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DNA Barcoding of Fish Eggs in the Gulf of Mexico

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by

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ABSTRACT

DNA barcoding of fish eggs is a relatively new technique that enables more accurate identification of early life stages of ecologically and economically important fish species. Using DNA barcoding of individual planktonic percomorph eggs, this thesis determines putative spawning locations of neritic and oceanic fish species in the Gulf of Mexico (GoM). Surveys at 40 stations in the Gulf of Mexico showed a clear delineation of spawning sites, with neritic fish eggs generally found on continental shelves, and oceanic fish eggs found at the surface of deeper waters. However, samples collected between Florida and Cuba revealed exceptions to this trend driven by physical oceanographic processes, with mesoscale eddies transporting eggs of neritic fishes off the Florida continental shelf into the deep Florida Straits. This thesis highlights new spawning information for many marine species, including numerous economically important species (e.g., tunas, groupers, dolphinfishes, billfishes). Methods developed in this thesis allow new interpretation of the interplay of species life history, hydrodynamics of GoM waters and varying habitats. Better understanding of the distribution of fish eggs can help identify regions where additional protection of spawners and recruits may be appropriate.

CHAPTER ONE:

INTRODUCTION

The Gulf of Mexico

The Gulf of Mexico (GoM) is a large, deep, semi-enclosed sea found between the United States, Mexico and Cuba, that supports over 1,500 fish species (McEachran, 2009). The GoM is an economically important fishery region, yielding $35 billion in seafood sales, income, jobs, landing revenue, and value-added impacts in 2012 (Hale et al., 2015). Fish are also important ecologically, playing critical roles in food webs, nutrient cycling, bioturbation and as living mobile links between ecosystems (Holmlund & Hammer, 1999). For fisheries managers to make the most informed decisions, it is crucial to understand the complete life cycle of the target fish species (Cocheret de la Moriniere et al., 2002).

When making management decisions and creating new environmental protection regulations, it is crucial to establish the timing and fate of species occupying a given environment in the absence of anthropogenic or natural disturbances. The GoM has experienced numerous large-scale impacts, including the *Deepwater Horizon* (DWH) oil spill, devastating hurricanes (Irma, Harvey, Ike, Dennis), and Mississippi river floods, in which studies have been done to gauge recovery to pre-disturbance states (Beyer et al., 2016; Greening et al., 2006; Murawski et al., 2014; Rabalais et al., 1998). Without such baseline data, we cannot compare effects of disasters relative to “normal” conditions.

Finding Spawning Locations - Larvae Versus Eggs

Larvae of most species can be easily identified through visual inspection, allowing inference of spawning locations (Peebles & Tolley, 1988; Sassa et al., 2006). While larvae can be used to identify proximal spawning sites, they do not always provide the most accurate or direct location of spawning. A study done in Terra Ceia Bay, Florida, collected eggs and larvae from the same location and showed they did not belong to the same species (Burghart et al., 2014). The reason for this disparity is due to the movement of larvae for days to weeks prior to collection (Cowen & Sponaugle, 2009). It is possible for spawning location to be predicted from larval distributions via hindcasting with hydrodynamic simulations (Allain et al., 2007), but many factors must be considered when creating these models, including vertical migration (Vikebø et al., 2007) and the age of the larvae at collection time. To reduce error in determining spawning location prediction using hindcasting, fish eggs can be used instead of larvae. Fish eggs are generally hours old when collected and are considered mostly passive particles in the water column, except for their positive buoyancy in seawater (Fabra et al., 2005).

Collection of fish eggs has little bias, since eggs are collected with a plankton net towed along the water's surface, and unlike larvae, eggs have no ability to avoid oncoming nets. Since broadcast spawning is the most commonly used reproduction method in fishes, eggs from a variety of species can be captured with plankton tows, including eggs from fish species of commercial (i.e. tunas and groupers) and non-commercial importance (i.e. lizardfishes and goatfishes) (Burrows et al., 2019). Fishes that may not be well sampled with surface-towed plankton nets are primarily species that are not broadcast spawners, including certain species of the families Pomacentrinae, Apogonidae, Blennidae, Syngnathidae, and Sebastinae (Thresher, 1982; Wilson et al., 2003; Wourms, 1991). Planktonic fish eggs in the water column can be

collected at any time of day, in any season and in any location (Duke et al., 2018). However, collection of eggs from April to August in the GoM allows for spawning observation of most coastal species (Chancellor & Murawski, 2015; Claro et al., 2014).

DNA Barcoding

Even though the observation of eggs allows a more direct prediction of spawning grounds, eggs are more difficult to visually identify than larvae, since fish eggs have few morphological variations. However, this obstacle can be overcome using genetic barcoding, a method used to identify fishes from a variety of tissues, including muscle samples, fin clips, whole body specimens, larvae and eggs. DNA barcoding became a widely used method following the publication of a paper by Hebert et al. (2003) establishing a core marker for identification of all animals, the mitochondrial gene cytochrome *c* oxidase I (COI) subunit. Universal primers for polymerase chain reaction (PCR) amplification of a portion of the COI gene allow for successful identification of many animal species (Ward et al., 2005). These primers were adapted by Ivanova et al. (2007) by adding an M13 tail to enable simple direct sequencing of amplified genetic products. Gene sequences can then be compared against the Barcode of Life Database (BOLD; http://www.boldsystems.org/), a community driven species identification database, which contains sequences for over 20,000 different species of ray-finned fishes (Actinopterygii) (Ward et al., 2009). DNA barcoding has been used extensively to identify adult fishes (Ward et al., 2005), fish stomach contents (Smith et al., 2005), and fish larvae (Azmir et al., 2017; Hubert et al., 2010; Ko et al., 2013), but is less commonly applied to individual fish eggs from plankton samples (Ahern et al., 2018; Burghart et al., 2014; Burrows et al., 2019; Frantine-Silva et al., 2015; Harada et al., 2015; Kawakami et al., 2010; Leyva-Cruz et

al., 2016). DNA barcoding has also proven to be more accurate than distinguishing eggs morphologically. In one study, eggs were grouped morphologically into migratory and non-migratory species using perivitelline space, but the morphological method only correctly identified 25% of the eggs when compared to genetic results (Becker et al., 2015). DNA barcoding thus reports a breakthrough technology to genetically identify fish eggs while avoiding the more tedious and inaccurate process of morphological identification.

Thesis Goals

This thesis applies DNA barcoding to identify individual fish eggs, providing quantitative information about the relative abundance and spatial distribution of eggs belonging to specific taxa throughout the GoM. In addition, this thesis documents the encounter rates of eggs from economically valuable species in the GoM. These data serve as an important baseline to evaluate changes in fish spawning trends following natural and anthropogenic disasters, like hurricanes and oil spills.

CHAPTER TWO:

DNA BARCODING REVEALS CLEAR DELINEATION BETWEEN SPAWNING SITES FOR NERITIC VERSUS OCEANIC FISHES IN THE GULF OF MEXICO

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Abstract

We combined research-vessel cruises of opportunity with DNA barcoding to survey planktonic, percomorph fish eggs at 40 stations distributed across and around the Gulf of Mexico (GoM). The objectives were (a) to determine whether eggs of fishes that are potential candidates for the daily egg production method (DEPM) can be readily barcoded, (b) to identify taxa that are spawning in the GoM, (c) to determine encounter rates for eggs of economically valuable taxa, and (d) to characterize individual egg taxa as being primarily neritic, primarily oceanic, or primarily mixed (i.e., both neritic and oceanic). Of the 1,144 eggs that were individually barcoded, 709 (62%) were definitively identified to species (62 species from 42 families), with an additional 20 taxa identified to genus or subfamily level. The eggs of 15 economically important species were identified, most of which had intermediate encounter rates and moderately dispersed spatial distributions, as indicated by an index of aggregation. SIMPROF analysis of stationwise cluster analysis identified eight significant groups within the 35 stations that yielded percomorph eggs; a corresponding species wise analysis identified six groups of stations as having a neritic egg community and two groups as having an oceanic community,

with a community transition located at the shelf break. Although the neritic and oceanic stations did not share important species, it remains possible that coastal pelagic species have mixed neritic–oceanic distributions. Together, these results indicate DEPM fish-egg surveys based on DNA barcoding are feasible at the large marine ecosystem scale.

Introduction

Using the abundance of planktonic fish eggs to estimate the biomass of the parent fish stocks (daily egg production method, DEPM) has been hindered by practical limitations, including difficulty with visually identifying drifting eggs to species, and not knowing the appropriate spatial range for egg surveys that are required by DEPM (Borchers et al., 1997; Lo et al., 1996; Stratoudakis et al., 2006). Fish larvae are usually more visually distinctive than fish eggs, and thus, larval distributions have been used to identify spawning grounds (Peebles & Tolley, 1988; Sassa et al., 2006). However, the capture locations of planktonic larvae are not ideal proxies for spawning habitat because the larvae can drift long distances over days or weeks prior to capture, resulting in substantial spatial offsets from natal spawning grounds (Cowen & Sponaugle, 2009; Muhling et al., 2017); this drift is often behaviorally modulated rather than passive (Vikebø et al., 2007). The net effect of these spatial offsets can be egg and larval species compositions that are highly dissimilar (Burghart et al., 2014). In contrast, the progeny of live-bearing species (e.g., rockfishes) may live in close proximity to their locations of parturition.

In contrast to larvae, the eggs of broadcast-spawning fishes are often only hours old when collected and are passive particles (except for often being positively buoyant), and these two traits reduce error when hydrodynamic models are used to locate spawning areas (Burghart et al., 2014). Genetic barcoding has been used extensively to identify adult fishes (Ward et al., 2005),

fish stomach contents (Smith et al., 2005), and fish larvae (Hubert et al., 2010), but is less commonly applied to individual fish eggs from plankton samples (Burghart et al., 2014; Harada et al., 2015). Previous applications of genetics-based approaches have either used specific primers to search samples of fish eggs for species of interest (Chow & Inoue, 1993) or used degenerate primers and DNA barcoding to identify individual fish eggs to species level (Ivanova et al., 2007). Degenerate primers are mixtures of oligonucleotide sequences (primers) that produce a larger range of possible nucleotide matches during PCR amplification (Iserte et al., 2013). Studies that have compared visual identifications of percomorph eggs with identifications obtained through DNA barcoding have indicated visual identifications can be highly unreliable (Larson et al., 2016).

DNA barcoding of individual fish eggs alleviates DEPM limitations by allowing the spatial ranges of the drifting eggs to be identified with greater confidence (Burghart et al., 2014; Stratoudakis et al., 2006). In cases where fish eggs can be identified directly (whether visually or by barcoding), it has been observed that the eggs of some species occur on both the continental shelf and over deep, ocean waters, invalidating the shelf break as a natural boundary for egg surveys (Borchers et al., 1997). For other species, it has been suggested that spawning near the shelf break facilitates population connectivity (Cowen & Sponaugle, 2009) via dispersion of eggs and larvae by ocean currents. The literature supporting dispersion-based reproductive success is described by Karnauskas, Cherubin, and Paris (2011), who challenged this idea after their hydrodynamic models indicated predominant particle trajectories resulted in retention on the continental shelf, rather than offshore dispersion. Various other studies have also described biophysical interactions that retain the eggs and larvae of continental shelf (neritic) species on the continental shelf or near the upper slope (Hutchings et al., 2002; Muhling et al., 2017;

Weisberg et al., 2014). It thus appears that while some species have eggs and larvae that are widely dispersed in both neritic and deep-oceanic waters, other species have eggs and larvae that are generally retained either in the deep sea or on the continental shelf, inclusive of the upper slope. However, retention is not perfect, and the process may be accompanied by spillover of eggs and larvae into adjacent waters. In the case of neritic retention, spillover is not always aberrant in regard to survival, as offshore-displaced larvae may remain competent enough to reach advanced larval stages (Velez & Moore, 2018). In general, research activities that document spawning locations and then compare these locations with oceanographic features (currents, remotely sensed data, bottom physiography, etc.) are likely to provide insight regarding the factors that influence spawning locations, including factors such as the likelihood of retention.

The objective of this study was to combine DNA barcoding of individual fish eggs within plankton samples from cruises of opportunity in order to do the following:

1. determine whether eggs of fishes that are potential candidates for DEPM (i.e., species with economic value) can be readily barcoded from large batches containing multiple species;
2. identify economically valuable taxa that have the potential to be self-recruiting in the Gulf of Mexico (GoM), as opposed to being dependent on connectivity with populations outside the GoM;
3. determine encounter rates for eggs of economically valuable taxa; and
4. characterize the fish-egg taxa as being primarily neritic, primarily oceanic, or primarily mixed (i.e., both neritic and oceanic).

This effort intentionally addressed the large marine ecosystem (LME) scale and thus examines coarse scales of distribution.

Because DNA barcoding of individual fish eggs is a relatively new research activity that has not been widely applied geographically, the results of genetics-based egg surveys also have fundamental exploratory value and relevance to biogeographic studies. Collecting passively drifting fish eggs is one of the least biased methods of collecting fishes. It can be equally effective at collecting small, cryptic species as it is for large, evasive species; most economically valuable species are at least moderately large and moderately evasive. The fourth objective provides linkages between biodiversity studies of fishes in continental shelf (Murawski et al., 2018), deep benthic (Wei et al., 2012), mesopelagic (Sutton et al., 2017), and epipelagic (Habtes et al., 2014) habitats of the GoM.

Methods

Study Site and Sample Collection

Planktonic fish eggs were collected during three cruises by the R/V *Weatherbird II* during 2015–2016 (Figure 1). The first cruise crossed the GoM from Tuxpan, Mexico to St. Petersburg, Florida (USA), during fall 2015 (September 27–October 1). The second cruise focused on a smaller region of the Northeastern GoM during spring 2016 (April 8–12), and the third cruise circumnavigated the GoM during late summer 2016 (August 4–September 10). Egg sampling was conducted on cruises where the primary activity was collecting adult fishes using demersal long lines (Murawski et al., 2018), and thus, plankton tows were only conducted at times when other shipboard research activities were not ongoing and long steaming periods were anticipated. As a result, potential transects were preselected during the cruise planning stage.

Given objective 4, effort was made to include stations that either crossed the shelf break or ran parallel to it. All stations (n = 40, Figure 1) were sampled using a bongo-type (double conical) plankton net (333 μm mesh, 61 cm mouth diameter) equipped with General Oceanics 2030R mechanical flowmeters and 1-liter plastic cod-end jars. The net was towed obliquely, starting at depth (~100 m) followed by haulback to the surface by hydraulic winch while the vessel was underway; tow duration was 15 min, which resulted in a mean filtered volume of 310 m^3. After retrieval and net washdown, one of the two bongo samples was preserved in 50% isopropanol in ambient seawater (the other sample was used in a different study). Upon return to the laboratory, percomorph eggs (eggs of spiny-finned fishes) were visually distinguished from clupeoid eggs (eggs of anchovies, herrings, and sardines) via stereomicroscopy at 9-108X. All percomorph eggs from each plankton sample were transferred to 70% isopropanol in a glass vial, except when the total number of percomorph eggs was >96; in such cases, a subsample of 96 eggs was transferred. The total number of percomorph eggs was recorded for all samples.

Genetic Identification of Fish Eggs

Individual eggs were placed in 0.2-ml polymerase chain reaction (PCR) tubes with a sterile pipette tip, and all excess isopropanol was removed. The Chelex DNA extraction method adapted from Hyde et al. (2005) was used for stations 101–120 and 201, but HotSHOT DNA extraction (Truett et al., 2000) was used for all subsequent samples. The change was based on a previous study that demonstrated that DNA extracted from zooplankton eggs with the HotSHOT method was stable for longer periods of time than DNA extracted using the Chelex method (Montero-Pau et al., 2008). No noticeable differences in the overall success of DNA barcoding were noted between the methods.

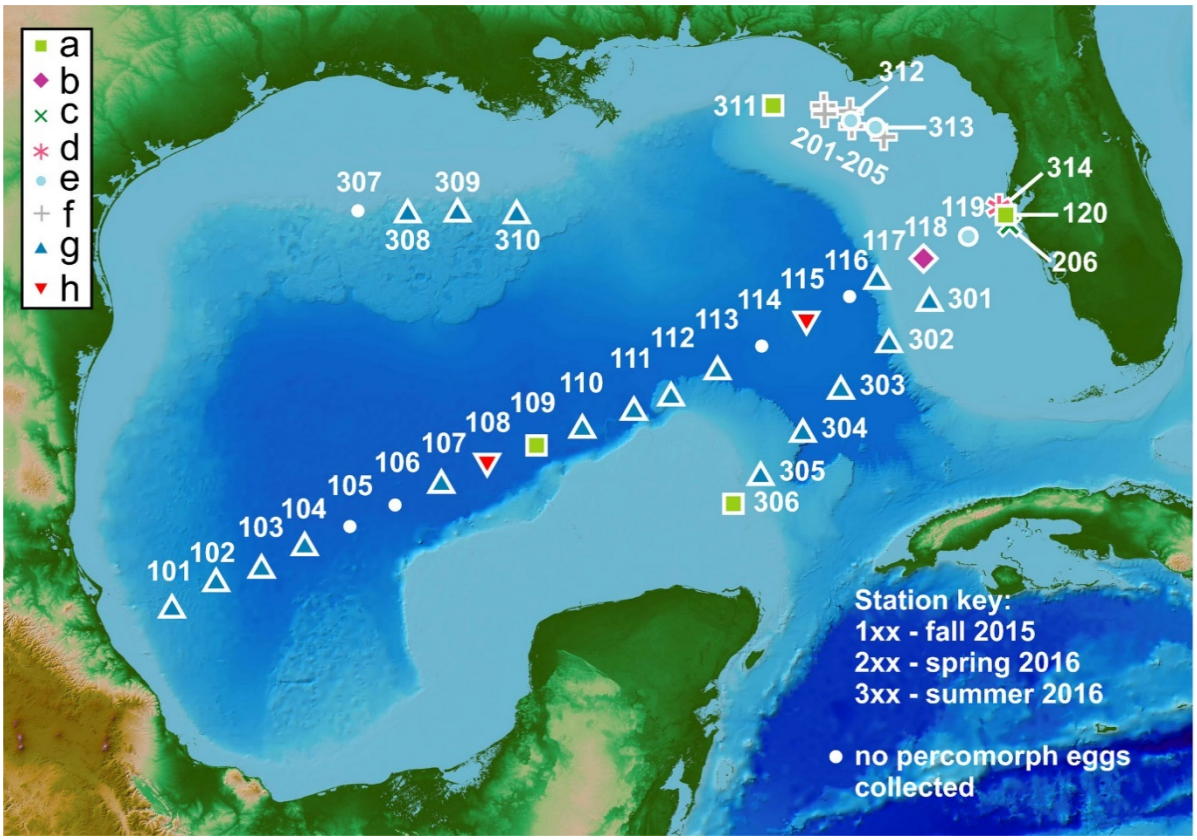

Figure 1: Map of plankton stations, identified by SIMPROF group. The symbols for stations 206 and 314 were jittered for clarity, but were much closer to station 120 than depicted here

For Chelex extraction, 150 µl of 10% Chelex 100 molecular biology grade resin (Bio-Rad Laboratories) was added to each tube containing an individual fish egg, followed by crushing the egg in the resin with a sterile toothpick. The tubes were then incubated in a thermocycler (Eppendorf 6321) at 60°C for 20 min, 99°C for 25 min, 37°C for 1 min, and 99°C for 15 min. For the HotSHOT extraction method, 50 µl of alkaline lysis buffer (25 mM NaOH, 0.2 mM disodium EDTA, pH 12) was added to each tube and the fish egg was crushed in the buffer using a sterile toothpick. Tubes were then heated in a thermocycler for 30 min and cooled on ice for 3 min. Finally, 50 µl of neutralization buffer (40 mM Tris–HCl, pH 5) was added and the sample was vortexed quickly to complete the extraction.

Each fish egg was then genetically identified by PCR amplification and Sanger sequencing (DNA barcoding) of the mitochondrial cytochrome c oxidase I (COI) gene using the COI-3 universal fish primer cocktail described by Ivanova et al. (2007). Each 50 µl PCR contained final concentrations of 1x Apex NH_4 buffer, 1.5 mM Apex $MgCl_2$, 0.2 µM Apex dNTPs, 1 U Apex RedTaq (Genesee Scientific), 0.2 µM primer cocktail, 10 µg/µl bovine serum albumin (New England BioLabs Inc.), and 2-5 µl of target DNA. At first, 2 µl of target DNA was used; if the PCR failed, then 5 µl of target DNA was used in a second reaction. The PCR was heated to 94°C for 2 min, followed by 45 cycles of (94°C for 30 s, 52°C for 40 s, 72°C for 1 min) and 72°C for 10 min. Successful PCR amplification was confirmed by running products on a 1.5% agarose gel stained with ethidium bromide. Successful PCR products were sent to TACGen (tacgen.com) for purification and Sanger sequencing using the M13 forward primer, which is contained within flanking tails of the primer cocktail (Ivanova et al., 2007). Sequences were then trimmed for quality using Sequencher™ 5.3 (Genecodes) and compared against the species-level records in the Barcode of Life Database (BOLD; http://www.boldsystems.org/) for identification. The lowest level of taxonomic assignment confidently predicted by BOLD is reported here. Barcodes ranged from 608 to 645 base pairs in length (Ivanova et al., 2007).

In several cases, the COI-3 region did not provide sufficient resolution for discriminating between related species, only allowing identification to genus. To achieve definitive species-level identifications for eggs belonging to economically important groups, additional PCRs were performed. The ATCO region between the ATPase6 and COI-3 genes was amplified from the DNA of 23 fish eggs originally identified as either *Thunnus thynnus* or *Katsuwonus pelamis* (Chow & Inoue, 1993). With the exception of different primers (L8562 and H9432), the reaction mixture was the same as above, with cycling conditions of heating to 94°C for 2 min, followed

by 45 cycles of (94°C for 30 s, 50°C for 40 s, 72°C for 1.5 min) and 72°C for 10 min. Another PCR was performed on DNA from a single fish egg initially identified as either *Scomberomorus cavalla* or *Acanthocybium solandri* to achieve a definitive identification. The same PCR composition from above was used with different primers to amplify a longer section of the COI gene (Paine et al., 2007) and was heated to 94°C for 2 min, followed by 45 cycles of (94°C for 30 s, 57°C for 40 s, 72°C for 2 min) and 72°C for 10 min. A total of 24 products were cleaned with a Clean & Concentrator-25 kit (Zymo) and sent to TACGen for bidirectional Sanger sequencing.

Statistical Analyses

Several multivariate community analyses were conducted to identify taxonomic communities, and the collection locations of the resulting communities were plotted on a map of the survey area; the objective was to determine the degree of spatial overlap between neritic and oceanic communities. For each station, the density (effort-corrected abundance) of eggs in the water column was calculated by dividing the total number of percomorph eggs in the sample by the volume filtered by the plankton net, as determined from flowmeter readings. The total egg density at each station was then apportioned to individual taxa according to proportional representations in the sample of barcoded eggs relative to the total number of percomorph eggs in the sample.

The resulting catch table was analyzed using PRIMER 7 software (v. 7.0.13, PRIMER-E, Auckland, New Zealand), wherein the density values were square-root-transformed and used to compute a Bray–Curtis similarity matrix. Stations and taxa were independently grouped using hierarchical cluster analysis based on the group-average cluster mode, with the resulting

dendrogram for stations being subdivided into statistically significant clusters (groups of stations) via SIMPROF analysis (Clarke et al., 2008); SIMPROF-group identities were then plotted on the map of stations. To describe major taxonomic trends in community structure, dendrograms for both stations and taxa were arranged into a seriated heatmap (*shade plot* routine in PRIMER 7), with abundance represented by the square root of density. Note that PRIMER 7 limits heatmap depictions to the most "important" taxa, which are taxa that have the highest percentage contributions to any of the samples, with a maximum depiction of 50 important taxa.

The similarity matrix was also used to generate a nonmetric multidimensional scaling (nMDS) plot for station associations, which included an overlay of SIMPROF groups. SIMPROF groups were classified as being neritic or (deep) oceanic by examining taxon-specific native distribution maps in FishBase (http://www.fishbase.org/search.php) and by comparing these with the species-wise cluster analysis. The nMDS plot was used to compare relative station similarities.

A species accumulation curve was generated to gauge the extent to which the surveys represented the fish-egg species richness of the GoM. Stochastic species accumulation curves were created using the *specaccum* function in the *vegan* package implemented in R (Oksanen et al., 2017); the jackknife procedure (n = 1,000) used selection of stations without replacement. These results provide a rarefaction curve that depicts the number of cumulative species encountered as a function of the number of stations sampled. Egg patchiness was described using Bez's (2000) index of aggregation (I_a) to characterize the relative spatial dispersion of individual egg taxa.

Results

Five of the 40 samples (13%) did not contain percomorph eggs. Of the 1,144 successfully barcoded eggs, the BOLD database definitively identified (>97% certainty) 62 species from 42 families. An additional 18 taxa were identified at genus level, and two taxa (one species of scad and one species of tuna) were identified at the subfamily level. The species accumulation curve (Figure 2) was nonasymptotic, indicating substantially more taxa would have been encountered with additional sampling.

Summary statistics for the egg catch are presented in Table 1. The eggs of 15 economically important species were identified; in decreasing order of mean abundance (eggs 10^3 m^{-3}), these were *Sciaenops ocellatus* (red drum), *Katsuwonus pelamis* (skipjack tuna), *Thunnus atlanticus* (blackfin tuna), *Mycteroperca phenax* (scamp), *Rhomboplites aurorubens* (vermilion snapper), *Lutjanus campechanus* (northern red snapper), *Euthynnus alletteratus* (little tunny), *Mycteroperca microlepis* (gag), *Pagrus pagrus* (red porgy), *Coryphaena hippurus* (common dolphinfish), *Thunnus albacares* (yellowfin tuna), *Auxis thazard* (frigate tuna), *Caulolatilus cyanops* (blackline tilefish), *Scomberomorus cavalla* (king mackerel), and *Istiophorus albicans* (Atlantic sailfish).

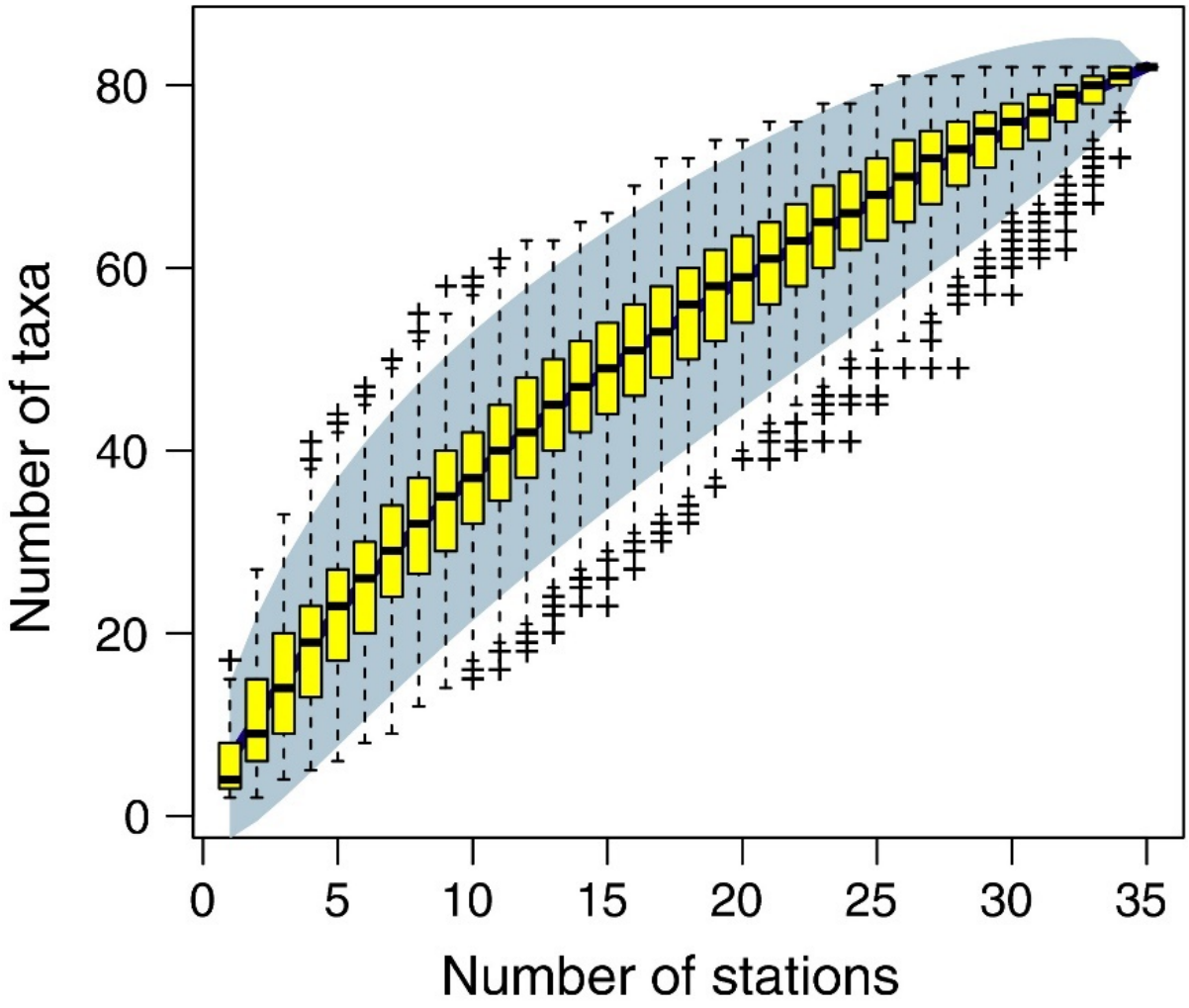

Figure 2: Stochastic species accumulation results for fish-egg sampling in the Gulf of Mexico during 2015 and 2016 based on 1,000 jackknifed iterations that sampled the station data without replacement. Yellow bars are the interquartile ranges of species richness from each experiment; light blue is the confidence interval for the mean species richness as a function of the number of stations sampled, and crosses and "Ts" are the ranges from jackknife iterations

Table 1: Habitat, economic importance, catch statistics, and Bez's (2000) index of aggregation (I_a) for all fish-egg taxa encountered during the survey

Taxon	FishBase common name	Habitat	Economic importance	Encounter frequency	Mean density	Maximum density	Mean nonzero density	Aggregation (I_a)
Acanthostracion quadricornis	Scrawled cowfish	neritic	no	1	0.08	2.96	2.96	1
Auxis sp.	Frigate tuna	oceanic	yes	5	5.24	98.1	36.69	0.35
Auxis thazard	Frigate tuna	oceanic	yes	1	0.18	6.23	6.23	1
Bellator militaris	Horned searobin	neritic	no	1	0.84	29.52	29.52	1
Bothus robinsi	Twospot flounder	neritic	no	1	0.18	6.23	6.23	1
Brama sp.	Pomfret	oceanic	no	2	0.4	8.18	6.96	0.52
Calamus sp.	Porgy	neritic	yes	1	0.69	24.15	24.15	1
Caranx crysos	Blue runner	both	no	1	0.08	2.64	2.64	1
Caulolatilus cyanops	Blackline tilefish	neritic	yes	1	0.13	4.63	4.63	1
Centropristis ocyurus	Bank seabass	neritic	no	2	0.37	7.03	6.47	0.5
Coryphaena hippurus	Common dolphinfish	oceanic	yes	2	0.34	9.5	5.97	0.67
Cubiceps sp.	Driftfish	oceanic	no	8	10.58	286.87	46.28	0.62
Cyclopsetta fimbriata	Spotfin flounder	neritic	no	2	0.62	17.76	10.78	0.71
Cyclopsetta sp.	Flounder	neritic	no	1	0.18	6.23	6.23	1
Decapterus sp.	Scad	both	minor	8	11.27	216.12	49.32	0.35
Diplectrum formosum	Sand perch	neritic	no	1	1.38	48.31	48.31	1
Diplospinus sp.	Escolar	oceanic	no	11	4.47	26.88	14.23	0.12
Echeneis naucrates	Live sharksucker	both	no	1	0.08	2.96	2.96	1
Echeneis sp.	Remora	both	no	3	0.28	3.71	3.21	0.34
Echiophis intertinctus	Spotted spoon-nose eel	neritic	no	1	0.17	5.92	5.92	1
Etrumeus sadina	Red-eye round herring	both	no	2	1.5	47.23	26.27	0.82
Eucinostomus sp.	Mojarra	neritic	no	1	0.69	24.15	24.15	1
Euthynnus alletteratus	Little tunny	both	yes	2	0.43	12.46	7.55	0.71
Fistularia tabacaria	Cornetfish	neritic	no	1	0.08	2.96	2.96	1
Gempylid	Snake mackerel	oceanic	no	1	0.49	17.29	17.29	1
Gymnachirus sp.	Gom fringed sole	neritic	no	1	0.11	3.71	3.71	1
Haemulon aurolineatum	Tomtate	neritic	no	1	0.2	7.03	7.03	1
Haemulon sp.	Grunt	neritic	unknown	1	13.8	483.08	483.08	1
Halichoeres bathyphilus	Greenband wrasse	neritic	no	1	0.8	28.1	28.1	1
Halichoeres bivittatus	Slippery dick	neritic	no	1	0.69	24.15	24.15	1

Table 1 (Continued)

Taxon	Fishbase common name	Habitat	Economic importance	Encounter frequency	Mean density	Maximum density	Mean nonzero density	Aggregation (I_a)
Istiophorus albicans	Atlantic sailfish	oceanic	yes	1	0.08	2.88	2.88	1
Katsuwonus pelamis	Skipjack tuna	oceanic	yes	4	18.5	570.79	161.86	0.79
Lepidophanes guentheri	Günther's lanternfish	oceanic	no	1	0.54	18.75	18.75	1
Lutjanus campechanus	Northern red snapper	neritic	yes	1	0.59	20.57	20.57	1
Malacanthus plumieri	Sand tilefish	neritic	no	1	0.18	6.23	6.23	1
Menticirrhus littoralis	Gulf kingcroaker	neritic	minor	1	0.69	24.15	24.15	1
Menticirrhus saxatilis	Northern kingfish	neritic	minor	1	0.69	24.15	24.15	1
Mugil sp.	Mullet	neritic	yes	1	0.4	14.06	14.06	1
Mullus auratus	Red goatfish	neritic	no	2	1.84	59.05	32.18	0.85
Mycteroperca microlepis	Gag	neritic	yes	2	0.38	7.03	6.63	0.5
Mycteroperca phenax	Scamp	neritic	yes	2	0.7	14.06	12.33	0.51
Nesiarchus nasutus	Black gemfish	oceanic	no	2	0.69	15.12	11.99	0.53
Ophichthus gomesii	Shrimp eel	neritic	no	1	0.08	2.96	2.96	1
Orthopristis chrysoptera	Pigfish	neritic	minor	1	1.38	48.31	48.31	1
Oxyporhamphus micropterus	Bigwing halfbeak	oceanic	no	2	0.26	6.22	4.62	0.56
Pagrus pagrus	Red porgy	neritic	yes	1	0.36	12.46	12.46	1
Paraconger caudilimbatus	Margintail conger	neritic	no	1	0.08	2.96	2.96	1
Pareques iwamotoi	Blackbar drum	neritic	no	1	0.13	4.48	4.48	1
Prionotus martis	GoM barred searobin	neritic	no	2	1.89	62.37	33.04	0.89
Prionotus ophryas	Bandtail searobin	neritic	no	1	0.69	24.15	24.15	1
Prionotus sp.	Searobin	neritic	no	5	3.85	56.21	26.94	0.26
Pristipomoides aquilonaris	Wenchman	neritic	minor	2	1.99	67.25	34.85	0.93
Pterycombus petersii	Prickly fanfish	oceanic	no	1	0.14	4.78	4.78	1
Remora osteochir	Marlin sucker	oceanic	no	1	0.09	3.05	3.05	1
Rhomboplites aurorubens	Vermilion snapper	neritic	yes	2	0.63	14.8	11.11	0.56
Rypticus bistrispinus	Freckled soapfish	neritic	no	1	0.08	2.64	2.64	1
Rypticus maculatus	Whitespotted soapfish	neritic	no	1	0.22	7.57	7.57	1
Rypticus sp.	Soapfish	neritic	no	1	1.78	62.37	62.37	1
Saurida brasiliensis	Brazilian lizardfish	neritic	no	4	3.48	98.37	30.43	0.67
Saurida normani	Shortjaw lizardfish	neritic	no	1	0.9	31.4	31.4	1
Scad	Scad	both	minor	2	0.2	4.48	3.46	0.54
Sciaenops ocellatus	Red drum	neritic	yes	1	19.6	686.09	686.09	1

Table 1 (Continued)

Taxon	Fishbase common name	Habitat	Economic importance	Encounter frequency	Mean density	Maximum density	Mean nonzero density	Aggregation (I_a)
Scomberomorus cavalla	King mackerel	neritic	yes	1	0.11	3.71	3.71	1
Serranus notospilus	Saddle bass	neritic	no	1	0.19	6.52	6.52	1
Sphyraena borealis	Northern sennet	neritic	no	1	0.34	11.81	11.81	1
Stomias sp.	Dragonfish	oceanic	no	1	0.89	31.09	31.09	1
Syacium papillosum	Dusky flounder	neritic	no	8	14.95	194.84	65.39	0.23
Symphurus plagiusa	Blackcheek tonguefish	neritic	no	1	0.69	24.15	24.15	1
Symphurus urospilus	Spottail tonguefish	neritic	no	1	0.11	3.79	3.79	1
Synodus foetens	Inshore lizardfish	neritic	no	4	2.37	62.37	20.77	0.6
Synodus sp.	Lizardfish	neritic	no	7	6.11	89.17	30.53	0.26
Thunnus albacares	Yellowfin tuna	oceanic	yes	2	0.28	6.22	4.97	0.53
Trachinocephalus myops	Snakefish	neritic	no	8	13.22	156.96	57.83	0.22
Trachurus lathami	Rough scad	neritic	minor	3	10.66	360.06	124.33	0.93
Tuna	Tuna	oceanic	yes	4	2.74	57.46	23.99	0.44
Urophycis floridana	Southern codling	neritic	no	1	0.15	5.3	5.3	1
Vinciguerria sp.	Bristlemouth	oceanic	no	2	0.68	18.66	11.96	0.66
Xyrichtys novacula	Pearly razorfish	neritic	no	5	19.38	267.86	135.66	0.32

Note

Encounter frequency is number of stations. Densities are eggs 10^3 m^{-3}, with the mean calculated from 35 stations with positive catches. GoM, Gulf of Mexico. Columns 2–4 are derived from FishBase (http://www.fishbase.org/search.php).

The index of aggregation (I_a, Figure 3) was negatively correlated with encounter frequency (Pearson's $r = -0.81$, $n = 82$, $p < 0.0001$). By mathematical definition, taxa with a frequency of encounter of 1 have high I_a values. At the other extreme, the most frequently encountered taxon, *Diplospinus* sp. (escolar), had the lowest I_a value, indicating its spawning was broadly dispersed relative to other taxa. Many of the economically valuable species (listed

above) had intermediate I_a values (Figure 3), indicating their spawning was moderately dispersed.

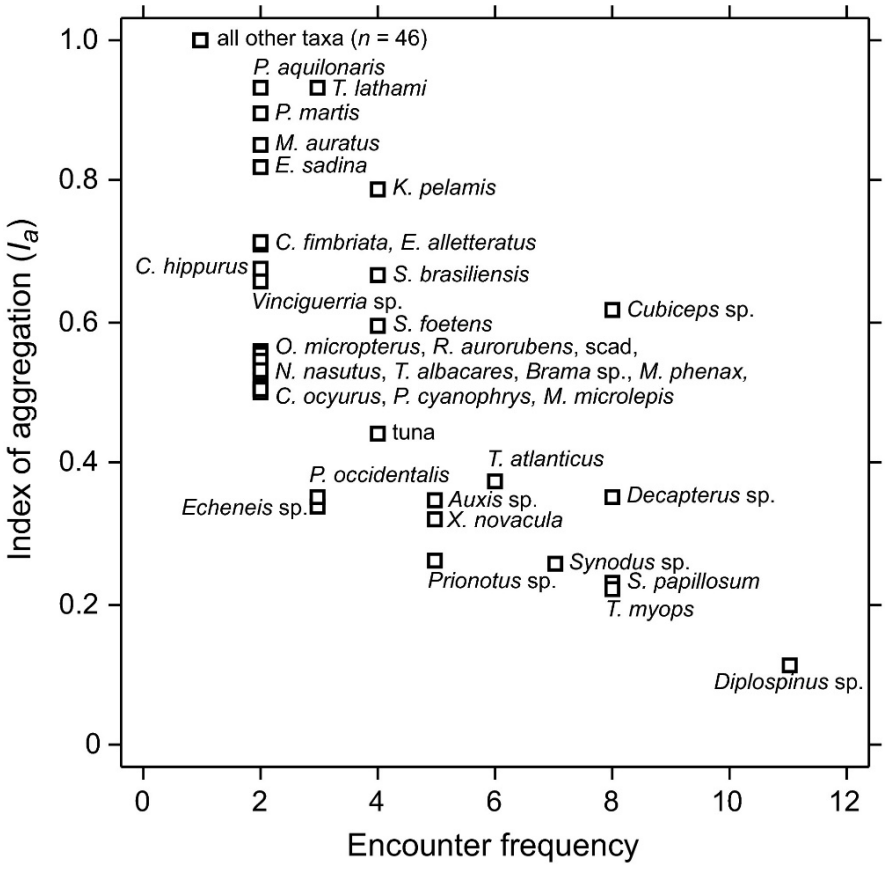

Figure 3: Comparison of fish-egg encounter frequency (number of stations) with Bez's (2000) index of aggregation, I_a. The two metrics were strongly correlated (Pearson's $r = -0.81$, $n = 82$, $p < 0.0001$)

The SIMPROF analysis produced eight significant groups within the 35 stations that yielded percomorph eggs (Figure 1). The compositions of these groups (top 50 most important taxa) are presented in Figure 4, which includes a species-wise dendrogram that has a major division that corresponds with the interface between neritic and oceanic station-wise (SIMPROF)

groups. A minor oceanic group, group *h*, consisted of the eggs of two mesopelagic species at two stations. Together, Figures 1 and 4 indicate a community transition at the shelf break. The nMDS plot (Figure 5) and Figure 4 indicate SIMPROF group *a* was most similar to the major oceanic group (group *g*), but there was no overlap between these groups in low-stress 2D nMDS space, and they did not share important species.

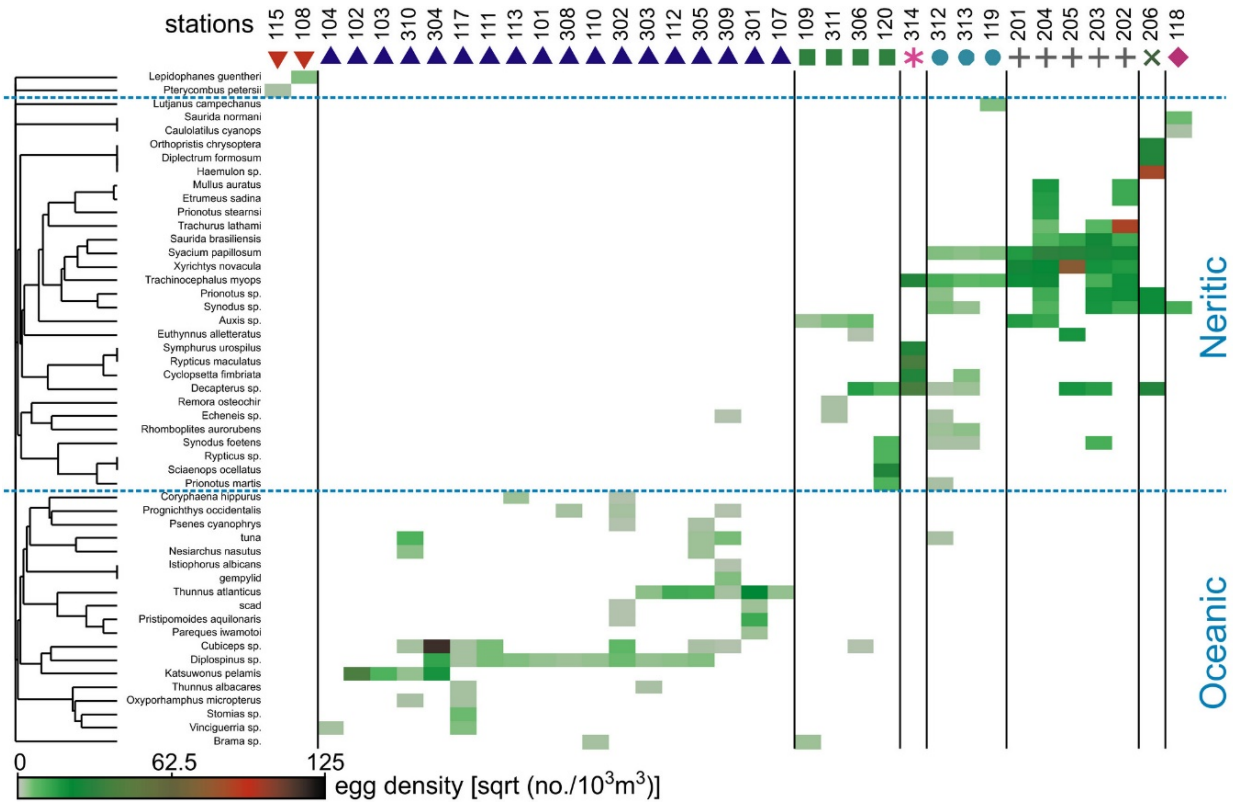

Figure 4: Heatmap of the 50 most important fish-egg taxa, with a dendrogram indicating species associations and vertical lines identifying statistically significant station associations (SIMPROF groups). For clarity, the station-wise dendrogram is not shown

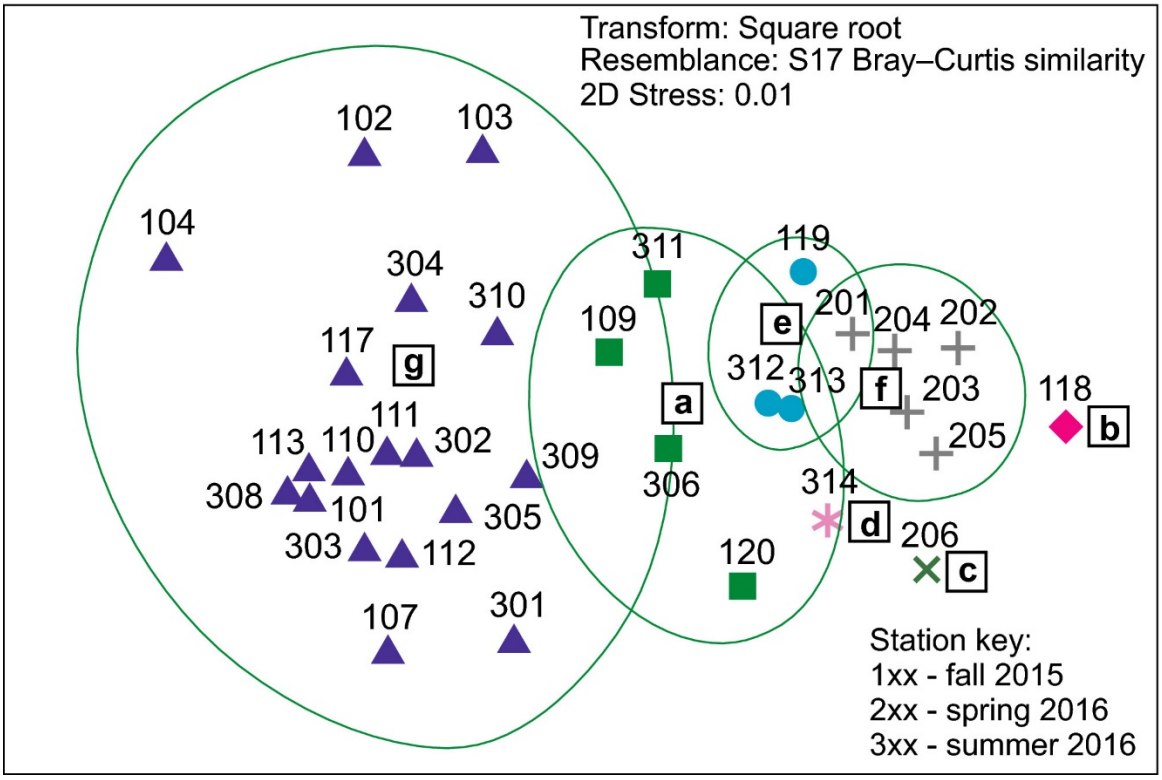

Figure 5: nMDS plot of station similarities, with ellipses drawn around SIMPROF groups, which are labeled with letters within boxes. The minor oceanic group, group *h* (two mesopelagic species), is not shown because the two stations in this group were spatial outliers that made the overall scale illegible for the other groups

Discussion

Factors That Affect Egg Distribution

The distribution of planktonic fish eggs is influenced by the biogeography of broadcast spawners, the spatial patchiness of spawning habitats, temporal variation in spawning activity, and advection of eggs after spawning. Regarding biogeography, the fundamental distinction among egg communities was the separation of neritic species from deep-ocean species (Figures 1, 4, and 5). This distinction is not entirely explained by position in the water column,

as both groups included a mixture of pelagic and demersal species. The oceanic group included epipelagic species (e.g., *C. hippurus* and *Istiophorus albicans*), mesopelagic species (e.g., Günther's lanternfish, *Lepidophanes guentheri*, and prickly fanfish, *Pterycombus petersii*), and demersal species that live near the shelf break (e.g., *Pristipomoides aquilonaris* and blackbar drum, *Pareques iwamotoi*). In general, however, the neritic group included far more demersal species, including cryptic burrowers (e.g., the eels *Paraconger caudilimbatus* and *Echiophis intertinctus*).

Although detailed biogeographic histories are not available for most of the fish families in the GoM, demersal neritic species such as lutjanids (snappers) appear to be derived from eastern Pacific ancestors that became isolated after the Panamanian Gateway closed 4.5 million years ago (Gold et al., 2011). Since then, vicariant and ecological speciation has produced a large number of species that are now endemic to the tropical and temperate waters of the western Atlantic, inclusive of the GoM. This contrasts with the oceanic egg group, which was dominated by species that have much larger ranges, including ranges that are circumglobal within tropical and temperate waters (e.g., *C. hippurus* and *K. pelamis*) and ranges that extend across large areas of both the north and south Atlantic Oceans (e.g., *Lepidophanes guentheri* and *T. atlanticus*).

To some extent, the patchiness of spawning habitat is indicated by the percent of stations at which a given taxon occurred (Figure 3). Frequently encountered taxa (e.g., *Diplospinus* sp. and driftfish, *Cubiceps* sp.) appear to be more general in their spawning habitat than taxa that were encountered in large numbers at relatively few locations (e.g., *K. pelamis* and rough scad, *Trachurus lathami*). Higher levels of spawning-habitat selectivity may relate to the geography of egg and larval transport (Cowen & Sponaugle, 2009; Weisberg et al., 2014) or to

orientation with dynamic physical processes that support biological productivity (E. Peebles et al., 1996; E. B. Peebles, 2002; Reglero et al., 2014).

The species–accumulation curve for the present survey was not asymptotic (Figure 2), indicating that adding stations to the survey (more potential spawning sites) would have added many more species to the overall catch. There are more than 1,500 identified fish species in the GoM (McEachran, 2009), and a large proportion of these are broadcast spawners with buoyant eggs. Our study identified 82 egg taxa, and thus, there are a considerable number of additional species known from the continental shelves and deep ocean that were not collected. This pilot study, however, did not systematically sample the spatial or temporal (seasonal) range of fish spawning in the GoM. Nevertheless, the success of the method indicates that a systematic survey of the entire GoM using these methods would provide a more comprehensive data set with which to evaluate spatial and temporal patterns in fish biodiversity. Such a study could also evaluate population connectivity among different continental shelf areas and the deep-oceanic GoM using particle (fish eggs and larvae) tracking studies by seeding models with identified fish-egg densities. Furthermore, a more comprehensive survey would provide genetic materials for discerning potential subpopulation connectivity.

Temporal variation in spawning season is widespread among species, but tends to be consistent within species (Cushing, 1969). Phenological studies indicate species-specific spawning seasons are responsive to climate change, generally starting earlier in the year when sea surface temperatures are warmer (Jansen & Gislason, 2011). In the present survey, cruises at different times of year collected the eggs of spring spawners (e.g., *M. microlepis*and *M. phenax*), summer spawners (e.g., *L. campechanus* and *R. aurorubens*), and fall spawners (e.g., *S. ocellatus*). Within spawning seasons, there can be substantial variation in egg production

in association with changing moon phases, and the same species may spawn more heavily during different moon phases at different locations (Farmer et al., 2017). Note that we arranged for each of the three cruises to visit a common location during different seasons (stations 120, 206, 314, Figure 1), and this common location was classified as having different egg communities during each of the three seasons. When spawning season is targeted by egg cruises, encounter rates can be substantially higher than indicated in Table 1. For example, we collected eggs of each of the spring-spawning groupers *M. microlepis* and *M. phenax* in two of the six spring samples (33% encounter rate).

Geographic Delineation of the Neritic and Oceanic Egg Communities

Within the observed fish-egg distributions, there was little evidence of a community gradient (coenocline) associated with the rapid increase in depth that occurs beyond the shelf break. The fish-egg community transition thus resembled the abrupt transition of an ecotone (DiCastri et al., 1988; Figures 1, 4 and 5), rather than the gradual transition of an ecocline (Kent et al., 1997). However, this perception is partly attributable to the scale of the survey and its sampling resolution, as the benthic fish communities of the GoM clearly exhibit depth zonation on the continental slope (Wei et al., 2012) that would have been difficult to resolve with the widely spaced stations used in this study (Figure 1).

On the continental shelf, an economically valuable assemblage of reef fishes known as the “grouper–snapper complex” (e.g., serranids, lutjanids, sparids, haemulids; sensu Coleman et al., 2000) is one potential target for DEPM. Reef fishes adhered to the abrupt neritic–oceanic transition at the shelf break, with minor exceptions. The circumglobal snapper genus *Pristipomoides*, which favors deeper water (upper slope to depths > 500 m) more than

most other snapper species (Allen, 1985), was one of two reef-associated taxa to be classified within the oceanic fish-egg community (Figure 5; the second species was a poorly known species, *Pareques iwamotoi*). Leis and Lee (1994) describe the genus *Pristipomoides* as favoring rocky bottoms of the upper continental slope, while also being semipelagic. This genus is ancestral and zooplanktivorous, in contrast to more derived snappers that eat larger prey (Frédérich & Santini, 2017). *Pristipomoides* larvae attain relatively large sizes (>2 cm) and become fully scaled, while remaining translucent, before developing the dense body pigmentation that is associated with settlement from the water column onto benthic habitats (Leis & Lee, 1994). This genus and several other snapper species, including some that are considered to be neritic species (e.g., *L. campechanus*), are present as relatively large, translucent larvae within the water column seaward of the GoM's shelf break (Velez & Moore, 2018); the large size of these presettlement individuals and their position in the water column suggest some reef fish larvae remain competent even while existing in a deep, pelagic environment. It is unclear whether the presence of these advanced-stage larvae in deep waters adjacent to the slope is the result of involuntary spillover or active habitat selection. Among groupers, there are a number of species that occupy slope waters (e.g., the diverse group of anthias serranids), although the eggs of this group were not identified during the present survey. Thus, while the eggs of reef fishes were essentially confined to the continental shelf, it is also known that reef fishes use habitats on the upper slope and in the adjacent water column seaward of the upper slope.

One important consideration when examining the biogeography of species associated with the neritic–oceanic comparison is the occurrence of ocean currents, warm- and cold-core eddies, and associated fronts (Schmitz Jr, 2005). During the 2015 and 2016 cruises, oceanic conditions in the GoM were substantially different (Figure 6). In 2015, the Loop Current was

well-established, with intense eddies present in the western GoM. The eastern boundary of the Loop Current often interacts with the outer shelf, potentially providing a mixing zone for neritic and oceanic species and transport of coastal species from the northern GoM, southeast to the Florida Keys. The cross-Gulf transect occupied during 2015 crossed the Loop Current, which extended deep into the GoM to the Northwest. During the 2016 sampling, however, the Loop Current was weak and was eventually cut off, and thus, there was not a sharp water column boundary among faunas. There was, however, a cold-core eddy at the center of the Florida–Yucatan transect, potentially upwelling cold, nutrient laden water, yet there were no apparent egg community trends associated with this feature. In general, ocean circulation did not appear to be responsible for the community transition at the shelf break (Figure 6).

None of the 50 important taxa (as defined in statistical analyses section) that occurred in both the oceanic and neritic groups (Figure 5) were reef fishes. The (relatively few) overlapping taxa were *Echeneis* sp. (a remora that travels while attached to much larger nekton), unidentified tunas, a species of driftfish (*Cubiceps* sp.), and an oceanic pomfret (*Brama* sp.) that was collected near the Yucatan Shelf at the only deepwater station that was classified as neritic (station 109). Both of the mixed neritic–oceanic egg taxa described by Borchers et al. (1997) were coastal pelagics (Bakun & Parrish, 1991; Klima & Wickham, 1971), which include zooplanktivorous clupeids, engraulids, carangids, and scombrids. The Borchers et al. taxa were a scad (carangid) and a mackerel (scombrid). While we did not identify any mixed neritic–oceanic SIMPROF groups or observe substantive taxonomic overlap between the neritic and oceanic groups, there was taxonomic uncertainty within the scad genera *Trachurus* and *Decapterus*, and also among the tunas (inclusive of the genus *Auxis*), and so it is possible that some coastal pelagics spawned in both neritic and oceanic waters (note that clupeid and engraulid eggs were

visually identified and were excluded from DNA barcoding, with the exception of *Etrumeus sadina* eggs, which were mistaken for percomorph eggs).

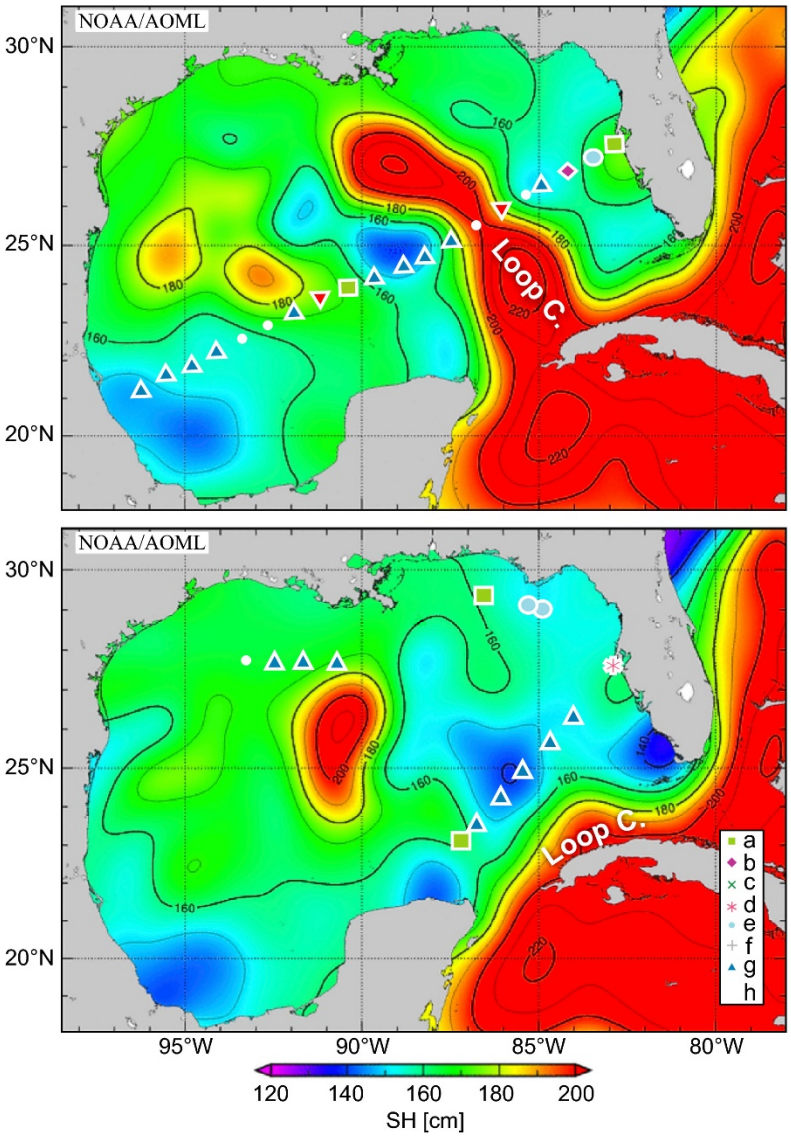

Figure 6: Sea heights (SH) from satellite altimetry during the fall (upper panel; September 9, 2015) and summer 2016 (lower panel; August 22, 2016) cruises. Data indicate strong northward intrusion of the Loop Current (Loop C.) into the GoM during 2015, with warm- and cold-core eddies present in the western Gulf. During 2016, the Loop Current was essentially cut off and

flowed more directly through the Florida Straits. Graphics modified from http://www.aoml.noaa.gov/phod/dhos/altimetry.php#SHA

DEPM and Egg Mortality

Daily egg production method requires consideration of egg mortality when estimating the abundance of eggs at the time of spawning (vs. the time of collection). Microscopy can be used to visually stage the eggs of a given species, and the abundances of successive egg stages can be fit to catch curves to estimate egg mortality for that species (e.g. Lo et al., 1996). Because the DNA barcoding process destroys the eggs, the eggs must be visually staged before barcoding, and a protocol for assigning species proportions to egg-stage distributions in the entire sample must be developed. Visual staging, however, may be more difficult for eggs that have been directly preserved in alcohol (ethanol or isopropanol) than for eggs that have been fixed in formalin first. Formalin cannot be used in conjunction with barcoding because it damages DNA by fragmentation, base modification, and by cross-linking the DNA with itself or proteins (Hykin et al., 2015). Fragmentation, in particular, interferes with the Sanger sequencing method. We observed variation in the preservation quality of eggs preserved in isopropanol. While many of the isopropanol-preserved eggs still contained detailed anatomical features that would allow staging, others did not. Additional research into the cause of these variable preservation results is needed.

Conclusion

We found the eggs of fishes that are potential candidates for DEPM were readily barcoded and were encountered at high enough rates to make egg surveys practical for many

species. Eggs from 15 economically important fish species were definitively identified by DNA barcoding. These and other taxa that spawn within the GoM are likely self-recruiting to some extent. Population connectivity may subsidize these GoM populations, but the taxa encountered in the egg survey are less likely to be dependent on such connectivity. At the LME scale, the eggs reflected spatial variation in the community structure of spawners, which had a distinct community transition at the shelf break. Together, these results indicate DEPM fish-egg surveys based on DNA barcoding are feasible at the LME scale.

CHAPTER THREE:

DNA BARCODING OF FISH EGGS COLLECTED OFF NORTHWEST CUBA AND ACROSS THE FLORIDA STRAITS DURING MAY 2017

Note: This chapter has been prepared as a manuscript to be submitted for publication.

Abstract

Fish eggs can be used as a tool to identify spawning sites for any broadcast spawning species. Since eggs are hard to morphologically identify under a microscope, genetic techniques were employed to identify them down to the species level. In this study, fish eggs were collected at 23 stations along the northwest coast of Cuba and across the Florida Straits. A total of 564 fish eggs were successfully identified to 89 taxa within 30 families. Fifty-six of the taxa were identified down to the species level, 23 to the genus level, and the remaining 10 to the family level or higher. This study demonstrates an exception to the results of previous work showing a clear delineation of neritic fish species spawning on continental shelves and oceanic species spawning over deeper waters (Burrows et al., 2019). Satellite data showed the presence of a mesoscale cyclonic eddy at the northern end of our Florida Strait transect, bringing eggs from reef-associated species off the continental shelf of Florida and into the Florida Straits. This study also gives new spawning information for *Luvarus imperialis* (Louvar), *Bothus lunatus* (Plate Fish), *Eumegistus illustris* (Brilliant Pomfret), and many economically important species. Fisheries managers of the United States and Cuba can use this information to make better informed decisions when creating new regulations and Marine Protected Areas.

Introduction

Due to the difficulty in accurately identifying fish eggs based on morphological characteristics, many studies have inferred spawning locations of a given species based on the presence of larvae (Peebles & Tolley, 1988; Sassa et al., 2006). However, fish larvae can be days to weeks old before capture (Cowen & Sponaugle, 2009), therefore hindcasting spawning locations based on surface currents and the length of time larvae are in the water column may be uncertain due to a variety of factors of larval behavior, including vertical migration (Vikebø et al., 2007). Alternatively, fish eggs are typically only hours old before hatching into larvae, and they behave as relatively passive particles once they have floated to surface waters, reducing the error in predicting spawning location using hydrodynamic models. The only study that has directly addressed this issue demonstrated the dissimilarity of fish egg and larval species compositions in Terra Ceia Bay, FL (Burghart et al., 2014).

Broadcast spawning is the most common type of reproduction strategy among fishes, allowing eggs to be dispersed into the water column, where the majority float to the surface due to positive buoyancy (Fabra et al., 2005). Identification of passively drifting fish eggs collected with plankton nets, combined with hydrographic modeling of surface currents can therefore be used to predict spawning locations for a diversity of fish taxa. However, this method is not applicable to some fish groups, especially those with demersal eggs like some species of damselfishes (Pomacentrinae), cardinal fishes (Apogonidae), and blennies (Blenniidae); (Thresher, 1982) brooding fishes such as seahorses and pipefishes (Syngnathidae; Wilson et al., 2003), and ovoviviparous fishes such as rockfishes (Sebastinae; Wourms, 1991). Planktonic fish eggs can be collected in any season and any time of the day; however, to capture eggs from fish with more selective breeding seasons it is important to know timing of egg release. Peak

spawning season for coastal species in the Northern Hemisphere is typically from April to August (Claro et al., 2014); however, there are a wide variety of spawning strategies, including aggregations and spawning migrations to and from the continental shelf.

Fish eggs are very difficult to visually identify to the species level, due to their morphological similarities. Most fish eggs have been identified based on color, size of perivitelline space, oil globules, yolk and shape, but there are very few distinguishable characteristics among taxa, with the exception of clupeoid (anchovy, herring, and sardine) eggs, which are easily distinguished from percomorph (spiny-finned fish) eggs. Some visual features used for identification, including coloration, are typically lost during the preservation process (Smith, 1995). Previous studies comparing visual identifications of percomorph eggs with results from genetic barcoding have shown that visual identification is unreliable (Larson et al., 2016). Genetic barcoding of the mitochondrial gene cytochrome *c* oxidase I (COI) allows for identification of fish eggs, often to the species level (Ahern et al., 2018; Burrows et al., 2019; Duke et al., 2018). The Barcode of Life Database (BOLD; http://www.boldsystems.org/) contains reference sequences for over 20,000 ray-finned fish species (Actinopterygii), serving as an excellent community-driven resource for the identification of fish sequences (Ward et al., 2009). Accessions to BOLD have increased significantly as the fish taxonomic community has entered these resources. Separating each fish egg into individual DNA extractions allows for a quantitative measure of the number of eggs contributed by each species, from a given multispecies egg sample.

Understanding the complete life cycle of targeted fish species of interest, including their spawning locations, is a necessity for proper fisheries management. Gulf of Mexico (GoM) fisheries in Florida and Cuban waters are important economically for both commercial and

recreational purposes, but they are also important ecologically (Holmlund & Hammer, 1999). The fishing industry is ever growing and has to be regulated in order to sustain fish populations.

The main location for this study is Cuban waters bordering the southeast region of the GoM. This region has a multitude of habitats supporting marine fishes including a steep shelf area, well-developed shallow and mesophotic reefs, and many oceanic and nearshore habitats that host over 1,000 recognized fish species (including subspecies) (Claro et al., 2014). Cuba also has many commercially significant finfish species including tunas *(Thunnus atlanticus, Katsuwonus pelamis*), Swordfish (*Xiphias gladius),* snappers, grunts, jacks and groupers. The northwest coast of Cuba is of particular interest because it has a narrow continental shelf with a very steep shelf slope (Claro et al., 2014). In a previous study (Chapter Two), the species composition of fish eggs found on and off the continental shelves of the GoM showed a clear delineation of neritic species spawning on the continental shelves, and oceanic species spawning in deeper waters (Burrows et al., 2019). This study aims to determine if this pattern holds true for another location in the GoM, using DNA barcoding of individual fish eggs collected in May 2017. This study also identifies spawning locations for a diversity of broadcast spawning fish in this area, providing valuable new data for fisheries managers of both the United States, Cuba, and potentially Mexico.

Methods

Study Site and Sample Collection

Planktonic fish eggs were collected with a 0.333 mm mesh bongo net, towed at the surface for 15 minutes from the *RV Weatherbird II* (http://www.fio.usf.edu/vessels/rv-weatherbird) in May 2017 (Table 2) across the Florida Straits and along the northwestern Cuban

coast. One of the two bongo net samples was preserved immediately with 30% isopropanol and returned to the laboratory. It should be noted that this was not the intended method of preservation (see Discussion). Plankton samples from sites F1-F9, C7, C8, C10, C11 and C14 were held in the original preservative (30% isopropanol) for approximately 2 months, while sites C1-C6, C9, C12 and C13 were held in the original preservative for over a year. The percomorph eggs were then picked out of the sample with forceps under a stereomicroscope at 9-108X magnification, while clupeoid eggs were excluded from further work. A subsample of 96 or more percomorph eggs from each sample was placed into a glass vial with 50% isopropanol until DNA was extracted from individual eggs.

Genetic Identification

Using a sterile pipette tip, individual fish eggs were placed in individual 0.2 ml polymerase chain reaction (PCR) tubes, and excess isopropanol was removed. DNA extractions were performed using the HotSHOT method (Truett et al., 2000). To lyse the fish eggs, 50 µl of alkaline lysis buffer (0.2 mM disodium EDTA, 25 mM NaOH, pH 12) were added to each tube and each egg was crushed with a sterile toothpick. All PCR tubes were set in a thermocycler at 95ºC for 30 minutes then moved onto ice for 3 minutes to cool to room temperature. To complete the extraction, 50 µl of neutralization buffer (40 mM Tris-HCL, pH 5) was added and the samples were vortexed to mix thoroughly.

The PCR technique was used to amplify a portion of the mitochondrial COI gene with the COI-3 universal fish primer cocktail (Ivanova et al., 2007). Each 50 µl PCR reaction contained 2 µl of DNA template and final concentrations of 1x Apex NH_4 buffer, 1.5 mM Apex $MgCl_2$, 10 µg/µl bovine serum albumin (New England BioLabs Inc.), 0.2 µM Apex dNTPs, 0.2 µM primer

cocktail, 1 U Apex RedTaq (Genesee Scientific). The thermocycling protocol consisted of heating to 94ºC for 2 minutes, 45 cycles of (94ºC for 30 seconds, 52ºC for 40 seconds, 72ºC for 1 minute), followed by extension at 72ºC for 10 minutes. To confirm successful amplification, the PCR products were run on a 1.5% agarose gel (60 minutes, 120 V) and stained with ethidium bromide for visualization. Successful PCR products were sent to TACGen (tacgen.com) to be purified and Sanger sequenced using the M13 forward primer (Ivanova et al., 2007).

Table 2: Cruise station locations

Station	Date (YYMMDD)	Total # eggs in sample	Latitude	Longitude
C1	170513	3136	23.19	-82.08
C2	170513	1899	23.19	-82.11
C3	170514	236	23.03	-82.76
C4	170514	88	23.04	-82.75
C5	170515	1637	23.02	-83.02
C6	170515	155	23.03	-82.97
C7	170518	10	22.16	-84.81
C8	170518	-	22.10	-84.85
C9	170519	23	22.45	-84.53
C10	170519	51	22.49	-84.53
C11	170521	4381	22.73	-84.07
C12	170521	936	22.71	-84.09
C13	170522	3251	22.91	-83.56
C14	170523	2152	23.00	-83.16
F1	170524	83	23.17	-82.77
F2	170524	160	23.34	-82.77
F3	170524	131	23.50	-82.77
F4	170524	39	23.67	-82.77
F5	170524	7	23.83	-82.77
F6	170524	44	23.99	-82.77
F7	170525	34	24.17	-82.76
F8	170525	2735	24.35	-82.75
F9	170525	1096	24.50	-82.76

In order to differentiate between certain economically important species that are closely related, a second PCR was performed. The same PCR mixture was used as mentioned above, with the exception of different primers used for each of the following. Primers L8562 and H9432 were used to differentiate between *Thunnus thynnus* or *Katsuwonus pelamis* (72 fish eggs), with the following thermocycler conditions: 2 minutes at 94°C, followed by 45 cycles of (94°C for 30 s, 50°C for 40 s, 72°C for 1.5 min), and 10 minutes at 72°C (Chow & Inoue, 1993). Primers LCOI 121 and HCOI 1199 were used to differentiate between *Scomberomorus cavalla* or *Acanthocybium solandri* (5 fish eggs), with the following thermocycler conditions: 2 minutes at 94°C, followed by 45 cycles of (94°C for 30 s, 57°C for 40 s, 72°C for 2 min), and 10 minutes at 72°C (Paine et al., 2007). All products from these PCRs were cleaned with a Zymo Clean & Concentrator -25 kit and sent for bidirectional Sanger sequencing at TACGen (tacgen.com).

Data Analysis

Sequencher™ 5.3 (Genecodes) was used to trim the sequences for quality. Successful sequences were compared to the species-level records on the Barcode of Life Database (BOLD; http://www.boldsystems.org/) for identification to the lowest level of taxonomic assignment. If there was no match in the BOLD Database, sequences were compared to the species-level records on GenBank using the Basic Local Alignment Search Tool (BLAST; https://blast.ncbi.nlm.nih.gov/Blast.cgi). If the sequence identity was less than 97%, the sequence was considered unidentified.

Multivariate community analyses were completed as described in Burrows et al. (2019) to compare species composition of eggs among collection sites.

Satellite Imagery

MODIS/A Chlorophyll-a data were obtained from NASA (https://oceancolor.gsfc.nasa.gov). The sea surface height anomaly (SSHA) were produced and distributed by Archiving, Validation and Interpretation of Satellite Oceanographic data (AVISO; http://www.aviso.altimetry.fr/). Satellite imagery figures were plotted using MATLAB R2017a (http://www.mathworks.com/) with M_Map (a mapping package, http://www.eos.ubc.ca/~rich/map.html).

Results

From the 23 sites, a total of 1562 eggs were processed for DNA barcoding. Due to poor sample preservation (see Discussion), a large number of these eggs did not yield PCR products (n=832) or did not yield high quality sequences (n=101). Addition, 65 sequences were most similar to invertebrates or had <97% identity to sequences in the BOLD or BLAST databases (n=65). The 564 fish eggs successfully identified included 89 taxa within 30 fish families. Fifty-six of the taxa were identified to the species level, 23 to genus, and the remaining 10 to family level or higher.

Of the 89 taxa identified, 51 were reef-associated. Reef species included *Acanthurus* spp. (surgeonfishes), *Chaetodon* spp. (butterflyfishes), *Diodon holocanthus* (Longspined Porcupinefish), *Diplectrum formosum* (Sand Perch), *Gymnothorax moringa* (Spotted Moray), *Haemulon* spp. (grunts), *Halichoeres* spp. (wrasses), *Holacanthus* sp. (angelfish), *Kyphosus* sp. (chub), *Lactophrys* spp. (trunkfishes), *Lutjanus* spp. (snappers), *Ocyurus chrysurus* (Yellowtail Snapper), *Pomacanthus* spp. (angelfishes), *Sparisoma viride* (Stoplight Parrotfish), *Syacium*

papillosum (Dusky Flounder), *Synodus* spp. (lizardfishes), *Thalassoma bifasciatum* (Bluehead Wrasse), *Trachinotus falcatus* (Permit), and *Xyrichtys novacula* (Pearly Razorfish).

From the 21 stations with species identifications, seven significant groups were produced from the SIMPROF analysis. Figure 8 represents the composition of each significant group, revealing that in general eggs along the coast of Cuba group together and eggs along the Florida Straits transect group together.

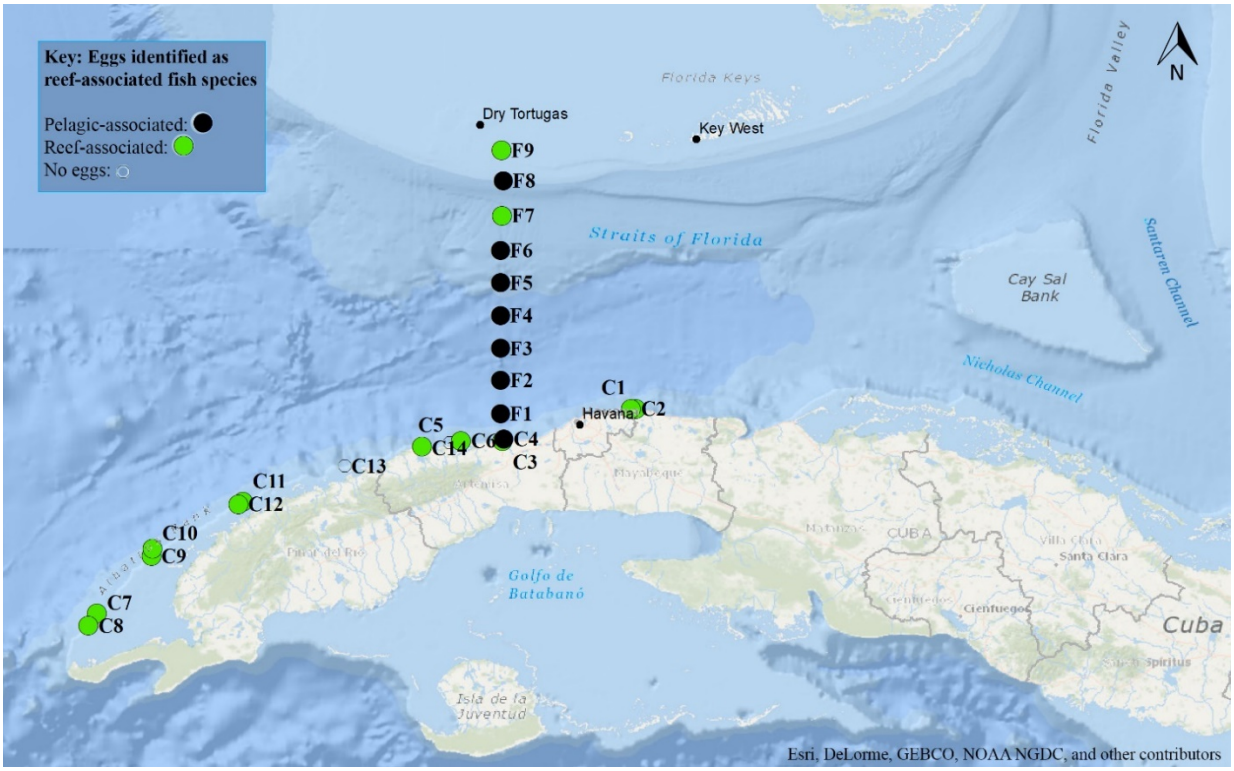

Figure 7: Bongo net deployment stations from May 2017, indicating stations where fish eggs were identified as mostly reef-associated species. Plankton tows were performed at stations along the coast of Cuba between demersal longline fishing sites on the same cruise (Murawski et al., 2018). Green symbols indicate stations with fish eggs identified as reef-associated species. F= Florida Straits, C=Cuba.

Table 3: Fish egg species identification

Taxon	FishBase Common Name	Habitat	Economic Importance	Encounter Frequency (Stations)
Acanthurus bahianus/ A. tractus	Ocean Surgeonfish/ Five-band Surgeonfish	reef-associated	no/minor commercial	1
Acanthurus chirurgus	Doctorfish	reef-associated	minor commercial	5
Acanthurus coeruleus	Blue Tang Surgeonfish	reef-associated	minor commercial	3
Atule mate/Selar crumenophthalmus	Yellowtail/Bigeye Scad	reef-associated	minor commercial; gamefish/ commercial; gamefish	1
Atule mate/Selar crumenophthalmus/ Selar boops	Yellowtail/ Bigeye/ Oxeye Scad	reef-associated	minor commercial; gamefish/ commercial; gamefish/ commercial	1
Auxis thazard/A. rochei	Frigate/Bullet Tuna	pelagic-neritic	commercial; gamefish	1
Auxis thazard/Sarda orientalis	Frigate Tuna/ Striped bonito	pelagic-neritic	commercial; gamefish/minor commercial	1
Auxis thazard/Sarda orientalis/A. thazard thazard/A. rochei	Frigate tuna/Striped bonito/Bullet tuna	pelagic-neritic	commercial; gamefish	1
Bothus lunatus	Plate fish	reef-associated	minor commercial	1
Brama dussumieri	Lesser Bream	pelagic-neritic	commercial	1
Brama orcini/ B. cf. dussumieri	Bigtooth Pomfret/ Lesser Bream	benthopelagic/ pelagic-neritic	minor commercial/ commercial	1
Chaetodon sedentarius/ C. sanctaehelenae	Reef/Saint Helena Butterflyfish	reef-associated	no	1
Chaetodon striatus	Banded Butterflyfish	reef-associated	no	1
Coryphaena hippurus	Common Dolphinfish	pelagic-neritic	commercial; gamefish	1
Coryphaena hippurus/ C. equiselis	Common/Pompano Dolphinfish	pelagic-neritic	commercial; gamefish	3
Decapterus macarellus/ D. tabl	Mackerel/ Roughear Scad	pelagic-oceanic/ reef associated	commercial; gamefish/minor commercial	3
Decapterus punctatus/ D. tabl	Round/ Roughear Scad	reef-associated	minor commercial	3
Diodon holocanthus	Longspined Porcupinefish	reef-associated	minor commercial	1
Diplectrum formosum	Sand Perch	reef-associated	commercial; gamefish	1
Diplospinus multistriatus	Striped Escolar	benthopelagic	subsistence fisheries	2

Table 3 (Continued)

Taxon	FishBase Common Name	Habitat	Economic Importance	Encounter Frequency (Stations)
Diplospinus multistriatus/Nealotus tripes	Striped Escolar/Black Snake Mackerel	benthopelagic/ bathypelagic	subsistence fisheries	4
Epinephelus adscensionis	Rock Hind	demersal	commercial; gamefish	1
Eucinostomus argenteus	Silver Mojarra	reef-associated	minor commercial	1
Eucinostomus argenteus/E. harengulus	Silver/Tidewater Mojarra	reef-associated/demersal	minor commercial/no	2
Eucinostomus argenteus/ E. harengulus/E. gula	Silver/Tidewater/ Jenny Mojarra	reef-associated/ demersal/ reef-associated	minor commercial/no/ minor commercial	1
Eucinostomus argenteus/ E. harengulus/E. lefroyi	Silver/Tidewater/ Mottled Mojarra	reef-associated/ demersal/ reef-associated	minor commercial/ no/ minor commercial	2
Eumegistus illustris	Brilliant Pomfret	bathypelagic	no	2
Euthynnus alletteratus	Little Tunny	reef-associated	commercial; gamefish	1
Euthynnus alletteratus/ Auxis thazard	Little Tunny/ Frigate Tuna	reef-associated/ pelagic-neritic	commercial; gamefish	1
Gymnothorax moringa	Spotted Moray	reef-associated	minor commercial	1
Haemulon aurolineatum	Tomtate Grunt	reef-associated	minor commercial	3
Haemulon carbonarium	Caesar Grunt	reef-associated	minor commercial	1
Haemulon flavolineatum	French Grunt	reef-associated	commercial	2
Haemulon flavolineatum/ H. aurolineatum	French/ Tomtate Grunt	reef-associated	commercial/ minor commercial	2
Haemulon plumierii	White Grunt	reef-associated	Minor commercial; gamefish	1
Halichoeres bivittatus	Slipper Dick	reef-associated	no	3
Halichoeres dimidiatus	Yellowcheek Wrasse	reef-associated	no	1
Halichoeres garnoti	Yellowhead Wrasse	reef-associated	no	1
Halichoeres radiatus	Puddingwife Wrasse	reef-associated	minor commercial	3
Holacanthus ciliaris/ H. bermudensis	Queen/Bermuda Blue Angelfish	reef-associated	minor commercial	1
Istiophorus albicans	Sailfish	pelagic-oceanic	commercial; gamefish	1
Kajikia albida	White Marlin	pelagic-oceanic	minor commercial; gamefish	1
Katsuwonus pelamis	Skipjack Tuna	pelagic-oceanic	commercial; gamefish	4
Katsuwonus pelamis/ Thunnus atlanticus/ T. thynnus	Skipjack/Blackfin/ Bluefin Tuna	pelagic-oceanic	commercial; gamefish	1
Kyphosus vaigiensis/ K. incisor	Brassy/ Yellow Sea Chub	reef-associated	commercial; gamefish/ minor commercial; gamefish	1
Lactophrys bicaudalis	Spotted Trunkfish	reef-associated	no	1

Table 3 (Continued)

Taxon	FishBase Common Name	Habitat	Economic Importance	Encounter Frequency (Stations)
Lactophrys trigonus	Buffalo Trunkfish	reef-associated	commercial	2
Lactophrys triqueter	Smooth Trunkfish	reef-associated	minor commercial	1
Lampris guttatus	Opah	bathypelagic	minor commercial; gamefish	1
Lutjanus analis	Mutton Snapper	reef-associated	commercial; gamefish	1
Lutjanus analis/ L. purpureus	Mutton/ Southern Red Snapper	reef-associated/demersal	commercial; gamefish/ commercial	2
Lutjanus apodus	Schoolmaster Snapper	reef-associated	commercial	4
Lutjanus griseus	Mangrove Snapper	reef-associated	commercial; gamefish	2
Lutjanus mahogoni/ L. synagris	Mahogany/ Lane Snapper	reef-associated	commercial; gamefish	1
Lutjanus synagris	Lane Snapper	reef-associated	commercial; gamefish	2
Luvarus imperialis	Louvar	pelagic-oceanic	minor commercial	1
Makaira nigricans	Blue Marlin	pelagic-oceanic	commercial; gamefish	1
Makaira nigricans/ Istiompax indica	Blue/ Black Marlin	pelagic-oceanic	commercial; gamefish	1
Malacanthus plumieri	Sand Tilefish	reef-associated	minor commercial	1
Neoepinnula Americana	American Sackfish	benthopelagic	no	1
Nesiarchus nasutus	Black Gemfish	benthopelagic	minor commercial	1
Ocyurus chrysurus	Yellowtail Snapper	reef-associated	commercial; gamefish	4
Peristedion truncatum	Black Armoured Searobin	bathydemersal	no	1
Pomacanthus arcuatus	Gray Angelfish	reef-associated	minor commercial	1
Pomacanthus arcuatus/ P. paru	Gray/ French Angelfish	reef-associated	minor commercial	1
Prognichthys occidentalis	Western Bluntnose Flyingfish	pelagic-neritic	no	4
Psenes cf. nigrescens/P. maculatus/Cubiceps baxteri	Driftfish/ Black Flathead	pelagic-oceanic	no	3
Psenes cyanophrys/P. pellucidus	Freckled/ Bluefin Driftfish	bathypelagic	no/commercial	1
Psenes pellucidus/ P. maculatus/ Cubiceps whiteleggii/ Centrolophus niger	Bluefin/Silver/ Shadow Driftfish/ Rudderfish	bathypelagic/ pelagic-oceanic/ benthopelagic	commercial/no/no /gamefish	1
Remora albescens	White Suckerfish	pelagic-oceanic	no	1
Remora osteochir	Marlin Sucker	pelagic-oceanic	no	1
Rhomboplites aurorubens	Vermillion Snapper	Demersal	minor commercial	1
Scomberomorus cavalla	King Mackerel	reef-associated	commercial; gamefish	1

Table 3 (Continued)

Taxon	FishBase Common Name	Habitat	Economic Importance	Encounter Frequency (Stations)
Selar crumenophthalmus	Bigeye Scad	reef-associated	commercial; gamefish	1
Sparisoma viride	Stoplight Parrotfish	reef-associated	minor commercial	2
Syacium papillosum	Dusky Flounder	reef-associated	minor commercial	2
Synodus intermedius/ S. foetens	Sand Diver/ Inshore Lizardfish	reef-associated	minor commercial /subsistence fishery; gamefish	1
Synodus synodus	Diamond Lizardfish	reef-associated	commercial	4
Taractichthys steindachneri	Sickle Pomfret	benthopelagic	commercial	1
Thalassoma bifasciatum	Bluehead Wrasse	reef-associated	no	3
Thunnus atlanticus	Blackfin Tuna	pelagic-oceanic	commercial; gamefish	8
Thunnus atlanticus/ T. albacares	Blackfin/ Yellowfin Tuna	pelagic-oceanic	commercial; gamefish	4
Thunnus atlanticus/ T. albacares/T. obesus	Blackfin/Yellowfin/ Bigeyed Tuna	pelagic-oceanic	commercial; gamefish	3
Thunnus atlanticus/ T. albacares/T. thynnus/ T. obesus	Tuna	pelagic-oceanic	commercial; gamefish	1
Thunnus atlanticus/ T. thynnus	Blackfin/ Bluefin Tuna	pelagic-oceanic	commercial; gamefish	3
Thunnus atlanticus/ T. thynnus/T. albacares	Blackfin/Bluefin/ Yellowfin Tuna	pelagic-oceanic	commercial; gamefish	3
Trachinotus falcatus	Permit	reef-associated	commercial; gamefish	1
Xiphias gladius	Swordfish	pelagic-oceanic	commercial; gamefish	1
Xyrichtys novacula	Pearly Razorfish	reef-associated	minor commercial; gamefish	3

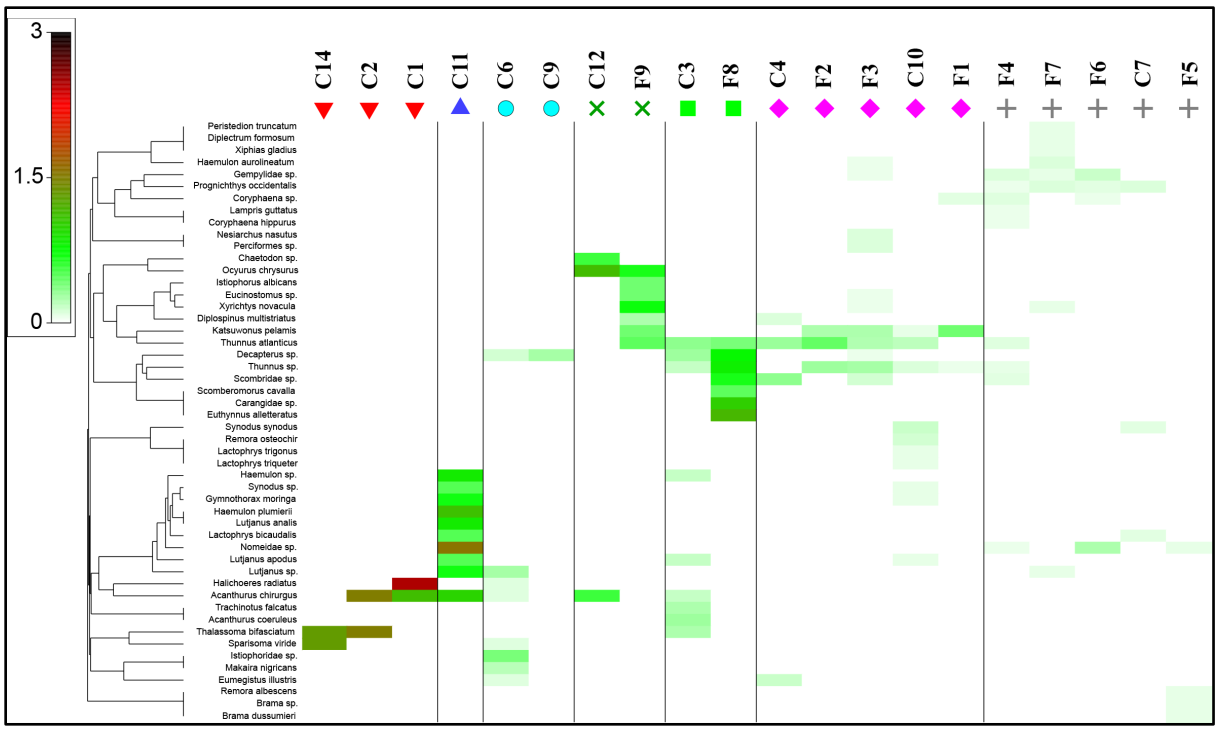

Figure 8: Heatmap showing the 50 most statistically significant fish-egg taxa. SIMPROF site groups are indicated by different symbols and are separated by vertical lines.

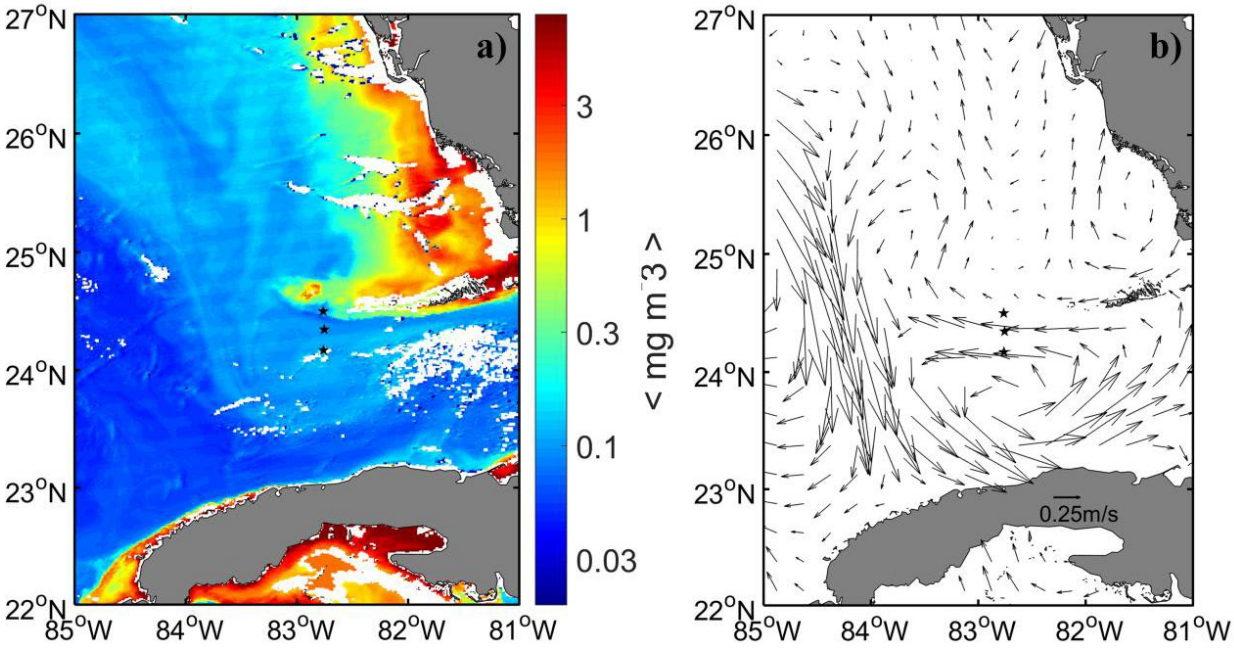

Figure 9: Mesoscale cyclonic eddy in the Florida Straits. Left: A snapshot of MODIS Chlorophyll-a concentration on May 10, 2017 (white regions are clouds). Right: A snapshot of geostrophic velocity anomaly field derived from AVISO sea surface height anomaly (SSHA) data on May 24, 2017. Black stars indicate three fish egg collection sites F9-F7, from top to bottom.

Discussion

In order to protect fish nurseries and spawning areas we need to know where and how often fish are spawning. This study is the first to genetically identify fish eggs in waters surrounding Cuba and across the Florida Straits. The identified fish eggs can also be used to identify spawning locations of economically important fish species and can aid in fisheries management decisions.

This study demonstrates spawning activity for species of commercial and recreational importance including *Auxis* spp. (tunas), *Coryphaena* sp. (dolphinfish), *Decapterus* sp. (Scad), *Epinephelus adscensionis* (Rock Hind), *Euthynnus alletteratus* (Little Tunny), *Istiophorus albicans* (Sailfish), *Katsuwonus pelamis* (Skipjack Tuna), *Thunnus* spp. (tunas), *Lutjanus* spp. (snappers), *Makaira nigricans* (Blue Marlin), *Scomberomorus cavalla* (King Mackerel), *Ocyurus chrysurus* (Yellowtail Snapper), *Trachinotus falcatus* (Permit), and *Xiphias gladius* (Swordfish). Valderrama et al. (2018) outlined the importance of adding new Marine Protected Areas (MPA) in Cuba. MPAs are chosen based on their conservation value and the presence of marine species of ecological and economic importance. Fishes of importance to Cuban people and the integrity of the local ecosystem were also found in our study, including Lutjanidae (snappers) and *Epinephelus adscensionis* (grouper). Currently there are some protected areas along the northwest coast, close to where this study was conducted (Valderrama et al., 2018). Closing fishing seasons around times of spawning could decrease mortality of spawning adults because some fish species, especially snappers and groupers, aggregate when spawning and are therefore easier to catch (Claro et al., 2014).

Murawski et al. (2018) previously described adult fishes caught using longline gear along the northwest coast of Cuba at the same time these eggs were collected. The adult fish species matching our egg identifications were *Gymnothorax moringa* (Spotted Moray), *Thunnus atlanticus* (Blackfin Tuna), *Ocyurus chrysurus* (Yellowtail Snapper), *Lutjanus analis* (Mutton Snapper), *Epinephelus adscensionis* (Rock Hind), and *Haemulon plumierii* (White Grunt) (Murawski et al., 2018). Lutjanidae is the most economically important fish family in Cuba, comprising 21% of total fish catches (Salas et al., 2011). Many of these catches occur during spawning season, because of spawning aggregations, making them easier to catch (Salas et al.,

2011). Collection of matching adult species near the locations of egg collection is additional evidence that these eggs were spawned off the northwest coast of Cuba.

Eggs from many of the commercially important species found during this study were not present in the longline fishing data. For example, species such as *Coryphaena hippurus* (Common Dolphinfish), *Euthynnus alletteratus* (Little Tunny), *Makaira nigricans* (Blue Marlin), *Scomberomorus cavalla* (King Mackerel), and *Xiphias gladius* (Swordfish) can be caught using longlining, but were not recovered by Murawski et al. (2018). Any time eggs are present, we can assume that the fish that spawned the egg is nearby. Not only can DNA barcoding of fish eggs be used to identify spawning locations, but it can also be used to identify the location of economically important species that avoid fishing gear or may not be captured with a given amount of sampling effort. With longline fishing, the fish must be in the right place at the right time and be over a certain size to be caught (depending on the species). Capturing eggs via plankton net at the surface allows for a reduction in catch bias but does not always show the full number of species in an area, because not every species is spawning at the same time, in the same place. Combining longlining and egg tows can increase the overall number of species identified in one area.

This study has shown us new spawning information for a number of species not generally assumed to occur in the area including: *Luvarus imperialis* (Louvar), *Bothus lunatus* (Plate Fish) and *Eumegistus illustris* (Brilliant Pomfret). There is little information on the spawning habits or the length of larval development of *Luvarus imperialis*. Based on the collection of larvae, this species is predicted to spawn in the northwest Pacific Ocean near Japan (Nishikawa, 1987). In a separate study, larvae, juveniles, and a single specimen with mature gonads were collected in the southwest Atlantic Ocean (Domingues et al., 2015), indicating spawning is occurring there, but

larval development can take months and larvae can travel great distances away from spawning locations. Since eggs of *Luvarus imperialis* were collected at station F9, we can say with more confidence that this species spawns near south Florida in addition to the locations previously found. *Bothus lunatus* is another species with little known about length of egg and larvae development, and spawning locations (van der Veer et al., 2018). Spawning of *Bothus lunatus* has been observed near Bonaire, Netherlands Antilles (Konstantinou & Shen, 1995), but our data shows spawning is also occurring near northwest Cuba (station C6). *Eumegistus illustris* is a rare species of fish, with very little published spawning information. Juveniles of various sizes have been collected and studied from the Line Islands in Kiribati, and Japanese waters (Moteki & Mundy, 2005). Juveniles were also collected in the waters north of New Guinea (Okiyawa, 1988). In this study, *Eumegistus illustris* eggs were found at stations C4 and C6 in northwestern Cuban waters, suggesting a new spawning area for this species. While these three species have been highlighted, this study provides new spawning information on numerous fish species.

This study had a higher failure rate (64%) during DNA barcoding compared to previous studies (Ahern et al., 2018; Burrows et al., 2019; Harada et al., 2015; Lewis et al., 2016; Leyva-Cruz et al., 2016). This low success rate was most likely due to poor preservation, since the plankton tow biomass was stored in 30% isopropanol instead of the intended 70% isopropanol. The low isopropanol concentration and the long-term storage prior to processing could have negatively affected the long-term stability of the fish egg DNA (Michaud & Foran, 2011), which led to the higher failure rate in the second batch of samples processed (which were not extracted for over a year) compared to the first batch of samples processed (which were extracted within two months of collection). The first batch of fish eggs processed (sites F1-F9, C7, C8, C10, C11 and C14) had a 58% success overall, while the second batch (C1-C6, C9, C12 and C13) had a

14% success overall. A previous study showed that DNA degradation occurs around 6 months of storage in alcohol solutions (Michaud & Foran, 2011). In future studies, proper preservation and quick processing (no longer than 6 months after collection) should be of highest priority.

In our previous work in the GoM, there was a clear delineation between neritic fish species spawning on continental shelves, while oceanic species spawned in deeper waters (Burrows et al., 2019). While this trend was generally true for this study as well, eggs from some neritic (reef-associated) species were found in the deep water of the Florida Straits and eggs from pelagic-associated species were found in shallower waters, as seen in Figure 7. One explanation for this transportation is the steepness of the continental slope off the northwest coast of Cuba (Claro et al., 2014) at the station closest to Cuba (station C4), eggs were mostly pelagic-associated. When eggs are spawned near the shelf edge, they could easily drift into deeper waters away from the reef where they were presumably spawned. In order to avoid predation, some reef fishes may swim to the edge of a reef to release their eggs (Johannes, 1978). An explanation for station F7 having mostly reef-associated fish egg species identifications (Figure 7) is the apparent formation of a cyclonic eddy off the coast of the Florida Keys, coinciding with the egg collection (Figure 9). Figure 9a shows a relatively high Chlorophyll-a concentration on May 10, 2017 near the Dry Tortugas region indicating a mesoscale cyclonic eddy entraining waters from the Florida continental shelf and moving them to the Florida Straits. Figure 9b shows ocean surface current patterns derived from altimetry data on May 24, 2017, also showing a mesoscale cyclonic eddy. Station F7 was toward the distal end of the jet of water entraining eggs offshore. This eddy appears to have entrained eggs of *Lutjanus* sp. (snapper), *Diplectrum formosum* (Sand Perch), *Xyrichtys novacula* (Pearly Razorfish), and *Haemulon aurolineatum* (Tomtate Grunt) that are typically found in shallow waters (Burrows et al., 2019). An approximate calculation of egg

longevity (~24 hours) and speed of the Florida Current (average of 1 m/second) shows that a fish egg could last in the water column for about 86.4 km. Following the shortest path of arrows from the West Florida Shelf to station F7 on Figure 9b gives a distance of approximately 81 km using Google Maps (maps.google.com). This calculation shows it is possible for eggs to be transported from the West Florida Shelf to station F7, where they were collected, before hatching into larvae. After these fish eggs hatch to larvae, it is possible they could be transported back to the continental shelf of southern Florida (Florida Keys) or swept up closer to the east coast of Florida. A study by Sponaugle et al. (2005) showed that larvae can be concentrated by these mesoscale eddies and transported from lower to upper Florida Keys.

The Florida Straits is relatively a narrow channel linking the GoM and the Atlantic, where waters are dynamic, with a volume transport of 30 Sverdrups (Sv) of flowing water (Richardson, 2001). In addition to the fast-moving Florida Current, both mesoscale and sub-mesoscale eddies frequently occur (Kourafalou & Kang, 2012; Lee et al., 1995; Shay et al., 1998). The cyclonic eddies are highly productive with abundant nutrients, phytoplankton, and copepods (Hitchcock et al., 2005; Lee et al., 1994), which can also influence cross-shelf transport of fish larvae (Lane et al., 2003; Lee et al., 1992; Limouzy-Paris et al., 1997; Shulzitski et al., 2017; Sponaugle et al., 2005). Ocean fronts are discontinuities in the marine environment that can influence the ecology of marine organisms (Alemany et al., 2014; Leichter & Witman, 2009). Fronts are typically associated with enhanced productivity at all trophic levels, including fishery grounds (Lohmann & Belkin, 2014). In particular, fronts play an important role in reproduction, feeding and migration of fish and squids (Olson, 2002). Mesoscale eddies are ubiquitous in the ocean, and they play crucial roles in the transport of salt, heat, nutrients, plankton, dissolved oxygen and carbon (Dong et al., 2014; Zhong et al., 2017). Also, they have a

profound influence on biological productivity, upper ocean ecology and biogeochemistry, and thus in elemental cycling and fluxes (Falkowski et al., 1991; McGillicuddy Jr et al., 1998). The cyclonic eddies in the Dry Tortugas region and Florida Straits are known to be highly productive with abundant nutrients, phytoplankton, and copepods (Hitchcock et al., 2005; Lee et al., 1994), which also have major implications on cross-shelf transport of biological and chemical materials (e.g., fish larvae, pollutants) and in offshore operations (e.g., sewage outfalls, search and rescue) (Kourafalou & Kang, 2012; Lane et al., 2003; Limouzy-Paris et al., 1997; Shulzitski et al., 2017; Sponaugle et al., 2005).

Conclusion

In summary, this data collected in this study can help provide valuable new reproductive information on commercially and non-commercially important species spawning along the southwest Florida and northwest Cuban coasts and in the Florida Straits. The mesoscale eddy observed in the Florida Straits demonstrated that physical processes can move reef-associated or neritic fish eggs away from spawning locations and into deeper waters. It is important for us to combine our knowledge of biological and physical processes to better estimate spawning locations. While this was a pilot study, we anticipate these new data will allow fisheries managers to identify critical habitats to conserve economically important species. There is considerable potential for finding new spawning areas for many species through egg identification; therefore, future studies expanding the seasonal and horizontal scope can be used to define spawning locations and seasonality of spawning in the waters surrounding Florida and Cuba.

CHAPTER FOUR:

ADDITIONAL DISCUSSION

Comparison of the Two Studies

In Chapter Two, a broad approach was taken looking at the Gulf of Mexico (GoM) as a whole. This study revealed a clear delineation between oceanic and neritic fishes spawning sites, on and off the continental shelves of the GoM (Burrows et al., 2019). In contrast, Chapter Three focused on the southeast section of the GoM, the southwest tip of Florida and the northwest coast of Cuba and identified neritic fish eggs in deeper waters than expected based on the prior results from the GoM. Examination of ocean color patterns during the sampling period revealed a mesoscale eddy that was pulling neritic fish eggs off the continental shelf of Florida and into the deeper waters of the Florida Straits. These two studies go hand-in-hand to help us understand the physical and biological processes that affect the spatial distribution of fish eggs in the GoM. In order to fully understand these processes, future research should be conducted to replicate these studies on varying temporal and spatial scales.

A Note on Preservation and Processing

Preservation of plankton samples is a vital part of the barcoding process. The most commonly used preservation method is fixing plankton samples in 95% or 96% ethanol, then storing eggs in 95% or 96% ethanol after they are picked out of the ichthyoplankton samples. Lewis et al. (2016) obtained successful sequences for 93% of their samples, while Leyva-Cruz et

al. (2016) had 46% success with almost identical preservation methods. In addition to storing in ethanol, some studies store picked eggs at 4ºC, leading to 48-62% success (Ahern et al., 2018; Harada et al., 2015). No percent of success was published, but eggs preserved and stored in 50% isopropanol and ambient seawater had no obvious genetic identification issues (Burghart et al., 2014). While results of successful DNA extractions vary between studies, it is clear that alcohols are the preservation method of choice. In order to get the best results, it is important to preserve samples quickly, and extract DNA within 6 months of preservation.

In this thesis, preservation was completed with 70% isopropanol in Chapter Two, while 30% isopropanol was used in Chapter Three by mistake. In Chapter Three, the low percentage of alcohol used for preservation and a delay in the processing of fish eggs led to a low success rate in DNA barcoding. There are too many variables within the available literature to determine what percentage of alcohol is the best option for high sample success, but Chapter Three shows that the combination of slow sample processing and low alcohol percentage led to low success. In future studies, ichthyoplankton samples should be fully drained of seawater and fixed in a high percentage of alcohol (70% or greater ethanol or isopropanol), and eggs should be picked out and placed in new alcohol within 6 months of collection (the sooner the better), to avoid problems with excess biomass. When samples are processed quickly, there is less risk of DNA degradation, leading to higher success rates.

Future Work

In this thesis, each fish egg was crushed with a toothpick in an individual tube and processed separately. Individual extractions take time and labor to complete, while metabarcoding allows for pooling of a subset of eggs from each sample location, highly reducing

the amount of lab work needed, while retaining the spatial data on where species are spawning. Metabarcoding (i.e., DNA barcoding on DNA extracted from all constituents in a mixed community of fish eggs obtained from a plankton tow) enables description of the presence or absence of the species contained in the sample, representing a potential alternative to the time-consuming approach of individual egg extractions used in this thesis. However, this approach is not quantitative and thus does not allow for assessment of species dominance and egg abundance. During metabarcoding, the number of target DNA sequences for PCR is linked to the number of copies of that gene (and thus cells) in a given sample. Therefore, eggs at different developmental stages containing different numbers of cells will yield unequal numbers of sequences. Although many attempts have been made to reduce the bias of PCR primers within different templates, there are always biases associated with amplifying a mixture of targets which could cause certain sequences to be over- or under-represented relative to the true number of eggs (Elbrecht & Leese, 2015). Metabarcoding will be the future of this field, because of the tedious nature of the DNA extraction method and the higher cost of sequencing individual fish eggs.

DNA barcoding of fish eggs is a relatively new method, so very few studies have assessed the variability of egg community composition over time. A previous study in the southeastern Gulf of California demonstrated seasonal changes in the abundance and species richness of fish eggs (Ahern et al., 2018). Another recent study, done over a period of three years in the Marine Protected Areas of San Diego, showed a 50% reduction in fish egg abundance during an El Niño event (2014-2016). In the same study, they linked interannual variation of egg abundance to cold water temperature and upwelling zones (Duke et al., 2018). Studying temporal variation in fish egg abundance and community composition in the GoM will allow for broader

management solutions and help us understand changes associated with anthropogenic and natural events. Future projects should include seasonal samples over multiple years to properly document baseline spawning information for the GoM. Concurrent collection and analysis of physical oceanographic data would strengthen these observations, as demonstrated in this thesis where an eddy was shown to transport fish eggs.

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