Quick Look Report: *Dendrogyra cylindrus* spawning research – July 2018

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Contributing Partners:

Nova Southeastern University (NSU), including funding from Florida Department of Environment (DEP)
Keys Marine Lab (KML), including divers from University of Florida (UF)
Florida Aquarium Center for Conservation (FLAQ)
Fish and Wildlife Research Institute – South Florida Regional Lab Restoration Ecology Team (FWC)
Fish and Wildlife Research Institute – Coral Team
Florida Keys National Marine Sanctuary (FKNMS)
Mote Marine Lab (Mote)
SEZARC & Disney cryopreservation team
Volunteers

Abstract:

In July 2018, Nova Southeastern University led a multi-agency effort to observe and collect gametes from spawning pillar coral (*Dendrogyra cylindrus*) colonies in the Looe Key region of the Lower Florida Keys. Five boats and 23 divers representing six agencies/institutions recorded data and collected eggs and sperm for propagation of the species. Additional observations were conducted on *ex situ* fragments at Mote Marine Lab, Keys Marine Lab, and Florida Aquarium. Field spawning matched predicted dates, but spawning of males was significantly earlier than in previous years while egg release remained the same. Cryopreservation efforts documented sperm motility of only 1% after one hour. Estimated fertilization rates were consequently much lower than in previous years (< 10% compared to 90%). Laboratory fragments at Keys Marine Lab released eggs in timings consistent with laboratory releases in previous years: later in the night and over an extended number of nights. We posit that these asynchronous releases are the result of a missing environmental cue. In both field and laboratory colonies, observations of inter-year and/or inter-night hermaphroditism was observed. Recently thought to be rare within the species, production of both male and/or female gametes by an individual may be common. Mixed gamete solutions were transferred to Florida Aquarium and Mote Marine Lab; low levels of cell division and larval swimming were documented, but no settlers have yet been found. Mote will further assess settlement tiles in coming weeks.

Background:

The pillar coral *Dendrogyra cylindrus* is a rare but conspicuous coral on Caribbean reefs. It is unique in its taxonomy as the only species within its genus, and it is unique in its structure as the only columnar coral in the Caribbean. It has been listed as “threatened” under the US Endangered Species Act, “threatened” by the State of Florida, and “vulnerable” by the IUCN.

Baseline surveys of the population on the Florida Reef Tract (FRT) from Dry Tortugas to West Palm Beach were conducted in 2013-2014 and described a population of approximately 745 *D. cylindrus* colonies representing approximately 158 genotypes. Back-to-back bleaching events in 2014 and 2015 and a geographically progressing multi-species hard coral disease that causes nearly 100% mortality in *D. cylindrus* have all contributed to mass

mortality of this species (Fig 1). Surveys between April and August 2018 document only 130 remaining colonies (83% loss) and 68 genotypes (57% loss). While new colonies and tissue can be propagated by natural or human fragmentation of colonies, new genotypes can only be produced through sexual reproduction.

D. cylindrus is generally gonochoric, meaning that individuals are either male or female (but see results/discussion). Gametes (sperm and eggs) must encounter each other to produce fertilized eggs that develop into swimming planula larvae that must then find a place to successfully settle and grow into new colonies. Because the remaining adult genotypes are widely dispersed throughout the FRT, the likelihood of gamete encounters is extremely low. Juveniles of this species have not been documented, and it is suspected that this species is reproductively extinct in Florida.

Since 2012, annual observations of male and female *D. cylindrus* spawning on the FRT have allowed for accurate timing predictions. Spawning in the wild occurs 2-4 nights after the full moon of August, and 58-122 minutes after sunset. Males generally spawn a few minutes before females. Identifying the sex of a colony is not visually possible until the moment of spawning.

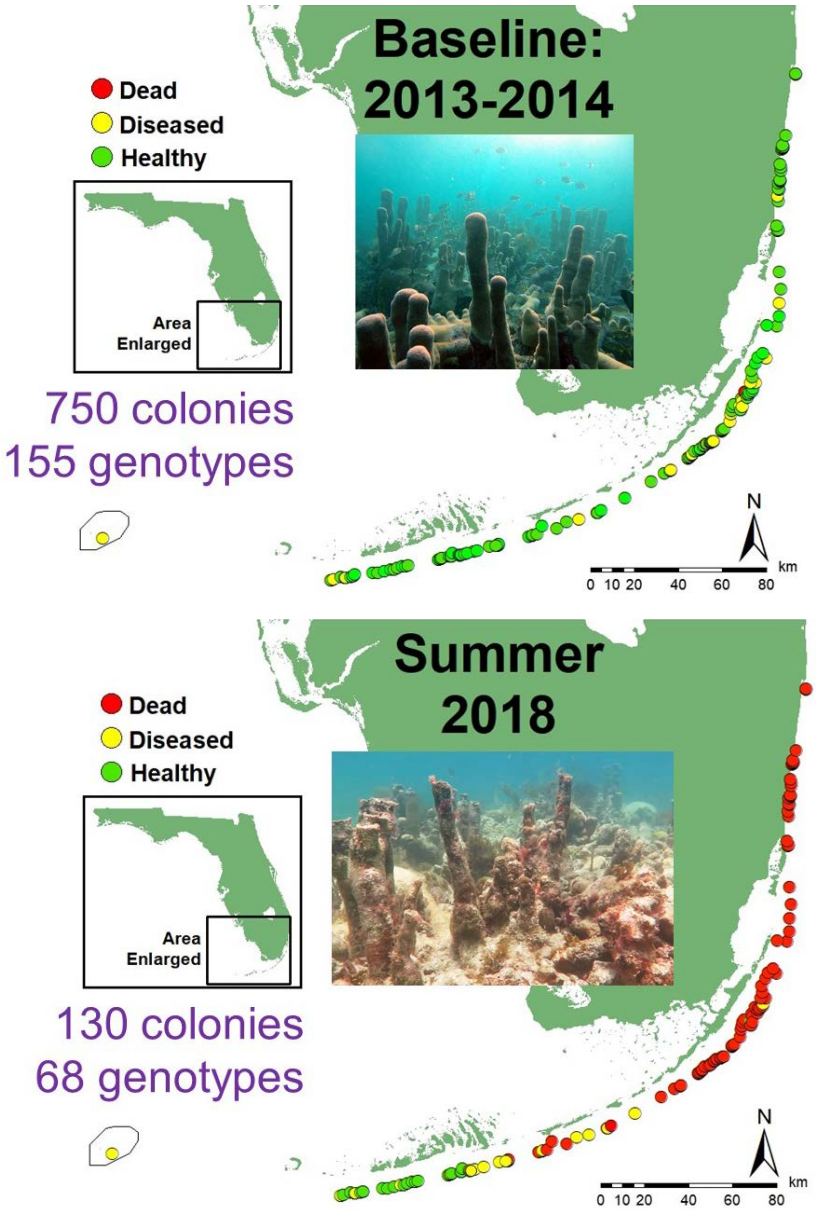

Fig 1. Distribution and status of all known *D. cylindrus* colonies on the Florida Reef Tract. Baseline 2013-2014 surveys show a background level of disease. Surveys four yearslater document almost complete mortality within the Upper Keys and Southeast Florida, with disease progressing through the Middle and Lower Keys. Photos are taken from the same location at a site in the Upper Keys.

Since 2016, a genetic rescue project has collected and housed fragments of *D. cylindrus* genotypes in onshore aquarium facilities. At three of these (Mote, Florida Aquarium, and Keys Marine Lab), corals are exposed to natural light regimes and thus receive cues to induce spawning. In 2016 and 2017, onshore fragments were observed spawning.

Successful gamete collection from wild colonies, egg fertilization, and settlement of *D. cylindrus* larvae was first accomplished in Florida in 2016. From that settlement event, 3 individual genetically distinct juveniles have survived to 2 years of age. Fertilization of spawning onshore coral fragments has been attempted, but has not been successful. One potential bottleneck identified has been short-term sperm motility; this could limit the

ability of sperm transported at sea between sites or between onshore facilities to be able to successfully fertilize eggs.

Goals for the 2018 spawning event were as follows:

1. Record date and time of gamete release in the field to add to and confirm predictive models of spawning.
2. Record gender of observed wild colonies to determine whether colonies observed over multiple years exhibit inter-year hermaphroditism
3. Record date and time of gamete release in laboratory settings to build predictive models of lab spawning and compare with wild gamete release.
4. Record genders of laboratory fragments to continue to build a database of genders of Florida genotypes (KML, Mote, FLAQ).
5. Attempt fertilization, larval rearing, and settlement of *D. cylindrus* colonies by two different facilities (Mote and FLAQ)
6. Attempt cryopreservation of *D. cylindrus* sperm for potential use as a tool to improve fertilization across sites or days/years (SEZARC/Disney).

2018 Protocols

The Looe Key region was targeted for observations and collections due to the quantity of genetically distinct colonies as well as a progressing disease boundary that will likely cause mortality to these colonies before the next spawning event. Looe Key SPA contains six genetically distinct colonies, and an additional ten genotypes are located within 10 km to the west (Fig 2). A subset of these sites were selected in order to position multiple dive teams within swimmable distances of boats. A total of eight genotypes were monitored the first night and nine on the second night. Most of these genotypes were also observed spawning in 2017, so inter-year gender comparisons were possible.

Field surveys were conducted on July 29 and July 30. Teams of SCUBA divers were deployed on each colony from 9:00-10:05 pm on July 29, and 8:50-10:05 pm on July 30. Each team recorded the timing of spawning, proportion

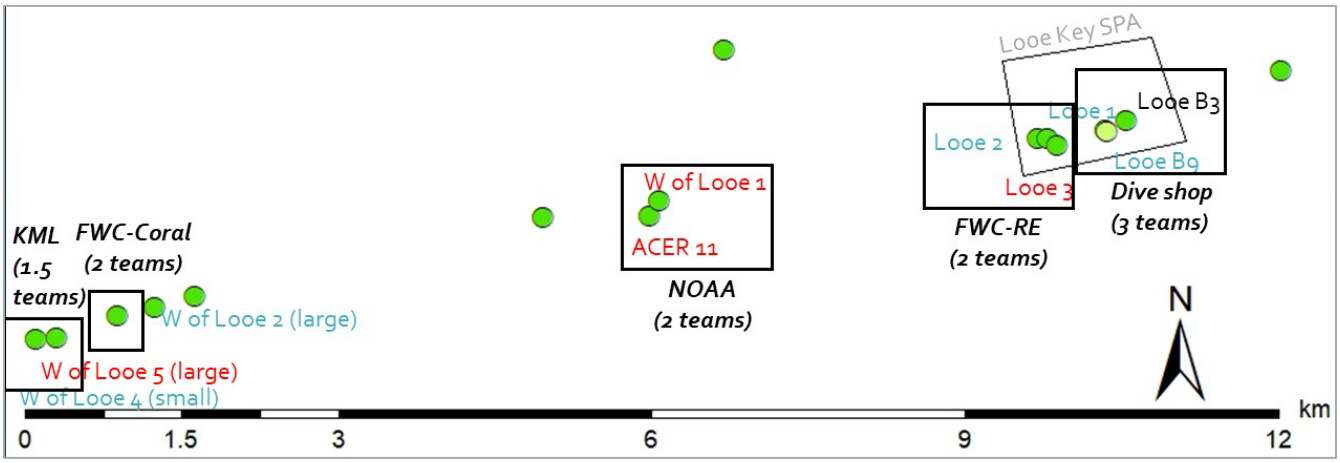

Fig 2. Location of *D. cylindrus* colonies (green circles) between American Shoals and Looe Key SPA (grey box in top right corner). Boxes and text represent the colonies and teams deployed for observation on spawning night one.

of colony spawning, and gender. They also collected gametes using large plastic bags weighted with rigid rims. In addition to bag collections, small amounts of sperm were also collected with 60 cc syringes to provide concentrated samples for cryopreservation. All gametes were returned to boats immediately after spawning. Eggs were concentrated using gravy separators (night one) or purpose-built sieves (night 2). Sperm samples in syringes were filtered onboard by SEZARC staff through a nylon mesh stallion gel filter unit, added directly to preloaded cryovials containing combinations of DMSO and DMA, and dropped directly into a dry shipper for cryopreservation. All wild-collected gametes were transported to a single vessel where at-sea fertilization occurred by dumping concentrated eggs into sperm collections. Samples were then transported back to shore where they were split between Mote and FLAQ staff for larval development.

	2017	7/29/2018	7/30/2018
W of Looe 4	X	X	X
W of Looe 5	X	X	X
W of Looe 3 (4 colonies)			X
W of Looe 2	X		
ACER 21 (2 colonies)	X		
ACER 11	X		X
W of Looe 1	X	X	
Looe 2	X	X	X
Looe 3	X	X	X
Looe 1	X	X	X
Looe Buoy 9	X	X	X
Looe Buoy 3	X	X	

Fig 3. Sites observed in 2017 and on nights one and two of spawning in 2018. Observed sites are marked with an X. Nights in which spawning was observed are shaded green.

Teams of personnel were also stationed at onshore facilities (FLAQ, Mote, and KML) to observe spawning of *ex situ* fragments. Nightly observations were conducted from July 29 through August 1 (Mote), July 29 through August 2 (FLAQ), and July 29 through August 3 (KML).

2018 Field Results

Field spawning was observed on 5 of 8 field colonies on July 29 and 6 of 10 field colonies on July 30. Both nights yielded gametes from male and female colonies. Males spawned between 21:06-21:37 (58-86 minutes after sunset) and females between 21:40-21:55 (89-104 minutes after sunset).

Fig 4. Observations of *D. cylindrus* spawning within the Florida Reef Tract from 2012-2018. Yellow circles and times represent the date of the full moon. Blue clouds indicate the presence of spawning, with size indicative of the intensity. Red crosses are nights when colonies were observed but did not spawn. Text notes the location of the observation as well as the number of minutes after sunset that spawning occurred. Red text is males, blue text is females, yellow text is both, with timing not differentiated.

While the nights of spawning line up with predictions based on observations from previous years (Fig 4), males spawned abnormally early in 2018 (Fig 5). Average time of male spawning was 20-32 minutes earlier than averages from previous years, while female spawning was not significantly different. This increased gap between male and female gamete release resulted in a much longer time to fertilization. Pre-freeze motility estimates by SEZARC indicate 10-20% motility 20-30 minutes after sperm release, 1% motility after 60 minutes, and 0% motility after 90 minutes. We suspect that the additional minutes added to pre-fertilization time in 2018 led to reduced fertilization rates. Egg fertilization was estimated at < 10% (fertilization rates in 2016 and 2017 were estimated around 90%).

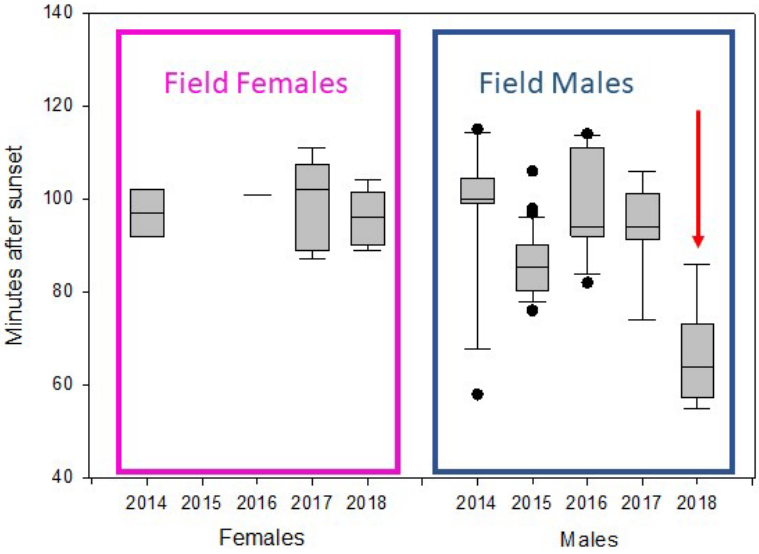

Fig 5. Spawning times of wild colonies across all observation sites by year. A one-way ANOVA showed no difference in annual spawning times among females, but did identify 2018 males as spawning significantly earlier than in all other years ($p < 0.001$)

D. cylindrus has traditionally been considered a gonochoric species. However, observations at a clonal site in the Upper Keys between 2012-2014 documented males and females on genetically identical colonies, sex switching between years, and even production of eggs and sperm within a single colony. It was suspected that this hermaphrodism was rare, as three other large clonal sites have been gonochoric throughout colonies and over multiple years. However, multi-night and multi-year observations of the Looe colonies documented one colony that produced eggs and sperm on different nights of the 2018 spawn, and another that produced eggs in 2017 but sperm in 2018 (Fig 6).

2018 Laboratory Results

Coral fragments were observed over multiple nights at Keys Marine Lab, Mote Marine Lab, and Florida Aquarium. Fragments at Mote spawned in 2016 and 2017, including fragments that had been in tanks through two spawning seasons (Fig 7). However, in 2018 these corals are generally fragmented and some were exhibiting partial bleaching or tissue recession, which may have inhibited the production of gametes.

There was also no spawning of Florida Aquarium fragments. These fragments

	8/9/2017	8/10/2017	7/29/2018	7/30/2018
W of Looe 4	0%	15%	99%	80%
W of Looe 5	15%	80%	80%	100%
W of Looe 3 (4 colonies)				0%
W of Looe 2	35%	65%		
ACER 21 (2 colonies)	0%	0%		
ACER 11	0%	25%		25%
W of Looe 1	0%	50%	10%	
Looe 2	0%	30%	30%	15%
Looe 3	0%	50%	0%	100%
Looe 1	5%	100%	0%	0%
Looe Buoy 9	15%	10%	30%	5%
Looe Buoy 3	0%	0%	0%	

Fig 6. Gender and proportion of each colony releasing gametes on each night of observation in 2017 and 2018. Of colonies observed spawning across both years, two were consistently male, three were consistently female, and two were hermaphroditic either between years or between nights in 2018 (shaded in yellow).

were generally larger with good tissue growth and polyp extension. However, FLAQ staff surmise that the lack of temperature gradient in their artificial seawater between winter and summer may not have triggered gamete development. Two FLAQ coral spawning labs that can control lighting and temperature are nearly completed and will be used to develop *in situ* coral spawning techniques to hopefully increase and improve gamete production in future years.

At Keys Marine Lab, 40 fragments representing 17 genotypes were observed for spawning. Of these, 13 different fragments representing 6 genotypes spawned (Fig 7). All spawning fragments were collected from the wild between April 17 and May 15 of 2018, and three of the genotypes are since extinct or nearly extinct in the wild. Four of the thirteen fragments spawned over multiple nights (Fig 7). All spawning fragments were female, including a fragment from Sombrero in which the wild parent colony had spawned as a male in 2016. No males spawned, and so cryopreservation at this site was not possible. Some eggs were combined with thawed sperm that was cryofrozen from wild colonies on earlier nights. Six cryo samples, representing holdings preserved in a variety of concentrations of DMSO and Glycerol, were used. However, sperm motility in all of them was 0%, and no eggs were successfully fertilized.

Comparisons between field spawning times and lab spawning times have showed that laboratory corals spawn later in the evening, on later nights, and with more variation in both minutes and nights than field corals (Fig 8). One hypothesis for this is that settlement cues in laboratory settings are lacking or not as precise as they are in the field. Perhaps gametogenesis progresses, but the cue for release is not present and so corals continue to wait until they have no other option but to release. If this is the case, it suggests that larval spawning in captivity will require further efforts to determine what those release cues are in order to trigger more coordinated release times between nights and minutes, particularly between males and females.

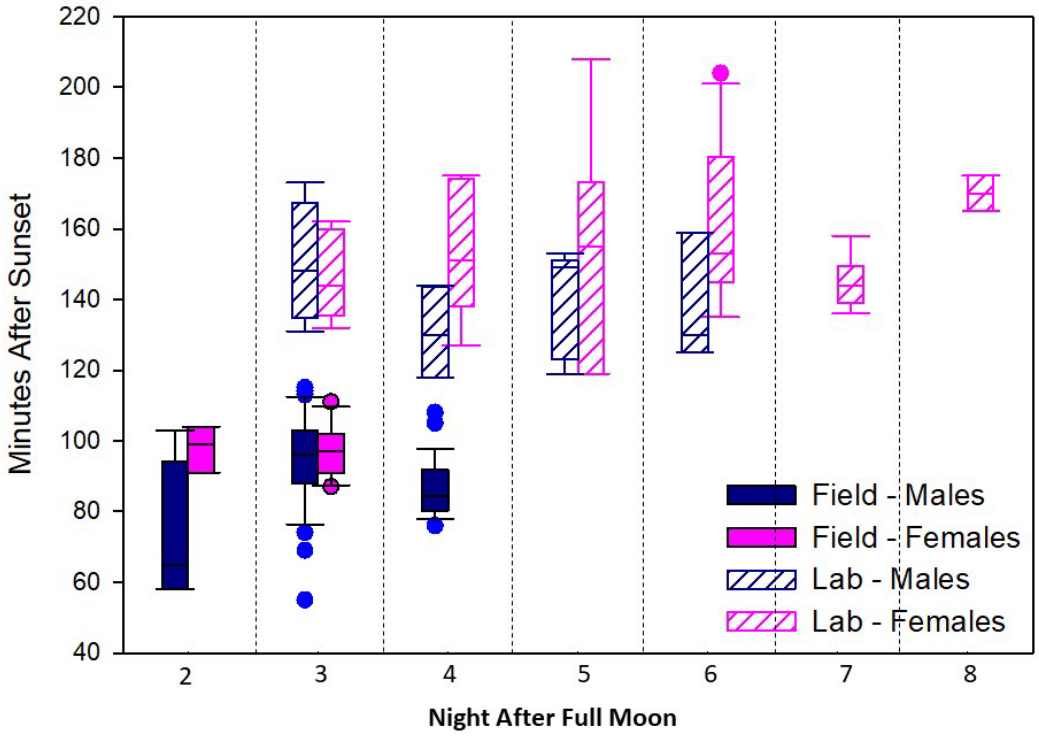

Fig 8: Box plots comparing spawning times after sunset of field (solid) and laboratory (striped) *D. cylindrus* colonies. Plots show the median (center line), 25%-75% (box), 10% - 90% (whiskers) and outliers (dots). Data are compiled from all field observations on the Florida Reef Tract (2012-2018) and from all laboratory observations of FRT-collected corals (KML and Mote, 2016-2018). Field corals spawn on earlier days, earlier times, and in a smaller time range than laboratory corals.

Fig 7. Data from laboratory spawning observations from Mote and KML from 2016-2018. Data presented are the minutes after sunset in which release of eggs (pink) or sperm (blue) was observed. Multiple entries on the same night at Mote represent gamete release from different fragments of the same genet. “Collected” column shows the date fragments were moved from the field into laboratory settings. Note that some fragments spawned through two consecutive years ex situ.

			2016					2017							2018			
			Night 2	Night 3	Night 4	Night 5	Night 6	Night 2	Night 3	Night 4	Night 5	Night 6	Night 7	Night 8	Night 4	Night 5	Night 6	Night 7
MOTE	Site	Collected	8/19/2016	8/20/2016	8/21/2016	8/22/2016	8/23/2016	8/9/2017	8/10/2017	8/11/2017	8/12/2017	8/13/2017	8/14/2017	8/15/2017	7/31/2018	8/1/2018	8/2/2018	8/3/2018
BH4	DC-0025	8/9/2016	136	144	138	119	135						158					
W of Looe 3	DC-2	7/22/2016					145	146	139, 162	151		164	140, 144	176				
Looe 3	DC-8	7/30/2016				132	145		132	127, 138, 163	155	204	136, 138, 144	166				
WDR2-2	DC-10	8/10/2016					135											
WDR2-1	DC-11	8/10/2016				119												
W of Looe 2	DC-1	7/22/2016										186						
Mid Sambo	DC-16	8/23/2016										163						
Sand North	DC-4	7/26/2016							158		163	152, 194	150					
W of Looe 5	DC-6	7/28/2016									208	149						
MK32-1	DC-13	8/10/2016			118		125		131, 150, 173	142, 144	151	159 (m), 152 (f)	143					
Looe B9	DC-7	7/30/2016							146		153							
MK32-2	DC-0024b	1/31/2016									144		149					
BH1-1	DC-0016	8/9/2016				134												
	DC-0031	8/9/2016					130											
BH1-8	DC-006	7/30/2016			118	123												
Coffins	DC-1006	1/14/2016																
Pelican 2	DC-17	8/23/2016									148, 149, 149							
KML																		
Crocker 3		8/20/2016		87	134													
LKL2	DC-215	4/6/2017								154								
	DC-216A	4/6/2017								155								
	DC-217	4/6/2017								154								
Coffins	DC-247A	6/15/2017								155								
Pickles 3	DC-250C	6/29/2017									152							
W Washerwoman	294B	5/8/2018													102			
	294C	5/8/2018													102			
	292B	5/8/2018														148		
	295	5/8/2018														154	161	
	291	5/8/2018															133	149
	293	5/8/2018																150
Sambo	296	5/10/2018													142	145	154	
	297	5/10/2018													149	147		
	298	5/10/2018																152
Sand Key 1-7	303	5/10/2018													92			
9' Stake	299	5/10/2018														130	137	
Sombrero 2-3	263	4/17/2018														146		
Pickles 3	304A	5/15/2018														147		

Larval rearing:

Mixed eggs and sperm were provided to Mote Marine Lab and FLAQ personnel upon return to the dock.

Florida Aquarium protocols and results (courtesy of Keri O'Neil):

Florida Aquarium staff transported the mixtures to Keys Marine Lab. Upon arrival, the solution was scooped through a 1000 micron sieve to remove large plankton. Egg solution was then slowly siphoned into a 75 micron mesh sieve to concentrate. The sieve was sitting in a bowl filled with water, the diameter of the bowl being larger than the height of the sieve. The bowl was placed over a 5 gallon bucket or a floor drain so that excess water could overflow the bowl. At no point were eggs allowed to rest on the sieve out of the water. The sieve was up-righted into the bowl without allowing eggs to compress on the sieve.

Eggs were transferred into eight replicate polystyrene deli containers with 1 micron filtered seawater at pH 8.1 and temp 80 deg F. A large number of zooplankton was still present in the polystyrene containers and were removed by hand using a pipette when possible. No egg division was observed and eggs were presumed unfertilized. Many eggs were seen to "burst" by the next morning (confirmation of unfertilized). However, by 5:00 pm the day after spawning, larvae were observed to be spinning/circling. Actively moving larvae were transferred into clean filtered seawater in new polystyrene containers. An estimated 150 larvae were present, so overall fertilization is estimated to have been about 5-10% based on an estimated number of eggs at ~2000. These are gross visual estimates and were not verified by replicate sub-samples as we presumed the developing eggs to be very fragile and did not want to homogenize them.

After movement was observed, larvae were offered pre-conditioned settlement tiles of different shapes and material (ceramic and aragonite) and orientations. The following morning, some small chips of live CCA were obtained from a local live rock supplier (KP Aquatics) and also offered as settlement substrate. Many larvae were observed probing the bottom under a dissecting scope. Larvae were allowed to search for settlement substrate for 36 hours undisturbed. After this time, a scan of the settlement substrates was completed by two trained coral biologists. No settled larvae were found, however ~10 larvae were still moving and not settled. Their movement was greatly reduced (slow). They were transferred into a clean petri dish with clean filtered seawater and offered 4 different CCA chips. No settlement was observed and larvae eventually stopped moving and began decreasing in size (decaying). Settlement substrates were retained and transported back to Florida Aquarium's Center for Conservation facility. They were scanned again for recruits, but none were found.

Mote protocols/results (courtesy of Chris Page):

Upon receiving DCYL gametes from the field teams, collection bins containing mixed gametes were left static and undiluted for approximately 1 hour to facilitate fertilization. During this period swimming invertebrates were removed via pipette from collection bins. Immediately after this period, sperm was diluted by draining water from collection bins through a 48um sieve small enough to exclude fertilized embryos using a ¾" siphon. Once collection water was significantly reduced, embryos were split into 10 portions (per collection night) and placed into clean 5 gallon buckets filled with clean well seawater such that only a few hundred embryos were present in each culture bucket. After 30 minutes, these culture buckets were then drained to 25% their volume through a 48um sieve and refilled with clean seawater to further dilute remaining sperm and opportunistic microbes

12 hours post fertilization: culture buckets were again drained to 10% their volume. The remaining seawater and embryos were then poured directly into a clean 5 gallon bucket filled with clean seawater. This process was repeated 2-3 times a day as necessary for 2 days post fertilization.

2-3 days post fertilization: Because resulting larvae were deemed less fragile than earlier in development, culture buckets were drained down to 10% their volume. The remaining water was poured through a 48um sieve half-submerged in a container of seawater. The sieve containing larvae was then rinsed with two full 2-liter containers of seawater to flush opportunistic microbes through the sieve while retaining viable larvae. Larvae were then transferred to clean 5 gallon culture buckets. This process was performed 1-2 times/day

4-5 days post fertilization: Larvae were sieved and transferred to settlement tanks containing ceramic substrates. They were given one or a combination of the following preparations:

- Substrate dusted with live CCA collected from Mote microfragmenting raceways
- Substrate dusted with preserved CCA (via desiccation)
- Substrate encased in preserved CCA
- Substrates with live or preserved CCA super glued to the substrate.

This was done to prevent settlers attached to the CCA dust from being dislodged once substrates were moved for examination.

15 days post to 1 month post-fertilization: Potentially settled substrates were transferred to flow-through raceways that provided stable conditions. Prior to this, substrates were not moved in order to prevent settler dislodgement. Multiple attempts to find recruits have been made and potential recruits have been observed, but objects defy positive identification and quantification at this stage as potential recruits are nondescript and clear in color, consistent with early developmental stages of DCYL recruits witnessed in previous years. In previous years, accurate quantification was possible roughly 2-3 months post settlement

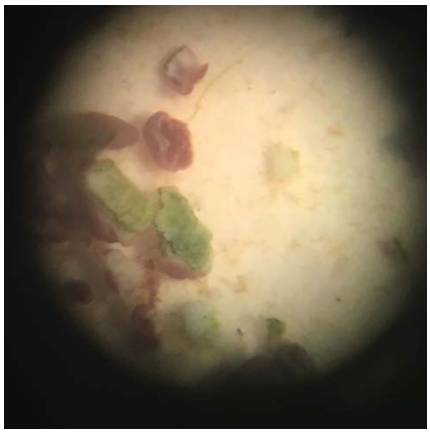

Fig 9: Potential *Dendrogyra cylindrus* recruit from 2018 larval rearing at Mote Marine Lab (photo: Chris Page)

General notes on fertilization/rearing:

Despite improved on-water protocols to minimize dilution by concentrating eggs, and to minimize zooplankton predation by sieving, 2018 fertilization rates were low (< 10%), particularly in comparison to previous years (~90% in 2016 and 2017). We suspect this is largely due to the early release of sperm from males, which led to decreased sperm motility when combined with eggs. We can not specify what may have caused these early releases, but if they are to continue in future years, successful and rapid cryopreservation and control over synchronous laboratory based gamete release will become increasingly important.

Late August Updates:

Spawning research was further conducted on *D. cylindrus* fragments housed at Keys Marine Lab on August 28, 29, 30, and into September. On August 28, spawned gametes were mixed and yielded approximately 4000 swimming larvae 24 hours later which will be transported to Florida Aquarium. Additional gametes were mixed on August 29; fertilization rates were around 50% and viable larvae will also be moved to Florida Aquarium. On August 30, SEZARC re-visited to collect and cryopreserve sperm from the spawning fragments.

Acknowledgements:

Funding for these activities was partially provided by DEP Award B3852C. Activities occurred under FKNMS permit FKNMS-2016-062-A1. Extensive personnel time as well as boat and dive support were provided by all of the partners as well as volunteers. The nature of this endeavor would not be possible without these collaborations.