Diffusion tubes: a method for the mass culture of ctenophores and other pelagic marine invertebrates.

Wyatt L. Patry[1]*, Mac Kenzie Bubel[1], Cypress Hansen[1], Thomas Knowles[1]

[1]Animal Care Division, Monterey Bay Aquarium, Monterey, California, United States of America

* Corresponding author

E-mail: wpatry@mbayaq.org

Abstract

The culture of pelagic marine invertebrates, especially the ctenophore *Mnemiopsis leidyi*, has been demonstrated in past studies dating back to the 1960's, however the *mass* culture of delicate pelagic invertebrates has remained elusive. By using a pair of acrylic tubes and enabling water diffusion between them, we have been able to reliably and cost effectively mass culture several genera of ctenophores (*Pleurobrachia*, *Hormiphora*, *Bolinopsis*, *Mnemiopsis*, and *Leucothea*), one species of siphonophore (*Nanomia*) and one species of larvacean (*Oikopleura)*. The simple,

compact method is effective enough to support two permanent exhibits of ctenophores at the Monterey Bay Aquarium while minimizing live food culture requirements with the potential to support further investigation of pelagic marine invertebrate ontogeny, ecology and genomics.

Introduction

Interest in ctenophore culture and other pelagic marine invertebrates as model organisms has surged recently (Howes *et al*., 2014; Jaspers *et al.*, 2015, Jaspers *et al.*, 2018, Martí-Solans *et al*., 2015; Presnell and Browne, 2019). Basic ontogeny of ctenophores has been observed since at least the turn of the 19th century by Chun, Agassiz and Mayer however maintaining cultures in the laboratory remained elusive until later in the 20th century. Pioneering efforts in developing rearing vessels for pelagic culture were made by Wulf Greve with his invention of the planktonkreisel (Greve 1968 and 1970) and other variations such as the double cuvette design (Greve, 1975). Greve achieved several generations of *Pleurobrachia pileus* in these studies. The kreisel was later formalized by Hamner (1990) for use aboard ships and then adapted for use in public aquariums by the Monterey Bay Aquarium (Raskoff *et al*., 2003).

The method presented here originated at the Monterey Bay Aquarium in July 2015 to support the temporary exhibition, *The Jellies Experience*, and while collaborating with W. E. Browne of University of Miami on *Mnemiopsis leidyi* culture (Presnell *et al*. 2019). After successfully culturing *Mnemiopsis*, we turned our focus to culturing other ctenophores such as *Pleurobrachia bachei* and *Bolinopsis infundibulum*. The young cydippids of these species are so delicate that they do not survive in traditional plankton kreisels. They are susceptible to being damaged by the

seawater flow in the tank and sticking to the outflow screen. Additionally, juveniles do not survive in standing seawater dishes due to rapid accumulation of ammonia from waste, and high mortality from being transferred into new seawater. Therefore, we needed a rearing tank with minimal or no mechanical driven water flow, no outward pressure on the outflow screen, and the ability to passively exchange new seawater. We experimented with rearing tanks featuring seawater flow just outside of the outflow screen, such that seawater could passively exchange without creating outward pressure on the screen. While these methods were effective at eliminating buildup of nitrogenous wastes and minimizing forces on juvenile ctenophores caused by seawater flow, the tank shapes were not appropriate for the swimming and feeding behavior of the animals. In traditional rectangular aquaria, juveniles were observed actively consuming prey at or near the surface and then sinking to the bottom, where significant biofouling occurs. Initially we designed and constructed a cylindrical tank with more vertical space for feeding, minimal biofouling surface area with mesh covering the bottom and secured in a pseudo-kreisel such that water flowed up through the bottom. This initial design proved somewhat effective, however juveniles still spent significant time contacting the bottom mesh. An improved design providing even more vertical space, while reducing the volume of the tank was implemented. Our final version utilizes a double cylinder configuration in which an elongated inner cylinder is surrounded by an outer cylinder ‘sleeve’, affectionately referred to as a “cteno-tube” in the lab.

Over the past four years, we have experimented with this new double cylinder rearing tank design and have refined a protocol for raising fragile pelagic ctenophores. We observed a number of physical parameters including; feeding rates, biofouling, and analyzed fluid flow rates within the ‘cteno-tube’ in relation to adult ctenophore yield. Here we present these results,

additional variations on the double-cylinder method and provide a best practices protocol for the mass culture of pelagic ctenophores and other gelata.

Method

Materials and design

Diffusion tubes were constructed using two 0.9 m long acrylic tubes. The outer and inner tubes had volumes of ~35 L and 28 L with inner diameters of 21.59 cm and 20 cm respectively, so that one could be placed inside the other (an inner and outer cylinder); each cylinder has a thickness of 0.3175 cm (Fig. 1). The bottom of the inner cylinder was fitted with 55 µm nylon screening (Model# M55 PentairAES, Inc.) using silicone sealant (Dowsil 795 or 999-A) (Fig. 1c). This inner cylinder was then placed on a 3 cm tall riser made of rigid plastic mesh (Model# N1020 PentairAES, Inc.) such that the top of the inner cylinder rises above the outer cylinder (Fig. 1a). The outer cylinder was glued to a ~1 cm thick square acrylic baseplate, 15-20 cm^2, using acrylic cement (Weld-On #16 Fast Set solvent cement). Rigid PVC tubing, 0.635 cm inner diameter, (Part# 48855K41, McMaster-Carr) was placed in the outer cylinder, between tubes, providing water flow near the bottom. Filtered seawater (5 µm) was pumped through the tubing at rates of 1.1, 2.2 and 4.5 Liters per minute (Lpm) based on ½, 1, and 2 theoretical turnovers of total cylinder volume per hour. Three identical pairs of diffusion tubes were constructed and placed on a wet table with recirculating filtered seawater (5 µm) such that water overflowed out of the outer cylinder and on to the table (Fig. 2a). Diffusion between tubes through the 55 µm nylon screening was observed by the addition of dye (McCormick blue food color) to the incoming

water. In the 4.5 Lpm treatment, flow was such that a slight suction vortex pulled the ctenophore eggs into the bottom screen which prevented hatching. To resolve this, a PVC pipe tee fitting (Part# 4881K47, McMaster-Carr) was added to the bottom of the inflow tube to reduce water velocity and divert the incoming water away from directly underneath the screen, which proved successful (Fig. 2b).

Characterizing physical parameters

A set of probes (Hach HQ40d with LDO probe) for measuring pH, dissolved oxygen, and temperature was deployed within the inner cylinder of each set of tubes and reads were taken every 12.7 cm at seven discrete depths. Tubes were allowed to equilibrate for at least 24 hours prior to testing and the probes were lowered slowly to read depths using a simple pulley system. A minimum settling time of two minutes at each depth was necessary to take accurate reads of each parameter. We also used a dye to observe mixing/stratification within the tube and found that the inflow tube caused flow disturbance resulting in water primarily diffusing in from the opposite side of the tube inflow. In order to account for discrepancies caused by the inflow tube's position on one side vs. the other, probe readings were established twice, once closest to the inflow tube and once on the opposite side.

Methods of spawning and culturing

Three adult *Hormiphora californensis* were placed in each inner cylinder (Fig. 3a). To induce spawning (Fig. 3b), the tubes were shrouded in a black, opaque cover for total darkness

overnight, followed by 2 hours of bright light the next morning (Kessil A360 Tuna Blue LED aquarium light placed ~5 cm above the inner cylinder) (Pang & Martindale 2008). When eggs were observed throughout the water column, usually ~12 hours post spawn or 24 hours after setup, the adults were removed from the tubes using a spoon attached to a short pole or by slowly swirling them to the surface.

Hatching of the eggs was observed 12 - 48 hours post spawning. Juvenile cydippids were fed on the first day post hatch (dph). Each tube was given a standardized concentration (~30 nauplii mL^{-1}) of live *Parvocalanus crassirostris* (Reed Mariculture Inc.) nauplii mixed with live algae (*Isochrysis galbana*, *Rhodomonas lens*, *Dunaliella tertiolecta*, or *Tetraselmis chuii*, Florida Aqua Farms, Inc.) in a 1:1 ratio, both cultured in the lab. *P. crassirostris* nauplii were added at the surface of the tube water column for six weeks using the following regimen: 25 mL 3 times per week (Sunday, Tuesday, Thursday) for the first 14 days, increasing to 50 mL for the next 14 days, and 100 mL for the last 14 days.

The ctenophores were observed throughout the water column and sampled once a week for six weeks to be photographed and measured. For photo documentation and measurement, 15 - 30 juvenile cydippids were randomly pipetted out of the water column down to 30 cm depth and transferred to a glass crystallizing dish filled with filtered seawater for photographing under a microscope (Zeiss Stereo Zoom V16, Canon EOS Rebel T5i). Each photograph was scaled and measured using ImageJ 1.52A (Schneider et al., 2012).

When cydippids reached ~0.5 - 1 cm in diameter (~6 weeks post hatch) they were transferred using widened disposable polyethylene pipettes (tips cut off, Transfer pipets 13-711-7M, FisherBrand) or small polypropylene beakers (50 - 100 mL Nalgene Griffin low form beaker)

from the diffusion tubes to kreisel tanks (Raskoff et al., 2003) modified to provide for better longevity of ctenophores. The kreisel screens were replaced with a polyethylene mesh with 4.0 mm openings (Model #N1670 PentairAES, Inc.). Slot openings in the supply boxes were reduced in width using a single piece of 4 mm corrugated plastic (high-density polyethylene and polypropylene) instead of two 6 mm pieces (Model #24244SC CorrugatedPlastics.Net). Pumps were set to provide ~ 10 liters per minute (Lpm) of volumetric flow to the lower supply box and ~7.5 Lpm to the upper box (Model DCW-2000 Jebao, Inc.) using flow meters (Blue-White Industries, Ltd, Model #F-44500L-8). The upper box provided ~0.8 Lpm of filtered seawater (5 µm) at 13°C. The diet was diversified to include 1 - 2 mysid shrimp (*Mysidopsis bahia*, Aquatic Indicators, Inc.) per comb jelly, usually ~50 per kreisel and 200 mL of *P. crassirostris* adult copepods, fed daily around 1200 PST.

Statistical analysis

A single factor ANOVA was used in Microsoft Excel to compare differences in mean total length between treatments. Weeks 5 and 6 were non-parametric and assessed further with a Kruskal-Wallis test. Growth rates were adapted to the data using the R (R Core Team, 2013) package Growthcurver (https://cran.r-project.org/package=growthcurver).

Results

Physical parameters and depth profiling

Temperature

Under the highest turnover rate (4.5 Lpm), the water column exhibited a significant thermocline within 12-24 cm of the surface. With a surface temperature between 16-17°C, the surface layer was approximately 3-4 degrees warmer than the water below 24 cm in depth (Fig. 4a). This was in agreement with dye observations, which indicated slower turnover in the top half of the water column under the 4.5 Lpm treatment. Although the other two treatments exhibited less dramatic thermoclines, the mean surface temperature exceeded the mean bottom temperature by at least 1.5 °C in all three treatments. Temperatures reported are the mean of the measurements taken on the two opposite sides of the inner cylinder, nearest and farthest from the inflow line. No significant difference was found between the near and far side measurements.

pH

pH was the most consistent of all parameters measured, ranging between 7.84 and 7.90 across all flow rates and depths, with the low flow treatment yielding the lowest pH at the surface of the water column (Fig. 4b). The low and high flow treatments exhibited slightly decreased pH at the surface layer.

Dissolved Oxygen

Dissolved oxygen measurements exhibited no significant differences with regard to depth, flow rate, or temperature. The high flow treatment showed slightly decreased dissolved oxygen at the

surface layer in conjunction with decreased pH, however the measurements were within the standard error of the probe (Fig. 4c).

Culturing success

All three treatments were successful in raising >50 adults of *H. californensis* over a six-week period. The high flow (4.5 Lpm) treatment yielded the highest survival (89), followed by medium flow (1.1 Lpm) (87) and low flow (2.2 Lpm) (75). High flow and low flow treatments reported the following growth curves respectively, K = 33.705, N = 0.026, r = 0.574 and K = 33.759, N = 0.013, r = 0.635. Growthcurver failed to find a best fit for the medium flow treatment. The growth rate under each treatment varied little for the first 4 weeks of growth (each $p > 0.07$) and then greatly increased during the fifth week (Fig. 5). Ctenophores in the high flow treatment had the fastest growth rates during weeks 5 and 6 ($p = 2.1$ E-3and 3.8 E-8, respectively), while the medium treatment had the slowest growth rate (Fig. 6).

Discussion

Our method utilizes a cylindrical tank shape which maximizes the ratio of water volume to the surface area of tank bottom, which has several important effects. By minimizing the bottom biofouling area, contact with these fouled surfaces is greatly decreased, vertical swimming or migration space for juveniles is maximized and a thermocline is produced where the juvenile cydippids can feed furthest from the tank bottom. Another key feature of our design is the passive exchange of seawater through the mesh which generates much lower water velocities

than in a traditional plankton kreisel. These lower flow rates allow delicate juvenile ctenophores to develop in a very gentle environment while still facilitating the exchange of clean filtered seawater. In contrast to traditional aquaria for gelatinous zooplankton, our method provides a superior pelagic environment for young ctenophores and other gelata larvae that is inexpensive and easily replicable.

Using a higher turnover rate generated a more pronounced vertical thermocline and resulted in higher survival and significantly faster growth rates among the treatments. It is not clear why the medium flow out performed the low flow treatment, perhaps further replication would provide resolution. Greater concentrations of *P. crassirostris* were observed at the surface of the inner cylinder in the high flow treatment and this appeared to be optimal for ctenophore feeding, helping to keep cydippids actively feeding near the surface and away from settled particulates at the bottom of the inner cylinder. Fouling was limited to minor algal compaction on the bottom mesh and cydippids did not appear to spend significant time there or become impacted.

Overall, fewer *H. californensis* were produced in this experiment than previous and subsequent trials with the diffusion tubes. We believe this is due to the restricted quantities of food used. When feeding quantity was increased from 50 mL to 100 mL at week 5 there was a notable increase in growth rate across treatments. It is also possible that lower yields may reflect using fewer parents (n = 3) than previous/subsequent trials (n = 10). Ultimately, we adopted a feeding regime that doubled the amounts of copepod nauplii (see Conclusions). An F4 population was achieved shortly after the experiment using 10 parents of the F3 population, yielding >600 cydippids.

Further development of this method has the potential for increased efficiency and enable use of the tube method with new species of pelagic gelata. Changing cylinder height and width may allow for more variation in thermocline profiles and vertical space, however taller tubes will require more volumetric flow to the outer cylinder. For larger ctenophore species diffusion tubes with larger diameters may have increased utility, for example *Leucothea pulchra* was successfully cultured to F2 using diffusion tubes 2 m high and 0.35 m diameter with a flow of ~4 Lpm (Fig. 7). *Bolinopsis infundibulum* and *Nanomia bijuga* were grown using an inner cylinder measuring 0.9 m high and 0.25 m diameter (outer cylinder was 0.9 m high and 0.30 m diameter) (Fig. 8). Changing the mesh size may impact diffusion rates between the two tubes, thermocline formation and perhaps be better fitted to a particular target species. For example, a 22 µm mesh was originally used for *P. bachei* but we found the mesh fouled quickly. When replaced with 55 µm mesh, diffusion and flow performance were enhanced and fouling no longer a problem.

Conclusions

After four years of trials culturing various gelata using different sizes of the diffusion tubes, we derived a standard procedure for mass culture of ctenophores from 1-10 parental adults using 1 m diffusion tubes (*Hormiphora* and *Pleurobrachia*) and a feeding regime based on results yielding >100 adult ctenophores in 4-5 weeks was adopted as follows:

I. To start a culture from wild collected adults, use 1 - 10 adult ctenophores depending on desired genetic diversity. Adults should be fed a fish or mysid meal 24 hours prior to spawning and again 1 hour before the dark period begins (Presnell et al., 2019).

II. To induce spawning, place all adults in an equilibrated diffusion cylinder (for inner diameter 20 cm, a flow rate of 4.5 Lpm), shrouded in opaque material to ensure complete darkness for a 4-hour period (or overnight). Uncover the tube and expose to bright lighting for at least 2 hours (Presnell et al., 2019).

III. When adults have spawned and eggs are visible in the water column, gently remove them from the tube using ladles or dishes.

IV. Eggs hatch within 12-48 hours, first feeding should ideally take place within the first 24 hours of hatching to ensure that cydippids are able to begin feeding immediately.

V. Feeding Table:

Time	Week 1	Week 2	Week 3	Week 4	Week 5
Frequency	3 times per week	3 times per week	3 times per week	3 times per week	3 times per week
Copepod nauplii	50 mL	100 mL	200 mL	400 mL	400 mL
Adult copepods	None	50 mL	100 mL	200 mL	400 mL

Feeding Notes:

a) Feeds may be skipped when copepods remain in the tube uneaten.

b) When copepods remain uneaten in the tube, live algae (*Isochrysis*, *Tetraselmis*, *Rhodomonas* etc.) may be added to the next feed as an equal volume to the feed. This helps keep the remaining feed enriched and growing.

c) Feed may be placed in a beaker and slowly dripped into the top of the inner tube using a drip emitter or similar device (DYNALON clamp, part #670715)

VI. At 4-5 weeks post hatching the cydippids may be moved to a pseudo-kreisel or kreisel tank. If cydippids have not achieved a total body size of 0.5 - 1 cm diameter by this time, their residence in the tube may be increased to as long as 6 weeks. However uniformly slow growth rates are a sign of a weak hatch, not enough food and/or significant disruption in water flow/diffusion through the tube. As residence time increases in the tube, so will impaction of the screen.

Gelatinous zooplankton require physically stable conditions to grow *en masse*. Our tube design maximizes water exchange through an efficient and gentle diffusion profile, reduced fouling, increased residence time of live prey, and significant total water turnover with very little or no mechanical turbulence. The diffusion cylinder method can be a critical tool for laboratory use in the cultivation of a variety of pelagic gelata species and thus contribute to a range of research in these fragile organisms on population genetics, zooplankton plastivory, behavior and ecology.

Acknowledgements

We thank Dr. W.E. Browne for his enthusiastic collaboration, comments on draft manuscript and support of ctenophore culturing here at the aquarium. The DEEP-C project for creating a joint ctenophore project that introduced us to Dr. Browne and other ctenophore researchers. Shannon Johnson for her comments on the draft manuscript. John Negrey for instrument calibration and

support. John Hoech, Paul Clarkson, Marcus Zevalkink, Sarah Halbrend, Evan Firl, the Drifters gallery staff and volunteers that contributed their time and resources to this project.

Funding

Funding was provided by the Monterey Bay Aquarium Foundation.

References

Dunn CW, Hejnol A, Matus DQ, Pang K, Browne WE, Smith SA, Seaver E, Rouse GW, Obst M, Edgecombe GD, Sørensen MV. Broad phylogenomic sampling improves resolution of the animal tree of life. Nature. 2008 Apr;452(7188):745.

Greve W. The “planktonkreisel”, a new device for culturing zooplankton. Marine Biology. 1968 Mar 1;1(3):201-3.

Greve W. Cultivation experiments on North Sea ctenophores. Helgoländer wissenschaftliche Meeresuntersuchungen. 1970 May;20(1):304.

Greve W. The “Meteor Planktonküvette”: a device for the maintenance of macrozooplankton aboard ships. Aquaculture. 1975 Jul 1;6(1):77-82.

Gyllenberg G, Greve W. Studies on oxygen uptake in ctenophores. InAnnales Zoologici Fennici 1979 Jan 1 (pp. 44-49). Finnish Academy of Sciences, Societas Scientiarum Fennica, Societas pro Fauna et Flora Fennica and Societas Biologica Fennica Vanamo.

Hamner WM. Design developments in the planktonkreisel, a plankton aquarium for ships at sea. Journal of Plankton Research. 1990 Jan 1;12(2):397-402.

Hirota J. Quantitative natural history of Pleurobrachia bachei in La Jolla Bight. Fish. Bull. 1974 Jan 1;72:295-335.

Howes EL, Bednaršek N, Büdenbender J, Comeau S, Doubleday A, Gallager SM, Hopcroft RR, Lischka S, Maas AE, Bijma J, Gattuso JP. Sink and swim: a status review of thecosome pteropod culture techniques. Journal of Plankton Research. 2014 Feb 10;36(2):299-315.

Jaspers C, Møller LF, Kiørboe T. Reproduction rates under variable food conditions and starvation in Mnemiopsis leidyi: significance for the invasion success of a ctenophore. Journal of Plankton Research. 2015 Mar 18;37(5):1011-8.

Jaspers C, Marty L, Kiørboe T. Selection for life□history traits to maximize population growth in an invasive marine species. Global change biology. 2018 Mar;24(3):1164-74.

Martí□Solans J, Ferrández□Roldán A, Godoy□Marín H, Badia□Ramentol J, Torres□Aguila NP, Rodríguez□Marí A, Bouquet JM, Chourrout D, Thompson EM, Albalat R, Canestro C.

Oikopleura dioica culturing made easy: A Low□Cost facility for an emerging animal model in E vo D evo. genesis. 2015 Jan;53(1):183-93.

Pang K, Martindale MQ. Mnemiopsis leidyi spawning and embryo collection. Cold Spring Harbor Protocols. 2008 Nov 1;2008(11):pdb-rot5085.

Presnell JS, Browne WE. Krüppel-like factor gene function in the ctenophore Mnemiopsis suggests an ancient role in promoting cell proliferation in metazoan stem cell niches. bioRxiv. 2019 Jan 1:527002.

Presnell JS, Bubel M, Knowles T, Patry W, Browne WE. Multigenerational laboratory culture of pelagic ctenophores with special focus on the invasive coastal lobate *Mnemiopsis leidyi*. Nature Protocols, in revision. 2019.

Team RC. R: A language and environment for statistical computing.

Raskoff KA, Sommer FA, Hamner WM, Cross KM. Collection and culture techniques for gelatinous zooplankton. The Biological Bulletin. 2003 Feb;204(1):68-80.

Schneider CA, Rasband WS, Eliceiri KW. NIH Image to ImageJ: 25 years of image analysis. Nature methods. 2012 Jun 28;9(7):671.

Sprouffske K, Wagner A. Growthcurver: an R package for obtaining interpretable metrics from microbial growth curves. BMC bioinformatics. 2016 Dec;17(1):172.

Figure 1

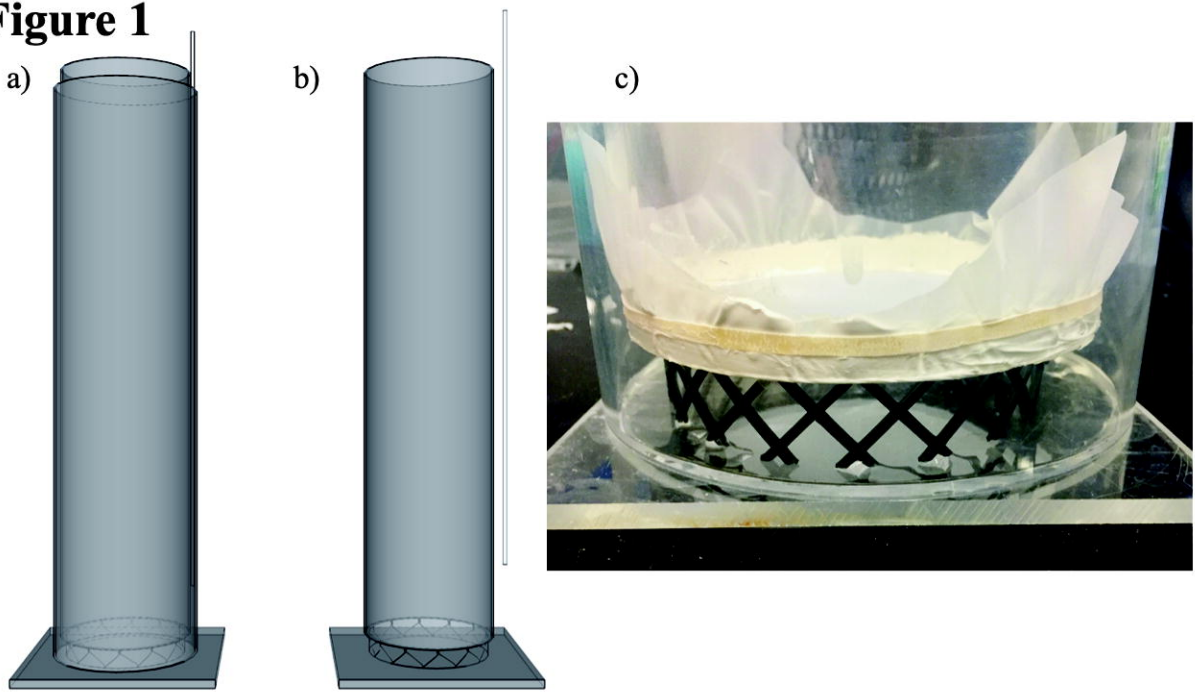

Fig. 1 Rendering and construction of the diffusion tubes. (**a**) both tubes are visible, with supply tubing at the top right. (**b**) outer tube has been removed to reveal the inner tube resting on the riser, made with rigid plastic mesh. Water supply tubing is entirely visible on the right, note how tubing does not reach to the bottom to avoid entrainment of eggs into the screen. (**c**) bottom portion of tube shown with plastic riser. A rubber band may be used to secure the mesh for gluing with silicone sealant, excess mesh can be cut off. Care should be taken when lowering the inner tube to avoid tearing the mesh on the riser. Riser should be cut to the exact diameter of the inner tube so the mesh does not rest on the riser.

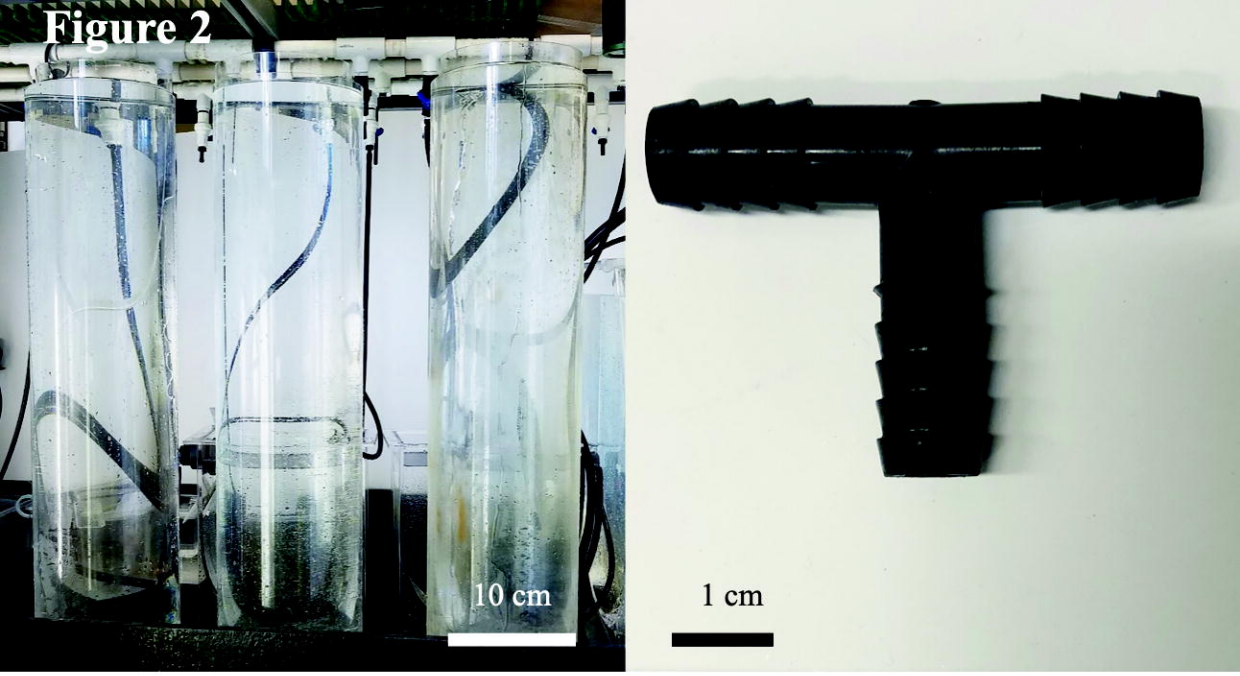

Fig. 2 (**a**) Experimental setup; three sets of diffusion cylinders on a wet table. (**b**) Tee fitting used to prevent entrainment of eggs on screen.

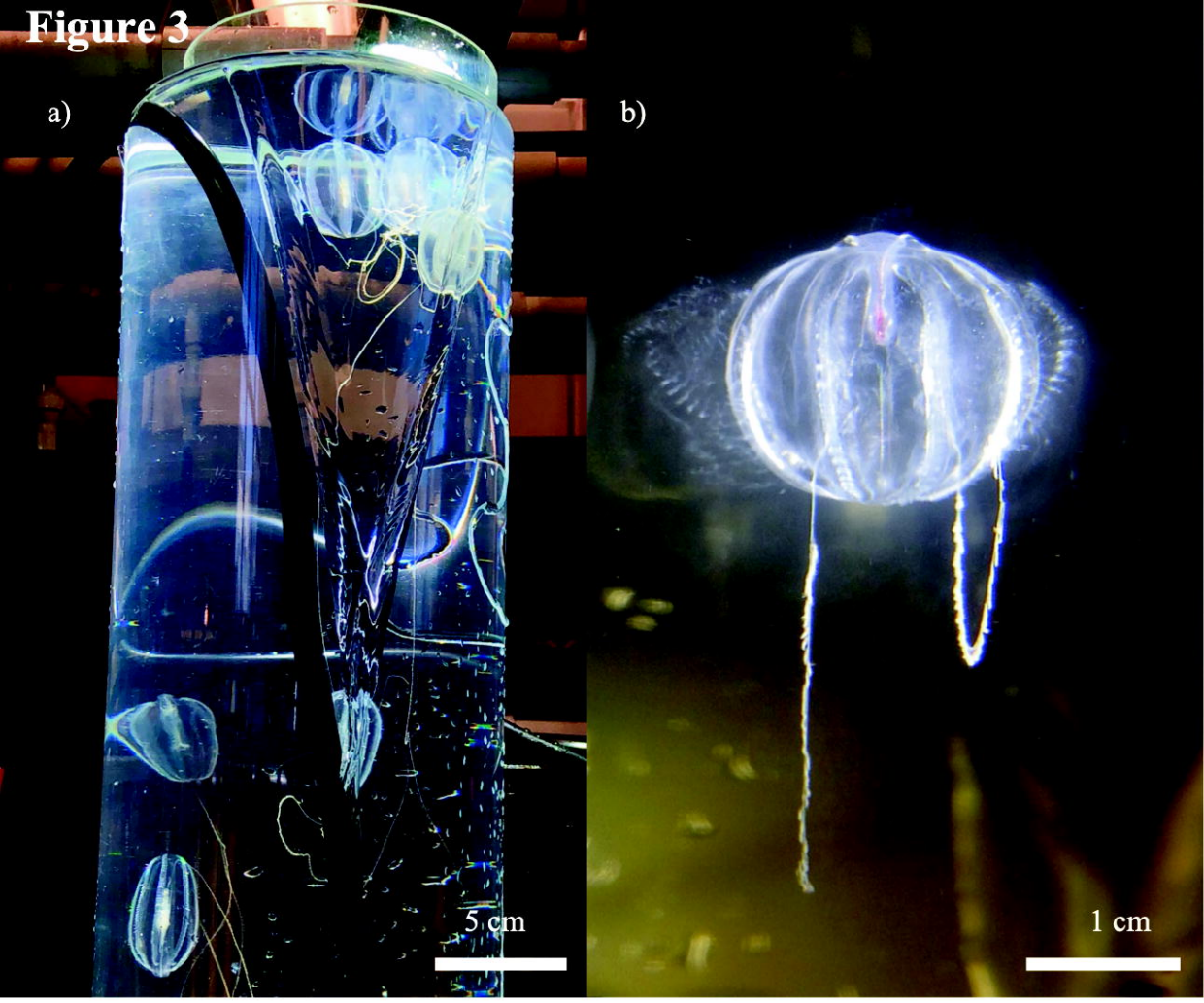

Fig. 3 (**a**) *Hormiphora californensis* spawning in the inner cylinder with a bright LED light. (**b**) *Pleurobrachia bachei* releasing sperm from the meridional canals beneath the ctene rows.

Figure 4

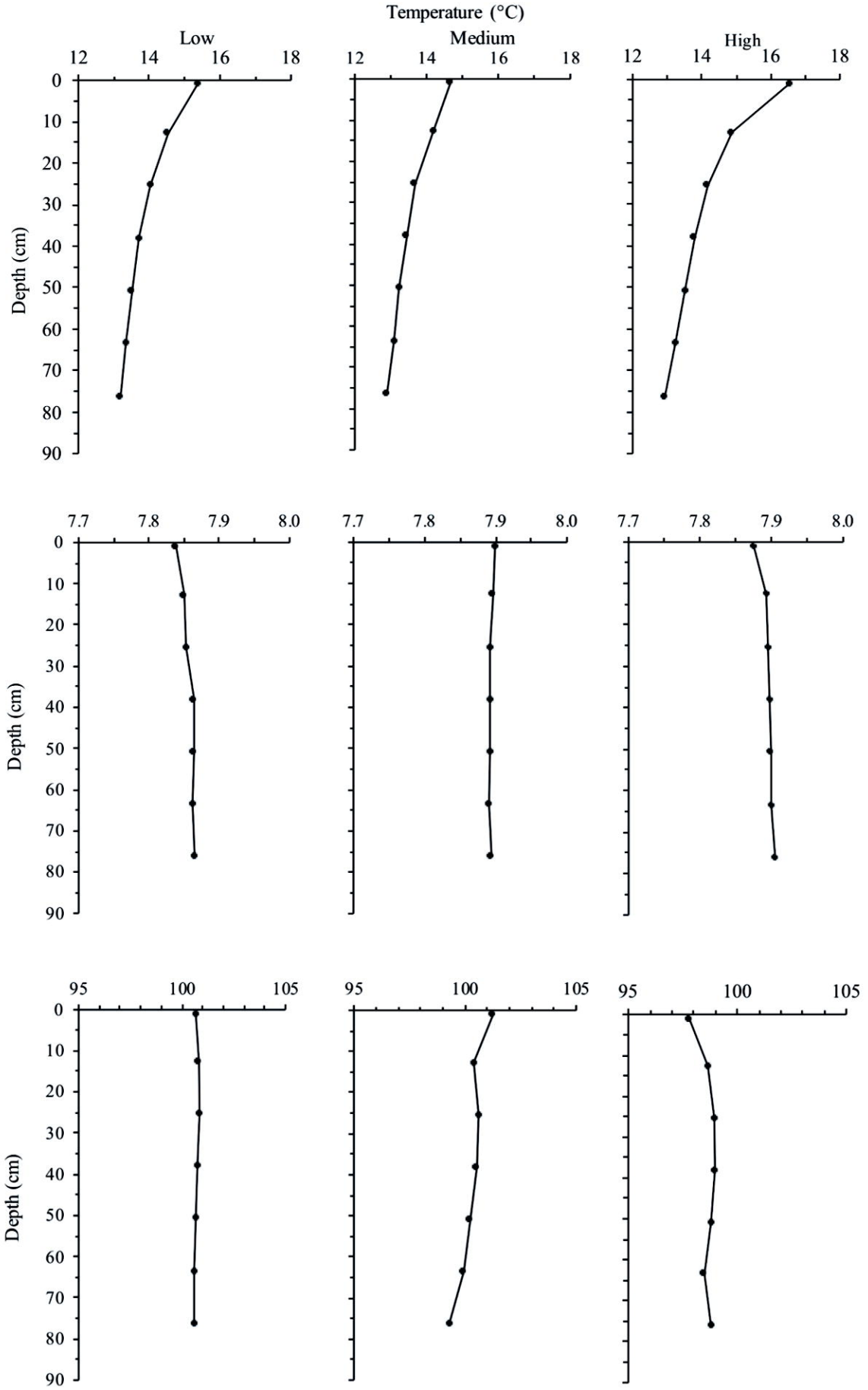

Fig. 4 Depth profiles of each treatment for temperature, pH and dissolved oxygen. Line is the mean of the two measurements taken on opposing sides of the inner tube.

Figure 5

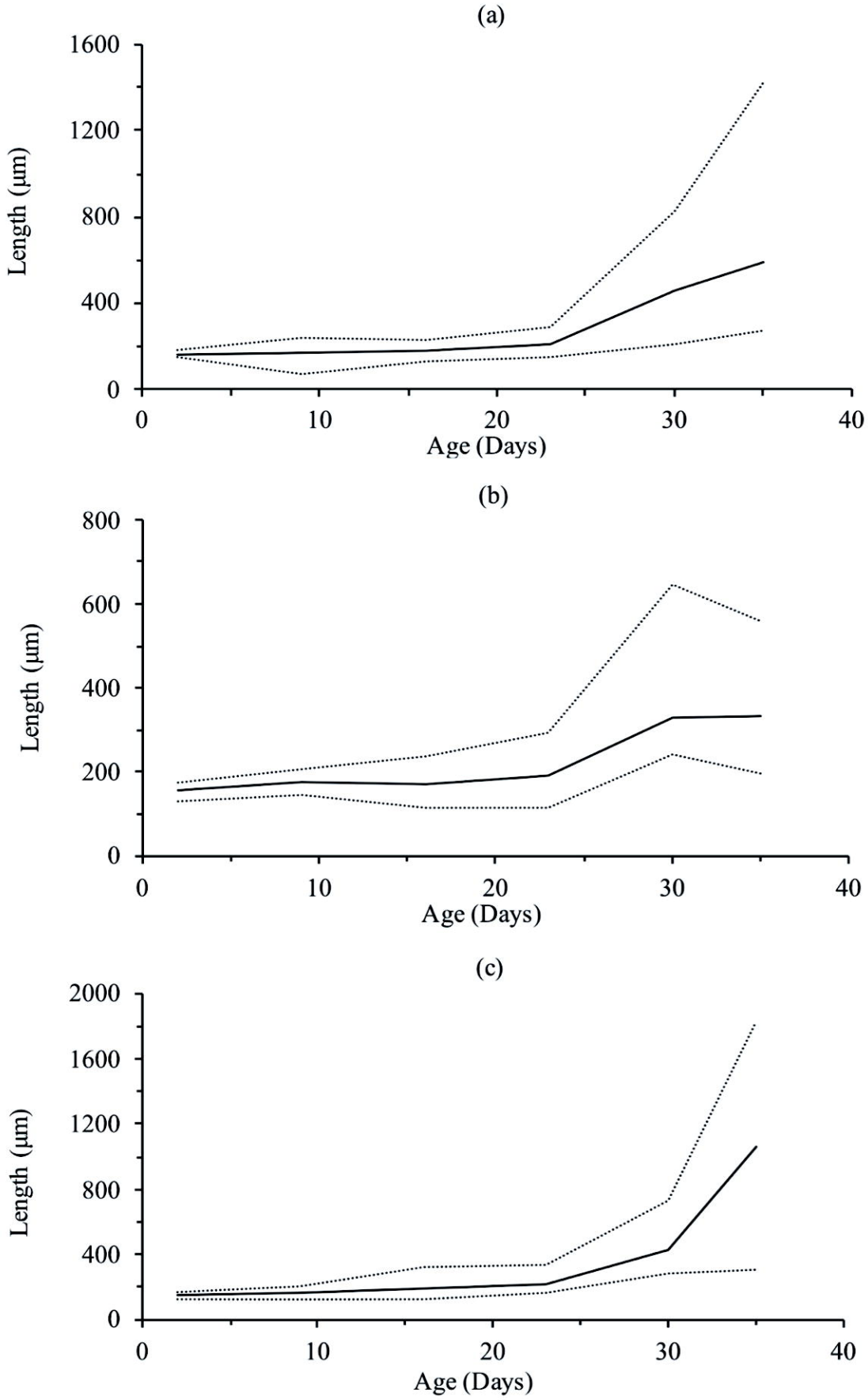

Fig. 5 Average (solid line), minimum, and maximum (dotted lines) growth rates of *H. californensis* in low (**a**), medium (**b**), and high (**c**) flow treatments.

Figure 6

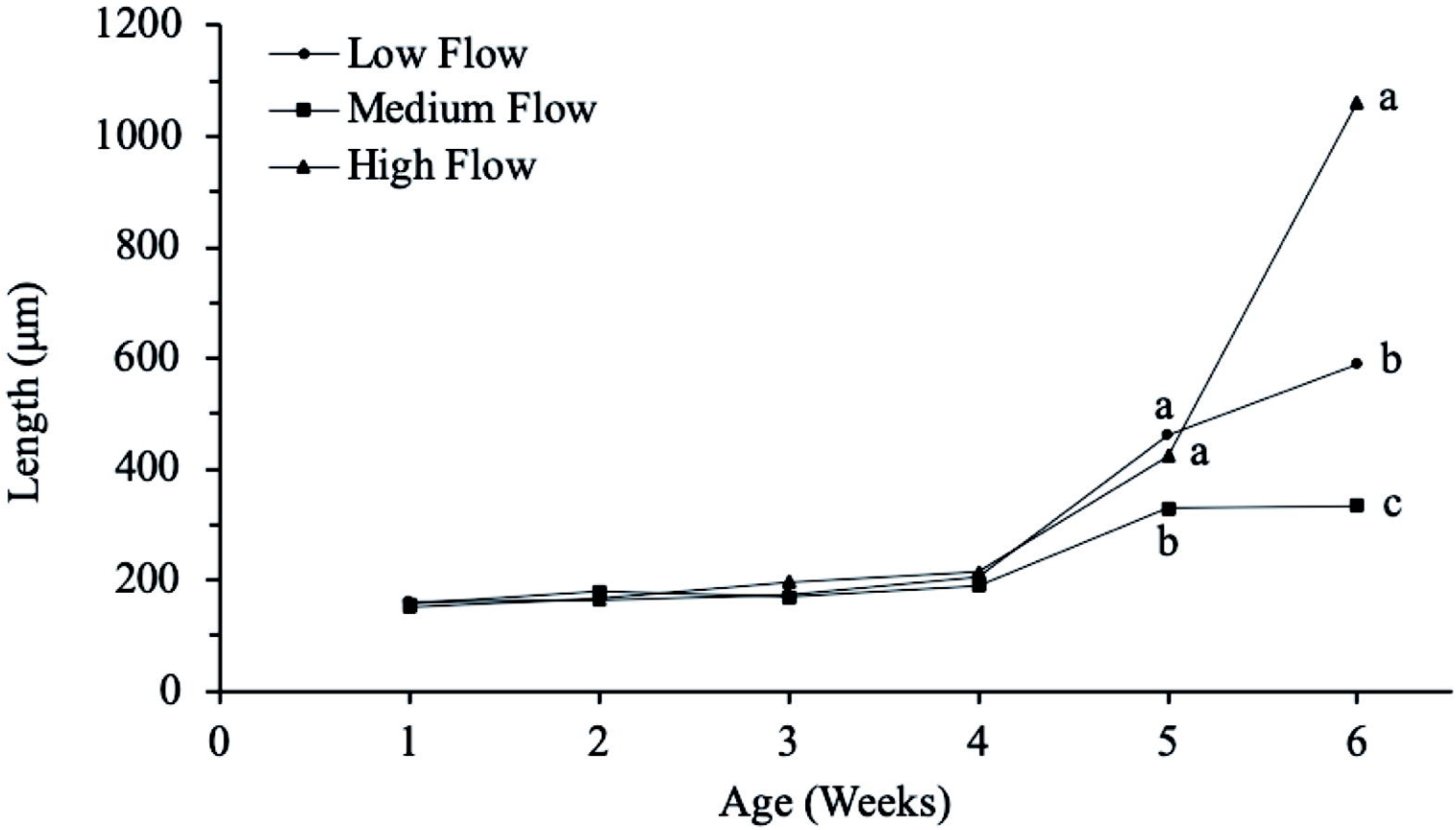

Fig. 6 Mean growth rate for the low (circles), medium (squares), and high (triangles) flow treatments. At week 5, high and low treatments (**a**) were significantly higher than medium flow (**b**) ($p = 0.0021$). At week 6, all three treatments were significantly different from each other (**a**,**b**,**c**) ($p = 0.00000004$).

Figure 7

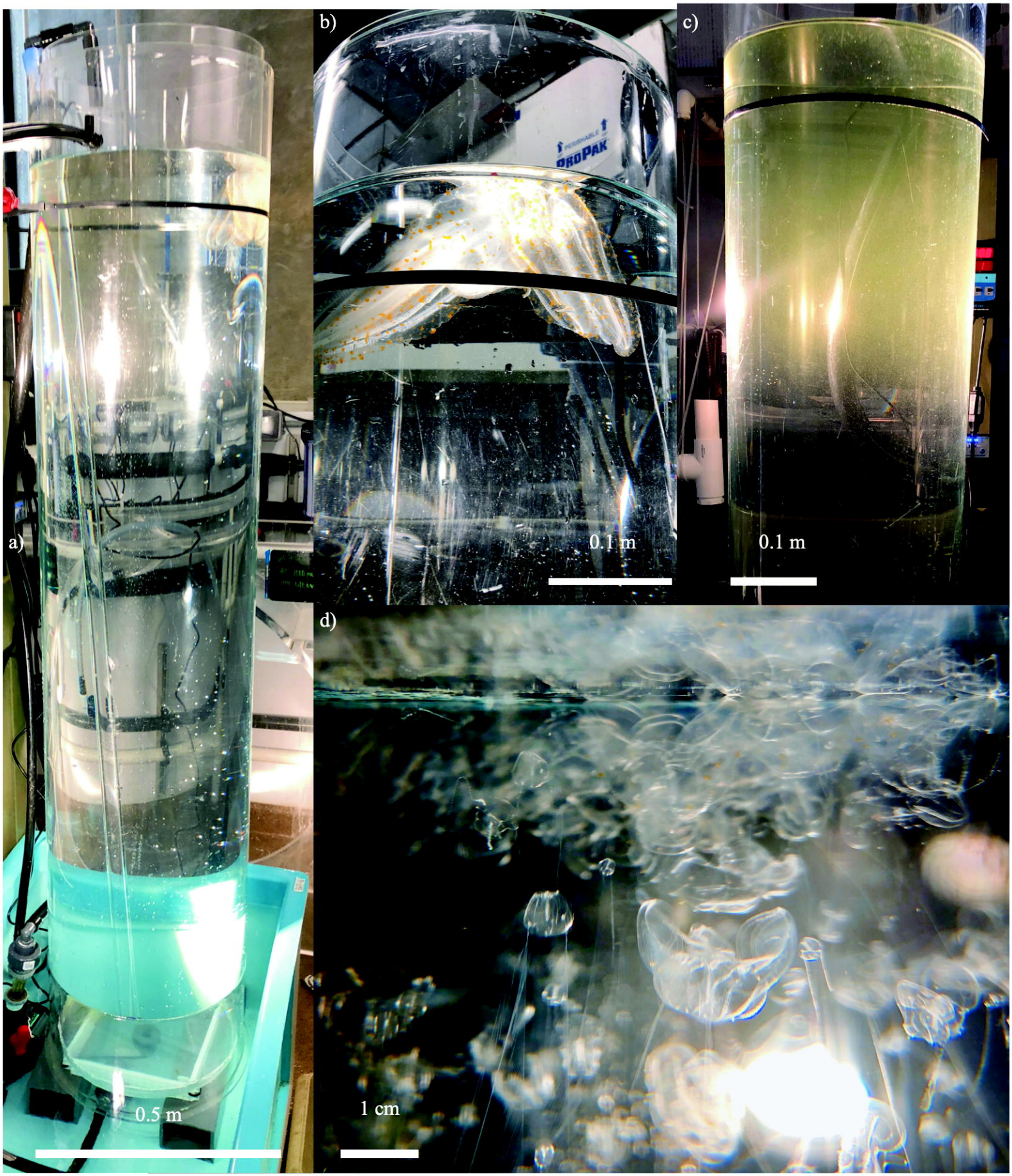

Fig. 7 (**a**) Two meter tall diffusion tubes for rearing *Leucothea pulchra* sitting inside of a 200 L reservoir. (**b**) three large adults were spawned to produce the F1 generation. (**c**) thermocline visible at the top of tube, 9 dph (**d**) various srages of F1 larval *L. pulchra* visible at the surface of the inner tube, 21 dph.

Figure 8

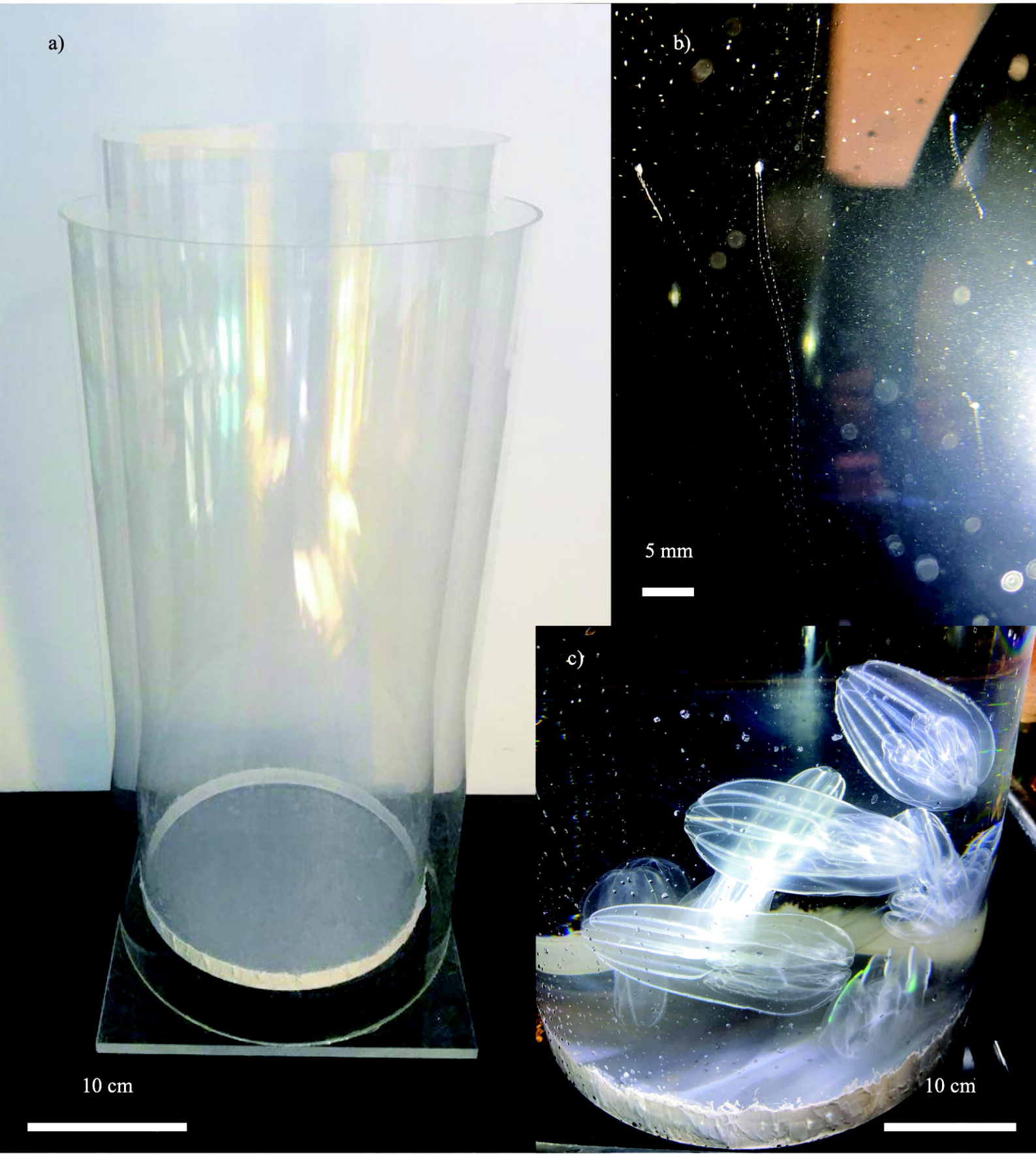

Fig. 8 (**a**) 30 cm wide diffusion tubes used for *Bolinopsis infundibulum* and *Nanomia bijuga* culture. (**b**) larval *N. bijuga* at 35 days post settlment visible near the top of the tube (**c**) mature *B. infundibulum* spawning under bright light, the wider and shorter tube makes it easier to place and remove the adult lobates.