be tested for each new site and associated set of environmental conditions.

4.5. Future directions

The assay presented here is highly effective for the detection of *S. lewini* eDNA presence, however the results are limited to a single pilot study in Apra Harbor, Guam. Increased sampling and analysis would allow for better spatial resolution and may reveal the occurrence of *S. lewini* in additional survey sites. Furthermore, temporal sampling and subsequent analysis would allow for better insight into anecdotal reports of Apra Harbor having been used as a nursery ground, with an expected peak in detections during pupping season (Duncan and Holland, 2006). Globally, the *S. lewini* eDNA assay developed may be best applied in regions similar to Guam, where low levels of abundance dictate that alternative survey methods are ineffective or cost prohibitive. Examples include additional regions within the Mariana Archipelago as well as American Samoa, where quantitative information on *S. lewini* presence is unavailable, but the species has been reported historically or anecdotally (NMFS, 2015; Zgliczynski et al., 2013). In regions predicted to have high abundance, the eDNA survey provides a highly efficient method for pilot studies to obtain preliminary data to assist in the identification of sites where more labour intensive, complementary survey efforts would be best applied. Furthermore, if the samples collected in these regions contain eDNA copy numbers that meet or exceed the modelled LOQ of 16 copies per reaction, this may allow for estimates of relative abundance and/or indications of seasonal migration, as is possible for many fish species (e.g., Shelton et al., 2019). Finally, further primer development (to include polymorphic regions of DNA) and the application of species-specific metabarcoding techniques may additionally allow for population genetic analysis and estimates of genetic diversity (e.g., Sigsgaard et al., 2017). In summary, the eDNA methods developed here offer a highly effective survey technique to complement existing and ongoing research on the global distribution of *S. lewini* and, with further application and development, may additionally allow for estimates of relative abundance and genetic diversity.

5. Conclusion

Scalloped hammerhead sharks have undergone alarmingly steep population declines, particularly in the Pacific and Indian Oceans, and at present there is no action recovery plan or systematic recovery scheme in place to relieve them of their critically endangered status (Pacoureau et al., 2021; Rigby et al., 2019). Here an optimised method for the detection of *S. lewini*, including adaptable field protocols and a highly sensitive and specific molecular assay is presented. A single one-day sampling effort using the workflow developed here resulted in the detection of *S. lewini* in Guam, where evidence of their presence had previously been restricted to anecdotal and historical reports, despite substantial visual survey effort (Kami, 1971; MacNeil et al., 2020; Martin et al., 2016; NMFS, 2015; Zgliczynski et al., 2013). If adopted and applied, the eDNA methods described here will provide much needed information on the global distribution of *S. lewini*, assist in the identification of conservation sites and allow for ongoing monitoring of the species' recovery into the future.

CRediT authorship contribution statement

Alyssa M. Budd: Data curation, Formal analysis, Investigation, Methodology, Project administration, Validation, Visualization, Writing - original draft. **Madalyn K. Cooper:** Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Writing - original draft. **Agnès Le Port:** Conceptualization, Funding acquisition, Methodology, Data curation, Investigation, Writing - review & editing. **Tom Schils:** Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Writing - review & editing. **Matthew S. Mills:** Data curation, Investigation, Project administration, Writing - review & editing. **Mari E. Deinhart:** Investigation, Project administration, Writing - review & editing. **Roger Huerlimann:** Conceptualization, Funding acquisition, Methodology, Supervision, Validation, Writing - original draft. **Jan M. Strugnell:** Conceptualization, Data curation, Funding acquisition, Project administration, Methodology, Resources, Supervision, Writing - original draft.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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