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Investigating the Impacts of Different Diets on Adult *Bolinopsis microptera* Fecundity and Spawning

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ABSTRACT

Bolinopsis microptera is a lobate ctenophore that is cultured at the Monterey Bay Aquarium (MBA) for display in the novel Into the Deep exhibit. It is also incorporated into the diets of deep-sea jellies featured in the exhibit. The purpose of this exploratory experiment investigated the impacts of three different diets on the fecundity of the adult *B. microptera*. Over a 14-day period, three *B. microptera* were fed different diets (larval *Menidia beryllina* less than 30 days old, adult mysids *Americamysis bahia*, and copepods *Parvocalanus crassirostris* and *Acartia tonsa*) to observe how these diets affected egg production per day and throughout the entire experimental period. The key findings from the single-trial experiment over 14 days showed that the larval fish-fed *B. microptera* displayed the most frequent and substantial egg production, particularly in the AM, with periodic mass spawning events throughout the trial period. The copepod-fed *B. microptera* had the second-highest egg production, contrary to initial expectations, while the mysid-fed *B. microptera* yielded the lowest egg production and showed major inconsistencies with several 0 AM/PM counts. The copepod diet demonstrated moderate spawning consistency with only one day of zero AM/PM egg counts. Both the larval fish-fed and copepod-fed groups exhibited higher egg counts in the AM, with fewer zero AM/PM counts compared to the mysid-fed group. The findings from this exploratory experiment can help to inform animal husbandry practices regarding the spawning of *B. microptera* and improve efficiency in egg production by feeding sexually mature adults with larval *M*and/or maintaining an adequate density of adult *P. crassirostris* and *A. tonsa* copepods to sustain them for at least two weeks to initiate and continue intermittent spawning.

INTRODUCTION

The phylum Ctenophora comprises marine gelatinous zooplankton invertebrates, most notable for containing the largest animals known to locomote using cilia, which gives them the common name “comb jellies” (Tamm, 2014). Ctenophores are found throughout the ocean, from the surface to depths of over 10,000 m, and inhabit environments with temperatures ranging from -2 to 30 ℃ (Lindsay & Miyake, 2007). The deepest recorded ctenophore was an undescribed species in the order Platyctenida, discovered at a depth of 10,040 meters in the Kermadec Trench of the Southwest Pacific Ocean in 2022. Ctenophores play a crucial role in influencing the population dynamics of their prey, by preying on small planktonic organisms (Swanberg & Båmstedt, 1991). These comb jellies are bi-radially symmetrical and are distinguished by their eight comb rows, composed of fused cilia that beat synchronously to propel them through the sea (Harbison, 2001).

The phylum Ctenophora includes two classes: Tentaculata, characterized by the presence of tentacles, and Nuda, characterized by their absence. Tentaculate ctenophores have tentacles lined with colloblasts that release sticky filaments to immobilize prey, unlike true jellies that use stinging nematocysts (Nagabushanam, 1959). The Tentaculata class further includes three orders: Beroida, Lobata, and Cestida (Harbison, 1985). Members of the order Lobata typically have laterally compressed, oval-shaped bodies and two oral lobes that extend beyond the mouth, which they use to ensnare prey with sticky mucus lining the inner membrane of the lobes (Matsumoto & Harbison, 1993). Many lobate ctenophores are also capable of producing bioluminescence under their comb rows to communicate and deter predators (Haddock & Case, 1999).

One species within the genus *Bolinopsis* includes the *Bolinopsis microptera* (Agassiz, 1865). This species was once considered synonymous with *Bolinopsis infundibulum*, which appears identical in appearance. However, Shannon Johnson, along with colleagues from MBARI, the MBA, and Friday Harbor Laboratories, reversed this classification using genetic sequencing, identifying morphological differences and confirming it as a separate species (Johnson *et al.*, 2022). According to MBARI's VARS system, *B. microptera* has been observed at depths ranging from 25 to 2,000 m (VARS).

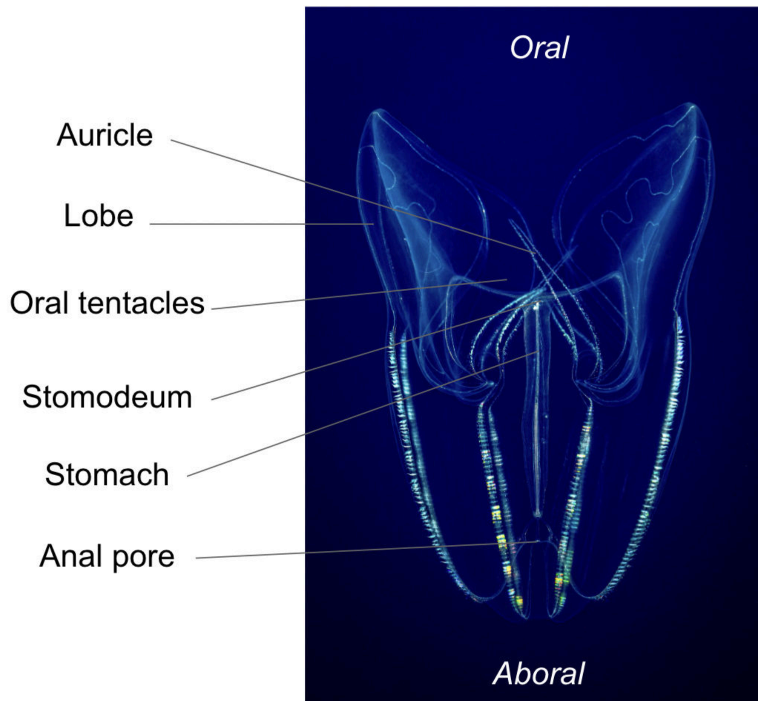

Figure 1: B. microptera basic digestive anatomy. Ciliated auricles create water currents to startle prey into sticky colloblasts on oral tentacles and lobes. Food is then moved through the stomodeum into the stomach and is then transferred and absorbed through the gastrovascular cavity, and waste is expelled through the anal pore at the aboral end (photo by Monterey Bay Aquarium).

Adult *B. microptera* have a polar diameter of approximately 50.8 mm and exhibit biradial symmetry. They are elongated in shape, with short lateral oral lobes and intricately coiled, long ambulacral tubes that facilitate food transport, digestion, and nutrient distribution (Agassiz, 1865). *B. micropterus* feed on ichthyoplankton prey along with small crustaceans that are able to fit within their feeding lobes (Bishop, 1968). They forage vertically with their oral lobes facing towards the surface (Figure 1) (Matsumoto & Harbison, 1993). According to MBARI's Video Annotation and Reference System, they have been sighted on average from 25 to 2,000 meters deep. They have been observed to consume smaller prey (200 to 3,200 µm) at a high rate (Bishop, 1968).

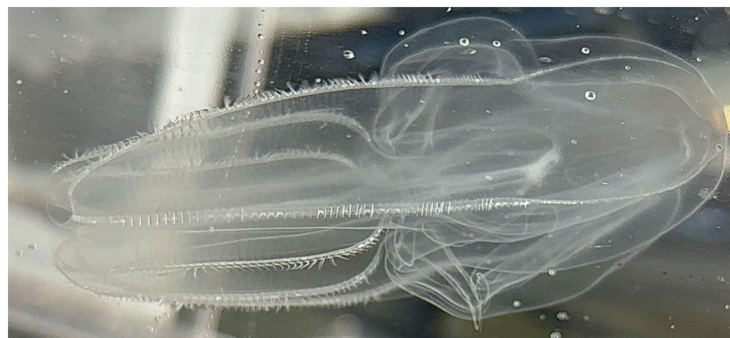

Figure 2: Lateral view of B. microptera before spawning (oral end on right side). The forming crescent-shaped white masses along the testis side of the meridional canal can be seen underneath the ctene rows.

Like many other ctenophores, the *B. micropterus* are self-fertile hermaphrodites and likely spawn seasonally (El-Bawab, 2020). Lobed ctenophores are known for their high fecundity; a single *B. infundibulum* with a gut length of 32 mm was observed to produce over 1,000 larvae in just three days (Schulze-Röbbecke, 1984). Along each of the ctene rows, the egg and sperm are stored along the meridional canals in opaque bands along the walls behind the eight ctene rows (Strathmann, 1987). Spawning in *B. microptera* can be initiated by exposing the animals to a dark period of six to ten hours, followed by light exposure, during which they have been observed to visibly prepare for spawning by forming crescent-shaped white masses along the testis side of the meridional canal (Figure 2) (Dunlap, 1966). Crowding individuals into a small, controlled space can also induce spawning, particularly when combined with dark periods followed by light exposure and a gradual increase in temperature (Mills & Strathmann, 1987). Spawning typically begins between 1-1.5 hours once exposed to increased temperature, and changes in light which can contribute to increasing stress to initiate spawning (Dunlap, 1966). Spermiation in *B. microptera* occurs 1.5 to 2 hours after light exposure, with sperm exiting the body through two pores on either side of the interctene muscle band (Dunlap, 1966). Within five minutes of spermiation, the eggs, approximately 300 µm in diameter and covered by a thick gelatinous coating, are released (Dunlap, 1966; Kamshilov, 1960). This process occurs simultaneously across the entire animal during spawning (Dunlap, 1966). After spawning, the cydippid larvae typically hatch a little over a day later (Baker & Reeve, 1974). Larval hatchlings look similar in morphology to animals of the Cydippida order as they have two tentacles to feed with, paired ctene rows, an apical sensory organ, and a simple four lobed gastrovascular system with a stomodeal pharynx (Schulze-Röbbecke, 1984).

At the MBA, *B. microptera* were first cultured and displayed in the Drifters exhibit. They are now kept as backup exhibit animals and are cultured primarily to feed other display jellies at higher trophic levels. With the opening of the novel Into the Deep exhibit, the team noticed that feeding chopped ctenophores to deep-sea animals such as *Beroe*, *Pandea rubra*, *Modeeria rotunda*, Red X, and *Lampocteis* visibly improved their health, making them appear more plump compared to their slightly shriveled appearance when being primarily fed copepods. As a result, the demand for *B. microptera* culturing has increased, leading to a challenge in maintaining a constant supply. There is a delicate balance between achieving high spawning quantities while managing high larval mortalities post-hatching, as the larvae are delicate and vulnerable between hatching and developing their feeding lobes.

Food quality plays a significant role in culturing these animals, as it can impact the fecundity of lobed ctenophores. However, the effects of different foods on ctenophore fecundity has not been well studied in a controlled laboratory setting. To address this, an exploratory experiment was conducted over 14 days, during which *B. microptera* individuals were fed three different diets consisting of larval fish, mysids, and copepods to investigate the effects of diet on adult fecundity. It was hypothesized that the *B. microptera* fed larval fish would have the highest fecundity, followed by mysids, then copepods.

MATERIALS AND METHODS:

COLLECTION

Figure 3: ROV Ventana *held above the ocean surface to be inserted or removed from the water. The ROV's 2,300 meter umbilical cable allows the ROV to be controlled remotely from the ship control room. Real time video is placed on screens in the control room in order to ensure accuracy and precision of animal collection (photo by MBARI).*

The MBA collected the original F1 generation using a beaker attached to a long pole at the water's surface, where they are occasionally found. This generation was then spawned at the aquarium to provide multiple subsequent generations and remains ongoing. The lab *B. micropterus* generations are occasionally enriched with wild caught every two years in order to prevent mortalities and quality issues from inbreeding. When restocking *B. micropterus*, which are occasionally collected during MBARI midwater cruises, ROVs like *Ventana* are equipped with low-impact detritus samplers. These samplers allow for the collection of delicate gelatinous animals, including ctenophores, from the midwater column (Figure 3). Their midwater toolsled has a suction sampler and 12 different containers that can be rotated upon use. Upon selection, each container is able to close the top and bottom simultaneously; the ROV must position itself accurately with its thrusters towards the animal inside the container, and can then close both ends to encapsulate the animal (MBARI). However, due to the higher demand for restocking other deep-sea jellies in the MBA's Into the Deep exhibit, whose life cycles are not yet fully understood in captivity, *B. micropterus* are typically a lower priority for collection and are instead spawned in a lab setting as often as possible.

WATER SYSTEM

For any tanks at the aquarium that house Ctenophora and Cnidaria—whether in the Drifters Lab, Into the Deep exhibit, Plankton Lab, Tiny Drifters exhibit, or the Drifters exhibit—the water is supplied through the "JSW" (Jelly Sea Water) system. The seawater, drawn from the ocean, is passed through sand filters, UV sterilized, and run through a heat exchanger to maintain a constant temperature of 12-13°C for the cold-water tanks. Salinity from the nearby ocean remains around 34 to 35 PSU. When the water exits these tanks, it is part of an "exotic system" that undergoes additional filtration and UV treatment to prevent non-native species from being introduced into the Monterey Bay. This experiment took place on a cold-water system wet table in the Drifters Lab that usually runs at 12 ℃.

CULTURING

Gelatinous zooplankton are transparent, fragile planktonic animals with mesoglea-like internal tissues that help regulate their buoyancy (Raskoff *et al.*, 2003). They can be fed a diverse range of prey items, including *Artemia salina* nauplii, krill, chopped squid, medusae, wild plankton, rotifers, trochophore larvae, agar-based foods, algae, bivalve hepatopancreas, and "grow-lights" for zooxanthellae (Raskoff *et al.*, 2003). At the MBA, *A. salina* nauplii, Rotifera, and copepods are cultured and harvested multiple times a week to feed gelatinous animals. In 1996, the MBA established a permanent jelly gallery in the Outer Bay Wing, featuring the Drifters Gallery, which highlights gelatinous zooplankton species native to Monterey Bay and the California coast (Knowles, 2015). The MBA has also contributed to scientific knowledge by describing the life cycles of various species and discovering new ones in the Monterey Submarine Canyon, culminating in the creation of the Into the Deep exhibit in April 2022. Additionally, the MBA has collaborated with institutions across the United States and globally to advance the understanding of animal husbandry for these delicate organisms.

The MBA continuously cultures various life stages of the majority of jellies on exhibit to ensure a consistent supply for display. Tanks used to hold, raise, and grow species of ctenophores, scyphozoans, and hydrozoans include, but are not limited to, kreisels, pseudokreisels (PKs), diffusion tubes, and rectangular tanks connected to outflow tanks. The kreisel and PK tanks originated from the first design of the “planktonkreisel”, a tank designed by biologist Wolf Greve in 1968 that addressed the challenges of housing delicate gelatinous planktonic marine animals that can easily be damaged by friction against walls, strong jet streams, and air bubbles. This tank design allowed culturing for planktonic animals using a rounded glass vessel, bottom sand filter, and center column that allowed water movement to enter the tank and circulate in a smooth directory flow (Greve, 1968). In 1990, Dr. Bill Hamner adapted this design into a more circular cylindrical design to create a “carousel” of water to minimize contact with the walls of the tank for planktonic animals (Hamner, 1990). This new design enabled the MBA to further adapt Hamner’s design to display jellies, receiving recognition for their innovative husbandry practices

and captivating the attention of millions of visitors (MBARI). The circular shape and gentle water flow keep jellies suspended without harming them, simulating their natural environment.

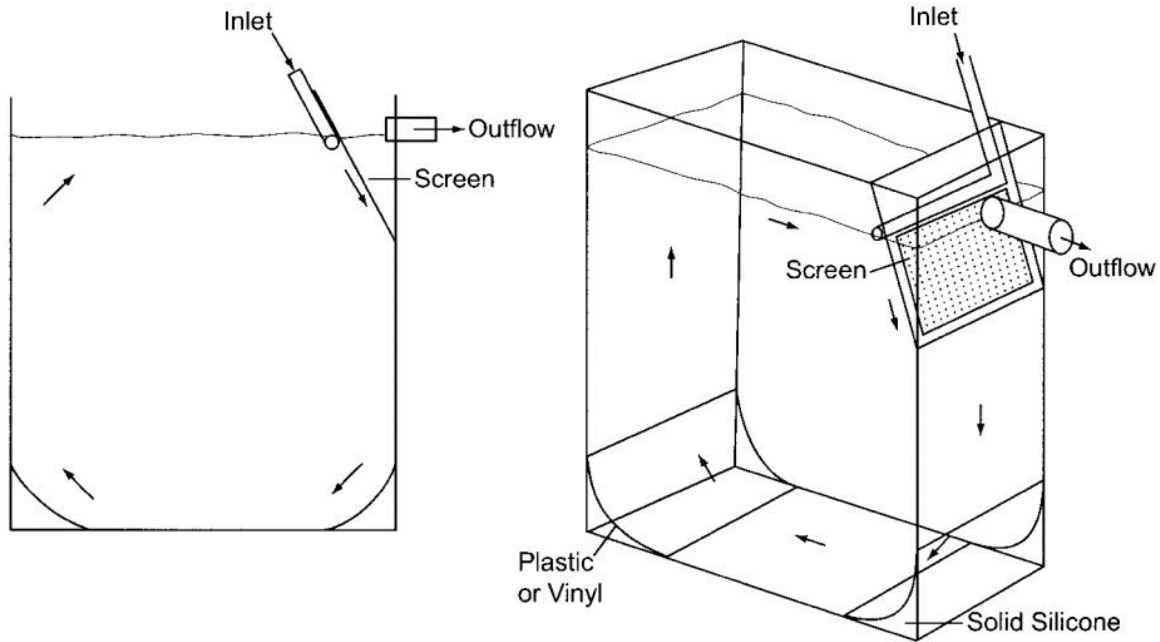

Figure 4: PK design created from the standard tank. The bottom corners are rounded with acrylic and outflow is separated from the tank through the inlet spray bar (Figure from Raskoff et al., 2003, used with permission).

Features in the PK such as a spray bar and central outflow drain behind a fine mesh frame help maintain water levels and cleanliness, while the smooth acrylic glass interior limits abrasive damage to the jellies upon contact, providing a safe, controlled environment for fragile marine life (Raskoff *et al*., 2003). The PK, builds on the kreisel design with enhancements for better efficiency and maintenance all while at a smaller scale (Figure 4). PKs offers a more affordable and simpler tank design compared to a kreisel, making it easier to set up high quantities in behind-the-scenes rooms and exhibits. However, kreisels are still preferred for raising many jelly species.

STANDARD CULTURING PROTOCOL FOR *B. MICROPTERA*

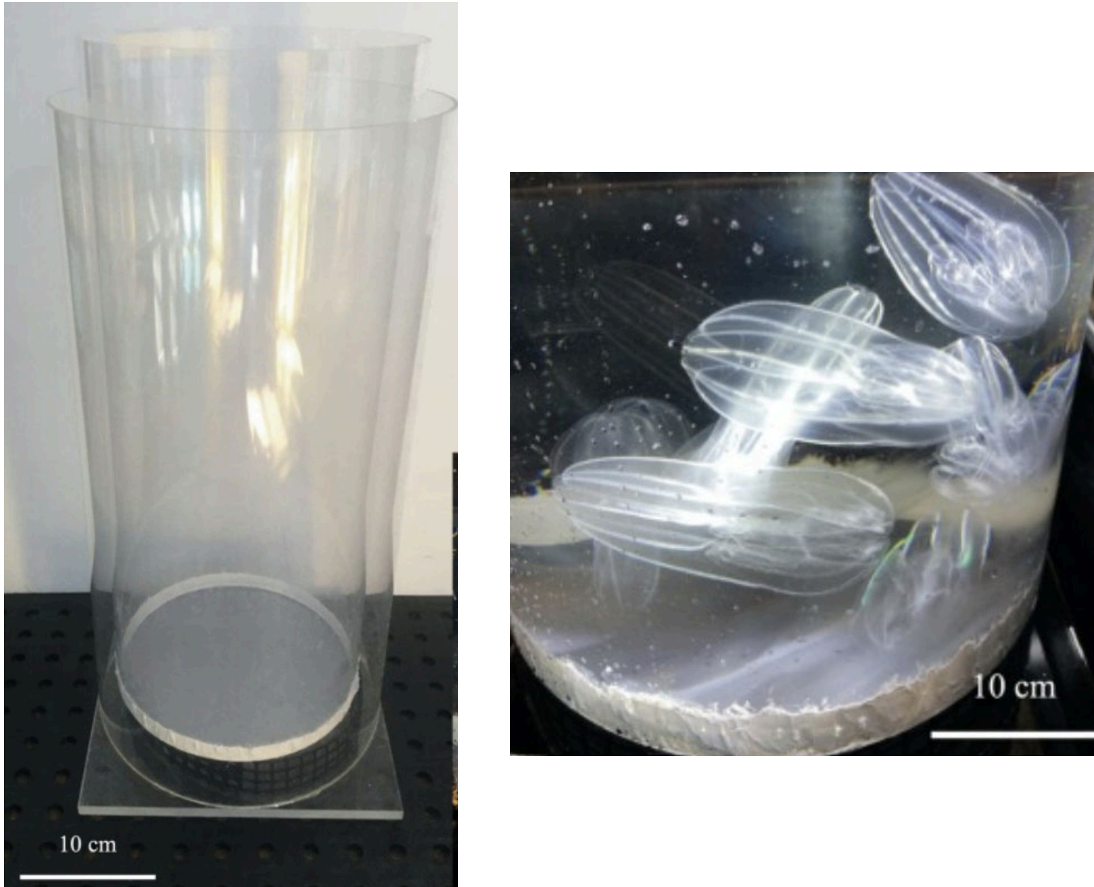

Figure 5: Diffusion tank used for spawning B. microptera at the Monterey Bay Aquarium (Patry, Bubel, Hansen, & Knowles, 2020)

The MBA found the most success with using diffusion tubes for spawning ctenophores (Figure 5). These tubes are designed to provide a stable, controlled environment for spawning jellies, with passive circulation through a bottom mesh to maintain gentle water flow. This setup allows the eggs to develop and hatch in a stable environment. Increasing size must be accommodated, as the newly hatched cydippid larvae exhibit an average instantaneous growth rate of 0.240 d^{-1} over a period of 4 weeks (Båmstedt & Martinussen, 2015). For the *B. microptera*, newly spawned individuals remain in the tube until they reach at least 10 mm, after which they are transferred to a PK (Patry *et al.*, 2020). Once the juvenile *B. microptera* reach adult size, they are moved to a small circular kreisel or PK (Raskoff *et al.*, 2003).

The feeding regimen for *B. microptera* varies depending on the size and life stage of the individuals in the cohort. Juvenile *B. microptera* cydippid larvae are typically fed live juvenile *P. crassirostris* nauplii and occasionally *Rotifera*. As they develop their oral lobes, they are fed adult *P. crassirostris* copepods, *A. tonsa* copepods, adult mysids (*A. bahia)*, and less than 30 day old inland silverside larval fish (*M. beryllina)* in preparation for spawning (Aquatic Indicators Inc.).

Drawing on the initial success of feeding larval fish to increase the spawn quantity of the sea walnut (*Mnemiopsis leidyi*), which led to its successful full-cycle culturing at the Monterey Bay Aquarium, similar techniques have been applied to *Leucothea pulchra* and *Bolinopsis microptera* with proven success. However, these techniques had not been observed in an experimental setting for *B. microptera* until now.

PSEUDOKREISEL CONSTRUCTION

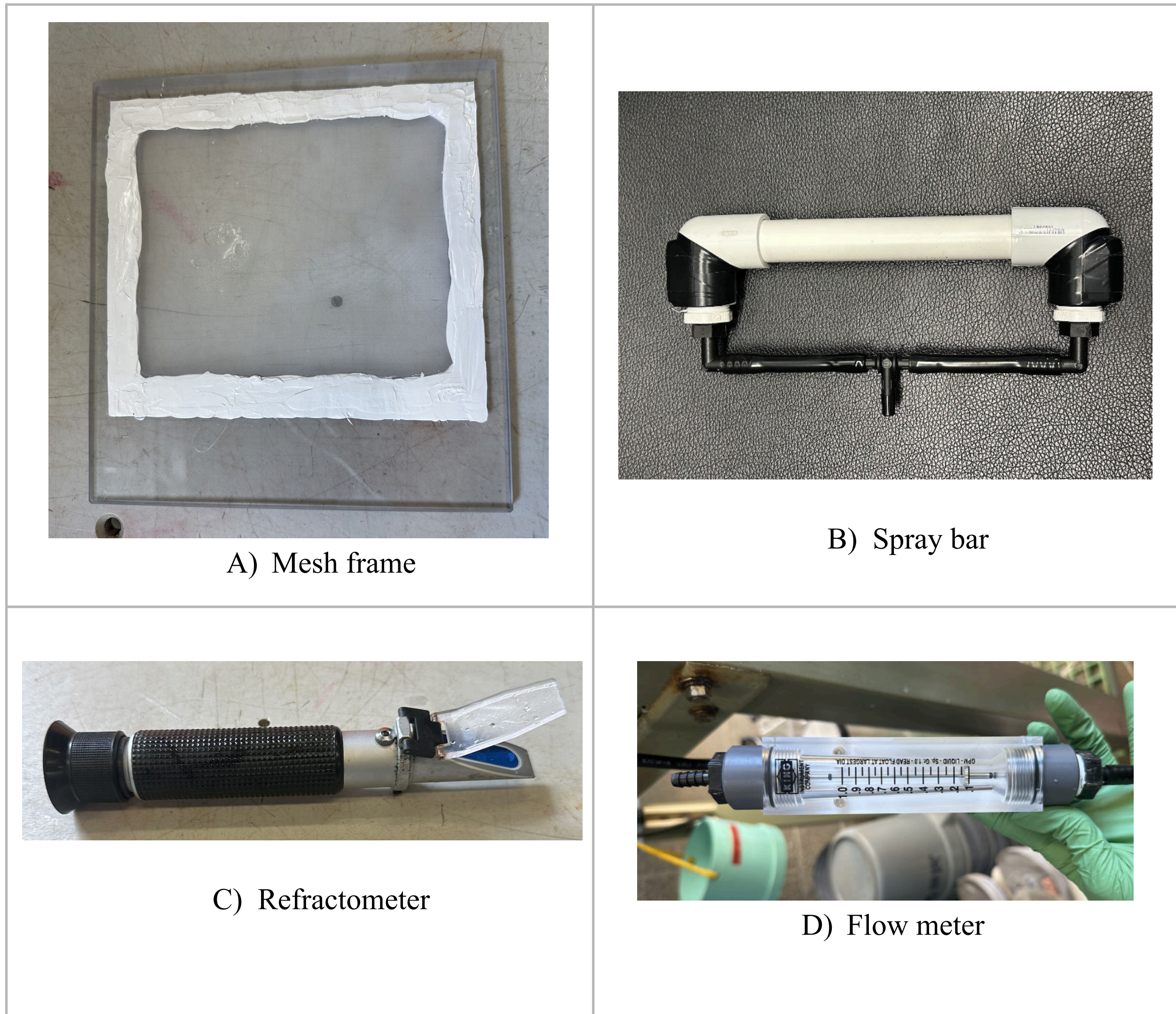

Figure 6: (A) mesh frame for filtration and (B) spray bar for water circulation were placed in each of the three PKs for the experiment. (C) Refractometer used to measure seawater salinity in tanks. (D) Flow meter used to measure flow rate in each of the 3 PKs. Flow rate = 0.4 GPM.

Three 18-inch PKs were used in this experiment (Figure 4). Each PK has a volume of approximately 40.29 liters of seawater. Before the experiment began, images of *B. microptera* eggs with a scale were uploaded into ImageJ, revealing that measurements from six comb jellies in a beaker indicated the hours old eggs averaged approximately 360 µm in diameter. Based on this, a 200 µm mesh was used to create one mesh frame for each tank for water filtration and to

prevent eggs from exiting the tank (Figure 6A). The mesh was glued to the acrylic sliding frame using Dowsil 799 silicone, covering any frays from the mesh edges. Before the glue fully dried, it was smoothed out with a scraper to ensure that the eggs or jellies would not become ensnared by rough areas on the frame.

One spray bar was constructed for each tank using a PVC pipe slightly shorter than the tank width of 21.59 cm (Figure 6B). Each spray bar had to be constructed identically to ensure consistency across the tanks. The inflow tube was connected to a ¼" barbed tee fitting, which split the water flow into black rubber tubing that was cut and fitted to both ends. These rubber tubes were connected to ¼" barbed elbow fittings, which connected with ½" PVC 90-degree elbows. The elbows were then inserted at each end of the white PVC pipe. Tiny holes were evenly drilled into the PVC pipe and elbows to allow water to enter the tank, circulating through the tank due to its circular bottom shape. A 1 cm square of neoprene was attached to each end of the elbows and wrapped in electrical tape to ensure the spray bar fit snugly in front of the mesh filter held by the opposing acrylic walls of the tank.

After the spray bars were tested, the three tanks were filled with seawater, and ran for 30 minutes to clear all debris from inside. The temperature was measured at 12°C using a temperature gun, and a salinity of 35 PSU was recorded using a refractometer (Figure 6C). After inserting the mesh frames and connecting the spray bars to their inflow tubing, the water flow was manually adjusted to 1.51 LPM and measured with a flow meter (Figure 6D).

FEEDING TRIAL

Once water flow was evenly adjusted and circulated for 30 minutes, for each PK, one adult *B. microptera* was scooped up using a 2 liter beaker from the F6 generation from a 18 inch PK. All individual jellies were selected based on size similarity and were the largest out of the F6 cohort, as the gut length of the jellies were T1 60 mm, T2 60 mm, and T3 45 mm. In their original tank they were fed a net of adult mysids (*A. bahia,* ~1 cm) and 2-3 < 30 day old inland silverside larval fish (*M. beryllina* ~1.5 cm) were target fed to each jelly, once a day *(*Aquatic Indicators Inc). The purpose of this was to provide these animals with more nutrient dense food for the comb jellies, which will allow enough energy reserves to produce and spawn eggs. This experimental method depends on self fertilization by the same adult jelly rather than multiple adult jellies in one volume of water, in order to keep count for individual egg production. This approach does not include factors of crowding, and manipulated light/dark periods. Instead, the natural light cycle is simply enhanced as at 8 AM an upper light is turned on, and then turned off at 4 PM. On the right side of the wet table there is a window that allows light into the nearby algae cultures.

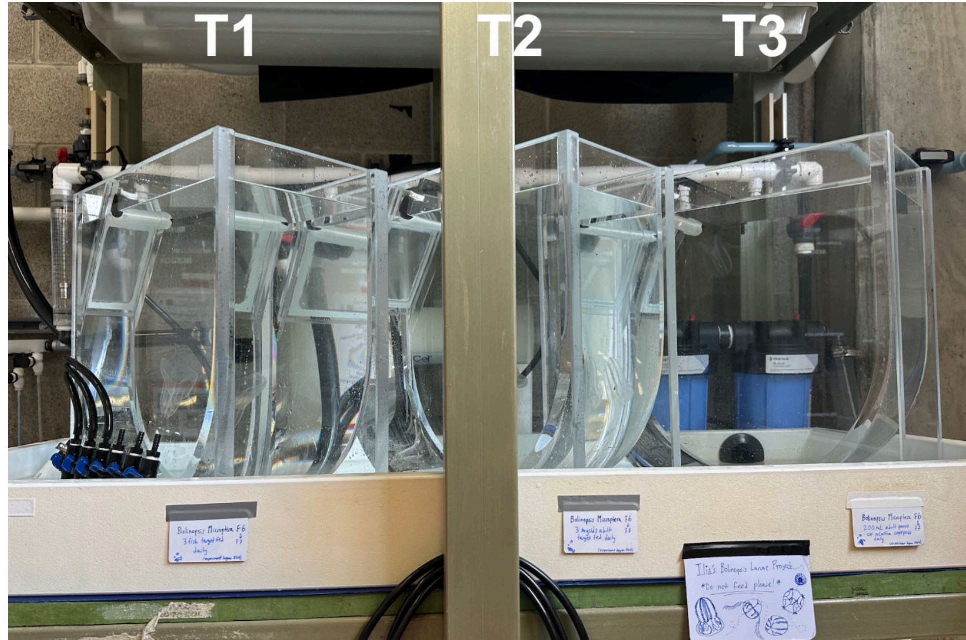

Figure 7: Three PKs used during the feeding trials.

In the experimental PKs (figure 7), T1 (left) was fed 3 inland silverside larval fish (*M. beryllina*), T2 (middle) was fed 3 adult mysids (*A. bahia*), and T3 (right) was fed 200 mL of *P. crassirostris* or *A. tonsa* copepods (both ~0.02-0.04 cm), depending on which copepod culture had the better yield that day. These diets were fed once a day in the AM, between AM and PM egg counts. In order to maintain the cultures of these live animals that were being fed to the *B. microptera*, larval fish were fed *A. salina* nauplii, adult mysids were fed *A. salina* nauplii and Rotifera, *P. crassirostris* were fed *Isochrysis galbana,* and *A. tonsa* were fed *Rhodomonas lens*. If there was a control in this experiment, that would have included an individual jelly in a fourth tank at one end of the wet table that would not get fed throughout the 14 day period, and egg counts would be measured over time along with the other tanks. However, due to animal welfare concerns for starving an animal, this control was voided from the experiment. Previous observations of this species have shown that starving *B. microptera* prevents them from spawning successfully. Rather than using their energy reserves for reproduction, they help to maintain survival over time. As a result, the jelly will shrink in size to adapt to the lack of food, making it highly unlikely for them to spawn (Granhag & Hosia, 2015).

EGG COUNT MONITORING

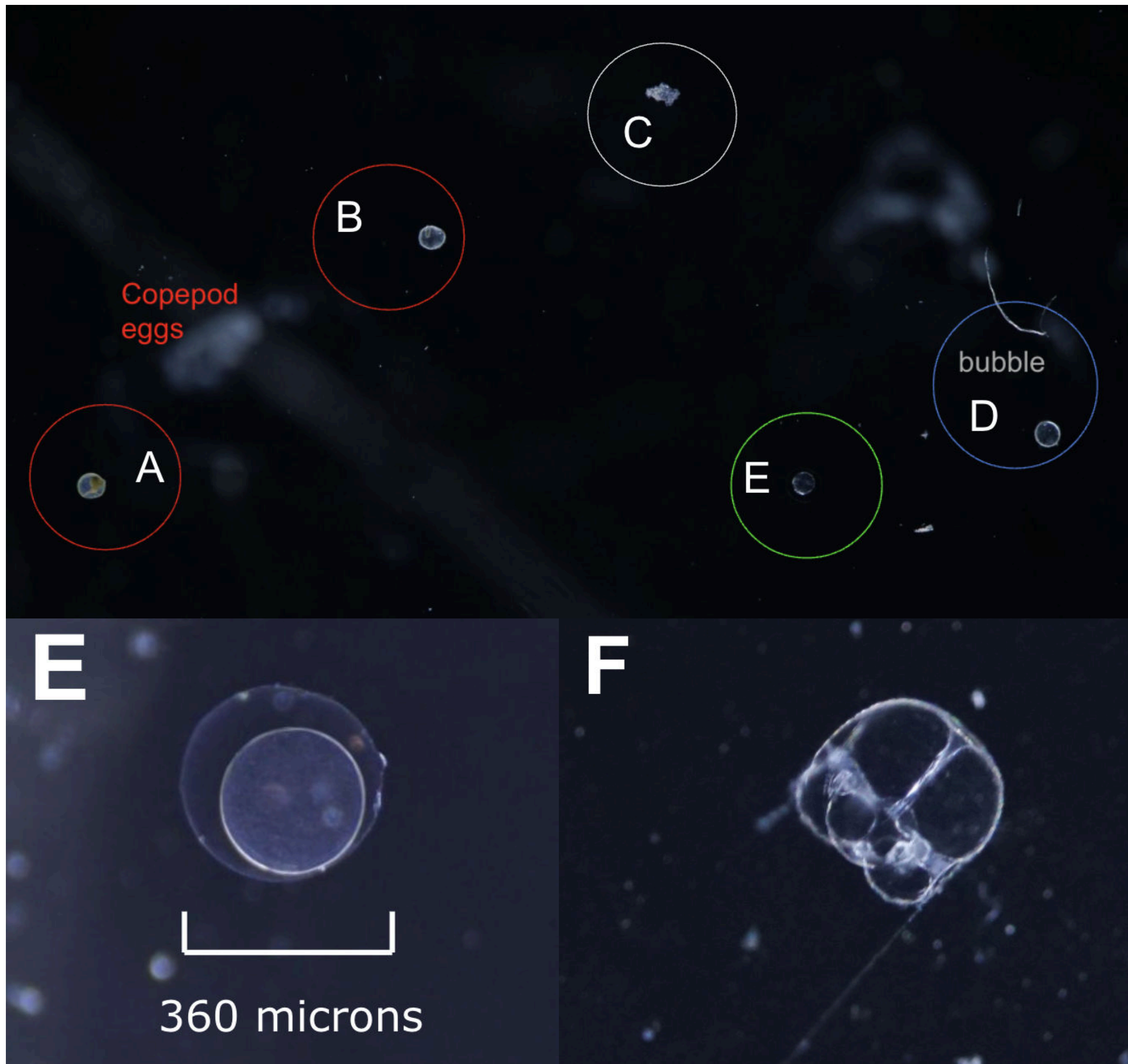

Figure 8: (A & B) Copepod eggs appear translucent light blue in coloration with green/brown spots. (C) Piece of debris. (D) air bubble. (E) B. microptera egg. (F) B. microptera larvae 24 hours post hatching.

Beginning on 7/29/24, eggs were counted twice daily, at 8 AM and 2 PM. After the morning count, *B. micropterus* were fed their respective diets. Counting eggs twice daily can provide insight into: 1) whether the jellies spawn overnight (AM) or during the day (PM) and if they spawn continuously (AM ≈ PM) or only once (AM or PM); 2) how food type affects egg production; and 3) whether reliable spawning occurs in the PKs.

For egg counts, the 360 µm diameter eggs are visible to the naked eye with the aid of a flashlight illuminating the water while the upper light switch is turned off. For 24-hour post-hatched cydippid larvae, the length from the statolith to the mouth averages 359.5 µm. Larval counts were adjusted to the day before to account for the likely hatching date. The eggs are removed using an elongated pipette with a wide mouth and placed into a deep petri dish filled with 1 cm of seawater. They are then transferred to a microscope for verification and tallying, using a glass

pipette to move them from the first dish to another. The total number of eggs and larvae is counted both in the morning and afternoon for 14 consecutive days. Care must be taken not to mistake the eggs for (A-B) copepod eggs, (C) debris, or (D) bubbles, as these can appear similar to the naked eye but are more easily differentiated under a microscope (Figure 8).

DATA ANALYSIS

For AM and PM egg counts over the 14-day experiment (7/29-8/11), paired t-tests, multivariable comparison analyses, and significance tests for differences in egg counts across different diet types were planned. Additionally, comparisons between the same tank types in the AM and PM were also set to be analyzed in R. However, due to the low statistical significance of the data — as there was only one sample per diet type — it was decided to exclude statistical analysis from the study. For the figures, the original data table used to monitor the egg counts during the AM and PM for the 14 days will be provided. Using this table, separate bar graphs for the following were created: the total egg quantities across each diet type for the two-week cycle, the total number of eggs spawned per day, the quantity of eggs spawned in the AM and PM, and the overall daily AM and PM counts over the 14-day period.

RESULTS:

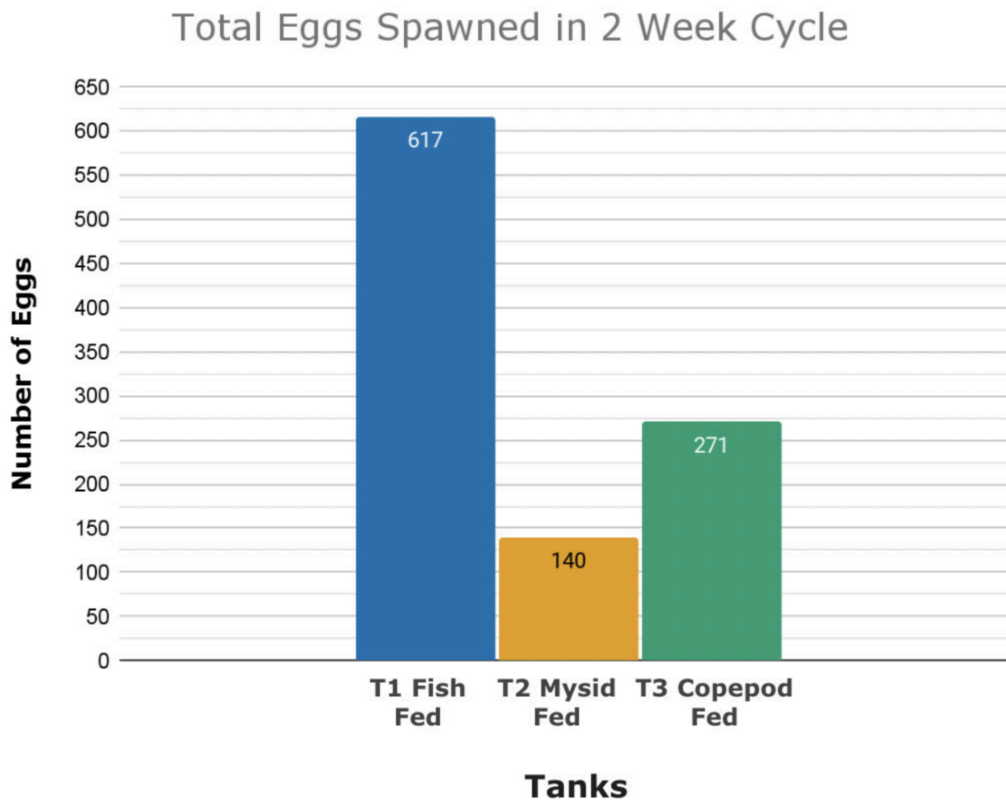

Figure 9: Total amount of eggs spawned during the 14 day experiment period. Tanks left to right are T1 fish fed, T2 mysid fed, and T3 copepod fed.

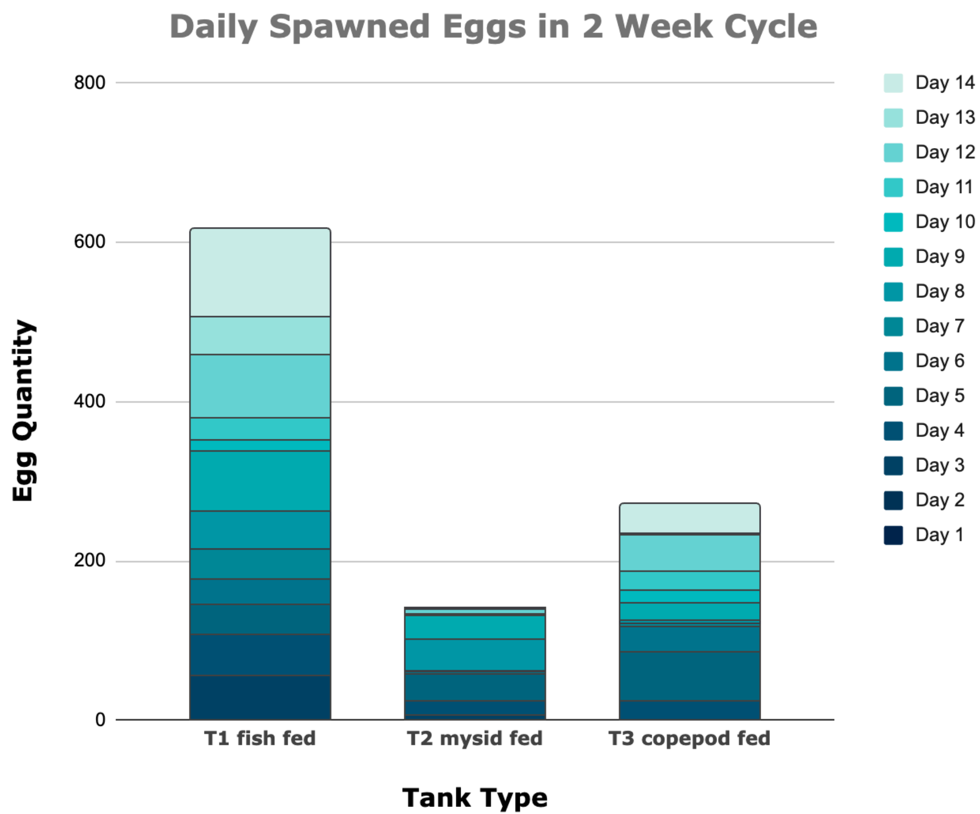

Figure 10: Daily spawned eggs for the 14 day experiment period. Each day is represented in different shades, with the wider bars indicating a higher egg count for that day. Some days with 0 counts are not shown on the graph.

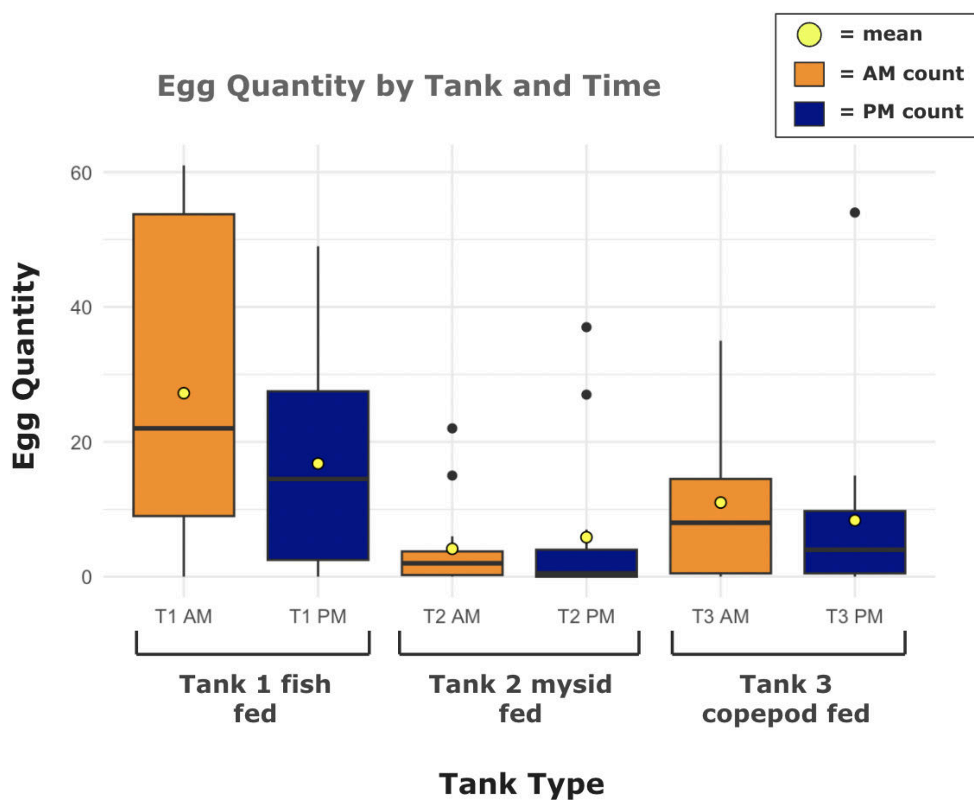

Figure 11: Egg quantity for each tank diet type for the morning (8AM) and afternoon (2pm). T1 = larval fish fed, T2 = adult mysid fed, T3 = copepod fed. Morning is indicated in orange, afternoon is indicated in blue. Yellow dots indicate mean count for that AM/PM time bar and black dots are outliers.

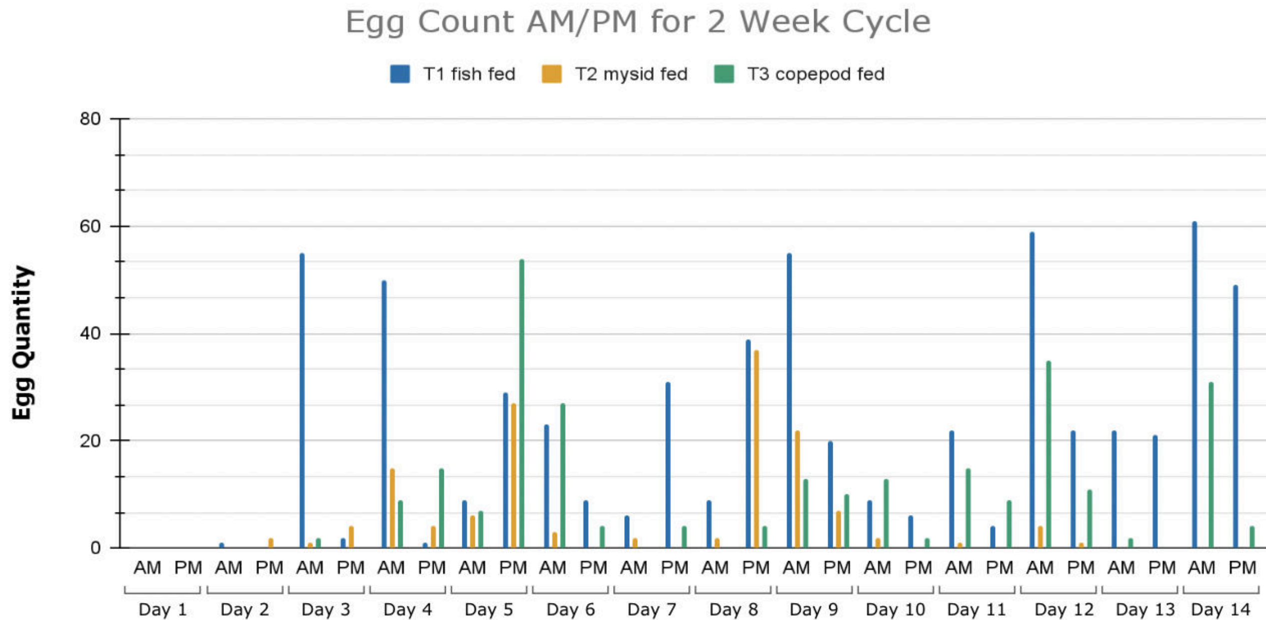

Figure 12: AM/PM egg counts to observe potential cyclical spawning patterns for the 14 day experiment period, with blue indicating T1 fish fed, yellow T2 mysid fed, and green T3 copepod fed. X axis represents specific morning (8AM) and afternoon (2PM) times for each of the 14 days in chronological order, and Y axis represents egg counts recorded for that given time of day.

	Eggs Collected Morning (~8AM)			Eggs Collected Noon (~2PM)		
	Tank 1 Count	Tank 2 Count	Tank 3 Count	Tank 1 Count	Tank 2 Count	Tank 3 Count
7/29/24	0	0	0	0	0	0
7/30/24	1	0	0	0	2	0
7/31/24	55	1	2	2	4	0
8/1/24	50	15	9	1	4	15
8/2/24	9	6	7	29	27	54
8/3/24	23	3	27	9	0	4
8/4/24	6	2	0	31	0	4
8/5/24	9	2	0	39	37	4
8/6/24	55	22	13	20	7	10
8/7/24	9	2	13	6	0	2
8/8/24	22	1	15	4	0	9
8/9/24	59	4	35	22	1	11
8/10/24	23	0	2	23	0	0
8/11/24	61	0	31	49	0	4
Total count	382	58	154	235	82	117

Figure 13: Table used to collect data for figures 9-12 for the 14 day experiment duration (7/29/24 - 8/11/24), with morning (8AM) and afternoon (2PM) counts recorded for each of the three food diet tanks.

Even though there were no statistical differences, observations from the single-trial experiment over the 14-day duration only supported one component of the original hypothesis: the larval fish-fed *B. microptera* produced the highest quantity of eggs compared to the other two groups. However, instead of the mysid-fed *B. microptera* having the second-highest egg quantity, the copepod-fed *B. microptera* produced the second highest egg quantity, ultimately rejecting the original hypothesis. The larval fish-fed diet (T1) resulted in the highest quantity of egg spawning, with a total of 617 eggs (Figure 9). This diet provided the longest consistent spawning duration, with no days of 0 AM/PM spawning counts from beginning spawning on day 2 through day 14 and occasional mass spawns reaching counts in the 50s and 60s (Figure 10). The adult mysid-fed diet (T2) yielded low egg production (Figure 9) throughout the experiment, with very few days showing counts above 40 eggs and major spawning inconsistencies, including multiple days with 0 AM/PM spawning counts (Figure 10). The copepod diet (T3) produced eggs with moderate consistency, showing only one day with a zero count following spawning (Figure 10). T3 demonstrated the potential to support increased egg quantities on the majority of days, but generally resulted in lower counts compared to the larval fish-fed T1 tank (Figure 9), and never reached egg counts above 40 in a single day (Figure 13). T1 and T3 displayed substantially higher counts in the AM, with six intermittent mass spawns above 40 eggs, but both dwindled in egg counts and showed fewer 0 AM/PM spawning counts throughout the experiment compared to T2.

Two of the three diets resulted in high egg counts observed in the AM (Figure 11). All three tanks displayed occasional mass spawning events throughout the entire experimental period, most notably with the highest quantities in T1 (Figure 11). When observing the outliers, it is important to consider how they represent collections of high mass spawns for that time of day across the different diets. The three highest outlier spawn counts across the tanks were: T2 AM with 22 eggs on day 9, T2 PM with 37 eggs on day 8, and T3 PM with 54 eggs on day 5. For the AM, T1 had the highest mean count of 27.2 eggs, followed by T3 with 11 eggs, and finally T2 with 7.2 eggs. For the PM, T1 again had the highest mean egg count with 16.8 eggs, followed by T3 with 8.4 eggs, and T2 with 5.9 eggs. All box plots were positively skewed, as the means for both AM and PM across all tanks were above the median. Notably, the means for T2 in both the AM and PM were greater than the 3rd quartile. Compared to T2 and T3, T1 had the highest egg production, particularly in the AM, with more consistent peaks in production (Figure 10). The highest T1 AM count was 61 eggs on the final day of the experiment (day 14), while the T2 PM counts were generally lower than the AM, with the highest PM count being 49 eggs on that same day (Figure 13). T2 had the lowest mean egg quantities for both the AM and PM when compared to the other two tanks. Within T2, the highest AM count of 22 eggs on day 9 was lower than the outlier PM count of 37 eggs on day 8.

Upon observing the data table (Figure 13) and the daily AM and PM spawning patterns (Figure 12), T1 began spawning on day 2 in the AM and continued spawning for 13 consecutive days, including both AM and PM counts from day 3 to day 14. T2 began spawning on day 2 in the PM and spawned consecutively from day 2 PM to day 6 AM. This was followed by a decrease in spawning from day 6 PM to day 7 PM and again from day 8 AM to day 10 AM. Afterward, egg counts dwindled to 0, with a low peak of 4 eggs on day 12 AM, followed by 0 eggs throughout both AM and PM on days 13 and 14. T3 began spawning on day 3 in the AM, spawning consecutively from day 4 AM to day 6 PM. It then dwindled to 0–4 counts in the AM and PM for days 7 and 8, spawned consecutively from day 9 AM to day 13 AM, and had a final peak on day 14 in the AM before decreasing in the PM.

When observing the jumps in mass spawning in Figure 12, it is noticeable that T1 generally had the most jumps in mass spawning in the AM. From day 3 to day 14, there were six high-count spawns above 40 eggs (excluding the T3 day 5 PM outlier), with five of these six mass spawns occurring in the AM. Across the 14 days, it is apparent that the majority of the AM counts for T1 are higher than the PM counts. For T2, all AM and PM counts never exceeded 40 eggs, unlike T1 and T3. With T2, there were two gradual peaks in spawning: one on the day 5 AM count and another on the day 8 PM count. Following day 8, the number of spawns notably declined, with 0 counts throughout both days 13 and 14.

DISCUSSION

Methodology Trial and Error, Changing the Experiment

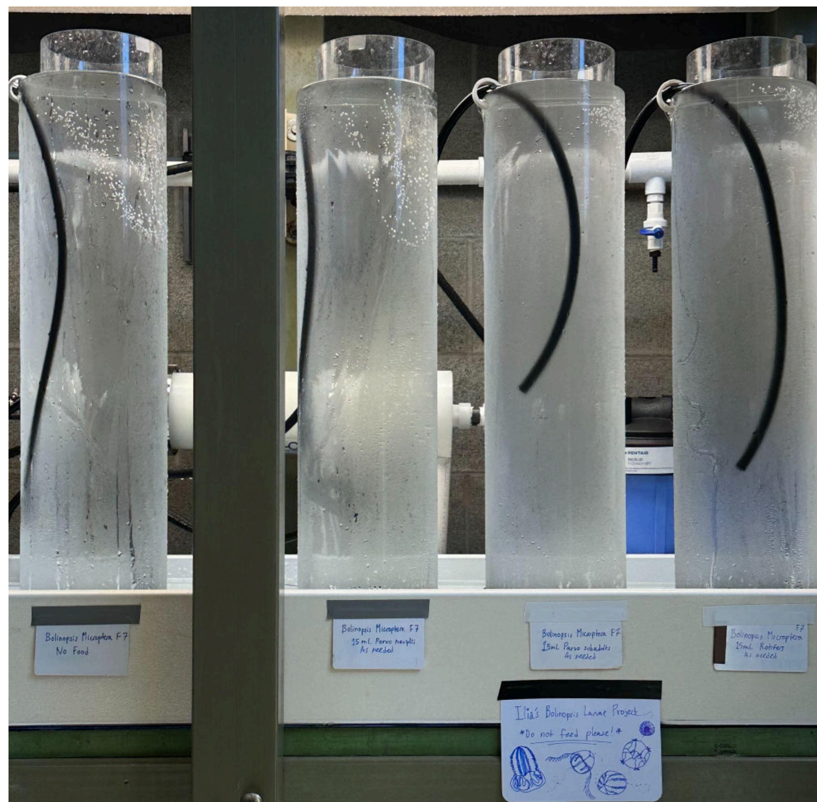

Figure 13: Original larval rearing experiment with different diets fed to three different diffusion tubes. Tubes from left to right were intended to be assigned as the control fed no food (left), 15 mL P. crassirostris copepods as needed (middle left), 15 mL P. crassirostris subadults as needed (middle right), and 15 mL Rotifera *as needed (right).*

The original experiment, which was not included in the results, focused on spawning larvae from the same F6 generation cohort of *B. microptera* from multiple jellies to yield the most eggs, with the intention of placing at least 30 eggs into 4 diffusion tubes. However, this attempt was unsuccessful (Figure 13). The goal was to experiment with the F7 generation larvae from hatching to metamorphosis, particularly during the growth of their oral lobes, which is the most crucial and delicate stage where they are highly prone to mortality. The aim was to determine if Rotifera could be relied upon to feed the larvae when the ideal food source of copepod nauplii cultures ran short or crashed, and whether larvae could be fed sub-adult copepods, as this had never been attempted before.

In Figure 13, the T1 tube (left) was set as a control by providing no food to the eggs to better understand mortality rates in the diffusion tubes. T2 and T3 would each be fed 15 mL of *P. crassirostris* nauplii as needed, while T4 (right) would be fed 15 mL of Rotifera as needed. It was crucial to maintain a balanced density of prey in the water volume to prevent larval starvation. However, if the prey density was too high, too many prey could get caught in a larva's tentacles, potentially tearing them apart, thereby lowering the larvae's chances of survival by depriving them of their feeding appendages, which could lead to starvation (Schulze-Röbbecke, 1984).

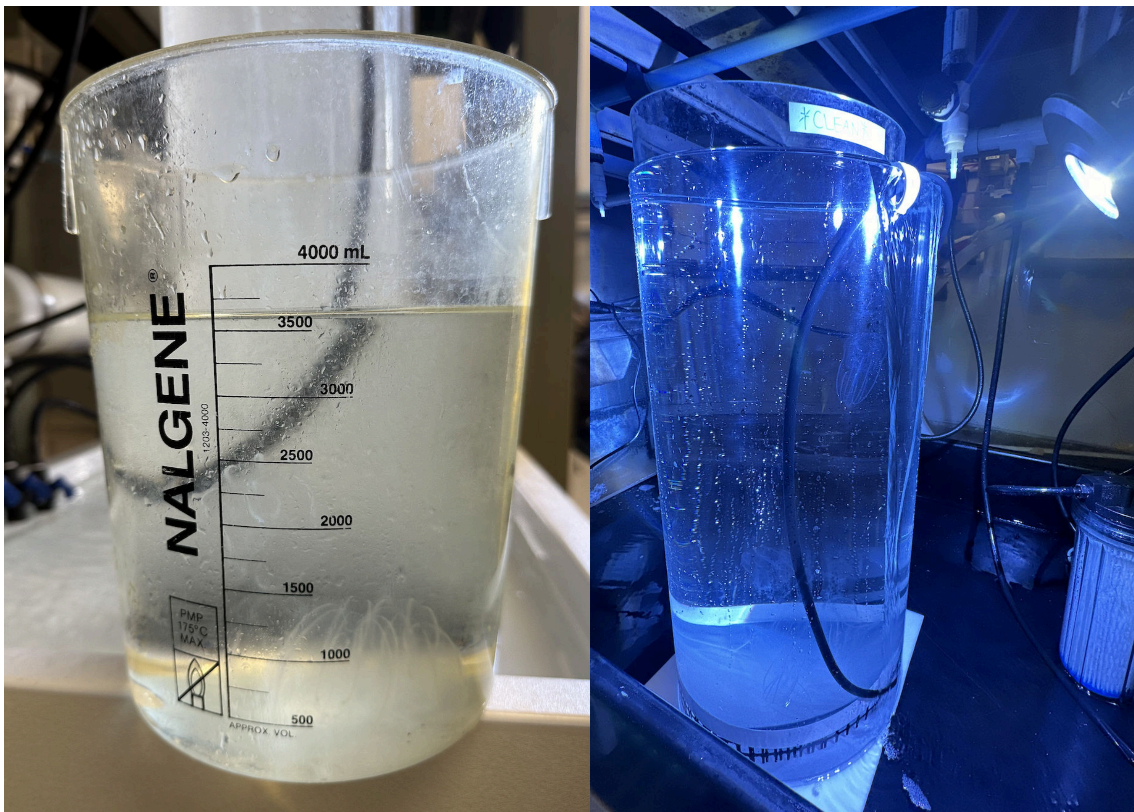

Figure 14: Spawning methods used to induce spawning using crowding and light manipulation with 4-6 B. microptera adults. Method 1 (left) included using a 4 liter beaker with no water circulation, and Method 2 included using a diffusion tube with low water circulation and introducing light following a dark period for multiple hours.

Two methods were used to induce spawning from 4–6 adults to collect the necessary eggs for this experiment. Method 1 involved placing the adults in 4-liter beakers, while Method 2 utilized a diffusion tube (Figure 14). In Method 1, the factors of crowding and light were expected to initiate spawning (Dunlap, 1966, pp. 15). The beaker's shallow height allowed for easier access to separate and remove the eggs once the adults were removed, as the negatively buoyant eggs would settle at the bottom. However, this method only allowed spawning to be induced for less than a day to prevent biofouling, which could negatively impact the health of the jellies. This method initially failed after four attempts with two holding tanks of the F6 generation, each fed different ratios of mysids and/or fish—one intended for feeding to deep-sea jellies and the other for spawning the F7 generation before this experiment began. While it worked twice, transferring to the trial diffusion tubes led to high mortality rates, resulting in an insufficient number of specimens for the experiment. Consequently, only the eggs were measured to gather some information about the F7 generation for the mesh tank frame. A 4-liter beaker was used to initiate spawning, but no eggs were found by the end of the day, so the beaker was left overnight (Figure 14). The next day, 30 eggs hatched, and 15 eggs were transferred into two containers: one with no food and the other with Rotifera and *Artemia salina* nauplii, to simply test survival after transfer following the high mortality rates. All but three eggs did not survive, possibly due to the pipette transfer being too harsh despite a modified wider mouth, suggesting transferring eggs should be avoided.

Method 2, using the diffusion tube for spawning, had advantages such as being left overnight and covered with a black wrap to prevent light exposure until the next morning when the cover was removed, which would help initiate spawning (Dunlap, 1966). However, it was more challenging to access the eggs due to the tube's narrow and tall shape, requiring a pipette modified with a long stick. With the low water circulation and the movement of adult comb jellies in a larger volume of water, the eggs settled at different heights within the column. The comb jellies had to be removed to allow the negatively buoyant eggs to settle to the bottom. Eight *B. microptera* were placed in the diffusion tube, which proved more successful as it allowed more time to initiate spawning. However, following a successful spawn, over the next few days, only a few larvae remained, with most disappearing in the trial tubes. Another spawning attempt was made, eggs were measured, and clumps were moved into diffusion tubes to test the transfer method again. By the end of the period, only 2–3 larvae per tube were observed, which was not enough for the experiment. Despite the factors of light and crowding, the spawning failures suggested that the F6 generation may have already spawned unprompted in a prior week, or that the pipette transfer once again was the cause of these mortalities while using both spawning methods.

Overall, spawning the F7 *B. microptera* generation for the larval feeding trials was delayed by a week because it required multiple attempts with these two spawning methods (Figure 14). Due to

the failure to produce substantial larval samples using these methods, the methodology had to be reconsidered, shifting the focus toward spawning adult *B. micropterus* rather than monitoring egg and larval mortalities.

Improvised Experiment with Adult *B. microptera*

There is minimal scientific literature that analyzes the spawning patterns of the *B. microptera*, considering their lengthy history of taxonomic confusion with the *B. infundibulum* that is quite similar in morphology with minute differences and confirmed a separate species through genetic sequencing (Johnson *et al*., 2022). Not much is known about the spawning patterns of the *B. microptera* in a lab setting, let alone through in-situ observations, due to the difficulties that follow with spawning this delicate species that spawn small, virtually transparent eggs and consistent monitoring is necessary to analyze any cyclical spawning patterns. However, it is known that the *B. infundibulum* spawns during the spring and early summer, however lab observations regarding whether the *B. microptera* spawns continuously or intermittently is still largely unanswered, and with only three sample individual adults in this observational single trial all given different diets cannot make us jump to conclusions. However with based on the results from these in lab observations from this diet experiment, the *B. microptera* is capable of spawning eggs daily in a presumably intermittent due to high quantities in mass egg spawning events, and that food plays an impact on the spawning quantity as well as time of day (Figure 12 & 13). Due to the nature of this being an exploratory experiment, it is important to identify the factors of the methodology that may have impacted the experimental results: tank structure, standardization and timing of the different diets given, aquarium setting restraints, and lack of replicability within the experiment that limits our conclusions to the observations of this 14 day experiment.

The total (Figure 9), daily (Figure 10), and AM/PM (Figure 11) spawning quantities across the different diets could best be explained by several factors: variability in the nutritional content of each diet, differences in the amount of each diet given per day due to the lack of a standardized feeding protocol, and the dependency on external factors such as the quality of food upon arrival from the vendor and culture quality. The T1 larval fish-fed tank had the highest total egg count of 617 eggs (Figure 9), and had the most consistent spawning counts throughout the experiment compared to the other two tanks as upon beginning spawning there were no 0 counts (Figure 13). T1 also had the highest average daily egg counts, ranging from 16.8 (PM) to 27.2 eggs (AM) (Figure 11), which aligns with past successes in feeding larval fish to other species at the aquarium to aid in increasing egg quantity for the *Mnemiopsis leidyi* and *B. microptera*. These past observations were further supported by the results of this experiment. When observing the T2 total egg count of 140 eggs, which averaged 4.1 eggs (AM) and 5.9 eggs (PM) (Figure 11), it had the lowest egg count in comparison to the other two diets. This suggests that the mysid diet is the least effective of the three, contradicting the original hypothesis that the mysid-fed *B. microptera* tank would have the second highest egg count, following the larval fish-fed tank. The mysid-fed T2 tank was the only diet out of the three that spawned more in the PM than in the

AM. However, there is only a difference of 1.8 eggs, with no statistical significance (Figure 11). The results of the T3 copepod-fed tank are highly dependent on both the nutritional quality and quantity of copepods in the Drifters Lab cultures on a day-to-day basis. For example, on days 8 and 9, there is a notable decrease in the total quantity of eggs spawned while being fed these copepod species (Figure 10). This could be due to a decline in the overall density of copepods or a reduction in algae density in the cultures prior to these days, which decreases the nutritional content of each copepod fed to T3. Additionally, both copepod species are distributed to many other invertebrates, and based on the quantity and quality of copepods available each day, certain animals may be prioritized. Compared to T1 and T2, the daily egg spawn count in T3 is the most consistent throughout the experiment, likely due to the more stable density of prey in the water (Figure 10). In lobed ctenophores, ingestion rates are directly proportional to food concentrations, and their ability to self-fertilize, combined with high fecundity and rapid growth, explains their potential for rapid population expansion when food is abundant (Kremer, 1976; Reeve *et al.*, 1978). Culture variation and crashes affect the daily nutritional content of prey animals fed to the jellies in this experiment, though the overall impact of prey quantity on their nutrition remains unquantified. This variability significantly affects T3, as the quality of the copepod diet depends on culture conditions that influence copepod quantity, nutritional value, and whether or not cultures crash. The *B. microptera* may prefer consuming smaller prey (200 to 3,200 μm) over larger prey at a high rate (Bishop, 1968). The copepods fed to the adult jelly in T3 are within the 200-400 μm range and are provided differently from T1 and T2, where they are target-fed. Instead, copepods are poured into the PK, allowing the jelly to feed at a steady rate throughout the day, which supplements its diet even into the next day. Despite this, the egg yield remains lower than that of T1, which is fed larval fish.

In Figure 12, the spawning patterns of tank T2 are shown in more detail and are consistent with the overall trends seen in Figure 10. The nutrition provided by mysids may explain these results, as they have the thickest exoskeleton in comparison to copepods, the only other diet type with an exoskeleton. This may potentially cause mysids to be more difficult to digest, potentially leading *B. microptera* to obtain fewer nutrients. In addition to this, there is a large disparity in the size quantity between the mysids and larval fish given to T1 and T2. Although both tanks received three individuals, a larval fish individual is much larger than an adult mysid individual, as *M. beryllina* is about 0.5 cm longer in length.

When considering the structure of the PKs, despite their efficient shape and water circulation allowing for generally healthy care of delicate species such as *B. microptera*, mortalities and injuries to eggs, larvae, and adult individuals are still possible. Confined to a walled tank, pelagic species can occasionally bump into or rub against the sides of the acrylic and mesh, or experience sudden jet propulsion from the spray bar when coming into contact with the upper corner of the tank. Additionally, stress from transferring them from their larger holding PK to the experimental PK could have influenced earlier spawning, despite efforts to keep the delicate species intact. When transferring *B. microptera*, regardless of their stage, they are generally more delicate compared to other ctenophores and are highly prone to injury. The jellies were moved to

the PKs, and experiment recordings began that same day. An adult jelly in T2 had a small piece of the oral lobe separate from the body on day 2, likely due to the initial tank transfer, but this had a negligible effect on hunting success; throughout the 14 days, this jelly could still trap the mysid adults provided. One suggestion from mentor Dr. George Matsumoto for future experiments is to place the spray bar behind the mesh frame rather than in front and adjust the spray bar angle to maintain the circular water flow in the tank, thereby preventing damage from the jet propulsion of water entering through the tiny holes of the PVC bar, even though the flow rate is very low.

Before the experiment began, the three *B. microptera* used in these PKs were already fed the same species of mysids and larval fish that were given to T1 and T2 to ensure a healthy and sexually mature cohort to spawn the next F7 generation. This may have had a higher impact on the egg counts in the first week. The spawns for week two are more reliable as the food diets from the experiment have run through their gut for 7 days. Although food can be fully digested and excreted from the body in several hours, it is more important to consider the nutrient accumulation in the body from these diets over time, which impacts egg spawn quantity.

No scale was included to standardize the amount of food given to each *B. microptera*. This was necessary for the experiment to make the results more applicable to animal husbandry contexts for the Jelly Care team. With many tanks in both the holding and exhibit areas, food is not weighed out to jellies to increase efficiency in completing all tasks. At the MBA, daily tasks must be completed in both the AM and PM, including feeding, cleaning/general tank maintenance, and exhibit rounds in the Drifters exhibit, Open Sea exhibit, and Into the Deep exhibit. Therefore, each tank receives a specified amount of food measured in nets of larval fish, krill, mysids, volume of Rotifera, *A. salina* nauplii, and copepods. This specified amount per tank changes over time as the animals increase in size and nutritional requirements.

One disadvantage of this experiment was not knowing the nutritional content of each food type given. The *M. beryllina* larval fish and adult *A. bahia* are delivered to the aquarium from a vendor (Aquatic Indicators Inc.), and their quality varies based on their condition upon arrival at the Drifters Lab. This is also important for *P. crassirostris* and *A. tonsa* copepods, which are delivered by a vendor but are "fattened up" in the Drifters Lab with Rotifera and algae to maximize their nutritional content before being fed out. As copepods depend on live algae, their cultures can vary in quality and may crash. Low-quality copepod cultures can impact the nutritional content given to the animals that day, as there will be a lower density of copepods. In this experiment, the density of copepods fed to T3 varied, and measuring this density was not feasible due to time constraints and the impracticality of counting copepods in 200 mL twice a day.

Lastly, the overall setting of the experiment constrained its scope and materials, as well as the 10-week summer internship duration. Within the aquarium, only three PKs could fit on the wet table (one per food type). Adding a fourth PK as a control would have complicated egg collection with the modified long pipette, as it would require tanks to be fully facing sideways,

making it difficult to view the sides with a larger surface area. With only one 14-day trial, this limited the ability to verify results with reliable statistics.

FUTURE WORK / RECOMMENDATIONS

One significant way to improve this experiment would have been to send the three food types to a food lab to obtain the nutritional profiles for each diet. This would allow for a better understanding of the caloric content, minerals, and other nutrients present in each food type, which greatly impact fecundity in many animals. If there were scales involved to measure out the food evenly between the different food type tanks, this would have improved the standardization of the food variables.

Additionally, when planning a larval rearing project for this species, it is important to allocate more preparation time to establish the correct methodology for successfully spawning *B. microptera* larvae and to account for potential sample mortalities before the experiment begins. Seventy percent of the internship duration was spent on methodological trial and error before the improvised 14-day experiment could start. If the appropriate methodology had been determined before the internship began, it could have allowed for a much longer experimental period, potentially up to 8 weeks (and 1 additional week for analysis/presentation and project report preparation) if everything had gone smoothly. With a longer experimental period beyond the 10 week internship duration, this would have allowed more reliable conclusions regarding the overall spawning cycles, and the question of how many eggs a single *B. microptera* can produce over its whole lifespan could be answered.

In the standard spawning protocol for this species at the aquarium, adults are typically group-spawned in a diffusion tube. After spawning, the adults are removed, and the eggs remain in the tube from hatching to the juvenile stage, during which the lobes develop. Valuable lessons were learned regarding how delicate the eggs and larvae of this species are, and that egg transfer is not recommended to be included in the experiment even for the experimental preparation. This was evident when we repeatedly attempted to transfer multiple batches of eggs or larvae into the diffusion tubes; no matter how gentle the transfer, the high mortality rate prevented us from achieving a sufficient sample size of 30 for each of the four diffusion tubes, ultimately making the larval rearing experiment with different diets unsuccessful.

When reconsidering a larval rearing experiment with different diets, it is also worth noting the findings of the Ikeda 2022 paper, which reported that *B. mikado* larvae can survive by feeding on the excrement of adults. The main advantages of this culture method are its simplicity and reduced physical intervention, requiring only feeding and observation until the cydippid larva are large enough to hunt. This minimizes damage from water exchange and supports stable breeding of *B. mikado* (Ikeda *et al.*, 2022). Perhaps a similar experiment could be conducted by comparing larvae raised with the same *B. microptera* adults that spawned them against those isolated and

provided with different diets. It is unclear whether this approach would work for *B. microptera*, but it could offer a way to optimize food resources by feeding both generations in the same tank simultaneously. Since individual *B. microptera* jellies are typically removed from food tanks when preparing to feed them to deep-sea jellies, combining the current and new generations could allow the new generation to feed on the leftover food from the older jellies.

When considering both experiments with adults and larva for this species, for future experiments the *B. microptera* should be kept and spawned in the same PK in order to reduce physical interference for this delicate species. Dr. Kevin Raskoff suggested a PK can be made with a mesh frame that allows eggs to exit the main tank holding area for the *B. microptera*, and the eggs could be counted or removed from an outflow tank; similar in the way how certain scyphozoans are spawned at the aquarium, for instance with the *Aurelia aurita* moon jelly polyps kept in one tank and upon strobilation when the mobile ephyra separate, flow into an outflow tank (Raskoff *et al*., 2003).

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