A Guide to the Meso-Scale Production of the Copepod *Acartia tonsa*

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This manual is based on research supported by three separate agencies: the United States Department of Agriculture-Agricultural Research Service (ARS) through the Harbor Branch Oceanographic Institution (HBOI) via a sub-contract (#20021007) to N. Marcus, G. Buzyna, and J. Wilcox , the State of Florida Department of Agriculture through a grant to the Mote Marine Laboratory and a sub-contract (MML-185491B) to N. Marcus; and a grant from the Florida Sea Grant College Program (project R/LR-A-36) to N. Marcus.

Appreciation is also expressed for the labors of Alan Michels, Patrick Tracy, Chris Sedlacek, Cris Oppert, Laban Lindley, Guillaume Drillet, and Glenn Miller, as well as for the support of the Florida State University Marine Laboratory staff.

This publication was supported by the National Sea Grant College Program of the U.S. Department of Commerce's National Oceanic and Atmospheric Administration (NOAA), Grant No. NA16RG-2195. The views expressed are those of the authors and do not necessarily reflect the view of these organizations.

TP 156 January 2007

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I. Introduction

This manual is intended as a guide for the daily production of a few million *A. tonsa* nauplii for feeding to marine vertebrates and invertebrates. This scale of production is greater than most research would require, but smaller than commercial production, hence the term meso-scale production. This manual will briefly describe the biology of *Acartia tonsa* Dana that is relevant to culture, the culture methodology for meso-scale production of their eggs and nauplii, the system components utilized in production, and how to construct a few simple tools useful for this scale of production. Commercial production of copepods requires much greater feed production than is described, or the development of an efficient artificial feed, and, therefore, is not the focus of this manual.

Throughout this manual "*Acartia*" and "*Acartia tonsa*" are used interchangeably; as if there were only one species of *Acartia*. This is for simplicity of writing only. It is understood that there are other species in this genus; and procedures for culturing *A. tonsa* may or may not work for any other species of *Acartia*. We make no claims either way.

Meso-scale production, in this manual, refers to the process of batch culturing adult *A. tonsa* copepods with the goal of harvesting a few million eggs per day, during peak production; and to the staging of batch cultures to provide a continuous supply of nauplii. Depending on the species being fed and the hatch ratio of nauplii from eggs, sustaining from 5,000 to 20,000 fish larvae from the productivity of a half dozen 200L tanks is possible. Research conducted at FSU has shown for southern flounder (*P. lethostigma*), that copepods may be "diluted" with rotifers while still retaining the beneficial effects of a copepod-rich diet (Wilcox et al 2006). Thus, the potential to sustain even greater numbers would exist for other species that can consume both effectively.

While copepod nauplii are typically used to feed early life stages of larval marine fishes, they are appropriate for many other culture purposes. *A tonsa* are also readily consumed, both as nauplii and as adults, by economically important invertebrates, such as corals and stone crabs (*M. mennippe*).

A. tonsa was selected as the target species for culture at Florida State University due to their cosmopolitan distribution, their relatively hardy nature in culture, and their beneficial nutritional profile. *A. tonsa* freely release eggs that are slightly negatively buoyant, therefore collection of eggs is simple; and the eggs from our local stocks tolerate storage for two to six weeks. A different copepod species may be more nutritious, or even required, for the growth of a specific fish or invertebrate; but few other copepods are eaten by as many different species as *A. tonsa.* In North Florida, *A. tonsa* is the dominant coastal copepod during the summer months, thriving in dense populations, which makes wild collection easy, and indicates a tolerance for crowding that is useful in culture conditions.

II. Biology of *Acartia tonsa* Dana 1849

Figure 1. *Acartia tonsa*

Acartia tonsa (Figure 1) are pelagic calanoid copepods (Crustacea/Copepoda/Calanoida/ Acartiidae). *A. tonsa* are distributed worldwide, occurring in the Atlantic, Indian, and Pacific oceans, and the Azov, Baltic, Black, Caspian (a recent invader), and Mediterranean seas. They are euryhaline and eurythermal; and tolerate salinities ranging from 1 ppt to 38 ppt, and temperatures ranging from 0^0C to 30^0C (Mauchline, 1998). They are neritic, being most commonly found near-shore in 0 to 20 m depths, but have been reported from depths to 600 m. It is the dominant estuarine calanoid copepod in our region of the Gulf of Mexico, being present year-round, but exhibiting seasonal population density fluctuations. *A. tonsa* reproduces throughout the year in North Florida.

Adults are approximately 1.5 mm in length, and their N1 nauplii are approximately 70 μm in length. *Acartia* eggs are 70-80 μm in diameter, spherical, covered with short spines, and are slightly heavier than seawater. At 25^0C, most eggs hatch to nauplii within 48 h. Nauplii progress through six stages (N1 through N6) to become copepodites, which then progress through six stages (C1 through C6) to become sexually mature adults (C6).

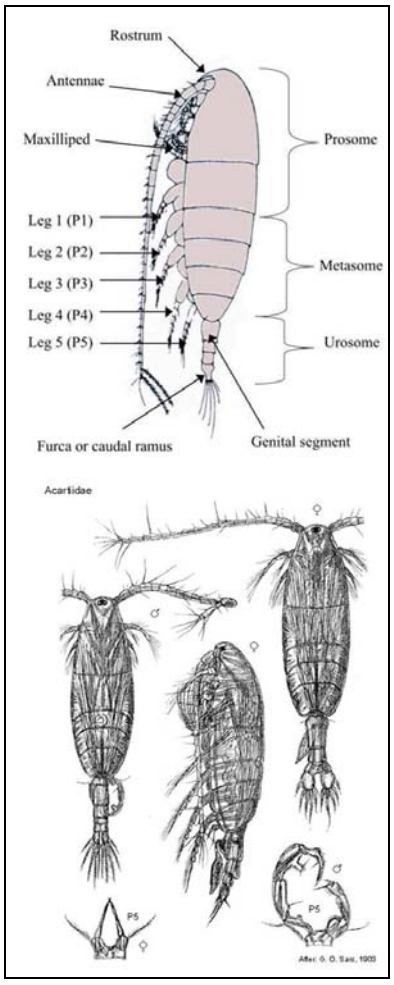

Figure 2. *Male and female morphological feature.*

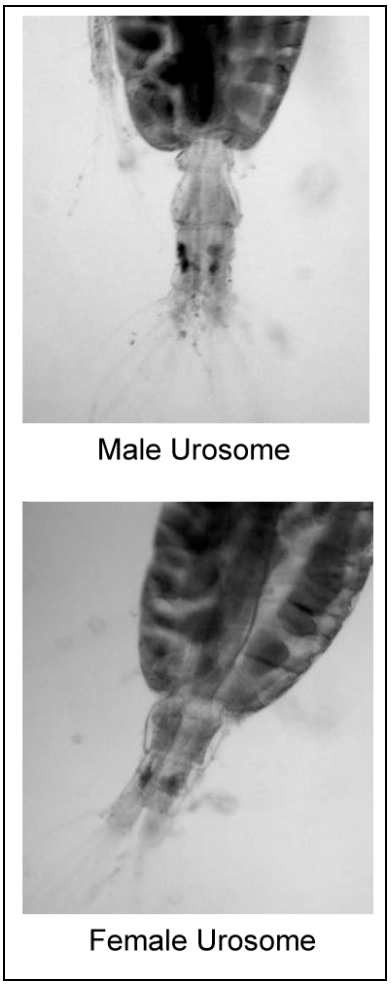

Figure 3. *Male and female urosomes.*

Adult males and females are visually distinguished by conformation of their antennae, urosome, and swimmerets (Figure 2, 3, Sabatini 1990). Males use their antennae to clasp a female to allow them to deposit a spermatophore on the female urosome (Figure 3) for fertilization. Males live shorter lives than the females (Parrish and Wilson 1978, Sazhina 1987) which has an impact on egg viability in maturing batch cultures. Females release an average of 18-50 eggs per brood (Mauchline, 1998) every 5-6 days (Sazhina 1971), and can produce up to 718 eggs in their lifetime (Parrish and Wilson 1978). *A. tonsa* are broadcast spawners, and do not carry their eggs as some copepods do. This allows for easier egg collection and storage.

Acartia primarily feed on phytoplankton, but also consume ciliates, rotifers, and their own eggs and nauplii (Mauchline, 1998). Consumable prey size ranges from 5 μm to 100 μm (Petipa, 1959). *Acartia* are typically fed phytoplankton, because of the ease of culturing this food source. Our laboratory uses two species of cryptomonads

(*Rhodomonas spp.*) and some dinoflagellates (*Akashiwo, Oxhyrris,* and *Prorocentrum*), and this has worked well. *Acartia* have been reported to survive on microalgal cultures of *Chaetoceros*, *Isochrysis*, and *Tetraselmis* (Apeitos et al 2004).

Copepod populations were selected from plankton collections done in Apalachee Bay, Florida ($29^0$52'N, $84^0$26'W). *A. tonsa* can be collected here by plankton tow any time of the year, but are most common during the warmer months. Adult *Acartia* were manually selected from plankton concentrate with the aid of a dissecting microscope.

III. Use of Copepods in Aquaculture

Many marine fishes produce tiny larvae following an abbreviated hatching cycle. These exceedingly small larvae necessarily require even smaller prey to fit in their developing mouth and gut. Unlike the brine shrimp, *Artemia salina,* and the marine rotifers, *Brachionus spp.*, marine fish larvae regularly encounter *Acartia* nauplii in the wild, and many species are adapted to digest these nauplii and similar organisms. Due to the abundant populations, size, and nutritional profile of calanoid copepod nauplii, they are the primary first-feed for many marine species, both vertebrate and invertebrate (Lee et al, 2005). Additionally many adult vertebrate and invertebrate species of great economic value, such as the seahorses (an ornamental and medicinal commodity) or the corals (national treasures and tourist commodities) readily consume copepods.

Studies have shown that for a number of marine fish species, diets including copepod nauplii result in better development, growth, nutritional content, and survival of the first-feeding larvae compared to diets consisting solely of rotifers and *Artemia* (Watanabe et al. 1983; Støttrup et al. 1986; Kraul et al. 1992; Støttrup and Norsker 1997; Schipp et al. 1999; Shields et al. 1999; Støttrup 2000; Payne et al. 2001; Evjemo et al. 2004). Copepods have been shown to have a natural omega-3 profile, which exceeds that provided by enriched rotifers or enriched *Artemia* (Sargent et al., 1997).

The recent publication of *Copepods in Aquaculture* by Lee, O'Bryen, and Marcus (2005) consolidates much of the recent information on copepod culture and use. To quote Blackwell Publishing:

> "(It)… includes review articles and papers presented by leading international experts in copepod biology and aquaculture. It … integrates the most up-to-date information on selecting copepod species, effects of algal species on reproduction, ways to increase production, the nutritional value of copepods, behavioral characteristics of copepods, potential use of copepod nauplii and eggs, and their application to larval rearing of various marine finfish species."

While the majority of aquaculture of *Acartia* is for early feeds for fish larvae and, to a lesser extent, adult feeds for ornamental species; there also exists a niche market for bioassay *Acartia tonsa*. These protocols, as presented, do not conform to bioassay production standards; but only slight modifications would be required to achieve bioassay standards.

IV. Production of *Acartia tonsa*

A. Overview

Batch culture of *A. tonsa* copepods is relatively straightforward, once proper environmental and nutritional conditions are met. Start with a clean 300L tank, algae, and filtered, UV-treated seawater. Tanks can be stocked with nauplii or copepodites. Clean and feed the tank daily. Adults will begin producing eggs and sperm in 9-12 days; thereafter egg production will rise, peak, and fall. Separate eggs, nauplii, and adults from the detritus daily, using graded sieves, then return adults and nauplii to the tank. Clean the eggs and discard detritus. Prepare eggs for immediate use or storage.

Once the hatching success falls below 75%, it is time to terminate the culture batch. For continuous production of nauplii, start sequential batch cultures at 5-7 day intervals. When timed correctly, one tank of a series will be at maximum productivity at any given time.

B. Production Stipulations

1. *A. tonsa* (and copepods in general) are fragile aquatic organisms that do not respond well to rough treatment, vigorous aeration, or the crushing effect of gravity.
2. Tank aeration creates bubbles which pop and launch copepods onto the tank cover and sidewalls. Water surface tension holds them there until dead. Aeration should be the least volume able to maintain >4 mg/L dissolved oxygen (DO). If the surface has an apparent "boil" over the airstone, air volume is too high.
3. Male *Acartia* utilize their antennae to grasp the female for mating. Rough handling, resulting in breakage of antennae, will reduce the apparent number of males in the batch and the overall production of viable eggs. Handle animals carefully.
4. Locally acclimated, wild collected *Acartia tonsa* may have slightly different thermal and salinity preferences. Adjust the protocols accordingly.
5. *Acartia* culture is not a monoculture, expect ciliates and other organisms.
6. Algae is cultured at 30 ppt, if culturing at lower salinity, add freshwater proportionately to the total feed volume to maintain stable salinity.
7. Artificial seawater is as productive as natural seawater for copepod culture, but is usually more expensive.
8. *A. tonsa* nauplii are usually fed 15,000 cells/ml *Rhodomonas lens* and 15,000 cells/ml *R. salina* once daily. Advanced copepodites and adults receive 25,000 cells/ml of each *Rhodomonas* species, with an occasional feeding of *Akashiwo sanguinea* at 150 cells/ml.

C. Harvest Stipulations

1. Always fill the stacked-sieve holder and wash bottles with treated seawater at culture tank temperature. Do not siphon copepods onto a dry freestanding sieve.
2. Always wet the Nitex mesh and remove entrapped air bubbles, before siphoning.
3. Always keep the sieves submerged. Copepods were not designed to tolerate gravity.
4. Water velocity through the sieves should never crush the copepods against the mesh.
5. As the tank water level drops, frequently rinse down copepods stuck to the sidewalls.
6. Water quality, DO, and temperature must remain stable for culture times to remain consistent and predictable. Use digital controllers for all heaters and chillers.
7. Exchange 30% of the tank volume weekly with new treated seawater to prevent metabolite build-up.

D. Air Quality Control

1. Microfilter all air entering algae and copepod culture vessels.
2. Draw cool, filtered, interior air for aeration, whenever possible. Contamination issues will increase when using exterior air, increasing filter replacement costs.
3. Use disposable in-line 0.2 μm pore anti-bacterial air filters (Pall 37mm or Gelman Acro50 vent filters) for all aeration.
4. Maintain *Acartia* at >4 mg/L DO while trying to cause minimal damage to or loss of the organisms.
5. Aerate to maintain algae culture CO_2 saturation, pH stability, and uniform mixing; while trying to cause minimal damage to or loss of the organisms.

E. Water Quality Control

1. Monitor incoming seawater for salinity, pH, DO, color, and scent (particularly the "rotten egg" smell of H_2S) prior to collection and treatment. Log all coastal "Red Tide" and other harmful algal bloom advisories. Record all readings in a waterproof journal.
2. Serially filter raw seawater through 50 μm, 10 μm, and 1 μm mesh bags then pass through an ultraviolet sterilizer (at no more than half the maximum flow rating). Filter set and ultraviolet sterilizer are mounted on a garden cart for portability around the facility (Figure 4).
3. Filter bags are cleaned, then sanitized overnight in hypochlorite solution once a week under normal usage (more often, if needed).
4. Follow manufacturer recommendations regarding UV bulb replacement.

Figure 4. *Filter set and ultraviolet sterilizer.*

5. Maintain a ten-day reserve of filtered seawater and a reserve of artificial seawater mix. Never use questionable seawater for cultures, use the reserve or artificial mix.
6. Filtered, UV treated seawater can be used directly to culture copepods (once aerated to saturation with 0.2 μm filtered air) or chlorine treated prior to other use.
7. Treat container of filtered seawater with 10% commercial hypochlorite solution at 0.2 ml/L, let stand overnight without aeration. Dechlorinate with stock thiosulfate solution volume for volume (V/V) at 0.2 ml/L. Use this water for filling all wash bottles, stacked sieve holders, harvest samples, population counts, egg hatching trials, etc.

F. Production of Eggs and Nauplii

1. System and Equipment Preparation

1. The copepod culture tank is shown in Figure 5. If starting with a brand new tank, wash the interior of the 300 L Fiberglas tank with a low-residue laboratory detergent (e.g. Alconox or Sparkleen) and water. Rinse thoroughly. Then treat with 100% muriatic acid (HCl) solution *outdoors*. Rinse thoroughly.
2. Whenever working with acids, observe all appropriate safety precautions, review acid safety protocols, maintain flowing freshwater within reach at all times. Be very careful and wear proper protective gear. (Do not use muriatic acid indoors: chlorine gas (Cl_2) may be generated.)

Figure 5. *300L production tank*

3. Leach the tank three times (24 h each time) to remove all water-soluble remnants of the manufacturing process.
4. If using an aged tank, wash thoroughly with low-residue laboratory detergent (e.g. Alconox or Sparkleen) and water. Clean with 100% muriatic acid, as needed (see precautions: E.1.1). Rinse thoroughly.
5. Initiate hatching of *A. tonsa* eggs 48 h prior to projected stocking time. Hatch approximately 50,000 eggs per liter in a clean 20L carboy.
6. Assemble all needed items: tank, cover and brace, air line and stone, harvest siphon, stacked sieves, sieve holder, drain line, wash bottles, beakers, egg storage tubes, dual 40W Cool-White fluorescent fixture, light timer, digital temperature controller, aquarium heater(s).

7. Prior to chlorination of the system, place aquarium heaters (dial heater to hottest settng or to "always on"), digital controller thermal probe, airline with airstone mounted, and harvest squeegee in the tank for chemical sanitary treatment of all immersed plumbing and wiring.
8. Fill the tank to the rim with filtered, UV-treated seawater, adjust the salinity as needed, and chlorinate the tank with 60 ml (0.2 ml/L) commercial 10% Hypochlorite solution per liter. This yields ~1 ppt free chlorine. Do not start aeration.
9. Filling the tank to 200 L working depth prior to chlorination is unacceptable, the freeboard sidewalls and cover underside will not be sanitized.
10. Allow treated system to stand for 24 h. Thereafter, de-chlorinate with 60 ml of stock Thiosulfate solution. Start vigorous aeration.
11. After an hour, dip a 'free chlorine' test strip and read to verify zero 'free chlorine' remaining. Aerate the treated seawater to at least 6 mg/L DO.

2. Culture Start-up Protocol

Once the equipment is clean, sanitized, and ready to use, complete the following steps:

1. Plug in the digital thermal controller and set to 25^0 C (or other selected temperature), check that the thermal probe for the controller is located 40 cm away from heater. Plug aquarium heater(s) into the digital controller.
2. Plug airline into 0.2 μm air filter, attach airline from pump. Turn on pump and adjust air to vigorously agitate the tank (while no copepods are present).
3. Allow tank to come to stable 25^0 C temperature. In continuously hot conditions, replace the heater with a chiller; the stable temperature concept is the same.
4. Plug in a timer for the fluorescent lights set to 14 h light /10 h dark (summer). Lights are mounted directly above the tanks to maximize light intensity for prolonged alga survival. Remember that light intensity drops by the square of the distance from the source.
5. Count cell density of algae in production using a hemocytometer. Follow hemocytometer instructions. Count three samples for each species of feed, calculate averages, and compute cells/ml by container. Record cells/ml by species of algae, container, and date in an Algae Log Book.
6. Compute volume of each species of algae required to feed a 200 L tank at a concentration of 15,000 cells/ml for each algal species (naupliar feed density). Add each required feed volume to obtain total volume of feed needed to add to the tank to achieve proper feed concentrations.
7. Carefully fold back tank cover (using alcohol sanitized hands). Drain tank to 200 L working depth; continue to drain below 200 L by total volume of feed to be added.
8. Minimize aeration and align air stone and heater in tank center.
9. Add the correct volume of each algal species and stock hatched nauplii at one per ml (i.e., for 200 L this requires 200,000 nauplii).
10. Cover tank. Verify that lights are on and timer is set to "automatic". Verify that the digital controller is on and set to the proper temperature.
11. Nauplii develop to copepodites in less than a week at 25^0C, and copepodites mature into adults in less than a week. Inspect the population's developmental

stage regularly and record appropriate data (see Weekly Protocol for sampling technique).

3. Daily Culture Protocol

1. Count algae concentrations and compute required feed volumes to be added.
2. Siphon detritus from tank bottom daily.
3. Remove sufficient water volume to allow addition of new feed volume(s), while maintaining 200 L total volume. As the population matures to stage C-III and C-IV, this volume can be increased to 300 L. Adjust feed computations accordingly.
4. Connect siphon drain hose to siphon and clamp hose to stacked sieves.
5. Discharge siphon drain hose into submerged stacked sieves (150 μm on top, 53 μm on bottom). Mesh screens on stacked sieves must remain submerged during the entire collecting cycle.
6. Discharge effluent from culture system into filtration or appropriate treatment system.
7. 150 μm upper sieve will retain feces, advanced copepodites, and adults. Rinse upper sieve gently over lower to pass eggs through, then into the beaker marked "adult".
8. 53 μm lower sieve will retain feces, nauplii, and eggs. Rinse sieve gently into separate collection beaker marked "nauplii".
9. Beakers are allowed to stand for 10 minutes to allow settling.
10. Adults and nauplii are then attracted to the upper layer of the beaker with light and decanted for return to the tank.
11. Eggs are washed free of feces as thoroughly as possible and then processed as appropriate for immediate hatching or storage for future hatching.
12. The collection from the 48 μm Nitex harvest sieve (the lower one) is first rinsed through an 100 μm Nitex sieve into a beaker to help pulverize the feces and detritus present in this fraction. This collection is then rinsed thoroughly on a 70 μm Nitex sieve, until virtually all feces are removed. Eggs to be stored should be counted, and placed in 30 ml seawater in clearly labeled 50 ml Falcon centrifuge tubes and held at 1ºC.
13. Count a subset of eggs and allow them to hatch for 48 h. The nauplii hatching in 48 h divided by the eggs collected is used to determine the hatch ratio for this cohort of eggs.
14. Rinse sieves vigorously in hot water, submerge in a 10% muriatic acid bath for 30 minutes, rinse again, and allow to air dry.

4. Weekly Culture Protocol

1. Population counts should be done weekly for feeding adjustments.
2. Population counts are obtained by thoroughly mixing the tank *after* siphoning eggs for the day and feeding the tank (for return to full 200 L working volume).
3. Remove three 250 ml samples from the thoroughly mixed tank.
4. Sieve each sample individually through 53 μm mesh, rinse into a 50 ml beaker, bring to 10 ml volume, stain with Lugol's solution and count nauplii, copepodites, and adults. Determine sex of adults for male/female counts. Pool the three counts

and average. Compute total population (average/250 ml x 4 x tank volume). Record result.

5. As the population matures (and biomass and tank volume increase), increase feeding concentration to 25,000 cells/ml for each species of algae.
6. The tank should retain a minimum red "cast" to the tank culture water (if using *Rhodomonas spp.*) after 24 h to imply moderate excess feed availability. If not, increase feeding frequency and/or feed concentrations above 25,000 cells/ml.
7. Excess feeding is unnecessary and unwise. Serious water quality problems can occur. Monitoring must be ever more vigilant and cleaning more thorough at increased feeding concentrations. Additionally, mature algal cultures often display elevated pH (in the 9 – 10 pH range). Adding large volumes of algae culture can cause dramatic pH shifts in the tank, with potentially disastrous results.
8. As the tank approaches senescence, hatching ratios will drop below 75%, irrespective of egg production. Males live shorter lives than females (Parrish and Wilson, 1978), thus females can produce non-viable eggs once the males die out and the attached spermatophores are depleted. Terminate the tank when hatch ratios drop below 65%.
9. If continuous culture of *Acartia* is desired, alternating tanks must be started on a staggered schedule to allow one population to be maturing, while the other population is providing peak production. Do not wait until one tank is senescent prior to starting the next or you will have a week or longer refractory period with no egg production.

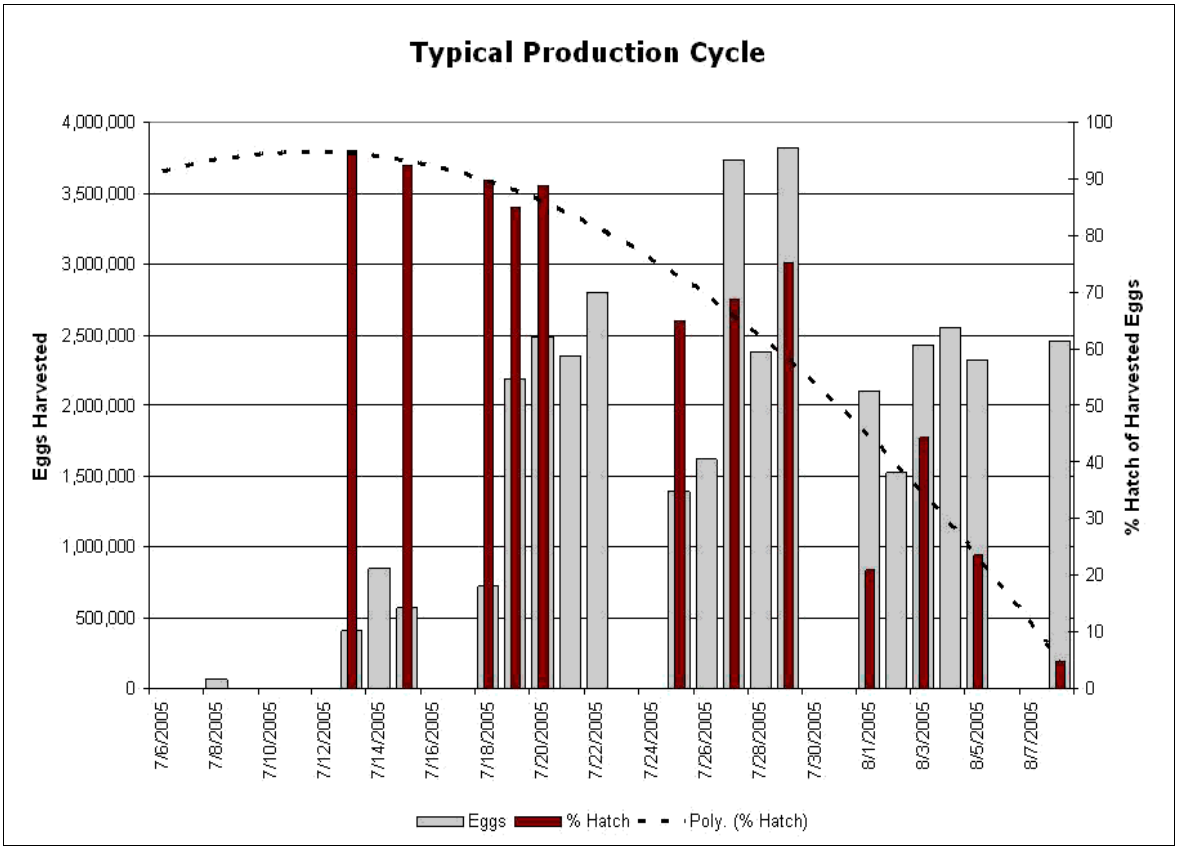

Figure 6. *Typical Production Cycle. July 6 – August 8. Tank stocked with N3 – C1* A. tonsa *at 1 per ml on July 6th. One week later egg production started. Two weeks later egg production began to peak. Three weeks later, egg production remained high, but the hatch % was declining. After four weeks of batch culture, hatch was below 25%.*

G. Egg Counting Protocol

1. Wash freshly collected eggs free of debris, and deposit in a 50 ml Falcon tube, filled to the 30 ml waterline.
2. Vigorously agitate the tube and extract a 0.5 ml sample, dilute to 10 ml.
3. Extract and count a 0.5 ml sample of the diluted stock. This represents 1/1,200 of the total egg collection (1/60 x 1/20). The goal is to count 60-100 eggs per 0.5 ml of diluted sample.
4. If the sample is still too concentrated, remove 0.5 ml of the first dilution and bring to 10 ml.
5. Extract and count 0.5 ml of the second dilution. This represents 1/ 24,000th of the total egg collection (1/60 x 1/20 x 1/20).
6. Count three replicates at a suitable dilution, average the three replicates, and compute the total egg collection for the 50 ml tube (use the correct dilution factor!).
7. Set out three counted replicates of 60-100 eggs each in 100 mm Petri dishes with covers for 48 h hatch-ratio determination. Fill 85% full with treated seawater, label, and cover
8. Label and store remaining eggs or set out to hatch. Label should include date, species, egg count, and tank number.

H. Egg Storage Protocol

1. Storage of eggs is useful, both as a reservoir of nauplii to restart production tanks, and as a supply of nauplii for feeding fish or invertebrate larvae and adults.
2. *Acartia* non-diapause eggs can be stored for the two to six weeks. Shelf life may vary between *Acartia* from different locations.
3. To successfully store eggs, they must be fastidiously cleaned of all feces and detritus. Wash eggs through a 100 μm sieve into a beaker (to fracture the feces), pour that slurry through a 70 μm sieve, and wash carefully until little detritus remains.
4. Count eggs prior to storage and label the storage vessel to indicate species, collection date, egg count, and tank.
5. Store (up to) 5,000,000 eggs per 50 ml Falcon tube.
6. Storage is most successful when temperatures are stable around 1^{0} C.
7. Egg viability from the Gulf of Mexico strain of *A. tonsa*, fed Cryptomonads exclusively, drops rapidly during storage. Presuming a 90% hatch ratio on the initial storage date, one week's storage will reduce this to ~45% hatch, and two week's storage will further reduce this to ~25%, presuming well cleaned eggs.

I. Egg Hatching Protocol

1. Compute the number of nauplii required for hatch (nauplii required divided by the presumed hatch ratio plus a surplus for security). For example, to hatch 200,000 nauplii to start a 200 L tank:

   Divide 200,000 by the 50% presumed hatch ratio for eggs stored a week.

   200,000 eggs/ 0.50 hatch = 400,000 eggs

plus 25% more to avoid aggravation if the hatch ratio is lower

= 500,000 week-old eggs required

2. Count the eggs as previously described or read the label on the Falcon tube, compute the required portion of the 30 ml of stored eggs.
3. Rinse the eggs well on a 53 μm sieve.
4. Add approximately 50,000 eggs/L to treated, aerated seawater, cover the vessel, and use no aeration.
5. Incubate 48 h at 25^0 C.
6. Collect nauplii on a 53 μm sieve, dilute to 1000 ml, mix thoroughly, count nauplii in three 0.5 ml samples, average, and multiply by 2000 for total nauplii count. Record result.
7. For large naupliar counts serially dilute as for counting eggs. Keep accurate record of the dilutions used to facilitate population computation

V. Algae Culture

A. Overview

Feed an equal mix of *Rhodomonas lens* (CCMP 739) and *Rhodomonas salina* (CCMP 1319) to the copepods daily. Typical total feed concentrations are 30,000-50,000 cells/ml/day depending on population density and developmental stage.

The Provasoli-Guillard Center (Maine, USA) maintains alga cultures for sale, as does the University of Texas at Austin. Algae culture has its own set of protocols, be sure to review standard algal culture techniques prior to implementing the procedures described in this section.

The following algae species are utilized to boost egg production: *Akashiwo sanguinea* (strain *GSBL*), *Prorocentrum micans, Pavlova lutheri,* and *Oxyrrhis marina*.

Algal cell densities for feeding copepods are based on reported picograms carbon per cell, relative cell volumes and experiential judgement. Calculate what you predict will be required, then adjust based on residual feed density after 24 h.

Culture Conditions: *Rhodomonas lens* and *R. salina*
19-23^0 C +/-1^0
28-30 ppt
18 h light / 6 h dark
40 W Cool-White light bulbs
NaHCO3 Buffer
Aerate moderately
Feed F/2 solution regularly
Place carboys 10-20 cm from lights

Expect densities approaching 2,000,000 cells/ml without CO_2 enrichment, and >5,000,000 cells/ml with CO_2 enrichment.

To accommodate cultures in 20L carboys we constructed a shelf and light system in an indoor room and maintain the room at specific lighting and temperature conditions (Figure 7).

Figure 7. *Carboy algae culture. Lights are maintained on an 18 h on / 6 h off cycle.*

B. Algae Culture Protocol

1. Use sterile technique to maintain cultures effectively. Be fastidious.
2. Culture initial stocks in an incubator at 23^0 C, 18 h light/6 h dark, according to Provasolli-Guillard protocols, with progression from the 15 ml stock delivery vessel to covered 150 ml flasks to 1.5 L Fernbach flasks as culture densities allow.
3. Swirl flasks daily to maintain stocks in suspension.
4. Transfer to larger vessels as a function of cell density and culture age.
5. Transfer any cultures exceeding 500,000 cells/ml to the next larger vessel.
6. Transfer any cultures ten days old, irrespective of cell density, if its color remains healthy.
7. Fill all incubator vessels to working depth and microwave to 85^0 C for sanitation (Keller et al, 1988). Cool to 23^0C, inoculate vessels with F/2 (0.15 ml/L each of F/2 stocks A and B) and algae stock under a laminar flow hood to minimize contamination.
8. Scrub all carboys (20L) inside and outside with a brush, and rinse thoroughly with hot water. Acid-wash all carboys with muriatic acid, outdoors.
9. Fill all carboys completely to the top with 1 μm filtered, UV-sterilized seawater.
10. Add 10% hypochlorite at 0.2 ml/L and treat vessels for 24 h
11. Dechlorinate vessels with thiosulfate stock solution at 0.2 ml/L, and allow to stand for 4-6 h.
12. Filter all air to 0.2 μm with Gelman Acro50 air filters, to minimize contamination during culture.
13. Sterilize your hands with 70% isopropyl alcohol. Wipe the mouth and neck of the carboy with alcohol prior to inoculation.
14. Decant carboys to 15 L working depth.
15. Inoculate carboys with F/2 stocks A&B at 0.15 ml/L each, 6 g sodium bicarbonate, $NaHCO_3$ (3 millimole, final concentration), and swirl to dissolve the bicarbonate.
16. Inoculate carboys with 750 ml algae culture from incubator Fernbach vessels and label for species and starting date.
17. Start moderate aeration immediately.
18. Swirl carboys twice daily to maintain algae in the water column.
19. After 5 days, add additional F/2 A&B stock and slightly increase aeration.
20. When the pH elevates above 9, bubble CO_2 to further increase cell density. If CO_2 is unavailable, add a little more $NaHCO_3$ to buffer the solution.
21. When cultures are 5-7 d old, cell densities should exceed 1,000,000 cells/ml.
22. Maintain the culture as long as cell density continues to increase. Nutritive values of algae are greatest during the log-phase of growth. Feed to copepods, or use as an inoculant for larger scale algae culture.

Discard cultures when cell density ceases to rise. The algae culture has reached senescence and its nutritive value plummets. Use as feed for copepods only in emergencies.

For Each Species of Algae:

Feed concentration x liters of copepods cultured = Daily Production Demand
Algae concentration x liters of algae cultured = Daily Production Capacity

This demand specifies how many liters of a given species will be required daily (plus extra volume to maintain a production safety buffer).

Example:

Status: 200 L copepods @ 1 per ml, *R. salina* slightly higher cell count than *R. lens*

- 200 L of copepod culture requires:
  - 15,000 cells/ml x 200 L x 1000 ml/L = 3×10^9 cells *R. lens* per feeding
- *R. lens* culture at 1.5 million cells/ml requires:
  - 3×10^9 cells/ 1.5×10^6 cells/ml = 2 L of *R. lens* per feeding
- *R. salina* culture at 2 million cells/ml requires:
  - 3×10^9 cells/ 2×10^6 cells/ml = 1.5 L of *R. salina* per feeding
- Each week, 14 L of *R. lens* and 11 L of *R. salina* are required, at this culture density (more volume is required, if the density drops; less if it rises).

VI. References

Apeitos, A., J. Lotz, J. Ogle, J. Lemus, 2004. The effect of diet on fecundity of *Acartia tonsa*, a calanoid copepod. The Journal of the Mississippi Academy of Sciences, 49: 94.

Evjemo, J. O., K. I. Reitan and Y. Olsen. 2004. Copepods as live food organisms in the rearing of Atlantic halibut larvae (*Hippoglossus hippoglossus* L.) with special emphasis on nutritional value. Aquaculture 227(1-4):191-211.

Keller, M., W. Bellows, R. Guillard, 1988. Microwave treatment for sterilization of phytoplankton culture media. Journal of Experimental Marine Biology and Ecology 117(3):279-283.

Kraul, S., H. Ako, A. Nelson, K. Brittain, and A. Ogasawara. 1992. Evaluation of live feeds for larval and postlarval mahimahi, *Coryphaena hippurus*. Journal of the World Aquaculture Society 23(4): 299–306.

Lee, C.S., P. O'Bryen, N.H. Marcus, 2005. Copepods in Aquaculture. Blackwell Publishing, New York, pp 352.

Mauchline, J., 1998. The Biology of Calanoid Copepods: The Biology of Calanoid Copepods (Advances in Marine Biology, Vol. 33) Elsevier Academic Press, New York. pp 710.

Parrish, K., Wilson, D., 1978. Fecundity studies on *Acartia tonsa* (Copepoda: Calanoida) in standardized culture. Marine Biology 46: 65-81.

Payne, M. F. and R. J. Rippingale. 2000. Rearing West Australian seahorse, Hippocampus subelongatus, juveniles on copepod nauplii and enriched *Artemia*. Aquaculture 188(3-4):353-361.

Petipa, T.S., 1959. Feeding of the Copepod *Acartia clausi* Giesbrecht. Proceedings of the USSR AS Sevastopol Biological Station, Vol. 11:72-79.

Sabatini, M.E., 1990. The Developmental Stages (Copepodids I to VI) of *Acartia tonsa* Danae, 1849 (Copepoda, Calanoida). Crustaceana 59(1): 53-61.

Sargent J.R., McEvoy L.A., J.G.Bell, 1997. Requirements, presentation and sources of polyunsaturated fatty acid in marine fish larval feeds. Aquaculture 155:117-127.

Sazhina, L.I., 1987. Reproduction, Growth, Production of Marine Copepods. Naukova Dumka, Kiev. pp 156.

Sazhina, L.I., 1971. Fecundity of Mass Pelagic Copepoda of the Black Sea. Journal of Zoology 4:586-589.

Schipp, G. R., J. M. P. Bosmans and A. Marshall. 1999. A method for hatchery culture of tropical calanoid copepods, Acartia spp. Aquaculture 174(1-2):81-88.

Shields, R. J., J. G. Bell, F. S. Luizi, B. Gara, N. R. Bromage and J. R. Sargent. 1999. Natural copepods are superior to enriched *Artemia* nauplii as feed for halibut larvae (*Hippoglossus hippoglossus*) in terms of survival, pigmentation, and retinal morphology: relation to dietary essential fatty acids. Journal of Nutrition 129:1186-1194.

Støttrup, J. 2000. The elusive copepods: their production and suitability in marine aquaculture. Aquaculture Research 31(8-9):703-711.

Støttrup, J. G. and N. H. Norsker. 1997. Production and use of copepods in marine fish larviculture. Aquaculture 155(1-4):235-251.

Støttrup, J., K. Richardson, B. Kirkegaard, and N. J. Pihl. 1986. The cultivation of *Acartia tonsa* Dana for use as a live food source for marine fish larvae. Aquaculture 52(2):87-96.

Watanabe, T., C. Kitajima, and S. Fujita. 1983. Nutritional values of live organisms used in Japan for mass propagation of fish: a review. Aquaculture 34(1-2):115-143.

Wilcox, J.A., P.Tracy, and N.H.Marcus. 2006. Improving Live Feeds: effect of a mixed diet of copepod nauplii and rotifers on the survival and growth of first-feeding larvae of the Southern flounder, *Paralichthys lethostigma*. Journal of the World Aquaculture Society, Vol. 37(1).

VII. Selected Additional Readings

Chen, F. and N. H. Marcus. 1997. Subitaneous, diapause, and delayed-hatching eggs of planktonic copepods from the northern Gulf of Mexico: morphology and hatching success. Marine Biology 127: 587-598.

Doi, M., T. Singhagraiwan, S. Singhagraiwan and A. Ohno. 1994. An investigation of copepods being applied as initial food organisms for red snapper larvae. Thai Marine Fisheries Research Bulletin 5:21-26.

Hussain, N. A. and M. Higuchi. 1980 Larval rearing and development of the brown spotted grouper, *Epinephelus tauvina* Forskål. Aquaculture 19(4):339-350.

Jung, T. and C. Clemmesen. 1997. Effect of different food organisms on the development and nutritional condition of cod larvae (*Gadus morhua* L.) in laboratory rearing experiments. Ichthyoplankton Ecology: 24-25. Fisheries Society of the British Isles.

Marcus, N. H. and R. Lutz. 1998b. Longevity of subitaneous and diapause eggs of Centropages hamatus (Copepoda:Calanoida) from the northern Gulf of Mexico. Marine Biology 131: 249-257.

Marcus, N. H. and M. Murray, 2001. Copepod diapause eggs: A potential source of nauplii for aquaculture. Aquaculture 201: 107-115.

Murray, M.M., Marcus, N.H., 2001.Survival and Diapause Egg Production of the Copepod *Centropages hamatus* raised on dinoflagellate diets. Journal of Experimental Marine Biology and Ecology 270: 39-56.

Payne, M. F., R. J. Rippingale and J. J. Cleary. 2001. Cultured copepods as food for West Australian dhufish (*Glaucosoma hebraicum*) and pink snapper (*Pagrus auratus*) larvae. Aquaculture 194(1-2):137-150.

Rønnestad, I., S. Helland and Ø. Lie. 1998. Feeding *Artemia* to larvae of Atlantic halibut (*Hippoglossus hippoglossus* L.) results in lower larval vitamin A content compared with feeding copepods. Aquaculture 165(1-2):159-164.

Toledo, J. D., M. S. Golez, M. Doi and A. Ohno. 1999. Use of copepod nauplii during early feeding stage of grouper, *Epinephelus coioides*. Fisheries Science 65(3):390-397.

Appendices

Appendix 1

Abbreviations used

DO	dissolved oxygen
g	gram
h	hour
min	minute
L	liter
ml	milliliter
mm	millimeter
μm	micrometer
ppt	parts per thousand salinity
psu	practical salinity unit
PVC	Schedule 40 PVC potable water pipe
UV	ultraviolet light

Appendix 2

Chlorination / De-chlorination Water Treatment for Algae Culture

1. Obtain commercial 10% hypochlorite solution and prepare thiosulfate stock solution for neutralization.
2. Prepare a stock solution of sodium thiosulfate (25 g NaS_2O_3 in 250 ml seawater). It is used volume for volume (V/V) to neutralize the 10% hypochlorite solution.
3. To chemically sanitize 20 L carboys for algae culture, they must be completely filled in order to expose all interior surfaces to treatment.
4. Add 0.2 ml of commercial 10% hypochlorite solution (swimming pool chlorine) per liter seawater to reach 1 ppt free chlorine.
5. Fill carboys to the rim with 1 μm filtered, UV-treated natural seawater (NSW), add chlorinating solution, insert aeration tubing, and cap vessel securely.
6. Allow treatment to stand overnight without further aeration.
7. After 24 h, add 0.2 ml of the sodium thiosulfate solution/L to the carboys, and initiate vigorous aeration for three hours.
8. Dip a "free chlorine" test strip in a randomly selected carboy and read to verify zero free chlorine remaining in the carboys.
9. Apply this same treatment protocol (0.2 ml hypochlorite/L for 24h followed by 0.2 ml thiosulfate stock/L) to all carboys and tanks for sanitation prior to stocking copepods.

Appendix 3

Construction of the Squeegee Siphon Assembly

The squeegee is designed to gently scrape the bottom of the tank to retrieve the eggs produced since the last harvest. A siphon hose alone will not capture all eggs entrapped in the biofilm that builds up in living systems. The tool needs to conform gently to the bottom of the tank without damaging the Fiberglas gelcoat. Plumbing diameters need to be sized to maintain siphon velocity and flow without overwhelming the stacked sieves. Many alternate strategies could work. Our solution was to construct a simple siphon from PVC and install a section of Shop-Vac squeegee (an accessory for the wet vacuum head) as a scraper (Figures 8, 9, 10).

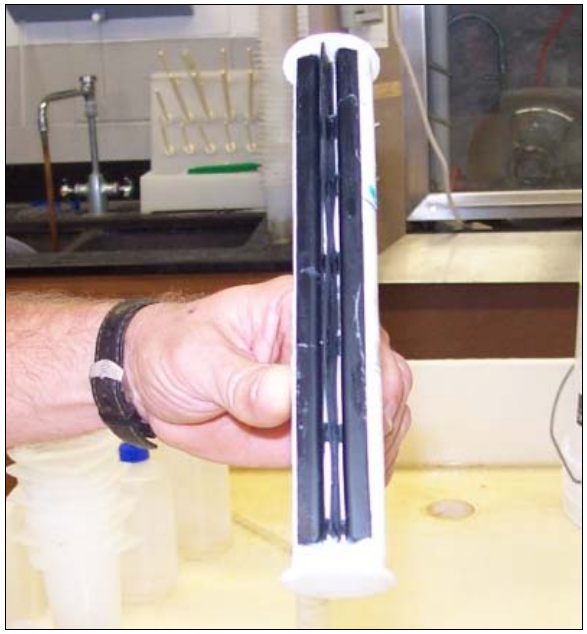

Figure 8. *Squeegee scraper.*

For a 300 L tank, construct a ¾" (1.9 cm) I.D. PVC siphon assembly:

¾" diameter PVC pipe, at least 60" long
¾" PVC tee fitting
¾" PVC couplings
¾" PVC 45^0 Elbow
¾" PVC female fitting
¾" PVC male to hose barb adapter
7' (2 m) section siphon hose
¾" Hose clamp for siphon hose
6"- 9" (15-23 cm) section of Shop-Vac® squeegee
PVC discs cut from 4" PVC test caps
Large report-style paper clamp

1. Glue the PVC tee and couplings together with sections of pipe to fill the interior voids as completely as possible. 6"- 9" (15-23 cm) siphon head width is the optimum width for this design.
2. Use a Dremel tool or hack saw to slice a slot opposite the tee inlet, cutting through both the fittings and the PVC pipe glued inside. The slot's interior faces are cut parallel, not splayed apart. The slot's width matches the width of the Shop-Vac squeegee, measured under the outer flange. Trim a section of squeegee to fit and cement in place with silicone aquarium sealant.

3. Cut discs of PVC and glue to the ends, positioned to allow the squeegee to just deform as it moves across the bottom of the tank.
4. Attach a length of PVC pipe sufficient to reach beyond the upper lip of the tank to the tee.
5. Glue a 45^0 elbow on to take the following sections by friction.
6. Glue on a short section of ¾" PVC pipe to the slip x female PVC fitting,
7. Screw into the female PVC fitting the male PVC to hose barb adapter.
8. Clamp the siphon hose to the barb adapter.
9. Slip the wire wings of a large report-style pinch clamp over the hose to allow the clamp to attach to the stacked sieves to hold the hose in place.
10. Let all parts stand for 48h to allow all volatile elements of the glue to dissipate.

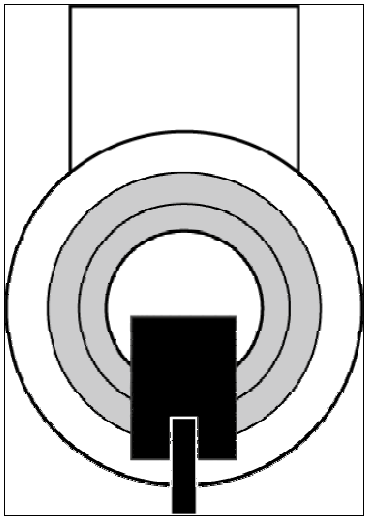

Figure 9. *Squeegee cross-section schematic.*

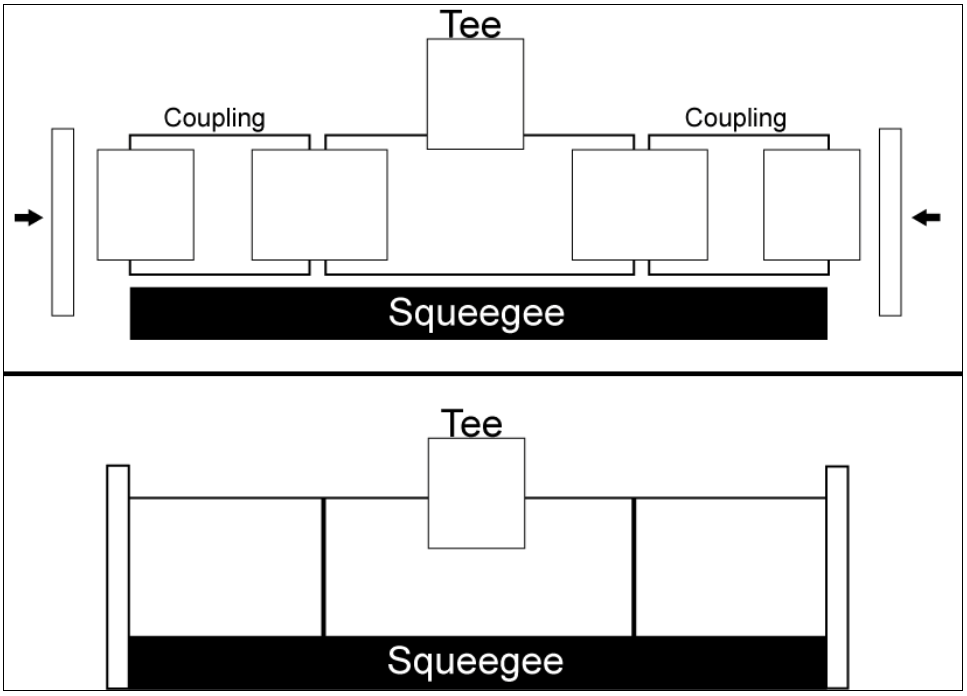

Figure 10. *Squeegee longitudinal section schematic.*

Use of the Squeegee Siphon

1. Sanitize your hands with 70% isopropyl alcohol or wear latex gloves.
2. The siphon head assembly remains in the tank, sanitized during tank treatment.
3. Connect the siphon hose assembly to the siphon head assembly (friction fit).
4. Initiate siphoning by providing suction.
5. Clamp the siphon hose to the stacked sieves.
6. The squeegee assembly is slowly swept across the tank bottom, starting from one corner and proceeding deliberately in a regular pattern.
7. Do not stir up the eggs and detritus on the bottom as you proceed.
8. When done, remove the siphon hose assembly; leave the siphon head in the tank.
9. Rinse the siphon hose with a wash bottle, drain effluent into the sieves.
10. Remove the siphon hose clamp from the stacked sieves and process the collection.

Construction of Stacked Sieves

We recommend that you construct a stacked filter set (Figures 11, 12) for each species of copepods you intend to culture. For a 300 L tank, we elected to use a 6″ diameter PVC coupling for the lower sieve, and a section of 6″ PVC pipe for the upper sieve.

Figure 11. *Stacked filter set.*

Upper Sieve: For Adult Capture

1. Cut a 9″ section of 6″ diameter PVC pipe. Cut squarely across the pipe on each end. Sand the edges smooth and flat.
2. Apply a thin coat of silicone aquarium sealant to the lower edge of the pipe section.
3. Stretch a piece of 150 μm Nitex screen carefully over the mouth of the 6″ pipe.
4. Apply a 0.5 cm ring of silicone aquarium sealant to the screen immediately above the pipe. Avoid smearing silicone over the free surface of the Nitex.
5. Work the silicone into the screen (over the PVC pipe only).
6. Sand the pipe exterior such that it fits closely into the coupling used as the lower sieve. It must be capable of being removed without loss of copepods or eggs from either sieve.

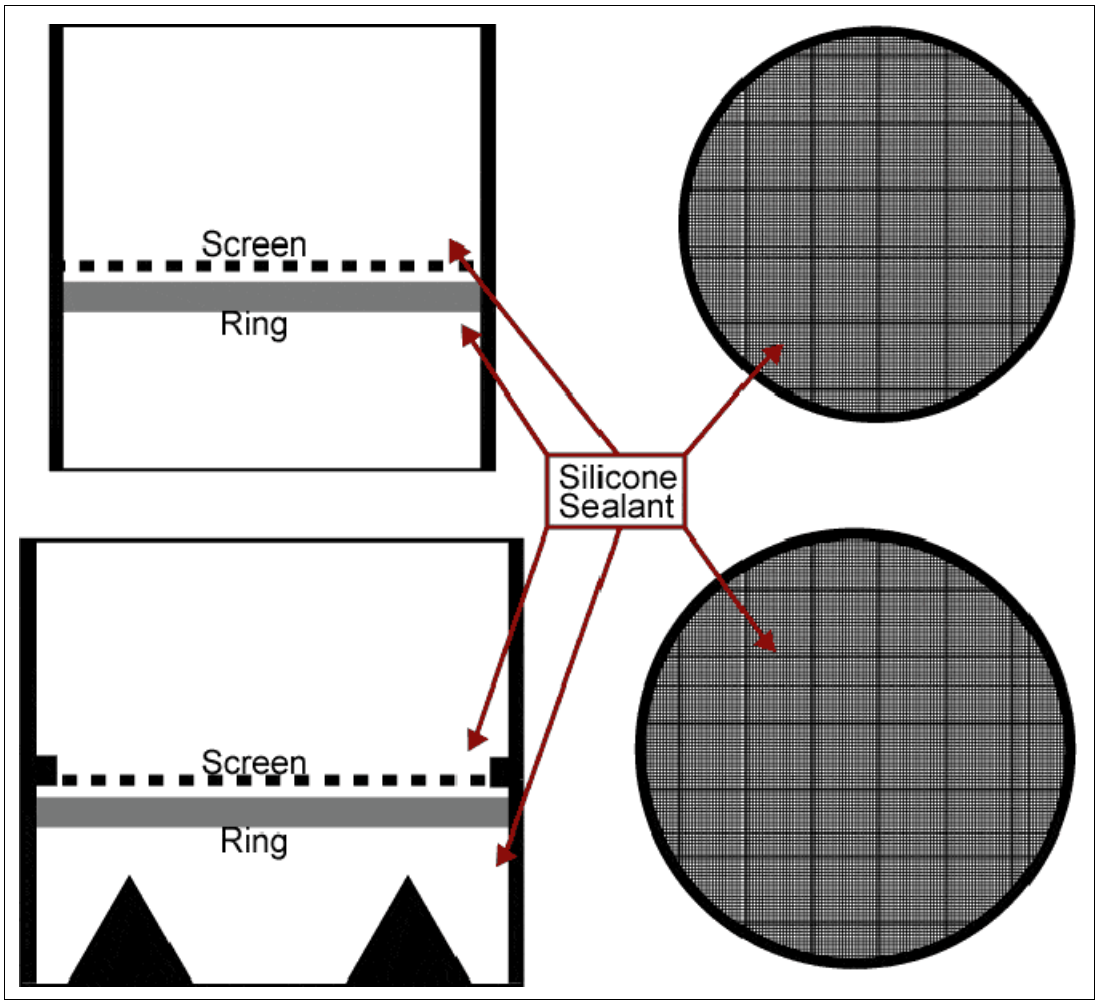

Figure 12. *Stacked sieves schematic.*

Lower Sieve: For Egg and Nauplii Capture

1. Use a Dremel tool, jig saw or hack saw to cut three triangles from the bottom edge of a 6" PVC coupling. This allows drainage while sieves are submersed.
2. Cut a ½" section of 6" diameter PVC pipe. Cut squarely across the pipe. Sand the edges smooth and flat.
3. Stretch a piece of 53 μm Nitex screen carefully over the 1/2" ring of 6"pipe.
4. Work the ring into the base of the coupling, capturing the Nitex screen smoothly.
5. Slide this in almost to the ridge in the center of the coupling; apply a 0.5 cm ring of silicone aquarium sealant immediately below the ridge, but above the screen.
6. Work the ring and screen up onto the silicone.
7. Smooth the bead generated into the screen over the ring. Avoid smearing silicone over the free surface of the Nitex.
8. Place a small bead of silicone on the seam where the lower edge of the ring meets the coupling.

Use of the Stacked Sieves

Figure 13. *Stacked sieves in collecting pan.*

1. Nest the sieves.
2. Immerse the stacked sieves in treated seawater; remove the air bubbles under the sieves.
3. Screens must remain submerged and wetted during the entire process. We use an oil collector from an automotive store (Figure 13).
4. Connect the siphon hose to the siphon head, initiate the siphon, insert into the hose into the stacked sieves. Clamp hose to the stacked sieves.
5. Clogging of the sieves may occur during collection and could result in the loss of adults and eggs. Be vigilant. Shake the sieves gently to improve flow during collection.
6. Rinse upper sieve (150 μm) over the lower sieve (53 μm) before collecting the adults and detritus in the upper sieve.
7. Collect contents of upper sieve into a 2 L beaker.
8. Collect contents of lower sieve into a separate 2 L beaker.

Appendix 4

Stock Solutions

Lugol's solution

1. Dissolve 10 g I_2, and 20g KI in 200 ml distilled water.
2. Add 20 ml concentrated glacial acetic acid.
3. Store solution in a dark glass bottle, preferably with a ground glass stopper
4. Use one or two drops on a slide for counting eggs or nauplii.
5. Discard all materials in a Hazardous Waste container.

Sodium thiosulfate:

1. Measure 10 g sodium thiosulfate.
2. Dissolve in 100 g of treated seawater.
3. Cap, date, and label container
4. Use volume/volume (to hypochlorite solution) to de-chlorinate treated water (0.2 ml/L hypochlorite is de-chlorinated with 0.2 ml/L thiosulfate)

Appendix 5

System Components	Supplier or Brand Used (Not an endorsement)
Water and Air Quality Control	
Refractometer	Aquatic-EcoSystems
DO/pH meter	YSI®, Orion®
Water analysis kit	Hach®, LaMotte®
Serial bag filter, 50μm, 10μm, 1μm	Aquatic-EcoSystems
UV-sterilizer, 40 gpm rating	Aquatic-EcoSystems
Hypochlorite solution 10%	Lowes
Sodium Thiosulfate	Fisher Scientific®
Alconox	Fisher Scientific®
0.2μm anti-microbial air filter	Pall® ACRO37, VRW
Copepod production	
300 L Fiberglas rectangular tank(s)	Aquatic-EcoSystems
Tank cover and brace	VisQueen®
Aerator	Sweetwater®
Aquarium heater	Aquatic-EcoSystems
Digital temperature controller	Process Control®
Light fixture and timer	Lowes
Stacked sieves	Lowes and Sefar®
Sieve holder with drain line	NAPA Auto Parts®
Squeegee for siphon	Shop-Vac®
Wash bottles	Fisher Scientific®
Dissecting microscope	Wild®, Olympus®
Manual Counter	Fisher Scientific®
Egg storage tubes	Falcon® 50ml & 15ml
Algae production	
Incubator with lights	Precision®
Erlenmeyer flasks 0.5L	Kimax®
Fernbach flasks 1.5L	Kimax®
Carboys 19L	Culligan®
Light bank and timer	Lowes
Aerator	Sweetwater®
CO_2 supply and rhodometer	AirGas
Algae cultures	
R. salina CCMP 1319	Provasoli-Guillard Center
R. lens CCMP 739	Provasoli-Guillard Center
Hemocytometer	Fisher Scientific®
Microscope	Olympus®
Manual Counter	
70% isopropyl alcohol (spray bottle)	Fisher Scientific®

Appendix 6

System Design Considerations

Ergonomic design	minimize wasted labor
Critical systems backups and alarms	avoid catastrophes
Clean and safe operation	avoid accidents
Site security and biosecurity	avoid contamination
Flexibility of operations	enhance opportunities
Scale of linked operations	assure success

FSU uses 300 L Fiberglas tanks for meso-scale culture and we are scaling up to 1000 L tanks for full-scale production. Tanks should be maintained independently and run as batch cultures to minimize risks. Batches of nauplii are stocked, fed, regularly cleaned, and monitored. Once egg production begins, tanks are siphoned and fed everyday. Water changes per day average 15-20% of the tank volume (from siphoning and feeding) done properly. System design should address minimizing contamination, regulating water quality, algae production requirements, daily maintenance requirements, efficient use of labor, and desired level of egg production.

Tanks must be designed and arranged to be independently cleaned, filled and drained, heated and cooled, and conveniently worked. Tanks should be covered, to prevent contamination; but the cover must not impede regular siphoning of the bottom of the tank. Labor must be allotted for tank maintenance, feeding, population and egg counting, sieving, and egg storage. Many small tanks will require disproportionately more labor than a few large ones. For research, this extra labor is acceptable. For commercial scale production the higher risks of catastrophic loss may be offset by the lower labor costs fewer tanks would require. Smaller tanks in replicate sets are more costly, but catastrophic loss impacts would be reduced by redundancy.

Tanks for culturing *A. tonsa* should have a flat bottom for ease of egg collection and tank cleaning. Depth and width are also dependent on human ergonomics, such that no more than 1 m depth or 1 m width/diameter is recommended when tanks are arranged in pairs along their long axis. Freestanding tanks may be up to 2 m in width and still be worked effectively from both sides. Acceptable length of tanks is a function of building design, fabrication costs, and production goals. Sufficient space must be provided between tanks to allow workers to perform effectively. Tanks ideally should be elevated off the floor, to increase control of tank thermal conditions and to allow for easy plumbing of the system.