Environmental cues inducing colony growth and medusa production in the invasive freshwater hydrozoan *Craspedacusta sowerbii*

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Introduction

The invasive freshwater hydrozoan *Craspedacusta sowerbii*, originating from the Yangtze River in China, has been recorded as an invasive species on every continent except Antarctica. In North America, *C. sowerbii* has been documented in 44 states and poses a potential threat to local ecosystems. Because of its invasive nature and little scholarly work, *C. sowerbii* has been the focus of our research for this past academic year.

The life cycle of *Craspedacusta sowerbii* consists of an asexual portion and a sexual portion (Figure 1); both portions start with sessile colonies of polyps, the most predominant form of *C. sowerbii*. In the asexual portion, polyp colonies bud rod-like structures known as frustules, which then transform into a new polyp colony. The sexual portion, on the other hand, involves polyp colonies budding motile medusae, which mature and produce gonads; much like other cnidaria, these gonads combine to form a planula larva, which develops into a new polyp colony. Polyp colonies are also able to regress into a dormant stage called a podocyst; to our knowledge, little work has been done regarding the formation and regeneration of podocysts, but current beliefs follow that low temperatures (including seasonal environmental changes) are a predominant factor for podocyst formation.

Preliminary work on *Craspedacusta sowerbii* growth and medusa production under certain environmental conditions has been performed - some preliminary results include that under higher temperatures, polyps tend to experience higher growth rates (Marchessaux et al., 2022), and medusae tend to appear at higher temperatures as well (McClary 1959). However, these studies were limited in that Marchessaux et al. (2022) only tested two temperatures, and medusa appearance relates to more than just temperature based on our personal observations. One factor seeming to influence medusa production is feeding rates, and to our knowledge no literature exists on this subject. Additionally, most laboratory work focuses exclusively on a Japanese strain of *C. sowerbii* (Marchessaux et al., 2022; Marchessaux and Bejean, 2020), with almost no laboratory work done on North American genotypes.

As such, our research for the past academic year has been focused on: a) expanding the temperatures of growth for *Craspedacusta sowerbii* colonies; b) quantifying medusa production based on feeding rates; and c) continuing to culture North American genotypes of *C. sowerbii* under laboratory conditions.

Methods

a) Growth and Temperature

Six cultures of *Craspedacusta sowerbii* from Coal City (Illinois, USA) and twelve cultures from Kamo (Japan) were started with 10 frustules in either a glass bowl or a glass or plastic petri dish. The animals were grown in Hydra Medium (HM) (Folino-Rorem et al., 2016) and kept covered in respective temperature chambers for 14ºC, 26ºC, and 28ºC treatments or in room temperature for 22ºC treatment. The Japan strain had three replicates for every culture, all in plastic petri dishes, while the Coal City strain had one replicate for each temperature in glass

petri dishes and one replicate for 26ºC and 28ºC in glass bowls. Frustules were obtained from stock cultures grown in the laboratory.

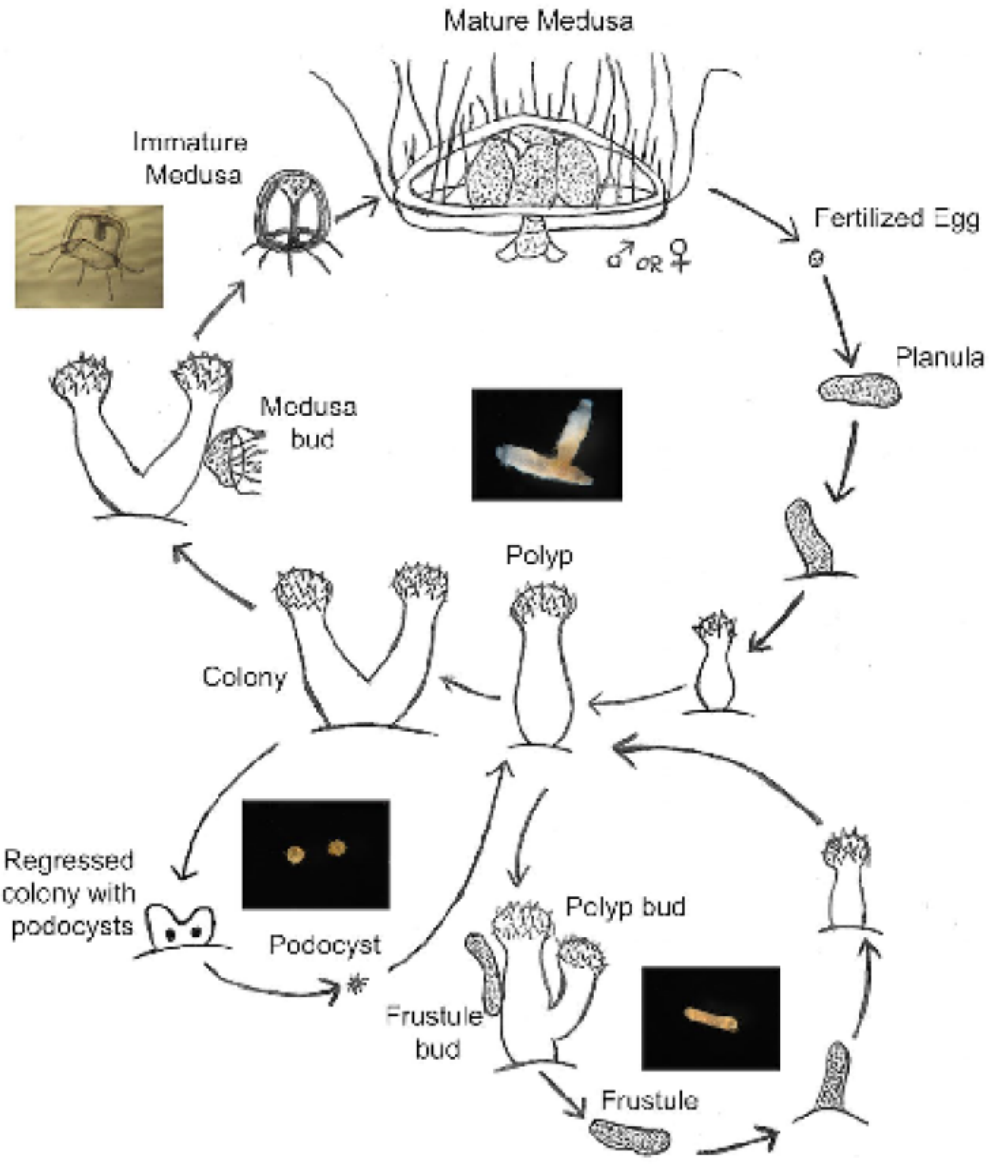

Figure 1: A visualization taken from Folino-Rorem et al. (2016) of the asexual, sexual, and dormancy stages of the life cycle of the freshwater hydrozoan *Craspedacusta sowerbii*.

Polyps were fed *Artemia salina* brine shrimp, rinsed three times in DI water and transferred to HM (Folino-Rorem et al., 2016, Marchessaux and Bejean 2020) approximately three times per week. Colonies from Coal City (CC) were fed directly using a P100 micropipette to deliver brine shrimp individually to polyps, and colonies from Japan were fed by flooding the petri dish with shrimp and changing water after approximately fifteen minutes.

Colony and frustule numbers were counted during feeding for CC and every day for Japan over the course of 3-4 weeks. Any medusae produced were removed with a manual pipette and were not counted, but were kept alive in a beaker filled with HM.

b) Medusa Production

Eight cultures of *Craspedacusta sowerbii* from Coal City (Illinois, USA) were started from 10 frustules each and grown in covered glass bowls kept at 26ºC. Four of these bowls were fed once a week, delivering a single *Artemia* brine shrimp to each colony with a micropipette, and the other four bowls were fed three times a week by the same method. Medusae were removed if possible and quantified; we quantified isolated medusae and medusae that were found (e.g., if a bowl had 4 medusae on a given day but one was attached to a colony and could not be removed, the isolated medusae were 3 and the found medusae were 4).

After two months of growth, the amount of colony growth in each bowl was quantified and used to standardize the number of medusae produced per colony.

c) Growth Methods

This portion of our research was more qualitative than the previous two. We examined the impact of substrate, filtration, and oxygenation on the growth of *Craspedacusta sowerbii* strains from Coal City, Lake Michigan, Barrington (all Illinois, USA) and Gatun Lake (Panama), the same strain used by Folino-Rorem et al. (2016) in first recording modern scientific literature on culturing *C. sowerbii* in a laboratory environment. We plan to perform quantitative experiments on growth under these conditions during the summer of 2023 based on the initial observations gathered over this year.

Firstly, we examined the difference of *Craspedacusta sowerbii* growth between a plastic substrate and a glass substrate. Typically, *C. sowerbii* has been grown in plastic vessels, but glass vessels seemed to have some better growth from some preliminary data. As such, we grew frustules from Coal City on glass slides and plastic slides: to do this, we seeded the slides with ten frustules each; once two or more had differentiated into polyps, we put the slides into slide boxes with the front and back walls cut out in a technique adapted from Folino-Rorem and Renken (2018) (Figure 2a). These boxes were then placed into a tank of HM with filtration by an AquaClear Small filter and aeration by air stone and bubbler. To feed, these boxes were transferred to interim containers of HM and flooded with brine shrimp before being returned to the original tank. Colonies were grown on these tanks for 2 months before being counted.

Secondly, based on the results of growing *Craspedacusta sowerbii* on slides in tanks with filtration, we put glass bowls containing *C. sowerbii* colonies from Coal City, Lake Michigan, Barrington, and Gatun Lake (Panama) into tanks with filtration and/or aeration. Filtration was done with the same AquaClear Small filters, while aeration was performed by attaching a long pipette tip to an air tube and securing the end of the tip to the bottom of the tank (Figure 2b). While growth was not quantified under these conditions, we have plans to do so over the summer in a similar manner to part a).

Finally, we found limited observations from Lytle (1961) and Dumont (1994) stating that submerged glass slides in the wild were used to collect *Craspedacusta sowerbii* polyps and frustules. As such, we submerged glass slides into the bowls mentioned above, and we also submerged slides and a myriad of other glass objects into samples collected from sites where

C. sowerbii medusae have been sighted, though for some the polyp has not been confirmed. Again, no quantitative data was collected, but we have plans to do so over the summer with actual field sampling. We also find evidence of plant material as a preferable substrate for *C. sowerbii* polyps and frustules (Amemiya, 1929; Gasith et al., 2011; Bushnell and Porter, 1967) and plan to perform additional sampling of plant material over the summer, as well as similar growth experiments to part a) with the presence of plants.

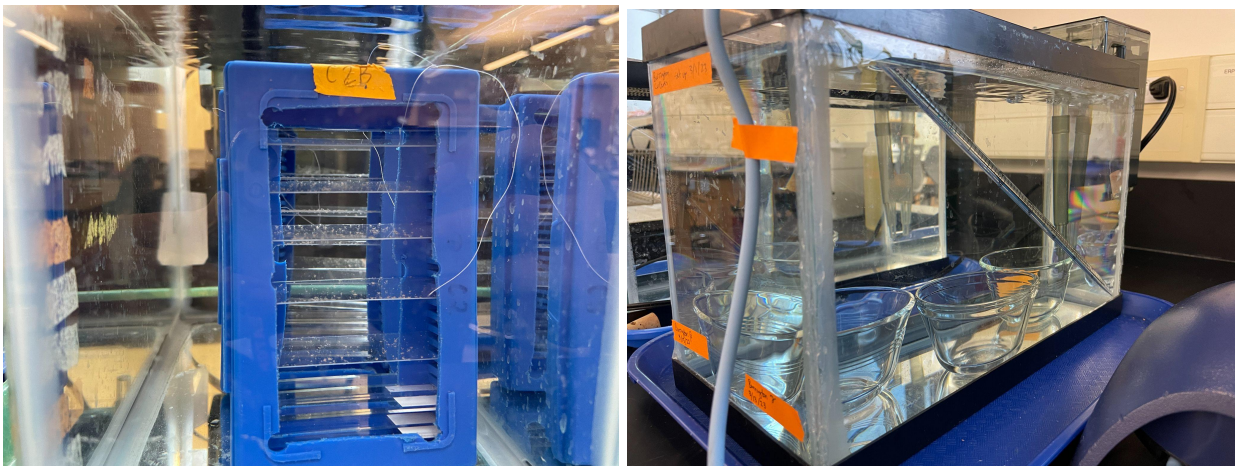

Figure 2: (a, left) Colonies of *Craspedacusta sowerbii* grown on plastic slides held in a slide box with windows cut in its front and back in a technique adapted from Folino-Rorem and Renken (2018). (b, right) Colonies of *C. sowerbii* grown in bowls in a 2.5 gallon (approx. 9.5L) tank with filtration and a long pipette tip for aeration.

Results

a) Growth and Temperature

All analyses and visualizations were conducted in RStudio using the packages ggplot2, dplyr, and janitor. For cultures from Japan, we averaged the number of polyps and frustules on each day of counting for each temperature before performing simple linear regression to model growth, with time (days) on the x-axis and number of polyps on the y-axis (Figure 3a-b) or frustules (Figure 3c-d). Generally, R^2 values consistently stayed within the 0.7-0.8 interval for regressions on both polyps and frustules, with the exception of polyps at 28C, which had an R^2 value of 0.33. This error is likely due to the fact that growth of polyps at 28C was not linear but better fit a polynomial curve.

Polyps grown at 26C saw by far the most proliferation, with an estimated growth rate (slope of the regression line) of 0.71 polyps/day. Proliferation then decreased for 22C, further for 28C, and 14C showed the least growth. However, frustule presence told a different story: for all temperatures, the number of frustules decreased over time, but 14C saw the least amount of decline, and 26C-28C saw equal amounts of high decline.

For CC cultures, since no replicates were used, we did not take averages and just performed simple linear regression, but we did so on colonies (not polyps) (Figure 4a-b) and frustules (Figure 4c-d). R^2 values stayed consistently high for polyps (around 0.7-0.9 interval)

but were much lower (some around 0) for frustules, indicating higher variability in the frustule counts over time.

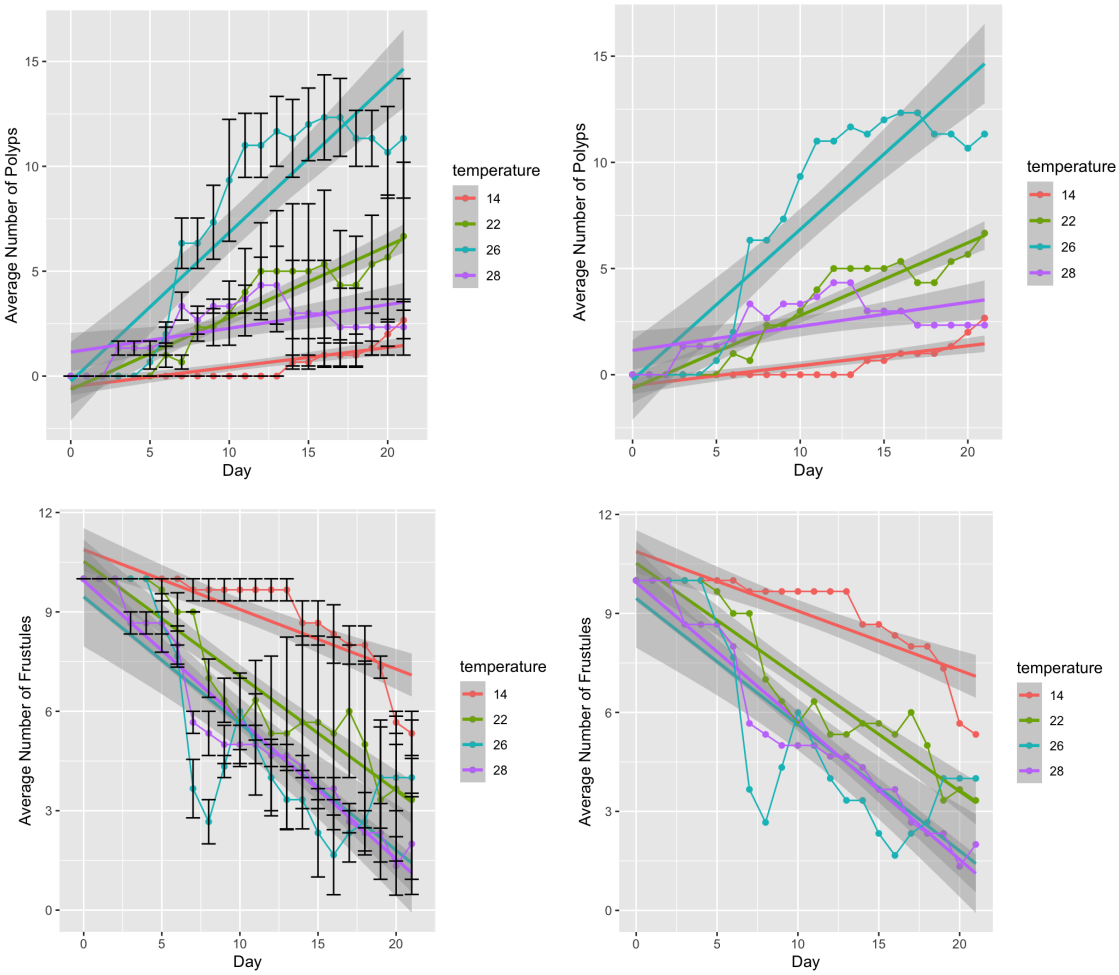

Figure 3: Linear regressions modeling growth of Japanese strains of *Craspedacusta sowerbii* polyps and frustules under controlled temperatures. Upper row: Linear regressions for the average number of polyps in a petri dish over time at four different temperatures (a) with error bars equaling 1 SEM, (b) without error bars. Bottom row: Linear regressions for the average number of frustules in a petri dish over time at four different temperatures (c) with error bars equaling 1 SEM, (d) without error bars.

Similar to the Japanese strain, the Coal City strain also showed the highest growth rate at 26C, at around 1.2 polyps/day. Proliferation was next highest in 28C, then 22C and lastly 14C, indicating that the Coal City strain is more tolerant of higher temperatures than the Japanese strain. Frustule numbers were also more inconsistent for Coal City, with none of their patterns being linear, unlike the Japanese strain that consistently decreased. However, any conclusions ought to be re-tested due to our lack of replicates.

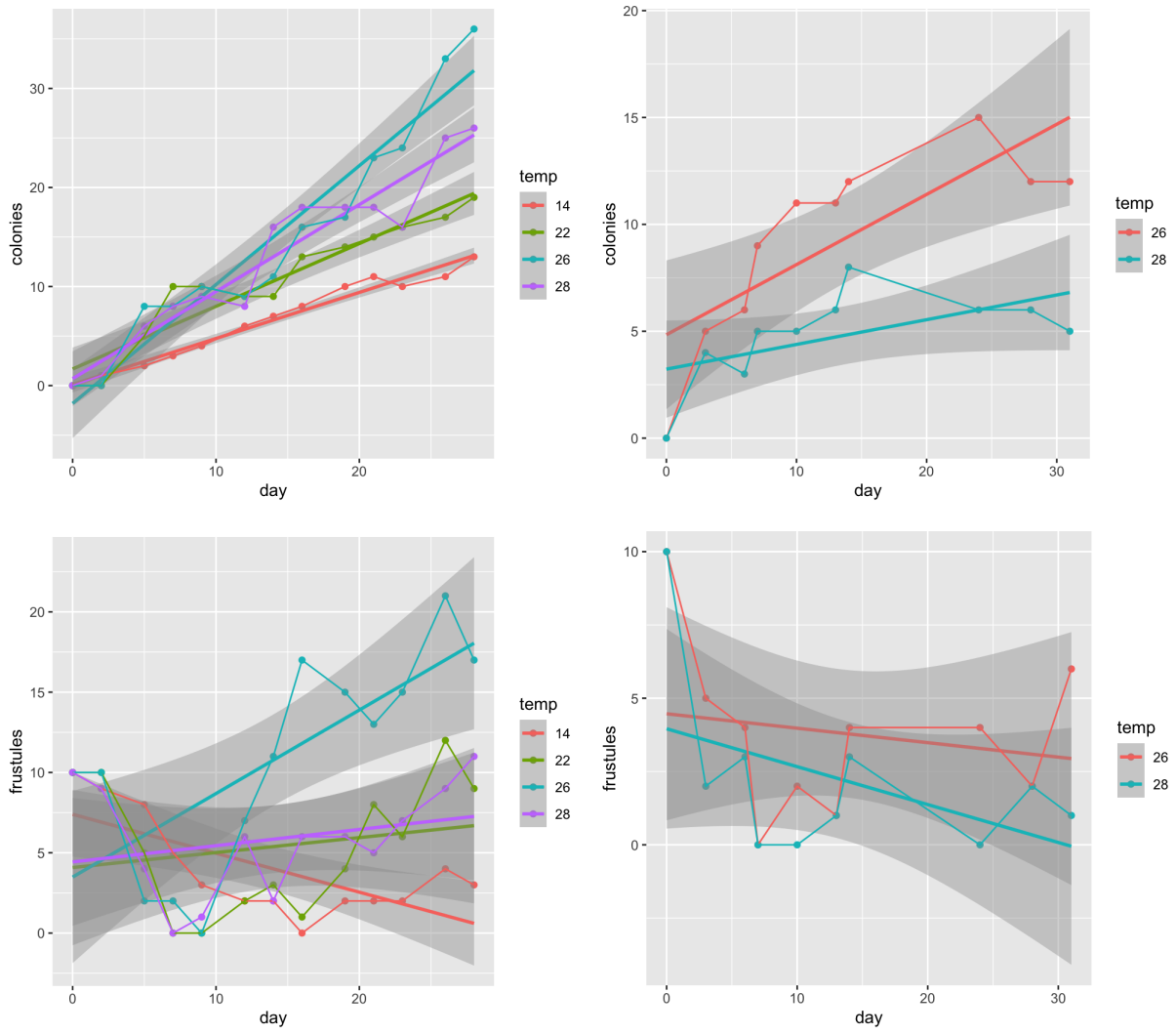

Figure 4: Linear regressions modeling growth of Coal City (Illinois, USA) strains of *Craspedacusta sowerbii* colonies and frustules under controlled temperatures. Upper row: Linear regressions for the average number of colonies in a petri dish over time at four different temperatures (a) grown in glass petri dishes (b) two different temperatures in glass bowls. Bottom row: Linear regressions for the average number of frustules in a petri dish over time at four different temperatures (c) grown in glass petri dishes, (d) two different temperatures in glass bowls.

b) Medusa Production

While at first glance, there seems to be a clear difference in medusae produced based on feeding rates with more medusae produced when fed three times per week, when standardizing for the number of colonies, we find that colonies fed only one time per week tended to produce more medusae per colony and that the higher amount of medusa production is related to the number of colonies (Table 1).

However, it should be noted that almost all of the medusae produced in the experimental (1x/week feeding) and in the control (3x/week feeding) groups were sourced from a single culture, preventing us from getting a meaningful t-test result ($p = 0.267$, $t = 1.2236$, $df = 6$). For the control group, 38 of the 44 found medusae came from a single bowl, and for the experimental group, 21 of the 25 were sourced from a single bowl. This would then be indicative of confounding factors influencing certain bowls differently from others.

Table 1: Summary of colony growth and medusa production for cultures of *Craspedacusta sowerbii* fed at differing rates.

Feeding Rate	**Total colonies**	**Average colonies/ bowl**	**Total medusae produced**	**Total medusae isolated**	**Medusae produced/ colony**	**Medusae isolated/ colony**
3x/week	1515	378.75	44	25	0.0290	0.0165
1x/week	165	41.25	25	16	0.1515	0.0970

c) Growth Methods

In general, we observed more colony and frustule growth on glass slides with more polyps per colony (Table 2), but due to the non-normal nature of the data we collected, we used the Mann-Whitney U-Test to quantify the exact difference between colony number, frustule number, and polyp per colony between samples on glass slides versus on plastic slides. We report no significant difference in the number of colonies per slide ($U = 84$, $p = 0.3734$) and no significant difference in the number of frustules per slide ($U = 97$, $p = 0.7414$). However, since polyps per colony is closer to a normal distribution, we used the t-test and again report no significant difference in the number of polyps per colony ($t = 0.948$, $p = 0.1759$, $df = 26$).

Table 2: Summary of colony growth for cultures of *Craspedacusta sowerbii* grown on glass slides versus plastic slides with filtration.

Slide Material	Colonies per slide	Frustules per slide	Polyps per colony
Glass	28.2857	24.7143	3.2374
Plastic	17.9333	19.0000	2.9331

For the additional genotypes we worked with (Lake Michigan, Barrington, and Panama), we found observationally that culturing colonies of these genotypes without aeration or filtration did not promote colony growth and frustule production; this was especially true of the Lake Michigan genotype, as it would disintegrate if kept in still water. However, when providing just filtration, we observed increased colony growth and frustule production in Lake Michigan and Panama genotypes, but not Barrington; Barrington only experienced significant colony growth and frustule production with the help of aeration.

Finally, preliminary observations of putting slides into existing cultures of Coal City *Craspedacusta sowerbii* colonies showed that frustules migrate onto the slides and morph into polyps. Such observations have not been seen with any other genotype we have worked with, but knowing that we have observed frustules crawling to different areas of a culture vessel, we hypothesize that it is still possible for frustules of other genotypes to propagate on slides.

Discussion

Our results provide slightly more nuance to the results obtained by Marchessaux et al. (2022), who showed that higher colony growth levels were achieved with a temperature of 29C. For both the Japanese strain (used by Marchessaux et al. (2022)) and the Coal City strain, we found 26C to be the optimal temperature for colony growth. This would indicate that temperature and growth rates are not directly correlated; rather, there is an optimum, but more genotypes would need to be tested to confirm that 26C is optimal for all strains of *Craspedacusta sowerbii.*

On this subject, though, while the optimal temperature was shared between Coal City and Japanese strains, we also noticed that Coal City had a higher growth rate at 28C than Japan did, and vice versa for 22C. This indicates to us that Coal City is a strain that can tolerate a wider range of temperatures and thus one that has a higher capacity to propagate in foreign environments. Our personal experience also validates this, suggesting that Coal City is a very hardy genotype that does not require any filtration or flow to experience massive levels of proliferation, and it can also survive for a very long time without being fed.

We also noted from the growth studies that the Japanese strain of frustules decreased over time, while the Coal City strain fluctuated. While the decreases are obviously explained in frustules morphing into polyps, it does not explain how more colonies will produce more frustules with time. Instead, I draw attention to the differing culture methods - the Japanese strain had frequent water changes, while the Coal City strain had none. Based on personal observations of frustules floating in culture water, it is likely that the decrease in frustules is based also on many being lost during water changes.

Our results also provide preliminary quantitative evidence for a feeding rate influence on medusa production. While this has been verified from personal experience and the experiences of others (Kent Winata and Dr. Paulyn Cartwright, personal communication), these results provide a numerical framework for this claim. However, there are some notable difficulties with the study that ought to be resolved in future experiments. Firstly, the number of colonies was counted at the end, not every day, while medusae were quantified every day. We attempted to resolve this by standardizing based on colony number, but this is not particularly accurate seeing as the medusae were produced over the entire experimental period. To alleviate this, we propose having an identical constant number of colonies in each replicate and removing any additional frustules or colonies, so that medusa production will only be based off a certain number in the beginning. Additionally, we do not know which colonies budded medusae; while we observed some budding, it was not enough to explain the numbers we recorded. Additional insight onto which colonies tend to bud more medusae will provide better insight into how the medusae appear in the wild, but this would require near constant observation of the colonies as we have observed medusae appearing in one day with no sign of budding the previous day. Finally, almost all of the medusae came from two replicates. Unfortunately, there is no way to alleviate this; some cultures are simply better at producing medusae than others. A genetic

analysis into medusa production would be labor-intensive, but very insightful into the medusa blooms in the wild.

Our final experiments on growth can be improved in that they simply need to be made more formalized. However, we have plans to do this over the summer and see whether glass or plastic is better for sampling *Craspedacusta sowerbii* colonies from the field. Nevertheless, insight into substrate affinity for *C. sowerbii* colonies would provide some better insight into growing them under laboratory conditions. While Marchessaux and Bejean (2020) provide a schematic for a dedicated system to grow colonies and medusae, we unfortunately lack the funds and materials to create such a system and are hoping that our explorations and observations can facilitate a simplified, inexpensive system for growing all stages of the *C. sowerbii* life cycle.

While our insights into the *Craspedacusta sowerbii* life cycle may not be enough to eradicate the species from its non-native environments, we hope that these results will provide understanding into how and why the medusae bloom as well as how the colonies can spread.

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