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Use of eDNA to test hypotheses on the ecology of *Chironex fleckeri* (Cubozoa)

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ABSTRACT: Considerable gaps in our understanding of cubozoan ecology exist due to challenges associated with their detection. Environmental DNA (eDNA) removes the need for physical identification, offering a new approach to detect and study these elusive taxa. The objective of this study was to utilise eDNA as an ecological tool to test hypotheses surrounding the ecology of the Australian box jellyfish *Chironex fleckeri*, through examining the presence of both polyp and medusa life history stages. Additionally, the utility of eDNA as a proxy of abundance was explored. This study was conducted within and outside of Port Musgrave, a semi-enclosed estuarine system in northern Australia. eDNA proved successful in detecting both life history stages. Polyps were detected during winter when medusae were absent. This detection allowed investigation into potential polyp habitat. Polyps were exclusively detected in habitats characterised by nearby patches of rocky substrata and shallow carbonate reefs, with no detection occurring in mangrove habitats. The highest frequency of medusa detections occurred within Port Musgrave, while detections outside were more sporadic. Through comparing the distributions of both life history stages, evidence suggests that Port Musgrave is likely a population stock of the species, aligning with predictions from biophysical models. Finally, use of eDNA as a proxy of abundance showed a poor relationship, which can be attributed to likely higher variance in eDNA concentrations resulting from the spatially dispersed nature of the jellyfish. We conclude that eDNA provides a new approach to study cubozoan ecology and will provide critical information needed to mitigate against their threat of envenomation.

KEY WORDS: Cubozoa · Box jellyfish · Environmental DNA · Detection · Life history · Polyps · Ecology · Population structure

1. INTRODUCTION

Cubozoans (box jellyfish) are a class of marine taxa which contain members described as the most venomous organisms on the planet (Chung et al. 2001, Kintner et al. 2005, Bentlage et al. 2010, Gershwin et al. 2013, Kingsford & Mooney 2014). Stings from these species can result in severe reactions, hospitalisation of the recipient and potentially death (Fenner et al. 1996, Fenner & Harrison 2000, Gershwin et al. 2013). The ability to manage this risk of envenomation is a global challenge faced by stakeholders and decision makers (Kingsford et al. 2018). To overcome this challenge, an increased understanding surrounding the ecology of these organisms is needed (Kingsford & Mooney 2014). Significant knowledge gaps exist surrounding cubozoan jellyfish life histories and population structures/dynamics (Kingsford & Mooney 2014). These gaps result from challenges associated with their detection due to the elusive nature of cubo-

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zoans, their transparency and their spatial and temporal variability (Kingsford & Mooney 2014, Kingsford et al. 2018). Environmental DNA (eDNA) has emerged as a formidable tool in ecological research which significantly enhances our ability to detect and monitor elusive species (Beng & Corlett 2020). Subsequently, it has been highlighted as a detection tool to overcome some of the above-mentioned challenges, as the approach removes the need to physically capture and morphologically identify individuals (Jerde et al. 2011, Sigsgaard et al. 2015, Barnes & Turner 2016, Smart et al. 2016, Evans et al. 2017). This genetic detection tool has recently been applied successfully to detect multiple jellyfish species (Bayha & Graham 2008, Gaynor et al. 2017, Minamoto et al. 2017, Ames et al. 2021, Bolte et al. 2021) and most recently, has been developed for *Chironex fleckeri* (Morrissey et al. 2022).

C. fleckeri is considered the most notorious cubozoan species (Gershwin et al. 2013, Kingsford & Mooney 2014). It is responsible for over 200 deaths to date in the Indo-Pacific region and contains the most potent venom of any organism on the planet (Gershwin et al. 2013). In an effort to understand more about the life history, presence and abundance of this jellyfish, Morrissey et al. (2022) developed a highly sensitive and specific eDNA detection assay for *C. fleckeri*. Morrissey et al. (2022) further showed that an eDNA approach is able to detect *C. fleckeri* medusae when they have been confirmed as present in an ecosystem. Additionally, as jellyfish eDNA has been found to decay rapidly (Minamoto et al. 2017, Bolte et al. 2021, Morrissey et al. 2022), comparable to that of multiple marine fish and invertebrate species (Sassoubre et al. 2016, Wood et al. 2020, Kwong et al. 2021), eDNA detection likely reflects the close proximity of an individual of the targeted species (Morrissey et al. 2022). eDNA therefore provides a spatially explicit detection tool which is significantly advantageous for studying cubozoan jellyfish ecology (Morrissey et al. 2022). In addition to detection, eDNA has previously been used as a proxy for species abundance (Thomsen et al. 2012b, Pilliod et al. 2013, Lacoursière-Roussel et al. 2016, Wilcox et al. 2016, Yamamoto et al. 2016). Although this relationship has not been validated for cubozoan jellyfish, if the relationship was established, then, in addition to detection, eDNA may allow for further investigation surrounding spatial and temporal abundance variation of cubozoan jellyfish (Morrissey et al. 2022). Thus, such a robust methodology would allow ecological hypotheses related to cubozoan population ecology to be tested (Kingsford et al. 2021, Morrissey et al. 2022).

Research has largely focused on detecting the medusae stage of cubozoan jellyfish due to their direct threat to human health and enterprise (Fenner et al. 1996, Fenner & Harrison 2000, Bordehore et al. 2011, Gershwin et al. 2013, Kingsford et al. 2018, Bolte et al. 2021). However, cubozoans have a polymorphic life history consisting of 2 major stages, the medusa and the polyp stages (Kingsford & Mooney 2014, Kingsford et al. 2018). Polyps, due to their tiny size (1–2 mm), are a challenge to detect and study in their natural environment, and to date, cubozoan polyps have only been located twice; Cutress & Studebaker (1973) found *Carybdea xaymacana* polyps in Puerto Rico, within mangrove channels, and Hartwick (1991) reported a few *C. fleckeri* polyps in Australia, within an estuarine river. An example of how difficult cubozoan polyps have been to find, Hartwick (1991) reported that he spent 7 yr undertaking intense and timely *in situ* searches within multiple Australian estuarine systems to locate polyps of *C. fleckeri*. Even after this intense surveying, only a few polyps were ever found. Consequently, to fully understand the ecology of *C. fleckeri*, a more effective approach to identify and narrow down areas of the habitat where *C. fleckeri* polyps reside is needed (Morrissey et al. 2022). eDNA has potential here, as Bolte et al. (2021) demonstrated the successful use of eDNA to detect habitat putatively holding polyps of *Copula sivickisi* near the substratum in seasons when medusae were absent. As *C. fleckeri* medusae are generally found in defined seasons (Kingsford & Mooney 2014, Kingsford et al. 2018), there is the potential to identify source locations of polyps when medusae are absent. Accordingly, this would provide information on a critical component of cubozoan jellyfish population dynamics.

The detection of *C. fleckeri* polyps in an estuary by Hartwick (1991) led to the assumption that polyps of *C. fleckeri* reside within estuarine environments. Recent evidence however, from the use of statolith microchemistry profiles (Mooney & Kingsford 2012), has questioned this assumption. Mooney & Kingsford (2012) concluded that suitable habitat for *C. fleckeri* polyps may extend beyond estuaries to marine environments. The thermo/osmotic tolerances of cubozoans highlight the ability of these organisms to endure a large range of conditions and hence their potential to reside in a range of environments (Courtney et al. 2016, Mooney & Kingsford 2016, Rowley et al. 2023). eDNA may provide more information beyond general environmental conditions to that of specific habitat types, such as mangroves, reefs and seagrass. This would advance our understanding of

the requirements of polyps, which can be quite specific (Cargo 1979, Brewer 1984, Svane & Dolmer 1995, Zang et al. 2023). Understanding habitat requirements and hotspots of polyps would significantly aid in furthering understanding on cubozoan ecology, specifically sources of medusae and their stock boundaries (Kingsford et al. 2021).

Mesopopulations, or stocks nested within metapopulations, are the population units of greatest interest to ecologists, as they are largely self-sustained and are relevant to understanding the ecology of an organism (Sinclair 1988). Despite the general assumption that jellyfish are planktonic and therefore should have high levels of connectivity, there is growing evidence that cubozoan stock boundaries are often at small spatial scales (Kingsford et al. 2021). Data on cubozoan distributions (Kingsford et al. 2021), statolith morphometrics (Mooney & Kingsford 2017b) and statolith microchemistry profiles (Mooney & Kingsford 2012, 2017a, Morrissey et al. 2020) support restricted distributions and discrete populations for some of the ~50 cubozoan species (Collins & Jarms 2018). Recently, biophysical modelling has also been utilised to make predictions on the dispersal of medusae and likely stock boundaries (Schlaefer et al. 2018, 2020, 2021). Schlaefer et al. (2020) reported that *C. sivickisi* medusae are likely to stay within 2 km of a bay, and that populations were at the scale of hundreds of metres to kilometres wide. Schlaefer et al. (2018) further reported that *C. fleckeri* medusae were retained within a semi-enclosed estuarine bay, when modelled both as passive and swimming, and therefore concluded that cubozoan stocks may often be at the scale of estuaries and bays with unlikely connectivity to other populations. As eDNA can be used as a spatially explicit detection tool, it allows for the testing of these model predictions.

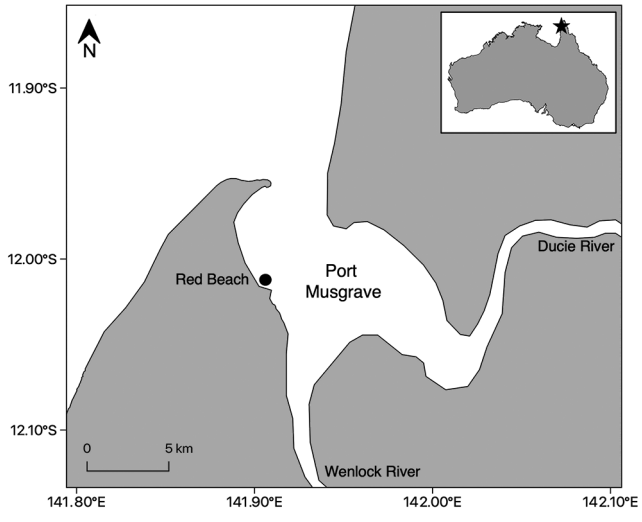

Fig. 1. Study location (star in the inset): Port Musgrave, Cape York Peninsula, Queensland, northern Australia

The objective of this study was to employ a box jellyfish (*C. fleckeri*) eDNA assay (Morrissey et al. 2022) to test hypotheses surrounding the ecology of this species. Specifically, this study examined the following: (1) *in situ* testing of eDNA concentrations as a proxy for *C. fleckeri* abundance, (2) the distribution of *C. fleckeri* medusae throughout and outside of a semi-enclosed estuarine system, (3) the use of eDNA to detect the elusive polypoid stage of this species, (4) how the distribution of medusae, based on eDNA, compares with that of the eDNA putatively detected from polyps, and (5) identifying habitats in which polyps are detected.

2. MATERIALS AND METHODS

2.1. Study area

This study was conducted in Port Musgrave, Cape York Peninsula, Queensland, Australia (11.99° S, 141.91° E). The area is a semi-enclosed shallow estuarine system with a ~3.5 km wide mouth (Fig. 1). Two major rivers, the Wenlock and Ducie Rivers, feed directly into Port Musgrave. Knowledge of *Chironex fleckeri* medusae abundance 'hotspots' (Red Beach) and strong ecological information surrounding the species exist for this area (Schlaefer et al. 2018). Qualitatively, water clarity nearshore in Port Musgrave ranged from 0.2 to 3 m, while along beaches outside of the port, water clarity was greater and estimated to be 3 to 10 m. At each sampling site, a conductivity, temperature and depth device (CTD; Seabird SBE 19 Plus) was used to measure both salinity and temperature among sites, and to examine the level of stratification. A high level of stratification could indicate that eDNA could be trapped below a halocline/thermocline (Gray & Kingsford 2003, Bolte et al. 2021, Littlefair et al. 2021). Given the absence of stratification, the temperature and salinity measurements for each site are reported as average values taken across the water column.

2.2. Field sampling

Schlaefer et al. (2018) studied the behaviour and likely dispersal of *C. fleckeri* in Port Musgrave and

Fig. 2. Sampling locations and sites located inside and outside of Port Musgrave, North Queensland, covering sandy beach (grey dots), mangrove (white dots) and outside port (black dots) habitats. Sampling sites are numbered. Black lines indicate location boundaries, which are labelled A–G

predicted that medusae were likely to remain in the Port due to favourable oceanography, strong swimming behaviour and an orientation to nearshore environments. We therefore hypothesised that abundance and eDNA detections would decrease towards the mouth of the Port and with distance along the coast to the north and south. Sampling for eDNA was undertaken along a gradient leading from a known hotspot, Red Beach (Sites 12–14), to the mouth of the Port, approximately 8 km away (Fig. 2). Sampling sites were located ~600 m apart, including both beach and mangrove habitats. In addition, samples were collected up both major river systems, the Wenlock and Ducie Rivers, to examine the extent to which the species occurred within the semi-enclosed estuary. Areas outside of the Port were also sampled, along a gradient leading into the Gulf of Carpentaria and at sites located along the shore both north and south of the mouth.

We also hypothesised that any detection of eDNA in the study area during the Austral winter, when *C. fleckeri* medusae are absent (no detection via seine netting or casual observations) (Hartwick 1991, Gordon & Seymour 2012, Mooney & Kingsford 2012, 2016), could only be explained by the presence of benthic polyps. Accordingly, sampling was undertaken both during Summer (December 2020) and mid-winter (July 2021), when medusae were respectively present or absent. To confirm the absence of medusae in mid-winter, sampling using seine net drags (mesh size of 3 cm) was undertaken at each sampling site. This ensured that no medusae individuals were still present following the previous Australian box jellyfish season, as water temperatures were within their tolerance levels (Hartwick 1991). Sampling during mid-winter had the potential to identify local hotspots of polyps.

For each sample site, 2 replicate 2 l water samples were collected. These samples were filtered (10 µm pore size) in the field immediately after collection and were stored in Longmire's buffer at a temperature of 4°C until processed. Prior to the collection of each replicate, an equipment control was also undertaken. Specific details surrounding collection, handling and storage of eDNA samples can be found in Morrissey et al. (2022).

2.3. Jellyfish abundance versus eDNA concentration

The estimated abundance of *C. fleckeri* medusae in the field was correlated with copies of eDNA to determine if eDNA could provide a robust proxy for jellyfish abundance. At each site, 2 methods, namely visual counts and a beach seine, were used to estimate abundance of jellyfish and to ground-truth detections using eDNA. eDNA samples were collected first to

avoid potential contamination from the fragments of jellyfish that could have been in a net. Visual transects (1–2 m deep) involved 2 trained observers located on the bow of the boat. Transect width was measured using a 3 m pole off the bow of the boat, and each observer counted in a 1.5 m lane over a 100 m distance. Jellyfish were collected with a 100 × 30 m beach seine net drag (mesh size of 3 cm) with an estimated sample volume of 1200 m^3. Seines and visual counts were centred where eDNA samples were collected. Both visual and seine net transects were undertaken for beach sampling sites, while only visual transects were undertaken for mangrove sites where obstacles prevented the use of nets. Number and sizes (inter-pedalia distance, IPD) of all captured *C. fleckeri* medusae were noted.

2.4. eDNA extraction and purification

The 'preserve, precipitate, lyse, precipitate and purify' method (Edmunds & Burrows 2020), adapted for extraction from filter papers (Cooper et al. 2021), was utilised to extract collected eDNA. Following extractions, the eDNA sample was purified through use of the Zymo One Step PCR Inhibitor Removal kit (Zymo IR; Zymo Research), as per the manufacturer's instructions. Purified eDNA was stored at −20°C until quantified. Specific details surrounding eDNA extractions and purifications can be found in Morrissey et al. (2022).

2.5. Quantitative PCR

A multiplexed TaqMan assay developed by Morrissey et al. (2022) (Table A1 in the Appendix) was used to detect, quantify and interpret presence of *C. fleckeri* eDNA via qPCR. QuantStudio 3 and 5 Real-Time PCR systems (Applied Biosystems, ThermoFisher Scientific) were utilised for this purpose. Each reaction consisted of 2 µl of eDNA template, 10 µl of TaqMan Environmental Master Mix 2.0, 0.7 µM sense and anti-sense *C. fleckeri* primers, 0.525 µM sense and anti-sense endogenous control primers and 0.25 µM of both *C. fleckeri* and endogenous control TaqMan MGB probes, and adjusted with MilliQ water to a final volume of 20 µl. A 2-step cycling profile was used: 95°C for 10 min, followed by 50 cycles of 95°C for 15 s and 60°C for 1 min. Six technical replicates were run per sample, and each plate contained at least 3 negative controls, extraction blanks (negative control monitoring contamination during extraction procedures), a positive control (*C. fleckeri* genomic DNA template) and synthetic DNA standards (10 000 to 1 copy μl^{-1}) which acted to ensure consistency among plates and allowed for quantification. Positive detection of *C. fleckeri* for a water sample was reported with minimum amplification of a single technical replicate (Trujillo-González et al. 2019, Budd et al. 2021, Cooper et al. 2021). Any positive detection was confirmed to be *C. fleckeri* through clean up and bidirectional Sanger sequencing of PCR product (undertaken by the Australian Genome Research Facility, Brisbane) and cross checking against reference sequences. Further, use of the endogenous control assay assures appropriate use of methods, their success, lack of false negative detection and PCR inhibitors (Furlan & Gleeson 2017).

2.6. Statistical analysis

Two measures of positive detection of *C. fleckeri* eDNA from a water sample were used. Each sampling site was utilised as the unit of measure for eDNA concentration (copies l^{-1}). Replicate filters (n = 2) were treated as sub-samples with positive technical replicates being averaged to represent eDNA concentration (copies l^{-1}) at each sample site (Thomsen et al. 2012a, Goldberg et al. 2013, Congram et al. 2022). Additionally, detections were also reported as number of positive technical replicates out of 12 per sampling site.

A robust comparison of eDNA concentrations inside and outside of the Port was obtained by analysing clusters of sites that were close together as the factor location. Accordingly, our sampling design addressed variation among locations (a = 7) separated by ~600 m to 20 km, and sites within locations separated by hundreds of metres to kilometres (b = 3). The data were tested with a fully hierarchical nested ANOVA that provided a critical test for each level of the design. Furthermore, the proportion of the variation explained for each level of the design was estimated with variance components using the raw data (Kingsford 1998). Following the recommendations of Underwood et al. (1997), the data were log transformed to satisfy the assumptions of the statistical test. A Kendall's tau correlation test was used to investigate the relationship between eDNA quantity/positive technical replicates and *C. fleckeri* medusa abundance. In all statical analyses, critical p was <0.05. All statistical analyses were undertaken using R (Version 4.1.2, R Core Team 2021) or SYSTAT (Version 13).

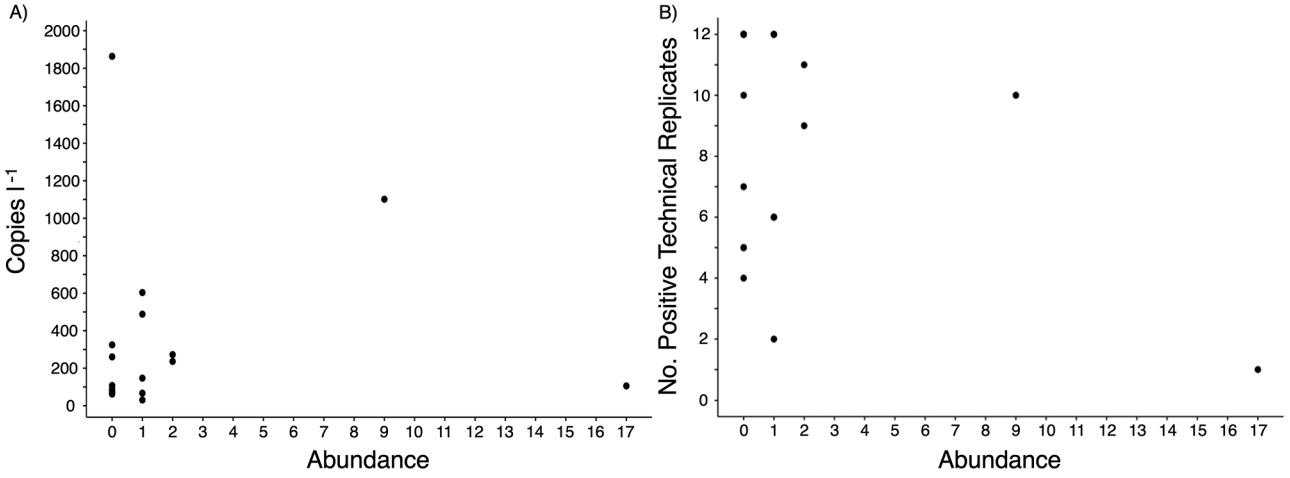

Fig. 3. Relationship between (A) eDNA copies (l^{-1}) and *Chironex fleckeri* medusa abundance, and (B) number of positive technical replicates and *C. fleckeri* medusa abundance, caught via seine net drags, at each sampling site

3. RESULTS

3.1. eDNA quantity as a proxy of *Chironex fleckeri* medusa abundance

There was a poor relationship between abundance of *C. fleckeri* medusae in seines and eDNA concentration (copies l^{-1}) ($tau_{(n = 17)}$ = 0.15, p = 0.45) and number of positive technical replicates ($tau_{(n = 17)}$ = −0.11, p = 0.59) via this *in situ* trial (Fig. 3). eDNA detection of *C. fleckeri* occurred at all sites where medusae were observed in counts and/or were captured in seines. A total of 34 *C. fleckeri* medusae were captured in beach seines within Port Musgrave. Further, densities of *C. fleckeri* medusae ranged from 0.7 to 21 ind. 1000 m^{-3} (Table A2). The poor relationship between densities in seines and eDNA quantity was attributed to the Red Beach sites where large numbers of medusae were collected in nets, but concentrations of eDNA were low (Fig. 3). Only 3 individuals were observed with visual transects in the low-visibility waters; only 1 individual was found in and around mangrove habitats (Site 10).

3.2. Detection and distribution of *C. fleckeri* medusae during the Australian box jellyfish season

C. fleckeri eDNA was found throughout the study area, both inside and outside of the Port, in summer. eDNA was detected at all 25 sampling sites and in 93% of field replicates (Figs. 4 & 5). Of the technical replicates, 63.9% displayed detection, and eDNA ranged from 21.91 to 2374.01 copies l^{-1} (Table 1). All equipment controls confirmed the absence of contamination, and the endogenous control demonstrated appropriate use and success of collection, handling, and extraction methods, as well as the absence of PCR inhibitors (Ct value of 35.7 ± 0.5) in analysed samples. There were large differences in eDNA concentrations among sites; however, there were no significant differences among close or widely separated locations, in both the concentration of eDNA (Table 2) and number of positive detections

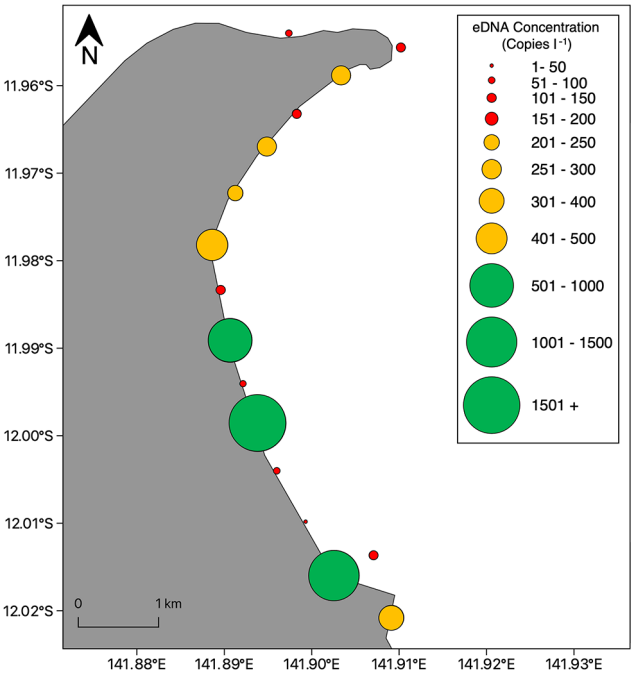

Fig. 4. Sampling sites within Port Musgrave with positive detections of *Chironex fleckeri* medusa eDNA. Bubbles indicate eDNA concentrations (copies l^{-1})

Table 1. Environmental DNA (eDNA) sample collection locations and sites (see Fig. 1), with site description, depth (m), depth-integrated temperature and salinity, number of positive technical replicates and eDNA quantity (copies l^{-1}) during the summer sampling period

Site no.	Location	Habitat description	Water depth (m)	Temperature (°C)	Salinity (ppt)	No. of positive detections/12	Copies l^{-1}
1	A	Sandy Beach with carbonate reef — mouth of Port Musgrave	5.53	35.7	30.3	6	120.7
2	A	Sandy beach with carbonate reef	0.9	35.7	30.3	12	260.3
3	A	Sandy beach with carbonate reef	0.93	35.7	29.2	6	147
4	B	Sandy beach with carbonate reef	0.95	35.8	29.2	9	272.4
5	B	Sandy beach with carbonate reef	1.08	35.7	29.1	11	236.1
6	B	Sandy beach with carbonate reef, ~600 m from mangroves	1.14	35.8	29.1	12	488.1
7	C	Mangroves	0.87	35.7	27.9	5	107.8
8	C	Mangroves	1.08	35.6	28.4	12	603.5
9	C	Mangroves	0.83	35.3	29.6	4	61.9
10	D	Mangroves	0.69	35.7	29.4	12	1863.5
11	D	Mangroves	0.7	35.5	29	10	80.8
12	D	Sandy beach with rocky substrate, bordering mangroves — Red Beach	0.9	35.5	28.7	2	30.4
13	E	Sandy beach with rocky substrate, bordering mangroves — Red Beach	0.67	35.8	31.6	1	1101.1
14	E	Sandy beach with rocky substrate, bordering mangroves — Red Beach	0.85	35.5	29	7	105.5
15	E	Mangroves	0.73	35.6	28.5	12	324.5
16	—	Open ocean outside of the Port	11.4	35.6	30	1	32.4
17	—	Open ocean outside of the Port	5.19	35.6	29.9	5	36.9
18	F	Sandy beach — south and outside of the Port, with reef, >6 km from Port mouth	0.97	35.7	30.3	9	225.3
19	F	Sandy beach — south and outside of the Port, with reef, <6 km from Port mouth	0.87	35.7	30.5	3	21.9
20	F	Sandy beach with rocky substrate — south and outside of the Port, ~1 km from the Port mouth	1.07	35.6	30.2	5	88.5
21	G	Open ocean outside of the Port	19.99	35.6	29.6	11	65.6
22	G	Sandy beach — north and outside of the Port, <6 km from Port mouth	1.14	36	29.6	12	2374
23	G	Sandy beach — north and outside of the Port, >6 km from Port mouth	0.91	36.3	29.7	12	1340.4
24	—	Estuarine river	0.5	26	30	12	1187.9
25	—	Estuarine river	4.4	30.5	29.5	1	34.3
Average	—	—	—	35.1 (30.5—36.3)	29.5 (27.9—31.6)	—	—

(out of the 12 technical replicates). Differences among locations only represented 0—3.5% of the variation (Table 2); this was especially obvious outside of the Port and near beaches where many copies were recorded at some sites and very few at others. Variation at the replicate level was also very high and explained 43—82% of the variation. Additional detections found outside of the Port were generally low and away from beaches. Detections were also found 21.5—28.5 km from the mouth of Port Musgrave up the Ducie and Wenlock Rivers, where salinities were 30 to 29.5 ppt, matching the other sample sites.

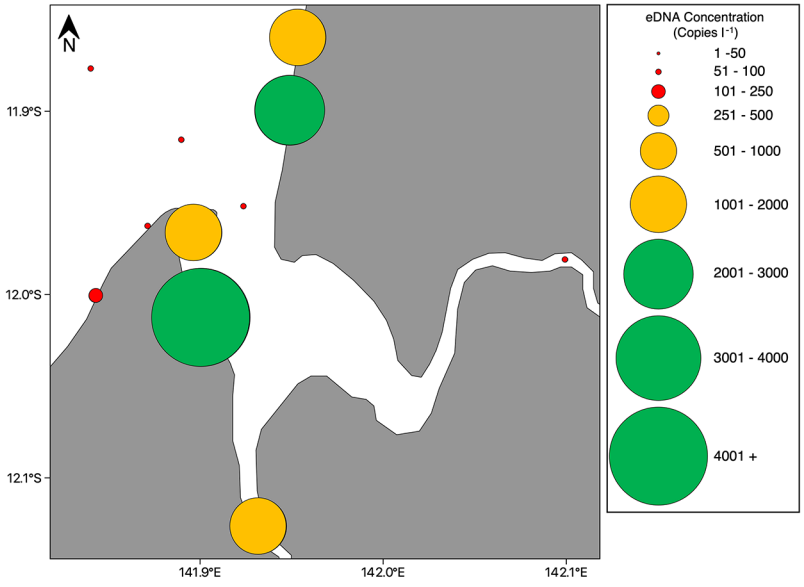

Fig. 5. Sampling sites outside and within (sites grouped) Port Musgrave with positive detections of *Chironex fleckeri* medusa eDNA. Bubbles indicate eDNA concentrations (copies l^{-1})

CTD profiles did not detect stratification of the water column in temperature or salinity where it was possible that eDNA could have been trapped below a thermocline/halocline even in water columns of up to 12 m deep. Temperatures and salinities were similar throughout the study area and along the estuarine gradient. Temperature and salinity were therefore unlikely to influence the patterns of eDNA described in this study (Barnes et al. 2014, Collins et al. 2018, Lamb et al. 2022). Temperatures generally ranged from 35.3–36.3°C; exceptions were low temperature readings (26 and 30.5°C) in shallow waters of the Wenlock and Ducie Rivers. Salinities ranged from 27.9 to 31.6 ppt (Table 1).

Table 2. Nested ANOVA, *Chironex fleckeri* environmental DNA copies (l^{-1}); data transformed (log $x+1$) and number of positive technical replicates (raw data) among locations and between sites nested in locations (ns: not significant; ***p < 0.001); all variance components (% var) were calculated from untransformed data

Source	df	Copies (l^{-1})			Positive technical replicates		
		MS	*F*	% var	MS	*F*	% var
Location	6	82.675	0.987 ns	3.6	5.802	0.776 ns	0
Site (Location)	14	83.75	26.49 ***	53.1	7.476	3.75 ***	18.1
Residual	231	3.161		43.3	2.048		81.9

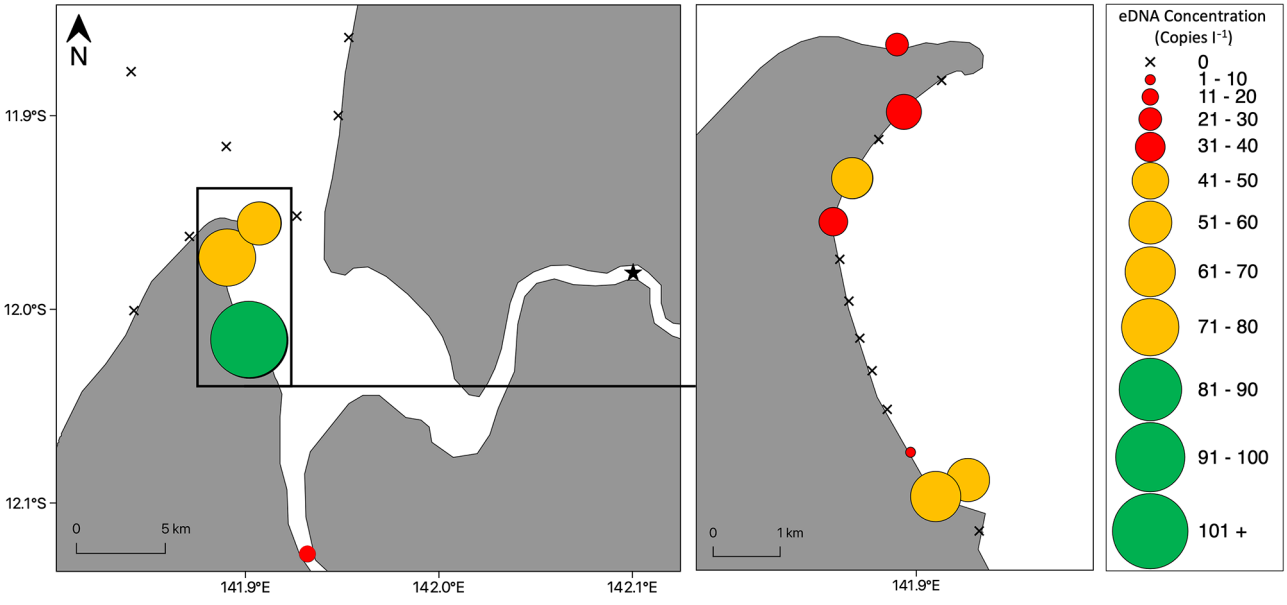

Fig. 6. Sampling sites within and outside of Port Musgrave with positive and negative detections of *Chironex fleckeri* polyp eDNA when medusae were absent. Bubbles indicate eDNA concentrations (copies l^{-1}). Star indicates that no data were collected at the site during this sampling time

3.3. Detection and distribution of *C. fleckeri* polyps

Positive detections of *C. fleckeri* were found within the Port outside of the recognised medusa season (Fig. 6). These detections could therefore only be due to the presence of the benthic polyp stage. No medusae were observed or collected in beach seines at the time of sampling. If medusae were present, eDNA detection would also be expected on the open coast, in higher concentrations across the study area, and within mangrove habitat, as was observed during summer sampling. *C. fleckeri* eDNA was detected at 9 out the 24 sampling sites and in 50% of field replicates. Of the technical replicates, 10.2% displayed detection, and eDNA ranged from 0.46 to 73.38 copies l^{-1} (Table 3). eDNA concentrations were found to be

Table 3. Environmental DNA (eDNA) sample collection locations and sites, with site description, depth (m), depth-integrated temperature and salinity, number of positive technical replicates and eDNA quantity (copies l^{-1}) during the winter sampling period

Site no.	Location	Habitat description	Water depth (m)	Temperature (°C)	Salinity (ppt)	No. of positive detections/12	Copies l^{-1}
1	A	Sandy Beach with carbonate reef — mouth of Port Musgrave	2.9	26.4	32.3	1	73.4
2	A	Sandy beach with carbonate reef	0.31	29.2	31.7	0	—
3	A	Sandy beach with carbonate reef	0.38	27.6	32	1	33
4	B	Sandy beach with carbonate reef	0.64	27.2	31.9	0	—
5	B	Sandy beach with carbonate reef	0.45	26.8	31.9	1	48.4
6	B	Sandy beach with carbonate reef, ~600 m from mangroves	0.27	29.1	31.8	1	29.7
7	C	Mangroves	0.15	27.1	31.7	0	—
8	C	Mangroves	0.3	27.3	31.7	0	—
9	C	Mangroves	0.65	27	31.2	0	—
10	D	Mangroves	0.65	27.2	31.4	0	—
11	D	Mangroves	0.47	27.1	31.3	0	—
12	D	Sandy beach with rocky substrate, bordering mangroves — Red Beach	0.38	28.1	31.5	1	0.5
13	E	Sandy beach with rocky substrate, bordering mangroves — Red Beach	0.54	27.4	31.5	1	52.6
14	E	Sandy beach with rocky substrate, bordering mangroves — Red Beach	0.51	28.3	31.6	1	48.4
15	E	Mangroves	0.77	26.8	31.7	0	—
16	—	Open ocean outside of the Port	4.83	26.7	33.7	0	—
17	—	Open ocean outside of the Port	5.2	26.6	33.6	0	—
18	F	Sandy beach — south and outside of the Port, with reef, >6 km from Port mouth	0.74	27.5	34.2	0	—
19	F	Sandy beach — south and outside of the Port, with reef, <6 km from Port mouth	0.88	27.2	34.2	0	—
20	F	Sandy beach with rocky substrate — south and outside of the Port, ~1 km from the Port mouth	0.41	28.3	33.4	3	18.2
21	G	Open Ocean outside of the Port	18.09	27	33.4	0	—
22	G	Sandy beach — north and outside of the Port, <6 km from Port mouth	1.23	26.3	33.4	0	—
23	G	Sandy beach — north and outside of the Port, >6 km from Port mouth	1.14	26.6	33.5	0	—
24	—	Estuarine river	0.5	28.5	16	1	12.4
Average	—	—	—	27.4 (26.4—29.2)	31.7 (16—34.3)	—	—

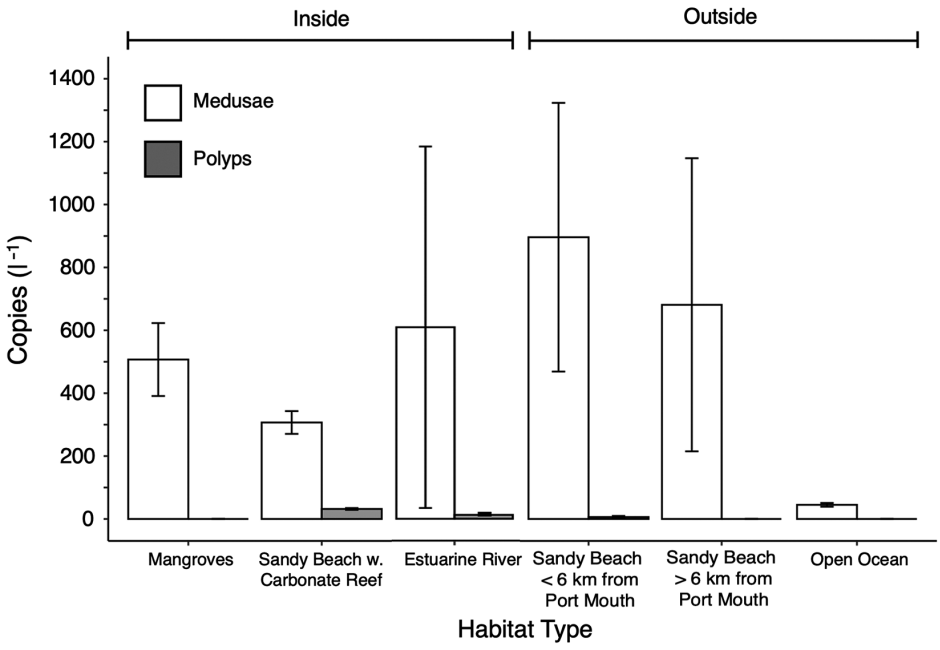

Fig. 7. Mean (±SE) environmental DNA copies (l^{-1}) for different habitat types, inside (mangrove, sandy beach with carbonate reef) and outside (sandy beach >6 km from the mouth of Port Musgrave, sandy beach <6 km from the Port mouth, open ocean) of Port Musgrave, for both *Chironex fleckeri* medusa and polyp stages. The detection of polyps outside of the Port was within 1 km of the Port mouth

considerably lower in comparison to those found in summer when the medusa stage was present (polyp mean eDNA quantity = 14.38 copies l^{-1}, medusa mean eDNA quantity = 504.86 copies l^{-1}) in addition to a lower detection rate in technical replicates (Tables 1 & 3). The equipment controls provided assurance of contamination-free conditions, while the endogenous control affirmed the proper application of collection, handling and extraction methods, with the absence of PCR inhibitors (Ct value [±SE] of 34.5 ± 0.37) in analysed samples.

Polyps were only detected at habitats with hard substratum, i.e. sandy beaches with carbonate reefs (Fig. 7). Detection of polyps did occur at 1 site outside of the Port that was close to the mouth (<1 km); it was also characterised by hard substrate (Site 1). Polyps were not detected in mangrove habitat.

CTD data showed that waters at all sample sites were uniform in temperature and salinity except within the Wenlock River, where salinity was considerably lower (16 ppt). Temperatures ranged from 26.4 to 29.2°C and salinities from 16 to 34.3 ppt.

4. DISCUSSION

4.1. eDNA as a proxy of abundance for cubozoan jellyfish

A poor association between eDNA concentration/number of positive technical replicates and *Chironex fleckeri* medusa abundance was found. This contrasts the strong positive associations between these 2 factors reported for numerous other taxa (Takahara et al. 2012, Thomsen et al. 2012b, Pilliod et al. 2013, Lacoursière-Roussel et al. 2016, Wilcox et al. 2016, Yamamoto et al. 2016), including scyphozoan jellyfish (Minamoto et al. 2017). The poor association may result from the spatially dispersed nature of *C. fleckeri* medusae. *C. fleckeri* medusae were spatially dispersed, with densities ranging from 0.7 to 1.7 ind. 1000 m^{-3} (as per this study), with highest densities occurring at known medusae 'hotspots' (21 ind. 1000 m^{-3}). The lower abundances of *C. fleckeri* medusae, in comparison to other taxa (e.g. *Chrysaora pacifica*, tens to hundreds of individuals per 150 m^2; Minamoto et al. 2017), would result in lower eDNA concentrations, as found in this study (in comparison to those reported by Minamoto et al. 2017 for *C. pacifica* medusae; 7.05×10^2 to 2.60×10^5 copies l^{-1}). The precision of eDNA-based abundance estimates is influenced by the amount of eDNA present (Pilliod et al. 2013). Additionally, as eDNA concentrations were lower, influence of both biotic and abiotic factors on eDNA presence and persistence would drive higher variation in eDNA concentrations, thereby further impacting this association (Barnes et al. 2014, Barnes & Turner 2016, Collins et al. 2018, Harrison et al. 2019, Jo et al. 2019, Stewart 2019, Huerlimann et al. 2020, Lamb et al. 2022).

Considerable variation in eDNA concentrations between neighbouring sample sites was found in this study. eDNA concentrations were significantly different between neighbouring sites within locations. This is prominently displayed at Red Beach, where samples (Sites 13, 14 and 15) were taken tens to hundreds

of metres apart. Varying abundances of *C. fleckeri* medusae were present at this location (1, 17 and 9 individuals, respectively) with associated eDNA concentrations (30, 105 and 1101 copies l^{-1}, respectively) which did not align with our estimates of abundance from seines. This variation is suggested to result from the above-mentioned factors, specifically, variability in release of eDNA from individuals (shedding rate), related dispersion from source individuals, transport of eDNA via oceanographic processes, and rates of decay. Therefore, the use of eDNA as a proxy for species abundance is likely to be more complex and variable for spatially disperse and rare taxa due to complex eDNA dynamics (Harrison et al. 2019), and more so in tropical environments (Huerlimann et al. 2020). We have, however, demonstrated that use of the method for detection is robust.

Mesocosm trials, controlling for the influence of biotic/abiotic factors should be undertaken to examine this use of eDNA within a controlled environment. Further, we require a greater understanding of variability in release of eDNA (shedding rates) from individuals of varying sizes and life-history stages (Klymus et al. 2015), and understanding of the related dispersion of eDNA in marine environments (Harrison et al. 2019). Increased replication would allow for less variation in eDNA concentrations (Pilliod et al. 2013). Further, clustering sites and using a nested sampling design provides more accurate measures of spatial variation in eDNA. Finally, there was potential for tiny medusae (<3 cm IPD) to avoid detection via seine netting, and we suggest that plankton tows, in areas where possible (lack of net snags and estuarine crocodiles), be utilised for further in-field exploration of this potential relationship. It should be noted, however, that *C. fleckeri* medusae smaller than the mesh size were on occasion captured, likely due to the pinching of the mesh, making it functionally smaller, and due to debris that was commonly found in the net.

4.2. Distribution of *C. fleckeri* medusae

We found great spatial variation in eDNA over spatial scales of hundreds of metres to tens of kilometres, which has not been documented for cubozoans. Within Port Musgrave, there was a high frequency of detection of eDNA and considerable variation in eDNA concentrations among sites within locations. The high detection rate of medusae within the Port is likely attributed to the presence of mangrove habitat. Post-larval, juvenile and small adult fish, along with juvenile crustaceans, which are common medusa prey (Carrette et al. 2002), are known to be of higher abundance within mangroves (Robertson & Duke 1987). Gordon & Seymour (2009) suggested that *C. fleckeri* medusae may remain in these areas of higher prey abundance to minimise energy expenditure. These findings further align with and support the biophysical models of Schlaefer et al. (2018). The models of Schlaefer et al. (2018) predicted medusae to aggregate in shallow waters within 10s to 100s of metres from Red Beach, which is where the presence of medusae was highest and where the highest concentrations of eDNA within the Port were found. Additionally, the models showed a decrease in medusae towards the Port mouth, further aligning with what was found *in situ*, suggesting that medusae are largely retained within the Port. *C. fleckeri* medusae were also detected with eDNA up both major river systems. As these areas provide suitable conditions for medusae (Mooney & Kingsford 2016), it is likely that the species is present throughout the entire estuarine system. As a result, our findings align with the predictions made by the biophysical models of Schlaefer et al. (2018) regarding the distribution of *C. fleckeri* medusae within the Port.

C. fleckeri medusae were also detected at sample sites outside of the Port, with strong detections along the beach north of the mouth of the Port. However, the frequency of detection was lower among sites outside of the estuary. It is suggested that these detected medusas are undertaking excursions outside and along these beaches. This suggestion is supported by Gordon & Seymour (2009) who, via acoustic tracking, observed a large adult medusa individual to move from estuarine habitat to open coastal beaches and then return. Further, the biophysical models of Schlaefer et al. (2018) showed high retention within the Port, with less than 2.5% of modelled medusae being advected from the Port, hence aligning with this suggestion. Regarding the transect leading from the Port mouth out into the Gulf of Carpentaria, it should be noted that there was some detection of eDNA at these sample sites. However, this detection may result from the aforementioned excursion, dispersal of eDNA from the Port via tidal currents or even from fragments of dead jellyfish from predation by turtles and other predators (although the frequency of predation is unknown). To further investigate these suggestions, models are required that combine diffusion of eDNA, accounting for the influence of tides and currents, and decay rates to determine likely distances of detection from the source. Further, the genetics of populations found in

different estuaries are required to determine if the low ecological connectivity argued in this study and that of Schlaefer et al. (2018) contrasts with multi-generation time scales.

4.3. Cubozoan polyp detection with eDNA

4.3.1. Ability of eDNA to detect cubozoan polyps

This study successfully detected the putative presence of *C. fleckeri* polyps outside of the jellyfish season, when their medusa stage was absent. Therefore, eDNA has the potential to provide an effective and resource-efficient technique to locate the source locations (polyp beds) of this and other cubozoan jellyfish species (Bolte et al. 2021). Despite the lack of visual confirmation of polyps in their natural habitat, the utilisation of the eDNA technique aims to remove the necessity for such confirmation, considering the challenges involved in detecting cubozoan polyps. Subsequently, the positive detections putatively confirm the presence of the *C. fleckeri* polyp stage. The accuracy of and confidence in these detections are ensured by the sensitivity and specificity of the detection assay, the use of optimised methodologies for elusive species and the implementation of best practice control measures (Morrissey et al. 2022). Arguments suggesting that the detection arose from the medusae stage are invalid. This is because sampling took place in July, 2 mo after the end of the Australian box jellyfish season, when medusae are known to be absent due to their seasonality (Hartwick 1991, Gordon & Seymour 2012, Mooney & Kingsford 2012, Kingsford & Mooney 2014). Additionally, *C. fleckeri* eDNA is known to decay rapidly (Morrissey et al. 2022), and no medusae were collected in seines or observed during the sampling period. The detected eDNA concentrations and presence were found to be significantly lower and highly localised compared to when medusae were present. This aligns with the understanding that medusae are free-swimming, while polyps reside on the benthos with likely minimal movement once they establish themselves in a suitable habitat, providing further evidence for the diminished and more localised eDNA detections. Bolte et al. (2021) also successfully used eDNA to detect the polyp stage of the harmless cubozoan species *Copula sivickisi*. In their study, they detected *C. sivickisi* exclusively in near-substrate samples outside of the Australian jellyfish season, where the polyp stage of this species would be expected to reside due to its diurnal swimming behaviour (Garm et al. 2012). This current study has successfully demonstrated the use of eDNA to detect this elusive life history stage. The ability to do so is 'game-changing' for the field, and our understanding of the ecology of this and other cubozoan species, and their subsequent management, will greatly benefit.

4.3.2. Implications of cubozoan polyp detection

The ability to detect and locate cubozoan polyps has numerous benefits for our ability to advance understanding of this elusive and dangerous taxon. Current understanding surrounding cubozoan polyp ecology is based upon laboratory investigations (Courtney et al. 2016, Boco et al. 2019). eDNA opens the door to studying the basic ecology of this life history stage in its natural environment, which, until now, has been logistically challenging to undertake. The potential to examine the environmental conditions which this life history stage endures, impacts of weather events, such as freshwater pulses due to rain events, potential habitat (see Section 4.5), and investigation into potential abiotic (temperature, salinity, pH) and biotic (food availability) drivers of polyp metamorphosis, and subsequent seasonality of medusae, in *in situ* conditions, are now within reach of ecologists. Further, a more in-depth understanding surrounding the population dynamics and population stock boundaries (see Section 4.4) of these taxa is now possible with understanding of polyp locations due to their role as the major driver of medusa abundance and periodicity (Arai 1997, Kingsford & Mooney 2014). This will further allow for the identification of jellyfish 'hotspots', which is of great benefit for furthering our understanding of cubozoan ecology and for management of the risk of envenomation to water users. Additionally, the ability to locate cubozoan polyps allows for further investigation into how cubozoan jellyfish will respond to future oceanic conditions resulting from climate change (Klein et al. 2014). With the development and emergence of environmental RNA (eRNA) detection (Yates et al. 2021), which enables finer-resolution detection of organisms due to the rapid decay of eRNA, a higher resolution of polyp occurrence may be possible and assist physical *in situ* location of this life history stage. eDNA hence is the best available tool to advance understanding of key aspects of cubozoan ecology and, due to the ubiquitous nature of DNA, can be applied to all cubozoan species including other dangerous species such as Irukandji jellyfish (~16 species within the family Carybdeidae) (Gershwin et al. 2013).

4.4. Comparison of *C. fleckeri* medusae and polyp distributions to inform stock boundaries

As both life history stages can be detected with eDNA, it allows, for the first time, a comparison of their distributions which may inform the spatial extent of population stocks. Polyps are the source of medusae, and likely play a key role in the dispersal patterns of medusae. Their location, combined with the swimming behaviours of medusae, would influence the likelihood of retention. Currently, for cubozoans, growing evidence suggests that some species have population stocks of small spatial scales, to the extent of bays and estuaries, but the localities of the polyp stage have yet to be incorporated and considered (Kingsford et al. 2021). As polyps have been putatively detected with eDNA in this study, it allows us to explore this for *C. fleckeri*, and to subsequently test predictions on the stock structure of this species in this area inferred from biophysical models (Schlaefer et al. 2018).

C. fleckeri medusae were found to occur extensively within the Port and were detected at some beaches outside. However, *C. fleckeri* polyps were only detected within the Port or close to the mouth, indicating that medusae in the study area likely originated from within the Port. These detections of polyps provide further evidence that medusae found outside were likely undertaking excursions from within the Port. Additionally, Red Beach, which is a known 'hotspot' of medusae, was utilised as a seeding location for the biophysical models of Schlaefer et al. (2018). This aligned well with where the largest concentrations of polyp eDNA were found and thus reflects a real-world medusa source location. Medusa distributions reported in this study subsequently align with and provide support to those predicted by the biophysical models of Schlaefer et al. (2018). Furthermore, from the extensive presence of medusae within the Port and within both river systems which flow into the Port, we agree with the suggestion by Schlaefer et al. (2018) that the estuarine system likely contains numerous local populations with high connectivity.

Enclosed bays and associated estuarine conditions are ideal for retention based on favourable currents, the behaviour of medusae and the presence of polyp source locations. To further explore the spatial scales of cubozoan stocks, the spatial robustness of populations needs to be investigated from coastal environments of different geomorphologies, ranging from enclosed to open.

4.5. Cubozoan polyp habitat

As eDNA can be used to detect *C. fleckeri* polyps, it provides a new approach to further our understanding of the ecology of this species and to assess hypotheses surrounding cubozoan polyp habitat. This study looked for the presence of the *C. fleckeri* polyp stage in beach, mangrove and estuarine river habitats and is the first study to directly examine this association. Prior to eDNA, inferences on cubozoan polyp habitat have largely been limited to broad environment categories such as estuaries. These inferences have resulted from the *in situ* reporting of *C. fleckeri* polyps (Hartwick 1991), statolith microchemistry profiles (Mooney & Kingsford 2012) and understanding on cubozoan thermo/osmotic tolerances (Courtney et al. 2016, Mooney & Kingsford 2016, Rowley et al. 2023). In this study, the polyp stage was detected primarily in beach habitats containing hard substratum within the Port. Detection also occurred within the Wenlock River and at a single site outside of the Port which was in close proximity to the Port mouth (1 km south, containing rocky substrata). These findings hence support those of Mooney & Kingsford (2012), who analysed *C. fleckeri* statolith microchemistry profiles and suggested suitable habitat of *C. fleckeri* polyps to extend beyond estuaries to marine environments. Most interestingly, no detection of polyps was found at mangrove habitats within this study. This finding aligns with Hartwick's (1991) *in situ* reporting of *C. fleckeri* polyps, where polyps were only located under stones and shells and not on nearby mangrove structures. As a result, it is possible that mangroves are not suitable habitat for *C. fleckeri* polyps. The findings of this study hence highlight the ability of *C. fleckeri* polyps to reside in environments of varying environmental conditions, as is supported by known cubozoan thermal/osmotic tolerances (Courtney et al. 2016, Mooney & Kingsford 2016, Rowley et al. 2023). Additionally, it showcases the need for eDNA to directly locate this life history stage, as suggestions based on physical tolerances provide only general environmental conditions. Currently, no other technique is able to efficiently detect polyps.

Regarding specific substrata, we note that expansive carbonate reefs often associated with oysters were present at sites where *C. fleckeri* polyps were detected. Oyster reef, due to its structural complexity, likely makes for suitable habitat where polyps can reside in crevices with appropriate water flow (bringing in food and removing waste) (Chapman 1973, Holst & Jarms 2006), while also providing shelter from

predators and sedimentation (Svane & Dolmer 1995). This suggestion is supported by the *in situ* finding of *C. fleckeri* polyps by Hartwick (1991), which were located on the underside of rocks, and from knowledge on scyphozoan polyp habitat preferences, which are often quite specific (Brewer 1984, Svane & Dolmer 1995, Holst & Jarms 2007, Zang et al. 2023). Multiple species of scyphozoan polyps have been reported to reside on the underside of rocks, below overhangs and in concealed habitats (Cargo & Schultz 1966, Brewer 1976, Kikinger 1992, Svane & Dolmer 1995, Pitt 2000), which additionally provides further support for the suggestion of mangroves being unsuitable habitat. We therefore suggest that any hard substrata (carbonate oyster reef, coral reef, rocky substrata and potentially artificial substrates), in both estuarine and marine environments, are likely suitable habitat for the polyp stage of this and other cubozoan species. Additionally, the highest concentrations of polyp eDNA in this study were found at the medusa 'hotspot' (Red Beach), suggesting that such 'hotspots' may be good predictors for the presence of polyps. This makes logical sense as, due to the nature of cubozoan sexual reproduction (broadcast spawning or internal development), and understanding of scyphozoan jellyfish planula larvae behaviour, which initially show a geopositive reaction once developed (Holst & Jarms 2007), cubozoan planula likely settle promptly following release from medusae and reside in close proximity to these areas. It is therefore suggested that eDNA sampling efforts to locate the polyp stage of cubozoan jellyfish should target areas of higher medusa abundances and areas where hard substratum is present.

5. CONCLUSION

Significant knowledge gaps surrounding the ecology of cubozoan jellyfish exist as a result of the challenges associated with their detection (Kingsford & Mooney 2014, Kingsford et al. 2018). This study has demonstrated the use of eDNA as an ecological tool to investigate and address these critical gaps surrounding the ecology of this dangerous cubozoan taxon. Through an *in situ* trial, the potential of eDNA as a proxy for species abundance was explored, but the relationship between abundance in seines and eDNA was weak for *Chironex fleckeri*. This highlights the need for a deeper understanding of eDNA dynamics in marine environments, especially for rare/spatially disperse taxa. Distributions of the medusoid stage of *C. fleckeri* were found to be of spatially broad occurrence within the Port, with a lower frequency of detection being found outside of the Port suggesting that medusae may be undertaking excursions from the Port. Additionally, eDNA proved successful in detecting the elusive benthic polyp stage of the species, hence revealing their locality, which is 'game-changing' for the field. The seasonality of the medusa stage of this species, being absent during the Austral winter (Hartwick 1991, Gordon & Seymour 2012, Mooney & Kingsford 2012, Kingsford & Mooney 2014), enabled this detection. This, for the first time, allowed investigation into potential cubozoan polyp habitat. Polyps were only detected in habitats that had nearby patches of rocky substrata and shallow carbonate reefs, not in mangrove habitats. Therefore, any hard substructure adjacent to the shore which provides appropriate shelter and water flow, likely is suitable habitat for cubozoan polyps. Comparison of the distributions of both life history stages allowed for investigating the spatial extent of *C. fleckeri* population stocks, with evidence found to support predictions made by biophysical models (Schlaefer et al. 2018), that Port Musgrave likely represents a population stock of the species. This finding subsequently validates the use of biophysical models to examine the movements and population structures of cubozoan jellyfish. Accordingly, eDNA offers a novel ecological tool to investigate hypotheses surrounding the ecology of dangerous cubozoan taxa which will subsequently benefit coastal managers to better understand and mitigate the threat these species pose to both human health and enterprise.

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Appendix

Table A1. Multiplexed assay information for detection of *Chironex fleckeri* and endogenous control (adapted from Morrissey et al. 2022)

Assay	Label	Sequence	Reference
Chironex fleckeri	Chironex_16S_F Chironex_16S_R Chironex_16S_P	ATCTTCCACTGTCTCAGCTTTACC CCTCAGTACTCGTGTCTCCCTA (FAM)-CTCGTCCTTCCAAGTATAAG-(MGB)	Morrissey et al. (2022)
Endogenous Control – Generic Fish	Fish_16S_F Fish_16S_R Fish_16S_probe	GACCTCGATGTTGGATCA CTCAGATCACGTAGGACTTTA (VIC)-ACATCCTAWTGGTGC-(MGB)	Furlan & Gleeson (2016)

Table A2. Densities of *Chironex fleckeri* medusae obtained via seine net drags

Site	No. medusae captured	Density (ind. 1000 m^{-3})
4	1	0.89
5	2	1.76
6	2	1.54
7	1	0.73
13	1	0.92
14	17	20.96
15	9	8.84

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