Supplementary information for A. Chaves-Fonnegra - Chapter 1- PhD Dissertation, corresponding to the published article:

Chaves-Fonnegra, A., M. Maldonado, P. Blackwelder, and J. V. Lopez. 2016. Asynchronous reproduction and multi-spawning in the coral-excavating sponge *Cliona delitrix*. *Journal of the Marine Biological Association of the United Kingdom* **96**:515-528.

A- Table 2. Histology protocol to look for reproductive structures in excavating sponges. Andia Chaves Fonnegra Oct 2009. Mix of sponge and coral histological techniques.

	Fix tissues overnight, 24h (or for extended periods up to 3 weeks) in Bouin´s fixative . Use gloves and a fume hood, or if in a boat or in the field station, do it outside.
	Rinse in distilled water 2x 10-15minutes.
	Decalcification : use Hydrochloric acid (HCl) 10% plus EDTA. Leave specimens for 2 to 3 days under the fume hood, use glass beakers of 200ml per each sample (Coral lab do it in a big plastic tray and separate samples with plastic rings) To prepare 3L (3000ml) of 10% HCl use: 300ml of HCl (Concentrated which usually is 37%) 3g of EDTA 2700ml of dH_2O. Mix well in a glass bottle, and put with a stir bar on a mixing plate for 20min to 1h. Depending of the quality of the EDTA it can take longer.
	After decalcification rinse specimens 3x30 min to 1hour in Distilled Water prior to ethanol.
	Dehydration: Transfer specimens to a glass vial, add and remove each of the following : 50% ethanol – 1 hour (can be stored in 50% ethanol for longer periods). 70% ethanol – 1 hour (then stop here for **Desilicification**: remove most of 70% ethanol and add 4% hydrofluoric acid prepared in 70% ethanol to plastic 50ml falcon tubes (**Prepare fresh every time under a fume hood**) and add until covering the specimen (usually 3-5ml, but depends on the size of the tissue). Leave the specimen in the 4 °C refrigerator overnight. The following day, remove the HF/Ethanol to a waste container and add fresh 70% Ethanol, enough to ensure the HF has been rinsed out, 20 min). Continue with dehydration series. [**HF waste was stored in a 4 °C refrigerator until it could be safely disposed as chemical waste, while carefully following all MSDS protocols**]. 95% ethanol – 15 min x 2 100% ethanol – 10 min x 2-3. Depending on the size of the specimen; longer for larger specimens). Note: absolute 100% ethanol causes shrinkage and hardening, so prolonged time in absolute is not good.

Draw off the 100% Ethanol and add Xylene or Toluene – 15 min x 2. Note: Xylene and Toluene melt plastics, use glass beakers or vials. I used Xylene.
Draw off the Xylene and place the tissue in molten paraffin in a beaker on the oven at 60 °C– 15min x 2.
Embedding: Transfer the tissue to a metallic or plastic mold with fresh molten paraffin. Orient the tissue on the bottom of the mold, press the tissue softly to release any trapped bubbles. Place the embedding rings. Pour in more wax, and move the boat off the hot plate to an ice cold plate.
Sectioning: Do sections between 0.4 and 0.5 µm, thicker sections at 10 µm can also be used, but usually are too thick to see the structures clearly. Use good microtome knifes, and be cautious that even desilicifing and decalcifing sometimes does not eliminate other minerals that can break the tissue. Sections should be done slowly and if you find minerals take a dissecting needle and pull them out. This can leave holes in the block and in your sections, so be careful. Use slides with a space to mark and proper markers or pencil that will not get erased during the staining procedure.

B- Table 3. Heidenhain´s Aniline Blue staining protocol used at the NSU Oceanographic Center Coral Histology Lab to stain corals, and used to stain marine sponges. Andia Chaves Fonnegra Oct 2009. Times may change depending on how fresh the stains are. This protocol is longer than the common Harris Hematoxylin/Eosin typically used for sponges.

Heat azocarimine to 56 °C and filter, then maintain in the oven at 60 °C while in use. If not in used keep it in the fridge at 4 °C
De-paraffinized 2 minutes each under hood 3 times xylene 3 times 100% ethanol 1 time 95% ethanol 1 time 80% ethanol 1 time dH_2O
Stain red 1 time Azocarmine- stain in oven at 60 °C – 25 minutes (species dependent) 2 times dH_2O (eliminate excess of stain) – 3 dips/plunges per container 1 time Aniline alcohol (distain) – (2-5 minutes but check under scope how much is distained) 1 time Phosphotungstic acid (fix) – (12-15 minutes) 1 time dH_2O (rinse) – (2 minutes)
Stain blue 1 time Aniline blue – stain in dark- 20-25 minutes 2 times dH_2O (rinse) – 2-3 dips/plunges per container

Dehydrate 1 time in 95% ethanol – 1 minute 3 times in 100% ethanol – 2 minutes 3 times in Xylene – 2 minutes
Cover slides Add two small drops of glue (Cytoseal) Put coverslide and let dry overnight.

C- Matlab code to quantify sponge tissue on pictures

```
tissue=[];

for i=1:1

eval(['pic=imread("C:\Users\Andia C Fonnegra\Desktop\pictures matlab\June 29
2011\pic',num2str(i),'.JPG");']);

% lines=size(trans,1);
% columns=size(trans,2);


level1=pic(:,:,1);
level2=pic(:,:,2);
level3=pic(:,:,3);

hole=[];
for i=1:3
eval(['hhole=find(level',num2str(i),'==255);']);
hole=[hole; hhole];
eval(['level',num2str(i),'(hole)=0;']);
end

picture=cat(3,level1,level2,level3);
figure;imshow(picture);

ttissue=100-(numel(hole)*100/numel(picture));
tissue=[tissue ttissue];

end
%close all;
```