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ANA CECÍLIA PINHO COSTA

ICHTHYOPLANKTON COMPOSITION AND DISTRIBUTION IN SEASCAPES OF EQUATORIAL ATLANTIC

FORTALEZA – CE

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Tese apresentada ao Programa de Pós-Graduação em Ciências Marinhas Tropicais da Universidade Federal do Ceará, como requisito parcial para a obtenção do grau de Doutor em Ciências Marinhas Tropicais.

Orientador: Prof. Dr. Marcelo de Oliveira Soares.
Coorientadora: Dra. Tatiane Martins Garcia.

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Aprovada em: __/__/____

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Aos meus pais, Eliomar e Fátima.

AGRADECIMENTOS

RESUMO

Os ovos e as larvas de peixe desempenham papel-chave no ciclo de nutrientes e no ciclo trófico dos oceanos e sua sobrevivência reflete diretamente sobre os estoques adultos. Apesar da importância de se identificar áreas e épocas de desova e berçário, poucos estudos sobre essa temática têm sido realizados em "seascapes" marinhos tropicais. A amostragem de ictioplâncton foi realizada em 12 estações, na plataforma continental interna, onde seis estações estavam localizadas nas áreas de banco de rodolitos e seis, no prado de fanerógamas marinhas. A amostragem foi realizada com auxílio de duas redes cônicas (diâmetro da boca: 50 cm e tamanho da malha: 300 μm e 200 μm). Um total de 8.094 ovos e 175 larvas foram coletados no prado de fanerógamas marinhas, e 2.014 ovos e 241 larvas foram coletados no banco de rodolitos (SRB). O número de ovos registrados foi maior no prado de fanerógamas marinhas, entretanto, o número de larvas de peixe foi ligeiramente maior em SRB. O prado de fanerógamas marinhas apresenta uma hidrodinâmica menos turbulenta, o que favorece a desova e a retenção dos ovos. Um habitat mais estruturado proporciona um melhor abrigo, especialmente para as larvas. Entretanto, conforme a ontogenia avança, os peixes podem explorar áreas menos abrigadas. Este comportamento foi observado neste estudo, onde o habitat SRB, menos estruturado, apresentou uma alta densidade de larvas na fase de desenvolvimento mais avançada. O domínio dos primeiros estágios larvais demonstra uma preferência por "seascapes" mais protegidos e menos turbulentos para desova e criação. Os resultados destacam que o mapeamento desses "seascapes" pode ajudar a estabelecer medidas de conservação para proteger a conectividade ecológica e as espécies tropicais importantes. Quanto aos ovos, pela primeira vez o método de DNA barcode foi usado para acessar a diversidade de ovos de peixes no Atlântico tropical. Trinta e um ovos de peixe foram sequenciados nesta análise. Um ovo foi identificado com sucesso em nível de ordem, três em nível de família, 20 em nível de gênero e sete em nível de espécie. O baixo número de ovos identificados a nível de espécie pode estar relacionado ao baixo número de DNAs codificados e depositados em bases de dados de espécies de peixes tropicais. Os resultados reforçam o uso desse método para fornecer dados confiáveis sobre a biodiversidade de peixes, desconhecida e subestimada em áreas marinhas de baixa latitude. Esta abordagem é altamente recomendada para gerenciamento futuro e os resultados apresentados podem ser aplicados em outras regiões costeiras equatoriais em todo o mundo, desde que um banco de dados genético confiável tenha sido desenvolvido.

Palavras-chave: ictioplâncton, "seascapes", berçário, pesca.

ABSTRACT

Fish eggs and larvae play key roles in nutrient and trophic cycling in oceans and their survival directly reflects on the adult stocks. Despite the importance of identifying spawning and nursery areas and seasons, few studies about them have been conducted in tropical seascapes. Ichthyoplankton sampling was carried out at 12 stations on the inner continental shelf, six located on the rhodolith bed areas and six in the seagrass meadows, with two conical nets (mouth diameter: 50 cm and mesh size: 300 μm and 200 μm). A total of 8,094 eggs and 175 larvae were collected from the seagrass meadow seascape, and 2,014 eggs and 241 larvae were collected in the rhodolith beds (SRB). The number of eggs was higher in the seagrass beds; however, the number of fish larvae was slightly higher in the SRB. Seagrass beds present less turbulent hydrodynamics and favor the retention of eggs and spawning. A more structured habitat provides better shelter, especially for larvae. However, as ontogeny progresses, the fish can explore areas with less shelter. This behavior was observed in this study, where the less structured SRB habitat had a high density of larvae in the later developmental stage. The dominance of earlier larval stages demonstrates a preference for more protected and less turbulent seascapes for nursery and offspring rearing. The results highlight that mapping of these seascapes (e.g., seagrass and rhodolith beds) will help to establish conservation measures to protect ecological connectivity and important tropical species. As for eggs, for the first time DNA barcode was used to access the diversity of fish eggs in the tropical Atlantic. 31fish eggs were successfully sequenced for the genetic analysis. One specimen was identified at order level, three at family level, 20 at genus level and seven specimens at species level. The low number of identified eggs may be related to the low number of barcoded DNAs of tropical fish deposited in databases. The results reinforce the use of DNA barcode to provide reliable data on unknown and belittled fish biodiversity in low-latitude marine areas. This approach is highly recommended for future management and the results presented can be applied in other equatorial coastal regions worldwide as long as a reliable barcode database has been developed.

Keywords: ichthyoplankton, seascapes, nursery, fisheries.

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General Introduction

The knowledge about the influence of the abiotic and biotic factors that drives the distribution and abundance of marine species remains a core question in ecology (Shuterland et al., 2013). It is known that physical variables, as temperature, salinity, currents, tides, depth, continental runoff (Hernández-Miranda et al., 2003; Zarrad et al., 2013) and/or biological processes, as spawning season and adult distribution (Hernández-Miranda et al., 2003; Zarrad et al., 2013), affect the spatial distribution and composition of ichthyoplankton. Nevertheless, there are still many gaps to be filled in the early life history of fishes especially in tropical coastal environments (Montgomery et al. 2006).

Most marine fish species are broadcast spawners and present a planktonic larval phase which are passively or actively transported (active transport given by swimming, when the ontogenetic development progresses) (Leis, 2006). Fish eggs and larvae play key roles in nutrient and trophic cycling in oceans (Hyndes et al. 2014) and their survival reflects on the adult stocks (Beck et al.,2001; Costa and Muelbert, 2017). Since these early stages respond promptly to disturbances, it provides a viable way to access human impacts and build fish stocks management actions (Ramos et al., 2015).

In tropical coastal regions, a great and belittled diversity of fishes can be found due to availability of habitats and unique ecosystems (Roberts et al., 2002). Coastal habitats like rhodolith beds, mangroves, reefs and seagrass beds are considered important habitats because of their complex three-dimensional structure (Lefheck et al., 2019). These interconnected habitats are economically and ecologically important as they provide a range of ecosystem services, acting in carbon and nutrient cycles, coastal protection, recreation (Barbier et al., 2011; Lefcheck et al., 2019) and also as nursery and reproductive zones by many species of invertebrates and vertebrates, such as fishes (Heck et al., 1997; Costa et al., 2020).

This knowledge about the distribution and composition of fish eggs and larvae is fundamental for the conservation of marine biodiversity and fishery resources (Menegotto and Rangel, 2018). Despite that, this knowledge is underestimated in equatorial marine areas even though in these low-latitude areas have been extremely impacted by both overfishing, local impacts, and climate change (Chaudhary et al., 2021).

These scenarios reinforce the urgency of studies targeted at the ichthyoplankton spatial distribution and abundance.

This PhD thesis aims to help to fill this gap through the evaluation of an important area in the Equatorial Southwestern Atlantic (Ceará coast, Brazil). This low-latitude shelf covers two important seascapes, such as seagrass and rhodolith beds, used by fishes in their life cycle. It is known that in these tropical seascapes there is a greater fish diversity and density than adjacent flattened areas (Heck et al., 1997; Horta et al., 2016). Because of this, these seascapes are classified as spawning and nursery grounds (Beck et al., 2001), including reef fishes (Moura et al., 2021). In these seascapes, larval fish can find food and protection throughout their development (Barletta-Bergan et al., 2002; Bonecker et al., 2007; Mota et al., 2017). Taking into account genetic and ecological aspects, the connectivity among the different seascapes is vital for fish species because they actually live in a mosaic of bottom and pelagic habitats according to the life cycle stage (Elliott et al. 2007; Teodósio et al. 2016; Santos et al., 2017).

As a consequence of the increase in near shore impacts due to human activities like eutrophication, fishing pressure and global climate change (Anderson et al. 2014; Freeman et al. 2019), it is essential to know the composition and spatial distribution of ichthyoplankton assemblages. In this regard, studies on fish eggs and larvae allow elucidate population dynamics (Amezcua et al., 2020); spawning areas, reproductive seasons (Macedo-Soares et al., 2009), and species composition (Mota et al., 2017).

In Equatorial South Atlantic, despite the species diversity, information on ichthyoplankton assemblages is still scarce (Hermes-Silva et al., 2009) and most of the research already carried out in this marine ecoregion were based only on morphological aspects (Macedo-Soares et al., 2012; Mota et al., 2017; Santana et al., 2020). Ichthyoplankton identification based on morphological descriptions is challenging because these organisms are small, fragile and some of the most commonly used taxonomic characteristics change greatly throughout ontogenetic development (Ré, 1999).

Morphological identification of eggs is even worse due to lack of diagnostic characters needed for species identification (Ahlstrom and Moser, 1980; Reynalte-Tataje et al. 2012) which can lead to errors in taxonomy (Larson et al., 2016). Fish eggs data provide even more reliable data about the distribution and abundance of spawning seascapes in comparison to the larval stages because it suffers less influence of transport

and mortality (Ouellet et al., 1997; Richardson et al., 2009). Despite their importance, few studies have focused on eggs identification on tropical Atlantic (Mota et al., 2014; Maltez et al., 2014; Lima et al., 2016; Santana et al., 2020) and the number of studies focus on the genetic identification of these specimens is ever smaller. In Brazil, studies using DNA barcode in the identification of fish eggs were carried only for continental fish species (Becker et al., 2015; Fratine-Silva et al., 2015; Nobile et al., 2019; Lima et al., 2020).

In this sense, this Ph.D. thesis has its originality and uniqueness in two core aspects. In the first, it has a spatial comparison of fish larvae and eggs in two distinct but interconnected seascapes. Second, it is the first study to use molecular identification to investigate marine fish assemblages in the equatorial Atlantic (Brazil). The present document is composed by two chapters:

Chapter 1. The aim of this chapter was to examine how the diversity and density of eggs and fish larvae at different developmental stages vary in shallow coastal seascapes (muddy sand with seagrass beds and gravelly sand with rhodolith beds). This first chapter was submitted and published recently in the journal Marine Environmental Research (Impact factor: 2.72) (Costa et al., 2020).

Chapter 2. The goals of this chapter were 1) to test the efficiency of this molecular identification method for the taxonomic identification of pelagic eggs from low-latitude regions with equatorial waters, and 2) to use the DNA barcode method for the identification of eggs under the hypothesis that the composition of the larval fish assemblage is reflected in the composition of planktonic eggs

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Chapter 1 - Seagrass and rhodolith beds are important seascapes for the development of fish eggs and larvae in tropical coastal areas
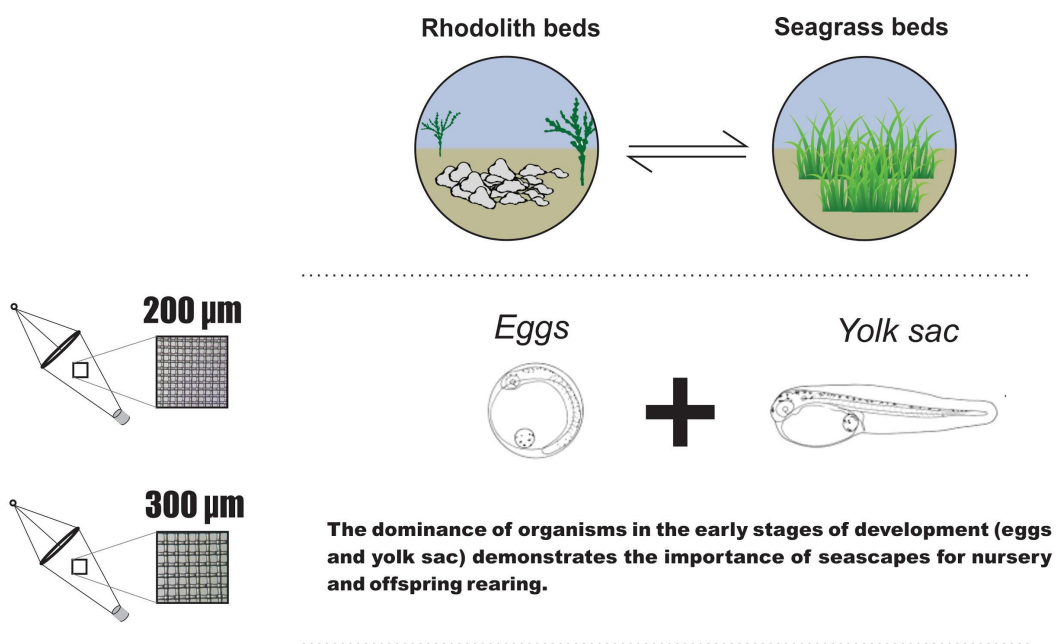
Figure 1. Graphical Abstract

Seagrass and rhodolith beds are important seascapes for the development of fish eggs and larvae in tropical coastal areas

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Abstract

In this study, the ichthyoplankton in two distinct tropical seascapes, gravelly sand with rhodolith beds (SRB) and muddy sand with seagrasses, were compared. The number of eggs was higher in the seagrass beds; however, the number of fish larvae was slightly higher in the SRB. Seagrass beds present less turbulent hydrodynamics and favor the retention of eggs and spawning. A more structured habitat provides better shelter, especially for larvae. However, as ontogeny progresses, the fish can explore areas with less shelter. This behavior was observed in this study, where the less structured SRB habitat had a high density of larvae in the later developmental stage. The dominance of earlier larval stages demonstrates a preference for more protected and less turbulent seascapes for nursery and offspring rearing. The results highlight that mapping of these seascapes (e.g., seagrass and rhodolith beds) will help to establish conservation measures to protect ecological connectivity and important tropical species.

Keywords: Ichthyoplankton; Seagrass meadow; Rhodolith beds; Connectivity; Fisheries

1.   Introduction

Shallow coastal areas provide important habitats for many species (Sánchez-Caballero and Borges-Souza 2018; Reis Filho et al. 2019). Essential fish habitats (EFHs) are environments that are necessary to maintain fish populations. Substrates within these habitats are used for multiple developmental stages, such as spawning, breeding, feeding, and growing to maturity (Benaka, 1999; Rosenberg et al. 2000).

One of the important types of coastal seascape, classified as EFHs, are seagrass, rhodolith, and seaweed beds (Amado-Filho et al. 2016; Eggerstsen et al. 2017). Seagrass beds (or seagrass meadows) and rhodolith beds have greater fish diversity and density than adjacent flattened areas (Bell and Pollard 1989; Heck et al. 1997; Horta et al. 2016), as they provide three-dimensional structures that are used as nursery, settlement, forage, and refuge areas (Berkström et al. 2012; Thiriet et al. 2014; Díaz et al. 2015). These structural complex areas are also used by important species that act as links in the marine food web and by fishery target species as spawning grounds (Bell et al. 1987; Huijbers et al. 2008; Nordheim et al. 2018).

Like terrestrial forests, seagrass meadows store large amounts of carbon, and there are initiatives to protect these blue carbon seascapes (Fourqurean et al. 2012; Macreadie et al. 2019). Moreover, rhodolith beds are important in global biogeochemical cycles, considering that they may form large biogenic carbonate deposits (Amado-Filho et al. 2016). However, because seagrass meadows and rhodolith beds are located in coastal areas, they have been under intense pressure from human activities, such as eutrophication, oil spills, habitat destruction, microplastics, and fishing (Kraufvelin et al. 2018; Huang et al. 2020; Magris and Tommaso 2020; Soares et al. 2020).

In coastal seascapes, larval fish can find food and protection throughout their development (Heck and Thoman 1984; Barletta-Bergan et al. 2002, Bonecker et al. 2007). The preference for habitats with specific types of substrates (Petry et al. 2003; Maynou et al. 2006) indicates that fish larvae can actively choose favorable habitats for their development (Grioche et al. 1997). Therefore, it is fundamental to understand the role of coastal seascapes in the recruitment and distribution of tropical fish eggs and larvae so that management strategies can be implemented (Hedberg et al. 2018). Important processes in the fish life cycle, such as settlement, distribution, and recruitment, are closely related to

characteristics of the seascapes they inhabit. For example, some organisms prefer seagrass beds that are less turbulent than unvegetated areas (Dorenbosch et al. 2004). When close to other connected shallow seascapes (e.g., coral reefs), seagrass and rhodolith beds can lead to increased density of some species and affect the assemblage structure (Gullström et al. 2008; Berkström et al. 2013; Carvalho et al. 2020). In this way, seagrass and rhodolith beds provide substrates and structural complexity that favor spawning and development (von Nordheim et al. 2018).

Despite the importance of identifying spawning and nursery areas, few studies about them have been conducted. In particular, a comparison of tropical ichthyoplankton in distinct seascapes, such as seagrass beds and unvegetated areas, has not yet been performed (Marcolin et al. 2010; Katsuragawa et al. 2011; Moussa et al. 2020). This kind of research will help to establish conservation measures to protect important fishes and may provide information about the ecological connectivity and overlap of species. The aim of this study was to examine how the diversity and density of eggs and fish larvae at different developmental stages vary in shallow coastal seascapes (muddy sand with seagrass beds *versus* gravelly sand with rhodolith beds).

2. Materials and Methods

2.1 Study area

The study area is in the Equatorial Southwestern Atlantic (Ceará coast, NE Brazil) (Figure 1) and is characterized by a marine ecosystem with warm waters (> 26 °C) and stable sea surface temperatures (< 5 °C of intra- and inter-annual variation) (Teixeira and Machado, 2013; Soares et al. 2019). This area is localized in the continuous subequatorial atmospheric circulation of the trade winds, which are persistent and intense throughout the year and show a mean wind velocity of 7.2 m/s (Maia et al. 2001; de Barros Corrêa et al. 2019). The climate is semiarid, and the estuaries are shallow with low river flow (Schettini et al. 2017). The Aracatimirim, Aracatiaçú, and Acaraú estuaries and the estuarine-lagoonal system related to coastal barriers are close to the study area (Figure 1). They are a *freshwater-deprived* system, due to irregular interannual rainfall distribution (e.g., 572.4 mm in 2005 and 2497.7 mm in 2009) and multiple dams along the watershed

(Pessoa, 2015). Moreover, precipitation is mainly influenced by the Intertropical Convergence Zone (ITCZ). The rainy season occurs between January and June, peaking from February to May, which was the period in which these samples were taken, and the dry season falls between July and December. Moreover, the sampling area is governed by a mesotidal regime and a westerly current (Maia et al. 2018).
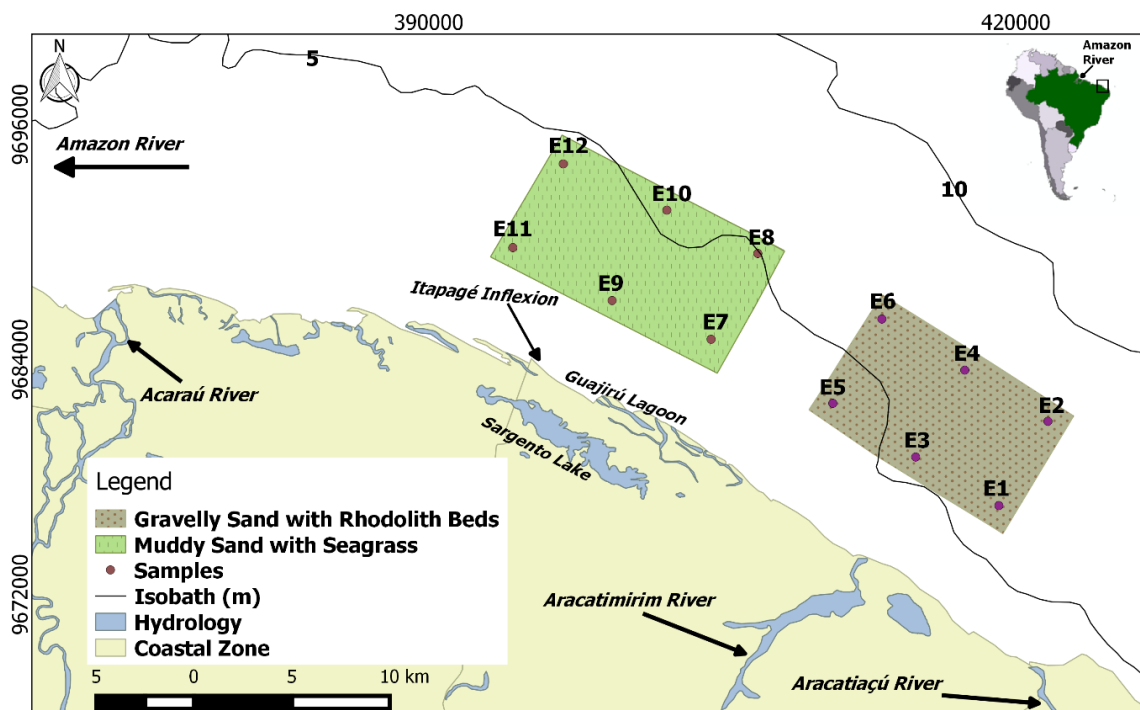
Figure 2. Map of the area assessed (Equatorial Southwestern Atlantic, Brazil): stations on the gravelly sand with rhodolith beds (E1 to E6) and muddy sand with seagrasses (E7 to E12) seascapes.

The pelagic zone is characterized by a well-mixed surface layer with a nutrient deficit in the euphotic layer. This means that this nutrient-poor area is classified as oligotrophic with low nitrate concentrations, chlorophyll-a biomass, and primary production (Ekau and Knoppers 1999; Souza et al. 2013; Queiroz et al. 2016). Moreover, the continental shelf in the study area is narrow (< 80 km) but supports high fish diversity (Freitas and Lotufo 2015; Freitas et al. 2019).

The equatorial southwestern Atlantic shelf (Ceará coast) comprises a mixed sedimentological pattern, including a significant carbonate supply, relict siliciclastic grains, and a mixing of carbonates and siliciclastics. The modern siliciclastic supply is

most common in nearshore bottoms, and discharge from shallow estuaries is low because of the semi-arid climate and multiple dams (Ximenes Neto et al. 2018a, b; Morais et al. 2019). In this way, different types of seascape habitats are found in the inner shelf, such as rhodolith beds, seagrass beds (Figure 2), *Halimeda* beds, and marginal coral reefs (Horta et al. 2016; Soares et al. 2017; Morais et al. 2019). Along the northeast coast of Brazil, there is a large area of seagrass meadow, composed of six different grass species (Barros et al. 2016; Copertino et al. 2016; Magalhães and Barros 2017). Along the entire coast, patches of seagrass meadows are found in the intertidal zones and in shallow waters (<10 m depth). *Halodule wrightii* Ascherson, 1868 is the most common habitat-forming species (Barros et al. 2016; Copertino et al. 2016), and it benefits from the input of nutrients and the reduced wind speed during the rainy season (Barros and Rocha-Barreira 2014). There is also an unvegetated seascape analyzed in this study that was formed by high modern carbonate sedimentation associated with calcareous red algae that consists of unconsolidated sediments (gravelly sand) and rhodolith beds. Rhodolith beds generally have epiphytes, such as seaweeds, and other organisms (Figure 3) (Ximenes Neto et al. 2018a, b).
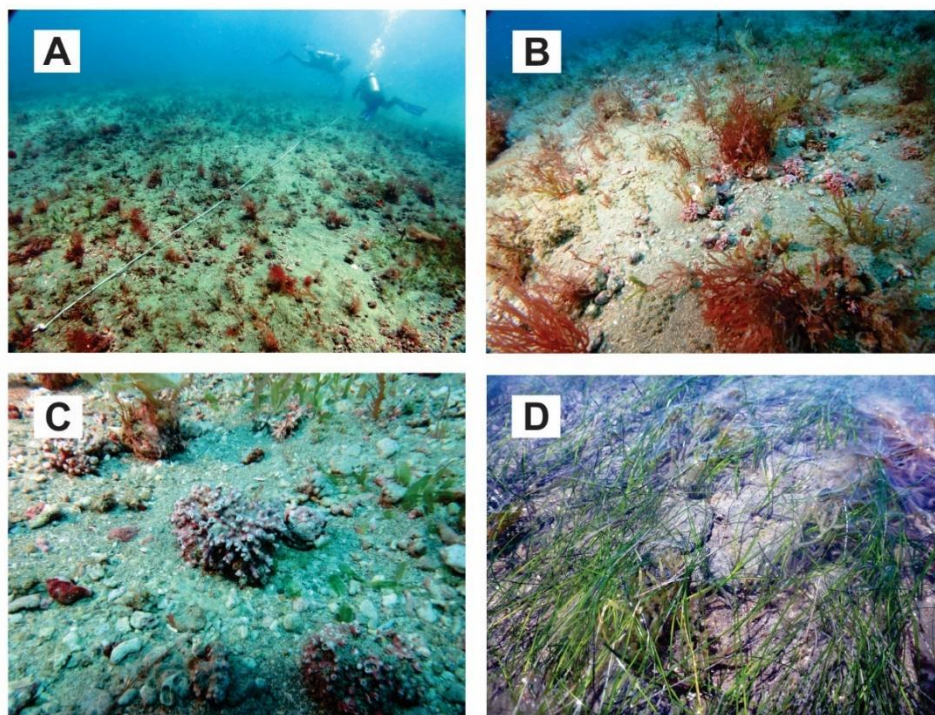
Figure 3. Benthic seascapes on the equatorial southwestern Atlantic shelf (Ceará coast, northeastern Brazil). A) General view of the seascape comprised of gravelly sand with rhodolith

beds; B) Subtidal seaweeds (*Rhodophyta*) populations growing on rhodoliths. In this view, we observed that rhodolith beds provide an important habitat for epibenthic communities, including macroalgae, invertebrates, and vertebrates; C) A detailed image of some rhodoliths in the study area; and D) Muddy sand with a seagrass (*Halodule wrightii*) bed.

The shallow shelf studied is related to the Itapagé Inflection that favored the development of the Acaraú High, which provided a low sloping seafloor (Ximenes Neto et al. 2018b). This geomorphologic pattern favors the occurrence of two different coastal seascapes in a mixed carbonate-siliciclastic system of shallow waters (<10 m depth): an extensive bank of *Halodule wrightii* seagrass in muddy sand, located near the Guajirú Lagoon, and an unvegetaded area composed of gravelly sand with rhodolith beds (Figure 2), located close to the Aracatimirim River estuary. The samplings were designed for the collection of ichthyoplankton inhabiting both the coastal seascapes (Figure 1).

2.2   Data samples

Ichthyoplankton sampling was carried out at 12 stations (distance of 5 km between stations in the same seascape) (Figure 1) on the inner continental shelf, six of which were located on the rhodolith bed areas and six in the seagrass meadows. Two campaigns were carried out in the same season (rainy period): the first in April 2017 and the second in March 2018. The tow was nocturnal, and a low-speed boat (no more than 3 knots) was used. Zooplankton samples were collected via 5 min sub-surface trawls with two conical nets (mouth diameter: 50 cm and mesh size: 300 μm and 200 μm), equipped with a General Oceanics flowmeter (General Oceanics, Miami, FL, USA). The samples were fixed in 4% formalin solution buffered with sodium tetraborate. Salinity and temperature were measured at each campaign and station using a YSI CastAway-CTD. Vertical profiles were made from the surface to the sea bottom (0 to 8 m depth, according to the station) to detect possible differences in temperature and salinity in these shallow waters.

2.3   Data analysis

In the laboratory, fish eggs and larvae were sorted and counted. The larvae were identified to the lowest possible taxonomic level, based on the specialized literature (e.g. Fahay 1983; Richards 2005; Bonecker et al. 2014). The identification of the larvae at the lowest possible taxonomic level was based on their morphometric and meristic characteristics and pigmentation patterns with the help of specialized literature (Fahay 1983; Richards 2005; Bonecker et al. 2014). The identification of fish eggs was difficult, because the egg stage descriptions were much less detailed than the larval stage descriptions. Thus, we analyzed the total abundance of eggs. The development stages were classified into yolk sac, preflexion, flexion, and notochord postflexion, according to Ré (1999). All organisms were deposited in the collection of plankton, "Professor Mariana Ferreira de Menezes (CPPMFM)" of the Federal University of Ceará (UFC).

The density of fish eggs and larvae was expressed as the number of organisms per 100 m³ of filtered water, and standardized according to the formula $D = N/V$, where N is the total number of each taxon in the sample, and V is the volume of filtered water recorded by the flowmeter. The non-parametric Kruskal-Wallis test ($p < 0.05$) was used to compare the densities of larvae at each stage of development between the two seascapes (muddy sand with seagrass beds and gravelly sand with rhodolith beds, hereafter, called seagrass and rhodolith beds, respectively) and the two sampling years (2017 and 2018). The Venn Diagram was drawn to show the number of families exclusive to each habitat and the number that occurred in both seascapes.

3.   Results

There was no variation in salinity (<0.50 g/kg between the maximum and minimum) and temperature (<0.8 ºC) between the seascapes or the years (Table 1). In addition, the salinity and temperature distributions were well mixed in the shallow water column at all stations, with no stratification (Figures S1 and S2).

Table 1 – Average and standard deviation of salinity (g/kg) and sea surface temperature (°C) in the seascapes for both years (Equatorial Southwestern Atlantic, Brazil).

	2017		2018	
	Seagrass beds	Rhodolith beds	Seagrass beds	Rhodolith beds

Salinity (g/kg)	36.13 ± 0.08	36.29 ± 0.11	36.87 ± 0.06	36.65 ± 0.19
Temperature (ºC)	29.56 ± 0.09	29.32 ± 0.09	29.88 ± 0.09	29.80 ± 0.07

A total of 8,094 eggs and 175 larvae were collected from the seagrass meadow seascape, and 2,014 eggs and 241 larvae were collected in the rhodolith beds using mesh nets (Table 2).

Table 2 - Total number of eggs and fish larvae collected in each seascape using mesh nets (200 versus 300 μm) (Equatorial Southwestern Atlantic, Brazil).

Seascape	Mesh nets	Eggs (n)	Larvae (n)
Gravelly sand with rhodolith beds	200 μm	768	122
	300 μm	1,246	119
Muddy sand with seagrass beds	200 μm	3,478	106
	300 μm	4,616	79

Meanwhile, the highest density ($P < 0.05$, Kruskal-Wallis) of eggs was recorded in the seagrass meadow area in both years and nets (Figure 3A and 4A). For the organisms collected with the 200 μm net, the eggs and yolk-sac larval stage showed a significant difference ($p < 0.05$) between years and seascapes, since this stage was not registered in rhodolith beds in 2018 or in seagrass beds in 2017 (Figure 3B). There was a significant difference ($p < 0.05$) between the eggs and yolk-sac stage, because they were absent in the seagrass beds in 2018 (Figure 4A and 4B).

Figure 4: Comparison between eggs and larval stages (A: eggs; B: yolk sac; C: preflexion; D: flexion; E: postflexion) collected with 200 μm net per seascape per year of the area assessed

(Equatorial Southwestern Atlantic, Brazil). Illustration of larval stages was adapted from Ré & Meneses (2008).
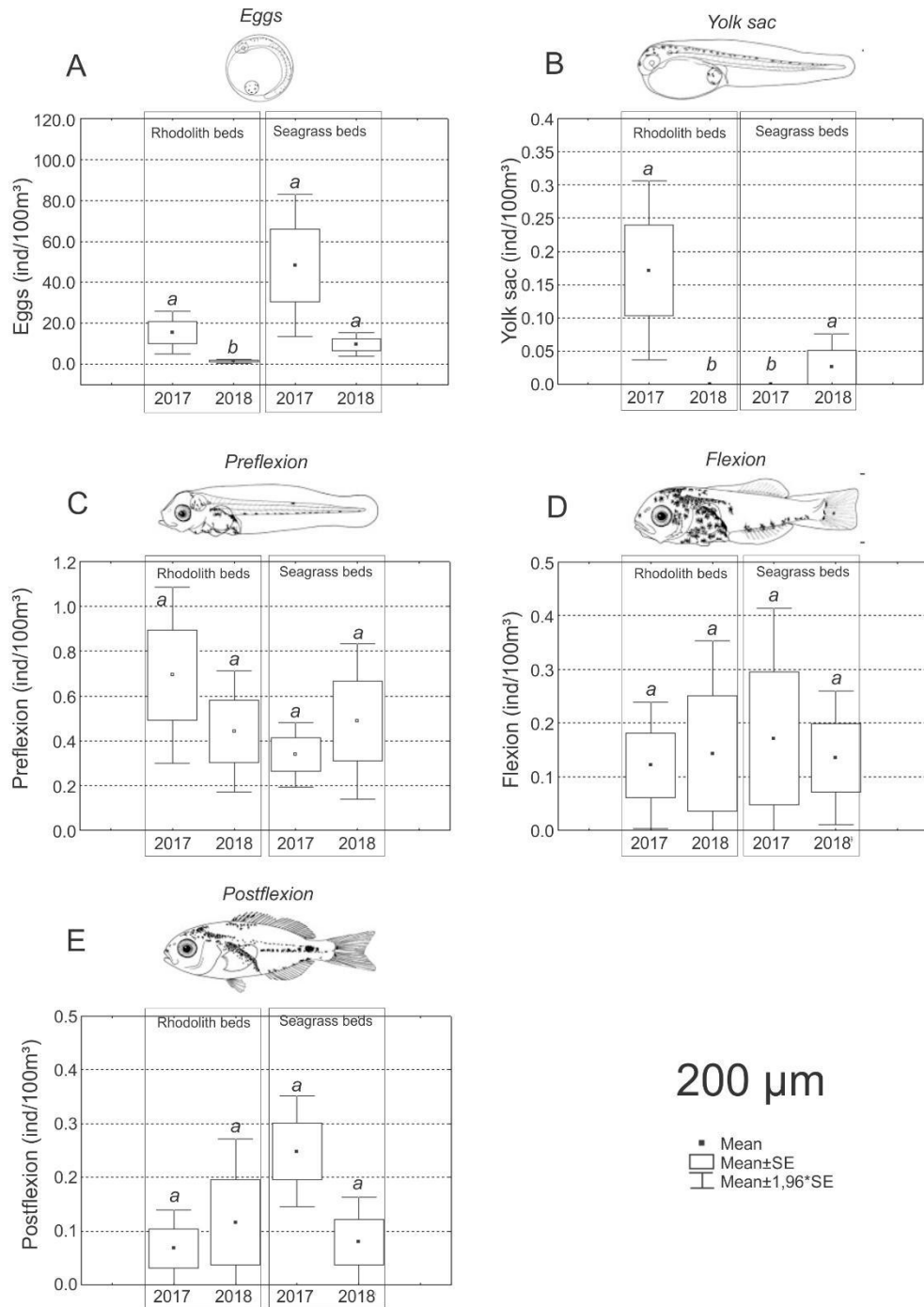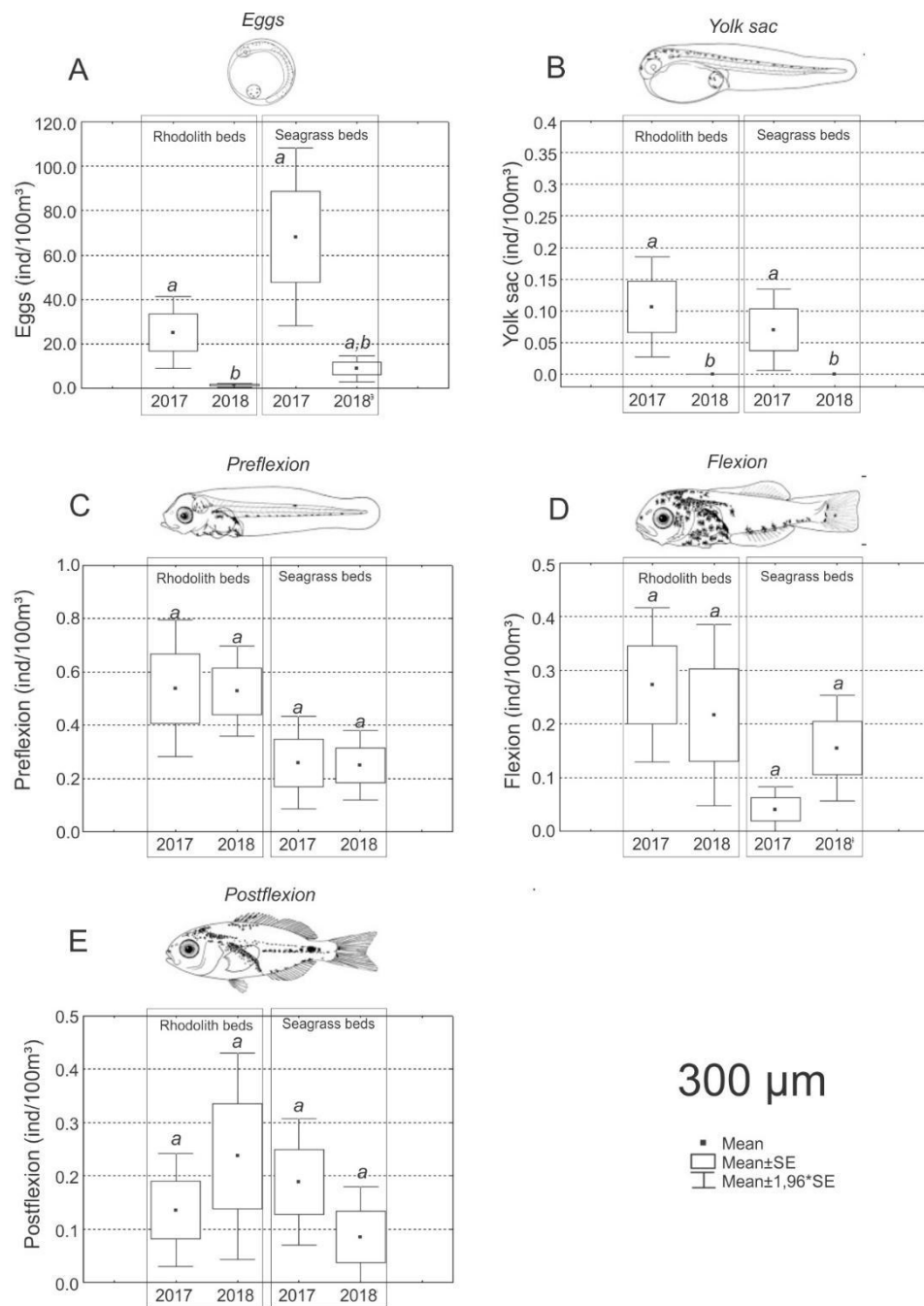

Figure 5: Comparison between eggs and larval stages (A: eggs; B: yolk sac; C: preflexion; D: flexion; E: postflexion) collected with 300 μm net per seascape per year of the area assessed

(Equatorial Southwestern Atlantic, Brazil). Illustration of larval stages was adapted from Ré & Meneses (2008).

A total of 15 families were identified in this study: Achiridae, Atherinidae, Atherinopsidae, Carangidae, Engraulidae, Gobiidae, Hemiramphidae, Lutjanidae, Microdesmidae, Paralichthyidae, Scaridae, Sciaenidae, Sparidae, Sygnathidae, and Tetraodontidae (Tables S1 to S4).

In the seagrass beds, the most abundant taxa collected with the 200 μm net in 2017 were the subfamily Syngnathinae and the family Atherinopsidae. In 2018, Atherinidae, Engraulidae (*Anchovia clupeoides* Swainson, 1839), and subfamily Syngnathinae (Table S1) were the most abundant. For organisms collected with the 300 μm net in 2017, the most abundant taxa were Syngnathinae, Atherinidae, and Hemiramphidae (*Hemiramphus* sp.). In 2018, the most abundant were Achiridae (*Achirus lineatus* Linnaeus, 1758), Engraulidae (*A. clupeoides*), and Atherinidae (Table S2).

In the rhodolith beds, among the organisms collected with the 200 μm net, the most abundant were representatives of the family Gobiidae, Engraulidae (species *A. clupeoides*) and Sciaenidae in 2017. In 2018, the most abundant organisms were members of the Engraulidae family (species *A. clupeoides*), Gobiidae, and Achiridae (species *A. lineatus*) (Table S3). For organisms collected with the 300 μm net in 2017, the most abundant organisms were Syngnathidae (subfamily Syngnathinae), Engraulidae (species *A. clupeoides*), and Gobiidae. In 2018, the most abundant taxa were Engraulidae (species *A. clupeoides*) and Gobiidae (Table S4).

The Venn diagram shows that the number of families that are common in each seascape was higher than the number of exclusive taxa (Figure 5). In the rhodolith beds, exclusive taxa were higher in 2017 (Figure 5A and 5B), while in the seagrass beds, exclusive taxa were higher in 2018 (Figure 5C and 5D).
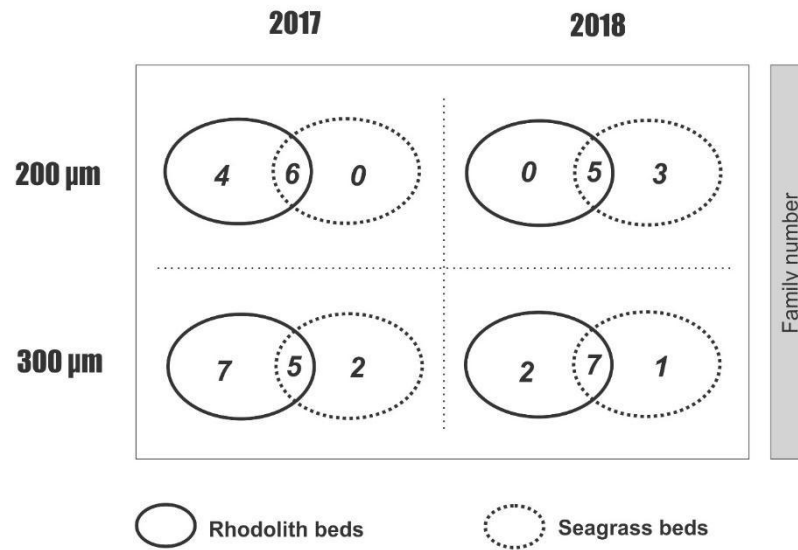
Figure 6: Venn diagram based on the number of families for 200 μm and 300 μm plankton nets in 2017 and 2018, considering the coastal seascapes studied (Equatorial Southwestern Atlantic, Brazil).

4. Discussion

The importance of coastal seascapes such as rhodolith and seagrass beds for the life cycle of tropical fishes is highlighted in this study. The homogeneity of environmental data on sea temperature and salinity between the two seascapes indicates that they are not outstanding in explaining the differences in diversity and density of eggs and larvae. Therefore, structural physical differences between seascapes (seagrass and rhodolith beds), ecological aspects of reproduction and connectivity of species, and other environmental factors (e.g., currents and tides) should be relevant to the patterns found. In this way, the study area of the muddy sand with seagrass beds is characterized by low depth and declivity (Figure 1). The flat geomorphology of this marine area favors colonization by the plant *Halodule wrightii,* providing the formation of an important seascape for the fish community.

There were more eggs recorded in the seagrass beds than in the rhodolith beds, reaffirming the role of this seascape in the initial phases of fish life cycles as spawning and nursery areas. In this sense, the larvae of Engraulidae, Gobiidae, and Syngnathidae were among the most abundant and are common inhabitants at some stage of their life cycle in

both seascapes. The most abundant taxa were common inhabitants of coastal shallow habitats, and were grouped as follows: *Chloroscombrus chrysurus* Linnaeus, 1,766 and *Achirus lineatus* in estuaries; organisms of genus *Lutjanus*, *Microdesmus,* and *Sparisoma* in coral reefs; *Hemiramphus* sp. and organisms previously registred as inhabitants of the coastal environments of Brazil's northern waters, like *A. clupeoides,* in seagrass beds (Menezes e Figueiredo 2003; Mafalda et al. 2008; Froese and Pauly 2019). These organisms are common in larval fish assemblages in tropical southwestern coastal ecosystems (Mafalda et al. 2008; Mota et al. 2017), suggesting that coastal seascapes are not only important for planktonic larvae but also for organisms that present other reproductive and spawning strategies, such as demersal eggs and parental care.

Recent studies have highlighted the role of seagrass and rhodolith beds as nursery and feeding areas for several organisms (Berkström et al. 2013; Bertelli et al. 2014; Amado-Filho et al. 2016; Moussa et al. 2020). Seagrass beds are associated with less turbulent hydrodynamics than unvegetated habitats, which favors the retention of organisms, especially in these initial phases (Bell et al. 1987; Björk et al. 2008). Seagrass beds may also act as important areas of ecological and genetic connectivity between mangroves, rhodolith beds, and marginal coral reefs (Soares et al. 2017; Soares 2020), influencing the survival of juvenile fish and increasing the abundance of reef species (Unsworth et al. 2008; Eggertsen et al. 2017; Mota et al. 2017). The number of families common to both seascapes suggests physical and ecological connectivity between the seagrass and rhodolith beds, which is an important finding for these seascapes, given their proximity to key areas, such as shallow and hypersaline estuaries with mangrove forests (Barroso et al. 2018) (Figure 1).

In the present study, the high number of eggs and yolk-sac larvae demonstrate a preference (and/or retention) for protected and less turbulent seascapes as nursery and offspring rearing areas. The distribution of eggs is influenced by the biogeography of broadcast spawners, spawning habitat characteristics, and regime flow that can act by transporting or retaining these organisms (Pörtner and Peck 2010). Tropical fish spawn continuously throughout the year, but some species have peaks associated with the rainy season (Johannes 1978). In this study, sampling was carried out in the rainy season under a tropical semiarid climate, which may explain the high number of eggs recorded. Moreover,

the current over the study area (equatorial western Atlantic continental shelf) flows predominantly westward, owing to the intense and persistent trade winds (Dias et al. 2013, 2018). In this sense, the inner shelf circulation is barotropic with wind-driven westward currents (Dias et al. 2018). However, during the rainy months of the sampling activities, the wind speed is the lowest throughout the year (< 2.5 m/s) (Soares et al. 2019). This reduces the current speed in the inner shelf and, consequently, the transport of eggs and larvae, favoring retention and habitat preference.

Despite the lower density of larvae found in the postflexion stage, compared to the preflexion stage in the evaluated tropical region, studies indicate that these larvae in the later developmental stages also use the seagrass beds to seek refuge from predators (Bell et al. 1987; Guidetti and Bussoti 2002). In this way, larvae can be passively transported to suitable habitats or actively guide and swim to such structurally complex vegetated habitats (Montgomery et al. 2006).

Habitat complexity and heterogeneity determine shelter and food availability (García-Charton et al. 2004; Carvalho and Barros 2017). Tolan et al. (1997) suggested that the greater the complexity of the coastal habitat, the greater the density of fishes. The authors also highlighted that seagrass beds not only serve as nursery areas for the early larval stages of estuarine fish, but the post-larvae and young juveniles of several species also appear to be strongly associated with specific seagrass and rhodolith habitats. In this way, microhabitat characteristics drive ontogenetic changes (Cuadros et al. 2016), and the greater the structural complexity of the spawning grounds, the greater the survival rates of fish in the early stages of life. This has already been demonstrated for Atlantic herring (von Nordheim et al. 2018). Thus, habitat connectivity is defined by the combination of dispersion and factors that favor the survival of dispersed organisms (Cowen and Sponaugle 2009). The more structured the habitat, the more shelter it provides, especially for larvae in the early stages of development, such as the present case in tropical shallow-water seascapes. However, as ontogeny progresses, larvae in the later stages can explore tropical areas with less shelter (Sogard 1997).

5.   Conclusions

The dominance of organisms in the early stages of development (eggs and yolk sac larvae) demonstrates a preference for the more protected and less turbulent seascapes for nursery and offspring rearing. The study of larval development phases is essential to characterize the environment and its use by organisms at each stage of their life cycle. The data collected in this study reinforced the idea that the bottom seascape may influence ichthyoplankton assemblage.

Many seagrasses and rhodolith beds worldwide are threatened (Amado-Filho et al. 2016; Kraufvelin et al. 2018; Huang et al. 2020; Magris and Tommaso 2020; Soares et al. 2020) and are not yet adequately protected by fishery regulations, restoration efforts, marine protected areas (MPAs), or marine spatial planning (MSP) (Copertino et al. 2013; Horta et al. 2016; Macreadie et al. 2019). Moreover, our results highlight the importance of understanding the physical and biological connectivity and movement of fish life stages among tropical seascapes (Berkström et al. 2013; Endo et al. 2019). Thus, the establishment of MPAs and MSP can only be effective if connectivity among seascapes is recognized. This research reaffirms the need for management of different tropical coastal seascapes and the need for knowledge of local processes for the conservation of marine biodiversity.

Supplementary material

Table S1. Identified taxa in seagrass meadows with the 200 µm net.

Seascape	Year	Family	Genus	Species	Developmental stage	Density (larvae.100 m³)
Muddy sand with seagrass beds	2017	Atherinopsidae			preflexion	0.7180
		Atherinopsidae			flexion	0.4561
		Carangidae	Chloroscombrus	Chloroscombrus chysurus Linnaeus, 1766	preflexion	0.7053
		Engraulidae	Anchovia	Anchovia clupeoides Swainson, 1839	flexion	0.2132
		Engraulidae	Anchovia	Anchovia clupeoides Swainson, 1839	postflexion	0.1768
		Engraulidae	-	-	preflexion	0.1626
		Gobiidae			preflexion	0.1797
		Hemiramphidae	Hemiramphus	Hemiramphus sp.	flexion	0.3593
		unidentified			preflexion	3.4142
		Syngnathidae			postflexion	3.9953
		Syngnathidae			flexion	3.0543
	2018	Achiridae	Achiurus	Achiurus lineatus Linnaeus, 1758	flexion	0.3630
		Atherinidae	-	-	flexion	4.3626
		Atherinidae	-	-	preflexion	0.4729
		Engraulidae	Anchovia	Anchovia clupeoides Swainson, 1839	flexion	2.9968
		Engraulidae	Anchovia	Anchovia clupeoides Swainson, 1839	preflexion	0.8256
		Gobiidae	-	-	preflexion	0.3586
		Hemiramphidae	Hemiramphus sp.		flexion	0.7055
		Lutjanidae	Lutjanus	Lutjanus sp.	flexion	0.4128
		Lutjanidae	Lutjanus	Lutjanus sp.	postflexion	0.2684
		unidentified	-	-	preflexion	2.8822
		unidentified	-	-	yolk sac	0.3630
		Sciaenidae	-	-	preflexion	0.5382
		Syngnathidae	-	-	postflexion	0.8640

Table S2. Identified taxa in seagrass meadows with the 300 µm net.

Seascape	Year	Family	Genus	Species	Developmental stage	Density (larvae.100 m³)
Muddy sand with seagrass beds	2017	Achiridae	*Achirus*	*Achirus achirus* (Linnaeus, 1758)	postflexion	0.1963
		Achiridae	*Achirus*	-	flexion	0.3485
		Achiridae	-	-	preflexion	0.1589
		Atherinidae	-	-	preflexion	0.9391
		Atherinopsidae	-	-	preflexion	0.3729
		Hemiramphidae	*Hemiramphus*	*Hemiramphus* sp.	flexion	0.3739
		Microdesmidae	*Microdesmus*	*Microdesmus* sp.	postflexion	0.1865
		unidentified	-	-	yolk sac	1.6138
		unidentified	-	-	preflexion	0.9519
		Sciaenidae	-	-	preflexion	0.3457
		Sygnathidae	-	-	postflexion	2.8750
	2018	Achiridae	*Achirus*	*Achirus lineatus* Linnaeus, 1758	flexion	1.6563
		Atherinidae	-	-	preflexion	0.6600
		Engraulidae	*Anchovia*	*Anchovia clupeoides* Swainson, 1839	preflexion	0.8114
		Engraulidae	*Anchovia*	*Anchovia clupeoides* Swainson, 1839	flexion	0.2620
		Gobiidae	-	-	preflexion	0.6258
		Hemiramphidae	-	-	flexion	0.5152
		Hemiramphidae	-	-	postflexion	0.2917
		Hemiramphidae	-	-	preflexion	0.2917
		unidentified	-	-	preflexion	0.8878
		Scaridae	-	-	preflexion	0.2917
		Sciaenidae	*Menticirrhus*	*Menticirrhus* sp.	postflexion	0.6258
		Syngnathidae	-	-	postflexion	0.3325

Table S3. Identified taxa in unconsolidated carbonate sediments bottoms with the 200 µm net.

Seascape	Year	Family	Genus	Species	Developmental stage	Density (larvae.100 m³)
Gravelly sand with rhodolith beds	2017	Atherinopsidae	-	-	preflexion	0.8612
		Carangidae	*Chloroscombrus*	*Chloroscombrus chrysurus* Linnaeus, 1766	preflexion	0.7622
		Engraulidae	*Anchovia*	*Anchovia clupeoides* Swainson, 1839	flexion	2.3016
		Engraulidae	*Anchovia*	*Anchovia clupeoides* Swainson, 1839	preflexion	0.5432
		Gobiidae	-	-	preflexion	2.5472
		Hemiramphidae	*Hemiramphus* sp.	-	preflexion	0.2716
		unidentified	-	-	yolk sac	11.8568
		unidentified	-	-	preflexion	1.9383
		Paralichthyidae	-	-	preflexion	0.8735
		Scaridae	*Sparisoma*	*Sparisoma* sp.	postflexion	0.2676
		Scaridae	*Sparisoma*	*Sparisoma* sp.	preflexion	0.2541
		Sciaenidae	-	-	preflexion	1.2158
		Syngnathidae	-	-	postflexion	0.4510
		Syngnathidae	-	-	preflexion	0.2676
		Tetraodontidae	-	-	preflexion	0.7289
		Tetraodontidae	*Canthigaster*	*Canthigaster rostrata* Bloch, 1786	preflexion	0.2676
	2018	Achiridae	*Achirus*	*Achirus lineatus* Linnaeus, 1758	postflexion	1.4442
		Engraulidae	*Anchovia*	*Anchovia clupeoides* Swainson, 1839	preflexion	5.2450
		Engraulidae	*Anchovia*	*Anchovia clupeoides* Swainson, 1839	flexion	2.4869
		Gobiidae	-	-	preflexion	1.4730
		Lutjanidae	*Lutjanus*	*Lutjanus* sp.	postflexion	0.5881
		unidentified	-	-	preflexion	2.3157
		Sciaenidae	-	-	preflexion	0.5881
		Sciaenidae	-	-	flexion	0.4215

Table S4. Identified taxa in unconsolidated carbonate sediments bottoms with the 300 µm net

Seascape	Year	Family	Genus	Species	Developmental stage	Density (larvae.100 m³)
Gravelly sand with rhodolith beds	2017	Achiridae	-	-	preflexion	0.2386
		Atherinopsidae	-	-	preflexion	0.4158
		Carangidae	*Decapterus*	*Decapterus* sp.	preflexion	0.4158
		Carangidae	-	-	preflexion	0.2716
		Engraulidae	*Anchovia*	*Anchovia clupeoides* Swainson, 1839	flexion	2.8201
		Engraulidae	*Anchovia*	*Anchovia clupeoides* Swainson, 1839	preflexion	0.8650
		Engraulidae	*Anchovia*	*Anchovia clupeoides* Swainson, 1839	postflexion	0.9544
		Gobiidae	-	-	preflexion	1.6733
		Hemiramphidae	*Hemiramphus*	*Hemiramphus* sp.	flexion	0.2491
		Microdesmidae	*Microdesmus*	*Microdesmus* sp.	postflexion	0.2877
		unidentified			yolk sac	0.9870
		unidentified			preflexion	1.2534
		unidentified	-	-	flexion	0.2716
		Paralichthyidae	*Citharichthys*	*Citharichthys macrops* Dresel, 1885	flexion	0.4158
		Paralichthyidae	*Etropus*	*E. crossotus*	preflexion	0.2877
		Paralichthyidae	-	-	preflexion	0.2716
		Scaridae	*Sparisoma*	*Sparisoma* sp.	postflexion	1.6456
		Scaridae	-	-	flexion	0.2386
		Sparidae	*Sparisoma*	*Sparisoma* sp.	flexion	0.2749
		Syngnathidae	-	-	postflexion	4.1885
		Tetraodontidae	*Canthigaster*	*Canthigaster rostrata* Bloch, 1786	preflexion	1.2406
		Tetraodontidae			preflexion	0.2419
	2018	Achiridae	*Achirus*	*Achirus lineatus* Linnaeus, 1758	postflexion	0.3327
		Atherinidae	-	-	preflexion	0.6596
		Engraulidae	*Anchovia*	*Anchovia clupeoides* Swainson, 1839	preflexion	3.9035
		Engraulidae	*Anchovia*	*Anchovia clupeoides* Swainson, 1839	flexion	3.5169
		Engraulidae	*Anchovia*	*Anchovia clupeoides* Swainson, 1839	postflexion	3.0991
		Gobiidae	-	-	preflexion	2.8016
		Gobiidae	-	-	flexion	0.3104
		Gobiidae	*Gobiosoma*	*Gobiosoma* sp.	flexion	0.2895
		Hemiramphidae	-	-	preflexion	0.3929
		Lutjanidae	*Lutjanus*	-	preflexion	0.3104
		unidentified	-	-	preflexion	1.0392
		Scaridae	-	-	preflexion	0.4892
		Scaridae	-	-	flexion	0.3808
		Sparidae	-	-	flexion	0.2895
		Syngnathidae	-	-	postflexion	0.3808

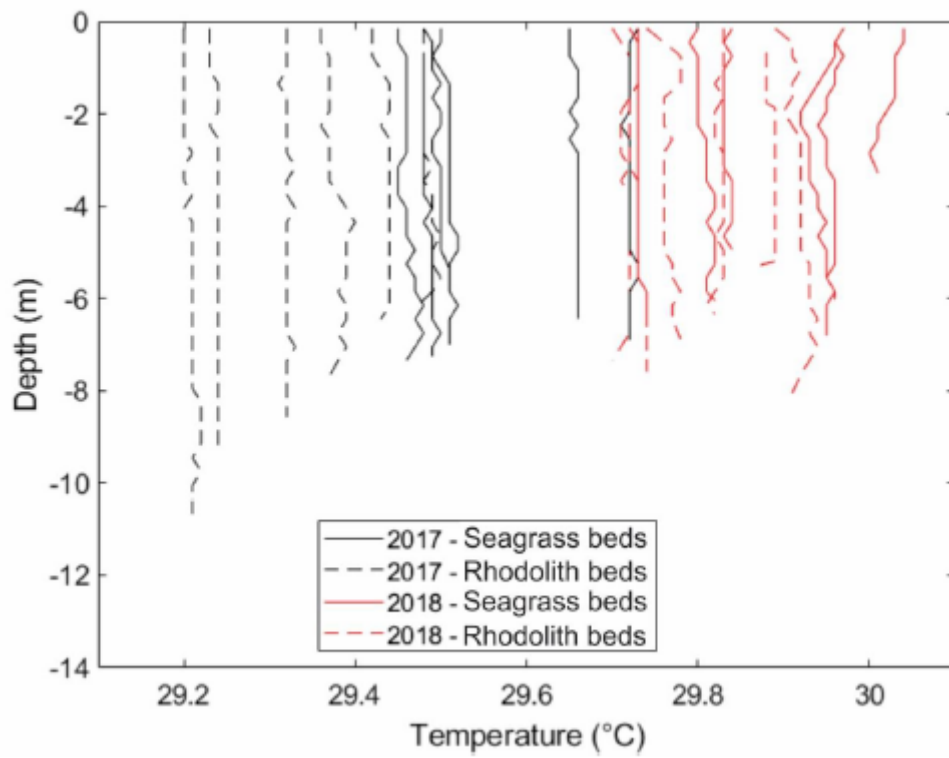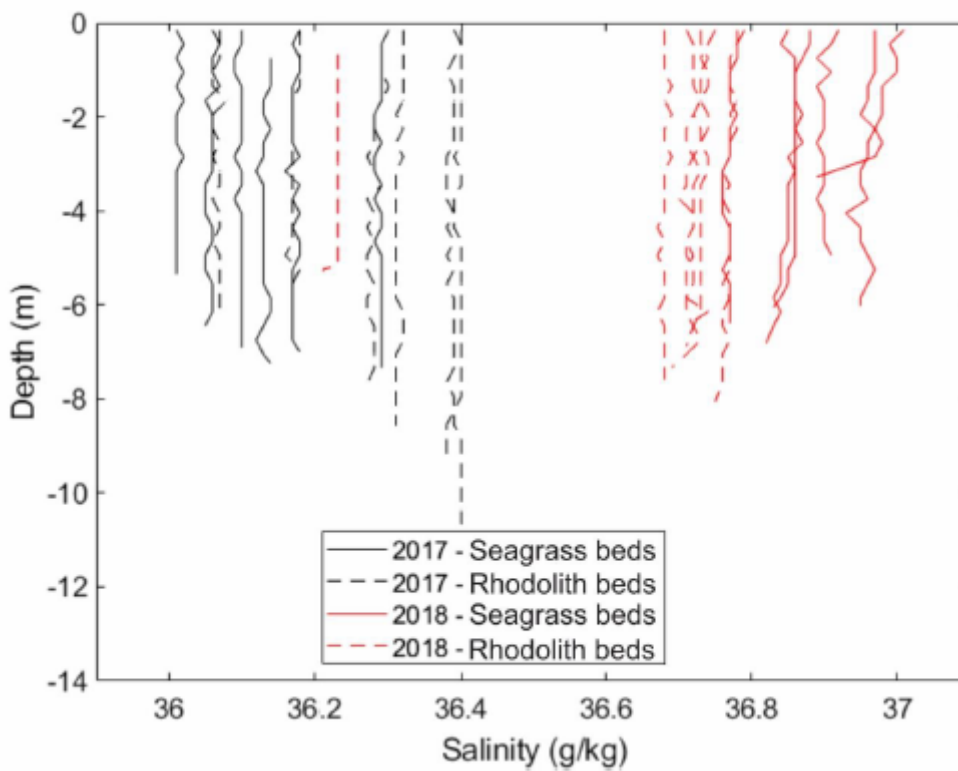Figure S1 – Vertical profiles of temperature (°C) between seascapes and years.

Figure S2 – Vertical profiles of salinity (g/kg) between seascapes and years.

Acknowledgements

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Chapter 2 - DNA barcode reveals cryptic diversity of fish eggs in Equatorial Southwestern Atlantic
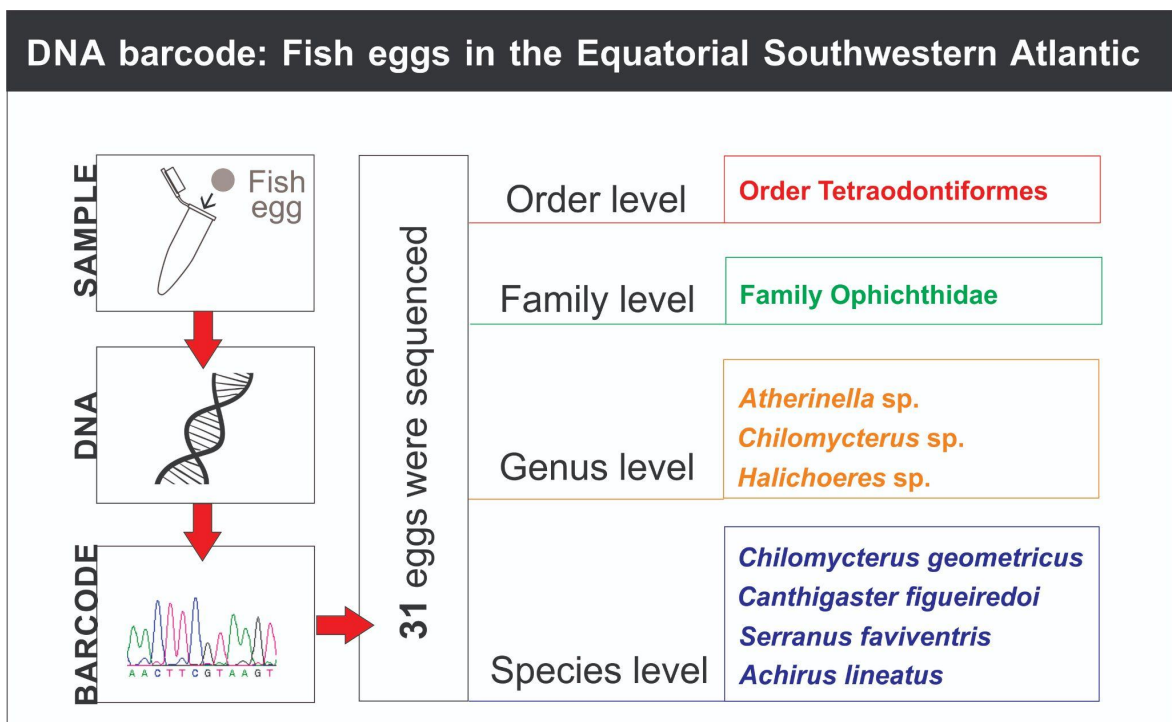

Figure 1. Graphical Abstract

DNA barcode reveals cryptic diversity of fish eggs in Equatorial Southwestern Atlantic

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Abstract

DNA barcoding presents a reliable and universal tool for species identification. However, few studies have used this methodology to analyze fish egg diversity in low-latitude seascapes. In this study, DNA barcodes were used to assess the diversity of fish eggs in the Equatorial Southwestern Atlantic; a rich and poorly-studied region. We sampled two seascapes for fish life cycle; seagrass and rhodolith beds. In total, 112 fish eggs were sorted for the genetic analysis and 31 were successfully sequenced. One egg was successfully identified at order level, three at family level, twenty at genus level and seven at species level. Since many tropical fish species have not yet been barcoded and deposited in genetic databases, our new research increased the diversity knowledge of fish eggs in low-latitude areas. The molecular identification approach revealed cryptic diversity among the types of eggs collected in important tropical seascapes. It is important to highlight that efforts should be made to improve the fish eggs and larvae Barcode database worldwide. This baseline information is important mainly to assess the global environmental changes on fish diversity in low-latitude coastal seas.

Keywords: Genetic, Molecular identification, Icthtyoplankton, Fish communities, South Atlantic.

1.   Introduction

Knowledge about taxonomic identification, spatial distribution and abundance of ichthyoplankton (e.g., fish eggs and larvae) are useful for identifying spawning areas, reproductive seasons, and migration routes (Nakatani et al., 2001; Goodsir et al., 2008; Hofmann et al., 2015). However, research involving taxonomy of fish eggs is scarce and only a low number of species have their early developmental stages described (Bialetzki et al., 2016). In lower latitudes, where species diversity is significant, the lack of data about morphological identification of eggs is even larger compared to subtropical and temperate regions (Kendall and Matarese, 1994). In this regard, the knowledge about ichthyoplankton diversity of equatorial fishes is strikingly insufficient, especially when we consider that this oceanic region presents high species diversity (Hermes-Silva et al., 2009) and vulnerability to global climate change (Auth et al., 2018; Nielsen et al., 2021).

The small number of fish eggs studies available are mostly based only on traditional techniques that involve the analysis of morphological characteristics (Macedo-Soares et al., 2012; Mota et al., 2017; Santana et al., 2020). However, morphological identification is challenging because fish larvae and eggs are small in size, and notably fragile, and some of the characteristics used in taxonomic identification change greatly throughout ontogenetic development (Ré, 1999; Leyva-Cruz et al., 2016).

Fish egg identifications traditionally are based on morphological characteristics, such as egg shape and diameter, presence of oil globules, width of the perivitelline space, aspect of the egg yolk and chorion, and embryonic pigmentation (Ahlstrom and Moser, 1980). However, fish eggs generally lack clearly distinct morphological characters needed for species identification (Ahlstrom and Moser, 1980; Ward et al., 2009). The visual identification method is prone to mistakes in classification (Larson et al., 2016), not only because eggs from taxonomically close species can be very similar, but because the eggs present natural variation in morphological features (Chambers and Leggett, 1996; Kucera et al., 2002). Moreover, the sample preservation process usually causes substantial egg shrinkage (Hiemstra, 1962) and loss of natural pigmentation, making it difficult to identify the developmental stage of the embryo by morphological approach (Valdez-Moreno et al., 2010). In this sense, DNA barcoding presents a more reliable and universal tool for species identification, based on the premise

that the mitochondrial gene cytochrome c oxidase subunit 1 (COI) is an efficient way for identifying animal species (Ward et al., 2005; Curry et al., 2018). Some studies have indeed demonstrated the efficiency of DNA barcoding in identifying fish eggs and larvae (Gleason and Burton, 2012; Becker et al., 2015; Lima et al., 2020), but it is less commonly applied to individual eggs from plankton samples (Burghart et al., 2014; Frantine‑Silva et al., 2015; Ahern et al., 2018; Burrows et al., 2019).

DNA barcodes have been successfully used in taxonomic studies of fish eggs from continental waters in South America (Becker et al., 2015; Fratine-Silva et al., 2015; Almeida et al., 2018; Lima et al., 2020). However, the technique has never been used to approach identification of fish eggs from the Equatorial Southwestern Atlantic Ocean, a rich and poorly studied region in the South Atlantic Ocean. In particular, this equatorial region acts as an ecological corridor between the reefs of the Brazilian eastern coast, the Amazon reefs, and the Caribbean reefs (Carneiro et al., 2022). Thus, our study aims at starting to fill this knowledge gap on diversity in equatorial regions. This kind of information can have great exploratory, ecological and biogeographic value (Burrows et al., 2019), especially in low-latitude marine areas, where studies of planktonic eggs and their genetic identifications are rare (Menegotto and Rangel, 2018). The samples of this study were collected from two important seascapes; seagrass and rhodolith beds, whose importance for fisheries and fish diversity is belittled (Costa et al., 2020). In this regard, our short communications have two main aims. Firstly, we used DNA barcoding to explore the diversity of fish eggs from this equatorial region. Secondly, we used DNA barcoding to support and improve the morphological identification method, through the description of diagnostic characteristics that would allow finer taxonomic classification.

2. Material and methods

2.1. Fish eggs sampling and processing

Sampling of fish eggs took place at 12 stations located on the inner continental shelf of the Equatorial Southwestern Atlantic (Ceará coast, NE Brazil - Figure 1), covering two different tropical seascapes: rhodolith and seagrass beds. The sampling was done during the rainy season of 2018 at night using conical nets (50 cm mouth diameter and

either 300 μm or 200 μm mesh), together with the mechanical flowmeter of General Oceanics. After the oceanographic sampling, the samples were fixed onboard on the oceanographic vessel in 70% ethanol.

In the laboratory, the samples were sorted under a stereoscope microscope. The fish eggs were separated into morphotypes according to their morphological characteristics (e.g. size, shape, perivitelline space, appearance of yolk and chorion) (Ahlstrom and Moser, 1980). After this procedure, the eggs were packed in micro tubes (2 mL) containing 70% ethanol and stored in a freezer at -4 ºC. A total of 112 eggs were taken from the collected samples in the Equatorial Southwestern Atlantic (Figure 1) and followed to molecular analysis (DNA barcoding).
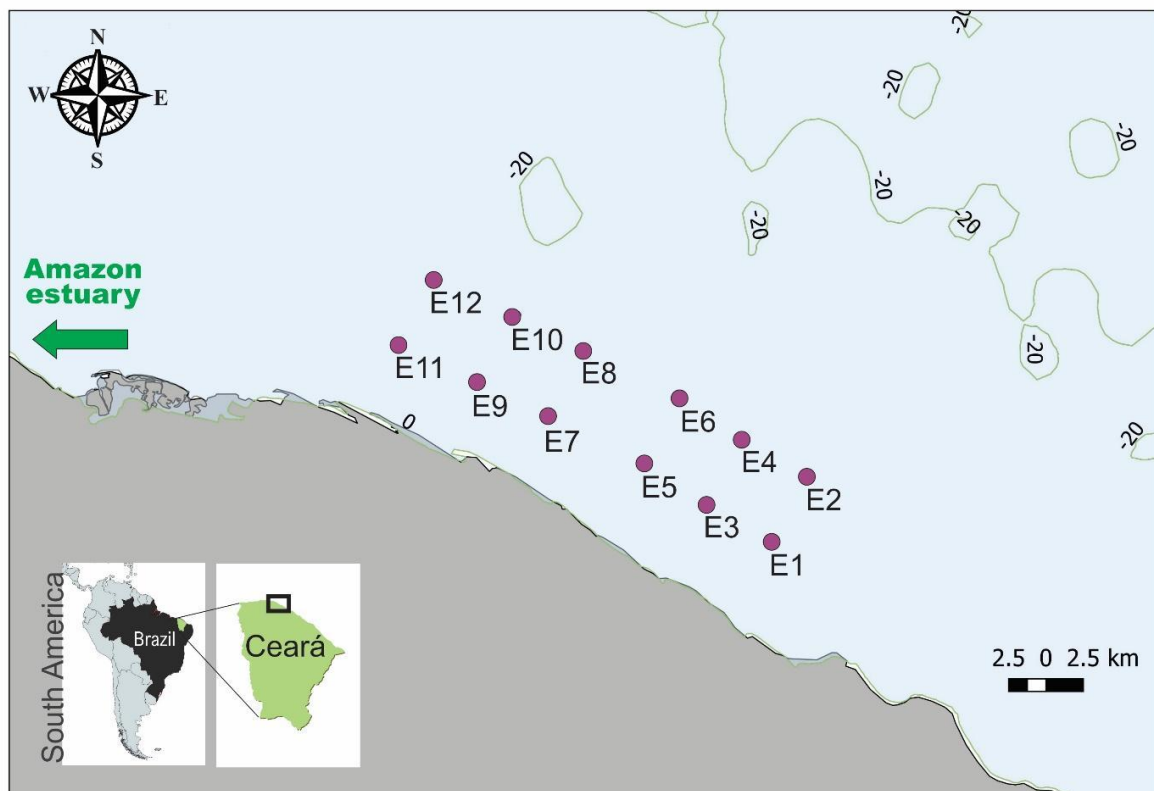

Figure 2. Study area with 12 stations (E1 to E12) and isobaths in Equatorial Southwestern Atlantic continental shelf (Ceará coast, northeastern Brazil).

2.2. DNA Barcode (Extraction, amplification and sequencing of DNA)

After the identification of morphotypes by traditional methods based on morphology, individual eggs from each morphotype were used for DNA Barcode analysis. The protocol chosen for the extraction of genetic material from fish eggs used Chelex100 resin (Hyde et al., 2005) and proteinase K. Each sample corresponded to an egg that was allocated in a 1.5 mL microtube with 100 μL of extraction buffer for DNA. Each 100 μL of buffer solution contained 100 mg of Chelex100 and 30 ng of Proteinase K. After being sealed, the microtubes were agitated in vortex for 10 seconds and then taken to the thermal cycler, in which the programmed extraction cycle was 60 minutes at 90 ºC, followed by 5 minutes at 60 ºC. Then the microtubes were centrifuged at 3,000 rpm for 1 minute. The supernatant was then recovered in a clean microtube and stored at -20 ºC.

After DNA extraction, amplification of the COI region was performed using the FishF1-5' TCA ACC AAA GAC ATT GGC AC 3' and FishR1-5' TAG ACT TCT GGG TGG CCA AAG CA 3' primers, following the protocol proposed by Ward et al. (2005). For each reaction, 1× of the proprietary buffer, 1.5 mM of $MgCl_2$, 0.2 mM of dNTP, 0.4 μM of each primer, 1 U of Taq DNA polymerase and 10 ng of DNA were used for a final reaction volume of 12.5 μL. Amplification was performed in a thermocycler (Applied Biosystems), under the following conditions: initial denaturation at 94 °C for 5 min, 35 cycles of 94 °C for 30 s, 54 °C for 30 s, 72 °C for 1 min, and final extension at 72 °C for 10 min. To confirm the amplification of the COI region, 1.5% (w/v) agarose gel electrophoresis was performed at 120 V for 60 minutes, with subsequent photo documentation in a KODAK system, under UV light.

Purified amplicons have been sent to Sanger sequencing, in both directions, at a third party partner. Resulting sequences have been individually checked and deposited in GeneBank (https://www.ncbi.nlm.nih.gov/genbank). Alignment of the sequences was performed using the MUSCLE application (Edgar, 2004) in the MEGA11 program, (Tamura et al., 2021). All sequences were submitted to the BOLD (https://boldsystems.org/) and BLAST database (http://https://blast.ncbi.nlm.nih.gov/Blast.cgi), to verify the correspondence and similarity of the submitted sequences with those stored in the database.

2.3. Identification Tree

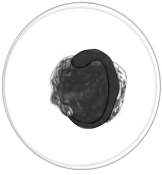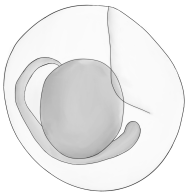We have used MEGA11 software (Tamura et al., 2021) to build a simple Neighbor-Joining identification tree including the species identified in the present work. Branch consistency was tested by 10,000 bootstrap replicates. The evolutionary distances were computed using the Kimura 2-parameter method (Kimura, 1980)
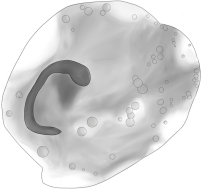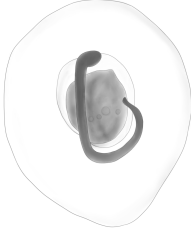
3. Results

The results indicated that the 112 fish eggs could be classified in 11 morphotypes. The morphotypes are described in Table 1.

Table 1. Morphological description and genetic identification (DNA Barcoding) of fish eggs sampled in Equatorial Southwestern Atlantic.

Representation	Morphotype description	Morphotype	BOLD top hit (Prob. of Placement)
	Medium, spherical eggs, measuring about 1.3 mm, chorion opaque.	Morphotype 1	Atheriniformes: Atherinopsidae: *Atherinella* sp. (species 99.5-100%)
	Small, spherical eggs, measuring about 0.8 mm, translucent chorion.	Morphotype 2	Pleuronectiformes: Achiridae: *Achirus lineatus* (genus 100%)

	Small, spherical eggs, measuring about 1.2 mm, translucent chorion, large perivitelline space, visible larvae.	Morphotype 3	Tetraodontiformes: Diodontidae: *Chilomycterus antillarum* (genus 100%)
	Small, spherical eggs, measuring about 0.8 mm, translucent chorion, presence of oil drops, narrow perivitelline space, visible larvae.	Morphotype 4	Tetraodontiformes: Diodontidae: *Chilomycterus antillarum* (genus 100%)
	Small, spherical eggs, measuring about 0.6 mm, translucent chorion, presence of oil drop, narrow perivitelline space, visible larvae.	Morphotype 5	Perciformes: Serranidae: *Serranus flaviventris* (species 100%)
	Small, spherical eggs, measuring about 0.7 mm, translucent chorion, presence of an oil drop, narrow perivitelline space.	Morphotype 6	Labriformes: Labridae: *Halichoeres* sp. (genus 100%)
	Large, spherical eggs, measuring about 2.2 mm, translucent chorion, presence of oil drops, large perivitelline space, visible larvae.	Morphotype 7	Anguilliformes: Ophichthidae: *Ichthyapus ophioneus* (species 100%)

	Medium, spherical shaped eggs, measuring about 1.6 mm, translucent chorion, presence of oil drops, narrow perivitelline space, visible larvae.	Morphotype 8	unidentified
	Medium, spherical eggs, measuring about 1.2 mm, translucent chorion, presence of oil drops, narrow perivitelline space, visible larvae.	Morphotype 9	unidentified
	Large, spherical shaped eggs, measuring about 2.4 mm, translucent chorion, presence of oil drops, large perivitelline space, visible larvae.	Morphotype 10	unidentified
	Ellipsoid-shaped eggs, translucent corion, narrow perivitelline space, presence of oil drops and apparent larvae	Engraulidae	unidentified

From the eggs selected for DNA barcode analysis, we obtained 31 sequences (GeneBank AN OP899780-OP899810) . One egg was identified at order level (mean similarity of 97.3 %), represented by order Tetraodontiformes. Three eggs were identified at family level (9.68 % of total) all belonging to family Ophichthidae and 20 eggs were identified at genus level (64.52 % of total), distributed among the genera *Chilomycterus* sp., *Halichoeres* sp., and *Atherinella* sp. Seven eggs (22.5 %) were identified at species level (4 species - *Achirus lineatus*, *Canthigaster figueiredoi*, *Chilomycterus geometricus* and *Serranus flaviventris*) with a mean similarity > 98 % (Figure 3; Table 1).
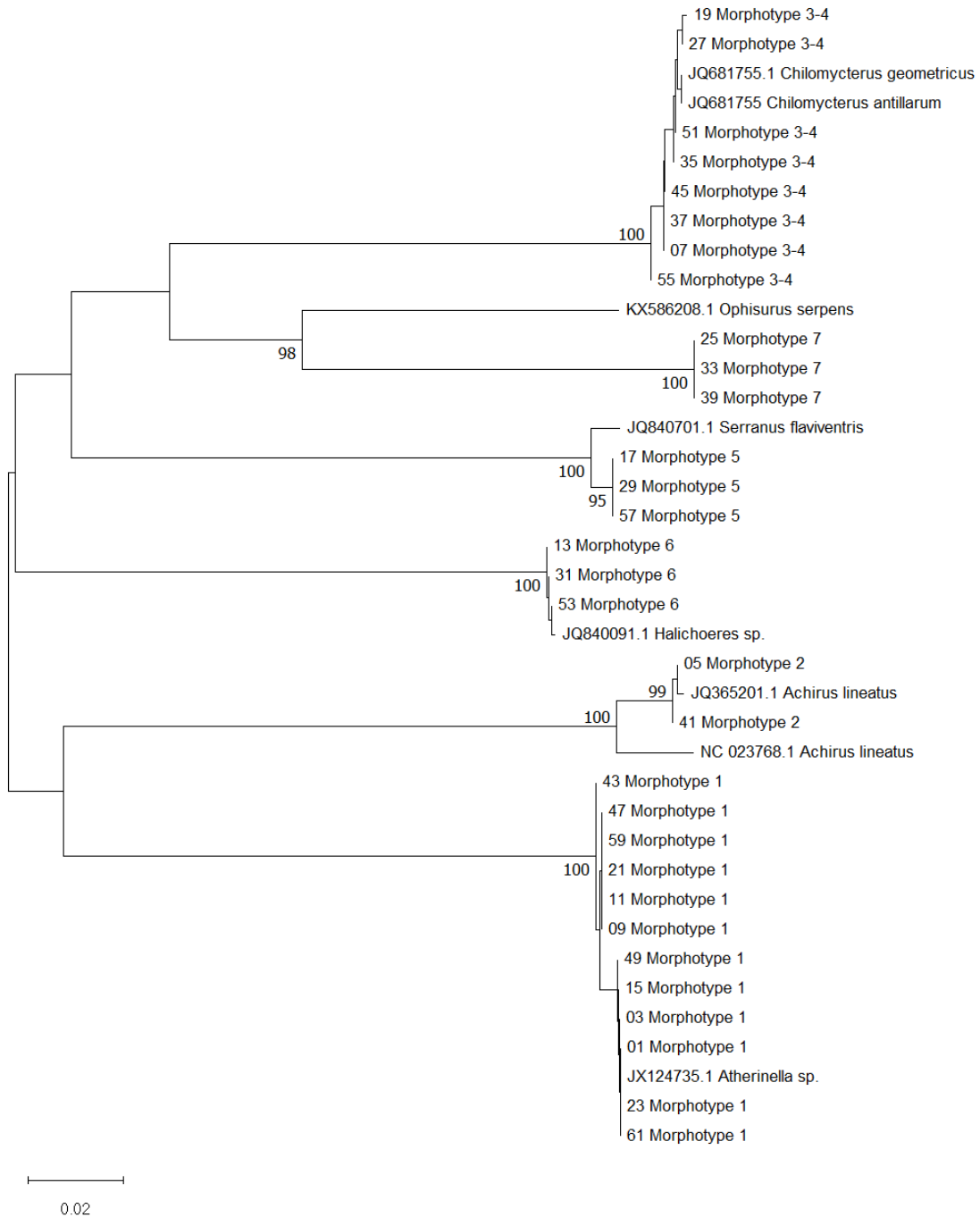
Figure 3. Neighbor-Joining identification tree of taxa found in Equatorial Southwestern Atlantic compared to selected published sequences. Egg morphotypes are numbered according to Table 1. Numbers near branches represent consistency after 10,000 bootstrap replicates The evolutionary distances were computed using the Kimura 2-parameter method, based on pairwise sequence comparisons. There were a total of 676 positions in the final dataset.

4.   Discussion

This baseline assessment represents the first molecular identification of fish eggs diversity in the Equatorial Southwestern Atlantic, providing useful and novel information. The fish eggs identified by the DNA barcode technique were different from the fish species identified from larval stages by traditional methods (Costa et al., 2020) in the same study area. This reveals a cryptic diversity not studied before by morphological techniques. Using the DNA barcoding approach, eleven egg morphotypes were identified comprising 6 genus from 4 families, and 4 species. These data increase the biodiversity knowledge in this low-latitude marine region.

Broadcast spawning is the most common reproduction method in fishes, therefore eggs from many species can be captured by plankton tows (Burrows et al., 2019) as we used in this study. Most studies identify fish spawning habitats using larvae data. However, eggs provide information that is more reliable about the spatial distribution and abundance of fish stocks in comparison to larvae, because they suffer less influence from transport and mortality (Ouellet et al., 1997; Richardson et al., 2009). Identification of fish eggs is crucial for recognizing and protecting spawning and breeding seascapes of not only commercially important species but also keystone species of the marine ecosystems and endangered fish species (Valdez-Moreno et al., 2010).

The success of DNA barcoding relies greatly on references deposited on global and open-access databases, which allow us to identify organisms to the lowest taxonomic level using the DNA sequence. Despite the existence of many COI barcodes deposited for fish species, they are still incomplete for several groups (Carvalho, 2022). In tropical coastal seas, there is a cryptic diversity that has not been identified or deposited in DNA databases yet (Garcia-Vazquez et al., 2021). This is especially true for low-latitude regions such as the study area in Equatorial SW Atlantic.

The eggs were initially grouped into 11 morphotypes according to characteristics such as size, presence of oil globules, length of perivitelline space and appearance of the chorion. The morphological identification of fish eggs to species level is difficult, especially for early stages, which has insufficient diagnostic features, besides its ontogenetic changes during development (Nishiyama et al., 2014). Also it is common to have an overlap in the characters used for morphological identification, among species and

families (Markle and Frost, 1985; Hou et al., 2020). In addition, samples preserved in ethanol can suffer protein denaturation, which can alter the morphological characters used for egg identification (Lewis et al., 2016; Vamos et al., 2017), making it even harder to accurately identify those eggs. This reinforces the importance of DNA barcoding method for fish egg species identification. It is necessary to emphasize that success of species identification using the DNA barcode technique depends on the presence of reference sequences available in databases (Becker et al., 2015) and primer's degree of taxon specificity (Kim et al., 2021).

Many of the identified eggs at species level belong to order Tetraodontiformes such as *Canthigaster figueiredoi* which is a pufferfish known as "Southern Atlantic sharpnose-puffer", a common inhabitant of tropical reefs and rocky bottoms from southern Caribbean to southern Brazilian coast along the South American reef system (Carneiro et al., 2022), including the oceanic islands of Atol das Rocas and Fernando de Noronha (Moura and Castro, 2002). This species is popular in aquarium activities (Gurjão and Lotufo, 2018). We also registered *Chilomycterus geometricus;* this is a burrfish distributed in western Atlantic, from southeastern Florida (USA) to the tropical SW Atlantic (northeastern Brazil). It is common to inhabit coral reefs near seagrass beds (Leis, 2006) such as those found in the study area (Costa et al. 2020) and it is also often traded in aquarium activity (Monteiro-Neto et al., 2003).

Another species identified was *Achirus lineatus*, a widespread flatfish, which belongs to an abundant group in tropical and subtropical regions worldwide (Richards, 2005; Guedes and Araujo, 2008). In Brazil, this fish is abundant nearshore, where their representatives are found in sandy bottoms, both marine and estuarine environments (Figueiredo and Menezes, 2000). Flatfishes are an economically important group because of the quality of their meat (Mendonça and Araujo, 2002). In southwestern Atlantic (northern Brazilian coast), yolk-sac and pre-flexion larvae of *A. lineatus* was registered on greater number in reef and beach habitats, while the post-flexion stage was mainly collected from the mangrove ecosystems, suggesting a migration among these coastal seascapes, from spawning to settlement ground, connecting them by ontogeny changes (Silva-Falcão and Araújo, 2013).

We also recorded *Serranus flaviventris* and *Halichoeres* sp. In this regard, *S. flaviventris* (order Perciformes) is a fish associated with rocky and coral reefs and seagrass

beds in western Atlantic, occurring from the Caribbean Sea to Uruguay (Lieske and Myers, 1994; Parenti and Randall, 2020). *Serranus* fishes possess colorful bodies and are used in aquarium and artisanal fisheries (Iwamoto and Wirtz, 2018). *Halichoeres* genus (family Labridae) were also present in the egg assemblage in Equatorial Southwestern Atlantic. This genus is a natural inhabitant of western Atlantic waters (Pinheiro et al., 2018; Freitas et al., 2019; Santana et al., 2020) and the species inhabits different habitats like tropical reefs (Gosline, 1965; Randall and Rocha, 2009) and oceanic islands (Randall and Rocha, 2009).

Atherinopsidae eggs, here identified at genus level *Atherinella* sp., present filaments to promote the fixation on the aquatic vegetation (Marín et al., 1995). The study area has a large seagrass bed (Costa et al., 2020) composed by marine plant *Halodule wrightii* which can provide protection and retention for those fish eggs. Also, Ophichthidae eggs were registered. Members of this family are marine, occasionally occurring in freshwater, inhabitant of tropical and temperate oceans worldwide (Bonecker et al., 2014). In western Atlantic, this group is known to breed and spawn in coastal seas (Barletta-Began et al., 2002; Pereira et al., 2015).

The characteristics of fish egg assemblage identified in our study area, located in Equatorial Southwestern Atlantic, reinforce the fact that many fishes use different and interconnected coastal habitats during their life cycle (Silva-Falcão et al., 2013; Eggertsen et al., 2017) acting as connectors between these nearshore seascapes (Bughart et al., 2014). Ichthyoplankton studies using the DNA barcode methodology provides cornerstone knowledge on composition, reproduction and spawning season and sites at relatively low cost and labor (Kim et al., 2021). That information is essential for conservation strategies, including design of Marine Protected Areas (MPAs) and fisheries management (Kerr et al., 2020).

Our study does not cover different times of the year, however, the success of the method indicates that this approach can be used to provide reliable seasonal and long-term data on fish diversity in low-latitude marine areas. In this regard, the DNA barcode analysis of ichthyoplankton emerges as a promising strategy to supplement information provided by conventional methods such as morphological taxonomic identification (also used in our study). DNA barcode method is rising as a paramount tool for identification, providing useful and novel information about reproductive biology of

fish species present in poorly-studied seascapes such as the equatorial SW Atlantic. It is important to know the community structure of fishes during early stages in order to effectively monitor and conserve these resources. Thus, it is recommended to use this molecular technique in studies of eggs for future management of fisheries and fish populations. However, it is necessary to highlight that efforts should be made to improve the fish Barcode database, especially for equatorial and tropical species.

In conclusion, DNA barcoding proved to be an effective method for fish biodiversity assessment, but genetic database limitations can reduce its success. Cryptic speciation and current taxonomic uncertainties add complexity to the current problem. In the present study we show that morphological and genetic techniques are complementary and can increase the knowledge on ichthyoplankton composition and they should be conducted to assess and predict fish population dynamics. Finally, we suggest the DNA barcoding is likely to play a central role in identifying important spawning habitats to conserve species of economic and ecologic interest in poorly studied low-latitude marine areas. This baseline information is important mainly to assess the global environmental changes on fish diversity and fisheries worldwide.

Acknowledgments

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General Conclusion

The results highlighted that the tropical Atlantic is an important nursery and offspring rearing where early stages of development look for protected and less turbulent seascapes. The coastal seascapes are also a spawning area for a diverse assemblage of marine fishes, with ecological and economically important species. DNA barcode technique was reliable to identify eggs of tropical fishes. This study can contribute to improve knowledge on fish population dynamics in shallow tropical areas, by setting a baseline for further morphologic and molecular studies in ichthyoplankton assemblage.