Chapter 5

Addressing data-deficiency of threatened sharks and rays in a highly dynamic coastal ecosystem using environmental DNA

Guido Leurs, Yvonne I. Verkuil, Nadia Hijner, Franziska Saalmann, Lilísio Dos Santos, Aissa Regalla, Samuel Ledo Pontes, Lei Yang, Gavin J. P. Naylor, Han Olff, Laura L. Govers

Published in Ecological Indicators (2023)

Abstract

Marine biodiversity loss is accelerating, leading to the elevated extinction risks of many species, including sharks and rays. To mitigate these losses, information on their distribution and community composition is needed. Monitoring these (often) mobile species is challenging, especially in remote, highly dynamic and turbid coastal areas. Here, we use an environmental DNA (eDNA) approach to (1) establish the presence and distribution of elasmobranch species, (2) compare this to a conventional fisheries-dependent approach, and (3) determine the influence of season, area-based protection and habitat on elasmobranch community composition in the highly dynamic Bijagós Archipelago in Guinea-Bissau (West Africa). We collected 127 seawater samples and detected elasmobranch DNA in 58 (45.7%) of these samples, confirming the presence of 13 different elasmobranch species (2 sharks, 11 rays), including seven threatened species. Eight species detected by the eDNA approach were also recorded in a fisheries observer program, which recorded another eight species not detected by the eDNA approach. The most commonly occurring species, based on the number of eDNA sampling locations, were the pearl whipray (*Fontitrygon margaritella*), smalltooth stingray (*Hypanus rudis*), scalloped hammerhead shark (*Sphyrna lewini*), and the blackchin guitarfish (*Glaucostegus cemiculus*). Species composition and richness differed significantly before (January-March) and after the rainy season (November-December). Furthermore, we showed that community composition and species richness did not differ between protected (MPA) and non-protected areas of the archipelago. Thus, we confirm that eDNA approaches are a valuable and non-invasive tool to study threatened shark and ray species in data-deficient and dynamic coastal areas, especially when combined with conventional monitoring methods such as fisheries-dependent information.

Introduction

Globally, coastal ecosystems are threatened by anthropogenic stressors, such as pollution and coastal development, causing a collapse in the richness and diversity of associated species (Worm *et al.* 2006, Cardinale *et al.* 2012). The loss of species may hamper the functioning and health of ecosystems and can lead to a loss of ecosystem services (Worm *et al.* 2006, Palumbi *et al.* 2009). Therefore, monitoring the status of biodiversity and individual species within ecosystems is essential to ensure future ecosystem health and the preservation of ecosystem services (Millenium Ecosystem Assessment 2005, Cardinale *et al.* 2012).

In marine ecosystems, top and meso-predators such as sharks and rays (i.e., elasmobranchs), can have important roles in coastal ecosystems (e.g., Heithaus 2010, Heupel *et al.* 2014, Roff *et al.* 2016, Heithaus *et al.* 2022). However, recent findings suggest that approximately 33% of all shark and ray species are threatened with extinction due to overfishing and habitat degradation (Dulvy *et al.* 2021). Due to their ecological roles, the loss of these species may influence ecosystem services of marine ecosystems, such as productivity of fisheries, detoxification of marine waters, and carbon sequestration ('blue carbon', Heithaus *et al.* 2008, Atwood *et al.* 2015, Küpper and Kamenos 2018).

Assessing species' conservation status (e.g., IUCN Red List status) is an important step toward implementing management actions that enable protection. However, specific information for the appropriate assessment of conservation status is missing for many shark and ray species or local/regional populations (Dulvy *et al.* 2021). This includes information on local presence, distribution and abundance of elasmobranch species. Monitoring biodiversity is costly and requires appropriate (research) capacity, causing data deficiency to be more profound in developing regions. The resulting deficiency of essential information impairs species' status evaluation and hampers the implementation of (cost-)effective conservation strategies.

A relatively novel approach to monitoring the occurrence of marine species is the use of environmental DNA (eDNA), which involves the metabarcoding of DNA traces of marine species in the water column or associated sediments (e.g., Thomsen *et al.* 2012). This approach simplifies species monitoring, increases species coverage (i.e., including cryptic, rare and highly mobile species and limiting misidentification), and is non-invasive and cost-effective compared to other traditional monitoring approaches (Thomsen *et al.* 2012, Miya 2022). Over the past years, the application of environmental DNA has been increasingly used to confirm the presence of fish species in both freshwater and marine waters and, more recently, to study the composition of elasmobranch communities (Bakker *et al.* 2017, Boussarie *et al.* 2018, Dunn *et al.* 2022). In addition, eDNA approaches have been successfully applied to determine seasonal abundance (Postaire *et al.* 2020), population sizes (Sigsgaard *et*

al. 2016), and the presence of highly cryptic species, for example, the presence of sawfishes (*Pristis spp.*) in estuaries (Lafferty *et al.* 2018, Schweiss *et al.* 2019, Lehman *et al.* 2020). Although these relatively novel approaches are promising to address elasmobranch communities in highly data-deficient regions, less is known about the success of this technique in studying elasmobranch communities in highly dynamic environments such as intertidal ecosystems experiencing strong (tidal) currents.

To determine if environmental DNA can be used to tackle data deficiency in highly dynamic, tropical coastal ecosystems, we aimed to study a highly data-deficient shark and ray community in the West African region. The coastal waters of the West African bioregion have a high occurrence of threatened endemic elasmobranch species (Derrick *et al.* 2020) and are a global hotspot for the most evolutionary distinct elasmobranch species (i.e., a measure of a species' evolutionary isolation) (Stein *et al.* 2015). However, the region currently also experiences one of the highest levels of fishing effort in the world (Kroodsma *et al.* 2018, Leurs *et al.* 2021). Industrial fisheries surrounding protected coastal areas in West Africa (Leurs *et al.* 2021) and small-scale fisheries within these areas both threaten elasmobranch populations due to their high targeted and non-targeted catches (Kyne *et al.* 2020, Lemrabott *et al.* in prep, Leurs *et al.* in prep., Moore *et al.* 2019). However, the presence and community composition of elasmobranch fishes in coastal areas within the region remains poorly understood, hampering adequate conservation of this threatened species group. In addition, the recent disappearance of species like largetooth sawfish (*Pristis pristis*) from the wider region and the occurrence of cryptic species, such as the African wedgefish (*Rhynchobatus luebberti*), asks for a more comprehensive approach to elasmobranch monitoring (Leeney and Poncelet 2015, Moore 2017).

Here, we determined if the environmental DNA approach can be used to successfully study elasmobranch communities in a tropical, data-deficient and highly dynamic intertidal environment. Specifically, we used an eDNA approach to (1) establish the presence and distribution of elasmobranch species within a highly dynamic tropical intertidal ecosystem, (2) compare the eDNA-based species richness and composition of the archipelago to preliminary small-scale fisheries data, and (3) determine if eDNA-based species richness and community composition differed across seasons (i.e., before and after the rainy season), across tidal phases, between protected and non-protected areas and with distance to mangrove forest. Although the Bijagós Archipelago is one of the largest intertidal areas in the region, supporting local (artisanal) fisheries and likely functioning as a nursery area for both coastal and pelagic fish species (including commercial species captured in the industrial fisheries in the wider region) (Correia *et al.* 2021), information on the distribution of elasmobranch species in lacking. The only information on elasmobranch species within this area originates from inferred species distributions (IBAP 2012), studies limited to a single species or island (Cross 2015, Leeney and Poncelet 2015), and recorded captures by industrial fishing fleets

operating outside the archipelago (Diop and Dossa 2011, Leurs *et al.* 2021). We aimed to provide information that is essential for the successful implementation of more efficient conservation measures for these threatened species, for future ecological studies focusing on the ecosystem functioning of the Bijagós, and to learn if and how this relatively novel approach can be used in remote, highly dynamic, and data-deficient environments to study sharks and rays.

Methods

Study area

The Bijagós Archipelago (11° 15' 0" N, 16° 5' 0" W) is located in Guinea-Bissau (Figure 5.1), in the extended estuary of the Geba River. The archipelago comprises 88 islands and islets lined by dense mangrove forests and intertidal mudflats connected through a complex system of gullies and channels.

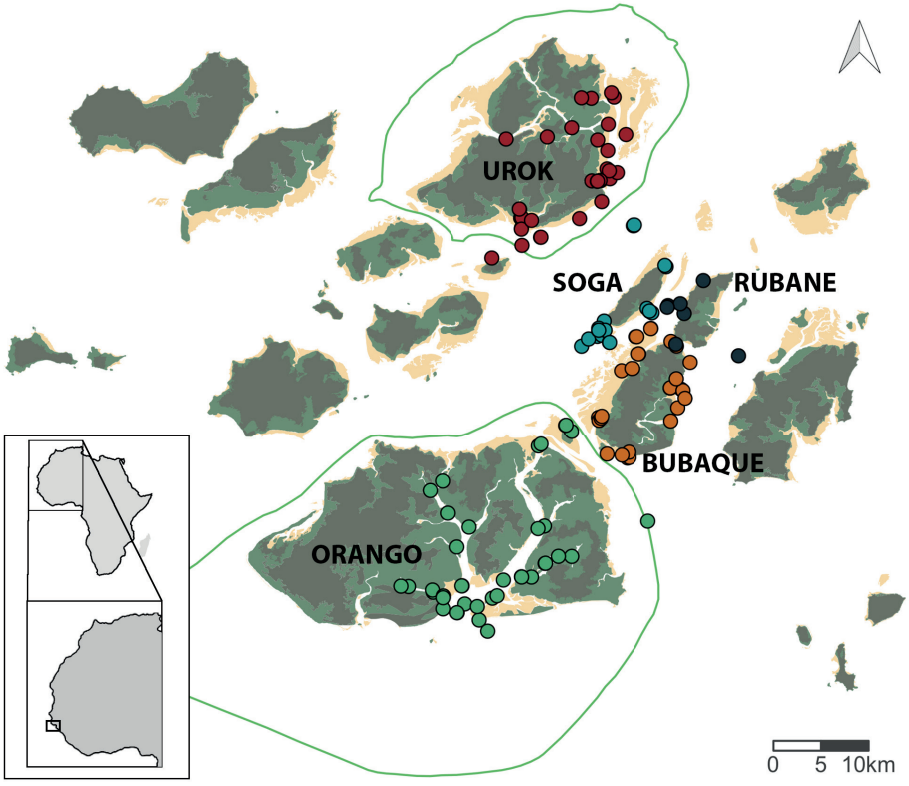

Figure 5.1 Overview of the sampling locations in the Bijagós Archipelago in Guinea-Bissau. Sampling was conducted in five different regions: Urok (n = 35; red), Soga (n = 19; light blue), Rubane (dark blue), Bubaque (n = 28; orange), and Orango (n = 38; green). The island's upland (dark green), mangroves (green) and intertidal areas (yellow) are shown. The marine protected areas (MPAs) are outlined in green.

With over 350 km^2 of mangrove forests and 760 km^2 of intertidal flats, the archipelago is recognized as an important area for (migratory) shorebirds (Salvig *et al.* 1994, Meijer *et al.* 2021), teleosts (Correia *et al.* 2021), and sea turtles (Catry *et al.* 2002), and was designated as a UNESCO Biosphere Reserve in 1966 (IBAP 2012, UNESCO 2020). In 2014, the archipelago was also recognized as an important wetland under the RAMSAR Convention (IBAP 2012, RAMSAR 2014).

Sample collection and preservation

We sampled surface water in five regions within the archipelago: Urok, Soga, Rubane, Bubaque, and Orango (Figure 5.1). Samples were collected before (January and February) and after (October to December) the rainy season in 2019. At each sampling location, we took a 2-liter water sample using a sterilized sampling bottle (i.e., using a 10% bleach solution) and by submerging the bottle completely underwater to prevent sampling the biofilm on the water surface. For each sample, we recorded the surrounding habitat (Table 5.1), geographic coordinates, and storage time (i.e., time between sample collection and filtration). Retrospectively, we determined the distance of the sampling location to the entrance of the Geba River, the distance to the nearest mangrove edge, and whether a sample was taken in or outside one of the two marine protected areas (Figure 5.1). Sampling time was used to determine the tidal phase and amplitude based on the tide table for Bubaque (11.33° N/15.87° W). We estimated that compared to the high tide in Bubaque, the high tide was one hour later in the Urok sampling region and one hour earlier in the Orango region. To account for potential variability in these high and low tide estimates, we considered samples taken within 30 minutes to or from high tide as 'high-tide samples' and similarly for low tide. Samples taken between low and high tides are referred to as 'receding tides' and 'incoming tides'. Straight after sample collection, samples were wrapped in aluminum foil and stored in an insulated cooling box until filtration. Upon return to the base camp or whenever the situation in the field permitted, samples were filtered as soon as possible using a portable, battery-operated vacuum pump (Makita 16V vacuum pump). The pump was connected to a Nalgene Erlenmeyer flask with a sterilized filter holder and funnel on top. Samples were filtered using sterile mixed cellulose ester filters (MERCK and PALL filters, 47mm Ø, 0.45μm pore size). We used multiple filters to filter a single 2-liter sample depending on the suspended material. As access to electricity during expeditions was not always guaranteed due to the remoteness of the field sites, each filter was subsequently stored in a Longmire's lysis buffer, which allows for sample storage without cooling (Williams *et al.* 2016, Spens *et al.* 2017, Taberlet *et al.* 2018). Sampling bottles, filter holders, and

funnels were sterilized using a 10% bleach solution between sampling efforts. To determine any contamination during sampling and equipment sterilization, we took a negative control sample for each sterilized batch of equipment by filtering bottled mineral water (i.e., equipment blank). The filters of these controls were stored the same way as filters used for sample filtration.

Table 5.1 The definitions of habitats assigned to each sample (see Leurs *et al.* 2023).

Habitat	Definition
Intertidal	Habitat that is submerged during high tide and emerges at low tide (generally shallower than 5 meters in depth).
Gulley	Water stream embedded in intertidal flats and/or mangrove forests (width of less than 250 meters).
Minor channel	A water stream that is more than 250m and less than 1 km wide and has no direct connection to the ocean.
Main channels/subtidal waters	Main water bodies with a width of over 1 km and a direct connection to the ocean.

Metabarcoding of samples

DNA extraction

In the lab (genetics lab of the University of Groningen), prior to DNA extraction, all filters (and buffer solution) belonging to the same field sample were pooled together in a sterile 50 ml vial and were stored submerged by adding Longmire's lysis buffer. Filter pooling was conducted in an ultra-violet (UV) box with sterilized forceps. Materials were sterilized using 50% bleach and subsequent rinsing with DNA-free water. Samples were then stored in the fridge (at about 2 °C) until DNA extraction. We applied a standard phase-separation and precipitation DNA extraction method based on phenol-chloroform (Minamoto *et al.* 2016). DNA quantities of every sample were determined using a spectrophotometer (Nanodrop 2000). Subsequently, DNA extracts were cleaned by gel extraction using the Promega Wizard® SV Gel and PCR Clean-Up System. This clean-up step was necessary because of the carry-over of PCR inhibitor originating from ingredients of Longmire's lysis buffer. The obtained clean DNA was then used as the PCR template.

Primer details

For species identification in elasmobranchs, the fast-evolving, mitochondrial protein-coding gene NADH dehydrogenase subunit 2 (NADH2) has been successfully applied (Naylor *et al.* 2005, 2012). The universal elasmobranch primers of Naylor *et al.* (2005), binding to the ASN and ILE tRNA regions, target a 1,044 bp fragment of NADH2. To amplify a shorter fragment from eDNA samples with potentially degraded DNA, we

used the ASN primer variant called 'ChimeraF' ('5-AAGGACTACTTTGATAGAGT-'3) (Naylor *et al.* 2005) in combination with two newly designed reverse primers yielding an amplicon of ca. 320 bp. The first reverse primer NADH2 'miniSharkR2' ('5-GGAATRATGGCTAATGTGTT-'3) targets both sharks and rays, and the second reverse primer 'miniSharkR5' ('5-CCTATTCAAACTAGGAGTC-'3) was specifically designed to target shark species. For subsequent sequencing, the following tails were attached to the primer: 5'-GATGTGTATAAGAGACAG_Forward-primer-3' and 5'-CGTGTGCTCTTCCGATCT_Reverse-primers-3'.

PCR and sequencing

Polymerase Chain Reaction (PCR) was set up in a DNA-free room. Each sample was amplified in triplicate to avoid PCR bias. AccuStart II PCR ToughMix© was used, as DNA in the collected samples may have been degraded due to biological processes or degradation caused by exposure to UV light. The reaction volume was 10 µl including 5 µl AccuStart, 1µl of each primer (10 µM), 1 µl ddH2O and 2µl DNA template. The PCR profile was 3 min at 94°C, followed by 35 cycles of 1 min at 94°C, 30 sec at 48°C, and 1 min at 72°C, and a final extension at 72°C for 10 min. The annealing temperature was set to 48°C to minimize taxonomic bias (Ishii and Fukui, 2001). PCR products were sequenced on a MiSeq© (Illumina) Sequencer at the Department of Human Genetics, Leiden University Medical Center, with the aim for a read-depth set at 50,000 reads per sample. Libraries were prepared with the MiSeq© V3 kit, generating 300-bp paired-end reads. Since the V3-kit does not normalize, i.e., leaves the relative presence of the initial PCR product intact, this library preparation method allows assessing the relative contribution of taxa to read abundance of each PCR product.

Lab controls

For each sampling period (before and after the rainy season) two negative extraction controls were included to test the Longmire's lysis buffer stock solution as a source of contamination: one with the first and one with the last batch of extractions. Additionally, negative control samples were taken from each PCR master mix to track possible contamination of PCR reagents.

Creation of OTU table

We extracted unique, high-quality barcode reads (molecular operational taxonomic units, abbreviated as OTU) using the software USearch 9.2 (Edgar 2010). First, paired-end reads were merged into a consensus sequence, removing the sequencing adaptors.

Primer sequences were removed by truncating each end by 25bp, the length of the longest PCR primer. The full dataset quality filtering was set at the default E-value of 0.4 and read-truncation to 220bp. To simplify clustering, truncated reads were de-replicated by assigning a count to unique reads, merging identical reads in both orientations. Subsequently, the singletons were removed (e.g., Frøslev *et al.* 2017). Using the UPARSE-OTU algorithm (Edgar, 2010), reads that were minimally 97% identical were clustered. This was replicated using a threshold of 100% similarity for clustering and yielded no differences in species detection. The consensus sequence of each cluster was assigned an OTU ID, resulting in an OTU sequence table. This algorithm also filters chimeras. For each sample, the number of reads (paired and with truncated primers) that matched with each OTU was determined, resulting in an OTU frequency table. The default identity match of 97% was used. The final OTU frequency table was adjusted for the negative extraction and PCR controls by deducting the number of reads found for an OTU in the pooled negative extraction controls from each cell in the OTU table. The final OTU table was blasted against the mitochondrial genome database of Chondrichthyes constructed and curated by the Florida Program for Shark Research (FPSR) at the Florida Museum of Natural History of the University of Florida (see Naylor *et al.* 2012). At the time of this analysis, the database contained 94% of known genera and 72% of known chondrichthyan species, plus potential new species and population-level variants. The database has been curated by taxonomic experts to exclude any wrongly identified haplotypes. Only the match with the lowest E-value was retained during blasting for each OTU.

Of the 127 samples we collected and sequenced, 58 (45.7%) contained elasmobranch DNA. Of 886,097 reads, 88.1% (780,581) could be taxonomically assigned to 110 unique OTUs; 40 OTUs were assigned with high taxonomic certainty using a percentage identity of ≥ 95% and query coverage of ≥ 85%. Of these, 25 OTUs were assigned to 13 elasmobranch species, accounting for 218,047 (24.6%) reads. The remaining 15 OTUs were assigned to teleosts (7.16% of reads; primarily *Sarotherodon melanotheron*), humans (0.04% of reads) and plant/bacteria (< 0.01% of reads). Of these elasmobranch species, 11 ray species were identified, accounting for 180,227 reads (82.7%) of the total number of reads. Two shark species were detected, accounting for the rest of the reads (17.3%). Elasmobranch reads per sample ranged from 0 to 6,521 (399.4 ± 976.2, mean ± standard deviation) after corrections for contamination.

Fisheries Observer Program

Data from a pilot fisheries observer program was used to compare the number of species detected in the eDNA survey. From February to September 2021, 122 fishing

boats operating within the Archipelago were sampled in the main fishing port of Bissau. Of each boat, the elasmobranch catches were identified to species level and information on the fishing trip (e.g., fishing location, duration) was documented. To compare the fisheries observer data to the eDNA results, only boats fishing within the Urok, Soga, Rubane, Bubaque, and Orango regions were included in the analyses (n = 44). Due to the limited sample size, species richness and composition between fisheries observer and eDNA data could only be compared on an archipelago level.

Statistical analyses

To minimize the influence of species presence due to cross-contamination, a species was considered present when the number of reads exceeded ten. To study the species composition across different variables (e.g., season, tidal phase, MPAs), we determined the frequency of occurrence for each species by dividing the total number of locations that a species was detected by the total number of sample locations. We used non-metric dimensional scaling to visualize the species composition of sampling locations at which at least one species was detected and determined significant differences in relative species composition across the different seasons, protected areas and tidal phases using a permutational analysis of variance (PERMANOVA). We determined the species richness for each sampling location as the number of detected species (S). We used a generalized linear model with a negative binomial error distribution to determine the relation between species richness and predictor variables. We conducted a Tukey's range test to test for differences among sampling season and tidal phases. Since the number of reads for a specific species can be influenced by PCR conditions (Taberlet *et al.* 2018) or ecological events (e.g., a deceased individual or reproduction/spawning, Barnes and Turner 2016**)**, we limited species-specific analyses to presence-absence. We used a general linear model with a binomial error distribution to determine the significance of independent variables in predicting the presence of a species. We included season, region, distance to the nearest mangrove, distance to the Geba River entrance, habitat, and tidal phase as independent variables. The presence of a species was only modeled for species detected at ten or more sampling locations, resulting in an exclusion of rare species from this analysis. Model selection was based on Akaike's Information Criterion (AIC) and Bayesian Information Criterion (BIC).

Results

Species presence and distribution

A total of 13 species were detected as part of our eDNA survey, with 7 (53.8%) of these species currently listed as threatened on the IUCN Red List. The four most common species in the study area based on the total number of sample points that a species

was detected are the pearl whipray (18.9%; N = 24 locations), smalltooth stingray (*Hypanus rudis*, 14.2%; N = 18 locations), scalloped hammerhead shark (12.6%; N = 16 locations), and blackchin guitarfish (11.8%; N = 15 locations) (Figure 5.2A). These four species were detected in the majority of study regions, except for the blackchin guitarfish and smalltooth stingray, which were not detected in the Rubane and Soga regions, respectively (Figure 5.3). Relatively rare species, such as the African cownose ray (*Rhinoptera peli*) and the marbled stingray (*Dasyatis marmorata*), were only detected in Urok and southern Orango (Appendix 5.1).

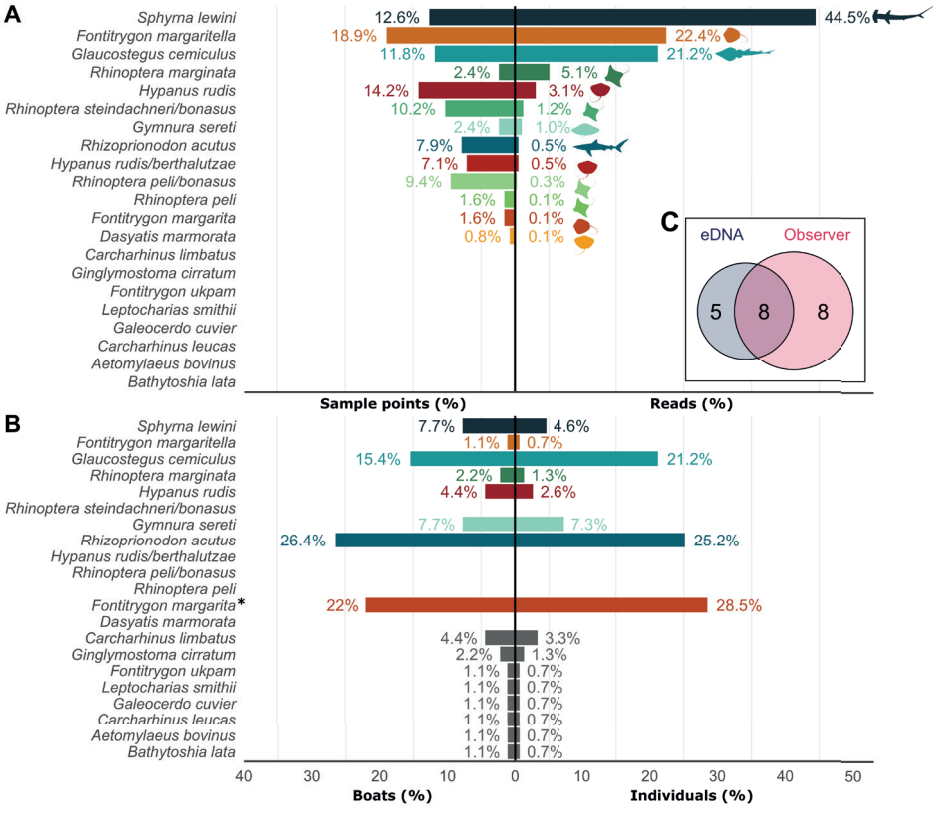

Figure 5.2 The species that were detected using environmental DNA in 2020 as a proportion of sampling points (n = 127) that a species was detected and as a proportion of the total number of reads (A). The species observed during the fisheries observer program in 2021 are shown as a proportion of the boats that captured the species and the proportion of the total number of individuals of elasmobranchs that were captured (B). Species were detected either by both methods or only by the eDNA survey or by the observer (C). Different colors indicate different species detected as part of the eDNA survey, with color tint indicating species group (sharks = blue, benthic rays = orange/red, benthopelagic rays = green, guitarfishes = light blue). Species only detected as part of the observer program are shown in gray.

**Possibly includes observations of F. margaritella due to misidentification.*

When considering the total number of reads per species, the most common species were the scalloped hammerhead shark (44.5%), pearl whipray (22.4%), blackchin guitarfish (21.2%), and the Lusitanian cownose ray (*Rhinoptera marginata*, 5.1%). This differed from fisheries-dependent information, as the most caught species were the milk shark (*Rhizoprionodon acutus*, 26.4%), daisy whipray (*Fontitrygon margarita* 22.0%, but likely includes *F. margaritella* due to frequent misidentification), blackchin guitarfish (15.4%), scalloped hammerhead shark (7.7%), and Seret's butterfly ray (*Gymnura sereti*, 7.7%) (Figure 5.2B). The eDNA approach and observer program overlapped in documenting the presence of eight species, whereas five additional species were only detected with the eDNA approach and eight other species were recorded only in the catches of local fishers (Figure 5.2C).

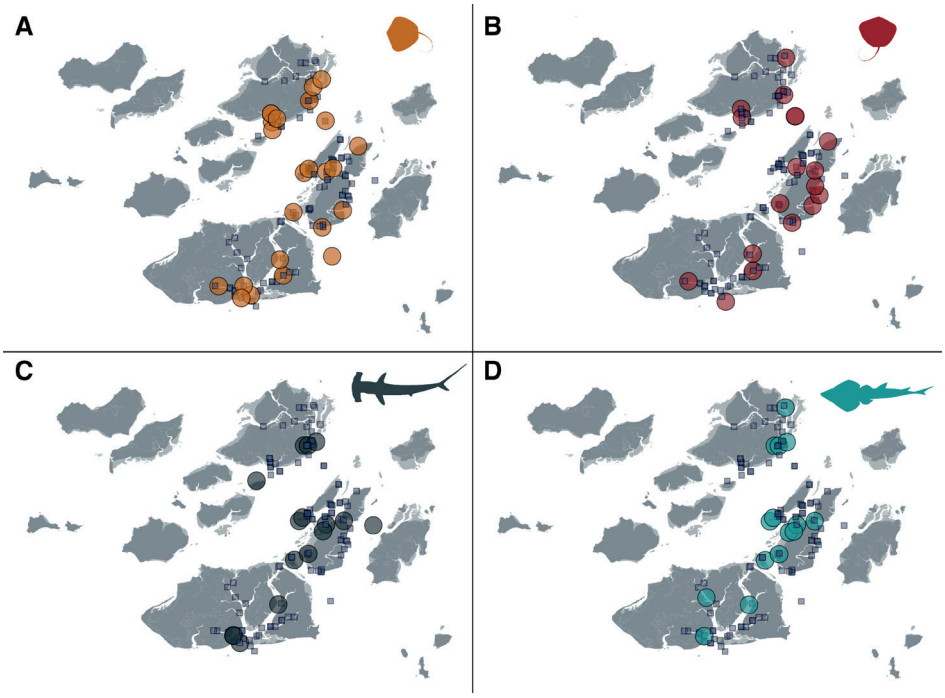

Figure 5.3 The sample points where the four most common species were detected using eDNA: (A) pearl whipray (*Fontitrygon margaritella*), (B) smalltooth stingray (*Hypanus rudis*), (C) scalloped hammerhead shark (*Sphyrna lewini*), and (D) blackchin guitarfish (*Glaucostegus cemiculus*). Grey squares indicate locations where the species was not detected. The distribution maps of the remaining species detected in this study are shown in Appendix 5.1.

We determined that eDNA-based species richness within the study area ranged from 0 to 7 species per location, with a mean of 1.0 spp. (95% CI: 0.75-1.27 spp.) (Figure 5.4). Seven of the species detected using the eDNA approach are classified as

threatened based on the IUCN Red List: The milk shark (*Rhizoprionodon acutus*) and pearl whipray (*Fontitrygon margarita*) are listed as Vulnerable, the Seret's butterfly ray (*Gymnura sereti*) as Endangered, and the scalloped hammerhead shark, blackchin guitarfish, Lusitanian cownose ray (*Rhinoptera marginata*), and the smalltooth stingray are listed as Critically Endangered. The fisheries observer recorded seven additional threatened species not detected using the eDNA approach. Of these, the blacktip shark (*Carcharhinus limbatus*), nurse shark (*Ginglymostoma cirratum*), barbeled houndshark (*Leptocharias smithii*), bull shark (*Carcharhinus leucas*), and brown stingray (*Bathytoshia lata*) are listed as Vulnerable, and the thorny whipray (*Fontitrygon ukpam*) and duckbill eagle ray (*Aetomylaeus bovinus*) as Critically Endangered.

Effects of season, protective status and habitat

We determined that both species richness and species composition differed significantly before and after the rainy season (Figure 5.5A-C; Richness: d.f. 1, F = 4.46, p = 0.04, composition: d.f. = 1, F = 7.79, $p < 0.01$) and that species composition differed between non-protected and protected areas when seasonality is taken into account. These seasonal differences are caused by a higher occurrence of the pearl whipray and the cownose ray *Rhinoptera steindachneri cf. bonasus* after the rainy season and a higher occurrence of the scalloped hammerhead shark and the blackchin guitarfish before the rainy season (Figure 5.5B). This was supported by a higher detection probability of the scalloped hammerhead (d.f. = 1, $X^2 = 10.4$, $p < 0.01$) and blackchin guitarfish (d.f. = 1, $X^2 = 11.1$, $p < 0.01$) before the rainy season (Appendix 5.6).

Although we determined that both species richness and composition across protected and non-protected areas did not differ significantly (Figure 5.5D-F), species composition differed significantly between protected and non-protected waters if seasonality is taken into consideration (d.f. = 1, F = 2.29, p = 0.04) (Figure 5.5G). After the rainy season, species composition within the MPAs significantly differed from locations outside the MPAs (d.f. = 1, F = 3.67, p = 0.03), but also from locations both in- and outside MPAs before the rainy season (d.f. = 1, F = 6.40, p = 0.001; d.f. = 1, F = 6.51, p = 0.002). These differences are caused by a higher occurrence of the pearl whipray within the MPAs after the rainy season and the higher occurrence of the scalloped hammerhead shark and guitarfish before the rainy season in both protected and non-protected areas (Figure 5.5H).

Species richness was influenced by the tidal phase (d.f. = 3, F = 3.75, p = 0.01), with the highest number of species detected in samples taken during incoming tide (1.59 ± 0.28 spp.) (Appendix 5.2). This coincides with the higher probability of detecting the most commonly detected species, the pearl whipray, during incoming tide (d.f. = 1, z = 2.18, p = 0.03) (Appendix 5.6).

Although the distance to the Geba River had no significant influence on the species richness and detection probability of a species, the distance to the nearest mangrove forest had a significant influence on the probability of detecting three ray species, the pearl whipray, blackchin guitarfish, and the cownose ray *Rhinoptera steindachneri cf. bonasus* (Figure 5.6). Samples taken further away from the mangrove edge had a higher probability of detecting the pearl whipray (d.f. = 1, X^2 = 4.5, p = 0.03) and *Rhinoptera steindachneri cf. bonasus* (d.f. = 1, X^2 = 5.9, p = 0.02). In contrast, the probability of detecting a blackchin guitarfish decreased when moving further from the mangrove edge (d.f. = 1, X^2 = 4.0, p = 0.05).

Sampling effort and storage

Lastly, increased sampling effort correlated with an increase in the number of species detected in our study. The maximum species richness (S = 13) was reached at 124 samples taken, which constitutes 96% of the total sampling effort of this study (Appendix 5.3). We also determined that extended storage times (0.03 - 7.2 hours) due to the remoteness of the study sites did not negatively impact the number of species detected (*Spearman* r = 0.09).

Figure 5.4 Species richness - the number of detected species - for every sampling point within the study area. Sampling points with a low species richness are indicated by a small yellow/orange point, and sampling points with a high species richness are indicated by dark purple colors and a larger point. Sampling points with no elasmobranch species detected are indicated with crossed dots.

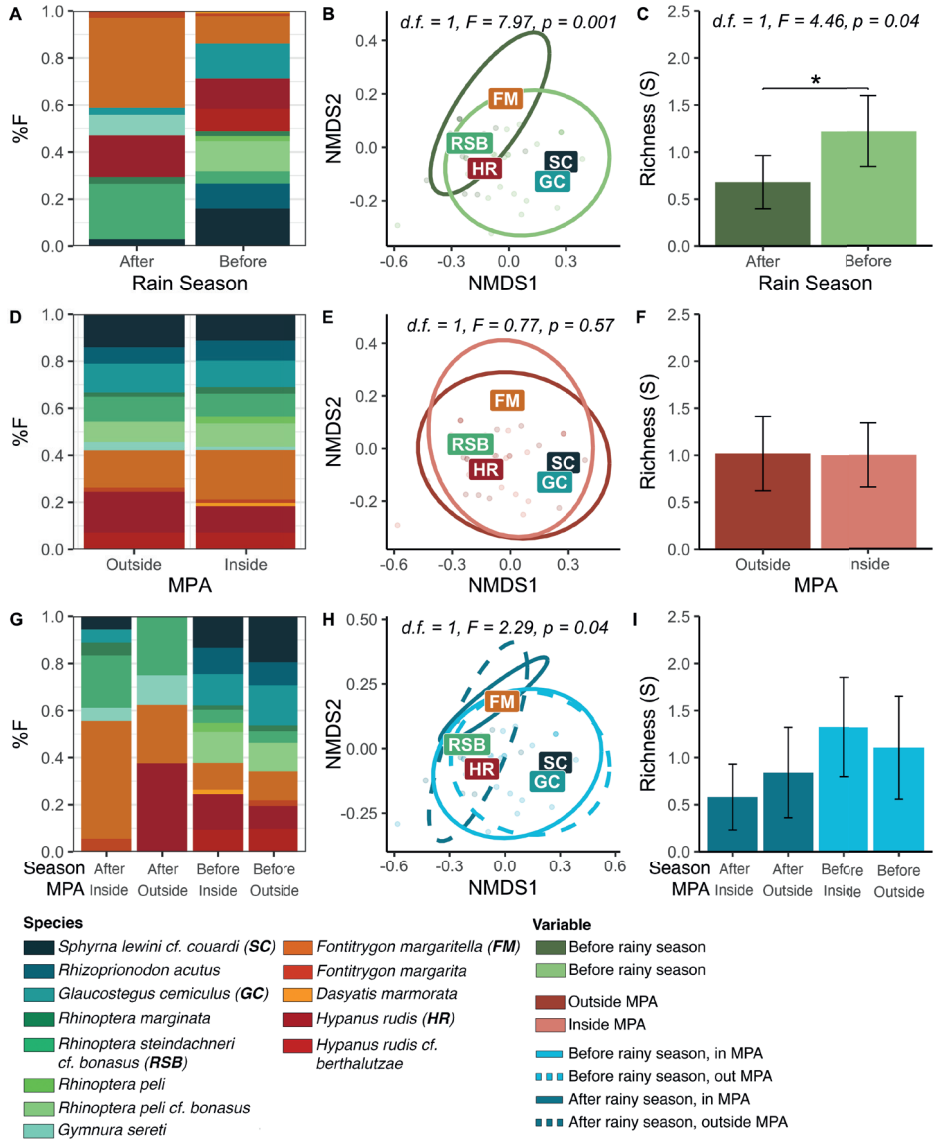

Figure 5.5 The influence of season (A-C), marine protected areas (D-F) and their interaction between (G-I) on the frequency of occurrence of a species (%F; left column), species composition (NMDS; center column), and the species richness (S; right column). Species are indicated by their different colors, with the five most common species indicated in the NMDS (FM = *Fontitrygon margaritella*, RSB = *Rhinoptera steindachneri cf. bonasus*, HR = *Hypanus rudis*, SC = *Sphyrna lewini cf. couardi*, GC = *Glaucostegus cemiculus*).

Discussion

For effective marine conservation, information on species presence, richness, and community composition is essential, especially in regions where resources for conservation are limited. In remote, highly dynamic and often highly turbid ecosystems like intertidal areas, resolving data deficiency of a species group can be challenging as many other observational methods are either unsuitable or require high research and financial capacity.

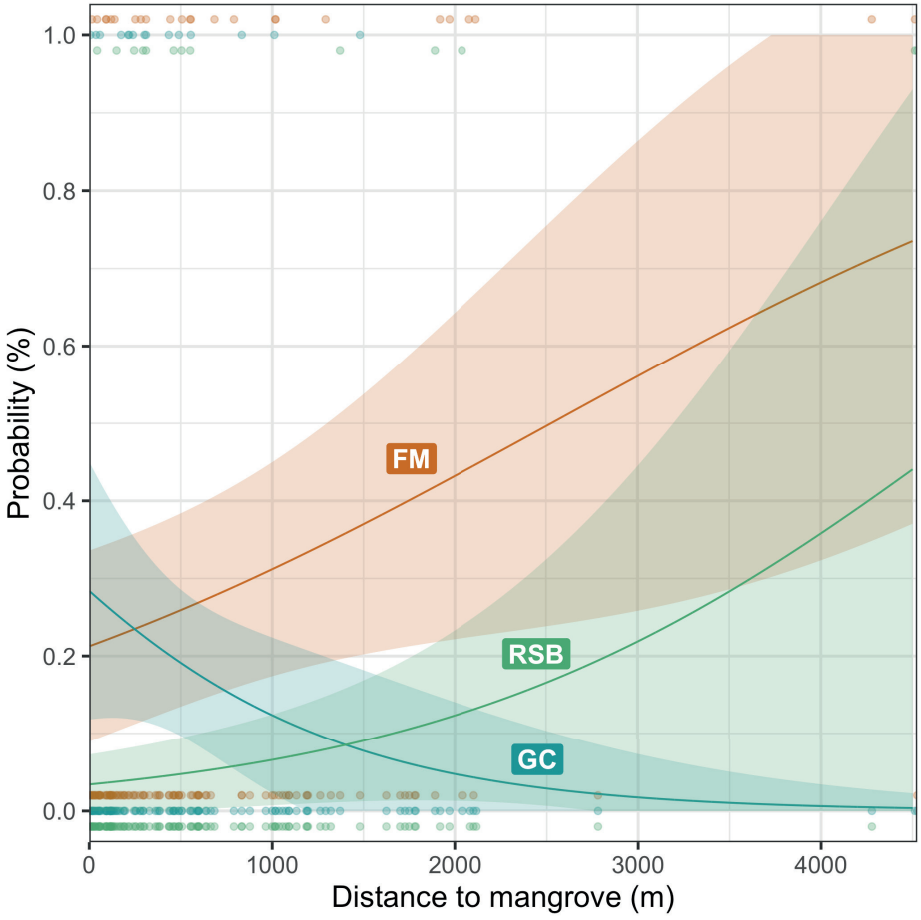

Figure 5.6 The probability of detecting the pearl whipray (Fontitrygon margaritella, FM), the cownose ray Rhinoptera steindachneri cf. bonasus (RSB), and the blackchin guitarfish (Glaucostegus cemiculus, GC) with increasing distance from the mangrove edge.

In this study, we aimed to solve data deficiency of elasmobranch species in the remote and dynamic Bijagós Archipelago in Guinea-Bissau, comparing an eDNA approach

with fisheries observer data. We confirmed the presence of 13 elasmobranch species (2 sharks and 11 rays, including 7 threatened species) in the Bijagós Archipelago using an eDNA approach, including the spatial distribution of these threatened species throughout the archipelago. An additional 8 species, including 7 IUCN threatened species, were solely detected by the fisheries observer program. In addition, our study confirms that species composition and richness of the elasmobranch community are mostly influenced by seasonal changes related to changes before and after the rainy season and less by differences between habitats (e.g., proximity to mangroves and estuary) or protective status of the sampling area. Our results show that an eDNA approach can successfully be used to tackle data deficiency on the presence of threatened shark and ray species on a local scale in highly dynamic coastal areas, including the indication of priority areas for the conservation of critically endangered species.

Species presence and distribution

The four most commonly detected species, the pearl whipray, scalloped hammerhead shark, smalltooth stingray and the blackchin guitarfish, were detected in sampling locations throughout the archipelago. These results suggest that the Bijagós Archipelago is an important area for these elasmobranch species. Coastal areas are known to be important nurseries or feeding areas for many elasmobranch species (Knip *et al.* 2010). Intertidal areas such as the Bijagós Archipelago and the habitats it provides can play an important role as (seasonal) feeding refugia for (early life stages of) sharks and rays (Leurs *et al.* 2023). For example, the scalloped hammerhead shark is known to use shallow coastal areas during early life stages before moving to a more pelagic habitat in deeper waters (Simpfendorfer and Milward, 1993, Zanella *et al.* 2019). This is confirmed by our preliminary results of the observer program, which shows that the majority of scalloped hammerheads captured within the archipelago are immature (Leurs, unpublished data).

Like many (early life stages of) elasmobranch species (Nagelkerken *et al.* 2008, White and Potter 2004), the blackchin guitarfish likely relies on the extensive mangrove forests of the Bijagós Archipelago. Our results show a higher probability of detecting this species close to the mangroves, which coincides with the catches of newborns and young-of-the-year individuals close to the mangrove edge, suggesting this species uses the mangrove edge as a nursery habitat (Leurs, unpublished data). Alternatively, for the pearl whipray, all life stages are likely to use coastal areas, including intertidal habitats, for feeding (Clements *et al.* 2022, Nauta *et al.* submitted).

The relative abundance of the species is potentially site-specific, as the pearl whipray is one of the most captured species in other coastal areas in The Gambia and Senegal (Moore *et al.* 2019, Jabado *et al.* 2021), but catches in the Banc d'Arguin in Mauritania are low (Lemrabott 2023).

Rare species like the largetooth sawfish (*Pristis pristis*) and African wedgefish (*Rhynchobatos luebberti*) were not detected in this study. Sawfishes are considered to be extinct from the West African region, with the last documented sawfish record originating from 2004 from the Bijagós Archipelago (Robillard and Seret 2006, Diop and Dossa 2011, Leeney and Poncelet 2015). Observations of the African wedgefish are increasingly rare within the region (Moore 2017). However, recent in-depth interviews and photographic evidence with fishers confirmed the capture of one specimen in March 2021 in the Bijagós (Leurs *et al.* unpublished). Novel eDNA approaches have a higher sensitivity for species-specific detection of rare and cryptic species compared to the approaches used in our study (e.g., Droplet Digital PCR, see Lehman *et al.* 2020).

Effects of season and protective status

We showed that species composition and richness of elasmobranch communities in the Bijagós Archipelago are mainly influenced by seasonality, with a higher species richness before the rainy season resulting in different species composition across the two seasons. The region's rainy season causes freshwater influx between June and October, significantly lowering salinity levels (Lafrance 1994, Cross 2014). As salinity can be one of the most important drivers of elasmobranch species composition in estuarine areas (Plumlee *et al.* 2018), it is likely that the observer changes are caused by changes in the abundance of species. In the Bijagós Archipelago, the differences between the two seasons are likely caused by the blackchin guitarfish and scalloped hammerhead shark, the presence of which was significantly lower after the rainy season. The fact that the movements of hammerhead sharks and guitarfishes can be influenced by changes in precipitation has been confirmed for other coastal areas (Hensley *et al.* 1998, Corgos and Rosende-Pereiro 2022). However, increased precipitation has also been linked to increased availability of crustaceans, the main prey of many guitarfishes (Lara-Mendoza *et al.* 2015). A decrease can also cause elasmobranch species to move away from coastal areas due to higher metabolic rates associated with maintaining osmoregulation (Meloni *et al.* 2002). Our results suggest that the blackchin guitarfish and scalloped hammerhead shark possibly move to waters with a relatively higher salinity during or right after the rainy season.

Furthermore, our results show that the richness and composition of elasmobranch species were similar between samples taken from protected and non-protected areas. This can be explained by the influences of horizontal water mixing due to tidal currents (Miya 2022) or by the mobility of shark and ray species, which likely move between protected and non-protected areas within the archipelago. Another explanation for the fact that no differences were found between protected and non-protected waters is the continued (targeted) fishing of elasmobranchs within both areas (Moranghajogo 2012**)**. These results suggest that the eDNA approach can successfully determine changes in species composition of elasmobranch communities across seasons and habitats in dynamic coastal areas.

eDNA-based monitoring of elasmobranch communities

The eDNA approach and fishery observer program differed in the number of species that were recorded, suggesting that a combination of monitoring methods is recommended for a complete overview of the elasmobranch community in highly dynamic (coastal) environments (Polanco Fernandez *et al.* 2021). eDNA-approaches have been described to resolve the phantom diversity of sharks and rays (Ip *et al.* 2021). However, in our study, no shark species of the genus *Carcharhinus* was detected using the eDNA approach, despite species from the genus being recorded amongst catches of the small-scale fisheries. Moreover, the large majority of eDNA reads assigned to elasmobranchs were assigned to ray species (82.7%). Possible explanations for differences in relative read abundance in eDNA approaches are differences in mobility or site fidelity of species, physiological differences, or the use of the eDNA methodology itself. Sharks are generally thought to move over larger distances compared to benthic ray species (Braccini *et al.* 2016). This may cause DNA concentrations of more mobile species to be low compared to species with high fidelity to the sample site. However, the differences in DNA shedding rates between species can also cause a bias in relative read abundance within marine communities (Tréguier *et al.* 2014, Stewart 2019). Benthopelagic myliobatid rays (i.e., eagle rays) excrete considerably more mucus compared to other elasmobranch species (e.g., guitarfish, sharks) (Meyer and Seegers 2012), possibly causing an imbalance in the detection of rays and shark species when the whole elasmobranch community is studied. However, another likely explanation for the differences could be caused by a lower occurrence of sharks within the archipelago due to their continued exploitation in and outside the archipelago. Differences in relative read abundances may also have a methodological origin. For example, PCR conditions might favor the DNA

amplification of specific species (Miya 2022). For this reason, we used two different primers to amplify ray and shark DNA in each sample separately and pooled the PCR products prior to sequencing. The storage time of samples (i.e., time between sample collection and fixation) can influence the read abundance (Eichmiller *et al.* 2016). However, storage time in our study was not correlated with a change in species richness. We emphasize that the translation of relative read abundance to relative species abundance should be done with caution and recommend that - if likely factors influencing relative read abundance are not addressed - eDNA-data should be translated into the presence/absence of species (Tréguier *et al.* 2014, Barnes and Turner 2016, Stewart 2019, Miya 2022).

Implications for elasmobranch monitoring and conservation

The eDNA approach used in our study successfully detected elasmobranch species throughout the study area but failed to detect some species that were detected in the fisheries observer program. Other studies have concluded that the combination of eDNA-approaches with conventional monitoring methods, such as the collection of fisheries data, underwater visual census and (baited) video monitoring, can improve the quality of collected data (Boussarie *et al.* 2018, Budd *et al.* 2021, Ip *et al.* 2021). Conventional methods often underestimate the presence of cryptic and rare elasmobranch species, are selective to specific species (e.g., due to elusiveness or selection bait used), or are less suitable to be used in specific areas (e.g., due to limited underwater visibility or a lack of fisheries to monitor). Hence, the locality of the study area and the elasmobranch community at hand determines which combination of monitoring methods is most appropriate, also considering the objectives of the monitoring program.

Our results suggest that the large majority (54%) of shark and ray species detected in this study are threatened with extinction on a global scale. In addition, in more than half of the samples collected, no shark or ray DNA was detected, and only two shark species were identified based on the eDNA approach: the milk shark and the scalloped hammerhead shark. Elasmobranchs within the wider West African region are at risk of being caught within coastal areas like the Bijagós Archipelago by artisanal small-scale fisheries (Lemrabott 2023, Moore *et al.* 2019) or by industrial fisheries on the outer edges of these areas once certain species leave their coastal habitats (e.g., ontogenetic habitat shifts/migrations) (Zeeberg *et al.* 2006, Leurs *et al.* 2021). The fishing effort of both types of fisheries has increased over the past decade and is a threat to the conservation status of sharks and rays within the wider West African region (Campredon and Cuq 2001, Dossa and Diop 2011, Kroodsma *et al.* 2018).

Our results show that an eDNA approach to elasmobranch monitoring can successfully be used in highly dynamic coastal areas with continued high exploitation of elasmobranchs to address the data deficiency on elasmobranch presence, distribution and community composition. Especially when this method is combined with conventional monitoring methods such as the collection of fisheries-dependent data. The information of this novel combination of techniques provides solid evidence on the distribution and status of threatened shark and ray species that benefits the more effective conservation of remote and highly dynamic coastal ecosystems.

Acknowledgments

This work was funded through the INNOfonds of the World Wildlife Fund Netherlands, the 'Waders of the Bijagós' project of the MAVA Foundation, and the Shark Conservation Fund. LG was funded by NWO grant 016.VENI.181.087. We would like to thank the entire crew of the Instituto da Biodiversidade e das Áreas Protegidas and the local fishers with whom we have collected the samples for this study for the close collaboration. We thank Peter de Knijff and Rick de Leeuw for their help with designing the sequencing protocol and bioinformatics analyses, and for sequencing our samples at the Department of Human Genetics, Leiden University Medical Centre (The Netherlands). We would like to thank Thimo Mulder for assisting in the laboratory and Jolanda Brons and Marco van der Velde for advice on lab procedures and the use of equipment. Lastly, the authors would like to thank Kasper Meijer for feedback on the analysis.

BOX D: SPECIAL OBSERVATIONS IN THE BIJAGÓS ARCHIPELAGO

Over the past five years, our team has studied the sharks and rays of the Bijagós Archipelago. During our expeditions, we worked with local fishers, conducted scientific fishing surveys (i.e., catch and release), interviewed fishers, and conducted landing site and market surveys. Below, we present important observations that have not (yet) been published.

Cryptic and elusive

The African wedgefish (*Rhynchobatus luebberti*) is the only species of wedgefish that occurs within the region (i.e., Mauritania to Democratic Republic of Congo). However, its range and abundance have significantly decreased over the past decades (Kyne and Jabado 2019). However, in March 2021, during our fisheries surveys (i.e., **Chapter 4**) with local researcher Assana Camará, we confirmed the first observation of this species within the waters of the Bijagós since 2006 (**Figure D1**; Moore 2017). This large male African wedgefish was captured near the island of Boloma and measured approximately 180 to 240 cm in total length. The species is likely captured more often in the Bijagós, as fishers referred to this species as 'casapai pintado' (spotted guitarfish) and indicated catching this species in recent years. This suggests that this species still occurs in the Bijagós and that the area may be an important refuge for this critically endangered species.

The relatively large thorny whipray (*Fontitrygon ukpam*) was initially only known from freshwater lakes and rivers from Nigeria to the Democratic Republic of Congo (Last *et al.* 2016). However, in February 2019, we sampled a fishing boat that had just captured four specimens in the (marine) waters around the island of Orango.

Figure D1 The African wedgefish (*Rhynchobatus luebberti*; left) and the thorny whipray (*Fontitrygon ukpam*; right) were captured in the Bijagós Archipelago.

We confirmed species identification by genetic sequencing in collaboration with the Florida Museum for Natural History (i.e., home to the curated database of the Chondrichthyan Tree of Life project). We determined this was a significant range and habitat extension for this species. Based on the known size-at-birth of this species (~30cm disc width), these four individuals represented early life stages (39-44 cm disc width). As a result, this range extension is now included in the latest IUCN Red List assessment of this species, which determined the species to be critically endangered (Jabado *et al.* 2021).

In addition, we confirm that large-bodied sharks are still present within the archipelago. Large (>2m total length) nurse sharks (*Ginglymostoma cirratum*) and tiger sharks (*Galeocerdo cuvier*) were observed during scientific research and landing site surveys. Large bull sharks (*Carcharhinus leucas*) were observed breaching out of the water completely, which has been described to be indicative of feeding behavior in juveniles of the species (Curtis and Macesic 2011).

Newborns and potential nursery areas

Coastal, shallow-water areas lined with mangrove forests such as the Bijagós Archipelago are often important refuge and nursery areas for the early life stages of elasmobranch species (Knip *et al.* 2010). However, the value of the Bijagós as a potential nursery area for sharks and rays remains unclear. Due to a large number of catches of newborn blackchin guitarfish (*Glaucostegus cemiculus*) in shallow-water mangrove habitats over the past years, we can conclude that these mangrove habitats are likely important nursery habitats for this critically endangered species. We regularly captured specimens between 30 to 35 cm in length with remnants of the umbilical cord, indicating birth within the last ~14 days (**Figure D2**; Debaere *et al.* 2023). Blackchin guitarfish can reach a maximum total length of 265 cm (Last *et al.* 2016). Similarly, we captured and documented newborn pearl whiprays (*Fontitrygon margaritella*), blacktip sharks (*Carcharhinus limbatus*), bull sharks (*Carcharhinus leucas*), and scalloped hammerhead sharks (*Sphyrna lewini*) of which umbilical scars were not fully closed (i.e., an indication of birth within the past <36 days, Debaere *et al.* 2023). In addition, we observed pregnant and near-term females of milk sharks (*Rhizoprionodon*) and *Fontitrygon*-species captured within the waters of the archipelago.

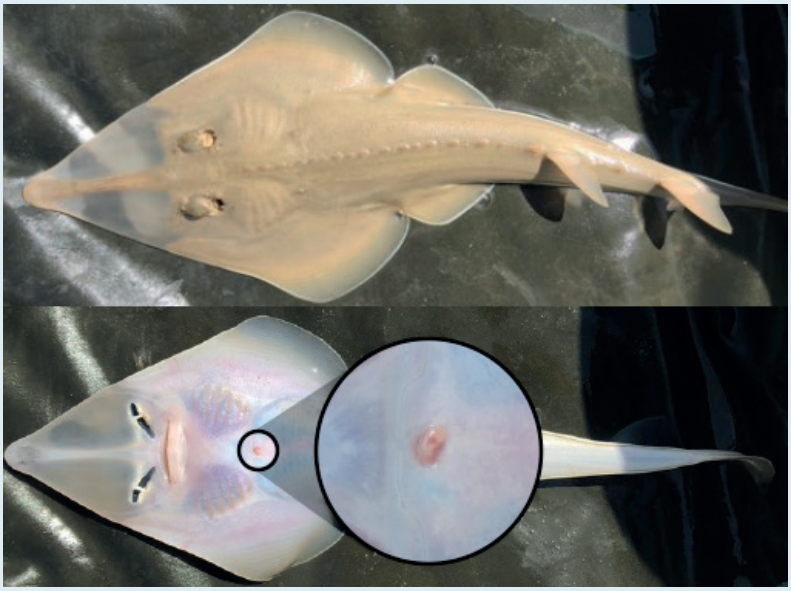

Figure D2 A newborn blackchin guitarfish (*Glaucostegus cemiculus*) with remnants of the umbilical scar visible (bottom).