

The Effect of *Photobacterium damsela* Availability on the Behavior of Choanocytes in the Marine Sponge *Amphimedon queenslandica*

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Abstract

Sponges are aquatic filter feeding animals that use internal chambers comprised of choanoflagellate-like cells called choanocytes to pump and filter water. In this study I investigated the behavior of choanocytes in juvenile *Amphimedon queenslandica*, a coral reef demosponge, when they were fed *Photobacterium damsela* subsp. *damsela* compared to when they were maintained in 0.22 μ m filtered seawater. Prior to these treatments I fluorescently-labelled choanocytes with Cm-DiI, which allowed tracking of the movement and behavior of these cells over 24 h. I found that choanocytes moved out of the chambers significantly more in unfed sponges, suggesting that choanocyte chambers and the aquiferous system are constantly being remodeled in unfed sponges. When fed however, the choanocytes were more prone to staying in their respective chamber, presumably collecting *P. damsela* from the water. Furthermore, when the juvenile sponges were exposed to *P. damsela* the choanocyte chambers grew in size, becoming 20% larger than the unfed juveniles. Lastly, there were significantly more cells being shed into the water column when the juveniles were fed. This suggests that under natural conditions, when the aquiferous system is fully functioning, replacement and shedding of choanocytes occur. I conclude that the aquiferous system of *A. queenslandica* is very dynamic, where the choanocytes have the ability to leave and join other pre-existing chambers or form entirely new chambers. However, when the choanocytes are exposed to food they are less likely to leave their chamber, resulting in development and growth of the choanocyte chamber.

Introduction

Sponges (Porifera) are aquatic filter feeding animals that have been ecologically important in benthic habitats across tropical, temperate and polar waters since they evolved over 600 million years ago (Knoll 2003). Sponges are extremely efficient filter feeders, being able to filter around 99% of the microorganisms and nutrients that pass through their system (Hadas, et al., 2009). Sponges feed by pumping water through a network of canals and chambers known as the aquiferous system. Scattered around the canals are chambers lined with a choanoflagellate-like cell type called choanocytes. The choanocytes form a sphere and each cell has a large beating flagellum pointed to the center of the chamber, generating water flow through the animal. The base of the flagellum is lined with microvilli which acts as a net through which microorganisms and nutrients in the water are caught (Mah et al., 2014). Through the process of phagocytosis, microorganisms like bacteria and even viruses are then transmitted to amoebocytes which delivers the food to other cells in the sponge (Reiswig, 1971; Hadas et al., 2006).

Amphimedon queenslandica is a marine demosponge found in tropical climates, often on oligotrophic reefs. *A. queenslandica* had its genome sequenced in 2010 (Srivastava et al., 2010). Therefore, while I will not be looking at transcriptional data in this study, *A. queenslandica* should be considered a model species and will be very useful in future studies. Marine bacteria make up the majority of a sponge's diet (Hadas et al., 2009; Maldonado et al., 2012). In an oligotrophic reef the concentration of bacteria in the water column is approximately $2-9 \times 10^5 \text{ ml}^{-1}$ (Gast et al., 1998). It was observed recently that the choanocytes of *A. queenslandica* are able to leave a choanocyte chamber and either join another pre-existing chamber or form an entirely new one (Sogabe et al., 2016). It is however, not known what governs this mobile behavior. As of date, studies regarding the choanocyte biology have mostly been descriptive with few quantitative studies being made regarding their mobile behavior. Furthermore, a study by de Goeij et al (2009) showed that there is a high cell turnover in sponges, where the choanocytes are frequently expelled into the water column. This is now theorized to be one of the major ways in which sponges recycle nutrients within the ecosystems, by releasing old choanocytes as particulate organic carbon (POC) into the water column (Maldonado, 2016). However, how the availability of food affects shedding of choanocytes is not known.

Given the importance of sponges in the nutrient cycle of benthic habitats I will investigate how the choanocyte behavior is altered depending on the availability of *P. damselae*, a marine bacterium. Due to the functional role of choanocytes as feeding cells, I hypothesize that the choanocytes will be less mobile and more inclined to stay in their relative chamber compared to when they are unfed, where they will be more mobile in order to find and catch food.

Methods

Sponge collection

In March 2017 ten fecund *A. queenslandica* sponges were collected from Shark bay, Heron island, Queensland Australia. The sponges were then transferred and kept in a flow-through aquarium at the University of Queensland. Temperature was around 22-23°C, they were fed an algal mix every day, and light schedule was set to 12 L – 12 D.

Larvae collection

Larval collection followed the protocol of Leys et al (2008). The adult sponges were induced to release larvae by increasing the water temperature in the tanks by 2-3°C and allowing the adults to release their larvae for a few hours. Larvae were collected using a Pasteur pipette. The larvae were induced to settle and undergo metamorphosis in the dark at 35°C in 0.22µm filtered seawater (FSW) onto live coralline algae *Amphiroa sp.* During metamorphosis, when the larvae settles onto the algae, the larvae were gently scraped off and placed on a ø12mm coverslip. Here, the larvae were allowed to re-settle and later placed in a 24well plate with 2ml of FSW per well. Once an osculum (a large vent through which the water leaves the sponge) is visible they are considered juveniles. The process from settlement to osculum formation takes approximately 72h (Degnan et al., 2015). I used 17 juveniles in total, 10 in the Unfed treatment and 7 in the Fed treatment. However, due to errors during the mounting of the juveniles onto slides, I was only able to use confocal microscopy on 10 individuals, 5 in Unfed and 5 in Fed.

Bacteria preparation

The marine bacterium *Photobacterium damsela* subsp. *damsela* was used to feed the sponges. The bacteria were grown in tryptic soy broth, with added 2% NaCl, (TSB2) due to their halophilic nature. The sponges were fed bacteria in the concentration of roughly 5×10^5 cells/ml, which was determined using optical density (OD₆₀₀) and a calibration curve. The juvenile sponges were fed *P. damsela* 1h before labelling the choanocytes with Cm-DiI, by adding 20µl bacteria culture to 2ml FSW. The Unfed group were given 20µl sterile TSB2.

Cell labelling

The choanocytes were stained using the cellular probe Cm-DiI, a red fluorescent dye with emission spectra 553/570nm maxima. The dye passes through the cell membrane where it becomes impermeable to the cell membrane. The dye is non-toxic and retained in daughter cells. Before labelling, the sponges were washed with FSW a number of times, mainly to remove excess *P. damsela* from the wells, to avoid accidentally labelling bacteria with Cm-DiI, thus confusing the image analysis. The dye was prepared by mixing 50µm Cm-DiI with 50µl DMSO. This stock solution was then diluted to a 1µM working concentration in FSW, which the juvenile sponges were exposed to for 15min. I found that 15 minutes was long enough to label the vast majority of the choanocytes, but still short enough to not label other cell types. This provided the clearest images of the choanocytes. After the 15-minute incubation period, the sponges were washed several times with FSW to prevent anymore cells being labelled during the experiment. The sponges were then kept in 2ml FSW, where the Fed treatment were fed *P. damsela*, while the Unfed group were given sterile TSB2. After this step, the sponges were kept at 25°C and in the dark throughout the experiment to avoid loss of the fluorescent signal.

After 24h, the sponges were fixed with a paraformaldehyde solution (4%). The samples were left in the fix at 4°C overnight and were subsequently washed several times with a 1× MOPS solution ((3-(*N*-morpholino) propanesulfonic acid). All the samples were then labelled with a DAPI stain, which dyes the nuclei a fluorescent blue, by incubating the samples in a 1µM DAPI solution for 10min. Afterwards, the samples were washed, again, with 1× MOPS, and were then mounted on microscope slides in ProLong™ Gold Antifade Mountant.

Imaging and Image analysis

Inverted fluorescent microscopy

The juveniles were imaged at 20x zoom using a fluorescent inverted microscope. I used imageJ to place a grid, with squares being $9000\mu\text{m}^2$, the mean number of choanocyte chambers were counted from three squares per individual. I was then able to compare the mean number of visible choanocyte chambers after 24h between the two treatments. To further quantify the difference in movement of Cm-DiI labelled choanocytes within the juvenile sponges, imageJ was set up to calculate the increase in number of Cm-DiI labelled choanocytes in the skirt of the sponge between 0h and 24h for both treatments. The skirt is defined as the area between the edge of the aquiferous system and the edge of the animal (fig 1). For this analysis, particle size was set to 1-20µm and sphericity to 0-1 in imageJ. In addition, the inverted fluorescent microscopy was used to image the juveniles post fixation once they had been labelled with the DAPI stain. These images were used, in similar ways as described above, to measure the density of DAPI labelled choanocyte chambers at 24h. By using the DAPI stain post fixation I got a snapshot of how the aquiferous system was

structured at the moment of fixation. The density of DAPI labelled chambers was determined by placing three $9000\mu\text{m}^2$ squares randomly on the juvenile and taking the mean number of choanocyte chambers. The images from the inverted scope was also used to compare the mean size difference of the choanocyte chambers between the two treatments at t0, by measuring the diameter of 10 random chambers per individual.

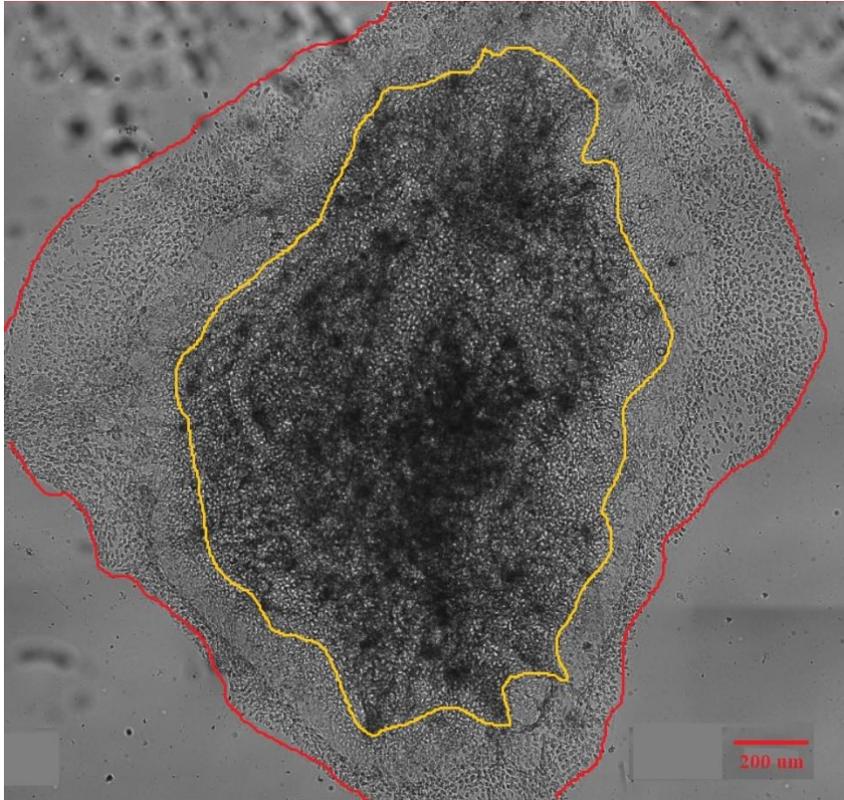


Figure 1. A light micrograph of a 4-day old juvenile *A. queenslandica*. The edge of the sponge is demarcated by a red line. The edge of the central body is demarcated by a yellow line. In between these lines is defined as the skirt of the sponge. Scale bar, 200 μm .

Confocal microscopy

The fixed samples were analyzed using the ZEISS LSM 510 META confocal microscope. For the data collection I used z-stacks placed towards the edge of the aquiferous system, using both 63x and 20x zoom, slices were set to 1-1.10 μm . The images were analyzed using the software Imagej. The confocal microscope produced detailed images on a cellular level of the choanocyte chambers. These images were used to calculate the difference in percentage make up of Cm-DiI labelled choanocytes per choanocyte chamber between the treatments. This was done by going through the z-stack counting the number of Cm-DiI labelled cells and dividing with the total number of choanocytes in the chamber (the DAPI labelled choanocytes). The confocal images were also used to compare the mean size difference of the choanocyte chambers between the treatments at t24 by measuring the diameter of 10 random choanocyte chambers per individual.

Cell shedding

To investigate whether there was a difference in cell shedding between the two treatments I collected the FSW from the individual wells after 24h, and fixed it in a paraformaldehyde

solution (4%) in 2ml Eppendorf tubes. The tubes were centrifuged, and the pellets were dissolved in 50µl fix solution. 10µl was added onto a slide and brought under the inverted fluorescent microscope. An area of 9mm² was imaged using the red fluorescent emission spectra. ImageJ was used to count the number of cells found in the area. In this test I also added a negative control, in the form of FSW.

Data analysis

The data was analyzed using IBM SPSS Statistics 24 using independent samples t-tests and a one-way ANOVA.

Results

Movement of the choanocytes

In order to determine the impact of feeding on choanocyte chamber structure and dynamics, I labelled choanocytes in the chambers of juvenile *A. queenslandica* with Cm-DiI and followed the fate of these cells through 24h. I found that juveniles fed with *P. damselae* had significantly more visible chambers at 24h compared to the unfed treatment ($P=0.02$, $t= -2.71$). The choanocyte chambers were more intact, i.e. less dispersal of the choanocytes labelled at t_0 , in the Fed treatment compared to the Unfed treatment (fig 2). Thus, the aquiferous system in the Fed treatment were kept structurally very similar throughout the 24h, while in the Unfed treatment the aquiferous system was undergoing larger structural changes (fig 3).

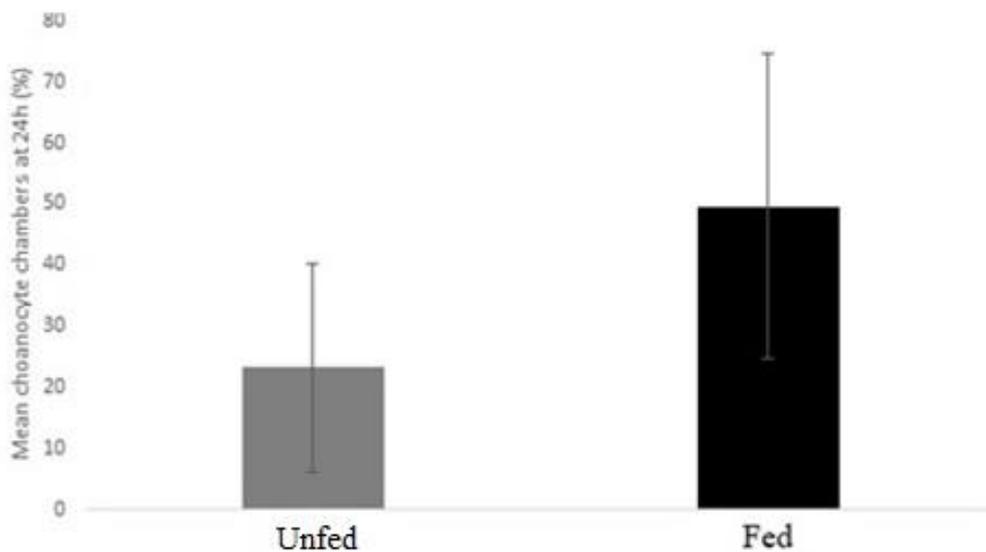


Figure 2. Mean percentage of Cm-DiI labelled choanocyte chambers detectable at 24h between the Unfed and Fed treatments. Error bars: standard deviation.

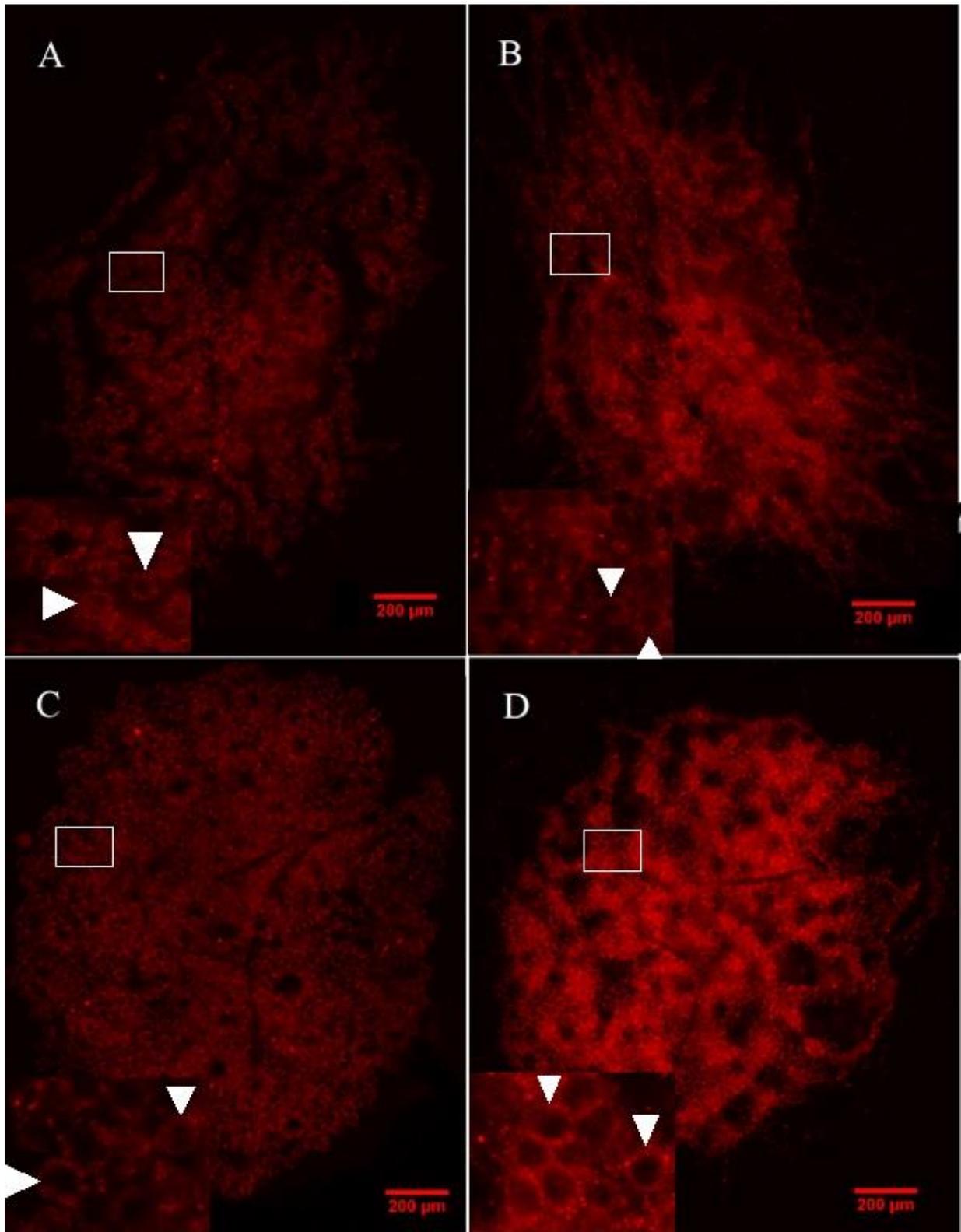


Figure 3. Four micrographs from two four-day old *A. queenslandica* juveniles stained with Cm-DiI to compare the change in choanocyte chamber dissolution between 0h and 24h. White rectangle marks the zoomed area with arrowheads showing the choanocyte chambers. The A and B individual was kept in FSW with no added food source, while the C and D individual was fed *P. damselae*. The A and C images were taken at 0h post Cm-DiI labelling, while B and D were taken 24h post labelling. Scale bar is 200µm.

Quantifying the increase in number of cells between t0 and t24 in the skirt of the sponge further illustrated the difference in choanocyte behavior between the treatments (fig 4). There are significantly more Cm-DiI labelled cells in the skirt of the Unfed juveniles compared to the *P. damselae* fed individuals ($P = 0.021$, $T = 2.634$). This shows that there is more cell movement towards the edge of the juvenile, and likely more restructuring of the aquiferous system when the juveniles are unfed.

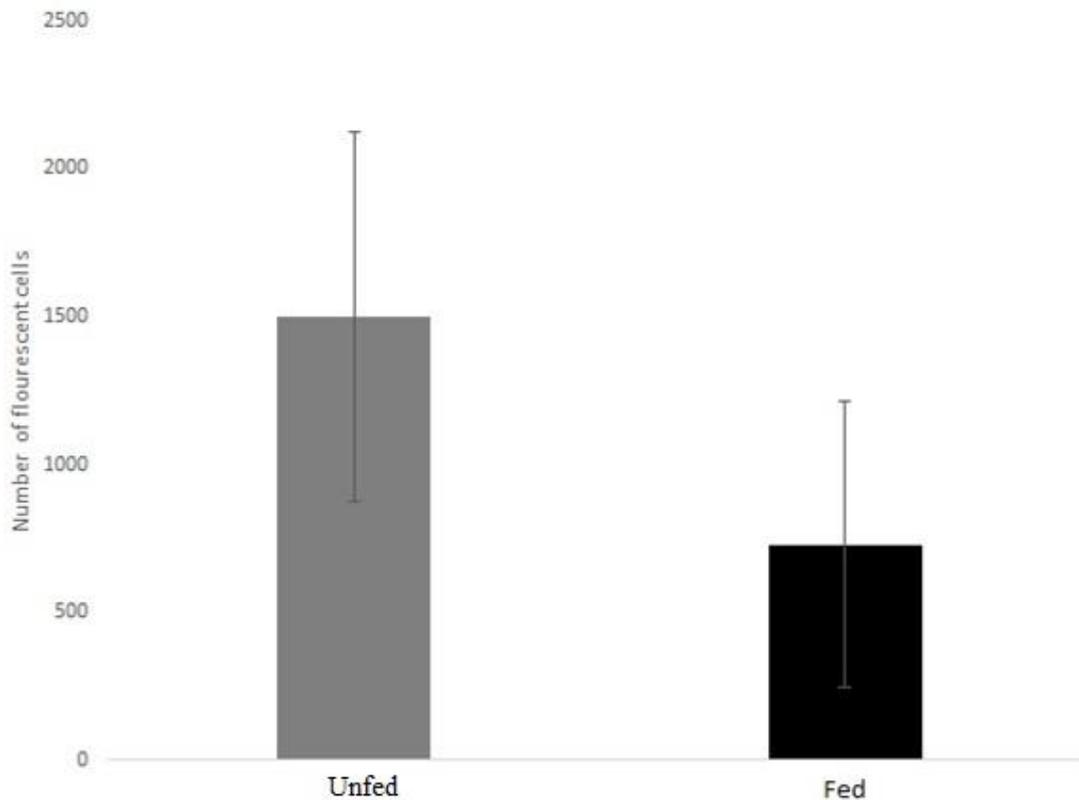


Figure 4. Illustration of the differences in net increase of Cm-DiI labelled cells in the skirt between the Unfed and Fed juveniles at 24h post labelling. Error bars: standard deviation.

Total number of choanocyte chambers

The DAPI staining gave a snapshot of how the aquiferous system was structured after 24h. Comparing the two treatments, it was evident that there was no difference in the total number of choanocyte chambers (fig 5) ($P = 0.28$, $T = 1.12$). Comparing the results from fig 2 and 5 we can see that, in the Unfed treatment, while the choanocyte chambers labelled at t0 have mostly dissolved by 24h, entirely new chambers have formed, and the density of chambers, (which can be extrapolated to total number of chambers in the juvenile) is not different between the two treatments.

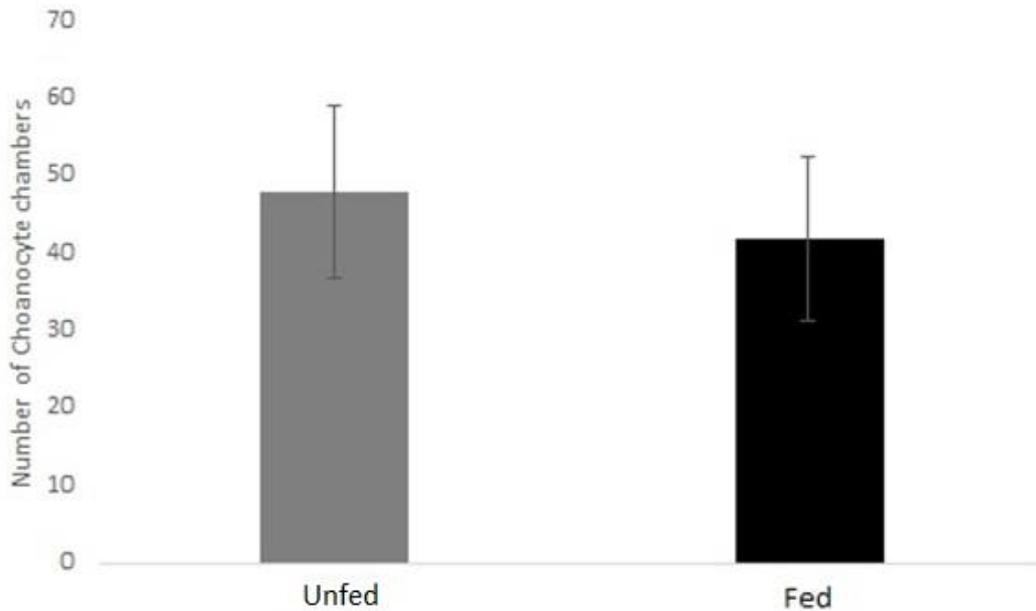


Figure 5. The bars explain the mean density of DAPI labelled choanocyte chambers at 24h per 9000µm². Error bars: standard deviation.

Percent Cm-DiI labelled choanocytes per choanocyte chamber after 24h

Confocal microscopy produced detailed z-stack images of the choanocyte chambers. The use of the red Cm-DiI at t0 and the DAPI stain at t24 allowed for comparisons between the treatments in the percentage make up of Cm-DiI labelled choanocytes per choanocyte chamber after 24h (fig 6 and 7). There was no significant difference in the percentage of Cm-DiI labelled choanocytes making up the choanocyte chambers between the treatments. The trend, however, is that there are more Cm-DiI labelled choanocytes making up the choanocyte chambers after 24h in the Fed group compared to the Unfed group. Cm-DiI labelled cells can be daughter cells from a choanocyte labelled at 0h. Furthermore, the images do not show whether the Cm-DiI labelled choanocytes have been stationary over the 24h or have translocated from a different chamber. Comparing the results from fig 2, 3 and 4 it seems like the choanocytes located in chambers of the unfed treatment are more likely to have originated from a different chamber.

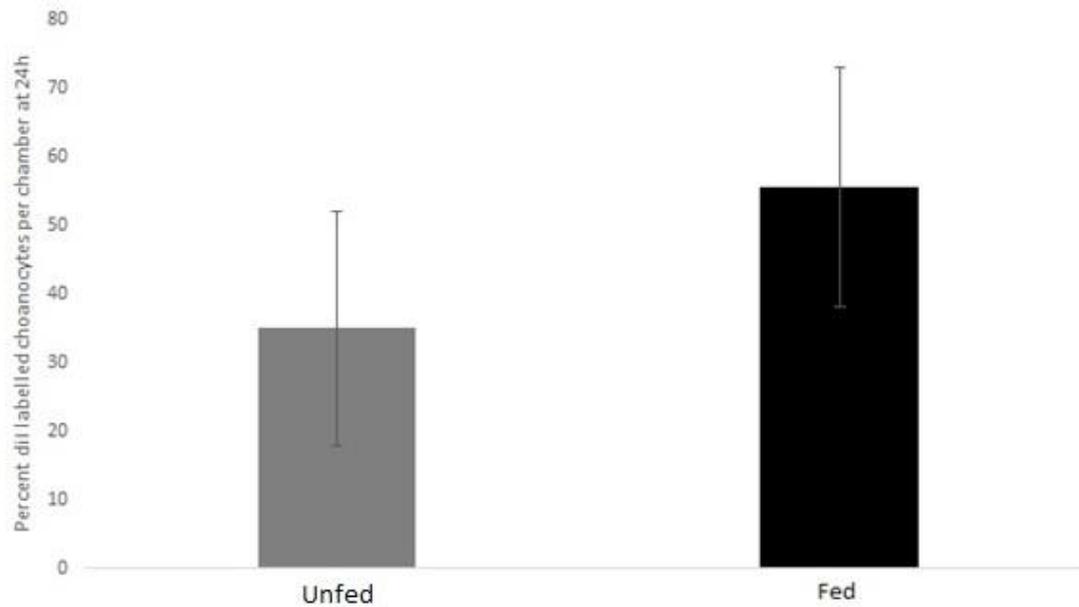


Figure 6. Bars shows the percentage of Cm-DiI labelled choanocytes in the choanocyte chambers at 24h. Error bars: standard deviation.

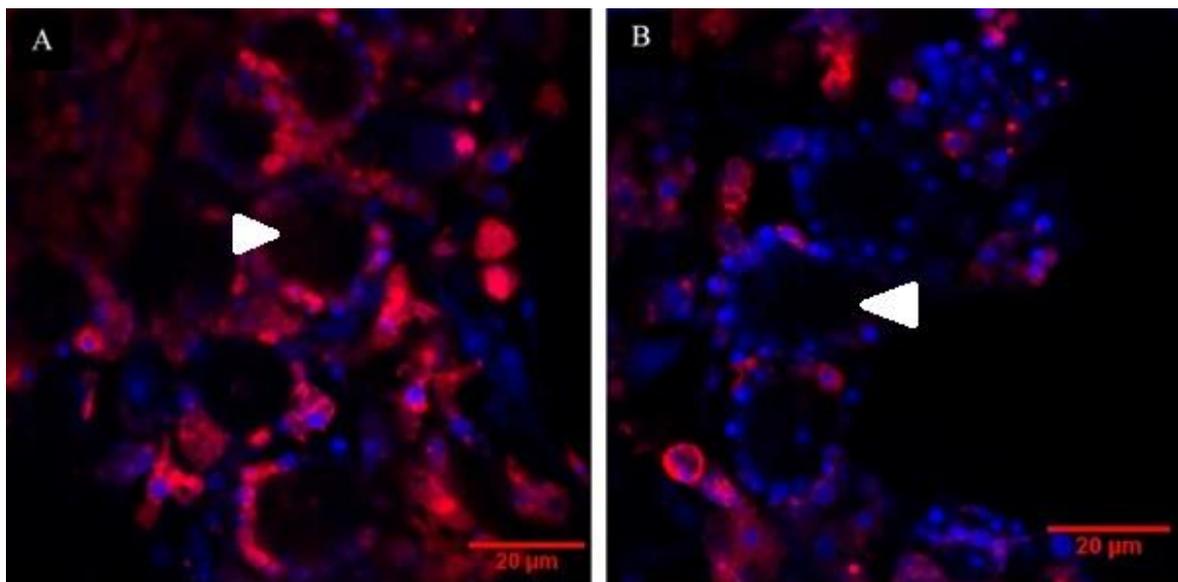


Figure 7. Two images from a confocal microscope of choanocyte chambers at 24h post Cm-DiI labelling. Arrowheads show the choanocyte chambers. Red cells were labelled with Cm-DiI at 0h, while the blue color comes from the DAPI staining made post fixation at 24h, which labels all nuclei. Clear blue choanocytes are new choanocytes formed post Cm-DiI labelling. A trend was visible where the fed juveniles (A) contained a higher percentage of Cm-DiI labelled choanocytes per chamber compared to the unfed juveniles (B). Scale bar is 20 μ m.

Size of the choanocyte chambers

The size of the choanocyte chambers is indicative of the age of the chamber, where a bigger chamber is generally older than a smaller one (Sogabe et al., 2016). Figure 8 and 9 compares the mean diameters of the choanocyte chambers at 24h and 0h respectively. The two results

are measured differently. Fig 8 was measured post fixation and mounting, using 20x zoom on the confocal microscope, while the chambers in fig 9 were measured in the juvenile's respective wells, containing 2mm FSW, using a fluorescent microscope at 20x. So while the diameter of the choanocyte chambers are comparable between treatments at the same time point, they are not comparable between time points, hence the results are shown in two separate figures. The results from fig 8 and 9 illustrates that the chambers did not differ in size at t0 ($P = 0.195$, $T = -1,379$) however at 24h, the mean diameter of the chambers were significantly larger (20%) in the Fed treatment compared to the Unfed ($P < 0.001$, $T = -4.643$).

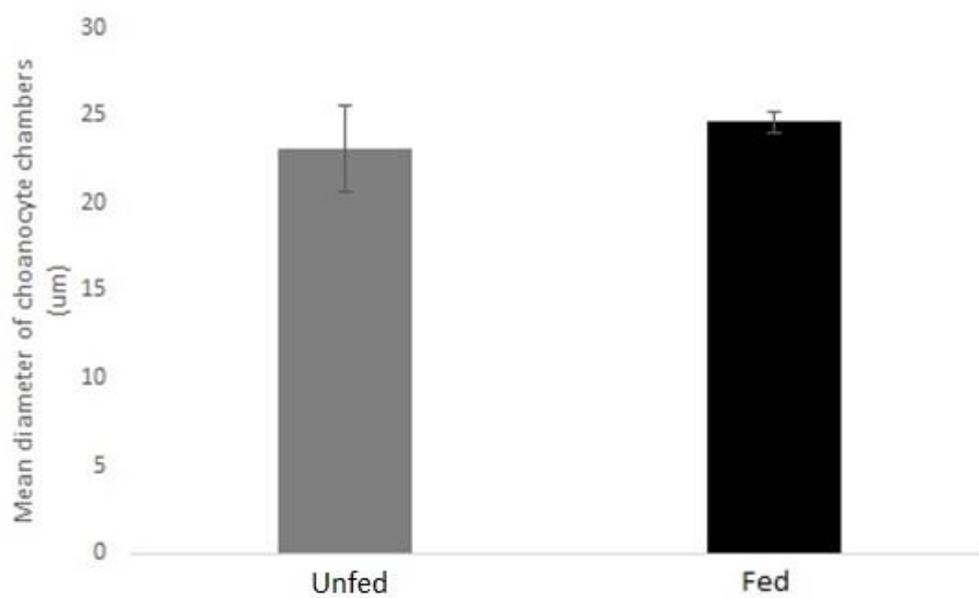


Figure 8. Differences in mean diameter of the choanocyte chambers for each treatment at 0h, using an inverted fluorescent microscope at 20x zoom. Error bars: standard deviation.

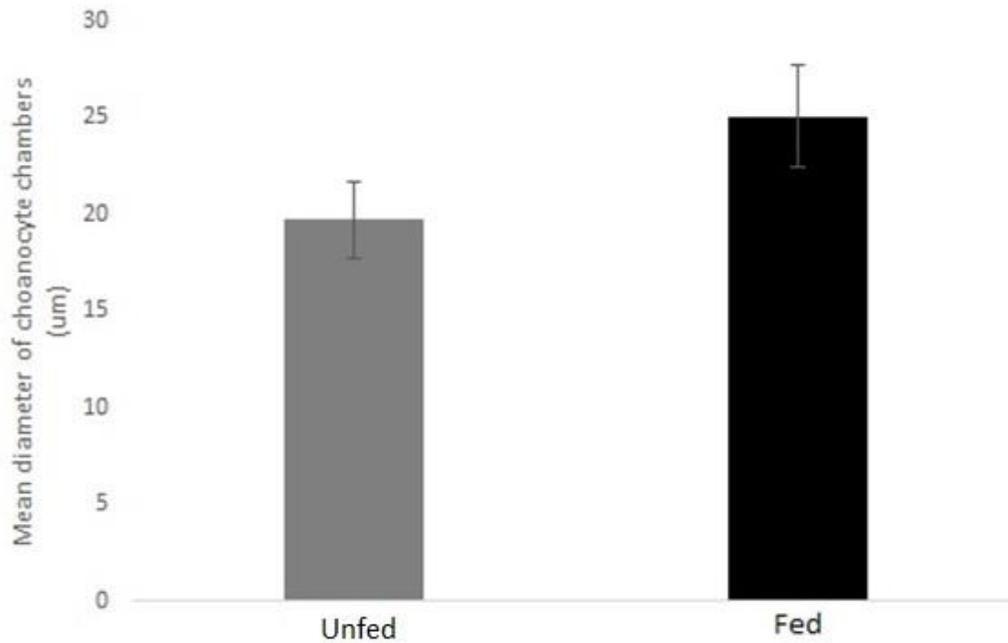


Figure 9. The plot shows the difference in mean diameter of the choanocyte chambers for each treatment at 24h, using a confocal microscope at 20x zoom. Error bars: standard deviation.

Cell shedding

Sponges have in recent years been hypothesized to be important nutrient recyclers in various ecosystems. The water column was examined for Cm-DiI labelled cells after 24h to investigate how food availability affects the shedding of choanocytes. A one-way ANOVA and a Tukey post-hoc test showed that there were significantly more Cm-DiI cells being shed from the fed sponges compared to the unfed sponges ($P = 0.016$) and the control group ($P = 0.010$). The results from the one-way ANOVA were $P = 0.007$, $F = 9.212$.

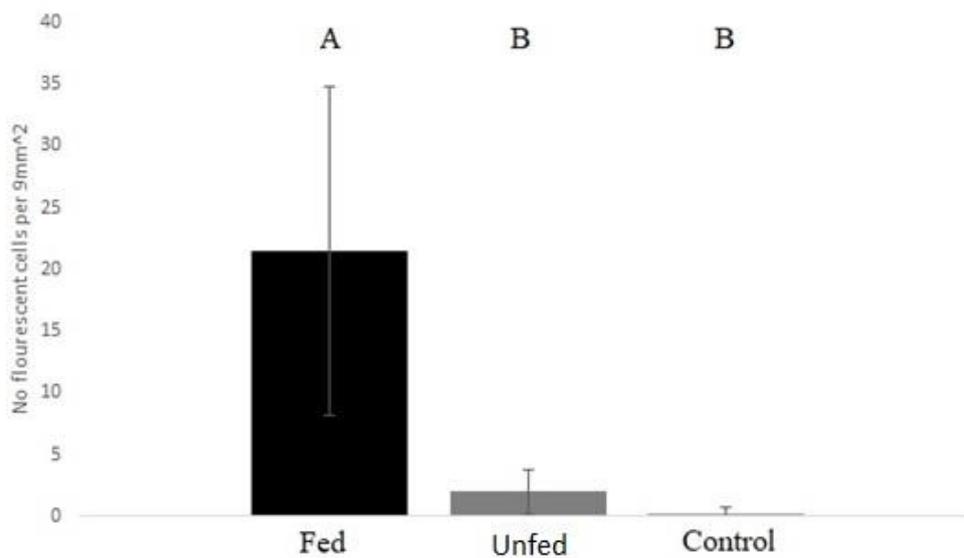


Figure 10. Mean number of Cm-DiI labelled cells in the water column after 24h for each treatment. Means with different letters are significantly different (Tukey, $P < 0.05$). Error bars: standard deviation.

Discussion

The aquiferous system of *A. queenslandica* is a dynamic system where the choanocytes readily leave the chamber to either join a pre-existing chamber, start a new chamber or differentiate into other cell types (Sogabe et al., 2016; Funayama et al., 2010). The results of this study suggest that the choanocytes are more content in their chambers and less prone to leave when they are fed *P. damselae*. When the juvenile sponge is kept in FSW however, the aquiferous system is in a constant state of remodeling. The question that arises from this highly mobile system is then, why do the choanocytes move? One proposal could be that the cells are lacking food which initiates a mobile behavior. However, the choanocyte will always be in contact with water, which in a natural environment has no reason to not contain food. However, it is demonstrated here that the unfed sponges contained significantly more Cm-DiI labelled cells in the skirt at 24h compared to the Fed treatment. This suggests that when a juvenile sponge does not receive food, the aquiferous system expands towards the edges of the animal. At the edges, it is possible that the surface of the animal provides the choanocytes with a higher rate of water flow, thus increasing the potential for food availability. Alternatively, the sponge itself might recognize the poor settling location, and chooses to move the entire animal, something that has been observed previously (Bond and Harris, 1988).

While there was no significant difference in the percent Cm-DiI labelled choanocytes per choanocyte chamber at 24h between the two treatments, the trend is that there are more Cm-DiI labelled choanocytes in the Fed treatment. This, I interpret as choanocytes that have been located in the same choanocyte chamber since the staining at 0h. However, there were chambers in the Non-fed treatment that had a high percentage of Cm-DiI labelled choanocytes in the chambers as well. This could be choanocytes that have stayed in the same chamber over 24h, or they are choanocytes that have come from different starting chambers, which to some extent is supported by the results in fig 2, 3 and 4, where it is clear that there is a lot of movement of these choanocytes when the sponge is unfed.

Furthermore, the chambers in the Fed treatment were on average around 20% bigger in diameter compared to the Unfed juveniles. A previous study by Sogabe et al. (2016) showed that choanocyte chambers can grow in size as it gets older. This suggests that the majority of the choanocyte chambers in the Fed treatment are the same chambers that were labelled with Cm-DiI at 0h, while the Unfed individuals most likely consists of newly formed chambers. The reason why the chambers in the Fed treatment did not contain a significantly higher percentage of Cm-DiI labelled choanocytes could be explained by new unlabeled choanocytes joined and expanded the pre-existing chamber. In addition, the increased cell shedding by the juveniles fed *P. damselae* suggests that once the sponge is able to extract food from the water column, the choanocytes are working as intended, which results in a natural turnover of cells with possible cell death and subsequent shedding. This theory would support the idea that sponges are important nutrient recyclers in the ecosystem (De Goeij et al., 2009).

The results indicate that if the choanocytes are processing food, they are less likely to leave their chamber. However, we do not know whether the same effect would have happened if the sponges were fed a non-organic substance. By labelling the bacteria with a fluorescent dye and adding a treatment fed with a fluorescent inorganic substance, such as plastic microbeads – one could investigate if there is a biological interaction between the choanocytes and the

bacteria, or if the choanocytes simply responds to the physical stimuli from an object. Recent studies suggest that the choanocytes of *A. queenslandica* have the ability to differentiate between symbiotic bacteria and “food” bacteria (Wehrl et al., 2007). Understanding how the mobile behavior of the choanocytes is affected by different types of bacteria and non-organic substances would be an apparent next step within this research.

It should be noted that this work was conducted on juvenile sponges, which naturally are in a development and growth phase. Thus, a certain amount of movement and restructuring might be expected. Therefore, in future studies it would additionally be interesting to follow the differences in development of the aquiferous system over a longer period of time, while also examining the movement of the choanocytes at a shorter interval. Furthermore, using a perfusion chamber to control the direction at which the *P. damselae* is supplied could potentially provide answers in how choanocytes choose their location in the aquiferous system. A previous study has already demonstrated that water flow and pressure can affect the structure of the sponge (Bond, 1992). The results from this study would suggest that we would find older and bigger choanocyte chambers closest to the food source.

One of the more interesting aspects of the choanocyte biology is their stem cell capabilities. Choanocytes have the ability to dedifferentiate into archeocytes, which is pluripotent cell type, with the ability to differentiate into new choanocytes or other cell types (Sogabe et al., 2016; Funayama et al., 2010). During my research, I observed plenty of Cm-DiI labelled archeocytes within the sponges of both treatments. These were archeocytes that most likely started out as choanocytes, since they were labelled with Cm-DiI. Unfortunately, I was not able to quantify the amount of Cm-DiI labelled archeocytes within the sponges. To examine this process, one needs to image more and larger areas of the sponge with a confocal microscope. If we could understand why some choanocytes differentiate into archeocytes in the presence and absence of food, it would provide further answers into choanocyte biology and their role in the sponge.

Finally, choanocytes are functionally and morphologically very similar to the free swimming unicellular group of choanoflagellates (Clark, 1866; Mehl and Reiswig, 1991; Maldonado, 2004). This has led to the theory that the last common ancestor to all metazoan were a colony forming organism not too different from a choanocyte chamber (Cavalier-Smith, 2017). Here, I have showed that food availability significantly alters the behavior of the choanocytes of *A. queenslandica*. When unfed, the choanocytes are more likely to leave their chamber to either join a pre-existing chamber or form an entirely new chamber. This suggests some amount of self-government from the choanocytes. In order to further bridge the knowledge gap about the evolution of multicellularity, I suggest a transcriptomic approach, comparing and contrasting gene regulation in choanocytes and choanoflagellates in the presence and absence of food.

Conclusion

In this comprehensive study focusing on the biology of choanocytes and the choanocyte chambers of juvenile *A. queenslandica* I have found, by the use of fluorescent cell trackers, that the aquiferous system of the sponge is a highly dynamic system. If the juveniles experience favorable conditions i.e. in the presence of food, individual choanocytes become less mobile, the aquiferous system is established and expanded. When the sponge is unfed however, the aquiferous system is in a state of constant remodeling, where choanocytes

migrate towards the edge of the body, most likely in search of more favorable feeding conditions. When the aquiferous system is functioning and filtering food, as in the feeding treatment, the chambers increase in size and there is a turnover of choanocytes, which are subsequently being shed into the water column. This system recycles valuable nutrients back into the ecosystem, which likely is a key component in many ecological communities such as coral reefs. Studying aspects of sponge biology further would provide more information about the importance of sponges on oligotrophic reefs around the world, from which suitable conservation methods could be developed. Going forward, I suggest a transcriptome analysis of the choanocytes, comparing and contrasting gene regulation in the presence and absence of food to help answer the question of how the intriguing and interesting choanocytes function.

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